Cellular Distribution, Post-translational Modification, and Tumorigenic Potential of Human Group III Secreted Phospholipase A2

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Human group III secreted phospholipase A2 (sPLA2-III) consists of a central group III sPLA2 domain flanked by unique N- and C-terminal domains. We found that the sPLA2 domain alone was sufficient for its catalytic activity and for its prostaglandin E2 (PGE2)-generating function in various cell types. In several if not all cell types, the N- and C-terminal domains of sPLA2-III were proteolytically removed, leading to the production of the form containing only the sPLA2 domain, which could be further N-glycosylated at two consensus sites. Immunohistochemistry demonstrated that sPLA2-III was preferentially expressed in the microvascular endothelium in human tissues with inflammation, ischemic injury, and cancer. In support of this, sPLA2-III was induced in cultured microvascular endothelial cells after stimulation with proinflammatory cytokines. Expression of sPLA2-III was also associated with various tumor cells, and colorectal cancer cells transfected with sPLA2-III exhibited enhanced PGE2 production and cell proliferation, which required sPLA2-III catalytic activity. When implanted into nude mice, the sPLA2-III-transfected cells formed larger solid tumors with increased angiogenesis compared with control cells. Moreover, small interfering RNA for sPLA2-III significantly reduced PGE2 production and proliferation of colorectal cancer cells. Taken together, these results reveal unique cell type-specific processing and N-glycosylation of sPLA2-III and the potential role of this enzyme in cancer development by stimulating tumor cell growth and angiogenesis.

In the phospholipase A2 (PLA2) family, secreted PLA2 (sPLA2) enzymes represent a group of structurally related, disulfide-rich, low molecular weight (typically 14–18 kDa) enzymes with strict Ca2+ dependence and a His-Asp catalytic dyad (1, 2). To date, 11 sPLA2 enzymes have been identified in mammals (IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIa, and XIIb). On a structural basis, these enzymes are further subdivided into three branches, namely groups I/II/V, group III, and group XII (1, 2). Individual sPLA2s exhibit unique tissue and cellular localizations and enzymatic properties, suggesting their distinct, tissue-specific roles in various pathophysiological events.

In an effort to address these questions, we herein examined the cellular localization and processing of sPLA2-III by immunohistochemistry and immunoblotting with an antibody raised against the S domain of sPLA2-III. Our results indicate that sPLA2-III is processed to the S domain-only form in several cell types that intrinsically express this enzyme. Furthermore, we show that sPLA2-III is expressed in the microvascular endothelium in various human pathologic tissues as well as in tumor cells. These observations, together with cell biological studies, reveal the unexplored tumorigenic potential of this unique sPLA2 enzyme.

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Human group III sPLA2 (sPLA2-III) is particularly unique among mammalian sPLA2s in that it consists of a central sPLA2 (S) domain and unique N-terminal (N) and C-terminal (C) domains, that the molecular mass of its full-length protein is estimated to be 55 kDa, and that the sPLA2 domain is homologous to bee venom group III sPLA2 rather than to other mammalian sPLA2s (3). Recombinant expression of this enzyme by bacterial and mammalian cell expression systems reveals that the central S domain alone is sufficient for its enzymatic functions (3, 4). On the basis of overexpression studies using HEK293 cells, the cellular arachidonate (AA)-releasing function of sPLA2-III is similar to that of sPLA2-X, which acts primarily on the phosphatidylcholine (PC)-rich outer leaflet of the plasma membrane (4–6). Because bee venom sPLA2 does not contain N and C domains corresponding to those found in human sPLA2-III, it was anticipated that these domains in the human enzyme would be removed proteolytically, resulting in the production of the form containing only the S domain, although no experimental data that directly support this hypothesis has yet been provided. Also, there is no information as to which cell types express sPLA2-III in human tissues and which functions this enzyme exerts in particular tissue microenvironments.

In an effort to address these questions, we herein examined the cellular localization and processing of sPLA2-III by immunohistochemistry and immunoblotting with an antibody raised against the S domain of sPLA2-III. Our results indicate that sPLA2-III is processed to the S domain-only form in several cell types that intrinsically express this enzyme. Furthermore, we show that sPLA2-III is expressed in the microvascular endothelium in various human pathologic tissues as well as in tumor cells. These observations, together with cell biological studies, reveal the unexplored tumorigenic potential of this unique sPLA2 enzyme.

sPLA2, secreted PLA2; TNFα, tumor necrosis factor α; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA; PC, phosphatidylcholine; WT, wild type.
EXPERIMENTAL PROCEDURES

Materials—Normal human pulmonary fibroblasts (NHPF), human pulmonary vascular smooth muscle cells (HPVSMC), human dermal microvascular endothelial cells (HMVEC), and their culture media and supplements were purchased from BioWhittaker. The human colorectal cancer cell line HT-29, the human prostate cancer cell line LNCaP, and the human epithelial bladder cell line 2B, the human prostate cancer cell line PC3, and the mouse Leydig cell line I-10 were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% (v/v) fetal calf serum, as described previously (7, 8). Rabbit antisera specific for individual human sPLA2s and pure recombinant human sPLA2s expressed by a bacterial expression system were generous gifts from Dr. M. H. Gelb (University of Washington) (9). Goat anti-human sPLA2-III antibody conjugated to horseradish peroxidase was purchased from Santa Cruz Biotechnology. Rabbit antibodies against human CD31 (an endothelial cell marker) and HAM56 (an alveolar macrophage marker) were purchased from Dako. Human sPLA2-III cDNA was kindly provided by Dr. G. Lambeau (CNRS-UPR 411, France) (3). cDNAs for truncated mutants (III-N + S (domain plus S domain), III-S + C (S domain plus C domain), and III-S (S domain alone)) for sPLA2-III (4), c-myc, and rabbit IgG A-chain (10) were described previously. Human interleukin (IL)-1β and tumor necrosis factor α (TNFα) were purchased from Genzyme. Tunicamycin, endoglycosidase H, and peptide N-glycanase F were obtained from Sigma. MTT cell counting kit was from Dojindo. Fluorescein isothiocyanate-conjugated and horseradish peroxidase-conjugated anti-IgG antibodies were purchased from Zymed Laboratories Inc. Primes for reverse transcriptase (RT)-PCR were from FASMAC. Oligonucleotides for small interfering RNA (siRNA) were from Sigma. Anti-sPLA2-III IgG antibody conjugated to horseradish peroxidase was purchased from Zymed Laboratories Inc. Anti-sPLA2-III antibody conjugated to horseradish peroxidase was purchased from Zymed Laboratories Inc. Hybridization and subsequent membrane washing were carried out as described previously (7, 8).

Expression of sPLA2-III by the Adenovirus System—Adenoviruses bearing sPLA2-III cDNAs were prepared with the ViraPower adenovirus expression system (Invitrogen) as described previously (7, 8). Briefly, the cDNAs were cloned into the pENTER Directional TOPO cloning kit (Invitrogen). After purification of the plasmids from the transformed Top10 competent cells (Invitrogen), the cDNA inserts were transferred to the pAd/CMV/V5-DEST vector (Invitrogen) by means of the Gateway system using LR clonase (Invitrogen). The plasmids were purified with a QIAxquick Giga Kit (Qiagen), and the linearized plasmids (1–2 μg) were then transfected into 293A cells (Invitrogen) in 1 ml of Opti-MEM in 6-well plates (Iwaki Glass). Then 293A cells were cultured for 1–2 weeks in RPMI1640 medium containing 10% fetal calf serum, with replacement of the medium every 2 days. When most of the transfected cells were detached from the culture plate, the medium were harvested together, freeze-thawed twice, and centrifuged to obtain the adenovirus-enhanced supernatants. Then aliquots of the supernatants were added to fresh 293A cells and cultured for 2–3 days to amplify adenoviruses. After 2–4 times of amplification, the resulting adenovirus-containing media were used as virus stocks. Viral titers were determined by the plaque-forming assay with 293A cells. As a control, the pAd/CMV/VP5/CMVlacZ vector (Invitrogen) was digested with Pael and transfected into 293A cells to produce lacZ-bearing adenovirus. Aliquots of the adenovirus-containing medium were added to the cells for subsequent analyses.

Distribution, Processing, and Functions of Group III sPLA2

Assays for AA Release and Prostanoid Generation—Assays for AA release and prostanoid generation were carried out as described previously (4–6). The primers used were as follows. III-N1 sense (N1-S) primer (5’-TCTGCTGGAAGCTCTCCGGAG-3’) and III-N1 antisense (N1-AS) primer (5’-CTCCGAGGACTCCACGACA-3’); III-N2 sense (N2-S) primer (5’-ACCTCTCTACAGTGGCTCTGG-3’) and III-N2 antisense (N2-AS) primer (5’-CACAAGGACGACTGTAAGATGT-3’); III-HQ sense (III-HQ-S) primer (5’-TGCGGGAAAAGACGCCGGT-3’) and III-HQ antisense (III-HQ-AS) primer (5’-CAGCGGCTTCTGTGTCGGCG-3’) as well as III-S sense (III-S-S) primer (5’-AACAGACTGAGCAAGCAACCT-3’) and antisense (III-S-AS) primer (4). To construct the III-S-N1 mutant, PCR was carried out with a set of III-S-S and III-N1-AS primers (for forward strand) and with a set of III-N1 sense and III-S-AS primers (for back strand) using Pyrobest polymerase (Takara Biomedicals) and sPLA2-III cDNA in pCR3.1 (Invitrogen) (4) as a template with 25 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. The III-N2 and III-S-N2 mutant cDNAs were constructed with a set of III-S-S and III-N2-AS primers (for forward strand) and III-N2-S and III-S-AS primers (for back strand) using cDNAs for sPLA2-III and III-S-N1 mutant as templates, respectively. To construct the III-HQ mutant, PCR was carried out with a set of III-S-S and III-HQ-S primers (for forward strand) and with a set of III-S-HQ-S and III-S-AS primers (for back strand) using sPLA2-III cDNA as a template. In each case, the forward and back forward were annealed, and subjected to second PCR with III-S-S and III-S-AS primers under the same thermal conditions. The PCR products were subcloned into the pENTER/D-TOPO vector for adenoviral expression, as described above. The plasmids were sequenced using a Taq cycle sequencing kit (Takara Biomedicals) and an automated DNA sequencer 310 Genetic Analyzer (Applied Biosystems) to confirm the sequences. Purification of the truncated sPLA2-III mutants was described previously (4).

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RT-PCR—Synthesis of cDNA was performed using 0.5 μg of total RNA from cells and tissues and avian myeloblastosis virus reverse transcriptase, according to the manufacturer’s instructions supplied with the RNA PCR kit (Takara Biomedicals). For amplification of cDNAs for III-N1-S and III-S-N2-S primers were used as templates, respectively. To construct the III-N1 mutant, PCR was conducted with a set of III-N1-S sense and III-S-AS primers (for back strand) using cDNAs for sPLA2-III and III-S-N1 mutan as templates, respectively. The PCR products were subcloned into the pENTER/D-TOPO vector for adenoviral expression, as described above. The plasmids were sequenced using a Taq cycle sequencing kit (Takara Biomedicals) and an automated DNA sequencer 310 Genetic Analyzer (Applied Biosystems) to confirm the sequences. Purification of the truncated sPLA2-III mutants was described previously (4).

Immunoblotting—Lytes were solubilized in Laemmli sample buffer (10 mM Tris-HCl [pH 7.4] containing 150 mM NaCl (TBS), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin) were subjected to SDS-PAGE with using 12.5% gels under reducing conditions with 2-mercaptoethanol. The separated proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) using a semi-dry blotter (Bio-Rad). After blocking with 5% (w/v) skim milk in TBS containing 0.05% (v/v) Tween 20 (TBS-T), the membranes were probed with anti-sPLA2-III antibody at 1:5,000 dilution in TBS-T for 2 h, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Zymed Laboratories Inc.) at 1:5,000 dilution in TBS-T for 2 h. After blocking with 5% (w/v) skim milk in TBS containing 0.05% (v/v) Tween 20 (TBS-T), the membranes were probed with anti-sPLA2-III antibody at 1:5,000 dilution in TBS-T for 2 h, followed by incubation with horseradish peroxi- 

dase-conjugated anti-rabbit IgG (Zymed Laboratories Inc.) at 1:5,000 dilution in TBS-T for 2 h, and were visualized using the ECL Western blot system (PerkinElmer Life Sciences), as described previously (4–6).

Immunohistochemistry—Immunohistochemistry of human lung was performed as described previously (12). Tissues were paraffin-embedded within 3 h postmortem from three cases with normal lung structure (all male, 61–76 years old). Synovial tissue sections were obtained (with approval from the ethical committee of the University) from three patients with rheumatoid arthritis (RA) (all female, 68–74 years old) having surgery at Toho University Omori Hospital. All patients were RA factor-seropositive according to the RA criteria (12) and had been treated intermittently with steroidal and nonsteroidal anti-inflammatory drugs for similar pe-
RESULTS

sPLA$_2$-III Is Capable of Augmenting PGE$_2$ Production in Various Cell Types—Although our previous study (4) demonstrated the AA-releasing and PGE$_2$-synthetic actions of human sPLA$_2$-III in HEK293 transfectants, it remained unclear whether this observation is a cell type–specific event or would be applicable to other cell types. To address this issue, we prepared adenoviruses bearing cDNAs for the full-length (FL) human sPLA$_2$-III and for its truncated mutants (N + S, S + C, and S), and we infected them into various mammalian cell types (including primary and transformed cells) to investigate their effects on PGE$_2$ production. Examples of these analyses are shown in Fig. 1, in which adenoviral overexpression of sPLA$_2$-III-FL or -S resulted in marked increases in IL-1$\beta$-stimulated PGE$_2$ production in normal human pulmonary fibroblasts (NHPF) and vascular smooth muscle cells (NHPVSMC), human colorectal (HCA-7) and prostate (PC3) cancer cells, and mouse Leydig cells (I-10). Appropriate expression of sPLA$_2$-III after sPLA$_2$-III-adenovirus infection was correlated with ongoing PGE$_2$ synthesis (Fig. 1).

N-Glycosylation of sPLA$_2$-III—To detect sPLA$_2$-III protein expressed in cells, we prepared an antibody against a synthetic peptide corresponding to the hydrophilic region near the C-terminal portion of the S domain of human sPLA$_2$-III (Fig. 2A).
To verify the specificity of the antibody, we initially used NHPF, which do not express sPLA₂-III endogenously (see below) and are therefore useful to evaluate the expression and dynamics of transfected sPLA₂-III protein. Thus, lysates of NHPF that had been infected with adenovirus for III-S or control (LacZ) were subjected to immunoblotting with the anti-sPLA₂-III antibody. Although the predicted size of III-S protein is ~16 kDa (3), our immunoblot revealed an intense 28-kDa band as well as a minor 16-kDa band in cells infected with III-S, but not with mock, adenovirus (Fig. 2B). Because there are two predicted N-glycosylation sites that are conserved in both human and mouse sPLA₂-III proteins (Asn-167 and Asn-280 in human sPLA₂-III and Asn-163 and Asn-276 in mouse sPLA₂-III; Fig. 2A), we anticipated that the 28-kDa species would represent an N-glycosylated form of III-S. Indeed, when the III-S adenovirus-infected cells were cultured in the presence of tunicamycin, an N-glycosylation inhibitor, there was a molecular mass shift of the main band from 28 to 16 kDa (Fig. 2B). Furthermore, when the lysates of III-S-transfected cells were incubated with endoglycosidase H (Fig. 2C) or peptide-N-glycosidase F (Fig. 2D), the 28-kDa band was again shifted to the 16-kDa band. These results indicate that III-S is expressed mainly as an N-glycosylated protein in NHPF. An additional 25-kDa band detected in the lysate of III-S-transfected cells after treatment with intermediate doses of peptide N-glycosidase F (Fig. 2D) may be a partially glycosylated form of III-S.

To determine which consensus N-glycosylation sites in III-S were glycosylated, adenoviruses containing N-glycosylation site mutants of III-S, in which either or both Asn residues were replaced with Ser residues (designated as III-S-N1 (N167S), -N2 (N280S), and -N1N2 (N167SN280S) mutants), were constructed and infected into NHPF. Under the condition where the expression levels of III-S-WT, -N1, -N2, and -N1N2 mRNAs were comparable (Fig. 3A), III-S-N1 and -N2 mutant proteins provided 22- and 25-kDa bands, respectively, relative to 28-kDa III-S-WT protein (Fig. 3B). This suggests that both the N1 and N2 sites are glycosylated and that a longer sugar chain is attached to the N1 site than to the N2 site. Most interestingly, the expression level of the III-S-N1 mutant protein was far lower than those of the III-S-WT and -N2 proteins (Fig. 3B). Moreover, the protein harboring mutations at both sites (N1N2) was barely detectable (Fig. 3B), even when 10 times more III-S-N1N2 adenovirus was added to the cells (data not shown). These results suggest that mutation at the N-glycosylation sites, particularly at the N1 site, influences the synthesis or stability of III-S protein in NHPF.

To assess if the N-glycosylation event would affect the enzymatic activity of III-S, lysates of NHPF that had been infected with adenovirus for III-S-WT, -N1, or -N2 (10 times more III-S-N1 adenovirus was added to the cells so that III-S-WT, -N1, and -N2 proteins were expressed almost equally, as assessed by immunoblotting (Fig. 3C)) were taken for PLA₂ enzyme assay. As shown in Fig. 3D, PLA₂ activities in the lysates of cells expressing III-S-WT, -N1, and -N2 were similar, indicating that the N-glycosylation does not influence the catalytic activity of III-S. However, we found that the PLA₂ activity secreted into the culture supernatant was significantly lower for the mutants than for III-S-WT; the activities of III-S-N1 and -N2 were about only 35 and 75%, respectively, of the activity of III-S-WT (Fig. 3E). This suggests that N-glycosylation, particularly at the N1 site, is important for proper secretion of III-S protein from the cells. PGE₂ synthesis in NHPF adenovirally transfected with III-S-WT, -N1, or -N2 is shown in Fig. 3F. Most interestingly, the PGE₂ biosynthetic abilities of III-S-WT, -N1, and -N2 (Fig. 3F) were correlated with their enzymatic activities in the culture supernatants (Fig. 3E) rather than with those remaining in the cells (Fig. 3D). These results suggest that, in this setting, III-S acts on cellular membranes to release AA mainly after secretion.

Collectively, our present results indicate that III-S can be N-glycosylated at two consensus sites and that this post-translational event affects the synthesis/stability and secretion of III-S protein in NHPF. However, subsequent studies (see below) revealed that sPLA₂-III can exist as a nonglycosylated
form in particular cell types that intrinsically express this enzyme. Therefore, the physiological and functional relevance of the N-glycosylation of sPLA2-III observed in NHPF (Figs. 2 and 3) and in several other cell types (data not shown) needs further elucidation. In addition, although there are other potential N-glycosylation sites in the C domain of human sPLA2-III (3), we did not focus on these sites in this study because these sites are not conserved between human and mouse enzymes.

Proteolytic Processing of sPLA2-III—When NHPF were infected with adenovirus for III-N + S and their lysates were then taken for immunoblot analysis with anti-sPLA2-III antibody, III-N + S protein (of which predicted size is 34 kDa (4)) appeared as multiple bands with molecular masses of ~34 and 45 kDa as well as 40 kDa at a higher adenovirus dose (Fig. 4A). Given that the S domain undergoes N-glycosylation (as noted above), the 40- and 45-kDa species may represent partially and fully N-glycosylated forms of III-N + S, respectively. In cells adenovirally transfected with III-S + C (of which the predicted size is 42 kDa (4)), a 55-kDa band was apparent at a higher adenovirus dose (Fig. 4A), suggesting that III-S + C protein is also N-glycosylated. Indeed, treatment of the cells with tunicamycin resulted in the shift of the bands for III-N + S and -S + C to their predicted molecular sizes (data not shown). Of interest, in cells expressing III-S + C, and to a much lesser extent III-N + S, an additional 28-kDa band, which co-migrated with the N-glycosylated form of III-S, was evident (Fig. 4A). In particular, III-S + C was almost entirely converted to the 28-kDa form when the cells were infected with a lower dose of III-S + C adenovirus. These results suggest that both the N and C domains can be cleaved leading to the production of III-S (or a closely related form) and that in NHPF the peptide bond between the S and C domains is more susceptible to this processing than is that between the N and S domains.

When immunoblotting with anti-sPLA2-III antibody was performed on parental (data not shown) or mock adenovirus-infected (Fig. 4B) BEAS-2B cells, a human bronchial epithelial cell line that expressed endogenous sPLA2-III mRNA (4), a 16-kDa band (likely corresponding to endogenous III-S protein) was faintly detected. The expression level of this band was unaltered after stimulation of the cells with IL-1β (data not shown). Following the infection of BEAS-2B cells with III-S adenovirus, the expression of the 16-kDa protein (exactly the same size as that detected in control cells) was markedly increased, with only a minor fraction expressed as a 28-kDa N-glycosylated form (Fig. 4B). In cells infected with adenovirus for III-N + S or -S + C, a major 16-kDa band and a faint 28-kDa band were again evident, whereas no immunoreactive bands with molecular masses of >35 kDa were detected. Neither the 16- nor the 28-kDa band was detected by control antibody.
These results suggested that both the N and C domains were entirely removed by certain processing proteases leading to the formation of III-S, the majority of which exists as a form with no or few sugar moieties, in BEAS-2B cells.

Immunohistochemistry.—To determine which cell types intrinsically express sPLA₂-III in human tissues, we performed immunohistochemistry with anti-sPLA₂-III antibody on several tissues. In normal human lungs, sPLA₂-III immunoreactivity was localized in bronchial epithelial cells (Fig. 5A, panels a and b), whereas aged, denucleated myocardiocytes were intensely positive for sPLA₂-IIA (panel e) and -V (panel g); and sPLA₂-IIA, but not -V, was also expressed in cardiovascular smooth muscle cells (green arrowheads). Repetitive results of three sections for each tissue are shown. No staining was found with control antibody in the tissues examined (not shown).

In human myocardial tissues with acute infarction, vascular endothelial cells adjacent to cardiomyocytes (Fig. 5C, panels a and c) and those in lesions with granulation (Fig. 5C, panel b) showed sPLA₂-III staining, whereas staining in cardiomyocytes was scarce. Distribution of sPLA₂-III in endothelial cells (Fig. 5C, panel c) was verified by staining of serial sections with anti-CD31 antibody (Fig. 5C, panel d). In normal human hearts, staining of sPLA₂-III in endothelial cells was poor, although a weak signal was found in some vessels (Fig. 5C, panel e). In comparison, sPLA₂-IIA (Fig. 5C, panel f) and sPLA₂-V (Fig. 5C, panel g) showed intense staining in cardiomyocytes that were devoid of nuclei, implying that these two enzymes are associated with severely damaged cardiomyocytes. Vascular smooth muscle cells in the coronary arteries were positively stained for sPLA₂-IIA (Fig. 5C, panel d) but not for sPLA₂-V (Fig. 5C, panel e). There was no appreciable staining of sPLA₂-IIA and -V in endothelial cells. Control antibody did not provide significant signals in the tissues tested above (data not shown).

We next examined the localization of sPLA₂-III in the human uterus (Fig. 6A), breast (Fig. 6B), and colon (Fig. 6C) cancer
Although no obvious staining of sPLA₂-III was found in the normal uterus (Fig. 6A, panel a), it was detected in tumor cells (green arrows) and neighboring microvascular endothelial cells (red arrows) (panels b and c). sPLA₂-V was detected in the interstitial fibroblasts (yellow arrows) beneath the tumor cells (panel d). No staining was found with control antibody (panel e).

B, staining of breast cancer. sPLA₂-III immunoreactivity was distributed in tumor cells and adjacent microvascular endothelial cells (panels a and b). Tumor cells were also positively stained with anti-sPLA₂-IIA (panel c) and -IID (panel d) antibodies but not with anti-sPLA₂-III (panel e) and control (panel f) antibodies. Vascular smooth muscle cells (blue arrow) were negatively stained for sPLA₂-IIID (panel e). C and D, staining of normal colon and colorectal cancer for sPLA₂-III (C) and other sPLA₂s (D). C, although sPLA₂-III was undetectable in normal colorectal glands (panel a), its positive staining was detected in cancer cells and adjacent microvascular endothelial cells (panels b and c). Control antibody yielded no signals in cancer tissues (panel d). D, sPLA₂-IIA was intensely positive in normal colorectal glandular cells (panel a), although its staining in colorectal cancer cells was scarce (panel b). sPLA₂-IID was detected in normal colorectal glandular cells, particularly in the surface mucosa (panel c), and was also intensely expressed in adenocarcinoma cells (panel d). sPLA₂-V was not detected in normal colorectal glands (panel e), although its scattered staining was found in adenocarcinoma cells (panel f). sPLA₂-X was predominantly located in the interstitium of normal colorectal glands (panel g) and cancer (panel h) tissues. Representative results of three sections for each tissue are shown.
Expression and function of sPLA2-III in primary human microvascular endothelial cells. A, HMVEC were stimulated for the indicated periods with 1 ng/ml IL-1β or TNFα, and the expression of transcripts for sPLA2-III and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by RT-PCR. B, HMVEC grown on collagen-coated dishes were stimulated for the indicated periods with IL-1β. Then the cells were fixed, permeabilized, and immunostained with anti-sPLA2-III or control antibody. C, HMVEC were infected with adenovirus for III-S, III-N + S, III-S + C, or LacZ for 36 h and then stimulated for 24 h with IL-1β. The cell lysates were subjected to immunoblotting with anti-sPLA2-III or control antibody (Ab). Positions of III-S, III-N + S, and III-S + C, which are N-glycosylated (glyco), as well as that of endogenous sPLA2-III are indicated by arrows. D, HMVEC were infected for the indicated periods with III-N + S or LacZ adenovirus (multiplicity of infection = 10) in the presence of IL-1β. Then the supernatants were taken for enzyme immunoassay for 6-keto-PGFlα (a stable end product of PGI2) (bottom panel), and the cells were subjected to RNA blotting with sPLA2-III cDNA as a probe and to immunoblotting with anti-COX-2 antibody (top panel). No induction of COX-2 and therefore no production of 6-keto-PGFlα were observed in these cells in the absence of IL-1β. Representative results of three independent experiments are shown.

Expression of sPLA2-III in Microvascular Endothelial Cells—The results from the immunohistochemical analyses (Figs. 5 and 6) prompted us to examine the expression of sPLA2-III in cultured primary HMVEC. RT-PCR revealed that the expression of sPLA2-III transcript was markedly elevated in HMVEC after stimulation with IL-1β or TNFα over 3–24 h (Fig. 7A). This induction of sPLA2-III mRNA was accompanied by increased expression of its protein, as assessed by immunocytostaining with anti-sPLA2-III antibody (Fig. 7B). Although the sPLA2-III signal was faint in unstimulated cells, it was markedly induced in the endoplasmic reticulum and perinuclear Golgi (as judged by the reticular staining pattern and the secreted property of sPLA2-III) in cells stimulated for 6–24 h with IL-1β. Control antibody did not provide fluorescent signals in IL-1β-stimulated cells (Fig. 7B).

Immunoblotting of IL-1β-stimulated HMVEC with anti-sPLA2-III, but not control, antibody revealed a single 53-kDa immunoreactive band (Fig. 7C). The size of this band was similar to that of III-N + S adenovirally overexpressed in HMVEC. Adenoviral transfection of III-S + C into these cells provided doublet bands around 60 kDa, and transfection of III-S resulted in the expression of a 28-kDa protein (Fig. 7C), implying that they undergo N-glycosylation. Although these observations suggest that endogenous sPLA2-III may be expressed as III-N + S (or a closely related form) with N-glycosylation, rather than III-S, in HMVEC, its precise entity needs further investigation. Infection of III-N + S adenovirus into HMVEC in the presence of IL-1β led to increased PGI2 synthesis (Fig. 7D), which was correlated kinetically with increased expression of III-N + S and IL-1β-dependent induction of endogenous COX-2 (Fig. 7D, top panel).

sPLA2-III Promotes Cancer Cell Growth—Because sPLA2-III expression is associated with tumor cells in various cancer tissues (Fig. 6), we next examined the expression and possible functions of this enzyme in the human colorectal cancer cell line HCA-7. Immunoblotting with anti-sPLA2-III antibody showed the expression of endogenous sPLA2-III protein, which appeared mainly as a 16-kDa nonglycosylated III-S form, in HCA-7 cells (Fig. 8A). The expression of this protein was unaltered when the cells were stimulated with IL-1β (data not shown). When III-SL cDNA was stably transected into HCA-7 cells by lentivirus-mediated gene transfer, there was a marked increase in the 16-kDa III-S protein band, as well as in minor N-glycosylated species with molecular masses of ~28–34 kDa,
whereas no larger immunoreactive bands corresponding to the III-FL, -N + S, and -S + C forms were detectable (Fig. 8B). These results indicate that III-FL is entirely processed to III-S in HCA-7 cells.

When these cells were prelabeled with [3H]AA for 24 h and then cultured for an additional 4 h, more [3H]AA was released from sPLA₂-III-transfected cells than from control cells in an FCS concentration-dependent manner (Fig. 8C). Accordingly, PGE₂ generation was also markedly increased in sPLA₂-III-transfected cells as compared with that in replicate control cells (Fig. 8C). It is known that PGE₂ facilitates the proliferation of several colorectal cancer cells, including HCA-7 cells (10). In fact, the proliferation of sPLA₂-III-transfected HCA-7 cells was apparently faster than that of control cells, as revealed by their photographs in culture (Fig. 8D). Control and III-FL-transfected HCA-7 cells were seeded at 10^4 cells/ml and cultured for 5 days, and their photographs (cultured in 5% FCS) (E) and cell numbers (cultured in medium containing 1 and 5% FCS), as quantified by MTT assay (F), are shown. G, expression levels of transcripts for sPLA₂-III, c-myc, rhoA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in control and III-FL-transfected HCA-7 cells were assessed by Northern blotting. H, control and III-HQ-transfected (a III-S mutant with a point mutation at the catalytic center His) HCA-7 cells were subjected to MTT cell growth assay. Immunoblotting of these cells with anti-sPLA₂-III antibody is shown in the inset. Representative results of 2–3 experiments are shown in A, B, E, G, and inset of H, and values are mean ± S.E. (*, p < 0.05 versus replicate control cells) of 3–4 experiments in C, D, F, and H.

To assess whether these PGE₂-biosynthetic and growth-promoting actions of sPLA₂-III depend on its catalytic activity, the catalytically inactive III-S mutant III-HQ, in which the catalytic center His was replaced with Gln, was lentivirally transfected into HCA-7 cells. Appropriate expression of III-HQ as a catalytically inactive III-S mutant III-HQ, in which the catalytic center His was replaced with Gln, was lentivirally transfected into HCA-7 cells. Appropriate expression of c-myc, rhoA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in control and III-FL-transfected HCA-7 cells were assessed by Northern blotting. H, control and III-HQ-transfected (a III-S mutant with a point mutation at the catalytic center His) HCA-7 cells were subjected to MTT cell growth assay. Immunoblotting of these cells with anti-sPLA₂-III antibody is shown in the inset. Representative results of 2–3 experiments are shown in A, B, E, G, and inset of H, and values are mean ± S.E. (*, p < 0.05 versus replicate control cells) of 3–4 experiments in C, D, F, and H.

Finally, we asked if endogenous sPLA₂-III contributes to PGE₂ production and thereby cell proliferation in HCA-7 cells. To this end, adenovirus harboring sPLA₂-III-directed siRNA was infected into HCA-7 cells, and accumulation of PGE₂ in the culture medium and cell proliferation were investigated. As shown in Fig. 10A, the expression of endogenous sPLA₂-III protein was considerably decreased in cells infected with sPLA₂-III siRNA adenovirus as compared with those infected with control (LacZ or random oligo) adenovirus. In this setting, there were partial but significant reductions in PGE₂ produc-
tion and cell proliferation in sPLA₂-III siRNA-transfected cells relative to those in control cells (Fig. 10B), suggesting the partial involvement of endogenous sPLA₂-III in these events.

**DISCUSSION**

In the present study, we reported the following novel aspects of sPLA₂-III, an sPLA₂ isozyme that has unique structural properties among the mammalian sPLA₂s. (i) sPLA₂-III is capable of augmenting the PG-biosynthetic response in various cell types, and the S domain alone is sufficient for this function. (ii) sPLA₂-III is often N-glycosylated at two consensus sites in the S domain, and mutations in these sites decrease the synthesis/stability and secretion of its protein. (iii) sPLA₂-III is proteolytically processed to the S domain-only form according to cell type. (iv) In human pathologic tissues, sPLA₂-III is preferentially expressed in microvascular endothelial cells as well as in tumor cells. (v) sPLA₂-III has the ability to facilitate the growth of human colorectal cancer cells both in vitro and in vivo, the latter being associated with increased angiogenesis.

**PG Biosynthetic Action**—We have shown that sPLA₂-III is capable of augmenting PG production in various primary and transformed cells (Figs. 1 and 7D). When the PG biosynthetic capacity of sPLA₂-III was compared with that of other sPLA₂s, increased PG synthesis was observed, in general, in the order sPLA₂-X > V > -IIF ≈ -III ≈ II A in many cell types tested (data not shown). This order appears to be correlated with the ability of these sPLA₂s to interact with the PC-rich outer plasma membrane (4–6, 17–21). Bee venom group III sPLA₂ contains a membrane-binding surface composed mainly of hydrophobic residues and two basic residues that come into close contact with the membrane, and interfacerial binding of this enzyme to PC-containing membrane vesicles occurs predominantly by a nonelectrostatic mechanism (22). Because of the structural similarity between human sPLA₂-III (S domain) and bee venom sPLA₂, the human enzyme may also interact with the PC-rich plasma membrane surface in a similar way. Although a recent report (23) has proposed that sPLA₂-IIA and -X release AA prior to secretion in HEK293 cells, this does not seem to be the case for human sPLA₂-III (at least in NHPF) because the PGE₂ synthetic capacity of sPLA₂-III mutants showed good correlation with their enzymatic activities released from the cells rather than those remaining in the cells (Fig. 3).

**Post-translational Modification**—We have provided evidence that human sPLA₂-III is processed to the S domain-only form in a cell type-related fashion. Thus, the linkage between the S and C domains of sPLA₂-III is more susceptible to cleavage than that between the N and S domains in NHPF (Fig. 4A), whereas the enzyme is converted almost completely to the S domain-only form in BEAS-2B (Fig. 4B) and HCA-7 (Fig. 8, A and B). This suggests that bronchial epithelial cells and colorectal cancer cells, which intrinsically express sPLA₂-III, may contain certain processing proteases that can sufficiently cleave the N-S and S-C domain linkages. In any case, it will be essential to determine the precise cleavage sites on sPLA₂-III in order to fully understand the mechanism for the unique proteolytic processing of this enzyme. The presence of a basic doublet KR at the end of the N domain of sPLA₂-III suggests that this part can be cleaved by subtilisin-like protein convertase in the Golgi (3). Unlike sPLA₂-IB and -X, which are activated only after proteolytic removal of the N-terminal propeptide (17, 24), the N and C domains do not influence the catalytic activity of sPLA₂-III (4). Therefore, by assuming that sPLA₂-III is indeed expressed as a III-N + S or closely related form in endothelial cells (Fig. 7C), the N domain may be associated with some endothelial cell-specific functions of this enzyme. For instance, the N domain might be important for spatiotemporal sorting of the enzyme to its proper target membrane compartments, as has been proposed in HEK293 cells (4).

We have also found that the S domain of sPLA₂-III transfected into NHPF (Fig. 2) and several other cell types (data not shown) undergo N-glycosylation at two consensus sites, and this post-translational modification is required for the optimal synthesis or stability and secretion, but not the catalytic activity, of the sPLA₂-III protein (Figs. 2 and 3). In relation to this, we have recently shown that the secretion of human sPLA₂-X can be affected by its N-glycosylation in certain if not all cell...
Expression and Potential Function in Tumor Cells—Our results argue that sPLA₂-III aberrantly expressed in tumor cells may have the potential to facilitate cancer development in a manner dependent on its catalytic activity (Figs. 8–10). The role of PGE₂ in the development of cancer by promoting tumor cell growth and angiogenesis has been demonstrated by studies employing mice deficient in cytosolic PLA₂α (32), COX-2 (33), and several PGE receptors (34, 35). Expression of COX-2 and microsomal PGE synthase is elevated in many, if not all, types of cancer cells (10), and overexpression of these enzymes promotes cancer development (36, 37). In addition, lysophosphatidylcholine and lysophosphatidic acid, as well as AA, can promote the migration of endothelial cells, and exposure of these cells to exogenous sPLA₂, such as bee venom sPLA₂, elicits the same response in a manner partially dependent upon their catalytic activity (38). In this context, sPLA₂-III expressed in tumor cells and adjacent microvascular endothelial cells can also be involved in this pathway leading to cancer development through augmenting the release of AA/PGE₂ and lysophospholipids. Thus, our observations provide the basis for future studies that will evaluate whether substances that specifically inhibit the activity of sPLA₂-III are applicable to prophylaxis and/or treatment of cancers.

Future Prospects—sPLA₂-III is also expressed in bronchial epithelial cells and macrophages in the human respiratory tract (Fig. 5A). Thus, sPLA₂-III, as well as sPLA₂-V and -X that are expressed in these cells (7), may participate in lung pathology by enhancing eicosanoid synthesis or by facilitating other events such as surfactant hydrolysis (39, 40) and antimicrobial defense (41). A recent study (42) has shown that both bee venom sPLA₂ and human sPLA₂-III induce dendritic cell maturation in culture. Given that the sPLA₂-III transcript is also detected in human brain (3), sPLA₂-III might also contribute to neuronal actions through catalytic activity-dependent (43) and -independent (receptor-mediated (44–46) fashions. Studies using sPLA₂-III gene-manipulated mice will help to clarify the physiological relevance of the present observations and speculations.

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FIG. 10. Down-regulation of endogenous sPLA₂-III expression in HCA-7 cells by siRNA. A, expression of sPLA₂-III protein in HCA-7 cells 5 days after infection with adenovirus harboring LacZ (Ad-LacZ), control oligonucleotide (Ad-control oligo), or sPLA₂-III siRNA (Ad-si-III), as assessed by immunoblotting. A representative result of three independent experiments is shown. B, PGE₂ production by (upper panel) and cell proliferation of (lower panel) HCA-7 cells infected with Ad-LacZ, -control oligonucleotide, or -si-III. Values are mean ± S.E. of three experiments (*, p < 0.05 versus Ad-LacZ and -control oligonucleotide).
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Cellular Distribution, Post-translational Modification, and Tumorigenic Potential of Human Group III Secreted Phospholipase A 2
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