Group X secretory phospholipase A$_2$ (sPLA$_2$-X) possesses several structural features characteristic of both group IB and IIA sPLA$_2$s (sPLA$_2$-IB and -IIA) and is postulated to be involved in inflammatory responses owing to its restricted expression in the spleen and thymus. Here, we report the purification of human recombinant COOH-terminal His-tagged sPLA$_2$-X, the preparation of its antibody, and the purification of native sPLA$_2$-X. The affinity-purified sPLA$_2$-X protein migrated as various molecular species of 13–18 kDa on SDS-polyacrylamide gels, and $N$-glycosidase F treatment caused shifts to the 13- and 14-kDa bands. NH$_2$-terminal amino acid sequencing analysis revealed that the 13-kDa form is a putative mature sPLA$_2$-X and the 14-kDa protein possesses a propeptide of 11 amino acid residues attached at the NH$_2$ termini of the mature protein. Separation with reverse-phase high performance liquid chromatography revealed that $N$-linked carbohydrates are not required for the enzymatic activity and pro-sPLA$_2$-X has a relatively weak potency compared with the mature protein. The mature sPLA$_2$-X induced the release of arachidonic acid from phosphatidylcholine more efficiently than other human sPLA$_2$ groups (IB, IIA, IID, and V) and elicited a prompt and marked release of arachidonic acid from human monocytic THP-1 cells compared with sPLA$_2$-IB and -IIA with concomitant production of prostaglandin E$_2$. A prominent release of arachidonic acid was also observed in sPLA$_2$-X-treated human U937 and HL60 cells. Immunohistochemical analysis of human lung preparations revealed its expression in alveolar epithelial cells. These results indicate that human sPLA$_2$-X is a unique $N$-glycosylated sPLA$_2$ that releases arachidonic acid from human myeloid leukemia cells more efficiently than sPLA$_2$-IB and -IIA.

Phospholipase A$_2$ (PLA$_2$)$^1$ comprises a diverse family of lipolytic enzymes that hydrolyze the sn-2 fatty acid ester bond of glycerophospholipids to produce free fatty acid and lysophospholipids (1, 2). PLA$_2$s participate in pathophysiological processes by releasing arachidonic acid from membrane phospholipids, leading to the production of various types of proinflammatory lipid mediators, such as prostaglandins (PGs) and leukotrienes (LTs) (3). Over the past two decades, a number of PLA$_2$s have been identified and characterized. From their biochemical features, these PLA$_2$s are classified into several families (4), including secretory PLA$_2$s (sPLA$_2$s) (5–11), arachidonoyl-specific cytosolic PLA$_2$ (cPLA$_2$) (12, 13), and Ca$^{2+}$-dependent PLA$_2$s (14).

Low molecular mass sPLA$_2$s (13–18 kDa) have several features including a high disulfide bond content, a requirement for millimolar concentrations of Ca$^{2+}$ for catalysis, and a broad specificity for phospholipids with different polar head groups and fatty acyl chains (15, 16). Mammalian sPLA$_2$s are classified into different groups depending on the primary structure characterized by the number and positions of cysteine residues. At present, five types of functional sPLA$_2$s (group IB, IIA, IID, V, and X) have been identified in humans (10, 15), whereas group IIC sPLA$_2$ found in rodents is a pseudogene in humans (17). Among them, group IIA sPLA$_2$ (sPLA$_2$-IIA) is thought to play a pivotal role in the progression of inflammatory conditions, since its local and systemic levels are elevated in numerous inflammatory diseases (18, 19). However, some inbred mouse strains have a natural frameshift mutation in the sPLA$_2$-IIA gene (20, 21). The phenotype of these deficient mice is similar to that of sPLA$_2$-IIA-expressing mouse strains in their responses to various inflammatory challenges that initiate arthritis (22, 23). In addition, we have recently shown that indoxam, one of the potent sPLA$_2$ inhibitors (24), suppressed the endotoxin-induced elevation of plasma tumor necrosis factor-α levels, with a similar potency for sPLA$_2$-IIA-deficient mouse strains (25). Transgenic mice expressing the human sPLA$_2$-IIA gene do not develop any overt inflammatory conditions (26). These findings point to the need to reevaluate the contribution of sPLA$_2$-IIA in inflammatory diseases and suggest that other types of sPLA$_2$s may play pivotal roles in place of or in concert with sPLA$_2$-IIA.

Group IB sPLA$_2$ (sPLA$_2$-IB) has been thought to act as a digestive enzyme, given its abundance in digestive organs (27).
However, a series of our studies have identified a variety of biological responses induced by sPLA2-IB via binding to its specific receptor, the PLA2-receptor (PLA2R) (28-31). Recent studies with PLA2R-deficient mice have demonstrated its potential role in the production of inflammatory cytokines during the progression of endotoxic shock (32). Group V sPLA2 (sPLA2-V) has been reported to be involved in the release of lipid mediators in P388D1 murine macrophages and mouse bone marrow-derived mast cells based on antisense experiments (33, 34). Recent studies have shown that this type of sPLA2 hydrolyzes phosphatidylethanolamine (PC) more efficiently than other sPLA2 groups and also induces a rapid release of arachidonic acid from several human alveolar epithelial cells by immunohistochemical analysis.

**Human group X sPLA2 (sPLA2-X)** has been cloned from fetal lung based on sPLA2-related sequences from DNA data bases (9). The sPLA2-X cDNA clone suggests a mature sPLA2 protein of 123 amino acids and the presence of a signal peptide with 32 amino acids. The presence of an arginine doublet and other polar residues preceding the mature sPLA2-X protein indicates that the signal sequence is a prepropeptide, although there is no biochemical evidence for the cleavage sites. sPLA2-X is the most acidic (pI 5.3) among human sPLA2s thus far identified and contains one potential glycosylation site. This sPLA2-X possesses 16 cysteine residues located at positions characteristic of both sPLA2-IB and sPLA2-IIA/IIID and also has an amino acid COOH-terminal extension that is typical of sPLA2-IIA and -IIID. A 1.5-kilobase transcript coding for sPLA2-X was detected in human spleen and thymus, suggesting its potential role in the immune system and/or inflammation (9, 16), although there are no data available with respect to its functional role in inflammatory processes.

**Preparation of Human sPLA2-X**—Preparation of sPLA2-X cDNA was as described previously (28). The sPLA2-X cDNA was subcloned into the BamH I and Not I recognition sites of the expression vector. The sPLA2-X expression plasmid was constructed by the same method as described above.

**Preparation of sPLA2-V cDNA**—The sPLA2-V cDNA was amplified by PCR from human placenta genomic DNA as a template. The sequences of the upstream and downstream primers were: hGX-AS, 5′-AAAGAAGGCTTCCACCATGAAAGGCCCTCTCCTCCACTGGCT-3′ and 5′-CTCAAGTGCGGCCGCCTAGTTGGTGATGGTGATGAGGGG-3′ (the underlined sequence corresponds to the His tag). The PCR-amplified fragment was digested with NotI and XhoI, which had a recognition site in the coding region of sPLA2-V by following the ligation of the corresponding region in the native sPLA2-X plasmid. The sequence of the PCR-amplified region was confirmed, and the CDnas were inserted into the mammalian cell expression vector under β-actin promoter.

**Chromogenic PLA2 Assay—**Spectrometric PLA2 assay was performed according to the method of Reynolds et al. (36). Briefly, 180 μl of the reaction mixture solution containing 1 μM diheptanoylthio-PC, 0.3 mM Triton X-100, 0.12 mM 5′-dithiothreitol/2-nitrobenzoic acid, 10 mM CaCl2, 0.1 mM KCl, and 0.1% BSA in 25 mM Tris-HCl buffer (pH 7.5) was preincubated in a 96-well plate for 15 min at 37 °C. The reactions were initiated by the addition of 20 μl of the enzyme preparation and continued for an appropriate time at 37 °C. The reaction was monitored by the absorbance at 405 nm.

**Recombinant Expression, Purification, and Characterization of Human sPLA2-X**—A recombinant plasmid containing sPLA2-X cDNA was subcloned into pET 15b (Novagen) and transformed into E. coli strain BL21 (DE3) cells. Expression of the recombinant sPLA2-X was induced with isopropyl-β-D-thiogalactopyranoside, and the conditioned medium (10% fetal calf serum (FCS)) was applied to a Ni2+ affinity column. After elution of the bound materials with Ni2+ buffer (10 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate buffer (pH 7.4)), the anti-sPLA2-X antibodies were sedimented by ultracentrifugation at 45% acetoniure. The receptor was analyzed by SDS-PAGE and Western blotting.

**Preparation of Anti-sPLA2-X**—The anti-sPLA2-X antibodies were then applied to a reverse-phase HPLC column (Cosmosil, 5C18 300AR, 4.6 × 150 mm, Nacarai Tesque, Japan) with a gradient of acetonitrile from 20% to 95% in 0.05% trifluoroacetic acid. The recombinant sPLA2-X was detected by Coomassie Brilliant Blue staining. The protein bands were excised, and NH2-terminal amino acid sequencing was performed with an Applied Biosystems Procise Sequencer.

**Recombinant Expression of Human sPLA2-V and sPLA2-IID**—Recombinant plasmid containing sPLA2-V or sPLA2-IID was transfected into CHO cells with LipofectAMINE reagent, and stably expressing clones were selected against hygromycin B (250 μg/ml). After immunoaffinity purification, the recombinant protein was determined by Western blotting with the anti-sPLA2-X antibody. The conditioned medium was dialyzed against 10 mM Tris-HCl (pH 7.4) and loaded on a HiTrap Q column (Amersham Pharmacia Biotech). The bound proteins were eluted with a gradient of NaCl from 0 to 0.5 M. The sPLA2-X-HisTag fraction was then applied to a reverse-phase HPLC column (Cosmosil, 5C18 300AR, 4.6 × 150 mm, Nacarai Tesque, Japan) with a gradient of acetonitrile from 20% to 95% in 0.05% trifluoroacetic acid. The recombinant sPLA2-X was detected by Coomassie Brilliant Blue staining. The protein bands were excised, and NH2-terminal amino acid sequencing was performed with an Applied Biosystems Procise Sequencer.

**Preparation of Anti-sPLA2-X Ab**—After purification of rabbits with purified sPLA2-X-HisTag (200 μg), the antisera was prepared, and the IgG fractions were purified with a Hitrap column.
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Purification and Characterization of Human sPLA₂-X—As a prompt approach to preparing the antigen, human sPLA₂-X was expressed in 293T cells as a COOH-terminal His-tagged form that retained PLA₂ activity. The sPLA₂-X-HisTag protein in the conditioned medium was purified with three steps of Ni²⁺-affinity column, HiTrap Q column, and a reverse-phase HPLC. As shown in Fig. 1A, SDS-PAGE analysis of the final preparations revealed the presence of a major component with an apparent molecular mass of 16 kDa as well as a minor component of 14 kDa. Treatment with N-glycosidase F, which releases N-linked oligosaccharides, shifted the 16-kDa band to a 14-kDa single band. The amino acid sequence analysis of the 14-kDa polypeptide revealed that the NH₂-terminal sequence (GILELAGT) precisely matched the putative mature form of human sPLA₂-X (9). In the sequence of human sPLA₂-X, there is one potential N-glycosylation site at position 71 (9). Thus, these findings demonstrate that the sPLA₂-X-HisTag protein is largely expressed as an N-glycosylated mature form in 293T cells.

Using purified sPLA₂-X-HisTag protein as an antigen, rabbits were immunized, and IgG fractions in the anti-serum were purified. As shown in Fig. 1B, the purified Ab recognized human sPLA₂-X-HisTag protein but did not react with the purified sPLA₂-IB and IIA. Conversely, the commercial Abs specific for human sPLA₂-IB or IIA were not reactive with sPLA₂-X-HisTag protein (data not shown). The prepared Ab did not react with unrelated His-tagged proteins including Axl-HisTag (data not shown), demonstrating that the COOH-terminal His-tagged portion is not an epitope for this Ab.

The neutralizing effects of anti-sPLA₂-X Ab on the activities of five types of human sPLA₂₅ were examined by the chromatogenic assay. The inhibitory potency of the Ab was examined at the times when each sPLA₂ reaction reached the sub-maximum at 37 °C. Recombinant Expression, Purification, and Characterization of Human sPLA₂-X Protein—Transfection with sPLA₂-X expression plasmid and generated sPLA₂-V-expressing CHO cells were incubated with anti-sPLA₂-X Ab for 2 h and then added to the reaction mixture solution, as described above. The inhibitory potency of anti-sPLA₂-X Ab was examined at the times when each sPLA₂ reaction reached the sub-maximum at 37 °C. Recombinant Expression, Purification, and Characterization of Human sPLA₂-X Protein—Transfection with sPLA₂-X expression plasmid and generated sPLA₂-V-expressing CHO cells were incubated with anti-sPLA₂-X Ab for 2 h and then added to the reaction mixture solution, as described above. The inhibitory potency of anti-sPLA₂-X Ab was examined at the times when each sPLA₂ reaction reached the sub-maximum at 37 °C. Recombinant Expression, Purification, and Characterization of Human sPLA₂-X Protein—Transfection with sPLA₂-X expression plasmid and generated sPLA₂-V-expressing CHO cells were incubated with anti-sPLA₂-X Ab for 2 h and then added to the reaction mixture solution, as described above. The inhibitory potency of anti-sPLA₂-X Ab was examined at the times when each sPLA₂ reaction reached the sub-maximum at 37 °C. Recombinant Expression, Purification, and Characterization of Human sPLA₂-X Protein—Transfection with sPLA₂-X expression plasmid and generated sPLA₂-V-expressing CHO cells were incubated with anti-sPLA₂-X Ab for 2 h and then added to the reaction mixture solution, as described above. The inhibitory potency of anti-sPLA₂-X Ab was examined at the times when each sPLA₂ reaction reached the sub-maximum at 37 °C.

RESULTS

Preparation and Characterization of Anti-sPLA₂-X Ab—As a prompt approach to preparing the antigen, human sPLA₂-X was expressed in 293T cells as a COOH-terminal His-tagged form that retained PLA₂ activity. The sPLA₂-X-HisTag protein in the conditioned medium was purified with three steps of Ni²⁺-affinity column, HiTrap Q column, and a reverse-phase HPLC. As shown in Fig. 1A, SDS-PAGE analysis of the final preparations revealed the presence of a major component with an apparent molecular mass of 16 kDa as well as a minor component of 14 kDa. Treatment with N-glycosidase F, which releases N-linked oligosaccharides, shifted the 16-kDa band to a 14-kDa single band. The amino acid sequence analysis of the 14-kDa polypeptide revealed that the NH₂-terminal sequence (GILELAGT) precisely matched the putative mature form of human sPLA₂-X (9). In the sequence of human sPLA₂-X, there is one potential N-glycosylation site at position 71 (9). Thus, these findings demonstrate that the sPLA₂-X-HisTag protein is largely expressed as an N-glycosylated mature form in 293T cells.

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The neutralizing effects of anti-sPLA₂-X Ab on the activities of five types of human sPLA₂₅ were examined by the chromatogenic assay. The inhibitory potency of the Ab was examined at the times when each reaction reached the sub-maximum levels. Pretreatment with anti-sPLA₂-X Ab led to a dose-dependent decrease in specific activity of the sPLA₂-X-HisTag protein. At 100 μg/ml, Ab blocked the enzyme activity by approximately 90% without suppression for the other four types of sPLA₂ activities (data not shown). Taken together, these results demonstrate that the prepared Ab was specific for sPLA₂-X among the known human sPLA₂ proteins.

Purification and Characterization of Human sPLA₂-X—Hu-
Preparation of sPLA₂-X-HisTag and anti-sPLA₂-X Ab. A, purification of sPLA₂-X-HisTag protein. The COOH-terminal His-tagged sPLA₂-X protein was purified as described under “Experimental Procedures,” and the purified material (1.4 μg) was analyzed by SDS-PAGE before (lane 1) and after (lane 2) N-glycosidase F treatment. Molecular weight markers (kDa) are indicated. B, titration of anti-sPLA₂-X Ab in ELISA. The 96-well plates were coated with 100 ng of sPLA₂-X-HisTag (A), affinity-purified sPLA₂-X (Δ), sPLA₂-IB (○), and sPLA₂-IIA (●) and then analyzed by ELISA with various concentrations of anti-sPLA₂-X Ab.

Reverse-phase HPLC of the affinity-purified materials resulted in their separation into three major protein peaks. SDS-PAGE analysis of the acid-eluted materials showed the presence of 15–18-kDa broad bands, 14-kDa and 13-kDa single bands (Fig. 2A, lane 1). Treatment with N-glycosidase F caused shifts to two bands (14 and 13 kDa) (Fig. 2B, lane 1). The NH₂-terminal sequence analysis revealed that the 13-kDa major protein (GILELAGT) is a putative mature form of human sPLA₂-X (9), whereas the 14-kDa minor protein possesses an additional 11 amino acid residues (EASRILRVRHRR) at the NH₂ termini of mature protein. Reactivity and susceptibility of affinity-purified sPLA₂-X with anti-sPLA₂-X Ab were virtually identical with its His-tagged form (Fig. 1B).

Substrate Specificity of Human sPLA₂-X—The substrate preference was examined with commercially available phospholipids, which possess palmitic acid at the sn-1 position and have different fatty acids at the sn-2 position as well as polar head groups. The hydrolysis rates of sPLA₂ proteins (three HPLC fractions of sPLA₂-X, sPLA₂-IB, and sPLA₂-IIA) were determined in micelle assays containing an individual substrate of 12 types of phospholipids. As shown in Table I, HPLC fraction 1 showed only 30–40% of the activity compared with fractions 2 and 3. This reduction was conceivably due to the presence of 14-kDa pro-enzyme in fraction 1 that might have no or relatively lower enzymatic activity compared with the mature protein. Absolute activities of fraction 3 were not considerably different from those of fraction 2, and the relative activity toward each phospholipid was essentially identical among the three HPLC fractions, indicating that the N-linked carbohydrates in the mature sPLA₂-X are not essential for its PLA₂ activity as well as the substrate specificity. Among the tested phospholipids, the mature form of sPLA₂-X (fractions 2 and 3) possessed the highest hydrolysis rates toward PC, whereas sPLA₂-IB and -IIA prefer POPG to other phospholipids as reported previously (40). sPLA₂-X also possessed higher hydrolysis rates for 2-arachidonyl PE and PA compared with the other two sPLA₂ types, whereas it showed weak hydrolyzing.

### Table I: Substrate specificity of sPLA₂-X, sPLA₂-IB, and sPLA₂-IIA activities

| Substrate sn-2 fatty acid | sPLA₂-X | sPLA₂-IB | sPLA₂-IIA |
|---------------------------|---------|----------|-----------|
| Fraction 1                | Fraction 2 | Fraction 3 |
| PE                        | 34.4    | 106.6    | 82.6      |
| C18:1                     | 38.2    | 155.0    | 116.7     |
| C20:0                     | 22.0    | 76.9     | 60.8      |
| PS                        | ND      | ND       | ND        |
| PA                        | ND      | ND       | 2.6       |
| PG                        | 24.0    | 85.7     | 61.1      |

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activity toward POPS and POPA similar to sPLA₂-IB and -IIA in this assay system. The sPLA₂-X activity toward POPG was dose-dependently suppressed by the addition of 1-oxamyo-in-dolidine derivative compound, indoxam, with an IC₅₀ value of 300 nM (data not shown), which also possessed strong inhibitory potency for sPLA₂-IB and -IIA (IC₅₀ = 54 and 1.2 nM, respectively) under the same assay conditions (25).

Next, five types of human sPLA₂ preparations were subjected to the reactions with the mixed substrates composed of four types of PCs or those of three types of PEs. Since each PLA₂ reaction showed different time dependence and hydrolyzing activity due to differences in substrate specificity and enzyme quantities, the percentages of hydrolyzed PCs and PEs were examined at the time (30 min) when each hydrolysis rate was within the linear range of enzymatic assays. Among the tested PCs, sPLA₂-X showed a preference for PAPC over the other three PCs, whereas the other four types of sPLA₂s possessed a lesser preference for this phospholipid species (Fig. 3A). Among the tested PEs, all of the five sPLA₂ groups showed the best preference for PLPE (Fig. 3B). However, sPLA₂-X showed relatively higher preference for PAPE compared with the other four sPLA₂s. A similar substrate preference was observed when the N-glycosylated mature form of sPLA₂-X (fraction 2) was used (data not shown).

Release of Arachidonic Acid from THP-1 Cells by the Action of Human sPLA₂-X—The preference of sPLA₂-X for 2-arachidonyl PC suggests its involvement in the release of arachidonic acid from intact cells, because the extracellular face of the plasma membrane of mammalian cells is largely composed of zwitterionic PC and sphingomyelin (41). As the expression of sPLA₂-X transcript was restricted in the immune tissues (9), several human myeloid leukemia cell lines were used to evaluate the potency of sPLA₂-X in the release of arachidonic acid. Human monocytic THP-1 cells were incubated with sPLA₂-IB, -IIA and the nonglycosylated mature form of sPLA₂-X (fraction 3), and the released fatty acids were quantified by reverse-phase HPLC analysis. As shown in Fig. 4A, sPLA₂-X induced a prompt and marked release of arachidonic acid from THP-1 cells compared with sPLA₂-IB and -IIA. This reaction reached a steady state level within 5 min, and the amount of arachidonic acid released within 10 min was about 12- and 17-fold more than that induced by sPLA₂-IB and -IIA, respectively. No release of lactate dehydrogenase activity was detected in

**FIG. 3.** Substrate specificity of human sPLA₂s in mixed phospholipids. Five types of human sPLA₂ (1 ng of sPLA₂-IB, 5 ng of sPLA₂-IIA, 2 ng of sPLA₂-X, 20 nl of partially purified sPLA₂-IIID and 40 nl of sPLA₂-V-CHO-conditioned medium) were subjected to reactions with the mixed phospholipids composed of four types of PCs (A) or three types of PEs (B) as described under “Experimental Procedures.” Results are expressed as the percentage of hydrolyzed phospholipids within a 30-min incubation. Each point represents an average of triplicate measurements.

**FIG. 4.** Effect of sPLA₂-X on fatty acid release from THP-1 cells. Human THP-1 cells were incubated with purified human sPLA₂s, and the released fatty acids were quantified as described under “Experimental Procedures.” Time-dependent release of arachidonic acid (A) and oleic acid (B) by 500 ng/ml sPLA₂-X (HPLC fraction 3) (●), sPLA₂-IB (▲), and sPLA₂-IIA (◆). Results are expressed after subtracting the values obtained from incubation in the absence of sPLA₂ at each time point. C, dose-dependent release of arachidonic acid by sPLA₂-X (●), sPLA₂-IB (▲), and sPLA₂-IIA (◆) during the 10-min incubation at 37 °C. Results are expressed after subtracting the values obtained from incubation in the presence of various concentrations of sPLA₂ inhibitor, indoxam (●) and cPLA₂ inhibitor, AACOCF₃ (◆), for 10 min at 37 °C, and the released arachidonic acid was quantified. Results are expressed as the percentage of released amounts in the absence of these inhibitors. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.
THP-1 cells during the incubation with these sPLA\(_2\)s for up to 80 min (data not shown). As shown in Fig. 4B, sPLA\(_2\)-X also induced remarkable release of oleic acid in contrast to slight releases by sPLA\(_2\)-IB and -IIA. The oleic acid release by sPLA\(_2\)-X was rather slow compared with the arachidonic acid release, and the maximum quantity of released oleic acids by sPLA\(_2\)-X (2.0 nmol/ml at 20 min) was larger than the amount of released arachidonic acid (0.60 nmol/ml). In contrast, the palmitic acid release was not detected within 10 min for any of three sPLA\(_2\)s groups (data not shown). Fig. 4C shows the dose dependence of sPLA\(_2\)-X in the release of arachidonic acid from THP-1 cells. The release was significant at 5 ng/ml and almost the same level as that induced by 5 µg/ml sPLA\(_2\)-IB and -IIA. The arachidonic acid levels released by 5 µg/ml sPLA\(_2\)-X (1.14 nmol/ml) were about 20% that of the maximum level induced by 3 µM A23187. The N-glycosylated sPLA\(_2\)-X (fraction 2) showed essentially the same responses as nonglycosylated sPLA\(_2\)-X (data not shown). As shown in Fig. 4D, a sPLA\(_2\) inhibitor, indoxam (25), blocked the sPLA\(_2\)-X-induced arachidonic acid releases in a dose-dependent manner with an IC\(_{50}\) value of 2.5 µM, whereas a known cPLA\(_2\) inhibitor, AACOCF\(_3\) (42), did not suppress the responses at up to 100 µM. The sPLA\(_2\)-X-induced release reaction was also blocked by anti-sPLA\(_2\)-X Ab (500 µg/ml) (data not shown).

As shown in Fig. 5A, the nonglycosylated mature form of sPLA\(_2\)-X (fraction 3) induced a significant production of PGE\(_2\), which contrasted with little, if any, production by the action of sPLA\(_2\)-IB and -IIA for up to 40 min. The sPLA\(_2\)-X-induced reaction of PGE\(_2\) production was slower compared with that in the arachidonic acid release (Fig. 4A), and reached the maximum level at 20 min. As shown in Fig. 5B, sPLA\(_2\)-X induced PGE\(_2\) production with a similar dose dependence as in the case of the arachidonic acid release (Fig. 4C), whereas sPLA\(_2\)-IB and -IIA evoked a slight PGE\(_2\) production even at 5 µg/ml. The amounts of PGE\(_2\) produced by 5 µg/ml sPLA\(_2\)-X (91 fmol/ml) at 37 °C, and the released [\(^3\)H]arachidonic acid was measured as described under “Experimental Procedures.” Results are expressed after subtracting the radioactivity obtained by incubating the cells in the absence of sPLA\(_2\)s at each time point. Each point represents the mean ± S.D. of triplicate measurements. The data are representative of three experiments.

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In contrast, there were no signals in type I squamous alveolar epithelial cells. These signals were specific for sPLA2-X, since pretreatment of Ab with a 10-fold excess of sPLA2-X protein resulted in abolishment of the signals (Fig. 7B). In addition, these positive signals could not be detected in parallel control samples with nonimmunized rabbit normal IgG (Fig. 7C).

**DISCUSSION**

Among the five groups of human sPLA2s thus far cloned, the biochemical properties of sPLA2-X have been poorly characterized. The present study describes the characteristics of sPLA2-X in terms of the structure and enzymatic activity as well as the expression in human lung. The recombinant sPLA2-X was released as a N-glycosylated form in the stably transfected 293T and CHO cells (Figs. 1A and 2). In the sequences of human sPLA2s, sPLA2-IB and -V do not possess any potential glycosylation site, whereas sPLA2-IIA, -IID, and -X have one (5, 9, 10). However, sPLA2-IIA is expressed as a single 14-kDa polypeptide with no sugars in human platelets and rheumatoid synovial fluids (6), and the purified sPLA2-IIA from the stably expressed CHO cells did not have the carbohydrates. Thus, sPLA2-X is the first example of glycosylated mammalian sPLA2. In the PLA2 assays (Table I), the substrate specificity and the hydrolyzing rates were not considerably different between glycosylated and nonglycosylated sPLA2-X (fractions 2 and 3). In addition, these two forms showed similar effects on the fatty acid release in THP-1 cells. These findings demonstrate that N-linked sugar chains in sPLA2-X are not essential for the PLA2 activity. Recently, the specific terminal oligosaccharide sequences of glycoproteins have been demonstrated to participate in various biological events, including the clearance from circulation and cell-cell interactions (43), and several types of mammalian lectins, such as the selectins, have been identified in various cell types (44). Although the precise structures of carbohydrates in sPLA2-X have not yet been clarified, some lectins in the specialized cell types might recognize its sugar chains and concentrate this sPLA2 in the cell surfaces to make it accessible to phospholipids or to make it become internalized into the cells. Since sPLA2-X was expressed in the transfected cell systems in the present study, its natural status must be examined to speculate its functional significance in vivo.

In the stably transfected CHO cells, a 14-kDa form was secreted as a minor component that possesses 11 additional amino acid residues at the NH2 terminus of the mature protein. SignalP computer analysis (45) for the potential cleavage positions in its signal sequence revealed that the most likely cleavage site is present between position −12 and −11 preceding the mature protein, thus demonstrating that the 11 amino acid residues are a propeptide. HPLC fraction 1 composed of the mixtures of pro- and mature forms showed considerably weak PLA2 activity compared with the mature sPLA2-X (Table I), suggesting that the cleavage of the propeptide at the arginine doublet, which is known to be efficiently catalyzed by subtilisin-like endoproteases (46), is critical for eliciting the maximum activity. This coincided with the case of sPLA2-IB, in which the proenzyme is inactive, and the release of propeptide by serine proteases such as trypsin and plasmin is required for the display of its PLA2 activity (47). Further studies on the identification of proteases related to the cleavage reactions should provide a clue to the precise maturation processing mechanisms of sPLA2-X.

The substrate preference of sPLA2-X, -IB, and -IIA toward PCs and PEs were similar between the assays with a single substrate (Table I) and the mixed substrates (Fig. 3). However, the specific activities of each sPLA2 were not identical between the two systems, as the sPLA2 activity is known to be dramatically changed by the bile salt/phospholipid molar ratio (48) and depend on the physical state of the substrate in the micelles, as demonstrated in the case of sPLA2-V (49). sPLA2-X was found to hydrolyze 2-arachidonyl PC and PE more efficiently than the other types of sPLA2s in the PLA2 assay with the mixed phospholipids and possesses the best hydrolysis rate for PAPC.

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2 K. Kawamoto, unpublished data.
among the 12 types of phospholipids examined. This substrate preference was quite a contrast with that of other sPLA₂ proteins. sPLA₂-IB and -IIA had the best hydrolysis rate for POPG, one of the phospholipid components of lung surfactants (40). sPLA₂-IID showed a substrate specificity similar to sPLA₂-IIA in mixed phospholipid assays but possessed the highest preference for PLPE in the individual phospholipid assay (10). In contrast, sPLA₂-V had little, if any, potency for hydrolyzing 2-arachidonyl-PC and -PE (Fig. 3). This is consistent with the findings of recent studies, in which sPLA₂-V showed preferences in the order of PLPC > PPG > PAPC (49). Among the five types of sPLA₂, sPLA₂-X is the most acidic (pI 5.3) and has the largest numbers of cysteine residues (9). Although the rationale of the substrate specificity of sPLA₂-X remains unclear at present, the substrate recognition mechanism can be addressed by mutation analysis, especially at the acidic and cysteine residues.

In several human myeloid leukemia cells, sPLA₂-X induced a rapid and marked release of arachidonic acid. Since the availability of cell membrane phospholipids as the substrates for PLA₂ is limited, the sPLA₂-X-induced arachidonic acid release could become saturable within 5 to 20 min. Alternatively, a portion of the released arachidonic acid could be esterified in the cell membrane phospholipids, as reported in murine P388D₁ cells (50), and the reaction rate of release and uptake of arachidonic acid might reach a steady state. In THP-1 cells, sPLA₂-X also elicited a remarkable release of oleic acid. In the assay with PC/DOC mixed micelles (Table I), sPLA₂-IB is more active than sPLA₂-X with regard to the hydrolytic efficiency toward POPC. In contrast, the oleate release from THP-1 cells by sPLA₂-X is much larger than that by sPLA₂-IB (Fig. 4B). We found that the enzymatic activity of sPLA₂-IB is negligible toward an aqueous dispersion of PC without DOC but is dramatically increased with PC/DOC mixed micelles. This is quite a contrast to sPLA₂-X, which showed an enzymatic activity toward POPC even in the absence of DOC at almost the same level of the activity in the DOC mixed micelles. These findings indicate that sPLA₂-IB activity is markedly affected by the physical state of the substrate compared with sPLA₂-X, which might explain the differences in their potencies in the intact cell membranes that contain a mixture of phospholipids.

Calculation of the rates of arachidonic acid release from THP-1 cells revealed that the reaction rate of sPLA₂-X (526 nmol/min/mg) was ∼66- and ∼155-fold higher than that of sPLA₂-IB and -IIA, respectively. From PAPC that is mostly present at the outer leaflet of the plasma membranes (41), sPLA₂-X released arachidonic acid at ∼5- and ∼58-fold higher rate than that of sPLA₂-IB and -IIA (Table I). The relative differences in the reaction rates between these two systems suggest that some specific machinery might be involved in the induction of the prompt release reactions of sPLA₂-X in the intact cells. The PLA₂R is known to mediate the sPLA₂-IB-induced biological responses including PGE₂ production in various cell types (32). Fonteh et al. (51) have recently reported the potential role of PLA₂R in the selective release of arachidonic acid by sPLA₂-IB in several inflammatory cells including THP-1 cells. Our preliminary studies revealed that the binding affinity of human sPLA₂-X for the mouse PLA₂R is about 100-fold lower than human sPLA₂-IB, although the species differences between the ligand and receptor must be taken into consideration (16). Since a marked arachidonic acid release was also observed in sPLA₂-X-treated U937 and HL60 cells where the PLA₂R mRNA was not detectable by reverse transcription-PCR analysis (51), the PLA₂R should not be involved in this process. It has been proposed that the interaction of sPLA₂-IB with cell surface heparan sulfate proteoglycan is important for its potency for the fatty acid release (52). However, mutagenesis studies have shown that the basic amino acid residues rather than the heparinoid binding regions are the main determinants for controlling the rate of fatty acid release from sPLA₂-IB-treated intact cells (53). Recently, sPLA₂-V was found to induce fatty acid release more efficiently than sPLA₂-IB in several mammalian cells (35), and Trp-31 on the putative interfacing binding surface was suggested to play an important role in its binding to PC vesicles as well as to the outer cell membrane (54). However, sPLA₂-X is the most acidic sPLA₂ and dose not possess the corresponding Trp residue in its sequence (9). The possible contribution of anionic residues in sPLA₂-X should be evaluated by the molecular level approach to understand its strong potency for fatty acid release from intact cells.

In the PLA₂ superfamily, cPLA₂ plays a potential role in the release of arachidonic acid during the cell activation process, since it possesses substrate specificity for 2-arachidonyl phospholipids (12, 13). In THP-1 cells, we could not detect the elevation of intracellular Ca²⁺ concentration by sPLA₂-X (data not shown), and the sPLA₂-X-induced arachidonic acid release was suppressed by sPLA₂ inhibitor but not blocked by a cPLA₂ inhibitor (Fig. 4D), indicating that fatty acid release by sPLA₂-X is not dependent on the cPLA₂ activation. sPLA₂-X also elicited a significant production of PGE₂ in contrast to little production by sPLA₂-IB and -IIA (Fig. 5), suggesting its potential role in the production of lipid mediators. We also detected a significant production of LTC₄ by the action of sPLA₂-X. However, the elevated amount of LTC₄ was as little as 0.05% that of the maximum level induced by 3 μM A23187, which made a contrast to 10% in the PGE₂ production. These findings suggest that the released arachidonic acid by sPLA₂-X was efficiently metabolized via cyclooxygenase but not by 5-lipoxygenase, although the precise mechanisms underlying the functional coupling between the arachidonic acid release and eicosanoid generation remain uncertain. The sPLA₂-X-induced fatty acid releases could be relevant to the production of bioactive lysophospholipids (55) as well as to the asymmetrical arrangement of membrane phospholipids, which might lead to higher susceptibility to interact with other types of sPLA₂ (56, 57). In addition, the accumulated arachidonic acid and oleic acid might activate phospholipase D to induce functional effects including apoptosis, as reported for Jurkat T cells (58).

In human lung preparations, the sPLA₂-X protein was detected especially in type II alveolar epithelial cells. Since these cell types are known to play a role in the secretion of lung surfactant, the release of various cytokines as well as the production of eicosanoids (59), the released sPLA₂-X might regulate these cell functions by releasing fatty acids from the cell membranes or by modulating the compositions of cell membrane phospholipids. Alternatively, sPLA₂-X released from epithelial cells might act on neighboring alveolar macrophages to elicit lipid mediator releases, such as reported for transcellular PG production in fibroblasts by the action of mast cell-derived sPLA₂-V (60). The restricted expression of sPLA₂-X transcripts in immunity-related tissues (9) also suggests its involvement in inflammatory responses. Analysis of the expression levels under pathological conditions should offer more information concerning the biological roles of this enzyme.

In conclusion, we have demonstrated here that sPLA₂-X

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3 T. Ono and K. Hanasaki, unpublished data.

4 K. Nakano, N. Suzuki, Y. Yokota, and K. Hanasaki, unpublished data.
possesses more powerful potency for releasing arachidonic acid compared with sPLA₂-IB and -IIA in human myeloid leukemia cells. We think that sPLA₂-X and cPLA₂ play essential roles in mobilizing arachidonic acid from the outside and the inside of the cells, respectively. Further studies are required to establish the physiological functions of sPLA₂-X as well as to understand the precise mechanisms underlying the arachidonic acid release from intact cells. Finally, elucidation of the biological roles of each group of sPLA₂ in disease states, especially in inflammatory conditions, should be of great value for the development of subtype-specific inhibitors as therapeutic drugs.

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