Structures, Enzymatic Properties, and Expression of Novel Human and Mouse Secretory Phospholipase A2s*

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Mammalian secretory phospholipase A2s (sPLA2s) form a family of structurally related enzymes that are involved in a variety of physiological and pathological processes via the release of arachidonic acid from membrane phospholipids or the binding to specific membrane receptors. Here, we report the cloning and characterization of a novel sPLA2 that is the sixth isoform of the sPLA2 family found in humans. The novel human mature sPLA2 consists of 230 amino acids (M r = 14,000) and is most similar to group IIA sPLA2 (sPLA2-IIA) with respect to the number and positions of cysteine residues as well as overall identity (51%). Therefore, this novel sPLA2 should be categorized into group II and called sPLA2-IIE following the recently identified group IID sPLA2 (sPLA2-IID). The enzymatic properties of recombinant human sPLA2-IIE were almost identical to those of sPLA2-IIA and IID in terms of Ca 2+ requirement, optimal pH, substrate specificity, as well as high susceptibility to the sPLA2 inhibitor indoxam. Along with the biochemical properties of proteins, genetic and evolutionary similarities were also observed among these three types of group II sPLA2s as to the chromosomal location of the human gene (1p36) and the exon/intron organization. The expression of sPLA2-IIE transcripts in humans was restricted to the brain, heart, lung, and placenta in contrast to broad expression profiles for sPLA2-IIA and -IID. In sPLA2-IIA-deficient mice, the expression of sPLA2-IIE was markedly enhanced in the lung and small intestine upon endotoxin challenge, which contrasted with the reduced expression of sPLA2-IID mRNA. In situ hybridization analysis revealed elevation of sPLA2-IIE mRNA at alveolar macrophage-like cells in the lung of endotoxin-treated mice. These findings suggest a distinct functional role of novel sPLA2-IIE in the progression of inflammatory processes.

Phospholipase A2 (PLA2)1 comprises a diverse family of enzymes that catalyzes the hydrolysis of glycerophospholipids at the sn-2 position to produce free fatty acid and lysophospholipids (1, 2). PLA2s 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 and leukotrienes (3, 4). To date, several mammalian intracellular and secretory PLA2s (sPLA2s) have been characterized and classified into different families according to their biochemical features (5, 6). Intracellular PLA2β comprise the Ca 2+-sensitive arachidonoyl-specific 85-kDa cytosolic PLA2 and a number of Ca 2+-independent sPLA2s (5). In contrast, sPLA2s have several common characteristics including a relatively low molecular mass (13–18 kDa), the presence of 6–8 disulfide bridges, an absolute catalytic requirement for millimolar concentrations of Ca 2+, and a broad specificity for phospholipids with different polar head groups and fatty acid chains (4, 7). At present, five distinct sPLA2s have been identified in humans and classified into different groups (group IB, IIA, IID, V, and X) depending on the primary structure characterized by the number and positions of cysteine residues (7), while group IIC sPLA2 found in rodents is a pseudogene in humans (8). Among them, group IIA sPLA2 (sPLA2-IIA) is thought to be one of the key enzymes in the pathogenesis of inflammatory diseases, since its local and systemic levels are elevated in diseases, such as septic shock, acute pancreatitis, and rheumatoid arthritis (9–11). However, some inbred mouse strains have a natural frameshift mutation in the sPLA2-IIA gene (12, 13) and are susceptible to arthritis in the antigen-induced model similar to sPLA2-IIA-expressing mouse strains (14, 15). In addition, a potent sPLA2-specific inhibitor, indoxam (16), was reported to suppress endotoxin-induced lethal effects as well as inflammatory cytokine production with a similar potency for sPLA2-IIA-expressing and sPLA2-IIA-deficient mouse strains (16). Transgenic mice expressing the human sPLA2-IIA gene do not develop any overt inflammatory conditions (17). These findings point to the need to reassess the role of sPLA2-IIA in inflammatory diseases and suggest that other types of sPLA2 including some unidentified isoforms may play a compensatory role. For example, the most classical sPLA2, group IB sPLA2 (sPLA2-IB), has long been thought to act as a digestive enzyme because of its abundance in digestive organs (18). However, recent studies have identified sPLA2-IB as a signaling molecule that induces the lipid mediator releases via binding to its specific receptor, the PLA2 receptor (19–25). Although sPLA2-IA acts as a ligand for murine PLA2 receptor (4), our studies with mice deficient in both PLA2 receptor and sPLA2-IA have demonstrated a potential role of sPLA2-IB/PLA2-receptor-mediated responses in the pro-POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; DIG, digoxigenin.

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† The abbreviations used are: PLA2, phospholipase A2; sPLA2, secretory PLA2; sPLA2-IB, IIA, IID, IIE, V, and X group IB, IIA, IID, IIE, V, and X sPLA2, respectively; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; RACE, rapid amplification of cDNA end; LPS, lipopolysaccharide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLPE, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine; POG, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphoglycerol; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine;
duction of inflammatory cytokines during the progression of endotoxic shock (26).

Recent advances in molecular biology and the growing amount of genetic information have led to the identification of several novel types of sPLA₂s. Chen et al. (27) discovered group V sPLA₂ (sPLA₂-V) from a human genomic DNA fragment similar to the sPLA₂-IIA gene. sPLA₂-V hydrolyzes phosphatidylcholine (PC) more effectively than sPLA₂-IIA to induce the release of lipid mediators in several mammalian cells (28). Cupillard et al. (29) have isolated the cDNA of group X sPLA₂ (sPLA₂-X) which possesses 16 cysteine residues at positions characteristic of both sPLA₂-IB and -IIA. We have recently shown its strong potency for the release of arachidonic acid from several human myeloid leukemia cells (30). More recently, we and other groups (31, 32) have reported the cloning of another sPLA₂ isoform, group IID sPLA₂ (sPLA₂-IID), which shares structural and enzymatic characteristics with that of sPLA₂-IIA. The expression of sPLA₂-IID mRNA is enhanced in the thymus of rats and sPLA₂-IIA-deficient mice upon endotoxin challenge, suggesting its potential role in the progression of pathological processes. We have recently searched the DNA data base and encountered an expressed sequence tag that could represent part of a new sPLA₂ isoform. Here, we report the cloning of a cDNA encoding a novel human sPLA₂, which we called group IIE sPLA₂ (sPLA₂-IIIE) based on its structural properties. We characterized the enzymatic properties of recombinant human sPLA₂-IIIE, and we compared its expression profiles in humans as well as in endotoxin-treated mice with those of the other sPLA₂s. Such comprehensive structural information and the availability of recombinant proteins of six types of human sPLA₂ should enable assessment of the relative contribution and closer study of the biological roles of each isoform in various disease states.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human genomic DNA was purchased from Roche Molecular Biochemicals. YAC clone (957 F 12) was distributed from Center d’Etude du Polymorphisme Humain (Paris, France). All oligonucleotides were purchased from Koken Shiyaku KK (Kobe, Japan). Lipopolysaccharide (LPS, Salmonella typhosa 0901) was obtained from Difco. 1-Palmitoyl-2-docosahexaenoyl-sn-glycero-3-phosphocholine and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (PLPE) were obtained from Sigma. Other synthetic phospholipids used in the assay for substrate specificity, including 1-palmitoyl-2-oleoyl-sn-glycéro-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoyl-sn-glycéro-3-phosphoethanolamine (POPE), were purchased from Avanti Polar Lipids. 1-Palmitoyl-2-(14C)-oleoyl-sn-glycéro-3-phosphocholine (14C)-POPC) (55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. Indoxam was synthesized at Shionogi Research Laboratories (33). Rat anti-mouse F(ab’)2 antibody was purchased from Serotec Co., Ltd. (Oxford, UK). Computational analysis on the isolated cDNAs and related sequences were performed with the GENETYX program (Software Development Co., Ltd.).

**Molecular Cloning of Mouse sPLA₂-IIIE**—Mouse gene fragment of sPLA₂-IIIE was discovered by a tBLASTn search (34) against GenBank™ data base using an 11-amino acid sequence (DRCVTHDCY) around the catalytic center of the mouse sPLA₂-IIA (13). A cDNA fragment corresponding to the identified gene sequence was amplified by polymerase chain reaction (PCR). Reverse-transcribed cDNAs from various tissues of B6Bl6J mice (one of the sPLA₂-IIA-deficient strains (12, 13)) were used as templates. Primers for amplification were 5’-ctctcaagaggagagacaag-3’, 5’-tcagaganggagagaaccgc-3’ (sense), and 5’-agctggggaggagg-3’, 5’-cttggtggtggagtcgc-3’ (antisense). Two rounds of amplifications (nested PCR) were carried out with these primers and ExTaq (Takara, Japan). Amplification conditions were 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min for 30 cycles. The PCR products were separated on agarose gel, and DNA of the expected size was isolated. The recombinant plasmid was then constructed with pCRII cloning vector (Invitrogen), purified with GFX Micro Plasmid Prep Kit (Amersham Pharmacia Biotech), and sequenced with Applied Biosystems PRISM 310 genetic analyzer. From the determined DNA sequence, new primers were designed for the PCR to isolate the genomic fragments of mouse sPLA₂-IIIE genes. Two rounds of amplifications (nested PCR) were carried out with these primers to PCR with two primers, 5’-catcaggctgctcatacctigtg-3’ and 5’-gctctgctcagcggctg-3’ and ExTaq. Thermal conditions were 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min for 30 cycles. All of the amplified bands were isolated, and their sequences were determined. Three of them were identified as corresponding to sPLA₂-IIIA, -IID, and the novel type of sPLA₂ genes (~350, 800, and 350 bp, respectively), all of which were cloned at the same position. Based on the determined sequence isolation, human sPLA₂-IIIE cDNA was attempted with the strategy used to clone the mouse sPLA₂-IIIE cDNA with marathon-ready cDNA from human small intestine (CLONTECH). Two sets of primers were designed to isolate the corresponding cDNA. For 5’-extension, 5’-ggtaacctacgctaggt-3’ and 5’-agttgccagcaggaagtctg-3’ were successively used with fixed primer corresponding to the adapter sequence attached to the 5’ terminus of the cDNA. For 3’-extension, four primers were used, 5’-ggctggagcttgcagcgg-3’ and 5’-ctcaccggtggtggagctg-3’ and 5’-ttcatcaccgggtggtggagctg-3’, 5’-agggtggtagtgctggtggagctg-3’, and 5’-ggctggtggtggtggagctg-3’. The latter two primers were derived from the mouse sPLA₂-IIIE cDNA sequence at the 3’-noncoding region.

The existence and positions of the intron of human sPLA₂-IIIE gene was analyzed using several primers. The sequence of the amplified DNA from human genomic DNA was compared with that of the cDNA. Separately amplified genomic portions were arranged to cover the whole region of the open reading frame. An identical amplification pattern of the genomic fragment was observed using the YAC (957 F 12) DNA as a template.

**Recombinant Expression of Human sPLA₂-IIIE**—Two successful PCR products were performed to amplify human sPLA₂-IIIE cDNA from small intestine cDNA. The first PCR was done with 5’-atgtaatcctccccacgtcct-3’ and 5’-ctcgctaatgagtcgagttg-3’ and the second with 5’-ataattggcatctgattct-3’, and the second with 5’-tcatcaccgggtggtggagctg-3’, and the second with 5’-tcaccgggtggtggagctg-3’, and the second with 5’-agttgccagcaggaagtctg-3’. The upstream primer had a NotI recognition site and Kozak sequence (in italics). The downstream primer had a XbaI recognition site. The sPLA₂-IIIE cDNA thus amplified was digested with NotI and XbaI and inserted into pcDNA3.1 (+) (Invitrogen) to construct the expression plasmids. After sequencing confirmation, 18 µg of recombinant plasmid was transfected into 50% confluent COS-7 cells grown in 148-cm² Petri dishes with FuGENE 6 transfection reagent (Roche Molecular Biochemicals), and the culture medium was collected after 3 days. PLA₂ activities in the culture medium and cell fractions were then measured with [3H]HDL-β labeled Escherichia coli membranes as a substrate (35). The recombinant human sPLA₂-IIIE partially purified from the culture supernatant by heparin-Sepharose affinity chromatography (Amersham Pharmacia Biotech), the sPLA₂ activity was eluted with 1 M NaCl and characterized with respect to the Ca²⁺-dependence and pH optimum as described previously (32).

**PLA₂ Assay for Substrate Specificity and Evaluation of Inhibitory Potency of Indoxam**—Partially purified human sPLA₂-IIIE was subjected to reactions with mixed phospholipids composed of four types of PCs or three types of PEs with different fatty acid chains at the sn-2 position (oleic acid, linoleic acid, and arachidonic acid in PC and PE or docosahexaenoic acid in PC), as described in our previous paper (32).
The released fatty acids were quantified according to the method of Tojo et al. (36).

The inhibitory potencies of sPLA₂ inhibitor, indoxam, against six types of human sPLA₂ were evaluated as follows. Purified recombinant proteins of human sPLA₂-IB and non-glycosylated sPLA₂-X were prepared from E. coli. The DNA sequences were identified as corresponding to IIA, IID, and the novel type (36). Therefore, we attempted to isolate the corresponding genomic clone using primers based on the sequence in the conserved region. After washing, the peroxidase activity was visualized by using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates. Immunohistochemical staining for mouse alveolar macrophages was performed as follows. The prepared slides of lung sections were de-waxed, incubated in methanol containing 0.3% H₂O₂, and treated with 5% normal rabbit serum for 20 min. The slides were then incubated with rat anti-mouse macrophage F4/80 antibody in PBS containing 0.1% bovine serum albumin and then incubated with biotin-conjugated rabbit anti-rabbit antibody followed by the treatment with peroxidase avidin-biotin complex reagent. After washing, the peroxidase activity was visualized by incubation with 50 μl Tris-HCl (pH 7.4) containing 0.1% 3,3′-diaminobenzidine and 0.05% H₂O₂. The slides were then counterstained with 1% methyl green dye, dehydrated, and mounted in Entellan new (Merck).

RESULTS

Molecular Cloning of Novel sPLA₂ and Characterization of Its Genomic Organization—In searching for novel sPLA₂ in the data base, we identified a cDNA fragment (GenBank accession number AF046275) that was derived from the sequence library created by the newly developed exon-trapping method designed for retrieval of the coding region distributed in the mouse genome (39). This fragment is composed of 320 bases including several sequence ambiguities. Translation of this tagged sequence presumed the existence of a novel sPLA₂ with a characteristic of the sPLA₂-IIA/IID subfamily, although the raw sequence in the database required framematches to code functional protein. The corresponding cDNA was amplified by PCR from reverse-transcribed cDNA isolated from several mouse tissues, and the sequence determined could encode a part of the functional sPLA₂. The remaining parts were isolated from mouse spleen cDNA, and the full-length cDNA (883 bp) thus identified encoded a novel sPLA₂ consisting of 142 amino acids.

To isolate its human counterpart, we initially designed degenerate primers based on the sequence in the conserved region of the mouse novel sPLA₂. However, extensive search by the PCR using cDNA derived from several tissues with various primer combinations was unsuccessful. Therefore, we attempted to isolate the corresponding genomic clone using primers that are capable of detecting the sPLA₂-IIA/IID subfamily based on their structural similarities with mouse novel sPLA₂. Three clones were amplified from human genomic DNA by PCR and identified as corresponding to IIA, IID, and the novel type of sPLA₂ genes, all of which contain one intron at the same position. The presumed exon part of the novel sPLA₂ sequence (encoding 29 amino acids) has 85.1% identity with the corresponding part of the mouse sPLA₂ cDNA. Because the same genomic fragments (sPLA₂-IIA, -IID, and the novel type) were amplified by PCR with the same pair of primers from the YAC strain (5875 P 12) which harbored a part (1.5 megabases) of the human chromosome around 1p36, these three types of sPLA₂ genes are closely located in the vicinity of this chromosome. The 5′ and 3′ parts of the cDNA connected to the isolated genomic sequence of the novel sPLA₂ were then isolated from a human small intestine cDNA library by PCR-based protocol. The 3′-noncoding region could not be isolated with the standard strategy using the fixed sequence for the adaptor attached to the poly(A) tail of the mRNA. We utilized...
the 3’-noncoding sequence of the mouse novel sPLA2 sequence to isolate human cDNA under the assumption that the noncoding part is also made up with a similar sequence in these two animals. A presumed open reading frame of the novel human sPLA2 cDNA clone thus obtained was composed of 426 bases that encoded 142 amino acids with 89% sequence identity against the mouse counterpart.

**Structural Features of Novel sPLA2 and Comparison with Other Mammalian sPLA2s**—Amino acid sequences of novel sPLA2s are shown in Fig. 1 aligned with those of other sPLA2s in human (A) and mouse (B). The sequence similarity and hydropathy profiles suggest that the N-terminal 19 residues are signal peptides. The calculated molecular masses of human and mouse mature sPLA2s are 14.0 and 14.1 kDa containing no potential N-glycosylation site, and the calculated isoelectric points are 8.02 and 7.73, respectively. Both protein sequences contain the consensus residues conserved among the functional sPLA2s thus far identified, including an active center (His48 and Asp49) as well as a Ca2⁺-binding loop, indicating that the newly cloned cDNAs encode catalytically active sPLA2s. The novel sPLA2s possess all of the structural features of sPLA2-IIA and IID, such as 7 disulfide bridges including the Cys50-Cys133 pair and the C-terminal extension structure. However, they do not contain the specific features of sPLA2-IB (7 disulfide bridges including Cys11-Cys77, a pancreatic loop, and a pre-propeptide sequence), sPLA2-IIC (8 disulfide bonds including Cys86-Cys92), sPLA2-V (6 disulfide bridges), and sPLA2-X (8 disulfide bridges including those characteristic of both IIA and IID).
IB, a prepropeptide sequence and the C-terminal extension). These characteristics are compatible with the composition of exon/intron boundary agreed with the GT-AG consensus rule (43). The positions of intron 1 and 3 were identical around the exon/intron boundary of human sPLA 2-IIE gene. Exon parts are indicated by capital letters and the introns are indicated by lowercase letters. The intron sequence near the exon is presented. The genomic fragment initially isolated by the PCR tron boundaries of human sPLA 2-IIE gene.

During the cloning process of human cDNA, we first identified the genomic fragment of sPLA 2-IIE containing the exon part between the Ca\(^{2+}\)-binding region and the catalytic center, as well as the intron sequence. Together with this clone, genomic fragments of sPLA 2-IIA and -IID were also isolated harboring an interrupting intron at the identical position (data not shown) (41, 42). Further PCR-based analysis revealed that the open reading frame of human sPLA 2-IIE gene was interrupted with three introns as shown in Fig. 2. The sequences around the exon/intron boundary agreed with the GT-AG consensus rule (43). The positions of intron 1 and 3 were identical to those of the sPLA 2-IIA gene (41, 42). Thus, similar genomic organizations among these group II sPLA 2s suggest that they are evolutionarily related.

Recombinant Expression of Novel sPLA 2s and Characterization of sPLA 2 Activity—In order to confirm that the novel cDNAs encode functional proteins, we expressed human and mouse sPLA 2-IIE using COS-7 cells as the host and characterized the recombinant products from various aspects. As shown in Fig. 3A, significant activity was detected in the supernatants of human sPLA 2-IIE-expressing cells using radiolabeled E. coli membranes as a substrate, whereas only 2% of the total activity was detected in the cell-associated fraction, indicating that human sPLA 2-IIE is a secreted enzyme. In contrast, hardly any activity was detected in the supernatants of mouse sPLA 2-IIE expressing cells, possibly due to a very low expression level and/or a low specific activity toward this substrate. We next constructed a C-terminal histidine-tagged mouse sPLA 2-IIE cDNA and expressed it in COS-7 cells in the same manner. We could detect significant PLA 2 activity in the affinity purified materials prepared from the culture medium (data not shown). For further characterization of enzymatic properties of human sPLA 2-IIE, we partially purified it with heparin-affinity chromatography, because group II sPLA 2s (IIA and IID) are known to have high affinity for heparin (32, 44).

The PLA 2 activity of recombinant human sPLA 2-IIE was completely dependent on Ca\(^{2+}\) and required 2 mM Ca\(^{2+}\) for the maximal level (Fig. 3B), and its optimal activity was detected within pH 7–9 (Fig. 3C). These characteristics are compatible with the common features of sPLA 2s proteins thus far identified.

The substrate preference of human sPLA 2-IIE was then determined individually with 13 types of commercially available phospholipids. The summary of the results (Table I) indicated the absence of a preference for the arachidonic acid-containing phospholipids. Among the phospholipids examined, sPLA 2-IIE showed preferences in the order of POPG > PLPE = POPE, which is compatible with the substrate specificity of sPLA 2-IIA. In the reactions with mixed substrates composed of various types of PCs or PEs with different fatty acid chains at the sn-2 position, sPLA 2-IIA showed a weak hydrolizing activity toward 2-arachidonoyl PC or PE and displayed the same preference profiles as sPLA 2-IIA and -IID (data not shown). The inhibitory potency of sPLA 2-specific inhibitor was then examined for six types of human sPLA 2s. In this experiment, we used one of the 1-oxamoylindolidine derivatives, indoxam (33), which has a powerful inhibitory potency for sPLA 2-IIA activity toward POPG as a substrate (IC\(_{50}\) = 1.2 nM) with no suppression against the activities of pancreatic lipase nor cytosolic PLA 2 at 50 \(\mu\)M (16). The inhibitory activities of indoxam were examined under the optimal conditions of each sPLA 2 reaction with POPG as a substrate. As shown in Fig. 3D, strong inhibition was observed against the activities of three types of group II sPLA 2s (IIA, IID, and IIE) with IC\(_{50}\) values within 1–2 nM, whereas the other isoforms (IB, V, and X) were less sensitive to the inhibitor with over 100-fold higher IC\(_{50}\) values. Taken together, these findings demonstrate that human sPLA 2-IIE has almost identical features with group II sPLA 2s (IIA and IID) in terms of both structural and catalytic properties.

### Tissue Expression Profiles of sPLA 2-IIE and Other sPLA 2s in Humans—Relative amounts of the transcript of each sPLA 2 isoform were analyzed in various human tissues by reverse transcription PCR (RT-PCR). The expression profiles of the known five types of sPLA 2s (Fig. 4) were generally compatible with those of the previous reports. For example, the positive expression of sPLA 2-IIA mRNA in various tissues and its absence in the brain, peripheral blood leukocytes, and testis previously observed by Northern analysis (32) were clearly observed in the present analysis. Specific expression of sPLA 2-IB in the pancreas, lung, and kidney (45), as well as abundant expression of sPLA 2-V in the heart and placenta (27), was also confirmed. However, in the case of sPLA 2-X, the expression in the spleen and thymus reported in the previous paper (29) could not be detected, possibly due to the usage of different tissue sources and/or different detection systems. Furthermore, an extra PCR product was observed in addition to the band at the expected size of sPLA 2-X, which was identified as an immature or improperly spliced transcript containing part of an intron. Compared with these known sPLA 2s, the expression of sPLA 2-IIE was quite different and restricted in the brain, heart, lung, and placenta. Since a single round of PCR did not produce visible DNA bands for sPLA 2-IIA, -IID, and -X in any samples of human tissues.
of the tissues examined, their expression levels should be lower than those of sPLA2-IB, -IIA, and -V.

**Enhanced Expression of sPLA2-IIE mRNA in Mice upon Endotoxin Challenge**—As the expression of group II sPLA2 isoforms (IIA and IID) is known to be changed in pathological states (9–11, 32), the expression levels of sPLA2-IIE were examined in endotoxin-challenged mice by Northern blot analysis. In this work, we used C57BL6/J strains in which the sPLA2-IIA gene is naturally disrupted (12, 13). In untreated mice, one major transcript of sPLA2-IIE (0.5 kb) was detected in the thymus, small intestine, lung, and spleen (Fig. 5). At 24 h after LPS injection, the expression level of this transcript was elevated in the thymus, small intestine, and kidney. In addition, upon endotoxin challenge, a distinct transcript (0.7 kb) was expressed in those tissues as well as in the lung but not in the spleen. In contrast, sPLA2-IIE mRNA was barely detected in the heart, liver, and pancreas with or without LPS stimulation (data not shown). Although the estimated size of the two observed mRNAs was apparently shorter than the isolated cDNA (883 bases), RT-PCR analysis confirmed an increase of the mRNA consisting of the open reading frame upon LPS stimulation in these tissues (data not shown). In the case of sPLA2-IID, the expression of two transcripts (1.0 and 2.0 kb) was up-regulated in the thymus but obviously decreased in the spleen, small intestine, and lung of LPS-treated mice. These findings suggest that the expressions of two related group II sPLA2s are differently regulated in the inflammatory processes taking place in various tissues of mice deficient in sPLA2-IIA.

In order to examine the sPLA2-IIE expressing cell types in the lung, *in situ* hybridization analysis was performed with a specific cRNA probe. In the control mice, few, if any, signals were detected with an antisense probe (Fig. 6A). However, upon LPS challenge, intense positive signals were detected with an antisense probe in some cell types surrounding the type II pneumocyte (Fig. 6B) in contrast to few signals with a sense probe (Fig. 6C). The sPLA2-IIE-expressing cells seemed to be alveolar macrophages judging from their morphologies, and some of the sPLA2-IIE signals coincided well with the

**Fig. 3. Recombinant expression of human sPLA2-IIE cDNA in COS-7 cells.** A, sPLA2 activity measured in cell supernatants and cell lysates of COS-7 cells transiently transfected with human sPLA2-IIE cDNA. sPLA2 activity in the culture supernatants or cell lysates prepared at 72-h post-transfection was measured by hydrolysis of [3H]oleate-labeled *E. coli* membranes as described under “Experimental Procedures.” Results are expressed as the mean value ± S.E. of triplicate determinations. B, Ca2⁺ dependence of human sPLA2-IIE activity. Enzymatic activity was determined in the presence of 2 mM EDTA (Ca2⁺-free) or increasing concentrations of CaCl2. Results are expressed as the mean value ± S.E. of triplicate determinations. C, pH dependence of human sPLA2-IIE activity. Specific sPLA2 activity was determined as described under “Experimental Procedures.” Results are expressed as the mean value ± S.E. of triplicate determinations. D, inhibitory potencies of indoxam against six types of human sPLA2s. Specific sPLA2 activity in the presence of various concentrations of indoxam was determined as described under “Experimental Procedures,” and results are expressed as the percentage of specific activity of each sPLA2 in the absence of indoxam. Each point represents the mean value of triplicate determinations.
sPLA2-IIE has a substrate preference similar to that of sPLA2-IIA and -IID, which contrasted with the preferred hydrolysis of 2-arachidonoyl PC by sPLA2-X (30). In addition, a susceptibility against the sPLA2 inhibitor indoxam divided the sPLA2 family into two subgroups: subgroup IIA, IID, and IIE, and subgroup IB, V, and X (Fig. 3D). Analysis of the crystal structure of the sPLA2-IIA complex with a closely related indolizine compound revealed the inhibitor to be located near the active site in sPLA2-IIA and bound to Ca^{2+} (48). Thus, a similar substrate specificity and a high sensitivity toward indoxam strongly suggest that the three group II sPLA2s form a similar three-dimensional structure around the catalytic center. In this context, sPLA2-IIE might play a compensatory role in the degradation of the endogenous phospholipids together with sPLA2-IID especially in sPLA2-IIA-deficient mice. Defensive roles against invading bacteria may also be functions of sPLA2-IIE like IIA and IID (49), because they preferentially hydrolyze phosphatidylglycerol and PE which are major components of bacterial phospholipids.

sPLA2-IIA is thought to be one of the key enzymes in the pathogenesis of inflammatory diseases, since its enhanced expression has been observed in various cell types stimulated by inflammatory cytokines and in various inflammatory models including rat endotox shock (50). In addition, its potential contribution to arachidonic acid release via binding to cell-surface heparan sulfate proteoglycan was demonstrated by transfection/overexpression experiments (44). In mice, however, sPLA2-IIA gene is naturally disrupted by a single nucleotide insertion in some inbred strains (12, 13). Furthermore, even in BALB/c mice that express sPLA2-IIA, its expression was limited to the intestine and increased little during endotox shock (51, 52). In sPLA2-IIA-deficient mice, the expression of sPLA2-IIA was markedly enhanced in various tissues upon LPS challenge, whereas the expression of another isoform, sPLA2-IID, was slightly enhanced in the thymus but remarkably decreased in the spleen, small intestine, and lung (Fig. 5). In this respect, sPLA2-IIE might play more pivotal roles against invading bacteria.

![Tissue distribution of sPLA2-IIE and five known sPLA2s in humans](image)

**Fig. 4. Tissue distribution of sPLA2-IIE and five known sPLA2s in humans.** Human multiple tissue cDNA panels were used as templates for PCR for each sPLA2 subtype. sPLA2-IID, -IIE, and -X cDNAs were amplified with a single round of PCR (35 cycles), and sPLA2-IIB, -IID, -IIE, and -X cDNAs were amplified by two rounds of PCR (30 cycles each). G3PDH was used as an internal standard. Arrowhead indicates the size expected from the cDNA sequence of human sPLA2-X. PBL, peripheral blood leukocyte.

**TABLE I**

| Substrate | sn 2 fatty acid | Activity (μmol/min/ml) |
|-----------|-----------------|-----------------------|
|           |                 | sPLA2-IIE | sPLA2-IIA |
| PC        | C16:0 (Palmitic acid) | 0.61 | 1.12 |
| C18:2 (Linoleic acid) | 0.44 | 0.36 |
| C20:4 (Arachidonic acid) | 0.58 | 0.37 |
| PE        | C18:2 (Linoleic acid) | 1.55 | 1.17 |
| C20:4 (Arachidonic acid) | 1.71 | 2.70 |
| PS        | C18:2 (Linoleic acid) | 0.03 | ND |
| PA        | C20:4 (Arachidonic acid) | ND | ND |
| PG        | C16:0 (Palmitic acid) | 0.15 | 0.32 |
| C18:1 (Oleic acid) | 2.59 | 0.66 |

**DISCUSSION**

Recent advances in molecular biology as well as accumulating DNA information have led to the notion that secretory and intracellular PLA2 activities are attributed to the growing number of PLA2 proteins in mammals (5, 29, 31, 32, 46). Therefore, the total set of family members needs to be identified in order to understand the precise role of each isoform in various biological events.

In the present study, we cloned and characterized a novel type of sPLA2 (sPLA2-IIE), which represents the sixth isoform of the sPLA2 family in humans. sPLA2-IIE has structural features common to the group II sPLA2 (sPLA2-IIA and IID) with respect to the characteristic distribution of cysteine residues (Fig. 1). In the C-terminal region of sPLA2-IIE, there is a relatively high content of basic amino acid residues, which is also characteristic of heparin-binding PLA2s including sPLA2-IIB, -IID, and -V (32, 44). In fact, the heparin-affinity column enabled a successful purification of human sPLA2-IIE. In addition to similarities in the primary structures, the genetic locus of sPLA2-IIE was identified on human chromosome 1p36 in the vicinity of IIA and IID genes. The sPLA2-V gene was also located close by at 1p34–36, whereas sPLA2-IB and -X genes were mapped on chromosomes 12 and 16, respectively (29, 47). Close relationships among sPLA2-IIA, -IID, and -IIE were also observed in the exon/intron structure. Taken together, these findings suggest that three types of group II sPLA2 genes constitute a gene cluster that is likely to have emerged from ancient gene duplication events.

Recombinant human sPLA2-IIE possesses enzymatic characteristics common to the known sPLA2s in terms of extracellular localization, Cu^{2+} requirement, and optimal pH range. It showed a substrate preference similar to that of sPLA2-IIA and -IID, which contrasted with the preferred hydrolysis of 2-arachidonoyl PC by sPLA2-X (30). In addition, a susceptibility against the sPLA2 inhibitor indoxam divided the sPLA2 family into two subgroups: subgroup IIA, IID, and IIE, and subgroup IB, V, and X (Fig. 3D). Analysis of the crystal structure of the sPLA2-IIA complex with a closely related indolizine compound revealed the inhibitor to be located near the active site in sPLA2-IIA and bound to Ca^{2+} (48). Thus, a similar substrate specificity and a high sensitivity toward indoxam strongly suggest that the three group II sPLA2s form a similar three-dimensional structure around the catalytic center. In this context, sPLA2-IIE might play a compensatory role in the degradation of the endogenous phospholipids together with sPLA2-IID especially in sPLA2-IIA-deficient mice. Defensive roles against invading bacteria may also be functions of sPLA2-IIE like IIA and IID (49), because they preferentially hydrolyze phosphatidylglycerol and PE which are major components of bacterial phospholipids.

sPLA2-IIA is thought to be one of the key enzymes in the pathogenesis of inflammatory diseases, since its enhanced expression has been observed in various cell types stimulated by inflammatory cytokines and in various inflammatory models including rat endotox shock (50). In addition, its potential contribution to arachidonic acid release via binding to cell-surface heparan sulfate proteoglycan was demonstrated by transfection/overexpression experiments (44). In mice, however, the sPLA2-IIA gene is naturally disrupted by a single nucleotide insertion in some inbred strains (12, 13). Furthermore, even in BALB/c mice that express sPLA2-IIA, its expression was limited to the intestine and increased little during endotox shock (51, 52). In sPLA2-IIA-deficient mice, the expression of sPLA2-IIA was markedly enhanced in various tissues upon LPS challenge, whereas the expression of another isoform, sPLA2-IID, was slightly enhanced in the thymus but remarkably decreased in the spleen, small intestine, and lung (Fig. 5). In this respect, sPLA2-IIE might play more pivotal roles against invading bacteria.

**Fig. 5. Expression of sPLA2-IIE and IID mRNAs in LPS-treated mice.** C57BL/6J mice were injected with S. typhosa LPS or saline. After 24 h, the tissues indicated in the figure were isolated, and mRNAs were prepared. The mRNA (5 μg) was analyzed by Northern blotting as described under "Experimental Procedures." Two mice were subjected to each experiment, and the typical result is shown.
roles than sPLA₂-II in place of sPLA₂-IIA. We have recently shown that indoxam suppressed murine endotoxic shock through sPLA₂-IIA-independent mechanisms (16). The potential contributions of indoxam-sensitive sPLA₂-IIE and -IID should be considered in this process. Since the expression of sPLA₂-V was also up-regulated in LPS-treated mice (52), these augmented sPLA₂ species might cooperatively play a role in the development of inflammatory conditions. Further analysis such as comparison of the promoter regions of sPLA₂ genes, especially between sPLA₂-IIE and -IID, could provide a clue to understanding the distinct regulatory mechanisms of expression.

In the lung of LPS-treated mice, enhanced expression of sPLA₂-IIE transcripts was detected in the alveolar macrophage-like cells (Fig. 6). Since alveolar macrophages are known to act as the source and target for a variety of inflammatory mediators produced during pulmonary inflammation (53), the secreted sPLA₂-IIE might regulate their functions in the defense against infectious agents and toxic particles in the airways. In the guinea pig model of LPS-induced acute lung injury, enhanced expression of sPLA₂-IIA was detected in the interstitial and alveolar macrophages (54). In this model, lysophospholipids exert a major injurious effect on lung tissue membranes, and administration of LY31127, an sPLA₂ inhibitor reported for various inflammatory models should be reevaluated after consideration of novel types of group II sPLA₂s.

The discovery of specific receptor for mammalian sPLA₂γ has led to the notion that sPLA₂ can exert various biological responses via binding to the receptor in addition to its digestive function (25). In rats and mice, sPLA₂-IB was identified as an endogenous ligand of the PLA₂ receptor to induce various physiological responses including cell proliferation and lipid mediator releases (24, 25). Our recent studies with PLA₂ receptor-deficient mice have demonstrated its potential role in the production of inflammatory mediators during LPS shock (26). Although sPLA₂-IIA does not act as a natural ligand for rat PLA₂ receptor, it can bind mouse PLA₂ receptor with ~5–10-fold lower affinity compared with sPLA₂-IB (4). In contrast, Valentin et al. (31) have recently shown that mouse sPLA₂-IIA does not bind the receptor. Since the receptor binding of sPLA₂ proteins does not depend on their enzymatic activities, mouse PLA₂ receptor could discriminate between sPLA₂-IIA and -IID despite their structural similarities around the catalytic center. To date, endogenous sPLA₂ ligands for PLA₂ receptor have not been clearly characterized in humans. Thus, the potency of sPLA₂-IIE as a natural ligand of the receptor needs to be evaluated. In addition to the cloned PLA₂ receptor, several binding proteins recognized by snake venom sPLA₂s have been reported, although their molecular structures, endogenous ligands, and biological functions remain uncertain (4, 56). Feuardent et al. (57) have recently reported the putative binding sites that might be involved in the venom sPLA₂γ-evoked suppression of HIV-1 virus entry into host cells. The possibility of sPLA₂-IIE acting as a ligand for these binding sites also deserves attention in future studies.

We have previously reported the cloning and characterization of mouse and human sPLA₂-II utilizing murine-expressed sequence tag as starting information. Isolation and characterization of human enzymes are important in terms of practical applications such as drug development. Human sPLA₂-IIE shares 89% identity with the mouse counterpart in the mature peptide region, whereas sequence identities of other sPLA₂s are below 80% with the lowest identity found in sPLA₂-IA (68%) (13, 41). Indeed, human and mouse sPLA₂-IIA have a number of distinct biological properties with respect to tissue distribution, induction levels under inflammatory conditions, a potential function as a genetic modifier of colorectal cancer, and binding affinity for the PLA₂ receptor (12, 58). In this regard, some specific and important functions assigned to sPLA₂-IIE might have been pressed pressure to maintain the required structure during evolution. Sequence alignment (Fig. 1) shows the presence of one distinct residue in sPLA₂-IIE (His²⁵) where all other mouse and human sPLA₂s have Gly. Although no
specific function(s) has been assigned to this residue at present, further functional analysis of sPLA2-IIE might reveal its biological significance. RT-PCR analysis revealed the expression of sPLA2-IIE transcripts in several human tissues in contrast to a broad distribution of sPLA2-IIA and -IIID (Fig. 4). Hybridization analysis with RNA Master blot membranes confirmed the expression of sPLA2-IIE in the same tissues, although discrete bands were not observed in any tissues examined by Northern analysis (data not shown). In physiological states, the expression levels of sPLA2-IIE were lower than the levels of Northern analysis (data not shown). In physiological states, the expression levels of sPLA2-IIE were lower than the levels of sPLA2-IIE, which agreed well with the fact that no expressed sequence tags corresponding to human sPLA2-IIE exist in the public data base. Considering its enhanced expression under endotoxic shock in mice, analysis for the expression of human sPLA2-IIE under some pathological conditions should be performed to evaluate its biological functions.

In conclusion, we isolated novel human and mouse sPLA2s (group IIE) and characterized the catalytic activities and expression. Abnormally high levels of PLA2 activity have been detected in association with various human diseases, especially in inflammatory conditions. Further studies on the biological functions of sPLA2-IIE are required to establish its fundamental roles in the progression of disease states. Finally, the discovery of the novel sPLA2-IIE should enable us to assign more precise functions to each sPLA2 family member which should be of great value for the development of sPLA2 inhibitors as therapeutics.

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Addendum—During preparation of this manuscript, a paper reporting the cloning of mouse sPLA2-IIE was published (61).
Structures, Enzymatic Properties, and Expression of Novel Human and Mouse Secretory Phospholipase A2

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