An emerging body of evidence suggests that type IIA secretory phospholipase A₂ (sPLA₂-IIA) participates in the amplification of the stimulus-induced cyclooxygenase (COX)-2-dependent delayed prostaglandin (PG)-biosynthetic response in several cell types. However, the biological importance of the ability of sPLA₂-IIA to bind to heparan sulfate proteoglycan (HSPG) on cell surfaces has remained controversial. Here we show that glycican, a glycosylphosphatidylinositol (GPI)-anchored HSPG, acts as a physical and functional adaptor for sPLA₂-IIA. sPLA₂-IIA-dependent PG₂ generation by interleukin-1-stimulated cells was markedly attenuated by treatment of the cells with heparin, heparinase or GPI-specific phospholipase C, which solubilized the cell surface-associated sPLA₂-IIA. Overexpression of glycican-1 increased the association of sPLA₂-IIA with the cell membrane, and glycican-1 was colocalized and colocalized by the antibody against sPLA₂-IIA. Glycican-1 overexpression led to marked augmentation of sPLA₂-IIA-mediated arachidonic acid release, PG₂ generation, and COX-2 induction in interleukin-1-stimulated cells, particularly when the sPLA₂-IIA expression level was suboptimal. Immunofluorescent microscopic analyses of cytokine-stimulated cells revealed that sPLA₂-IIA was present in the caveolae, a microdomain in which GPI-anchored proteins reside, and also appeared in the perinuclear area in proximity to COX-2. We therefore propose that a GPI-anchored HSPG glypican facilitates the trafficking of sPLA₂-IIA into particular subcellular compartments, and arachidonic acid thus released from the compartments may link efficiently to the downstream COX-2-mediated PG biosynthesis.

Stimulus-initiated arachidonic acid (AA)³ release, which is linked with the downstream cyclooxygenase (COX) and lipoxigenase pathways for eicosanoid biosynthesis, is a highly regulated cellular response that requires gene induction and/or posttranslational modification of a group of regulatory enzymes, namely phospholipase A₂ (PLA₂) (1). An expanding recognition of the structural and functional diversity of mammalian PLA₂ enzymes has revealed that the two major classes of Ca²⁺-dependent PLA₂s, namely 85-kDa cytosolic PLA₂α (cPLA₂; type IV) and 14-kDa secretory PLA₂ (sPLA₂) isozymes (types IIA and V), act as “signaling” PLA₂s, which contribute to the release of AA from agonist-stimulated cells, depending upon the phase of cell activation (2, 3). Among them, cPLA₂ has received much attention as a key regulator of stimulus-initiated eicosanoid biosynthesis, because it selectively releases AA, shows submicromolar Ca²⁺ sensitivity, and is activated by mitogen-activated protein kinase-directed phosphorylation (4, 5). cPLA₂ undergoes Ca²⁺-dependent translocation from the cytosol to perinuclear and endoplasmic reticular membranes (6, 7), where several downstream eicosanoid-generating enzymes, including two COX isozymes, are localized (8). Studies on cPLA₂-deficient mice have confirmed its critical role in lipid mediator generation during the acute allergic response, parturition, and postischemic brain injury (9, 10).

Among several members of the PLA₂ family, sPLA₂-IIA is the most widely distributed isozyme in humans and rats (11). The expression of sPLA₂-IIA is often dramatically up-regulated by proinflammatory stimuli, such as bacterial endotoxin, interleukin (IL)-1, and tumor necrosis factor (TNF) (12–15), and is down-regulated by glucocorticoids (16). Raised sPLA₂-IIA levels at inflamed sites suggest that it plays a crucial role in the propagation of inflammatory responses (17–19), which has been further supported by recent in vivo studies (20, 21). Current in vitro studies suggest that sPLA₂-IIA can amplify stimulus-initiated AA metabolism, particularly the delayed prostaglandin (PG)-biosynthetic response, which is accompanied by de novo synthesis of sPLA₂-IIA and COX-2 (2, 3, 12, 14, 22, 23). In the mouse, sPLA₂-V, a close relative of sPLA₂-IIA, may replace sPLA₂-IIA under certain conditions (2, 3, 24, 25). However, the molecular mechanisms whereby these sPLA₂s regulate AA metabolism are still poorly understood.

sPLA₂-IIA and -V have high affinities for heparanoids (2), and significant portions of these isozymes are associated with the cell surface, most likely through binding to heparan sulfate proteoglycans (HSPGs), which are expressed in most mammalian cells. We (2, 14, 22, 23, 26, 27) and others (28, 29) have shown that some of the cellular functions of these heparin-binding sPLA₂s depend on their cell surface HSPG-binding abilities. Association of sPLA₂-IIA with heparan or chondroitin
sulfate chains increases the hydrolytic rate of phosphatidylcholine present in lipoprotein particles modestly (30, 31). On the other hand, some reports have indicated that the actions of exogenous sPLA<sub>2</sub>-IIA on cells depend only on its interfacial interaction with substrate phospholipids rather than on its association with HSPGs (32).

The cell surface HSPGs fall into two families of molecules that differ in their core protein domain structures (33). The syndecans have core proteins with a transmembrane and a cytoplasmic domain, and they possess heparan and/or chondroitin sulfate chains near the N terminus distal to the plasma membrane (34). The glypicans, by contrast, lack a membrane-spanning domain, are anchored to the external surface of the plasma membrane via glycosylphosphatidylinositol (GPI), and have three heparan sulfate chains near the C terminus, which are close to the plasma membrane (35). Consistent with a GPI-anchored moiety, glypicans are mobile in the cell membrane and exhibit both apical and basolateral distributions, whereas syndecans are distributed basolaterally to be attached to extracellular matrix proteins (36). Interestingly, recent immunohistochemical studies have revealed that significant portion of glypican translocates to the nucleus in cells undergoing cell division and activation (37). There is extensive literature concerning glycosaminoglycans in the nuclear compartment (38–40). These findings appear to be compatible with the observations that several extracellular heparin-binding growth factors, such as fibroblast growth factor (FGF) and angiogenin, translocate into the nucleus via a HSPG-dependent route (41–44).

In an effort to clarify the role of sPLA<sub>2</sub>-IIA in the regulation of the PG-biosynthetic pathway, we have identified the cellular component that is functionally associated with sPLA<sub>2</sub>-IIA. We found that a GPI-anchored HSPG glypican acts as a cellular sPLA<sub>2</sub>-IIA-binding partner that contributed to enhancement of the sPLA<sub>2</sub>-IIA-mediated, cytokine-induced, delayed PG-biosynthetic response. In agreement with the emerging notion that factors, such as fibroblast growth factor (FGF) and angiogenin, translocate to the nucleus via a HSPG-dependent route (41–44).

**EXPERIMENTAL PROCEDURES**

**Materials—**Human embryonic kidney (HEK) 293 cells were obtained from the Health Science Research Resources Bank, rat liver-derived BRL-3A cells were from RIKEN Cell Bank, and rat fibroblastic 3Y1 cells were from Dr. Y. Uehara (National Institute of Health, Tokyo). The culture conditions for these cell lines have been described previously (2, 3, 14, 27). The cDNAs for mouse sPLA<sub>2</sub>-IIA and its heparin non-binding mutant KE4 (22), mouse cPLA<sub>2</sub>, human COX-1, and human COX-2 were described previously (2, 3). The cDNA for rat glypican-1 was provided by Dr. R. Margolis (New York University Medical Center, New York, NY). The rabbit anti-human cPLA<sub>2</sub> antibody was provided by Dr. R. M. Kramer (Lilly Research). Preparation of the rabbit anti-rat cPLA<sub>2</sub>-IIA antibody and its conjugation with cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) were described previously (51). The goat anti-human COX-2 antibody and rabbit anti-human cavin-2 antibody were purchased from Santa Cruz Biotechnology. The rabbit anti-human COX-1 antibody was provided by Dr. W. L. Smith (Michigan State University, Ann Arbor, MI). The PG E<sub>2</sub> enzyme immunoassay kit was purchased from Cayman Chemical. Human TNFα was provided by Dr. H. Ishimaru (Asahi Chemical Industries). Human and mouse IL-1β were purchased from Genzyme. LipofectAMINE PLUS reagent, Opti-MEM medium, and TRIZol reagent were obtained from Life Technologies, Inc. RPMI 1640 medium was purchased from Nissui Pharmaceutical. Bacillus cereus GSI-specific phosphatase C (GPI-PLC) was purchased from Roche Molecular Biochemicals. Heparin and Flavobacterium heparinum heparinase III were purchased from Sigma. Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG, FITC-rabbit anti-goat IgG, and FITC-goat anti-rabbit IgG antibodies were purchased from Zymed Laboratories Inc. Cy3-conjugated donkey anti-rabbit IgG antibody was from Chemicon.

**Establishment of Transfectants—**Establishment of 293 cell transfectants that stably expressed sPLA<sub>2</sub>-IIA, cPLA<sub>2</sub>, COX-1, and COX-2 was described previously (2, 3). Briefly, 1 μg of each cDNA subcloned into pcDNA3.1 (Invitrogen) was mixed with 5 μl of LipofectAMINE PLUS in 200 μl of Opti-MEM medium for 30 min and then added to cells that had been plated in six-well plates (Swak) containing 1 ml of Opti-MEM. After incubation for 6 h, the medium was replaced with 2 ml of fresh culture medium comprising RPMI 1640 containing 10% (v/v) fetal calf serum (FCS). After overnight culture, the medium was replaced again with 2 ml of fresh medium and culture was continued at 37 °C in a CO<sub>2</sub> incubator flushed with 5% CO<sub>2</sub> in humidified air. In order to establish stable transfectants, cells transfected with each cDNA were cloned by limiting dilution in 96-well plates in culture medium supplemented with 800 μg/ml Geneticin (Life Technologies, Inc.). After culture for 3–4 weeks, wells containing a single colony were chosen and the expression of each protein was assessed by immunoblotting. The established clones were cloned and used for the experiments as described below.

In order to establish sPLA<sub>2</sub>-IIA/glypican-1 double transfectants, 293 transfectants expressing sPLA<sub>2</sub>-IIA were subjected to a second transfection with glypican-1 cDNA, which had been subcloned into pcDNA3.1/Zeo+ (Invitrogen) at the EcoRI site. Three days after transfection, the cells were used for the experiments or seeded into 96-well plates to be cloned by culture in the presence of 50 μg/ml zeocin (Invitrogen) in order to establish stable transfectants overexpressing both sPLA<sub>2</sub>-IIA and glypican-1. The expression of each was examined by immunoblotting, RNA blotting, and, in the case of sPLA<sub>2</sub>-IIA, by measuring the PLA<sub>2</sub> activity of the supernatants.

**SDS-Polyacrylamide Gel Electrophoresis/Immunoblotting—**Cell lysates (10<sup>7</sup> cell eq) or culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis using 10% (w/v) gels for sPLA<sub>2</sub>-IIA and 10% gels for cPLA<sub>2</sub>, COX-1, and COX-2 under non-reducing and reducing conditions, respectively. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) using a semidy blotter (MilliBlot-SD system; Millipore), according to the manufacturer’s instructions. The membranes were probed with the respective antibodies and visualized using the ECL Western blot system (Amersham Pharmacia Biotech), as described previously (22).

**Cell Activation—**293 cells (5 × 10<sup>5</sup>/ml) were seeded into each well of 24- or 48-well plates. To assess AA release, 0.1 μM [H]<sub>2</sub>AA (Amersham Pharmacia Biotech) was added to the cells in each well on day 3, when they had nearly reached confluence, and culture was continued for another day. After three washes with fresh medium, 250 μl (24-well plate) or 100 μl (48-well plate) of RPMI 1640 with or without 1 ng/ml IL-1β and/or 10% FCS was added to each well and the amount of free [H]<sub>2</sub>AA released into the supernatant during culture for 4 h was measured. The percentage release was calculated using the formula <i>S</i> / (<i>S</i> + <i>P</i>) × 100, where <i>S</i> and <i>P</i> are the radioactivity in equal portions of the supernatant and cell pellet, respectively. The supernatants from replicate cells were subjected to the PG E<sub>2</sub> enzyme immunoassay. AA release and PG generation by [H]<sub>2</sub>AA-preloaded cells were also assessed by thin layer chromatography. Among the radiolabeled products released, >90% were AA and the rest (<10%) corresponded to PGs. Among PGs, PG<sub>E</sub> was the major product, foli...
lowed by modest production of PGD₂ and PGE₂. Therefore it is likely that the radioactivity released into the supernatants largely reflects [³H]AA release.

Culture and cytokine stimulation of 3YI (14) and BRL-3A (27) cells were performed according to our previous studies with slight modifications. Briefly, 3YI or BRL-3A cells that had attained 60–80% confluency in 12-well plates (Iwaki) were replaced with Dulbecco’s modified Eagle’s medium (Nissui) containing 2% FCS. After overnight culture, the cells were stimulated with 1 ng/ml mouse IL-1β and 100 units/ml human TNFα for 24 h in the medium containing 10% FCS.

**Immunofluorophtochromatography Using Anti-sPLA2-IIA Antibody—**BRL-293 cells coexpressing sPLA2-IIA and glypican-1 were grown in a 150-mm diameter dish, washed once with phosphate-buffered saline (PBS), and lysed in 10 ml of PBS containing 1% Nonidet P-40 (Nakalai Tesque), 50 μg/ml leupeptin (Sigma), 1.5 μM pepstatin (Peptide Institute), 1 mM phenylmethanesulfonyl fluoride (Wako), and 5 mM EDTA (cell lysis buffer). After incubation for 30 min at 4 °C, the crude nuclear fraction was obtained by low spin centrifugation at 450 g, as reported previously (92). The remaining supernatants were centrifuged for 1 h at 100,000 × g at 4 °C, and the resulting supernatants were applied to a rabbit anti-sPLA2-IIA antibody-conjugated Sepharose column. After applying the samples, the column was washed with the cell lysis buffer. The bound proteins were eluted with glycine-HCl buffer (pH 2). In separate experiments, the column was washed with the cell lysis buffer containing 1 M NaCl, followed by elution with glycine-HCl buffer.

**Immunocytoaining—**293 cells expressing sPLA2-IIA, 3Y1 cells, and BRL-3A cells were seeded onto collagen-coated cover glasses (Iwaki Glass) at 2.5 × 10⁶ cells/ml, cultured for 2 days, and activated with 1 ng/ml human IL-1β (for 293 cells) or with 100 units/ml human TNFα and 1 ng/ml mouse IL-1β (for 3Y1 and BRL-3A cells) for appropriate periods. In some samples, 1 mg/ml heparin was added temporally as required for the experiments. After removing the supernatants, the cells were fixed with 2% (w/v) parafformaldehyde in PBS for 30 min at 4 °C. Then the cells were treated sequentially at room temperature with 1% (w/v) bovine serum albumin with or without 1% (w/v) saponin for 30 min in PBS to block nonspecific binding and to permeabilize the membranes, appropriate first antibodies against sPLA2-IIA (1:500 dilution), cPLA2 (1:500), COX-1 (1:1,000), COX-2 (1:200), or caveolin-2 (1:200) in PBS containing 1% albumin for 2 h, and FITC- or rhodamine-Cy3-conjugated secondary antibodies (1:100 dilution for each) in PBS containing 1% albumin for 1 h. The coverslips were mounted on glass slides using Perma Fluor (Japan Tanner) and examined using a FLUOVIEW laser fluorescence microscope (Olympus).

**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**

**Binding of sPLA₂-IIA to GPI-Anchored HSPG Is Essential for Its PG-Biosynthetic Activity—**We have previously shown that HEK293 cells transfected with sPLA₂-IIA cDNA mainly express a cell membrane-associated form of sPLA₂-IIA, which appears to play an important role in the promotion of IL-1-induced, COX-2-dependent delayed PGE₂ generation (2, 3). To verify whether sPLA₂-IIA is indeed associated with the HSPG moiety on 293 cell surfaces, we treated the cells with heparinase or exogenous heparin and then looked for the appearance of sPLA₂-IIA in their supernatants (Fig. 1, A and B). There was modest sPLA₂-IIA release, which we detected by enzyme assay (Fig. 1A) and very faintly by immunoblotting (Fig. 1B), from the sPLA₂-IIA-transfected, but not control, cells. sPLA₂-IIA activity in the supernatant increased time-dependently when the sPLA₂-IIA-expressing, but not control, cells were cultured in the presence of heparinase, which degrades heparan sulfate chains (27, 28, 35–37) (Fig. 1A). Pretreatment of the sPLA₂-IIA-expressing, but not control, cells with heparin, which competes with cell surface HSPG for heparin-binding proteins (14, 22, 26–29), also caused an increase in sPLA₂-IIA activity in the culture media (Fig. 1A), the result essentially consistent with our previous observations (2). This heparinase (data not shown) or heparin (see Fig. 5) treatment solubilized the cell-associated sPLA₂-IIA almost completely. The increased release of sPLA₂-IIA into the supernatants of the sPLA₂-IIA-expressing cells after treatment with heparinase or heparin was confirmed by immunoblotting (Fig. 1B). sPLA₂-IIA mRNA expression in the sPLA₂-IIA-transfected cells was unaffected after treatment with heparinase or heparin (data not shown).

sPLA₂-IIA-expressing, but not control, cells treated with IL-1 produced a significant amount of PGE₂ (Fig. 1C), the production of which depended upon inducible COX-2, as reported previously (2, 3). Treatment of the sPLA₂-IIA-expressing cells with heparinase suppressed this PGE₂ generation markedly; the kinetics indicated an inverse relationship between augmented sPLA₂-IIA release (Fig. 1A) and inhibition of PGE₂ generation (Fig. 1C). Treatment of replicate cells with heparin also led to a nearly 80% reduction of IL-1-induced PGE₂ generation. These results, together with our previous findings that a heparin-nonbinding sPLA₂-IIA mutant KE4, in which a cluster of four lysine residues in the C-terminal domain is replaced by glutamic acid, is not associated with the cell surface and...
does not augment IL-1-induced AA metabolism (2, 3, 22), strongly suggest that cellular HSPG is required for the full action of sPLA$_2$-IIA in this experimental system.

As there are two families of cellular HSPGs, the integral syndecans and the GPI-anchored glypicans (33–35), we wanted to know which HSPG species is the major sPLA$_2$-IIA-binding target. To address this issue, we used GPI-PLC, which cleaves the GPI linkage and is thereby capable of solubilizing GPI-anchored plasma membrane proteins and their associated proteins. We found that treatment of the sPLA$_2$-IIA-expressing, but not control, 293 cells with GPI-PLC markedly increased the amount of soluble sPLA$_2$-IIA, as assessed by both enzyme assay (Fig. 2A) and immunoblotting (Fig. 2A, inset), and reduced sPLA$_2$-IIA-mediated PGE$_2$ generation in response to IL-1 by nearly 80% (Fig. 2B). These results suggest that binding to a GPI-anchored HSPG glypican is required for sPLA$_2$-IIA to exert its PG-biosynthetic function.

**Binding of sPLA$_2$-IIA to Glypican—** To obtain more convincing evidence for the interaction between sPLA$_2$-IIA and the GPI-anchored HSPG glypican, we attempted to establish sPLA$_2$-IIA/glypican-1 double transfectants. Fig. 3A depicts the expression levels of transcripts for sPLA$_2$-IIA and glypican-1 in HEK293 cells transfected with their cDNAs alone or in combination. Endogenous glypican-1 protein was faintly detected in 293 cells (Fig. 3A, clones 1, 3, and 5), and was significantly elevated in the glypican-1 transfectants (clones 2, 4, and 6). Several clones with different expression levels of sPLA$_2$-IIA were selected, including those expressing sPLA$_2$-IIA at a low level (clones 3 and 4), a very high level (clones 5 and 6), and without expression (clones 1 and 2).

The culture supernatants of 293 clones with high sPLA$_2$-IIA expression (clones 5 and 6) were collected after 3 days of culture, and the cells were then washed for 15 min with culture medium containing 1 mM NaCl to solubilize the cell surface-associated sPLA$_2$-IIA, by a procedure that was reported earlier (2). The ratio of sPLA$_2$-IIA released into the supernatants to that associated with the cell surface increased from 1:3 in cells overexpressing sPLA$_2$-IIA alone (clone 5) to 1:10 in cells overexpressing both sPLA$_2$-IIA and glypican (clone 6), as assessed by enzyme assay. Hence, glypican-1 overexpression revealed a more than 3-fold increase in the cells' capacity to capture sPLA$_2$-IIA.

The 100,000 × g supernatant of Nonident P-40-solubilized cells coexpressing sPLA$_2$-IIA and glypican-1 (clone 6) was analyzed by immunoprecipitation using an anti-sPLA$_2$-IIA antibody-conjugated Sepharose column, followed by immunoblotting with anti-sPLA$_2$-IIA and anti-glypican-1 antibodies (Fig. 4A). After washing the column with cell lysis buffer, proteins bound to the column were eluted with an acidic buffer (pH 2). As anticipated, a 14-kDa protein band corresponding to sPLA$_2$-IIA was detected in fractions eluted from the column, but not in the flow-through and washing fractions (Fig. 4A). Notably, a 64-kDa glypican-1 protein band was detected in the acid-eluted fractions in which sPLA$_2$-IIA was also recovered, whereas the flow-through and washing fractions contained only a trace level of glypican-1 (Fig. 4A). When the replicate immunoprecipitate was washed with the cell lysis buffer containing 1 mM NaCl, which was expected to facilitate the dissociation of sPLA$_2$-IIA
Interaction between sPLA$_2$-IIA and Glypican

from the heparan sulfate chains of glypican-1 without disturbing the interaction between sPLA$_2$-IIA and the anti-sPLA$_2$-IIA antibody, and then the antibody-bound protein was eluted with the acidic buffer, whereas sPLA$_2$-IIA was detected exclusively in the acidic buffer fractions (Fig. 4B). These results support the idea that sPLA$_2$-IIA physically interacts with glypican-1, most likely through its heparan sulfate chains, in 293 cells.

Functional Interaction between sPLA$_2$-IIA and Glypican—To assess the functional significance of the sPLA$_2$-IIA:glypican interaction, the established transfectants (Fig. 3A, clones 1–6) were prelabeled with [3H]AA and delayed [3H]AA release in response to IL-1 was investigated (Fig. 3B, upper panel). Cells expressing a high level of sPLA$_2$-IIA showed increased AA release up to 2.7% (clone 5) relative to the control cells, which released no more than 0.7% AA (clone 1). AA release by the sPLA$_2$-IIA high expression clones (clone 5) was augmented to 4.4% by glypican-1 coexpression (clone 6), whereas the expression of glypican-1 alone increased AA release only minimally (clone 2), revealing a synergistic action between the two. The augmented role of glypican-1 in sPLA$_2$-IIA-mediated AA release was more obvious when the sPLA$_2$-IIA expression level was low (clone 3); AA release by these cells was comparable to that of the control cells (clone 1), indicating that this level of sPLA$_2$-IIA was insufficient to modulate IL-1-induced AA release, yet introduction of glypican-1 into these cells dramatically enhanced the AA release to a maximal and saturable level (clone 4).

The role of glypican-1 in enhancing the functions of sPLA$_2$-IIA was further pronounced when IL-1-induced PGE$_2$ biosynthesis was assessed (Fig. 3B, lower panel). Cells expressing a high level of sPLA$_2$-IIA produced 2.5 ng of PGE$_2$/10$^6$ cells (clone 5), which was augmented up to 10 ng/10$^6$ cells by coexpression with glypican-1 (clone 6), whereas glypican-1 alone increased PGE$_2$ generation only modestly (clone 2) relative to the control cells (clone 1). Although PGE$_2$ generation by cells expressing a suboptimal level of sPLA$_2$-IIA was minimal (clone 3), it reached nearly 10 ng/10$^6$ cells when glypican-1 was coexpressed in these cells (clone 4).

Comparison of the increases in AA release and PGE$_2$ generation in the clones with high sPLA$_2$-IIA expression (clones 5 and 6) revealed an approximately 1.8-fold increase in AA and a 4-fold increase in PGE$_2$, following glypican-1 coexpression, reflecting greater sensitivity of PGE$_2$ generation than of AA release to the action of sPLA$_2$-IIA. This fact led us to examine whether overexpression of sPLA$_2$-IIA and/or glypican affected the expression of endogenous COX-2, a COX isozyme predominantly involved in the conversion of AA released by sPLA$_2$-IIA to PGE$_2$ during the delayed response (2, 3, 14, 22). COX-2 expression, which was induced modestly in the control cells by IL-1 stimulation, was significantly augmented in cells transfected with sPLA$_2$-IIA alone (Fig. 3C). Cells transfected with glypican-1 alone expressed COX-2 even before IL-1 stimulation at a level comparable to that observed in IL-1-stimulated control 293 cells, and IL-1 stimulation increased its expression further in the glypican-1-expressing cells. Most importantly, coexpression of sPLA$_2$-IIA and glypican-1 increased COX-2 expression in a synergistic manner in the presence, but not in the absence, of IL-1 stimulation (Fig. 3C). These results are reminiscent of our recent finding that sPLA$_2$-V, another heparin-binding sPLA$_2$ isozyme, augments IL-1 induction of COX-2 expression in a heparin-sensitive manner, whereas the heparin-nonbinding sPLA$_2$-IIA mutant KE4 and the heparin-nonbinding isozyme sPLA$_2$-X fail to do so (53).

Collectively, these results suggest that (i) glypican acts as an adapter for sPLA$_2$-IIA, probably by bringing it close to the cellular membrane through heparan sulfate chains near the C terminus; (ii) glypican facilitates sPLA$_2$-IIA-mediated AA release from IL-1-stimulated cells; (iii) glypican and sPLA$_2$-IIA act in synergy to enhance IL-1-induced COX-2 expression; and (iv) increased AA release and COX-2 induction by the coordinated actions of glypican and sPLA$_2$-IIA lead to marked elevation of PGE$_2$ generation.

Subcellular Localization of sPLA$_2$-IIA Overexpressed in HEK293 Cells—Immunostaining of permeabilized sPLA$_2$-IIA-expressing 293 cells showed that sPLA$_2$-IIA accumulated in punctate domains and in the perinuclear area (Fig. 5, A and B). The punctate domains were evident, whereas the perinuclear signal was barely found, in non-permeabilized cells (Fig. 5C). Incubation of sPLA$_2$-IIA-expressing cells with heparin abolished the punctate sPLA$_2$-IIA signal, whereas some perinuclear staining remained in permeabilized (Fig. 5D), but not in non-permeabilized (Fig. 5E), cells. Cells expressing the heparin-nonbinding sPLA$_2$-IIA mutant KE4, which was exclusively released into the supernatant (2, 22), were not stained appreciably by the anti-sPLA$_2$-IIA antibody (Fig. 5F), although RNA blotting showed that the expression level of KE4 was comparable with that of the native enzyme in the respective transfectants (2). Based on these observations, we hypothesize that sPLA$_2$-IIA is sorted predominantly into punctate microdomains on the plasma membrane that are sensitive to washing with exogenous heparin, and that a small portion is transported into the perinuclear area, possibly by the vesicular transport machinery. Importantly, double antibody staining of sPLA$_2$-IIA-expressing cells revealed that sPLA$_2$-IIA was colocalized with caveolin-2 (Fig. 5G). This result implies that the punctate domains in which sPLA$_2$-IIA resided are caveolae, organelas that contain GPI-anchored proteins and may be responsible for potocytotic vesicular transport (49, 50). In support of this speculation, subcellular fractionation studies showed the presence of both immunoreactive sPLA$_2$-IIA and glypican-1 in the nucleus-enriched fraction (Fig. 3A).

The subcellular localization of cPLA$_2$ and two COX isofoms, COX-1 and COX-2, in the respective HEK293 transfectants was also investigated. cPLA$_2$ was detected throughout the cytoplasm in unstimulated cells (Fig. 5H), and IL-1 stimulation promoted the translocation of cPLA$_2$ to the perinuclear region.
After fixation with paraformaldehyde, cells were treated with (A) and (J) cPLA2 and COX-1 were each visualized using FITC-conjugated anti-rabbit IgG antibody and goat anti-COX-2 antibody, respectively. cPLA2 and COX-1 were each visualized using FITC-conjugated anti-rabbit IgG antibody and COX-2 by FITC-conjugated anti-goat IgG antibody.

**Fig. 5. Subcellular distributions of sPLA2-IIA and other PG-biosynthetic enzymes in HEK293 transfectants.** Immunostaining of IL-1-stimulated 293 cells expressing native sPLA2-IIA (A–E) or its heparin-nonbinding mutant KE4 (F) with anti-sPLA2-IIA antibody. After fixation with paraformaldehyde, cells were treated with (A, B, D, and F) or without (C and E) saponin, followed by rabbit anti-sPLA2-IIA antibody and FITC-conjugated anti-rabbit IgG antibody. In D and E, the cells were cultured in the presence of 1 mg/ml heparin before fixation. G, double staining of sPLA2-IIA-expressing cells with rabbit anti-sPLA2-IIA and mouse anti-caveolin-2 antibodies, which were visualized using Cy3-conjugated anti-rabbit IgG and FITC-conjugated antimouse IgG antibodies, respectively. H and I, immunostaining for cPLA2. Cells incubated with (I) or without (H) IL-1 for 10 min were fixed, permeabilized, and then treated with rabbit anti-cPLA2 antibody. J and K, immunostaining for two COX isomers. Transfectants expressing either COX-1 or COX-2 were fixed, permeabilized, and then treated with rabbit anti-COX-1 antibody and goat anti-COX-2 antibody, respectively. cPLA2 and COX-1 were each visualized using FITC-conjugated anti-rabbit IgG antibody and COX-2 by FITC-conjugated anti-goat IgG antibody.

within 10 min (Fig. 5I). This perinuclear redistribution of cPLA2 continued over 4 h of culture with IL-1 (data not shown), during which cPLA2-mediated AA release continued to take place (2, 3). Both COX-1 (Fig. 5J) and COX-2 (Fig. 5K) were found in the perinuclear envelope and endoplasmic reticulum. These staining patterns were essentially consistent with current reports (6–8).

**Subcellular Localization of Cytokine-inducible Endogenous sPLA2-IIA—**To ensure that the preferential distribution of sPLA2-IIA in the caveolae and perinuclear location was not a peculiarity of the 293 transfectants, we next examined the subcellular distribution of endogenous sPLA2-IIA, which was induced by proinflammatory stimuli in several cell types. For this purpose, we used rat fibroblastic 3Y1 cells because the expression of sPLA2-IIA and COX-2 and attendant delayed generation of PGE2 are known to be strongly induced by stimulation with IL-1 and TNF in these cells (14). Whereas sPLA2-IIA immunoreactivity was weak in the punctate domains in unstimulated 3Y1 cells (Fig. 6, A and B), it increased markedly in the punctate and perinuclear compartments in replicate cells activated with IL-1 and TNF for 24 h (Fig. 6C), the period in which delayed PGE2 generation occurs at the maximal rate (14). sPLA2-IIA staining in IL-1/TNF-stimulated cells was markedly reduced when the cells were cultured in the presence of heparin (Fig. 6D), which solubilizes the cell surface-associated sPLA2-IIA without affecting its expression level and eventually causes a reduction in PGE2 generation (14). COX-2 was distributed in the perinuclear envelope and endoplasmic reticulum in IL-1/TNF-stimulated cells (Fig. 6E), but not in control cells (data not shown). Double antibody staining for sPLA2-IIA and caveolin-2 demonstrated their colocalization (Fig. 6F), providing further support for the accumulation of endogenous sPLA2-IIA in the caveolae of cytokine-stimulated 3Y1 cells.

The other cell type we examined was rat liver-derived BRL-3A cells, in which a heparin-sensitive, membrane-associated, cytokine-inducible sPLA2-IIA has been reported to be involved in cytokine-stimulated delayed PGE2 generation (27). Immunocytostaining of BRL-3A cells revealed again that sPLA2-IIA was localized in the punctate and perinuclear domains, in which caveolin-2 coexisted, in IL-1/TNF-stimulated, but not in unstimulated, cells (Fig. 7). As in the 3Y1 cells, COX-2 was found in the perinuclear envelope and endoplasmic reticulum in IL-1/TNF-stimulated BRL-3A cells (data not shown).

**DISCUSSION**

Several previous studies have demonstrated the dependence of cellular functions of sPLA2-IIA on HSPGs, particularly when delayed PG generation is accompanied by its concomitant expression in cells exposed to proinflammatory stimuli (2, 3, 14, 22, 26, 27). In a proposed model of this, sPLA2-IIA released from cytokine-stimulated cells is captured by the heparan sulfate chains of a HSPG and thus accumulates on the plasma membrane, from which the enzyme liberates AA. sPLA2-IIA bound to a HSPG on the surface of fibroblasts greatly enhances the PG-biosynthetic response in adjacent mast cells through a juxtacrine route (23). Furthermore, deposition of sPLA2-IIA into the matrix proteoglycan biglycan increases its hydrolytic efficiency of lipoprotein particles severalfold, an event that is implicated in the exacerbation of atherosclerosis (30, 31). As opposed to these positive regulatory effects of HSPG on sPLA2-IIA functions, however, there are also some contradictory reports that sPLA2-IIA acts independently of HSPG (32), which suggests that the relation between proteoglycans and sPLA2-IIA action is more complicated than previously thought. To reconcile this discrepancy, it seemed necessary to clarify what kinds of HSPG bind sPLA2-IIA and how they affect sPLA2-IIA...
functions in cells undergoing delayed PG generation.

The findings of the present study suggest that endogenously expressed sPLA2-IIA associates with the GPI-anchored form of HSPG, glypican. Removal of GPI-anchored HSPG from cell surfaces markedly attenuated sPLA2-IIA-mediated PGE2 production (Figs. 1 and 2). sPLA2-IIA and glypican were coimmunoprecipitated from the cells, implying their association in vivo (Fig. 4). Moreover, the ability of sPLA2-IIA to enhance IL-1β-induced AA metabolism was markedly enhanced by coexpression with glypican (Fig. 3). This effect was particularly evident when the expression of sPLA2-IIA was suboptimal. Importantly, the maximal response was produced even by low concentrations of sPLA2-IIA (ng/ml as estimated by enzymatic activity and the intensity of immunoblot bands) when combined with overexpressed glypican that were about 1,000 times lower than that required for exogenously added sPLA2-IIA to exhibit AA-releasing function (23, 28, 32, 54, 55). It is therefore likely that the role of glypican is to amplify the function of sPLA2-IIA expressed at physiological levels. As demonstrated by Gelb and co-workers (32), high concentrations of exogenous sPLA2-IIA could act on cells independently of HSPG to elicit rapid and transient AA release.

GPI-anchored proteins generally occur in microdomains of the cell membrane called caveolae or the caveolae-related domain (45–48). It has been shown recently that there are dynamic changes in the subcellular distribution of glypican, which moves to the nucleus and punctate caveolae-like domains, depending upon the cell cycle (37). Based on our present finding that sPLA2-IIA is functionally associated with the GPI-anchored HSPG glypican, it is tempting to speculate that glypican plays a specific role in the sorting of sPLA2-IIA into specific subcellular compartments; AA released by sPLA2-IIA in these compartments will be more accessible to the COX-2-dependent PGE2-biosynthetic pathway than if it was released randomly from the plasma membrane surface as was thought previously. Indeed, sPLA2-IIA appears to be localized in the caveolae in three different cell lines that exhibit cytokine-stimulated delayed PGE2 generation (Figs. 5–7), although final conclusion for this localization must be awaited until more detailed electron microscopic studies will be performed. In this regard, an earlier work using electron microscopic analysis showed a punctate staining pattern of sPLA2-IIA in TNF-stimulated rat vascular smooth muscle cells, where the surface of the cave-shaped compartments on the plasma membrane gave a strong signal for sPLA2-IIA (56). We expect that the cave-shaped compartments observed in the vascular smooth muscle cells also correspond to caveolae.

Caveolae are known to form a unique endocytic and exocytic compartment at the surface of most cells, capable of importing molecules, delivering them to specific locations within the cell, and compartmentalizing a variety of signaling activities (49, 50). They are not simply an endocytic device with a peculiar membrane shape but constitute an entire membrane system with multiple functions essential for the cell. Our detection of both sPLA2-IIA and caveolin-2 in the perinuclear region (Fig. 6) suggests that the caveolae-mediated endocytotic event called potocytosis occurs in cytokine-stimulated cells. Several previous subcellular fractionation studies demonstrated the pre-
It is likely that sPLA$_2$-IIA is able to associate with proteoglycans other than glypicans. The binding of sPLA$_2$-IIA to extracellular matrix HSPGs, such as perlecan and biglycan, may prevent its movement to the plasma membrane, eventually leading to inhibition of its cellular functions. This situation is reminiscent of FGF, which is stored in the extracellular matrix as a latent form; active FGF is released by the degradation of matrix HSPG by heparinase and proteases, and then interacts with a signal-transducing receptor subunit, which has been described for the FGF receptor system in which the ligand, its tyrosine kinase receptor, and HSPG form a stable ternary complex on the cell surface, thereby potentiating transmembrane signals (41, 42, 64). The participation of a receptor-like molecule(s) in this sPLA$_2$-IIA action should be examined because many receptor molecules as well as intracellular signal-transducing molecules are known to be associated with the caveolae (49, 50). Note that the M-type receptor, the only sPLA$_2$ receptor cloned to date, possesses a sequence stretch for receptor internalization in its short cytoplasmic domain (65, 66), and that sPLA$_2$-IB, a ligand for the M-type receptor, translocates to the perinuclear compartment through the receptor-dependent process (67).

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ence of significant sPLA$_2$-IIA in some intracellular organelle fractions including the nucleus (52, 57). The perinuclear localization of sPLA$_2$-IIA is also supported by the fact that glycian-1, an sPLA$_2$-IIA-adaptor protein, has a bipartite motif for nuclear localization signals (37). Since considerable evidence has indicated that caveolae are a site of Ca$^{2+}$ storage and entry into the cell (50), it is likely that sPLA$_2$-IIA, a Ca$^{2+}$-dependent enzyme, present inside caveola particles retains its enzyme activity even after internalization and translocation to the perinuclear domain. Moreover, sPLA$_2$-IIA has been reported to be active in the presence of only micromolar concentrations of Ca$^{2+}$ under certain assay conditions (58), raising the possibility that it is active even within the cell. The caveola membrane is enriched in sphingomyelin, which inhibits the enzymatic activity of sPLA$_2$-IIA in vitro (59). The high packing density of the bilayer leaflet enriched in this lipid hinders the penetration of sPLA$_2$-IIA into the plasma membrane, which may explain why quiescent cells are fairly resistant to sPLA$_2$-IIA. In keeping with the present finding that sPLA$_2$-IIA resides in caveolae, a sphingomyelin-rich microdomain (49, 50), a decrease in the cellular sphingomyelin content caused by sphingomyelinase in response to cytokines (60) may allow the otherwise silent sPLA$_2$-IIA to become active toward the caveola membrane. Cholesterol, which is also abundant in caveoiae (49, 50), counteracts the effect of sphingomyelin-based inhibition of sPLA$_2$-IIA (62) and may contribute to the temporal and spatial regulation of this enzyme during cell activation. Rather selective release of [3H]AA by sPLA$_2$-IIA, which we observed in sPLA$_2$-transfected cells (2), may be a reflection of the fact that [3H]AA is preferentially incorporated into the caveola and perinuclear membranes (63). It should be noted, however, that we have not yet conclusively shown that AA release by sPLA$_2$-IIA for PG production is due to enzyme on the inside of the cell. The enhanced AA metabolism by glypican expression could be due to enhanced internalization of sPLA$_2$-IIA or due to enhanced capture on the outside of the cell for AA release from caveolae on the plasma membrane. This point should be clarified in a future study.

Enhancement of cytokine-induced COX-2 expression is another intriguing feature of sPLA$_2$-IIA, which was synergistically augmented by coexpression with glypican (Fig. 3C). This result is consistent with our recent finding that COX-2 induction by sPLA$_2$-IIA and sPLA$_2$-V in 293 cells depends on their binding to HSPG as well as on their enzymatic activities (53). Thus, increased production of AA metabolites by the concerted actions of sPLA$_2$-IIA and glypican may lead to further amplification of COX-2 expression through a positive feedback route in this occasion. Alternatively, the binding of sPLA$_2$-IIA to the heparan sulfate chains of glypican facilitates its presentation to the putative sPLA$_2$-IIA receptor, which transduces signals leading to increased COX-2 expression. In line with this hypothesis, COX-2 induction by sPLA$_2$-IIA in rat serosal mast cells appears to involve a receptor-mediated pathway (23). This type of receptor system, utilizing both HSPG and a signal-transducing receptor subunit, has been described for the FGF receptor system in which the ligand, its tyrosine kinase receptor, and HSPG form a stable ternary complex on the cell surface, thereby potentiating transmembrane signals (41, 42, 64). The participation of a receptor-like molecule(s) in this sPLA$_2$-IIA action should be examined because many receptor molecules as well as intracellular signal-transducing molecules are known to be associated with the caveolae (49, 50). Note that the M-type receptor, the only sPLA$_2$ receptor cloned to date, possesses a sequence stretch for receptor internalization in its short cytoplasmic domain (65, 66), and that sPLA$_2$-IB, a ligand for the M-type receptor, translocates to the perinuclear compartment through the receptor-dependent process (67).

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proteoglycan (71), which may trap sPLA₂-IIA inside the proteoglycan-stimulated cells (autocrine route) or from extracellular microenvironments (paracrine or juxtacrine route) binds to GPI-anchored HSPG, glypican, which plays a crucial role in sorting of sPLA₂-IIA into the caveola signalosomes. A significant portion of sPLA₂-IIA may enter the cells through potocytosis and reach the perinuclear area, where the upstream (cPLA₂) and downstream (COX-2) activation of mast cells causes rapid exocytosis of sPLA₂-IIA, which may prevent unregulated hydrolysis of granule membranes. Activation of mast cells causes rapid exocytosis of sPLA₂-IIA, which may be released from serum to become active on target cells.

In summary, we have provided new insight into the mechanisms that regulate delayed PG biosynthesis via sPLA₂-IIA. sPLA₂-IIA produced by cytokine-stimulated cells (autocrine route) or from extracellular microenvironments (paracrine or juxtacrine route) binds to GPI-anchored HSPG, glypican, which plays a crucial role in sorting of sPLA₂-IIA into the caveola signalosomes. A significant portion of sPLA₂-IIA may enter the cells through potocytosis and reach the perinuclear area, where the upstream (cPLA₂) and downstream (COX-2) biosynthetic response. It is likely that sPLA₂-IIA utilizes the same regulatory machinery in some cells, because it can also has the ability to increase COX-2 expression, which thereby exhibit unique fatty acid-releasing properties different from sPLA₂-IIA (53).
Interaction between sPLA₂-IIA and Glypican

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