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Cellular Arachidonate-releasing Function of Novel Classes of Secretory Phospholipase A_2s (Groups III and XII)*

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Here we report cellular arachidonate (AA) release and prostaglandin (PG) production by novel classes of secretory phospholipase A_2s (sPLA$_2$s), groups III and XII. Human group III sPLA$_2$ promoted spontaneous AA release, which was augmented by interleukin-1, in HEK293 transfectants. The central sPLA$_2$ domain alone was sufficient for its *in vitro* enzymatic activity and for cellular AA release at the plasma membrane, whereas either the unique N- or C-terminal domain was required for heparanoid-dependent action on cells to augment AA release, cyclooxygenase-2 induction, and PG production. Group III sPLA$_2$ was constitutively expressed in two human cell lines, in which other sPLA$_2$s exhibited different stimulus inducibility. Human group XII sPLA$_2$ had a weak enzymatic activity *in vitro* and minimally affects cellular AA release and PG production. Cells transfected with group XII sPLA$_2$ exhibited abnormal morphology, suggesting a unique functional aspect of this enzyme. Based on the present results as well as our current analyses on the group I/II/V/X sPLA$_2$s, general properties of cellular actions of a full set of mammalian sPLA$_2$s in regulating AA metabolism are discussed.

Secretory phospholipase A_2 (sPLA$_2$)[1] comprises a family of Ca^{2+}-dependent lipolytic enzymes with a conserved Ca^{2+}-binding loop and His-Asp dyad at the catalytic site (1, 2). To date, 10 sPLA$_2$ enzymes (groups IB, IIA, IIC, IID, IIE, IIF, V, X, III, and XII) have been identified in mammals (1, 2). In general, sPLA$_2$s exhibit tissue- and species-specific expression, which suggests that their cellular behaviors and functions differ.

The group I/II/V/X sPLA$_2$s represent a class of enzymes with a molecular mass of 14–18 kDa and 6–8 conserved disulfides (1, 2). sPLA$_2$-IB and -X, but not the other enzymes, have an N-terminal prepropeptide, and the proteolytic cleavage of this prepropeptide is a regulatory step for generation of an active enzyme (3, 4). sPLA$_2$-IB is abundantly present in pancreatic juice, and its main function has been thought to be the digestion of dietary phospholipids, although recent data with sPLA$_2$-IB knockout mice have demonstrated no appreciable defects in this process (5). sPLA$_2$-IIA, a prototypic inflammatory PLA$_2$, and other group II subfamily sPLA$_2$s (IID, IIE, IIF, and V) are inducible in various tissues with inflammation or damage (6–13). On the bases of current biochemical and cell biological studies, sPLA$_2$s in the I/II/V/X branch may participate in various biological events, including arachidonate (AA) release from cellular membranes (see below) (9–18), host defense against bacteria (19, 20), atherosclerosis (21, 22), blood coagulation (23), and cancer (24).

Beyond the essential role of cytosolic PLA$_2\alpha$ (cPLA$_2\alpha$) in the initiation of stimulus-coupled AA metabolism, the I/II/V/X class of sPLA$_2$s also has the ability to augment AA metabolism by multiple mechanisms (1). sPLA$_2$s that show high interfacial binding to zwitterionic phosphatidylcholine (PC), such as sPLA$_2$-X and -V, are capable of releasing AA from the PC-rich outer leaflet of the plasma membrane of *quiescent* cells (the external plasma membrane pathway) (17, 25–27). Cationic, heparin-binding, group II subfamily sPLA$_2$s, such as sPLA$_2$-IIA, -IID, -IIE, and -V, show marked preference for anionic phospholipids over PC and utilize the heparan sulfate proteoglycan (HSPG)-shuttling pathway (14–18, 28). In this regulatory pathway, these enzymes are captured by HSPGs (typically glypican, a glycosylphosphatidylinositol-anchored HSPG) in caveolae or rafts on *activated* cells and then internalized into vesicular membrane compartments that are enriched in the perinuclear area, where downstream cyclooxygenases (COXs) are located (16, 17, 28). This spatiotemporal co-localization of sPLA$_2$s and COXs in the perinuclear compartments may allow efficient supply of AA between these enzymes. Recent evidence implies that the clathrin-independent, caveolae/raft-mediated endocytosis, and associated vesicular traffic is directed toward a rapid cycling pathway via the Golgi and endoplasmic reticulum (29). Occurrence of the HSPG-shuttling pathway appears to be cell type- and stimulus-specific, and in certain cases HSPGs exhibit a negative regulatory effect on the heparin-binding sPLA$_2$s by facilitating their internalization and subsequent lysosomal degradation (30, 31). sPLA$_2$-IIF, an anionic group II subfamily sPLA$_2$ with poor affinity for HSPG, may

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[1] The abbreviations used are: sPLA$_2$, secretory phospholipase A_2; PLA$_2$, phospholipase A_2; cPLA$_2\alpha$, cytosolic PLA$_2\alpha$; COX, cyclooxygenase; AA, arachidonic acid; OA, oleic acid; LA, linoelic acid; PG, prostaglandin; PGE$_2$, prostaglandin E_2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; HSPG, heparan sulfate proteoglycan; FCS, fetal calf serum; NDGA, nordihydroguaiaretic acid; RT-PCR, reverse transcriptase-PCR; IL-1, interleukin-1; TNF-α, tumor necrosis factor α; IFN-γ, interferon-γ; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; WT, wild type; 2-LA-PE, 1-palmitoyl-2-[^{14}C]linoleoyl-PE; 2-LA-PC, 1-palmitoyl-2-[^{14}C]linoleoyl-PC; 2-AA-PC, 1-palmitoyl-2-[^{14}C]-arachidonoyl-PC; 2-AA-PE, 1-palmitoyl-2-[^{14}C]archidonoyl-PE.

interact stably with the plasma membrane through its unique C-terminal extension and releases AA (11), although the possibility that this enzyme also functions after internalization cannot be ruled out. In addition, cellular actions of several sPLA$_2$s can be mediated by sPLA$_2$ receptors independent of their enzymatic activity (32). Targeted disruption of the M-type sPLA$_2$ receptor gene results in reduced inflammatory response in mice (33). The M-type sPLA$_2$ receptor can also act as a negative regulator for sPLA$_2$s by inhibiting their enzymatic functions in serum and by promoting their internalization and subsequent degradation (34, 35).

Besides the I/II/V/X branch, two distinct classes of sPLA$_2$, namely the group III and group XII branches, have been recently identified in mammals (36–38). Structurally, these two novel sPLA$_2$s show homology with the I/II/V/X sPLA$_2$s only within the Ca^{2+} loop and catalytic site His-Asp dyad. Human sPLA$_2$-III is a 56-kDa protein containing a long N-terminal domain, a central sPLA$_2$ domain that is homologous to bee venom group III sPLA$_2$, and a long C-terminal domain (36). sPLA$_2$-XII, which harbors an unusual Ca^{2+} loop, is distantly related to other classes of sPLA$_2$s (37, 38). This enzyme is expressed in antigen-activated helper T cells in the mouse (38). However, cellular functions of these two novel sPLA$_2$s have not yet been described. To expand our current understanding of the sPLA$_2$ actions on cells, we studied the cellular AA-releasing and prostaglandin (PG)-biosynthetic properties of human sPLA$_2$-III and -XII by expressing these enzymes in HEK293 cells, as we have previously done with the I/II/V/X sPLA$_2$s (9–12, 14–18).

EXPERIMENTAL PROCEDURES

Materials—Human embryonic kidney 293 (HEK293) cells (Human Science Research Resources Bank), human lung epithelial BEAS-2B cells (American Type Cell Collection, Manassas, VA), and human colon adenocarcinoma HCA-7 cells (a generous gift from Dr. M. Tsujii (Osaka University) and Dr. R. DuBois (Vanderbilt University Medical Center and VA Medical Center)) were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% (v/v) fetal calf serum (FCS; Bioserum). The cDNAs for human sPLA$_2$-III (36), human sPLA$_2$-XII (37), human COX-1 and COX-2 (15), and rat glypican-1 (16) were described previously. HEK293 cells stably expressing human sPLA$_2$-V, human sPLA$_2$-IIF, and human COX-2 were described previously (11, 14, 15). The enzyme immunoassay kits for PGE$_2$ and the COX-2 inhibitor NS-398 were purchased from Cayman Chemicals. The goat anti-human COX-1 and anti-human COX-2 antibodies were purchased from Santa Cruz Biosciences, Inc. (Santa Cruz, CA). A23187 was purchased from Calbiochem. Human interleukin (IL)-1β, interferon (IFN)-γ, and tumor necrosis factor α (TNF-α) were purchased from Genzyme. LipofectAMINE 2000 reagent, Opti-MEM medium, TRIzol reagent, geneticin, zeocin, and mammalian expression vectors (pCR3.1, pRc-CMV, and pcDNA3.1 series of vectors containing a neomycin- or zeocin-resistant gene) were obtained from Invitrogen. Fluorescein isothiocyanate-conjugated anti-mouse and anti-rabbit IgGs and horseradish peroxidase-conjugated anti-goat IgG were purchased from Zymed Laboratories Inc.. Mouse monoclonal anti-FLAG antibody, anti-FLAG antibody-conjugated agarose, and heparin were from Sigma. The lipoxygenase-inhibitory antioxidant nordihydroguaiaretic acid (NDGA) was purchased from BIOMOL. Heparin-Sepharose was purchased from Amersham Biosciences. Rabbit antiserum for human sPLA$_2$-XII was prepared as described previously (39).

Establishment of Transfectants—Establishment of HEK293 transformants was performed as described previously (12–16). Briefly, 1 μg of plasmid (sPLA$_2$ cDNAs subcloned into the pRc-CMV or pCR3.1 vector) was mixed with 2 μl of LipofectAMINE 2000 in 100 μl of Opti-MEM medium for 30 min and then added to cells that had attained 40–60% confluence in 12-well plates (Iwaki Glass) containing 0.5 ml of Opti-MEM. After incubation for 6 h, the medium was replaced with 1 ml of fresh culture medium. After overnight culture, the medium was replaced with 1 ml of fresh medium, and culture was continued at 37 °C in an incubator flushed with 5% CO_2 in humidified air. The cells were cloned by limiting dilution in 96-well plates in culture medium supplemented with 1 mg/ml geneticin. After culture for 3–4 weeks, wells containing a single colony were chosen, and the expression of each protein was assessed by RNA blotting. The established clones were expanded and used for the experiments as described below.

In order to establish double transformants expressing sPLA$_2$ and glypican, cells expressing each sPLA$_2$ were subjected to a second transfection with glypican cDNA subcloned into pCDNA3.1/Zeo(+) using LipofectAMINE 2000. Three days after the transfection, the cells were used for the experiments or seeded into 96-well plates and cloned by culturing in the presence of 50 μg/ml zeocin to establish stable transformants.

To assess functional coupling between sPLA$_2$ and either of the two COX isozymes, cells stably expressing sPLA$_2$ were transfected with COX-1 or COX-2 subcloned into pCDNA3.1 using LipofectAMINE 2000. Three days after the transfection, the cells were activated with A23187 to measure PGE$_2$ generation and were subjected to immunoblotting to examine COX-1 or COX-2 expression (see below).

Measurement of sPLA$_2$ Activity—sPLA$_2$ activity was assayed by measuring the amounts of radiolabeled fatty acids released from the substrate 1-palmitoyl-2-[^{14}C]arachidonoyl-phosphatidylethanolamine (2-AA-PE), 1-palmitoyl-2-[^{14}C]linoleoyl-PE (2-LA-PE), 2-AA-PC, or 2-LA-PC (Amersham Biosciences). Each substrate in ethanol was dried up under N_2 stream and was dispersed in water by sonication. Each reaction mixture (total volume 250 μl) consisted of appropriate amounts of the required sample, 100 mM Tris-HCl (pH 7.4), 4 mM $CaCl_2$, and 10 μM substrate. After incubation for 10–30 min at 37 °C, [^{14}C]AA or [^{14}C]LA was extracted, and radioactivity was quantified, as described previously (14–18).

Expression of Recombinant sPLA$_2$s by the Baculovirus System—Baculovirus expression of recombinant sPLA$_2$ proteins was performed using the BAC-to-BAC baculovirus expression system (Invitrogen). Briefly, sPLA$_2$ cDNAs were subcloned into the baculovirus expression vector pFASTBAC1 (Invitrogen) at appropriate restriction enzyme sites. Recombinant sPLA$_2$ proteins were first expressed in Sf9 insect cells and then amplified in High Five insect cells (Invitrogen) according to the manufacturer's instructions. Culture supernatants and cell lysates (4–5 days after infection) were used for subsequent experiments. Sf9 cells and High Five cells were maintained in Grace's insect medium (Invitrogen) supplemented with 10% FCS and Express Five SFM serum-free medium (Invitrogen), respectively.

Heparin Binding—Recombinant sPLA$_2$s (culture supernatants from baculovirus-infected High Five cells) were incubated with various amounts of heparin-Sepharose beads in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl (TBS) for 2 h at 4 °C, and PLA$_2$ activities remaining in the supernatants were assayed.

RNA Blotting—Approximately equal amounts (~5 μg) of total RNA obtained from the cells were applied to separate lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore Corp.). The resulting blots were then probed with the respective cDNA probes that had been labeled with [^{32}P]dCTP (Amersham Biosciences) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously (14–18).

SDS-PAGE/Immunoblotting—Lysates from 10^5 cells were subjected to SDS-PAGE using 7.5–12.5% gels under reducing conditions. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher and Schuell) using a semidry blotter (MilliBlot-SDE system; Millipore). After blocking with 3% (w/v) skim milk in TBS containing 0.05% Tween 20 (TBS-Tween), the membranes were probed with the respective antibodies (1:20,000 dilution for COX-1, 1:5,000 dilution for COX-2, and 1:20,000 for FLAG epitope in TBS-Tween) for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-goat (for COXs) or anti-mouse (for FLAG) IgG (1:5,000 dilution in TBS-Tween) for 2 h and were visualized using the ECL Western blot system (PerkinElmer Life Sciences) (14–18).

Reverse Transcription (RT)-PCR—Synthesis of cDNA was performed using 0.5 μg of total RNA from human cell lines and avian myeloblastosis virus reverse transcriptase, according to the manufacturer's instructions supplied with the RNA PCR kit (Takara Biomedical). Subsequent amplification of the cDNA fragments was performed using 1 μl of the reverse-transcribed mixture as a template with specific primers for each sPLA$_2$. For amplification of sPLA$_2$-IB, -IIA, -IID, -IIE, -IIF, -V, -X, and -XII, a set of 23-bp oligonucleotide primers corresponding to 5′- and 3′-nucleotide sequences of their open reading frames were used as primers (40–45). For amplification of sPLA$_2$-III, primers directed for the sPLA$_2$ domain and N-terminal domain were used (see below). The PCR condition was 94 °C for 30 s and then 35 cycles of amplification at 94 °C for 5 s and 68 °C for 4 min, using the Advantage cDNA polymerase mix (Clontech). The PCR products were analyzed by 1% agarose gel

electrophoresis with ethidium bromide. The gels were further subjected to Southern blot hybridization using sPLA$_2$ cDNAs as probe.

Construction of sPLA$_2$-III Mutants—sPLA$_2$-III mutants were produced by PCR with the Advantage cDNA polymerase mix using sPLA$_2$-III/pRc-CMV as a template. The condition of PCR was 25 cycles at 94, 55, and 72 °C for 30 s each. The primers used were as follows: III-5′ primer (5′-ATGGGGGTTCAGGCAGGGCTG-3′), III-3′ primer (TCACTGGCTCCAGGACTTCTG-3′), III-S-S primer (5′-GATGGACCATGCCTGGCACAC-3′), III-S-AS primer (TCAAGTTGGGGAGGTGGCCCG-3′), III-HQ-S primer (5′-TGCCGGGAACAAGACCGCTGC-3′), and III-HQ-AS primer (5′-GCAGCGGTCTTGTTCCCGGCA-3′). In order to obtain sPLA$_2$-III wild type (WT) and the truncated mutants III-S, III-N+S, and III-S+C (see "Results"), the primer sets III-5′ and III-3′, III-S-S and III-S-AS, III-5′ and III-S-AS, and III-S-S and III-3′, respectively, were used. In order for the mutants III-S and III-S+C to be secreted when expressed in cells, the signal sequence for human group IIA sPLA$_2$ (40) was linked to the 5′-end of the III-S-S primer and PCR-amplified. To attach the FLAG epitope at the C terminus, the FLAG antisense oligonucleotide 5′-TTACTTGTGATCGTCGTCCTTGTAGTC-3′ were directly linked to the 5′-ends of the antisense primers. In order to construct the catalytically inactive mutant III-N+S-HQ (see "Results"), the first PCR was conducted with III-5′ and III-HQ-AS primers or with III-HQ-S and III-S-AS primers using sPLA$_2$-III-WT cDNA as a template. The resulting two primary PCR fragments were mixed, denatured at 94 °C for 5 min, annealed at 37 °C for 30 min and then 55 °C for 2 min, and extended at 72 °C for 4 min during each cycle. The secondary PCR product with specific mutation was obtained after 25 additional PCR cycles with III-5′ and III-S-AS primers. A similar strategy was used to prepare III-S-HQ. Each PCR product was ligated into the pCR3.1 and was transfected into Top10F′ supercompetent cells (Invitrogen). The plasmids were isolated and sequenced using a *Taq* cycle sequencing kit (Takara Biomedicals) and an autofluorometric DNA sequencer 310 Genetic Analyzer (Applied Biosystems) to confirm the sequences.

Activation of HEK293 Cells—HEK293 cells (5×10^4/ml) were seeded into each well of 48-well plates. To assess fatty acid release (14–18), [^{3}H]AA or [^{3}H]oleic acid (OA) (both from Amersham Biosciences) (0.1 μCi/ml) was added to the cells in each well on day 3, when they had nearly reached confluence, and culturing was continued for another day. After three washes with fresh medium, 100 μl of RPMI 1640 with or without 10 μM A23187 with 1% FCS or 1 ng/ml IL-1β and/or 10% FCS was added to each well, and the amount of free [^{3}H]AA or [^{3}H]OA released into the supernatant was measured. The percentage release was calculated using the formula $(S/(S + P)) \times 100$, where S and P represent the radioactivity measured in the supernatant and cell pellet, respectively. The supernatants from replicate cells were subjected to the PGE$_2$ enzyme immunoassay.

To assess transcellular PGE$_2$ biosynthesis (11, 15), two cell populations (2.5×10^4 cells/ml for each) were added to the same wells of 48-well plates (100 μl/well) and cultured for 4 days. Then the cells were stimulated with IL-1β in medium containing 10% FCS for 4 h, and PGE$_2$ released into the supernatants was quantified.

Exogenous sPLA$_2$ Assay—Subconfluent cells grown in 48-well plates were incubated with recombinant sPLA$_2$s (culture supernatants from baculovirus-infected High Five cells) for 1 h, and PGE$_2$ released into the supernatants was quantified.

Confocal Laser Microscopy—Cells grown on collagen-coated cover glasses (Iwaki Glass) were fixed with 3% paraformaldehyde for 30 min in phosphate-buffered saline (PBS). After three washes with PBS, the fixed cells were sequentially treated with 1% (w/v) bovine serum albumin (for blocking) and 0.2% (v/v) Triton X-100 (for permeabilization) in PBS for 1 h, with anti-FLAG antibody (1:500 dilution) for 1 h in PBS containing 1% albumin and then with fluorescein isothiocyanate-goat anti-mouse IgG (1:500 dilution) for 1 h in PBS containing 1% albumin. After six washes with PBS, the cells were mounted on glass slides using Perma Fluor (Japan Tanner), and the sPLA$_2$ signal was visualized using a laser-scanning confocal microscope (IX70; Olympus), as described previously (16, 18).

Statistical Analysis—Data were analyzed by Student's t test. Results are expressed as the mean ± S.E., with $p = 0.05$ as the limit of significance.

RESULTS

Human sPLA$_2$-III

Enzymatic Properties—Human sPLA$_2$-III-WT and its truncated mutants, III-S, III-N+S, and III-S+C, (structures illustrated in Fig. 1*A*) were each transfected into HEK293 cells.

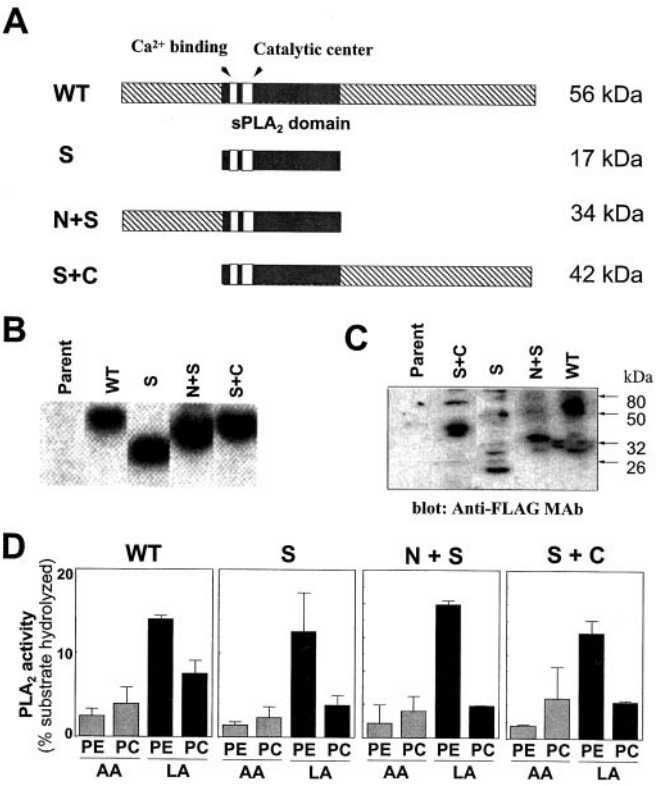

FIG. 1. **Expression of the WT and truncated forms of sPLA$_2$-III in HEK293 cells.** *A*, structures of the WT and truncated forms (S, N+S, and S+C) of sPLA$_2$-III. In the case of III-S and III-S+C, a signal peptide for human sPLA$_2$-IIA was fused at their N termini. The C terminus of each protein was tagged with the FLAG epitope as required for the experiments. *B* and *C*, expression levels of the WT and truncated forms of sPLA$_2$-III in their HEK293 transfectants were assessed by Northern blotting (5 μg of total RNA per lane) (*B*) and in the case of FLAG-tagged enzymes by SDS-PAGE/immunoblotting (10^5 cell equivalents/lane) (*C*). *D*, *in vitro* enzymatic activity of the WT and truncated forms of sPLA$_2$-III expressed in HEK293 transfectants. Aliquots (5–20 μl) of the culture supernatants were taken for PLA$_2$ assay using 2-AA-PE, 2-AA-PC, 2-LA-PE, and 2-LA-PC as substrates. Values (percentage of substrate hydrolysis equivalent to 10^7 cells) are means ± S.E. of five independent experiments.

Stable transfectants expressing WT and truncated enzymes, with or without C-terminal FLAG epitope, were screened by Northern blotting using a specific sPLA$_2$-III cDNA probe (Fig. 1*B*) or immunoblotting using an anti-C-terminal FLAG tag antibody (Fig. 1*C*), and clones in which their expression levels were almost comparable with one another were used in subsequent studies. As shown in Fig. 1*C*, III-WT, III-N+S, III-S+C, and III-S were expressed as major immunoreactive proteins with predicted molecular masses of 56, 32, 42, and 17 kDa, respectively. Flanking the C terminus with the FLAG epitope did not significantly affect *in vitro* and cellular functions, as described below.

Culture supernatants of these transfectants were assayed for PLA$_2$ activity using PE and PC bearing AA or LA at their *sn*-2-position as substrates. Under our PLA$_2$ assay condition, the WT and truncated enzymes exhibited comparable PLA$_2$ activity with similar substrate specificity (Fig. 1*D*). Of the four substrates tested, 2-LA-PE was the best substrate, being hydrolyzed 2–3 and 6–8 times more efficiently than 2-LA-PC and 2-AA-PE, respectively. 2-AA-PC was hydrolyzed ~2-fold faster than 2-AA-PE, whereas 2-LA-PE was hydrolyzed ~2-fold faster than 2-LA-PC.

Cellular Functions—To assess the fatty acid-releasing function of sPLA$_2$-III in cells, sPLA$_2$-III-WT-transfected and control HEK293 cells were preincubated overnight with [^{3}H]AA or

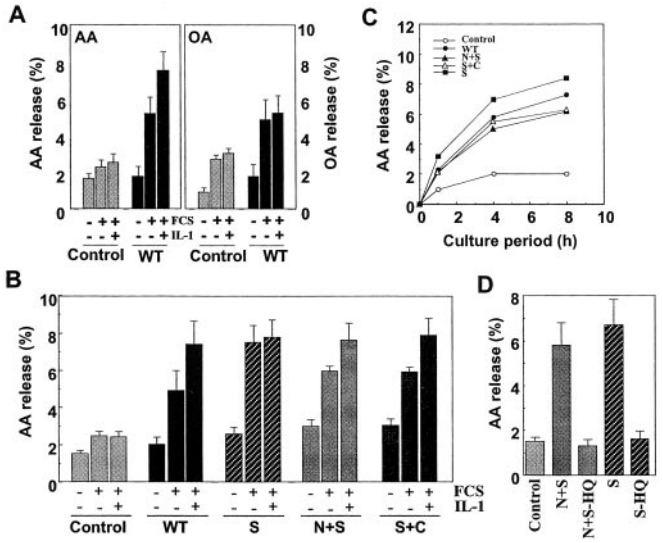

FIG. 2. **Cellular fatty acid release by sPLA$_2$-III.** *A*, control or sPLA$_2$-III-WT-expressing cells, which were prelabeled with [^{3}H]AA or [^{3}H]OA, were incubated for 4 h with 1% (−) or 10% (+) FCS with (+) or without (−) 1 ng/ml IL-1β to assess the release of these fatty acids. *B* and *C*, comparison of the [^{3}H]AA-releasing property between the WT and truncated (S, N+S, and S+C) sPLA$_2$-III. [^{3}H]AA-prelabeled cells were cultured for 4 h in varied combinations of FCS and IL-1β (*B*) or for the indicated periods in the presence of 10% FCS plus 1 ng/ml IL-1β (IL-1/FCS) (*C*). *D*, [^{3}H]AA release by III-N+S, III-S, and their point mutants (HQ), in which the catalytic center His was replaced with Gln, following 4-h incubation with IL-1/FCS. Values are mean ± S.E. of 3–5 independent experiments in *A*, *B*, and *D*, and a representative result of three independent experiments is shown in *C*.

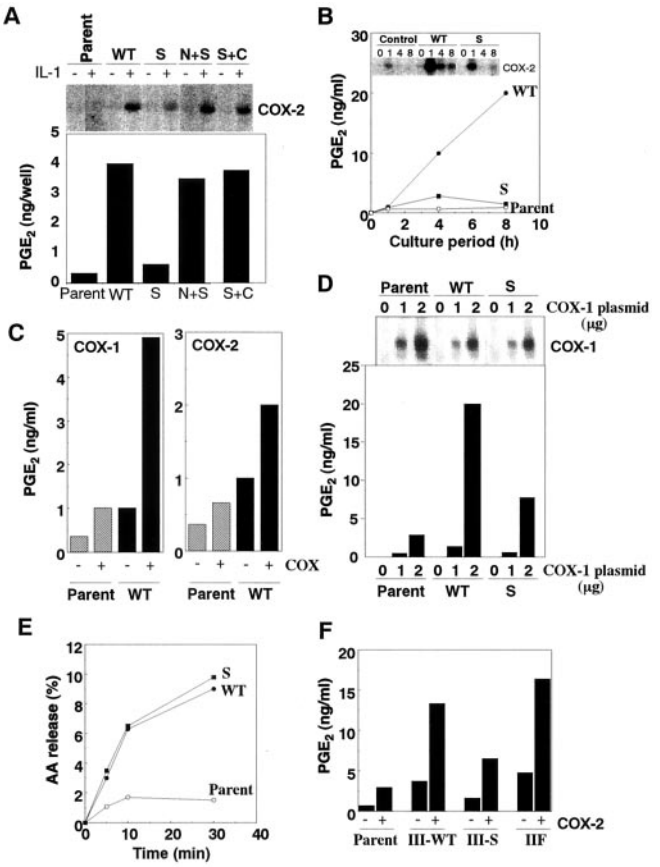

FIG. 3. **Coupling between sPLA$_2$-III and COXs.** *A*, PGE$_2$ generation by parent HEK293 cells and cells transfected with the WT or truncated (S, N+S, and S+C) sPLA$_2$-III after 4 h of incubation with IL-1/FCS. Endogenous COX-2 mRNA expression in the presence (+) or absence (−) of IL-1β was assessed by Northern blotting (*top*). *B*, time course of PGE$_2$ production by parental cells (*open circles*) and cells transfected with III-WT (*closed circles*) or III-S (*closed squares*) after incubation with IL-1/FCS. COX-2 mRNA expression in these cells at each time point, assessed by Northern blotting, is shown in the *inset*. *C*, parental and III-WT-transfected cells were transfected with mock (−) and COX-1 or COX-2 (+) plasmids (1 μg). Three days after transfection, the cells were stimulated with 10 μM A23187 for 30 min to assess PGE$_2$ release. *D*, parental cells and cells expressing III-WT or III-S were transfected with the indicated amounts of the COX-1 plasmid. Three days after transfection, A23187-stimulated PGE$_2$ production was assessed. COX-1 protein expression, as assessed by immunoblotting, is shown in the *top panel*. *E*, [^{3}H]AA release by parental cells (*open circles*) and cells expressing III-WT (*closed circles*) or III-S (*closed squares*) after stimulation with A23187 over 30 min. *F*, transcellular PGE$_2$ production. Parental cells and cells transfected with III-WT, III-S, or human sPLA$_2$-IIF were cocultured with COX-2-transfected (+) or parental (−) HEK293 cells, and PGE$_2$ production after 4-h incubation with IL-1/FCS was assessed. Values are mean ± S.E. of three independent experiments in *A*, *E*, and *F*, and a representative result of 2–4 reproducible experiments is shown in *B–D*.

[^{3}H]OA, washed, and then cultured for 4 h with or without 10% FCS and/or IL-1. As shown in Fig. 2*A*, in the presence of 10% FCS, the WT enzyme significantly increased the release of both [^{3}H]AA and [^{3}H]OA almost in parallel. The further addition of IL-1 resulted in an increase in [^{3}H]AA, but not [^{3}H]OA, release (Fig. 2*A*). Comparing these properties of sPLA$_2$-III with other sPLA$_2$s reported so far, FCS-dependent, fatty acid nonselective release is similar to that by the plasma membrane-acting enzymes, such as sPLA$_2$-X and -V, and IL-1 augmentation of AA release is reminiscent of that by the HSPG-shuttled enzymes, such as sPLA$_2$-IIA, -IID, -IIE, and -V (9, 10, 14–18).

In agreement with the *in vitro* enzymatic activity (Fig. 1), the release of [^{3}H]AA (Fig. 2*B*) and [^{3}H]OA (data not shown) by cells transfected with the three truncated enzymes (III-S, III-N+S, and III-S+C) was similar to that by cells transfected with the WT enzyme. As shown in Fig. 2*C*, [^{3}H]AA release proceeded gradually over 8 h of culture. Thus, the sPLA$_2$ domain alone is essential and sufficient for cellular fatty acid release. As a notable difference, [^{3}H]AA release by cells expressing III-WT, III-N+S, or III-S+C was significantly augmented by IL-1, whereas this augmentation was not observed appreciably in cells expressing III-S (*i.e.* the sPLA$_2$ domain alone) (Fig. 2*B*). When III-N+S-HQ and III-S-HQ, in which the putative catalytic center His in III-N+S and III-S was, respectively, replaced by Gln, were transfected into HEK293 cells, no *in vitro* PLA$_2$ activity was detected (data not shown), and an increase in cellular [^{3}H]AA release was not observed (Fig. 2*D*), despite their reasonable expression levels (data not shown). This result indicates that the catalytic activity is an absolute requirement for the enzymatic action of sPLA$_2$-III on both phospholipid vesicles and cellular membranes.

The AAs released by III-WT, -N+S, and -S+C following IL-1 stimulation were each efficiently converted to PGE$_2$ (Fig. 3*A*). This PGE$_2$ production was ablated by the COX-2 inhibitor NS-398 (data not shown), revealing functional coupling between sPLA$_2$-III and COX-2 in the IL-1-stimulated delayed response. Remarkably, PGE$_2$ production by cells transfected with III-S was less than that by cells transfected with III-WT, -N+S, and -S+C (Fig. 3*A*), despite the fact that all four proteins produced similar amounts of AA (Fig. 2*B*). Kinetic experiments demonstrated that PGE$_2$ production by III-WT increased linearly over 1–8 h, thus lagging behind AA release (Fig. 2*C*), whereas PGE$_2$ production by III-S was increased only modestly over the whole culture period (Fig. 3*B*). As shown in the *top panel* of Fig. 3*A*, III-WT, III-N+S, and III-S+C augmented IL-1-induced expression of COX-2 markedly relative to replicate control cells, whereas COX-2 induction by III-S was less than that by III-WT, -N+S, and -S+C (although it was still higher than control cells). Time course experiments showed

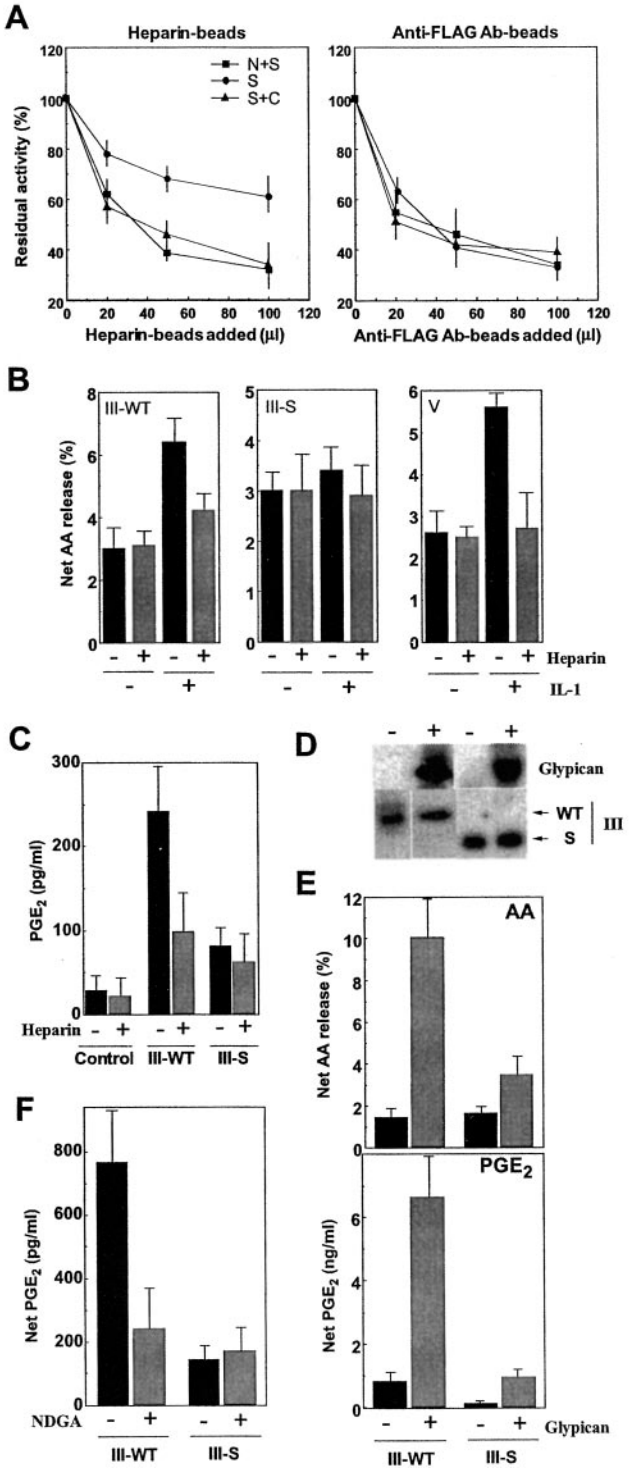

FIG. 4. **Heparanoid dependence of sPLA$_2$-III-mediated AA metabolism.** *A*, absorption of insect cell-derived recombinant FLAG-tagged sPLA$_2$-III (S, N+S, and S+C) by heparin- or anti-FLAG antibody-conjugated beads. The culture supernatants of baculovirus-infected High Five cells were diluted with TBS (5-fold for S+C and 50-fold for S and N+S to adjust their enzyme activities to be equivalent) and mixed with heparin- or anti-FLAG antibody-conjugated beads. After 2-h incubation, remaining PLA$_2$ activities in the supernatants were measured. *B* and *C*, effect of exogenous heparin on cellular AA release. [^{3}H]AA-prelabeled HEK293 cells expressing sPLA$_2$-III-WT or sPLA$_2$-III-S and human sPLA$_2$-V were incubated for 5 h with 0.5 mg/ml heparin and then cultured for an additional 4 h in medium containing 10% FCS with (+) or without (−) IL-1 in the continued presence of heparin to assess [^{3}H]AA release (*B*) and PGE$_2$ production (*C*). *D* and *E*, effect of glypican coexpression. HEK293 cells expressing suboptimal levels of sPLA$_2$-III-WT or III-S were subjected to second transfection with glypican. The expression of III-WT, III-S, and glypican was assessed by Northern blotting (*D*). These cells were prelabeled with [^{3}H]AA and analyzed for IL-1/FCS-dependent [^{3}H]AA release (*top*) and PGE$_2$ production (*bottom*) after 4-h treatment with IL-1/FCS. *F*, effect of NDGA. HEK293 cells expressing III-WT or III-S were cultured for 4 h with IL-1/FCS in the presence (+) or absence (−) of 10 μM NDGA, and PGE$_2$ released into the supernatants was quantified. Values are mean ± S.E. of 3–5 independent experiments.

that COX-2 mRNA induction in III-WT-expressing cells peaked at 1 h and declined to a plateau level after 4–8 h, whereas that in control and III-S-expressing cells reached a peak by 1 h and disappeared thereafter (Fig. 3*B*, *inset*). These results suggest that sPLA$_2$-III has the ability to enhance COX-2 expression, as in the case of several HSPG-binding group II subfamily sPLA$_2$s (16–18), and that the poor PGE$_2$-biosynthetic action of the sPLA$_2$ domain alone is, at least in part, due to its poor ability to induce COX-2.

To elucidate functional coupling between sPLA$_2$-III and COX enzymes more directly, we performed cotransfection experiments, in which HEK293 cells expressing sPLA$_2$-III were subsequently transfected with either COX-1 or COX-2, and PGE$_2$ production following A23187 stimulation was examined. Expression of COX-1 and COX-2 was verified by immunoblotting (data not shown). As shown in Fig. 3*C*, PGE$_2$ production by either COX-1 or COX-2 was markedly augmented by coexpression of sPLA$_2$-III-WT. When the PGE$_2$-biosynthetic activities of III-WT and -S were compared in this cotransfection analysis, PGE$_2$ production by III-WT via the overexpressed COX-1 (Fig. 3*D*) or COX-2 (data not shown) was significantly higher than that produced by III-S, although A23187-induced immediate [^{3}H]AA releases by III-WT and -S were comparable over 30 min of incubation period (Fig. 3*E*). These results suggest that the sPLA$_2$ domain alone is coupled with downstream COX enzymes less efficiently than is III-WT, even if COX enzymes are equivalently expressed in cells. III-N+S and III-S+C were coupled with the overexpressed COX-1 with the same potency as III-WT (data not shown), indicating that either the N- or C-terminal domain can confer onto the sPLA$_2$ domain the ability to efficiently couple to COX enzymes.

We next performed the transcellular PGE$_2$-biosynthetic assay (11, 15), in which sPLA$_2$-III-expressing and COX-2-expressing HEK293 cells were cocultured. As was observed with sPLA$_2$-IIF used as a positive control (11), coculture of sPLA$_2$-III-WT-expressing cells with COX-2-expressing cells resulted in a marked increase in the production of PGE$_2$ (Fig. 3*F*), indicating that the WT enzyme is capable of supplying AA to COX-2 in neighboring cells to propagate PGE$_2$ production in a paracrine manner. Coculturing sPLA$_2$-III-S-expressing cells with COX-2-expressing cells also increased PGE$_2$ production significantly, yet the amount of PGE$_2$ produced by III-S was again reproducibly lower than that produced by the WT enzyme (Fig. 3*F*).

Heparanoid Dependence—When recombinant FLAG-tagged sPLA$_2$-III-S, -N+S, and -S+C, which were expressed by the baculovirus/insect cell system (see below), were incubated with incremental amounts of heparin-conjugated beads, III-N+S and III-S+C were more efficiently absorbed than III-S to the beads (Fig. 4*A*, *left*) under the condition where they were equally precipitated by anti-FLAG antibody-conjugated beads (Fig. 4*A*, *right*), indicating that both N- and C-terminal domains facilitate the binding of sPLA$_2$-III to heparanoids. To assess whether sPLA$_2$-III action on cells depends on cell surface HSPG, as does the HSPG-shuttled group II subfamily sPLA$_2$s (14–18), the effect of exogenous heparin, which solubilizes the HSPG-bound pool of sPLA$_2$s and thereby suppresses their cellular functions (14–18), was examined. As shown in Fig. 4*B*, AA release by sPLA$_2$-III-WT in the absence of IL-1 (*i.e.*

FCS-dependent release) was insensitive to exogenous heparin, whereas IL-1-augmented AA release was reversed by heparin to a level comparable with FCS-dependent release (Fig. 4*B*, *left*). This heparin effect resembled its effect on sPLA$_2$-V-mediated AA release, where IL-1-stimulated (reflection of the HSPG-shuttling route), but not FCS-dependent (reflection of the plasma membrane route), AA release was suppressed by heparin (Fig. 4*B*, *right*). AA release by III-S, which was largely unaffected by IL-1 as noted above (Fig. 2), was unaffected by exogenous heparin (Fig. 4*B*, *middle*). Moreover, IL-1-stimulated PGE$_2$ production by WT-expressing cells was reduced to a level comparable with that by III-S-expressing cells following heparin treatment (Fig. 4*C*). In contrast, the inhibitory effect of heparin on PGE$_2$ production by III-S-expressing cells was only minimal (Fig. 4*C*).

Since the function of the HSPG-shuttled group II subfamily sPLA$_2$s is augmented by overexpression of glypican, a glycosylphosphatidylinositol-anchored HSPG that acts as a functional adaptor for these sPLA$_2$s (16, 18), we next assessed the effect of glypican coexpression on sPLA$_2$-III-mediated AA metabolism. Expression of glypican in III-WT- or III-S-expressing cells was verified by Northern blotting (Fig. 4*D*). As shown in Fig. 4*E*, coexpression of glypican markedly enhanced AA release and PGE$_2$ production by the WT enzyme. AA release and PGE$_2$ production by III-S were increased by glypican to a much lesser extent (Fig. 4*E*). A small augmentation of III-S function by glypican overexpression may be due to its weak affinity for heparanoids (Fig. 4*A*, *left*) or to some other unknown mechanisms. Overexpression of glypican alone did not affect AA release, as previously reported (16).

The function of the HSPG-shuttled group II subfamily sPLA$_2$s, but not that of the plasma membrane-acting sPLA$_2$s, is markedly attenuated by 12/15-lipoxygenase-inhibitable antioxidants (such as NDGA), leading to the suggestion that stimulus-induced membrane modification involves lipid-oxidative events (11, 46). In agreement with this notion, IL-1-augmented AA release (data not shown) and PGE$_2$ production (Fig. 4*F*) by sPLA$_2$-III-WT were reduced partially, to the level of those by III-S, by treatment of the transfectants with NDGA, whereas NDGA failed to affect PGE$_2$ production by III-S. This suggests that III-WT, as has been proposed for the other HSPG-shuttled sPLA$_2$s (11, 46–48), is accessible to particular membrane compartments that undergo oxidative modification after cytokine signaling.

Effect of Exogenous sPLA$_2$-III on AA Metabolism—The ability of the sPLA$_2$ domain of sPLA$_2$-III to elicit spontaneous (FCS-dependent) AA release suggests that it can act on the external plasma membrane, as does sPLA$_2$-X and -V (17, 25–27). To explore this possibility further, we aimed to examine the effects of exogenous sPLA$_2$-III on AA metabolism in mammalian cells. To this end, we expressed FLAG-tagged recombinant sPLA$_2$-III in High Five insect cells using the baculovirus system in order to obtain recombinant enzyme in abundance. As assessed by immunoblotting using the anti-FLAG antibody, recombinant III-S, III-N+S, and III-S+C were expressed abundantly in the insect High Five cells as the expected sizes (Fig. 5*A*). The expression levels of III-S and III-N+S were comparable, whereas that of III-S+C was approximately one-tenth that of III-S and III-N+S. III-WT was not expressed appreciably in High Five cells (possibly due to rapid proteolytic degradation). Similar results were obtained when the enzymes were expressed in Sf9 insect cells (data not shown). The *in vitro* PLA$_2$ activity of III-S was comparable with that of III-N+S and was about 10 times higher than III-S+C (Fig. 5*B*), in agreement with their expression levels (Fig. 5*A*). Enzymatic properties (*e.g.* substrate specificity, pH dependence, and Ca^{2+} requirement) of these insect cell-derived truncated enzymes were similar to those of enzymes expressed in HEK293 cells (data not shown).

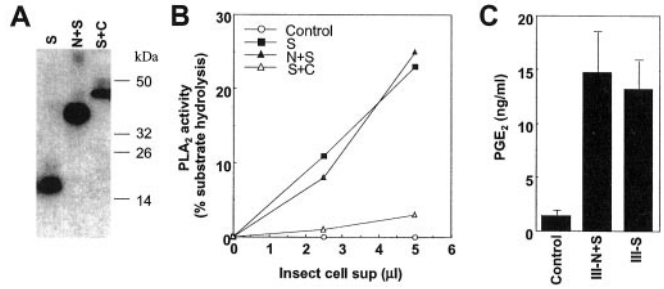

FIG. 5. **Recombinant expression of truncated sPLA$_2$-III mutants by the baculovirus system and their effects on PGE$_2$ production by HEK293 cells.** *A*, expression of the FLAG-tagged, truncated forms of sPLA$_2$-III in High Five insect cells. High Five cells grown in 12-well plates were infected with baculovirus bearing the truncated sPLA$_2$-III cDNAs for 5 days, harvested, and lysed in 1 ml of PBS, and 10-μl aliquots were subjected to SDS-PAGE/immunoblotting using anti-FLAG antibody. *B*, *in vitro* enzymatic activity of recombinant sPLA$_2$-III expressed in High Five cells. The indicated amounts of the culture supernatants of the baculovirus-infected High Five cells were taken for PLA$_2$ assay. A representative result of two reproducible experiments is shown. *C*, effects of recombinant III-S and III-N+S on PGE$_2$ production by COX-2-expressing HEK293 cells. The cells were incubated for 1 h with the culture supernatants of control or sPLA$_2$-expressing High Five cells that were diluted (1:10) with RPMI 1640 containing 10% FCS, and PGE$_2$ released into the supernatants was measured. A representative result of three independent preparations is shown. Values are mean ± S.E. of three independent experiments.

We then investigated the effects of recombinant III-S and III-N+S, which were secreted from the baculovirus-infected High Five cells at comparable levels (Fig. 5, *A* and *B*), on PGE$_2$ production by HEK293 cells. Thus, culture supernatants of control (*i.e.* no baculovirus infection) and of III-S- or III-N+S-expressing High Five cells were diluted 10 times with RPMI 1640 plus 10% FCS and added to COX-2-transfected HEK293 cells. As shown in Fig. 5*C*, both exogenous III-N+S and III-S markedly increased PGE$_2$ production by COX-2-transfected HEK293 cells after a 1-h incubation.

Subcellular Localization—We next performed indirect immunofluorescent confocal microscopy to assess subcellular distribution of sPLA$_2$-III-WT and truncated mutants in HEK293 transfectants. As shown in Fig. 6, signals for III-WT, III-N+S, and III-S+C were detected in the cytoplasmic punctate regions that excluded the nucleus, whereas III-S was distributed mainly on the plasma membrane but not in the cytosol. In addition, intense staining was seen at the spindle edges of cell adhesion sites in cells transfected with III-WT or III-N+S and to a lesser extent with III-S+C (Fig. 6).

Expression in Human Cell Lines—We next looked for human cell lines that expressed sPLA$_2$-III endogenously and found that BEAS-2B, a human lung epithelial cell line (Fig. 7, *A* and *B*), and HCA-7, a human adenocarcinoma cell line (Fig. 7*C*), expressed this enzyme in addition to several other sPLA$_2$s. RT-PCR for sPLA$_2$-III was carried out using two different sets of primers (III-5′/III-HQ-AS and III-5′/III-S-AS), each of which amplified a single band with a predicted size (Fig. 7*A*). Subsequent Southern hybridization using a sPLA$_2$-III-specific cDNA probe confirmed that these bands indeed corresponded to the expected portions of the enzyme (data not shown). In BEAS-2B cells, sPLA$_2$-III was constitutively expressed and decreased after stimulation with cytokines (TNF-α and IFN-γ) (Fig. 7*B*). In comparison, the expression of sPLA$_2$-IIA and sPLA$_2$-V was tightly controlled by cytokines in that sPLA$_2$-IIA was induced by TNF-α and suppressed by IFN-γ, and sPLA$_2$-V expression required both TNF-α and IFN-γ (Fig. 7*B*). sPLA$_2$-X was consti-

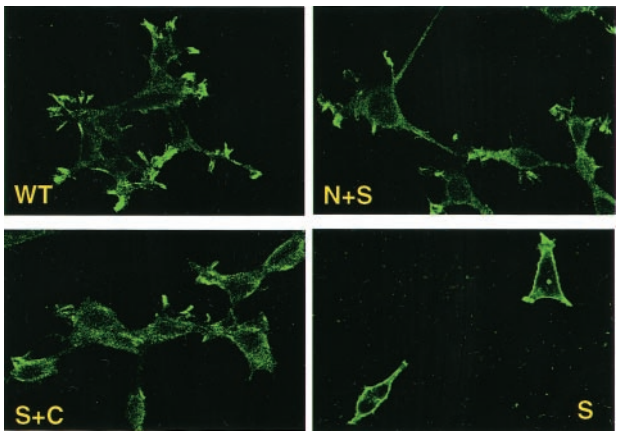

FIG. 6. **Subcellular distribution of sPLA$_2$-III.** HEK293 cells transfected with the FLAG-tagged sPLA$_2$-III-WT and truncated forms (III-S, III-N+S, and III-S+C) were subjected to immunofluorescent staining using anti-FLAG antibody and fluorescein isothiocyanate-conjugated anti-IgG. Detailed procedures are described under "Experimental Procedures."

tutively expressed (Fig. 7*B*), and other sPLA$_2$s (IB, IID, IIE, IIF, and XII) were undetectable (data not shown).

sPLA$_2$-III was also constitutively expressed in HCA-7 cells, in which its expression was unaffected by IL-1 (Fig. 7*C*). In this cell line, sPLA$_2$-IIA was absent in unstimulated cells and was strongly induced by IL-1; sPLA$_2$-IID, -IIF, -V, and -X were constitutively expressed, among which only sPLA$_2$-V was upregulated by IL-1; and other sPLA$_2$s (IB, IIE, and XII) were undetectable (Fig. 7*C*). When sPLA$_2$-III-S and sPLA$_2$-N+S, which were produced by the baculovirus system (Fig. 5), were exogenously added to HCA-7 cells, there was a substantial increase in PGE$_2$ production (Fig. 7*D*).

Human sPLA$_2$-XII

Enzymatic Properties—Human sPLA$_2$-XII cDNA was transfected into HEK293 cells, and the expression of the enzyme in stable transfectants obtained after drug selection was assessed by Northern blotting (Fig. 8*A*, *inset*) and Western blotting (see below). In our PLA$_2$ assay using the four substrates (PE and PC bearing *sn*-2-AA or -LA), sPLA$_2$-XII-expressing cells displayed no detectable PLA$_2$ activity in both culture supernatants and cell lysates. The enzyme activity was still below the detection limit even when recombinant sPLA$_2$-XII was overexpressed by the baculovirus/High Five cell system (data not shown). It is unlikely that sPLA$_2$-XII was inappropriately expressed in our system, since it was detected by Northern and Western blottings as readily as other sPLA$_2$s transfected in HEK293 or insect cells. Thus, sPLA$_2$-XII does not exhibit detectable enzymatic activity toward PE and PC in our PLA$_2$ assay, in line with a previous report that the activity of this enzyme is extremely low as compared with most other sPLA$_2$s (38).

Effects on AA Metabolism—[^{3}H]AA and [^{3}H]OA release by sPLA$_2$-XII-expressing cells in the presence of 10% FCS was increased minimally relative to that by control cells, even in the presence of IL-1 (Fig. 8*A*). There was no appreciable increase in A23187-stimulated immediate [^{3}H]AA release in sPLA$_2$-XII-expressing cells (data not shown). sPLA$_2$-XII-expressing cells did not display increased PGE$_2$ production relative to control cells even when cells were cotransfected with COX-1 (Fig. 8*B*) or COX-2 (data not shown), although sPLA$_2$-III and -IIF showed marked functional COX-1 coupling under the same experimental condition. sPLA$_2$-XII also failed to increase PGE$_2$ production when HEK293 cells expressing this enzyme were cocultured with those expressing COX-2 in the transcellular assay (data not shown).

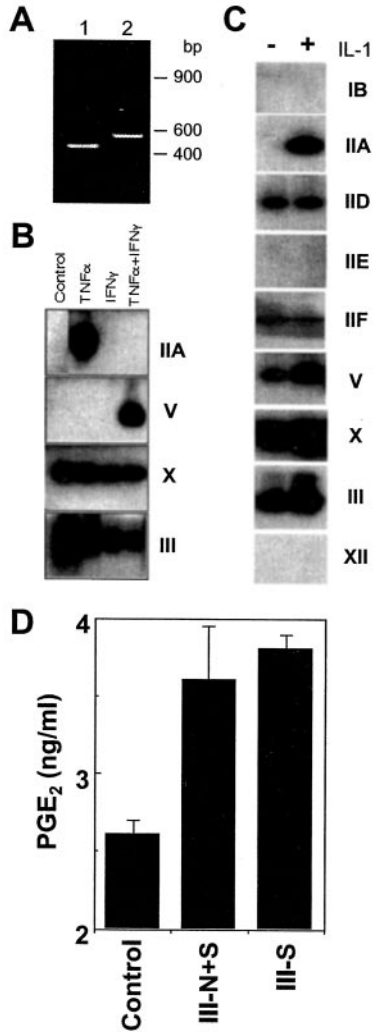

FIG. 7. **Expression of various endogenous sPLA$_2$s in human cell lines.** *A*, detection of endogenous sPLA$_2$-III in BEAS-2B cells by RT-PCR. Two different primer sets were used to amplify sPLA$_2$-III cDNA fragments in *lanes 1* (III-5′ and III-HQ-AS) and *2* (III-5′ and III-S-AS). Specific bands were visualized by ethidium bromide in agarose gels. *B* and *C*, expression of various sPLA$_2$s in BEAS-2B (*B*) and HCA-7 (*C*) cells with or without stimulation with 10 ng/ml IL-1α, IFN-γ, or TNF-α for 24 h. Specific bands were detected by RT-PCR followed by Southern hybridization. *A–C*, representative results of two reproducible experiments are shown. *D*, effects of recombinant III-S and III-N+S on PGE$_2$ production by HCA-7 cells (n = 3). The procedure is the same as in Fig. 5*C*.

Microscopic Analyses—Our antibody raised against human sPLA$_2$-XII specifically recognized a single 18-kDa protein in HEK293 transfectants on Western blotting (Fig. 8*C*). Using this antibody, we performed immunostaining of sPLA$_2$-XII expressed in HEK293 cells by confocal microscopy. We noted that a population of sPLA$_2$-XII-expressing cells exhibited unusual morphology; some cells appeared thin and long (Fig. 8*D*, *panel b*), whereas other cells were giant and multinucleated (Fig. 8*D*, *panel c*). In both situations, immunoreactivity of sPLA$_2$-XII was detected throughout the intracellular regions excluding the nucleus. These unique morphological features were not observed in cells transfected with other sPLA$_2$s (9–11, 14–18).

DISCUSSION

To complete our current understanding of the AA-releasing capacity of the full set of mammalian sPLA$_2$s in transfected cells (HEK293), we have herein examined the AA-releasing and PGE$_2$-biosynthetic functions of the two recently discovered

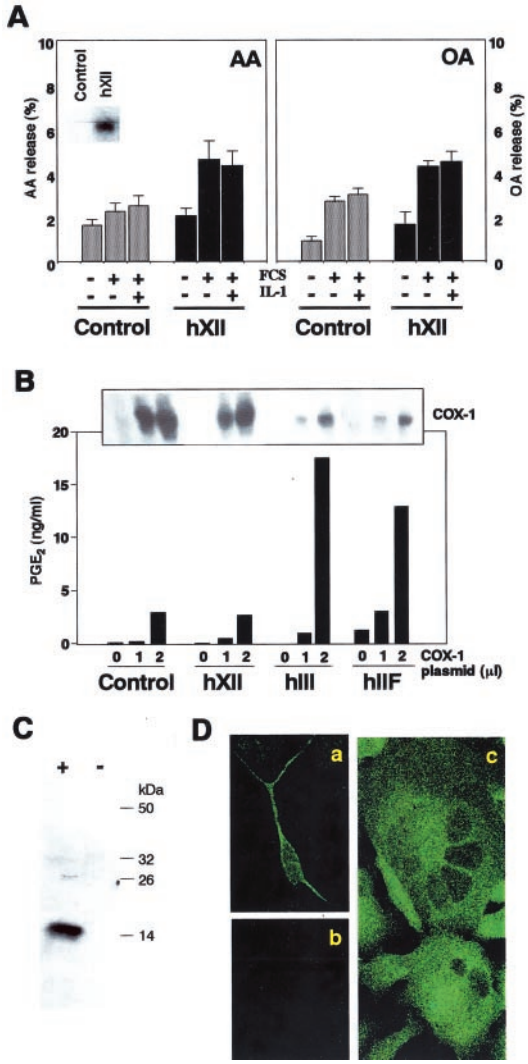

FIG. 8. **Properties of HEK293 cells transfected with sPLA$_2$-XII.** *A*, cellular fatty acid release. Control and sPLA$_2$-XII-transfected cells were prelabeled with [^{3}H]AA or [^{3}H]OA, and the release of these fatty acids after 4-h incubation with 1% (−) or 10% (+) FCS with (+) or without (−) 1 ng/ml IL-1β was assessed. The expression of sPLA$_2$-XII was assessed by Northern blotting (*inset*). *B*, PGE$_2$ production. Control cells and cells stably transfected with sPLA$_2$-XII, sPLA$_2$-III, or sPLA$_2$-IIF (positive control) were transfected with the indicated concentrations of the COX-1 plasmid. Three days after transfection, the cells were stimulated for 30 min with 10 μM A23187 to assess PGE$_2$ release. COX-1 expression was assessed by immunoblotting (*top*). *C*, detection of sPLA$_2$-XII protein in the sPLA$_2$-XII-transfected (+) and parental (−) HEK293 cells by immunoblotting using anti-sPLA$_2$-XII antibody. *D*, immunofluorescent staining on sPLA$_2$-XII-transfected or control cells using anti-sPLA$_2$-XII antibody. Two typical versions of sPLA$_2$-XII staining, in which the cells exhibited abnormal morphologies, are shown (*b* and *c*). Control cells did not show positive signals (*a*).

sPLA$_2$s, group III and XII. These two sPLA$_2$s show homology with other sPLA$_2$s only in the catalytic site and Ca^{2+}-binding loop (36, 38) and thus appear to have diverged from the group I/II/V/X sPLA$_2$s at early evolutional stages. Group III enzymes were originally identified in bee venom (49); subsequently in venom from scorpion (imperatoxin I and phospholipin) (50, 51), lizard (52), and jellyfish (53); and more recently in *Drosophila* (in which five distinct group III sPLA$_2$-related genes have been found) by scanning of public databases (2). Bee venom group III sPLA$_2$ has been shown to induce AA release when added exogenously to several cell types and elicits various biological effects *in vivo* (54–58). Imperatoxin I, a scorpion venom group III sPLA$_2$, inhibits ryanodine binding to Ca^{2+} release channels probably dependent upon its catalytic activity (50). However, it has remained unknown whether its mammalian homolog exerts a similar biological effect and, if so, how its function is regulated. Group XII sPLA$_2$s, first cloned from humans (37) and mice (38), have also been found in the genomic databases of various low vertebrate species, yet there has been no functional assessment of this group of enzymes.

sPLA$_2$-III—Human sPLA$_2$-III is made up of a central group III sPLA$_2$ domain flanked by N- and C-terminal regions (36). Although no data base entries with significant homology to the N- and C-terminal domains can be found, both domains are highly cationic and are predicted to fold separately from the sPLA$_2$ domain (36). Assessment of *in vitro* enzymatic activities of sPLA$_2$-III-WT and the truncated mutants (III-S, III-N+S, and III-S+C) expressed in HEK293 cells (Fig. 1) and High Five insect cells (Fig. 5) demonstrates that the central sPLA$_2$ domain alone is sufficient for catalytic function and neither N- nor C-terminal domain profoundly modulates the catalytic function of the sPLA$_2$ domain. This is in line with the observation that recombinant bee venom sPLA$_2$ expressed as an N-terminal fusion protein exhibits the same catalytic activity as the recombinant protein after removal of the N-terminal fusion peptide (59) and implies that the presence of the N-terminal extension (and presumably the C-terminal region, which is also not part of the catalytic site (36, 60)) does not interfere with the catalytic activity of sPLA$_2$-III. This contrasts with sPLA$_2$-IB and -X, for which proteolytic removal of the prepropeptide is essential for full enzymatic activity (3, 4). This difference exists most likely because, unlike the I/II/V/X sPLA$_2$s, which contain a hydrogen bond network linking the N terminus to catalytic residues (1, 2), the N terminus of bee venom group III enzyme (and probably human sPLA$_2$-III) does not form part of the active site structure (60).

The present cellular study suggests that sPLA$_2$-III-WT, as well as the mutants harboring either the N- or C-terminal domain, can act on cells through the HSPG pathway after IL-1 stimulation. The highly cationic nature of the N-terminal (pI 9.1) and C-terminal (pI 11.3) domains, in contrast to the central sPLA$_2$ domain that is acidic (pI 5.4) (36), may allow their electrostatic interaction with anionic heparin (or other anionic components). Although the affinity of III-N+S and III-S+C for heparin is weaker than that of sPLA$_2$-IIA, sPLA$_2$-IID, and sPLA$_2$-V (data not shown), it appears to be still sufficient to promote cellular function. Indeed, the IL-1-augmented components of AA release and PGE$_2$ production by sPLA$_2$-III-WT are sensitive to heparin treatment, and, conversely, overexpression of glypican, a glycosylphosphatidylinositol-anchored HSPG to which HSPG-shuttled sPLA$_2$s bind (16, 18), results in marked increases in AA release and PGE$_2$ production by III-WT (Fig. 4), providing strong support for a functional link between sPLA$_2$-III-WT and cellular HSPG. Moreover, the immunocytostaining study suggests that either the N- or C-terminal domain is essential for intracellular localization of the enzyme (Fig. 6). This is reminiscent of the previous finding that the heparin-binding group II subfamily sPLA$_2$s (IIA, IID, IIE, and V) can be internalized into cells in HSPG- and caveolae- or raft-dependent manners, followed by cytoplasmic vesicle formation and intracellular membrane hydrolysis (16, 18, 28).

Without cell stimulation, sPLA$_2$-III-WT and all of the truncated mutants elicit spontaneous (FCS-dependent), nonselective fatty acid release (Fig. 2). This pattern is very similar to that of sPLA$_2$-X, which acts on the PC-rich outer leaflet of the plasma membrane (4, 17, 25, 27), and suggests that sPLA$_2$-III, via its sPLA$_2$ domain, can act on cells through the external plasma membrane pathway. The facts that sPLA$_2$-III (and its truncated mutants) has significant activity toward PC (Fig.

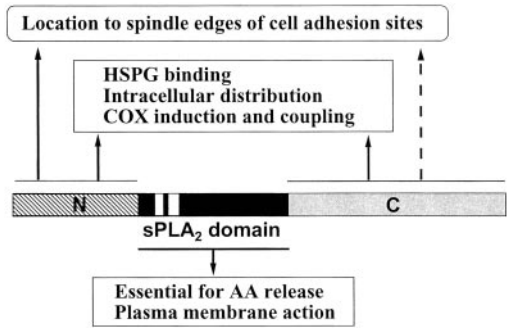

FIG. 9. **Diagram of domain-function relationship of human sPLA$_2$-III.** A central sPLA$_2$ domain is essential for the enzyme function and the plasma membrane action of the enzyme. Either the N- or C-terminal domain mediates HSPG binding, intracellular distribution, COX-2 induction, and efficient COX coupling. These domains, the N-terminal domain in particular, facilitate unique localization of this enzyme to the spindle edges of cell adhesion sites.

1*D*), that the cellular AA-releasing function of III-S is insensitive to exogenous heparin treatment or is poorly augmented by glypican overexpression (Fig. 4), that III-S is distributed on the plasma membrane (Fig. 6), and that exogenous enzyme is capable of increasing PGE$_2$ production in two cell types (Figs. 5*C* and 7*D*) support this notion.

Collectively, we conclude that sPLA$_2$-III can utilize the HSPG-shuttling pathway, where its unique N- and C-terminal domains play a role in entering the HSPG-shuttling route (and thereby affecting the targeting of the enzyme) and its core sPLA$_2$ domain exerts cellular membrane hydrolysis (Fig. 9). Although the N- and C-terminal domains have no sequence homology, both of them contribute to heparanoid binding based on the presence of basic residues. By comparison, the group II subfamily sPLA$_2$s have clusters of heparin-binding cationic residues on their core surfaces (61, 62). Strikingly, the N-terminal (and probably C-terminal) domain of sPLA$_2$-III also facilitates the distribution of this enzyme into the spindle edges of cell adhesion sites (Fig. 6). So far, none of the other sPLA$_2$s exhibit this unique localization (10, 11, 16–18). This suggests that some anionic components or binding molecules that preferentially associate with the N-terminal domain may exist in the spindle edges of HEK293 cells, although functional consequences of this localization remain to be elucidated. Note that the N- and C-terminal domains found in the five *Drosophila* group III enzymes have no sequence homology with those of human enzyme (2), suggesting that their roles are different.

Since group III enzyme purified from bee venom consists of only the sPLA$_2$ core (49), it may be anticipated that human sPLA$_2$-III also undergoes proteolytic processing and maturation in mammalian cells. The presence of a basic doublet KE at the end of the N-terminal domain and several basic residues including basic doublets in the C-terminal domain (36) suggests that their proteolytic removal by subtilisin-like protease in the Golgi (63). Indeed, besides the major bands with expected sizes, several shorter products are faintly detected in HEK293 cells transfected with sPLA$_2$-III (*e.g.* 30-kDa band in III-WT and III-N+S) in our immunoblot analysis (Fig. 1*C*). In addition, sPLA$_2$-III appears to be susceptible to endogenous protease(s) in High Five insect cells, in which III-S+C and III-N+S (and probably III-WT, which was almost undetectable) are rapidly degraded during storage (data not shown). At present, the maturation process of sPLA$_2$-III in mammalian cells remains obscure. It would be important to determine which forms of the enzyme (III-WT, III-N+S, III-S+C, III-S, or other processed forms) are truly present and functioning *in vivo*.

Interestingly, coexpression experiments demonstrate that III-WT (as well as III-N+S and III-S+C) is more efficiently coupled with COX than III-S (Fig. 3, *D–F*). Although the precise reasons for this result are unclear, it reminds us of the central dogma in the eicosanoid field that subcellular location of the biosynthetic enzymes is a critical determinant for their optimal functional coupling. It is now obvious that the perinuclear co-localization of sequential biosynthetic enzymes, including cPLA$_2\alpha$, COXs, and terminal PG synthases in the COX pathway and cPLA$_2\alpha$, 5-lipoxygenase, 5-lipoxygenase-activating protein, and terminal leukotriene synthases in the lipoxygenase pathway, is crucial for their functional coupling in activated cells (64–68). Indeed, our preliminary experiments have shown that the native cPLA$_2\alpha$, which translocates to the perinuclear membrane, is more efficiently coupled with COX than the cPLA$_2\alpha$ mutant, which moves to the plasma membrane.[2] In human neutrophils, the AA released by cPLA$_2\alpha$ at the perinuclear membrane, but not that released by sPLA$_2$-V at the plasma membrane, can be metabolized to leukotriene by the perinuclear 5-lipoxygenase (69). Considering the scenario that the HSPG-shuttled sPLA$_2$s can be internalized and cause membrane hydrolysis in the perinuclear region (16, 18, 28), it would be speculated that the AA released by the internalized sPLA$_2$s can be more efficiently supplied to adjacent COX in the perinuclear membrane than the AA released from cell surface by the plasma membrane-acting sPLA$_2$s (including III-S), although the latter AA can be accessible to the perinuclear COX (possibly by diffusion across the cytosol or with the aid of fatty acid transfer proteins), as has been observed with sPLA$_2$-X (4, 17, 25, 27) and even III-S (Fig. 3, *D* and *F*). Although intracellular membrane hydrolysis by the HSPG-shuttled sPLA$_2$s will need further study, our present data may shed light on the intracellular action of these sPLA$_2$s and its importance in efficient coupling with downstream enzymes.

The sPLA$_2$-III transcript is detected in the kidney, heart, liver, and skeletal muscle by Northern blotting (36). In two cell lines that endogenously express sPLA$_2$-III, sPLA$_2$-III expression is constitutive and not cytokine-inducible, unlike the group II subfamily sPLA$_2$s, sPLA$_2$-IIA and sPLA$_2$-V, that are induced by diverse sets of cytokines (Fig. 7). This implies that the transcriptional regulations and possibly functions of these sPLA$_2$s are distinct in these cells. Although exogenous sPLA$_2$-III-S modestly increased PGE$_2$ production by HCA-7 cells (Fig. 7*D*), it remains to be elucidated whether endogenous sPLA$_2$-III also participates in PGE$_2$ production or exhibits other functions. Expression of multiple sPLA$_2$s in a colon adenocarcinoma cell line is intriguing to note, since the COX-2-derived PGE$_2$ has been implicated in the exacerbation of colorectal cancer (70–72). Although targeted disruption of cPLA$_2\alpha$ (70), COX-2 (71), or the PGE$_2$ receptor EP2 (72) each reduces the incidence of colorectal cancer in *Apc* mutant mice, the phenotype of the cPLA$_2\alpha$ knockout mice is milder than that of the COX-2 or EP2 knockout mice, suggesting that some other PLA$_2$s can contribute to supplying AA to COX-2 in colorectal cancer. Possible involvement of sPLA$_2$s in colon cancer development is now under investigation.

Bee venom group III enzyme has been shown to evoke several cellular and *in vivo* responses via the N-type sPLA$_2$ receptor independently of its catalytic activity (2, 32). It is tempting to speculate that human sPLA$_2$-III may represent an endogenous ligand for the N-type receptor. Although the molecular entity of the N-type sPLA$_2$ receptor is still obscure, it has been recently shown that some neurotoxic sPLA$_2$s, including bee venom sPLA$_2$, bind to calmodulin with high affinity (73). Since calmodulin is a cytosolic protein, this finding supports the view that neurotoxic sPLA$_2$s have to be internalized to exert their

[2] M. Murakami, W. Cho, and I. Kudo, manuscript in preparation.

effect. Whether calmodulin indeed acts as a functional binding protein for sPLA$_2$-III and whether another high affinity binding site(s) for sPLA$_2$-III exists need to be addressed. Further elucidation of the N-type receptor may provide insights into the regulatory functions of sPLA$_2$-III. Additionally, a recent finding that a peptide derived from bee venom sPLA$_2$ inhibits replication of human immunodeficiency virus by blocking the virus entry into host cells (74) suggests a possible role of human counterpart in this process.

sPLA$_2$-XII—sPLA$_2$-XII has an unusual structure among the sPLA$_2$ family members in that only 3 of its 11 cysteines correspond to cysteines of other sPLA$_2$s (37, 38). Here we show that sPLA$_2$-XII fails to increase cellular fatty acid release and PGE$_2$ production despite its expression in HEK293 cells (Fig. 8, *A* and *B*). It is thus likely that sPLA$_2$-XII is incapable of mobilizing cellular AA due to its weak catalytic activity, although we cannot rule out the possibility that at higher expression levels it can influence AA metabolism. The putative sPLA$_2$-XII in zebrafish represented in genomic databases contains a leucine in place of histidine in the catalytic center, strongly suggesting that the zebrafish sPLA$_2$-XII has little or no catalytic activity (37). This supports the idea that the catalytic activity of sPLA$_2$-XII may not be critical for its cellular function. The appearance of multinucleated giant cells in a population of sPLA$_2$-XII-transfected cells (Fig. 8*D*) is noteworthy, which suggests a potential role of this enzyme in membrane fusion or cell division, although the molecular mechanisms are unclear. In these multinucleated cells, the main sPLA$_2$-XII immunoreactivity appears to be enriched in endoplasmic reticulum. Similar intracellular localization of mouse sPLA$_2$-XII has been reported in baby hamster kidney cells transfected with this enzyme (38). Immunohistochemistry using our anti-sPLA$_2$-XII antibody to determine cell types that endogenously express this enzyme will help us to understand its physiological and pathological functions.

CONCLUSION

Using the strategy of overexpression of AA-metabolic enzymes in HEK293 cells and several other cell lines, we have uncovered some of the functional properties of the full set of mammalian sPLA$_2$s. The ability of sPLA$_2$s to release AA from quiescent cells is highly dependent upon their interfacial binding to PC enriched in the outer leaflet of the plasma membrane (X > V > IIF ~ III > IB ≫ IIA; IIC, IID, IIE, and XII are almost inactive in this route). These sPLA$_2$s induce stimulus-independent, nonselective fatty acid release. sPLA$_2$s that poorly act on the PC-rich membrane can promote AA release from activated cells with support of HSPG as an adapter (IIA ~ V > IID > III > IIE; IB, IIC, X, and XII are nonfunctional in this route). These sPLA$_2$s prefer anionic membranes, bind heparanoids, and promote stimulus-dependent, AA-selective release and COX-2 induction. These sPLA$_2$s (an exception is sPLA$_2$-IIF, whose function does not depend on HSPG (11)) may be sorted into caveolae/rafts and internalized into particular membrane compartments that are assumed to be rich in AA and anionic membrane surfaces. Certain lipid-hydrolyzing products (fatty acids, lysophospholipids, or their derivatives) spatiotemporally generated by these sPLA$_2$s in these compartments may be linked to COX-2 induction. The occurrence of these distinct regulatory pathways depends on cell types and stimuli.

Although this series of studies has provided useful information about sPLA$_2$ behaviors and functions, much work is still needed to determine the complete set of functions of each sPLA$_2$ *in vivo*. Each sPLA$_2$ displays different tissue distribution and stimulus inducibility, implying that the sPLA$_2$ members exhibit nonredundant functions in each tissue. Some sPLA$_2$s may act as ligands (like cytokines), rather than enzymes, to transduce signals via the distinct classes of sPLA$_2$ receptors (32). Some sPLA$_2$s may play roles in defense against bacterial infection (which appears to be true for sPLA$_2$-IIA) (19, 20), lipoprotein metabolism (21, 22, 75, 76), and other biological events (23, 24, 77). Determining the precise localization of each sPLA$_2$ in various physiological and pathological tissues, developing inhibitors specific for each sPLA$_2$, and targeted disruption or transgenic expression of each sPLA$_2$ will yield more informative and conclusive answers.

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