Unique Membrane Interaction Mode of Group IIF Phospholipase A₂

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The mechanisms by which secretory phospholipases A₂ (PLA₂s) exert cellular effects are not fully understood. Group IIF PLA₂ (gIIFPLA₂) is a structurally unique secretory PLA₂ with a long C-terminal extension. Homology modeling suggests that the membrane-binding surface of this acidic PLA₂ contains hydrophobic residues clustered near the C-terminal extension. Vesicle leakage and monolayer penetration measurements showed that gIIFPLA₂ had a unique ability to penetrate and disrupt compactly packed monolayers and bilayers whose lipid composition recapitulates that of the outer plasma membrane of mammalian cells. Fluorescence imaging showed that gIIFPLA₂ could also readily enter and deform plasma membrane-mimicking giant unilamellar vesicles. Mutation analysis indicates that hydrophobic residues (Tyr¹¹⁵, Phe¹¹⁶, Val¹¹⁸, and Tyr¹¹⁹) near the C-terminal extension are responsible for these activities. When gIIFPLA₂ was exogenously added to HEK293 cells, it initially bound to the plasma membrane and then rapidly entered the cells in an endocytosis-independent manner, but the cell entry did not lead to a significant degree of phospholipid hydrolysis. gIIFPLA₂ mRNA was detected endogenously in human CD₄⁺ helper T cells after in vitro stimulation and exogenously added gIIFPLA₂ inhibited the proliferation of a T cell line, which was not seen with group IIA PLA₂. Collectively, these data suggest that unique membrane-binding properties of gIIFPLA₂ may confer special functional activity on this secretory PLA₂ under certain physiological conditions.

Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of the sn-2 ester bond of membrane phospholipids to liberate fatty acid and lysophospholipid. One of these products, arachidonic acid (AA) is transformed into potent inflammatory lipid mediators, collectively known as eicosanoids. Multiple forms of PLA₂, including secretory PLA₂ (sPLA₂) (1, 2) and Ca²⁺-dependent cytosolic PLA₂ (α, β, γ, δ, ε, and ζ) (3, 4) and Ca²⁺-independent intracellular PLA₂ (β and γ) (5), have been identified from mammalian tissues. Among intracellular PLA₂, group IVA cytosolic phospholipase A₂ has been shown to be involved in inflammation (6, 7), whereas intracellular PLA₂β has been implicated in spermatogenesis and insulin signaling (8, 9). However, physiological roles of other intracellular PLA₂ have not been fully defined.

10 sPLA₂ (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII) have been identified in mammals so far (1, 10). Many sPLA₂ have been shown to induce or augment cellular AA release and eicosanoid biosynthesis when overexpressed in or exogenously added to mammalian cells. However, it is not clear whether or not these sPLA₂ are directly involved in AA production and inflammation under physiological conditions. Among various sPLA₂, group V PLA₂ (gVPLA₂) has been implicated in inflammation by a recent gene knock-out study (11).

It has been reported that sPLA₂ can exert cellular effects through different mechanisms (12). Based on the earlier finding that the level of sPLA₂ was elevated in inflammatory exudates (13, 14), it was generally thought that sPLA₂ are released to the extracellular medium in response to specific stimuli and act on different target cells by a transcellular or paracrine mechanism. However, Kudo and co-workers found that many basic sPLA₂, glycerol-3-phosphocholine; HSPG, heparan sulfate proteoglycans; gIIFPLA₂, group IIA phospholipase A₂; gIIFPLA₂, group IIF phospholipase A₂; gVPLA₂, group V phospholipase A₂; gXPLA₂, group X phospholipase A₂; PC, phosphatidylcholine; PG, phosphatidylglycerol; PED6, N-[6-(2,4-dinitrophenyl)amino]hexanoyl-1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethylyl-bora-3,4a-diazid-s-indacene-3-pentanoyl)-sn-glycerol-3-phosphoethanolamine triethylammonium salt; POPC, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; pyrene-PG, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-sn-glycerol-3-phosphoglycerol; SM, brain sphingomyelin; sPLA₂, secretory PLA₂; NBD-cholesterol, 22-((7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol; DMEM, Dulbecco’s modified Eagle’s medium; PBS, fetal bovine serum; SPR, surface plasmon resonance; CHAPS, 3-(3-cholamidopropyl)dimethylammoniomion); 1-propanesulfonic acid; LUV, large unilamellar vesicle; GUV, giant unilamellar vesicles; PG, phosphatidylglycerol.
including group IIA PLAs (gIIAPLA2) and gVPLA2, remained bound to their parent cells after secretion due to their high affinity for cell surface heparan sulfate proteoglycans (HSPG) and were reinternalized to augment the stimulus-dependent AA release (15–21). This HSPG affinity has been shown to be important for the entry of different types of sPLA2s into mammalian cells (12, 22). More recently, it was reported that gIIAPLA2 and group X (gXPLA2) could also induce the cellular AA release during the secretory process (23). Among known sPLA2s, gVPLA2 (24, 25) and gXPLA2 (26, 27) can effectively bind and hydrolyze zwitterionic phosphatidylcholine (PC) that is rich in the external leaflet of mammalian plasma membranes. As a result, these sPLA2s are able to directly act on mammalian cells and catalyze the hydrolysis of cell surface phospholipids. Lastly, some sPLA2s have been reported to exert cellular effects through the binding to cell surface receptors (28).

First, gIIAPLA2 (gIIFPLA2) is unique among sPLA2s in two respects. First, gIIAPLA2 was shown to induce or augment the cellular AA and eicosanoid formation when overexpressed in mammalian cells despite having extremely low HSPG affinity (29) and low activity on PC vesicles (2). This suggests that gIIAPLA2 might have a unique mode of cellular action. Second, gIIAPLA2 is structurally unique in that it has an unusually long, proline-rich C-terminal extension (30, 31) (see Fig. 1A). To elucidate the mechanism by which gIIAPLA2 acts on mammalian cells, we built a model tertiary structure of gIIAPLA2 by homology modeling and measured the interactions of wild type and selected mutants of gIIAPLA2 with various model membranes and mammalian cells. Results show that due to its unique structural and membrane binding properties, gIIAPLA2 has an unprecedented ability to traverse the plasma membrane of mammalian cells, which is independent of binding to cell surface HSPG or phospholipid hydrolysis on the outer plasma membrane. These unique properties of gIIAPLA2 may allow this sPLA2 to perform some unusual functions under certain physiological conditions.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), cholesterol and brain sphingomyelin (SM) were from Avanti Polar Lipids, Inc. (Alabaster, AL), and 1,2-di-O-hexadecyl-sn-glycero-3-phosphocholine (DHPC) was from Sigma. 5-Carboxyfluorescein, 1-hexadecanoyl-2-(1-pyrenyldecanoyl)-sn-glycero-3-phosphoglycerocephyl-PG, N-((6-((2,4-dinitrophenyl)amino)hexanoyl)-2(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (PED6), Texas Red™ C2-maleimide, and 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3-ol (NBD-cholesterol) were purchased from Invitrogen. 1,2-Bis[12-(lipoxyloxy)dodecanoyl]-sn-glycero-3-phosphocholine (BLPC) and -glycerol (BLPG) were synthesized, and polymerized mixed vesicles (100 nm in diameter) were prepared as described previously (32, 33). Phospholipid concentrations were determined by a modified Bartlett analysis (34). Dulbecco’s modified Eagle’s medium (DMEM) and inactivated fetal bovine serum (FBS) were from Invitrogen. Human embryonic kidney cell line HEK293 and Zeocin™ were from Invitrogen. Recombinant human gIIAPLA2 (35) and gVPLA2 (25) were expressed in Escherichia coli and purified as described.

**Mutagenesis and Protein Expression**—The cDNA of full-length mouse gIIFPLA2 was cloned from the mouse testis cDNA library (Clontech) and subcloned into the pET-21a(+) vector (Novagen, Madison, WI) between the restriction sites Ndel and XhoI. Site-directed mutagenesis was carried out by the overlap extension PCR. All mutant constructs were transformed into DH5α cells for plasmid isolation, and their DNA sequences were verified. E. coli strain BL21 (DE3) was used as a host for the protein expression. 4 liters of Luria broth medium containing 100 μg/ml ampicillin was inoculated with 100 ml of the overnight culture from a freshly transformed single colony. The culture was grown at 37 °C. When the optical density of the culture at 600 nm reached 0.8–1.0, the culture was induced by 1 mM isopropyl-1-thio-β-d-galactopyranoside (Research Products, Mount Prospect, IL). After incubation for 4 h at 37 °C, cells were harvested at 5000 × g for 10 min at 4 °C and frozen at −20 °C. The cells were resuspended in the Celllytic B-11 (Sigma) bacterial cell lysis extraction reagent (5 ml/g of cell paste), and deoxyribonuclease was added to a final concentration of 5 μg/ml to reduce the viscosity of the suspension. The extraction suspension was shaken at room temperature for 15 min and centrifuged at 25,000 × g for 15 min. Pellets were dissolved in Celllytic B-11 diluted 20-fold in water, incubated, and centrifuged as described above, and these steps were repeated twice to obtain clear inclusion body pellets. Inclusion bodies were solubilized in 10 ml of 50 mM Tris buffer, pH 8.0, containing 6 M guanidinium chloride, 1 mM EDTA and stirred overnight at 4 °C. Any insoluble matter was removed by centrifugation at 50,000 × g for 40 min at 4 °C. The supernatant was loaded to a Superdex G-200 column (Amersham Biosciences) equilibrated with 50 mM Tris buffer, pH 8.0, containing 3 M guanidinium chloride and 5 mM EDTA. Fractions corresponding to the protein peak were pooled and added dropwise to 50 ml of 50 mM Tris, pH 8.0, containing 5 mM EDTA, 20 mM reduced glutathione, and 10 mM oxidized glutathione over 3 h. The solution was kept at room temperature for 20 h. The refolded protein solution was dialyzed against 4 liters of 25 mM Tris buffer, pH 8.0, containing 1 M urea for 4 h at 4 °C and against 4 liters of 25 mM Tris buffer, pH 8.0, containing 0.5 M urea, 0.1 mM dithiothreitol for 2 h at 4 °C, and finally against 25 mM Tris, pH 8.0, containing 0.5 M urea for 4 h at 4 °C. The protein solution was centrifuged at 50,000 × g for 40 min to remove insoluble matter, and the clear solution was loaded to a phenyl-Sepharose column (Amersham Biosciences) that was attached to an AKTA FPLC system (Amersham Biosciences) and equilibrated with 25 mM Tris, pH 7.4, containing 1 M ammonium sulfate. The column was eluted with a linear gradient of ammonium sulfate from 1 to 0 M and then with a linear gradient of 0–30% (v/v) acetonitrile in the same buffer. Fractions corresponding to the major protein peak were pooled and dialyzed against 25 mM Tris, pH 7.4, containing 160 mM NaCl, and stored at 4 °C. The purity of protein (>90%) was confirmed by SDS-PAGE. Protein concentration was determined by the
bicinchoninic acid method (Pierce) using bovine serum albumin (BSA) as a standard.

**PLA₂ Activity Assay**—The PLA₂-catalyzed hydrolysis of polymerized mixed vesicles (0.1 μM pyrene-PG inserted in 9.9 μM BLPC or BLPG) was carried out at 37 °C in 2 ml of 10 mM Tris buffer, pH 7.4, containing 0.16 M KCl, 1 mM CaCl₂, and 2 μM BSA (32, 33). The progress of hydrolysis was monitored as an increase in fluorescence emission at 378 nm using a Hitachi F4500 Fluorescence spectrophotometer with excitation wavelength set at 345 nm, and spectral bandwidth was set at 10 nm for both excitation and emission. The PLA₂-catalyzed hydrolysis of PED6 in the mixed vesicles of POPS/cholesterol/POPG/PED6 (107:31:20:1) was carried out at 37 °C in 2 ml of 10 mM HEPES, pH 7.4, containing 0.16 M KCl, 1 mM Ca²⁺. The progress of hydrolysis was monitored as an increase in fluorescence emission at 520 nm with the excitation wavelength set at 488 nm. Spectral bandwidth was set at 10 nm for both excitation and emission. Values of specific activity were determined from the initial rates of hydrolysis.

**Surface Plasmon Resonance Analysis**—Kinetics of vesicle-PLA₂ binding was measured by the surface plasmon resonance (SPR) analysis using a BIAcore X biosensor system (Biacore AB) and the L1 chip as described previously (36). All measurements were performed at 23 °C in 5 mM HEPES buffer, pH 7.4, containing 160 mM NaCl and 0.1 mM EDTA. The first flow cell was used as a control cell and was coated with 5400 resonance units of BSA. The second flow cell contained the surface coated with vesicles with varying lipid compositions at 5400 resonance units. After lipid coating, 30 μl of 50 mM NaOH was injected at 100 μl/min three times to wash out loosely bound lipids. Typically, no further decrease in SPR signal was observed after one wash cycle. After coating, the drift in signal was allowed to stabilize below 0.3 resonance units/min before any binding measurements, which were performed with a flow rate of 30 μl/min. 90 μl of protein sample was injected for an association time of 3 min, and the dissociation was then monitored for 10 min in running buffer. After each measurement, the lipid surface was typically regenerated with a 10-μl pulse of 50 mM NaOH. The regeneration solution was passed over the immobilized vesicle surface until the SPR signal reached the initial background value before protein injection. When needed, the entire lipid surface was removed with a 5-min injection of 40 mM CHAPS followed by a 5-min injection of 40 mM octyl glucoside at 5 μl/min, and the sensor chip was recoated for the next set of measurements. All data were analyzed using BIAevaluation 3.0 software (Biacore).

**Vesicle Leakage Experiments**—Appropriate amounts of lipids in chloroform were mixed, and the solvent was gently evaporated under a stream of dry N₂ to obtain the thin lipid film at bottom of a small thick-walled glass tube. To the dry lipid samples, 500 μl of 5 mM HEPES buffer, pH 7.4, containing 50 mM 5-carboxyfluorescein was added, and the mixture was vortexed. Large unilamellar vesicles (LUVs) were prepared by repeated extrusion through 100-nm polycarbonate filters using a Liposofast extruder (Avestin, Ottawa, Canada). Vesicles were separated from nonencapsulated 5-carboxyfluorescein by gel filtration using a Sephadex G-50 column eluted with 5 mM HEPES buffer, pH 7.4, containing 160 mM NaCl and 0.1 mM EDTA. 150 nm (final concentration) sPLA₂ proteins were added to 300 nm (final concentration) 5-carboxyfluorescein-containing vesicles in 2.0 ml of 5 mM HEPES buffer, pH 7.4, containing 160 mM NaCl and 0.1 mM EDTA, and the release of 5-carboxyfluorescein was measured using a Hitachi F4500 spectrophotometer with excitation and emission wavelengths set at 430 and 520 nm, respectively. After each leakage measurement, 20 μl of Triton X-100 (Pierce) was added to the mixture to achieve 100% release of 5-carboxyfluorescein. The percentage of leakage was calculated as \((F \times F₂)/(Fₘₐₓ \times F₂) \times 100\), where \(F_0\) is the fluorescence emission intensity before adding sPLA₂, and \(F\) and \(Fₘₐₓ\) represent the final fluorescence values after adding sPLA₂ and Triton X-100, respectively. All measurements were performed at 25 °C.

**Monolayer Measurements**—Surface pressure (π) of solution in a circular Teflon trough (4-cm diameter × 1-cm depth) was measured using a Wilhelmy plate attached to a computer-controlled Cahn electrobalance (model C-32) as described previously (37). 5–10 μl of phospholipid solution in ethanol/hexane (1:9 (v/v)) was spread onto 10 ml of subphase (25 mM Tris, pH 7.4, containing 0.16 M KCl and either 0.1 mM EGTA or 0.1 mM CaCl₂) to form a monolayer with a given initial surface pressure (π₀). Once the surface pressure reading of monolayer had been stabilized (after ~5 min), the protein solution (typically 40 μl) was injected into the subphase through a small hole drilled at an angle through the wall of the trough, and the change in surface pressure (Δπ) was measured as a function of time at 23 °C. Typically, the Δπ value reached a maximum after 30 min. The maximal Δπ value at a given π₀ depended on the protein concentration and reached a saturation value when [sPLA₂] = 2 μg/ml. Protein concentrations in the subphase were therefore maintained above such values to ensure that the observed Δπ represented a maximal value. The critical surface pressure (π_c) was determined by extrapolating the Δπ versus π₀ plot to the x axis (38).

**Fluorescence Labeling of sPLA₂**—Purified mouse gIIIFPLA₂ wild type and mutant proteins (H47Q and Y115A/F116A/V118A/Y119A) were dialyzed against 25 mM Tris, pH 7.2, containing 0.5 mM guanidine chloride for 4 h at 4 °C. Proteins were treated with a 10-fold molar excess of Texas Red™-C₂-maleimide for 3 h at room temperature. The reaction was quenched by incubating the mixture with an excess amount (10-fold excess of maleimide) of cysteine for 30 min. The solution of labeled protein was dialyzed against 25 mM Tris, pH 7.2, containing 15% ammonium sulfate for 2 h at 4 °C to remove excess reagents. The labeled proteins were purified using a phenyl-Sepharose column (Amersham Biosciences) as described above. Labeled protein fractions were collected and dialyzed against 25 mM Tris, pH 7.4, 160 mM NaCl for 24 h at 4 °C and then stored at −20 °C. W79C human gVPLA₂ was purified and labeled as described previously (22, 39).

**Microscopy Measurements on Giant Unilamellar Vesicles (GUVs)**—GUVs were prepared by the electroformation method using a home-built device as described previously (40, 41). Briefly, GUVs were grown in deionized water at 60 °C for 30 min by spreading ~3 μl of the lipid stock with various compositions on platinum wires. During GUV growth, the platinum wires were connected to a function generator (Hewlett-Pack-
ard, Santa Clara, CA) for 30 min, and a low frequency AC field (sinusoidal wave function with a frequency of 10 Hz and an amplitude of 3 V) was applied. After 45 min, the temperature was lowered to 40 °C, and the frequency generator was switched off after the system attained this temperature. All subsequent measurements were carried out at 40 °C in deionized water.

All microscopy measurements were carried out using a custom-built combination laser-scanning and multiphoton microscope that was described previously (42). Briefly, a 920-nm ultrafast pulsed beam from a tunable Tsunami laser, set up for femtosecond operation (Spectra Physics, Mountain View, CA) was spatially filtered and launched into the scan head. The beam was directed toward the primary dichroic mirror (Chroma Technology, Brattleboro, VT) and then toward the XY scan mirrors (model 6350, Cambridge Technologies, Cambridge, MA). A Prairie Technologies scan lens (Middleton, WI) was used to focus the laser light, collimated by the ×1 Zeiss tube lens and directed toward a ×40 water-corrected 1.2 numerical aperture Zeiss objective, mounted on a Zeiss 200 M platform (Carl Zeiss Inc., Thornwood, NY). Light excited by a 920-nm ultrafast pulse was collected on a nondescribed pathway by the Peltier-cooled 1477P style Hamamatsu photomultiplier tube. The light was reflected and filtered using appropriate optics. Instrument control was accomplished with the help of ISS amplifiers, an ISS three-axis scanning card (Champaign, IL), and two ISS 200-kHz analog lifetime cards. All of the microscopic experiments were controlled by a data acquisition program, SimFCS, kindly provided by Dr. Enrico Gratton.

Microscopy Measurements of sPLA2 Internalization and Activity—The labeling of cell membranes by PED6 was performed as described previously (39). A mixture of POPS/cholesterol/POPG/PED6 (107:31:20:1 in molar ratio, 300 nmol total) in chloroform was dried under N2 and resuspended in ethanol (10 μl), followed by the addition of DMEM (10 μl). The solution was dried again under N2 until the volume was reduced to ~7 μl to ensure that most of ethanol was evaporated. An additional 10 μl of DMEM was added to the mixture, and vesicles were prepared by sonication of the mixture on ice (20 min). Vesicles were incubated with HEK293 cells (25–50 min at 37 °C; 10 μl in each well) that had been placed into each of eight wells on a sterile NuncTM chambered cover glass and incubated for 24 h at 37 °C with 5% CO2 in the DMEM medium supplemented with 10% FBS and 250 μg/ml ZeocinTM. Viscle-treated HEK293 cells were rinsed five times with phosphate-buffered saline to remove the unincorporated dye. These cells were then treated with 250 nM Texas Red-labeled sPLA2, and imaging was performed with a Zeiss LSM510 laser-scanning confocal microscope with the detector gain adjusted to eliminate the background autofluorescence. The fluorescence signal from Texas Red-labeled protein was monitored with a 568-nm argon/krypton laser and a 650-nm line pass filter, whereas the BODIPYTM signal from the hydrolyzed PED6 was monitored with a 488-nm argon/krypton laser and a 530-nm band pass filter. A ×63 (1.2 numerical aperture) water immersion objective was used for all experiments. Images were analyzed using the analysis tool provided in Zeiss biophysical software package. For cholesterol depletion, HEK293 cells (1 × 104 cells/ml) were washed with the phosphate-buffered saline and incubated for 10–30 min at 37 °C with serum-free DMEM containing 5 mM methyl-β-cyclodextrin (Sigma). After incubation, the medium was removed, and cells were washed with phosphate-buffered saline to remove methyl-β-cyclodextrin. These cells were then treated with 200 nM Texas Red-labeled gIIFPLA2, and imaging was performed as described above.

AA Release from HEK293 Cells—Radiolabeling of HEK293 cells was achieved by incubating the cells (106) with 0.05 μCi/ml [3H]AA (Amersham Biosciences) for 20 h at 37 °C. Unincorporated [3H]AA was removed by washing the cells three times with DMEM containing 0.2% BSA. Radiolabeled cells (106) were resuspended in 160 μl of DMEM and 0.2% BSA and were stimulated with sPLA2. The reaction was quenched by adding 0.3 ml of ice-cold DMEM. The cell and the medium were separated by centrifugation, and then the radioactivity of pellet and supernatant, respectively, was measured by liquid scintillation.

Effects of gIIFPLA2 on Cell Growth—The effects of sPLA2 on cell proliferation and cell death were measured with the DO11.10 T cell hybridoma, using microscopic and cytofluorimetric readouts. Cells were cultured at 37 °C in RPMI 1640 medium (Mediatech, Herndon, VA) supplemented with heat-inactivated FBS (10% (v/v); HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and 50 μM 2-mercaptoethanol, as described (43). Physiological cell death (apoptosis) was induced by treatment with the macromolecular synthesis inhibitor actinomycin D (200 ng/ml, 12 h) (44). Dose-dependent effects on proliferation and viability were measured by seeding cells (5 × 104/well) into wells of a 24-well plate containing serial 2-fold dilutions of wild type or mutant sPLA2. Viable and dead cells (excluding and including trypan blue, respectively) were enumerated after varying periods of incubation. Viability also was confirmed cytofluorimetrically (FACSCaliber instrument and CellQuest software; BD Biosciences) by propidium iodide exclusion (1 μg/ml; λexcitation = 488 nm, λemission = 610 nm). Also the absence of externalized phosphatidylserine, as probed with fluorescein isothiocyanate-conjugated annexin V (BD PharMingen; λexcitation = 488 nm, λemission = 525 nm), and the light scatter properties of cells, relative to the characteristic profiles of viable and apoptotic cells, were assessed simultaneously (43).

Expression of gIIFPLA2 in CD4+ T Cells—Mononuclear cells from peripheral blood from healthy volunteers (with approval by the ethical committee of Showa University) were obtained using LymphoprepTM (NYCOMED) and were suspended in 5 ml of phosphate-buffered saline, pH 7.2, containing 5% FBS and 2 mM EDTA (MACS buffer). The cells were subjected to isolation of CD4+ T cells through negative selection using the MACS CD4+ T Cell Isolation Kit II (Miltenyi Biotech). Briefly, 107 cells were incubated with biotin/antibody mixture for 10 min and then with anti-biotin microbeads for 15 min on ice in 50 μl of MACS buffer. The cells were resuspended in 500 μl of MACS buffer and were applied to a MACS separator with LS column to obtain a CD4+ T cell-enriched fraction. These preparations were then applied to MACS CD25 Microbeads (Miltenyi Biotech) in a similar way to separate CD25high and CD25low T cells. Live CD4+CD25− cells (helper T cells) thus obtained were 90–95% pure as assessed by flow cytometry.
and were used in subsequent studies. The CD4⁺CD25⁻ T cells (5 × 10⁵) were cultured in 500 µl of RPMI1640 medium containing 10% FBS with or without 500 ng/ml anti-CD3 and anti-CD28 antibodies (BD Pharmingen) or 1 µg/ml phytohemagglutinin at 37 °C in a CO₂ incubator with 5% CO₂. After 24 h, total RNA was extracted from these cells using TRIzol (Invitrogen), and an aliquot (500 ng) was subjected to a reverse transcriptase reaction with Rever Tra Ace (TOYOBO) at 42 °C for 30 min and then 99 °C for 5 min. The resulting cDNA was subjected to PCR with a set of 23-bp oligonucleotide primers corresponding to the 5'- and 3'-nucleotide sequences of the open reading frame of human gIIIFPLA₂ using exTaq polymerase (Takara). The PCR conditions were 94 °C for 30 s and then 35 cycles of amplification at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, as described (45). The reaction products were applied to 1% agarose gel electrophoresis.

**RESULTS**

**Model Structure of gIIIFPLA₂**—To gain structural insight into the in vitro and cellular properties of gIIIFPLA₂, we built a homology model of the mouse gIIIFPLA₂. Three protein threading programs, which test the compatibility of the mouse gIIIFPLA₂ sequence with structures in the Protein Data Bank, identified a group II b sPLA₂ from *Agkistrodon halys pallas* (Protein Data Bank accession number 1JIA) as the template and the alignment obtained with BLAST as a guide. The sequence identity between these sPLA₂s is very high (41%). The structure of C-terminal extension was predicted by the ab initio modeling method in Nest. A calcium ion was added to the model by structurally aligning it to the template. The quality of the model is tested using Verify3D (48). The model structure is shown in a ribbon diagram with its putative membrane-binding surface pointing upward. Mutated residues (green), including active site His⁴⁷ and near-C-terminus hydrophobic residues, and near-C-terminus basic residues (blue) are shown in stick representations and labeled. The C-terminal extension is colored red. C, an electrostatic potential surface of gIIIFPLA₂. The molecular orientation is the same as in B. Red and blue grids indicate negative and positive electrostatic potential surfaces, respectively. Mutated hydrophobic residues (green), His⁴⁷ (yellow), and Lys¹¹¹ and Arg¹¹³ that generate a local cationic patch (blue) are shown in a space-filling representation. A Ca²⁺ ion is shown in magenta.

**Homology model building of mouse gIIIFPLA₂.** A, amino acid sequence alignment of *A. h. pallas* (1JIA) and mouse gIIIFPLA₂. Mutated residues of gIIIFPLA₂ (i.e. His⁴⁷ and hydrophobic residues) are colored green. Basic residues near the C-terminal extension are colored blue, and the C-terminal extension (residues 128–150) that is deleted in the ΔC¹²⁸⁻¹⁵⁰ mutant is underlined.

B, a model structure of mouse gIIIFPLA₂ is shown in a ribbon diagram with its putative membrane-binding surface pointing upward. Mutated residues (green), including active site His⁴⁷ and near-C-terminus hydrophobic residues, and near-C-terminus basic residues (blue) are shown in stick representations and labeled. The C-terminal extension is colored red.

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Group IIF sPLA₂

Unlike most basic sPLA₂s, such as gIIAPLA₂ and gVPLA₂, that for gIIFPLA₂ (see Fig. 1A), gIIFPLA₂ has a largely negative electrostatic profile with smaller cationic patches. Two most notable cationic patches are found near the Ca²⁺-binding loop and near the C-terminal extension (i.e. around Arg¹⁰⁹, Lys¹¹¹, and Arg¹¹³), respectively. These cationic patches on or near the putative membrane binding surface may account for the reported ability of gIIFPLA₂ to bind and hydrolyze anionic phospholipids, such as phosphatidylglycerol (PG) (2). On the other hand, the overall negative electrostatic property of gIIFPLA₂ may be attributed to its low HSPG affinity (29).

Vesicle Binding Properties of gIIFPLA₂.—To investigate how gIIFPLA₂ interacts with membranes, we employed POPC, POPG, and POPC/SM/cholesterol (1:1:2 molar ratio) LUVs for binding measurements. POPC/SM/cholesterol (1:1:2) was used as a mimetic of the outer plasma membrane of mammalian cells, because we were interested in investigating how gIIFPLA₂ interacts with the outer plasma membrane. We measured the interaction of gIIFPLA₂ with these vesicles by SPR analysis in the absence of Ca²⁺ to circumvent potential phospholipid hydrolysis during binding measurements. For comparison, binding of gVPLA₂ to these vesicles was also measured under the same conditions. As shown in Fig. 2, gVPLA₂ showed typical sensorgrams with association and dissociation phases for three types of vesicles. However, gIIFPLA₂ exhibited highly anomalous sensorgrams. For all three types of vesicles used, SPR signals steadily decreased to the base line during the association phase, indicating that lipid vesicles were detached from the sensor chip. This suggests that gIIFPLA₂ may disrupt the integrity of vesicles upon binding. This activity was not linked to the lipolytic activity of the protein, because Ca²⁺ was absent in the mixture. Due to its potential bilayer-disrupting activity, binding of gIIFPLA₂ to lipid vesicles could not be quantified by SPR and other conventional methods.

Vesicle Leakage Caused by gIIFPLA₂.—We therefore performed vesicle leakage experiments using LUVs encapsulating 5-carboxyfluorescein to assess the bilayer-disrupting activity of gIIFPLA₂. We first measured the release of 5-carboxyfluorescein from POPC/SM/cholesterol (1:1:2) LUV by gIAPLA₂, gIIAPLA₂, and gVPLA₂ in the absence of Ca²⁺. The vesicle leakage was monitored in terms of the increase in 5-carboxyfluorescein fluorescence emission due to the relief of self-quenching. The addition of gIIFPLA₂ to the vesicles caused the rapid release of 5-carboxyfluorescein from the vesicles in a concentration-dependent manner (see Fig. 3A). Under the same conditions, gIAPLA₂ induced little leakage, whereas gVPLA₂ showed 30% of the gIIFPLA₂ activity (Fig. 3B).

To understand the molecular basis of the unique vesicle-disrupting activity of gIIFPLA₂, we then varied the lipid composition of 5-carboxyfluorescein-containing vesicles. Fig. 3C shows that gIIFPLA₂ induced a significantly weaker leakage with POPC/SM/cholesterol (1:1:1) LUV than with POPC/SM/cholesterol (1:1:2) LUV. Furthermore, gIIFPLA₂ was not able to cause any leakage with POPC/SM (1:1) and POPC LUVs. These data thus indicate that the presence of cholesterol is essential for the unique ability of gIIFPLA₂ to disrupt the neutral lipid bilayers.
To elucidate the structural determinant of its unique membrane binding properties, we prepared a panel of gIIFPLA2 mutants and measured the vesicle leakage using POPC/SM/cholesterol (1:1:2) LUV encapsulating 5-carboxyfluorescein by wild type and mutants under the same conditions. In particular, we mutated aromatic/aliphatic residues near the C-terminal extension of gIIFPLA2, Y115A/F116A and Y115A/F116A/V118A/Y119A, and generated a C-terminal deletion mutant (Δ128–150), H47Q was prepared to confirm that the lipolytic activity is not involved in its vesicle-leaking activity. As shown in Fig. 3D, Y115A/F116A and Y115A/F116A/V118A/Y119A caused no detectable vesicle leakage, indicating that these aromatic and aliphatic residues (Tyr115, Phe116, Val118, and Tyr119) are essential for the vesicle-disrupting activity of gIIFPLA2. In contrast, Δ128–150 behaved essentially the same as the wild type, suggesting that this region plays no direct role in membrane interaction. As expected, H47Q behaved similarly to the wild type with respect to the vesicle leakage. The effects of basic residues, Lys111 and Arg113, on the interactions of gIIFPLA2 with various membranes were not investigated in this study because of low stability of corresponding mutants (e.g. K111A, K111E, R113A, and R113E).

**Enzyme Activity of gIIFPLA2 and Mutants**—To investigate the role of the clustered hydrophobic residues in the interfacial catalysis of gIIFPLA2, we also measured the activities of wild type and mutants in the presence of 1 mM Ca2+ toward polymerized mixed vesicles that have been used for substrates for many sPLA2s (32, 33, 52). In this model membrane system, a pyrene-labeled phospholipid (i.e. pyrene-PG) incorporated in the inert polymerized matrix of BLPG (or BLPC) is selectively hydrolyzed by sPLA2, which can be spectrofluorometrically monitored. This system was particularly useful for gIIFPLA2, because polymerized vesicles would not be easily disrupted by gIIFPLA2 during the activity assay. gIIFPLA2 had high specific activity for pyrene-PG incorporated in anionic BLPC vesicles (see Fig. 4). However, it showed much lower activity for pyrene-PG incorporated in zwitterionic BLPC vesicles (data not shown), showing that gIIFPLA2 prefers anionic to zwitterionic membranes in the absence of cholesterol. This finding is also consistent with our model structure (see Fig. 1C) showing the presence of cationic patches on the putative membrane-binding surface. Among gIIFPLA2 mutants, a good correlation between vesicle-disrupting activity and interfacial enzymatic activity was observed. In other words, Δ128–150 with the wild type-like vesicle-disrupting activity had essentially the same enzyme activity toward pyrene-PG/BLPG polymerized mixed vesicles as wild type, whereas Y115A/F116A and Y115A/F116A/V118A/Y119A with greatly reduced vesicle-disrupting activities showed drastically reduced enzymatic activity. Thus, the clustered hydrophobic residues seem to play an important role in the interfacial activity of gIIFPLA2, presumably by enhancing its membrane binding. As expected, H47Q showed no activity for any polymerized mixed vesicles.

**Monolayer Penetration of gIIFPLA2**—To understand the mechanism by which gIIFPLA2 causes the vesicle leakage and
Group IIF sPLA₂

the roles of the above residues in membrane binding of gIIFPLA₂, we measured the interactions of gIIFPLA₂ and mutants with various lipid monolayers at the air-water interface. This system has been used to measure the membrane-penetrating activity of a wide variety of proteins (38). The phospholipid monolayer was spread at constant area and the change in surface pressure (Δσ) was monitored after the injection of protein into the subphase. In general, Δσ is inversely proportional to π₀ of the lipid monolayer, and an extrapolation of the Δσ versus π₀ plot yields the critical surface pressure (πc), which specifies the upper limit of π₀ of a monolayer that a protein can penetrate into (38, 53). Because the surface pressure of cell membranes has been estimated to be in the range of 30–35 dynes/cm (54–56), the πc value for a protein that penetrates cell membranes should be above 30 dynes/cm.

We first measured the penetration of wild type gIIFPLA₂ into various monolayers (see Fig. 5A). Again, Ca²⁺ was removed from the subphase in most measurements to circumvent potential hydrolysis during monolayer measurements. gIIFPLA₂ showed high penetrating activity for the monolayer comprising POPC/SM/cholesterol (1:1:2) with πc slightly above 30 dynes/cm. This is consistent with the unique ability of gIIFPLA₂ to cause leakage from the POPC/SM/cholesterol (1:1:2) LUV. Furthermore, the monolayer-penetrating activity of gIIFPLA₂ greatly decreased when cholesterol was removed from the monolayer. The πc value was reduced to <25 dynes/cm for POPC and POPC/SM monolayers. We also used a nonhydrolyzable PC analog, DHPC, instead of POPC in the PC/SM/cholesterol (1:1:2) monolayer and measured its interaction with gIIFPLA₂ in the presence of 1 mM Ca²⁺ in the subphase. As shown in Fig. 5A, gIIFPLA₂ showed essentially the same affinity for the POPC/SM/cholesterol (1:1:2) monolayer without Ca²⁺ and for the DHPC/SM/cholesterol (1:1:2) monolayer with 1 mM Ca²⁺, validating our approach of measuring membrane interactions of gIIFPLA₂ in the absence of Ca²⁺ to circumvent the hydrolysis. DHPC was not used in vesicle leakage studies, because vesicles formed in the presence of this lipid had a high background leakage in the absence of gIIFPLA₂. It should be noted that LUVs used in our vesicle leakage measurements are known to have the surface pressure above 30 dynes/cm (54–56). This explains why gIIFPLA₂ did not cause a leakage with POPC or POPC/SM vesicles, although it had some affinity for POPC and POPC/SM monolayers with the initial pressure below 25 dynes/cm.

We then compared the interactions of gIIFPLA₂, gIIAPLA₂, and gVPLA₂ with the POPC/SM/cholesterol (1:1:2) monolayer. Fig. 5B illustrates that gIIAPLA₂ has significantly lower monolayer-penetrating activity than gIIFPLA₂. gVPLA₂ was more active than gIIFPLA₂ but was less active than gIIAPLA₂ with πc below 30 dynes/cm. These results are consistent with the different activities of the three enzymes to induce the vesicle leakage (see Fig. 3B). We then measured the penetration of gIIAPLA₂ mutants into the same monolayer. As shown in Fig. 5C, Y115A/F116A and Y115A/F116A/V118A/Y119A with little vesicle-leaking activity had πc values below 25 dynes/cm. In contrast, Δ128–150 and H47Q with wild type-like vesicle-disrupting activity showed monolayer penetration that was comparable with that of the wild type. Collectively, our monolayer measurements indicate that gIIFPLA₂ has a unique ability to penetrate the compactly packed zwitterionic lipid monolayers and bilayers, which accounts for its capability of inducing vesicle leakage, and that the presence of cholesterol is important for this activity.

Interaction of gIIFPLA₂ with GUV—GUVs (diameter >10 μm) are an excellent model system for cell membranes that allows direct visualization of various membrane processes, including structural changes of membranes (57). Since GUVs are devoid of proteins and carbohydrates, they allow for investigating if and how gIIFPLA₂ crosses the lipid bilayer based solely on its lipid-binding properties. We prepared GUVs composed of PC/SM/cholesterol/NBD-cholesterol (1:1:2:0.04), and the Texas Red-labeled gIIFPLA₂ was added to these GUVs in the absence of Ca²⁺. As shown in Fig. 6, Texas Red-labeled gIIFPLA₂ initially bound to the surface of GUVs and then entered the vesicles and was accumulated in high concentration inside the vesicles within 10 min, which led to the dramatic

![Figure 5](image1.png)

**Figure 5.** Interactions of sPLA₂ with monolayers of various lipid compositions. A, gIIFPLA₂ wild type was allowed to interact with POPC/SM/cholesterol (1:1:2) (○), POPC/SM/cholesterol (1:1:1) (□), POPC (▪), and POPC/SM (1:1) (▲) monolayers. 25 mM Tris, pH 7.4, 160 mM KCl, 0.1 mM EDTA was used as the subphase. Also, gIIFPLA₂ was incubated with the DHPC/SM/cholesterol (1:1:2) (□) monolayer formed over 25 mM Tris, pH 7.4, containing 160 mM KCl and 1 mM Ca²⁺. B, gIIFPLA₂ (○), gIIAPLA₂ (□), and gVPLA₂ (▲) were allowed to interact with the POPC/SM/cholesterol (1:1:2) monolayer. C, gIIFPLA₂ wild type (○), H47Q (△), Δ128–150 (■), Y115A/F116A (○), Y115A/F116A/Y118A/Y119A (▲) were allowed to interact with POPC/SM/cholesterol (1:1:2) monolayers. 25 mM Tris, pH 7.4, 160 mM KCl, 0.1 mM EDTA was used as the subphase for all but DHPC/SM/cholesterol (1:1:2) monolayers in A.
contraction and disruption of the vesicles. Essentially the same pattern was observed for >90% of GUVs characterized under the same conditions. Neither the Y115A/F116A/V118A/Y119A mutant gIIFPLA2 nor gVPLA2 caused a similar disruption of GUV under the same conditions (data not shown). These results thus confirm that gIIFPLA2 has a unique lipolytic activity-independent ability to cross the cholesterol- and SM-rich neutral lipid bilayer. This also suggests that this sPLA2 may be able to enter mammalian cells by directly crossing the plasma membrane without having to rely on any endocytic protein machinery.

**Action of gIIFPLA2 on HEK293 Cells**—Unusual membrane binding properties of gIIFPLA2 suggest that this sPLA2 may be able to traverse the plasma membrane in a lipolysis-, HSPG-, and endocytosis-independent manner. To explore this possibility, we chemically labeled gIIFPLA2 with Texas Red and exogenously added the fluorescently labeled gIIFPLA2 to HEK293 cells whose membranes are separately labeled with a fluorogenic PLA2 substrate, PED6. PED6 has been shown to be randomly distributed among various cellular membranes, including both leaflets of the plasma membrane, and display a large increase in fluorescence emission upon hydrolysis (39, 58). This approach, which has been successfully employed for the cell studies of gVPLA2 (12, 39), allowed simultaneous real time monitoring of the spatiotemporal dynamics and lipolytic activity of gIIFPLA2.

Prior to cell studies, we first measured the in vitro specific activities of fluorescently labeled and unlabeled proteins using POPS/cholesterol/POPG/PED6 (107:31:20:1) vesicles as a substrate. As listed in Table 1, gIIFPLA2 had 3-fold lower activity than gVPLA2 W79C (this mutant is essentially identical to the wild type gVPLA2 in all respects) (39) for the substrate at 1 mM Ca2+, and the difference was far greater at lower Ca2+ concentrations. This is because gVPLA2 has a lower Ca2+ requirement than gIIFPLA2 (2). Thus, gIIFPLA2 is expected to have much lower activity for PED6 than gVPLA2 in the cytosol, where Ca2+ is present in a submicromolar concentration. The presence of a single free cysteine (Cys137) in the C-terminal extension (see Fig. 1A) made it possible to specifically incorporate a single fluorescence probe into gIIFPLA2. The purified Texas Red-labeled gIIFPLA2 proteins had the same enzymatic activity toward PED6 (see Table 1) and vesicle-disrupting activity toward POPC/SM/cholesterol (1:1:2) vesicles (data not shown) as unlabeled proteins.

As shown in Fig. 7A, wild type gIIFPLA2 initially bound the plasma membrane and then readily entered HEK293 cells and accumulated on various intracellular locations, including the perinuclear region (see the red panel). A separate study using HEK293 cells expressing enhanced green fluorescent protein-tagged EEA1 protein showed that gIIFPLA2 was not located in endosomal structures (data not shown). Also, incubation of HEK293 cells on ice for 30 min before adding gIIFPLA2 did not have a significant effect on the cellular entry of gIIFPLA2 (Fig. 7B). These results suggest that gIIFPLA2 enters the cells by an endocytosis-independent mechanism. This notion is also consistent with our GUV data. As far as the rate of entry into

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**Table 1**
Relative activity of PLA2 on the PED6 substrate

| Enzyme                        | Relative activity |
|-------------------------------|-------------------|
| gVPLA2 W79C                   | 1.00              |
| Labeled gVPLA2 W79C           | 0.90              |
| gIIFPLA2                      | 0.37              |
| Labeled gIIFPLA2              | 0.30              |
| gIIFPLA2 Y115A/F116A/V118A/Y119A | 0.002           |
| Labeled gIIFPLA2 Y115A/F116A/V118A/Y119A | 0.002       |
| gIIFPLA2 H47Q                 | ND                |
| cPLA2                        | 0.0002            |

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**FIGURE 6.** Interactions of gIIFPLA2 with GUV. NBD-cholesterol (top row) and Texas Red-labeled gIIFPLA2 (bottom row) images of GUV before (A), right after (B), 8 min after (C), and 10 min after (D) the addition of 200 nM gIIFPLA2 to the GUV composed of PC/SM/cholesterol/NBD-cholesterol (1:1:2:0.04) in deionized water at 40 °C. Images were taken every 5 s. Bars indicate 10 μm.
Group IIF sPLA$_2$

A

0 min 3 min 10 min 18 min
gII$\text{FPLA}_2$ WT

PED6

B

0 min 18 min

C

0 min 18 min

D

0 min 3 min 8 min
gV$\text{PLA}_2$

PED6

E

0 min 18 min

protein

PED6

F

0 min 18 min

protein

PED6
HEK293 cells was concerned, gIIIFPLA2 was only slightly less effective than the Texas Red-labeled W79C-gVPLA2, that was shown to rapidly enter HEK293 and other mammalian cells due to its high HSPG affinity and PC activity (39) (see also Fig. 7D). However, unlike gVPLA2, which caused strong intracellular signals of PED6 hydrolysis, which were colocalized with protein signals, gIIIFPLA2 did not induce extensive PED6 hydrolysis (see the green panel of Fig. 7A). gIIIFPLA2 caused a minor degree of PED6 hydrolysis at the plasma membrane, but little PED6 hydrolysis was seen intracellularly. This is not unexpected, given the low enzymatic activity of gIIIFPLA2 toward PED6, particularly at low Ca²⁺. To explore the possibility that a rise in intracellular Ca²⁺ may enhance the activity of internalized gIIIFPLA2, we treated HEK293 cells with 10 μM ionomycin after gIIIFPLA2 entered the cells. However, little increase in PED6 hydrolysis was seen even after 30 min (data not shown), suggesting that gIIIFPLA2 has low cellular activity even at an elevated level of Ca²⁺.

To see if gIIIFPLA2 also shows much lower activity than gVPLA2 toward natural phospholipid substrates, we labeled HEK293 cells with [³H]AA and measured the release of [³H]AA from the cells after incubation with exogenously added gIIIFPLA2 and gVPLA2, respectively. Fig. 8 shows that gIIIFPLA2 has less than 5% of the gVPLA2 activity. Collectively, these results indicate that although gIIIFPLA2 can effectively enter HEK293 cells, it does not induce a significant degree of lipid hydrolysis due to its lower enzyme activity toward phospholipids in the plasma membrane and intracellular membranes.

Unlike the wild type gIIIFPLA2, Y115A/F116A/V118A/Y119A with much lower membrane-penetrating activities showed neither cellular entry nor PED6 hydrolysis when exogenously added to HEK293 cells under the same conditions (Fig. 7E). This indicates that the membrane-disrupting activity of gIIIFPLA2 is responsible for its unique cell membrane-traversing capability. A catalytically inactive mutant H47Q did enter HEK293 cells as well as the wild type gIIIFPLA2 without causing any hydrolysis (Fig. 7F), showing that lipolytic activity is not essential for the cellular entry of gIIIFPLA2.

Lastly, to measure the effect of cholesterol on the cellular entry of gIIIFPLA2, we treated HEK293 cells with 5 mM methyl-β-cyclodextrin. Although cells started to lose integrity after elongated incubation as a consequence of cholesterol depletion, about a half-population of cells still maintained the integrity within 20 min of incubation. Interestingly, when Texas Red-labeled gIIIFPLA2 was exogenously added to these methyl-β-cyclodextrin-treated HEK293 cells, it accumulated on the surfaces of the cells but did not enter the cells (see Fig. 7C). Essentially all red signals were removed from the cells when they were washed with the medium (data not shown). The same pattern was seen with virtually all methyl-β-cyclodextrin-treated cells. This result again underscores that the presence of cholesterol is essential for cell membrane-traversing activity of gIIIFPLA2.

**Effects of gIIIFPLA2 on Cell Proliferation**—The unique membrane-disrupting and -translocating properties of gIIIFPLA2 suggested that this sPLA2 might impact mammalian cell viability or even trigger cell death. To address this possibility, we looked for a particular cell type that intrinsically expresses gIIIFPLA2. By means of reverse transcription-PCR, we found that CD4⁺/CD25⁺ helper T cells isolated from peripheral bloods of human volunteers express gIIIFPLA2 mRNA (Fig. 9A). The expression of gIIIFPLA2 was markedly increased in CD4⁺/CD25⁺ T cells after immunologic (anti-CD3 + anti-CD28 antibodies) and nonimmunologic (phytohemagglutinin) stimulation, revealing a marked stimulus-coupled inducibility of this enzyme.

Based on this finding, we chose a murine T cell line, DO11.10, which proliferates rapidly and is particularly susceptible to the induction of apoptotic cell death, as a sensitive cell with which to test the effects of sPLA2. Over 40 h, the untreated cells underwent 2.5 population doublings (i.e. the doubling time is about 16 h). As shown in Fig. 9B, the wild-type gIIIFPLA2 significantly inhibited this proliferation; 50% inhibition was observed at ~0.3 μM. No appreciable cell death was triggered, although...
much higher doses of sPLA₂ (>1 μM) were toxic. These results were confirmed by flow cytometric analysis. With regard to forward and side angle light scatter, viable cells (Fig. 10A) were heterogeneous in size (because they were proliferating asynchronously) and showed low side scatter, whereas apoptotic cells (Fig. 10B) were smaller (less forward angle light scatter) and more granular (higher side angle scatter) and included cells that failed to exclude propidium iodide (data not shown). The population of cells treated with 0.25 μM wild-type gIIFPLA₂ (Fig. 10C) included fewer large, blasting cells but no appreciable numbers of apoptotic cells, consistent with an inhibition of proliferation and an absence of cell death. It is notable that the side angle scatter of these cells is elevated, suggesting that they may have some nonlethal membrane irregularity, perhaps as a consequence of membrane disruption by the enzyme. Under the same conditions, the Y115A/F116A/V118A/Y119A mutant of gIIFPLA₂ was much less effective than the wild type in inhibiting cell proliferation. Even at 1 μM, the mutant caused less than 40% inhibition (Fig. 9B), and the dose-dependent extent of apparent membrane irregularity was greatly reduced (Fig. 10D). Finally, gIIAPLA₂ had no effect on proliferation or cell integrity (Figs. 9B and 10D).

**DISCUSSION**

Despite intensive studies on sPLA₂s in the past decade, their physiological functions and the mechanisms by which these enzymes exert cellular effects still remain unknown and will require further genetic and cell studies. Recent studies on various sPLA₂s over the years have highlighted a good correlation between their biochemical/biophysical properties and their cellular actions. For instance, high activity of gVPLA₂ (24, 25) and gXPLA₂ (26, 27) toward PC membranes is well correlated with their unique capability to act directly on mammalian cells, the outer plasma membrane of which is rich in PC. Also, high heparin affinity of many basic sPLA₂s is linked to their binding to cell surface HSPG and cellular uptake (12, 15–22). Therefore, characterization of biochemical and biophysical properties of different sPLA₂ isoforms may provide an important new clue to their physiological functions and the mechanism of their cellular actions. The present study was performed to characterize biochemical and biophysical properties of gIIFPLA₂, with an aim of gaining new insight into its physiological functions.

Our structural modeling suggests that gIIFPLA₂ is a largely negatively charged molecule with smaller cationic patches on its putative membrane-binding surface (see Fig. 1C). gIIFPLA₂ prefers anionic PG membranes to zwitterionic PC membranes due to the presence of surface cationic patches, including one composed of Arg¹⁰⁹, Lys¹¹¹, and Arg¹¹³ near the C-terminal extension. Although the role of clustered cationic residues could not be assessed in this study due to the low stability of corresponding mutants, they are expected to play a role in binding to anionic membranes. Relatively weak interaction of gIIFPLA₂ with PC membranes is dramatically enhanced in the presence of cholesterol. In particular, gIIFPLA₂ demonstrates novel nonhydrolytic membrane-penetrating and disrupting activities on cholesterol-
containing membranes which have not been seen for any other mammalian sPLA₂s. Some basic sPLA₂s from snake venom have been reported to have vesicle-disrupting activities (59); however, these sPLA₂s typically require high protein concentrations (i.e. micromolar) and high contents of anionic lipids in the vesicles for such activities. Our results show that submicromolar concentrations of gIIFPLA₂ can cause major damage to electrically neutral PC/SM/cholesterol (1:1:2) vesicles. Monolayer measurements indicate that this vesicle-disrupting activity derives from the ability of gIIFPLA₂ to penetrate the compactly packed PC/SM/cholesterol (1:1:2) membrane. These results suggest that gIIFPLA₂ is ideally suited for attacking the outer plasma membrane of mammalian cells that comprise mainly PC, SM, and cholesterol. Indeed, gIIFPLA₂ can readily bind the outer plasma membranes of HEK293 cells and enter these cells. For all of these membrane-disrupting and -traversing activities of gIIFPLA₂, the presence of cholesterol is essential. This cholesterol requirement is quite intriguing in that cholesterol is known to stabilize the lipid bilayers and protect the mammalian outer plasma membranes against the lytic activity of antimicrobial peptides (60). It is not known at present whether gIIFPLA₂ directly binds cholesterol or recognizes cholesterol-induced structural changes in the membrane.

Several proteins, such as HIV-1 TAT, Drosophila Antennapedia protein, and HSV-1 VP22, have been shown to be able to rapidly (i.e. <15 min) cross the mammalian plasma membranes and reach the nucleus (61–63). These proteins contain so-called “protein transduction domains” or cell-penetrating peptides that are cationic peptides of 10–16 amino acids and responsible for their cell membrane-traversing activity. Although earlier studies indicated that protein transduction domains enter all cell types in a receptor- and endosome-independent mechanism, recent mechanistic studies have suggested that these domains are taken up by the cells through initial binding to HSPG and subsequent endocytosis and are localized in endosomes (64). Unlike these proteins, gIIFPLA₂ can traverse the mammalian plasma membranes by interacting exclusively with membrane lipids. gIIFPLA₂ is an acidic protein with extremely low affinity for HSPG (29). Also, our cell measurements suggest that it enters HEK293 cells in a lipolytic activity- and endocytosis-independent manner. This is further supported by the findings that gIIFPLA₂ is able to effectively go across the GUV composed of PC/SM/cholesterol (1:1:2).

A structural determinant of the unique membrane-disrupting and -traversing activities of gIIFPLA₂ turns out to be aromatic and aliphatic residues (Tyr⁰, Phe¹, Val¹, and Tyr¹) near the C-terminal extension. Our model structure suggests that these residues are located on the putative membrane-binding surface. It was reported that the unique C-terminal extension of gIIFPLA₂ is essential for its plasma membrane localization and optimal cellular functions (29). Our measurements showed that the C-terminal extension has no direct role in the in vitro membrane-disrupting activity and enzymatic activity of gIIFPLA₂. It is possible that in the previous study using HEK293 cells overexpressing gIIFPLA₂, the C-terminal deletion may have affected the secretion.

Previous immunostaining of the FLAG-tagged gIIFPLA₂ overexpressed in HEK293 cells has indicated that gIIFPLA₂ tightly binds the plasma membrane when secreted and augments the agonist-induced AA release and PGE₂ production from HEK293 (29). Our dual imaging of localization and enzyme activity of gIIFPLA₂, however, showed that exogenously added gIIFPLA₂ caused only a minor degree of PED6 hydrolysis at the plasma membrane (presumably the outer leaflet) and little to no hydrolysis at internal membranes. Furthermore, exogenously added gIIFPLA₂ caused much less [³H]AA release than gVPLA₂ from [³H]AA-labeled HEK293 cells under the same conditions. Therefore, it is likely that the previously reported AA-releasing activity of gIIFPLA₂ overexpressed in HEK293 cells occurs primarily before secretion, as has been reported for gIAPLAP₂, gVPLA₂, and gXPLA₂ (12, 23). Also, the site of the lipolytic action of overexpressed gIIFPLA₂ before secretion must be distinct from that of the internalized gIIFPLA₂ since the latter shows little intracellular lipolytic activity.

What, then, is the functional consequence of the internalization of gIIFPLA₂? Our data suggest that, at modest concentrations of gIIFPLA₂, plasma membrane disruption is not so severe as to cause cell death. However, the membrane-disrupting activity of gIIFPLA₂ can inhibit cell proliferation profoundly. Given that gIIFPLA₂ is markedly induced in CD4⁺ CD25⁺ T cells, gIIFPLA₂ induction and the inhibition of T cell proliferation might be functionally linked in T cell homeostasis. At this point, it is unclear under which condition this type of proliferative inhibition might occur. Undoubtedly, further studies are required to address the question. Nevertheless, our preliminary findings raise the intriguing possibility that, due to its unusual membrane disrupting and traversing activity, gIIFPLA₂ may perform unique functions under physiological conditions.

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