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On the Diversity of Secreted Phospholipases A_2

CLONING, TISSUE DISTRIBUTION, AND FUNCTIONAL EXPRESSION OF TWO NOVEL MOUSE GROUP II ENZYMES*

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Over the last decade, an expanding diversity of secreted phospholipases A_2 (sPLA$_2$s) has been identified in mammals. Here, we report the cloning in mice of three additional sPLA$_2$s called mouse group IIE (mGIIE), IIF (mGIIF), and X (mGX) sPLA$_2$s, thus giving rise to eight distinct sPLA$_2$s in this species. Both mGIIE and mGIIF sPLA$_2$s contain the typical cysteines of group II sPLA$_2$s, but have relatively low levels of identity (less than 51%) with other mouse sPLA$_2$s, indicating that these enzymes are novel group II sPLA$_2$s. However, a unique feature of mGIIF sPLA$_2$ is the presence of a C-terminal extension of 23 amino acids containing a single cysteine. mGX sPLA$_2$ has 72% identity with the previously cloned human group X (hGX) sPLA$_2$ and displays similar structural features, making it likely that mGX sPLA$_2$ is the ortholog of hGX sPLA$_2$. Genes for mGIIE and mGIIF sPLA$_2$s are located on chromosome 4, and that of mGX sPLA$_2$ on chromosome 16. Northern and dot blot experiments with 22 tissues indicate that all eight mouse sPLA$_2$s have different tissue distributions, suggesting specific functions for each. mGIIE sPLA$_2$ is highly expressed in uterus, and at lower levels in various other tissues. mGIIF sPLA$_2$ is strongly expressed during embryogenesis and in adult testis. mGX sPLA$_2$ is mostly expressed in adult testis and stomach. When the cDNAs for the eight mouse sPLA$_2$s were transiently transfected in COS cells, sPLA$_2$ activity was found to accumulate in cell medium, indicating that each enzyme is secreted and catalytically active. Using COS cell medium as a source of enzymes, pH rate profile and phospholipid headgroup specificity of the novel sPLA$_2$s were analyzed and compared with the other mouse sPLA$_2$s.

Phospholipase A_2 (PLA$_2$)[1] catalyzes the hydrolysis of glycerophospholipids, producing free fatty acids and lysophospholipids (1–7). Since the pioneering studies of PLA$_2$ activity in pancreatic juice and cobra venom more than a century ago (8), PLA$_2$ has recently emerged as a superfamily of intracellular and extracellular enzymes, which have been classified into 10 groups (3, 9). Intracellular PLA$_2$s comprise the well known group IV cPLA$_2$ (10), novel paralogs of this enzyme (11, 12), and several Ca^{2+}-independent PLA$_2$s (13–18). Extracellular PLA$_2$s include the 45-kDa platelet-activating factor-selective PLA$_2$ (19), and six structurally related secreted PLA$_2$s (sPLA$_2$s,[2] 13–16 kDa), which have been classified into groups I, II, V, and X (3, 4, 9, 20). A sPLA$_2$-like protein has also been described in humans and mice, and belongs to the otoconin family (21–23).

Until now, only one mammalian group IB sPLA$_2$, known as the pancreatic-type sPLA$_2$, has been identified (24). This sPLA$_2$ is found in large amounts in pancreas and at lower levels in lung, liver, spleen, kidney, and ovary (9, 25–27). Besides a role in lipid digestion, this sPLA$_2$ has been involved in cell proliferation, lipid mediator release, acute lung injury, and endotoxic shock (28–31). On the other hand, three mammalian group II sPLA$_2$s have been characterized. Group IIA sPLA$_2$ is also referred to as the inflammatory-type sPLA$_2$, as it is expressed at high levels during inflammation and associated diseases, at least in rat and human species (5, 32). This sPLA$_2$ is thought to be a potent mediator of inflammation (5, 32–34) and a potent antibacterial agent (35–37). It is also expressed at high levels in various human gastrointestinal cancers (38, 39), and the mouse group IIA (mGIIA) sPLA$_2$ has been proposed to act as a tumor suppressor gene in colorectal cancer (40, 41). Group IIC sPLA$_2$ has been cloned from rat and mouse species (42), but appears as a non-functional pseudogene in humans (43). Group IID sPLA$_2$ was very recently cloned from mouse thymus, and has specific tissue distribution and catalytic properties (20). A unique group V sPLA$_2$ was originally cloned from human brain and found to be prevalent in heart (44, 45). This sPLA$_2$ was also detected in murine macrophages and mastocytes, where it plays a role in lipid mediator production (46–48). Finally, group X sPLA$_2$ was cloned in humans and found to be ex-

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[1] The abbreviations used are: PLA$_2$, phospholipase A_2; sPLA$_2$, secreted phospholipase A_2; EST, expressed sequence tag; RACE-PCR, rapid amplification of cDNA ends by polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; DOPC, 1,2-dioleoyl-*sn*-glycerol-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine [^{3}H]-DPPC, [9,10-^{3}H]-1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine; [^{3}H]-DPPG, [9,10-^{3}H]-1,2-dipalmitoyl-*sn*-glycerol-3-phosphoglycerol. POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; lod, logarithm of odds.

[2] A comprehensive abbreviation system for the various mammalian secreted phospholipases A_2 (sPLA$_2$s) was used. Each sPLA$_2$ was abbreviated with a lowercase letter indicating the sPLA$_2$ species (b, d, gp, m, h, p, r, and rb for bovine, dog, guinea pig, mouse, human, porcine, rat, and rabbit, respectively), which is followed by uppercase characters identifying the sPLA$_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GV, and GX for group IB, IIA, IIC, IID, IIE, IIF, V, and X sPLA$_2$s, respectively).

pressed in spleen, thymus, and peripheral leukocytes, suggesting functions linked to inflammation or immunity (9).

Several sPLA$_2$s have also been characterized from snake, insect, and molluscan venoms, and classified into groups I, II, III, and IX (1, 3, 6, 49–51). These sPLA$_2$s share with mammalian sPLA$_2$s a number of structural and enzymatic properties (1–3), and can display a wide array of toxicities (49, 50, 52). Specific high affinity receptors for venom sPLA$_2$s have been identified and are likely to play a role in their toxicities (6, 53). To date, two main types of sPLA$_2$ receptors (M and N) have been identified and binding studies with endogenous sPLA$_2$s have shown that M-type receptors can be physiological targets for mammalian group IB and/or group IIA sPLA$_2$s, depending on the animal species (6, 27, 28), suggesting that these receptors can be involved in the biological effects of group IB and IIA sPLA$_2$s (28, 30, 31).

The ongoing diversity of mammalian sPLA$_2$s, the large diversity of venom sPLA$_2$s, and the identification of specific sPLA$_2$ receptors that are likely to have mammalian sPLA$_2$s as endogenous ligands suggest that additional mammalian sPLA$_2$s may exist and prompted us to search for novel sPLA$_2$s. Here, we report the cloning, chromosomal localization, tissue distribution, and recombinant expression of several novel mouse sPLA$_2$s, increasing their number in mice to eight distinct enzymes. A comparison of the tissue distribution and catalytic properties of the eight sPLA$_2$s is also presented.

EXPERIMENTAL PROCEDURES

Molecular Cloning of mGIIE sPLA$_2$—Searching for sPLA$_2$ homologs in gene data bases stored at the National Center for Biotechnology using the tBLASTn sequence alignment program (54) resulted in the identification of an exon trapped sequence (OST327, GenBank™ accession no. AF046275; Ref. 55) that was derived from a mouse embryonic stem cell cDNA library and that codes for a partial sequence of a novel sPLA$_2$. The 320-nucleotide sequence was then used to clone the entire cDNA sequence coding for this sPLA$_2$ by 5′- and 3′-RACE-PCR experiments as described previously (20). Briefly, total mouse thymus RNA (10 μg) was reverse transcribed, and double-stranded cDNA was ligated to adaptors containing sequences for the universal primers SP6 and KS. PCR reactions were performed using KS primer and a specific forward or reverse primer, for 3′- or 5′-RACE-PCR, respectively. PCR products were subcloned into pGEM-T easy vector (Promega), and colonies were screened using an internal ^{32}P-labeled oligonucleotide probe. 5′-RACE-PCR experiments resulted in the cloning of a 480-nucleotide sequence that was identical in its 3′ end (nucleotides 248–480) to the expressed sequence tag (EST) sequence and contained in its 5′ end (nucleotides 161–247) all the expected features of a sPLA$_2$, including a signal peptide sequence preceded by an initiator methionine. 3′-RACE-PCR experiments on the same cDNA resulted in the cloning of a 545-nucleotide sequence that was identical in its 5′ end (nucleotides 1–232) to the EST sequence and contained in its 3′ end (nucleotides 233–545) an in-frame extension of 7 amino acids, a stop codon, and a 3′-noncoding region of 288 nucleotides containing two putative polyadenylation sites and a poly(A) sequence. RT-PCR experiments on mouse colon cDNA were performed using primers derived from the RACE-PCR sequences and resulted in the cloning of a full-length cDNA containing an open reading frame of 429 nucleotides. The C-terminal portion of mGIIE sPLA$_2$ was also confirmed by cloning a ~2-kilobase pair mouse genomic DNA fragment, which was partially sequenced.

Molecular Cloning of mGIIF sPLA$_2$—Two ESTs (IMAGE Consortium clone identification 1498615 5′, GenBank™ accession no. AI173890; IMAGE Consortium clone identification 1498564 5′, GenBank™ accession no. AI173803) derived from a 14-day-old mouse embryo cDNA library were found to code for the N-terminal sequence of mGIIF sPLA$_2$. 3′-RACE-PCR experiments were then performed on mouse thymus cDNA as described above to clone the full-length sPLA$_2$. This led to the identification of a 599-nucleotide sequence that was identical in its 5′ end (nucleotides 1–204) to the EST sequences and contained in its 3′ end (nucleotides 205–599) an in-frame extension of 81 amino acids, a stop codon, and a 3′-noncoding region of 149 nucleotides. A new set of primers was designed to amplify the full-length sPLA$_2$ sequence and led to the cloning of a complete open reading frame of 507 nucleotides from thymus cDNA. Using the same primers, a ~5-kilobase pair genomic fragment was amplified, partially sequenced, and found to confirm the cDNA sequence.

Molecular Cloning of mGX sPLA$_2$—Three ESTs (IMAGE Consortium clone identification 922225 5′, GenBank™ accession no. AA512293; IMAGE Consortium clone identification 1052745 5′, GenBank™ accession no. AA607557; and IMAGE Consortium clone identification 1053472 5′, GenBank™ accession no. AA611431) derived from mouse irradiated colon cDNA library were found to code for the C-terminal portion of mGX sPLA$_2$. A first set of 5′-RACE-PCR experiments with mouse thymus cDNA led to the identification of a 385-nucleotide sequence that was identical in its 3′ end (nucleotides 307–385) to the EST sequences and that contained in its 5′ end (nucleotides 1–306) all of the expected features of a mature sPLA$_2$, including a Ca^{2+} loop region and a catalytic domain. Using this sequence, new primers for 5′-RACE-PCR experiments were designed and used on mouse testis cDNA. This led to the identification of the full-length cDNA sequence, including the signal prepropeptide and the initiator methionine. The full-length sequence was then confirmed by performing new RT-PCR amplifications from mouse testis cDNA.

Chromosomal Localization of Mouse sPLA$_2$ Genes—The localization of the different mouse sPLA$_2$ genes was performed by PCR analysis, using a mouse/hamster radiation hybrid panel (catalog no. RH04.02) from Research Genetics (56). For these experiments, various sets of sPLA$_2$ specific primers were designed to allow the amplification of DNA fragments (ranging from 168 to 276 nucleotides) from mouse genomic DNA template without amplification or with different patterns of amplification from hamster genomic DNA template. PCR reactions were performed in 10 μl containing 25 ng of DNA template, 50 ng of each primer, 1.5 mM $MgCl_2$ and 0.25 unit of *Taq* polymerase (Life Technologies, Inc.). PCR conditions were: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. PCR products were analyzed on a 2.5% agarose gel, transferred onto positively charged nylon membranes, and probed at high stringency with an internal ^{32}P-labeled oligonucleotide probe. Scoring of the results (logarithm of odds (lod) score) were analyzed using the Jackson Laboratory mouse radiation hybrid data base.

Analysis of the Tissue Distribution of Mouse sPLA$_2$s—A mouse Northern blot (CLONTECH, catalog no. 7762-1) and a mouse RNA master blot (CLONTECH, catalog no. 7771-1) were successively probed with ^{32}P-labeled riboprobes corresponding to the sequence of the different mature sPLA$_2$s in ULTRAHyb hybridization buffer (Ambion, catalog no. 8670) for 18 h at 70 °C. High-sensitivity strippable antisense riboprobes were synthesized using the Strip-EZ RNA Ambion kit (catalog no. 1360). Blots were washed to a final stringency of 0.1× SSC (30 mM NaCl, 3 mM trisodium citrate, pH 7.0) in 0.1% SDS at 70 °C and exposed to Kodak Biomax MS films with a Transcreen-HE intensifying screen. After each hybridization, blots were stripped as specified in the Strip-EZ RNA Ambion kit, checked for remaining radioactivity, and hybridized with the next sPLA$_2$ riboprobe. The absence of cross-hybridization of each sPLA$_2$ riboprobe to other mouse sPLA$_2$s was checked by performing parallel hybridization of Southern blots containing the eight full-length mouse sPLA$_2$ cDNAs using the same conditions as above.

Recombinant Expression of Mouse sPLA$_2$s in COS Cells—The full-length cDNAs coding for mGIB and mGIIE were subcloned into the expression vector pRc/CMV neo (Invitrogen), and those of mGIID, mGIIF, and mGV sPLA$_2$s were subcloned in pCI-neo (Promega), pcDNAI-ampi (Invitrogen), and pcDNAI-SupF (Invitrogen), respectively. Chimera cDNA constructs containing the hGIIA sPLA$_2$ signal peptide followed by the mGIIA, mGIIC, or mGX mature proteins were subcloned into the pRc/CMV neo vector. All of these constructs were sequenced after subcloning and transiently transfected into COS cells using DEAE-dextran as described (20). Five days after transfection, cell medium was collected and analyzed for sPLA$_2$ activity. When low sPLA$_2$ activity was detected (mGIIC, mGIID, mGIIE, and mGX transfections), the COS cell medium was concentrated about 16-fold with a Centriprep 10 concentrator (Millipore) and then used for substrate specificity studies.

Substrate Specificity Studies—Small unilamellar vesicles were prepared by sonication in assay buffer as described (57). Initial velocities for the hydrolysis of these vesicles were carried out with 17 μM phospholipid (see Table III for vesicle compositions) in 100 mM Tris-HCl, pH 8.0, with 2.5 mM $CaCl_2$ at 37 °C in a total volume of 50 μl (typically 10–20 μl of COS cell supernatant plus 30–40 μl of buffer). After 10 and 30 min, reactions were quenched with organic solvent and analyzed for free fatty acid as described (58). The sources of phospholipids are: [^{3}H]DPPC (89 Ci/mmol, NEN Life Science Products), [^{3}H]DPPG (400 Ci/mol, prepared as described; Ref. 20). For enzymatic assays, all phospholipids were used at ~50 Ci/mol, [^{3}H]DPPC was diluted with DOPC,

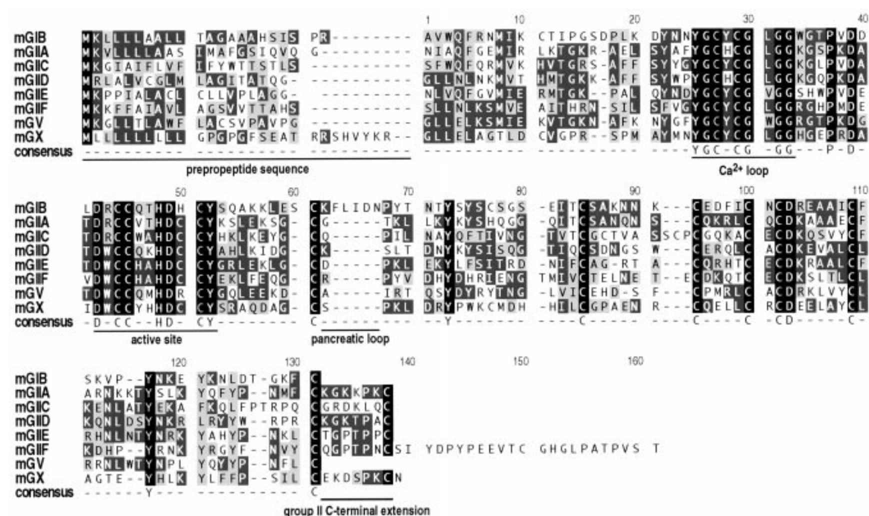

FIG. 1. **Alignment of the amino acid sequences of mouse $sPLA_2$s.** Sequences of full-length $sPLA_2$ proteins are shown. $sPLA_2$ sequences are from Refs. 20, 27, 40, 42, 43, and 64. $sPLA_2$s are *numbered* from the mature protein sequences, and the consensus sequence of the eight mouse $sPLA_2$s is shown. Putative *N*-glycosylation sites for mGIIF $sPLA_2$ are found at positions 79, 89, and 137. No *N*-glycosylation sites for mGIIE and mGX $sPLA_2$s have been found.

and [^{3}H]DPPG was diluted with POPG (both unlabeled phospholipids from Avanti). For all enzymatic assays, three time points were taken to ensure that the reaction progress was linear over the period of time in which the velocities were measured. This velocity was found to vary linearly when the amount of enzyme was reduced or increased 2-fold. Control reactions were carried out using supernatants from mock-transfected COS cells, and dpm were subtracted from the values obtained in the presence of mouse enzymes. pH rate profiles were obtained using the buffers described previously (20) and using the assay with [^{3}H]DPPG/POPG described above.

RESULTS AND DISCUSSION

Molecular Cloning of Novel $sPLA_2$s—As the number of released sequences in nucleic data bases is exponentially increasing, the search for protein homologs by using the tBLASTn sequence alignment program (54) is becoming a very useful tool to identify new proteins. In the PLA_2 field, this strategy has previously led to the cloning of hGX $sPLA_2$ (9), mGIID $sPLA_2$ (20), human paralogs of $cPLA_2$ (11, 12), and splice variants of human Ca^{2+}-independent PLA_2 (16). Using the same strategy, we have now identified novel ESTs that display significant homology to known $sPLA_2$s and that correspond to novel low molecular mass $sPLA_2$s. Three groups of ESTs coding for partial sequences of $sPLA_2$s were identified and used to clone by RACE-PCR three novel mouse enzymes called mouse group IIE (mGIIE), mouse group IIF (mGIIF), and mouse group X (mGX) $sPLA_2$s.

In the first group, a single EST derived from a mouse embryonic stem cell library (55) was identified. The sequence of this EST was found to show considerable identity to group II $sPLA_2$s and to code for the middle portion of mGIIE $sPLA_2$ (Glu-10 to Cys-131; see Fig. 1). 5′-RACE-PCR experiments on mouse thymus cDNA led to the identification of a nucleic sequence containing 161 nucleotides of 5′-noncoding sequence and the N-terminal region of mGIIE $sPLA_2$ including the signal peptide sequence. 3′-RACE-PCR experiments on the same cDNA led to the identification of the C-terminal sequence of mGIIE $sPLA_2$ and a 3′-noncoding region of 288 nucleotides containing two putative polyadenylation sites and a poly(A) sequence. Based on the RACE-PCR sequences, a new set of primers was designed and used to amplify the full-length mGIIE cDNA from mouse colon cDNA. Sequencing of the amplified fragment revealed complete identity with the EST sequence and the RACE-PCR products. The final cDNA sequence resulting from the alignment of the amplified PCR products and the EST sequence is made up of 870 nucleotides (GenBank™ accession no. AF166098).

A second group of two ESTs derived from a mouse embryo cDNA library was found to code for a 5′-noncoding region of 250 nucleotides, the signal peptide, and the first 67 N-terminal amino acids of the mature mGIIF $sPLA_2$ (Fig. 1). 3′-RACE-PCR experiments on mouse thymus cDNA led to the identification of an in-frame extension of 81 amino acids corresponding to the C-terminal portion of mGIIF $sPLA_2$, followed by a stop codon and a 3′-noncoding region of 149 nucleotides. mGIIF $sPLA_2$ thus appears as a mature protein of 148 amino acids containing an unusual extra C-terminal extension of 23 residues (Fig. 1). To confirm the presence of this extension, a new set of primers flanking the full-length mGIIF $sPLA_2$ sequence was designed and used for RT-PCR on mouse thymus cDNA. A DNA fragment of the expected size was obtained and found to code for the predicted 148 amino acids of mGIIF $sPLA_2$. The same set of primers was used to amplify a mouse genomic DNA fragment of ~5 kilobase pairs, and its partial sequencing was found to confirm the cDNA sequence. All together, the final cDNA sequence resulting from the alignment of amplified PCR products and EST sequences comprises 906 nucleotides (GenBank™ accession no. AF166099).

A third group of three ESTs were derived from a mouse irradiated colon cDNA library and found to code for the 3′ end of mGX $sPLA_2$. An initial set of 5′-RACE-PCR experiments from mouse thymus cDNA led to the identification of a sequence that codes for the full-length mGX $sPLA_2$ protein except for a portion of its signal peptide. Since mGX $sPLA_2$ was found to be highly expressed in adult mouse testis (Fig. 3), a second set of 5′-RACE-PCR experiments was performed on mouse testis cDNA using new upstream primers, and this led to the identification of the mGX $sPLA_2$ signal peptide sequence. The full-length cDNA coding for mGX $sPLA_2$ was finally amplified from mouse testis cDNA and found to be identical to the sequences of ESTs and 5′-RACE-PCR products. The consensus cDNA sequence from the alignment of the amplified PCR products and the EST sequences is made up of 1040 nucleotides (GenBank™ accession no. AF166097).

Structural Features of the Cloned $sPLA_2$s—An alignment of the amino acid sequences of the eight mouse catalytically active $sPLA_2$s that have been cloned so far is presented in Fig. 1, and their respective level of identity is shown in Table I. Based on their structural features, mGIIE and mGIIF $sPLA_2$s clearly are members of the group II collection of $sPLA_2$s (1, 3, 4, 6, 9, 20). Indeed, both $sPLA_2$s display the specific features of group II $sPLA_2$s, including a cysteine at position 50 and a cysteine that terminates the group II-specific C-terminal extension of 7 residues (Fig. 1). Furthermore, both $sPLA_2$s lack the specific features of group IA (disulfide 11–77, no C-terminal extension), group IB (same as group IA but with a pancreatic loop), group

Table I
Level of amino acid sequence identity (%) between the different mouse $sPLA_2s$

$sPLA_2$	mGIIA	mGIIC	mGIID	mGIIE	mGIIF	mGV	mGX
mGIB	37	34	33	34	23	32	32
mGIIA		42	48	51	31	41	38
mGIIC			42	40	32	37	33
mGIID				42	37	44	36
mGIIE					37	41	36
mGIIF						33	31
mGV							34

V (12 cysteines, no C-terminal extension), or group X $sPLA_2s$ (16 cysteines, prepropeptide sequence, C-terminal extension of 8 residues). The level of identity of mGIIE and mGIIF $sPLA_2s$ to other mouse group II $sPLA_2s$ (namely mGIIA, mGIIC, and mGIID $sPLA_2s$) is, however, less than 51% (Table I), indicating that all of these enzymes are not isoforms. The two novel $sPLA_2s$ have thus been given the names mouse group IIE (mGIIE) and mouse group IIF (mGIIF) $sPLA_2s$.

mGIIE $sPLA_2$ is made up of a signal peptide of 19 amino acids, followed by a mature protein of 127 residues (calculated molecular mass 14,467 Da) that contains all of the residues found in catalytically active enzymes (9). Similar to mGIIA, mGIIC, and mGIID $sPLA_2s$, mGIIE $sPLA_2$ is a basic protein with a calculated isoelectric point of 8.06 (Table II). mGIIF $sPLA_2$ is made up of a signal peptide of 20 amino acids, followed by a mature protein of 148 residues (calculated molecular mass 16,820 Da) that also contains all of the amino acids conserved in active $sPLA_2s$ (9). Interestingly, mGIIF $sPLA_2$ appears to be the most acidic mouse group II $sPLA_2$ so far identified (calculated isoelectric point 5.86), and is also slightly more acidic than mGIB and mGX $sPLA_2s$ (Table II). Another particular feature of mGIIF $sPLA_2$ is a long C-terminal extension of 23 amino acids containing an extra cysteine, in addition to the group II-specific C-terminal extension of 7 residues (Fig. 1). The presence of this odd cysteine raises the possibility that mGIIF $sPLA_2$ occurs as a covalent dimer. So far, none of the mammalian $sPLA_2s$ has been reported to occur as a covalent multimer, and only the pancreatic $sPLA_2$ has been proposed to dimerize after autocatalytic acylation (59) or treatment by transglutaminases (60). On the other hand, several venom $sPLA_2s$ are known to occur as homo- or heteromultimeric enzymes with or without an interchain disulfide (49, 50), suggesting that multimeric $sPLA_2s$ may also exist in mammals.

A blast search for homology with the mGX $sPLA_2$ protein sequence revealed that this protein has the highest level of identity (72%) with hGX $sPLA_2$ (9). In contrast, the level of identity of mGX $sPLA_2$ to other mouse $sPLA_2s$ is lower than 38% (Table I). In addition, the mouse protein shares with hGX $sPLA_2$ the same structural features (9). mGX $sPLA_2$ consists of a 28-amino acid prepropeptide sequence ending with a basic dipeptide and a mature protein of 123 residues (calculated molecular mass 13,899 Da), and is acidic (Table II). Like hGX $sPLA_2$, it has eight disulfides including group I and group II specific disulfides, and has a group II-like C-terminal extension of eight residues. Taken together, it is likely that mGX $sPLA_2$ is the mouse ortholog of hGX $sPLA_2$.

A search for other sequences related to mGIIE and mGIIF $sPLA_2s$ in EST data bases was unsuccessful, and no human or rat sequences corresponding to the orthologs of these enzymes were found. On the other hand, a search with the group X $sPLA_2$ sequence resulted in the identification of a novel EST (EST 194611, GenBank™ accession no. AA851843) derived from a rat spleen cDNA library. The EST clone was obtained from the American Type Cell Collection and found to consist of 910 nucleotides (GenBank™ accession no. AF166100) with 48 nucleotides of 5′-untranslated region, an open reading frame of 456 nucleotides, and a 3′-untranslated region of 406 nucleotides containing two putative polyadenylation sites and a poly(A) sequence. The open reading frame was found to code for a novel rat $sPLA_2$ of 151 amino acids that displays highest levels of identity with mGX (94%) and hGX (72%) $sPLA_2s$ (9). Furthermore, like the two group X enzymes, the rat protein is made up of a prepropeptide of 28 amino acids ending with a basic doublet, a mature protein of 123 residues (calculated molecular mass of 13,952 Da), and is acidic (calculated isoelectric point 5.58). All together, it is most likely that this protein corresponds to the rat ortholog (rGX) of mGX and hGX $sPLA_2s$.

Fig. 2 presents a phylogenetic dendrogram derived from the alignment of all known mature protein sequences of mammalian $sPLA_2s$. These sequences include those of catalytically active $sPLA_2s$ and those of the two $sPLA_2$-like domains of the $sPLA_2$-related proteins which have been cloned in humans (21), and more recently in mice (22, 23). This dendrogram separates the mammalian $sPLA_2s$ into four groups, including $sPLA_2$-like domains, group IB $sPLA_2s$, group X $sPLA_2s$, and group II and V $sPLA_2s$. It is likely that the different group II and V $sPLA_2s$ have arose from successive gene duplication events from one common ancestral gene. Interestingly, similar gene duplication events have also been reported for snake venom group II $sPLA_2s$ (51, 61, 62).

Chromosomal Localization of the Mouse $sPLA_2$ Genes—The mapping of genes for mGIB, mGIIA, mGIID, mGIIE, mGIIF, and mGX $sPLA_2s$ was carried out by PCR screening of a mouse/hamster radiation hybrid panel (see "Experimental Procedures" for details). Using this panel, the mGIB $sPLA_2$ gene was mapped on the central part of chromosome 5 (Table II), with highest lod score of linkage to the marker D5Mit136. This result fits well with the chromosomal localization of the hGIB $sPLA_2$ gene on chromosome 12q23–24 (63), a region exhibiting synteny with mouse chromosome 5. Using the same radiation hybrid panel, the mGX $sPLA_2$ gene was mapped to chromosome 16 (Table II), with highest lod score of linkage to the D16Mit154 marker. This result is also in agreement with the mapping of the hGX $sPLA_2$ gene to human chromosome 16p13.1-p12 (9). Finally, we mapped all four genes for mGIIA, mGIID, mGIIE, and mGIIF $sPLA_2s$ on mouse chromosome 4 (Table II), with highest lod score of linkage to the D4Mit54 marker. These results fit well with the previous mapping of the mGIIA $sPLA_2$ gene to mouse chromosome 4 (40, 43) and with that previously found for the mGIID $sPLA_2$ gene (20). Since mGIIC and mGV $sPLA_2$ genes have already been mapped to chromosome 4 (43), it is now known that six of the eight mouse $sPLA_2$ genes are colocalized on this chromosome. It was previously observed that the genes for mGIIA, mGIIC and mGV $sPLA_2s$ are tightly linked and are all located in the distal part of chromosome 4 (43). Furthermore, the human genes for group IIA and group V $sPLA_2s$ were found to be very close together, while the human gene for group IIC $sPLA_2$ was found to be slightly more distant (43). In good agreement with this later observation, the isolation of a mouse cosmid revealed that the genes for mGIIA and mGV $sPLA_2s$ are actually very close together and separated by only ~25 kilobase pairs (41). All together, these data indicate that the genes for mGIIA, mGIIC, and mGV $sPLA_2s$ lie within a gene cluster. Although there is a strong likelihood that the three other genes for mGIID, mGIIE, and mGIIF $sPLA_2s$ may also occur within the same gene cluster, it remains to be determined whether this is really the case. Finally, the gene for the mouse $sPLA_2$-like protein called otoconin-90 has been mapped on chromosome 15 (22, 23), *i.e.* on a chromosome that is different from those where the other mouse $sPLA_2$ genes have been mapped (Table II).

TABLE II
The different mouse sPLA$_2$s

sPLA$_2$	Major sources	Molecular mass[a]	pI[a]	Cysteine no.	Specific features	Substrate specificity	Chromosomal localization
		kDa					
mGIB	Pancreas, lung, liver, small intestine	14.1	6.71	14	Pancreatic loop propeptide	PG > PC	5
mGIIA	Small intestine	13.9	9.22	14	C-terminal extension[b]	PG >> PC	4
mGIIC	Testis, pancreas	14.6	8.33	16	C-terminal extension[b]	PG >> PC	4
mGIID	Pancreas, spleen, thymus, lung	14.2	8.71	14	C-terminal extension[b]	PG ~ PC	4
mGIIE	Uterus, thyroid, testis	14.4	8.06	14	C-terminal extension[b]	PG > PC	4
mGIIF	Embryo, testis	16.8	5.86	15	C-terminal extension[c]	PG > PC	4
mGV	Eye, heart, pancreas	13.8	8.08	12		PG, PC	4
mGX	Testis, stomach	13.9	5.88	16	C-terminal extension[d] putative propeptide	PG, PC	16

[a] As determined from the sequence of mature proteins.
[b] 7 amino acids.
[c] 23 amino acids.
[d] 8 amino acids.

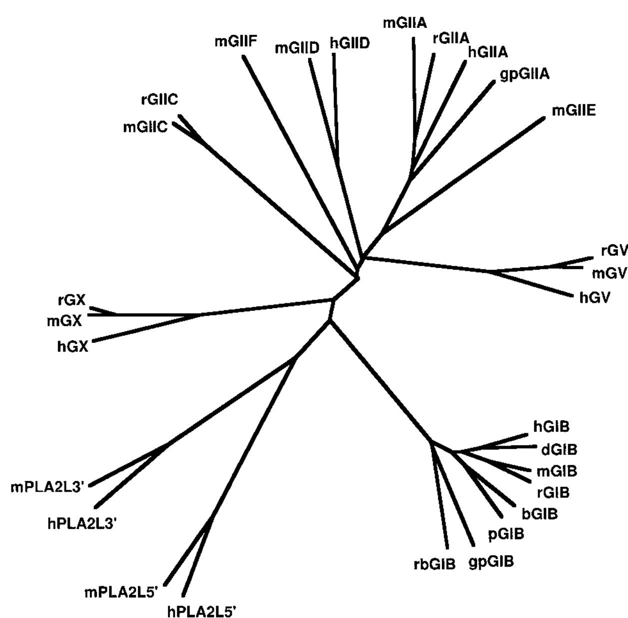

FIG. 2. **Phylogenetic dendrogram of mammalian sPLA$_2$s.** Sequences of mature sPLA$_2$ proteins were aligned using Clustal W, and the phylogenetic dendrogram was generated using Treeview. The sPLA$_2$ sequences have been retrieved from GenBank™ accession numbers.

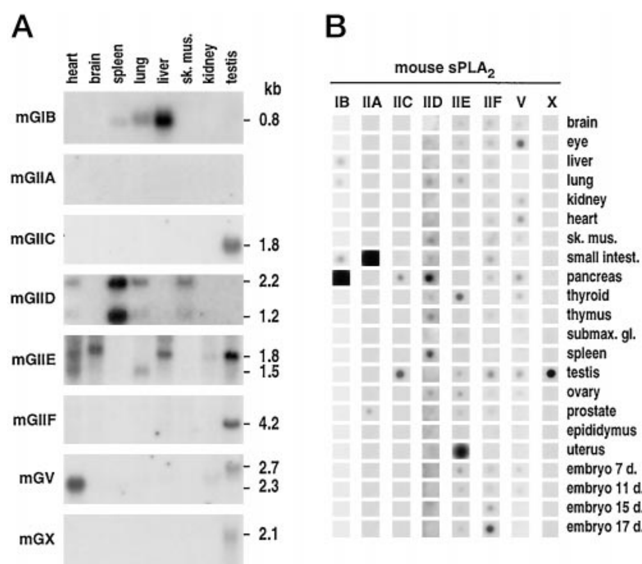

FIG. 3. **Northern blot and master blot analysis of the tissue distribution of mouse sPLA$_2$s.** A commercial Northern blot (*panel A*) containing 2 μg of poly(A)$^+$ RNA from different BALB/c mice tissues and a commercial master blot (*panel B*) containing normalized loading of 42–423 ng of poly(A)$^+$ RNA from tissues from Swiss Webster/NIH (embryos), BALB/c (pancreas and small intestine), or Webster (other tissues) mice were hybridized at high stringency with ^{32}P-labeled sPLA$_2$ RNA probes as described under "Experimental Procedures." *sk. musc.*, skeletal muscle; *small intest.*, small intestine; *submax. gl.*, submaxillary gland; *embryo 7 d.*, 7-day embryo. Blots were exposed for 2–7 days depending on the hybridization signal. Note that for mGIB and mGIIA sPLA$_2$s, exposure times were chosen to visualize expression of the sPLA$_2$s in tissues such as liver, lung, small intestine (mGIB sPLA$_2$), and prostate (mGIIA PLA$_2$). This leads to a very strong signal in pancreas and small intestine for mGIB and mGIIA sPLA$_2$s, respectively.

Tissue Distribution of the Mouse sPLA$_2$s—The tissue distribution of the three novel mouse sPLA$_2$s was analyzed by hybridization at high stringency of a mouse multiple tissue Northern blot and a mouse RNA master blot containing normalized loading of poly(A)$^+$ RNA from 22 different tissues. These blots were successively hybridized with the probes for the three novel sPLA$_2$s and then with the five other mouse sPLA$_2$ probes to directly compare the tissue distribution of the eight different mouse enzymes. Northern blot analysis indicates that mGIIE sPLA$_2$ is expressed from different transcripts, which are found in various tissues including testis (Fig. 3*A*). More detailed analysis with the mouse RNA master blot revealed a very high expression of mGIIE sPLA$_2$ in uterus as compared with the other mouse tissues (Fig. 3*B*). Finally, mGIIE sPLA$_2$ was found to be expressed at significant levels in thyroid and at lower levels in various other tissues including embryo (Fig. 3*B*). Northern blot analysis indicates that mGIIF sPLA$_2$ is expressed as a single transcript of 4.2 kilobases found in testis (Fig. 3*A*). Further analysis with the master blot indicates a very high expression in mouse embryo, and this expression increases with the age of development (Fig. 3*B*). Lower but significant expression of mGIIF sPLA$_2$ is also observed in testis, small intestine, pancreas, eye, and brain (Fig. 3*B*). Finally, mGX sPLA$_2$ was found to be expressed as a 2.1-kilobase transcript that is only detected in testis in both Northern and master blot analysis (Fig. 3). A fairly high expression of mGX sPLA$_2$ was also observed in stomach upon hybridization of another commercial mouse Northern blot (Origene, catalog no. MB1012; data not shown), indicating that mGX sPLA$_2$ expression is not restricted to testis, but limited to a low number of tissues. Surprisingly, the tissue distribution pattern of mGX sPLA$_2$ appears very different from that of hGX sPLA$_2$, which is expressed in spleen, thymus, blood leukocytes, lung, colon, and pancreas (9).

Fig. 3 also shows the tissue distribution of the various other mouse sPLA$_2$s. The pancreatic-type group IB sPLA$_2$ is ex-

pressed in large amounts in pancreas and at lower levels in liver, lung, spleen, and small intestine (Fig. 3). This expression pattern fits well with previously published data (26, 27). On the other hand, the expression of mGIIA sPLA$_2$ was found to be very narrow, in agreement with previous data (64). Indeed, mGIIA sPLA$_2$ is expressed at very high levels in small intestine, at relatively modest levels in prostate, and is not detected in all other analyzed tissues (Fig. 3). As described previously (42, 65), mGIIC sPLA$_2$ is expressed at high levels in testis (Fig. 3). Fairly high expression was also detected in pancreas (Fig. 3*B*), suggesting that this enzyme may function in tissues other than testis (65). mGIID sPLA$_2$ expression was found in pancreas, spleen, and various other tissues (Fig. 3). In agreement with previous data showing strong expression of hGV and rGV sPLA$_2$s in heart (44, 45), Northern blot analysis shows that mGV sPLA$_2$ is also expressed at high levels in heart, while lower expression is observed in testis and kidney (Fig. 3*A*). However, a more detailed analysis with the RNA master blot indicates that mGV sPLA$_2$ is expressed at very high levels in eye compared with heart, and is also expressed in pancreas, thyroid, ovary, and 11- and 15-day-old embryos (Fig. 3*B*). Interestingly, group IIA sPLA$_2$ has been found in large amounts in human tears and displays strong bactericidal activity against Staphylococci and other Gram-positive bacteria (37, 66). Whether mGV sPLA$_2$ is also capable of bactericidal activity will be interesting to analyze in the future.

Taken together, the obtained data clearly show that all eight mouse sPLA$_2$s have different patterns of expression, suggesting distinct functions for each of these enzymes. On the other hand, it also appears from Fig. 3 that several sPLA$_2$s can be found in the same tissue. For example, mGIB, mGIIC, mGIID, mGIIF, and mGV sPLA$_2$s are all expressed in pancreas. Pancreatic group IB sPLA$_2$ has been shown to be secreted through both exocrine and endocrine pathways (24, 67, 68), but those used for the other sPLA$_2$s are unknown. Furthermore, mGIIC, mGIIF, mGV, and mGX sPLA$_2$s are all expressed in testis and mGIIA is found in prostate. So far, only the expression of mGIIC sPLA$_2$ has been analyzed in testis, and the results indicate expression in meiotic cells (65). We also found by RT-PCR analysis that mGIB, mGIIA, mGIID, mGIIF, and mGX sPLA$_2$s are all expressed in stomach, while mGIID, mGIIE, mGIIF, and mGV sPLA$_2$s are expressed in skin (data not shown). Finally, other tissues such as small intestine, lung, thymus, heart, or eye and embryos of different ages also contain several sPLA$_2$s (Fig. 3). Whether the sPLA$_2$s colocalize at cellular level in these tissues and have redundant or specific functions remains to be determined.

Enzymatic Properties of Mouse sPLA$_2$s—The three novel mouse sPLA$_2$s and the five previously cloned enzymes were transiently expressed in COS cells, and crude cell medium containing sPLA$_2$ activity was used to test the ability of the various sPLA$_2$s to hydrolyze phosphatidylglycerol, and phosphatidylcholine vesicles (Table III). [^{3}H]DPPG/POPG was the most preferred substrate for all enzymes. Hydrolysis of [^{3}H]DPPC/DOPC by mGIIA and mGIIC sPLA$_2$s could not be detected, indicating a strong preference of these two enzymes for phosphatidylglycerol over phosphatidylcholine vesicles. Besides these two sPLA$_2$s, mGIB sPLA$_2$ shows the highest preference (~70-fold) for [^{3}H]DPPG/POPG over [^{3}H]DPPC/DOPC, while mGIID sPLA$_2$ shows the lowest preference (~2-fold).

Substrate specificity of sPLA$_2$s is controlled by the affinity of the enzyme for the vesicle interface and by the active site preferences for the enzyme at the interface (69). The dissection of these two components is not possible with the low amounts of mouse sPLA$_2$s present in COS cell supernatants. The general trend from the data in Table III is that all eight mouse sPLA$_2$s are more active on vesicles of pure phosphatidylglycerol than on vesicles of pure phosphatidylcholine. Based on the fact that previously characterized sPLA$_2$s bind much more tightly to anionic vesicles than to charge neutral vesicles (70), the relatively low activity of the eight mouse enzymes on phosphatidylcholine vesicles is likely to be due to poor interfacial binding. To date, the only sPLA$_2$s that display high affinity for phosphatidylcholine vesicles are the cobra venom enzymes (71). Of all the mammalian sPLA$_2$s characterized to date, hGIIA sPLA$_2$ binds weakest to phosphatidylcholine vesicles. The lack of detectable activity of mGIIA sPLA$_2$ on phosphatidylcholine vesicles (Table III) suggest that its interfacial binding behavior is similar to that of hGIIA sPLA$_2$. hGV sPLA$_2$ binds much tighter to phosphatidylcholine vesicles than does hGIIA enzyme (72). The fact that mGV sPLA$_2$ is one of the most active mouse enzymes on phosphatidylcholine vesicles suggests that it also binds more tightly to the zwitterionic interface than does mGIIA, and the same is true for mGX sPLA$_2$ and especially for mGIID sPLA$_2$. Further studies with recombinant mouse enzymes will allow independent analysis of interfacial binding and preferences of the active sites for phospholipids with different polar head groups.

TABLE III
Initial velocities for the hydrolysis of small unilamellar vesicles of phospholipids by mouse sPLA$_2$s relative to the rate of hydrolysis of phosphatidylglycerol vesicles

sPLA$_2$[a]	[^{3}H]DPPG/POPG[b]	[^{3}H]DPPC/DOPC[b]
mGIB	1.0 (5,460)[c]	0.014
mGIIA	1.0 (1,650)	<0.01
mGIIC	1.0 (2,460)	<0.01
mGIID	1.0 (19,560)	0.43
mGIIE	1.0 (13,230)	0.039
mGIIF	1.0 (14,400)	0.025
mGV	1.0 (7,230)	0.083
mGX	1.0 (4,080)	0.073

[a] Concentrated (16-fold) COS cell supernatants were used for mGIIC, mGIID, mGIIE, and mGX sPLA$_2$s.

[b] The indicated unlabeled phospholipid is the major component in the vesicles.

[c] Numbers are the initial velocity for the hydrolysis of the vesicle relative to that for [^{3}II]DPPG/POPG. Numbers in parentheses are the background-corrected dpm of product measured for the 30-min time point. All reactions were carried out in triplicate, and the standard errors are ±20% or less.

Fig. 4 shows the pH rate profiles for mGIIE, mGIIF, and mGX sPLA$_2$s. mGX sPLA$_2$ shows the typical pattern for sPLA$_2$s, a rise in activity as the pH is increased from 5 to 7 and then a fall in activity at higher pH. Surprisingly, mGIIE and mGIIF sPLA$_2$s retain considerable catalytic activity at pH 5, with activity falling as the pH is raised above 6, suggesting that these two sPLA$_2$s may function in weakly acidic cellular compartments. Finally, as expected, no catalytic activity was detected for mGIIE, mGIIF, and mGX in the absence of calcium (1 mM EGTA, data not shown). A more detailed analysis of the concentration of calcium required for optimal activity was not carried out because of the presence of calcium in the COS cell culture medium.

Concluding Remarks—With the cloning of three novel mouse sPLA$_2$s in this paper and the previous cloning of five mouse sPLA$_2$s (20, 27, 40, 42, 43, 64), it is obvious that a diversity of sPLA$_2$s exist in mice (Table II). Our current knowledge indicates that group IB, IIA, IID, V, and X sPLA$_2$s also exist in humans (9, 25, 44, 73, 74).[3] However, group IIC sPLA$_2$ appears as a pseudogene in humans (43), and it remains to be analyzed whether group IIE and group IIF sPLA$_2$s are expressed in this

[3] E. Valentin, F. Ghomashchi, M. H. Gelb, M. Lazdunski, and G. Lambeau, unpublished data.

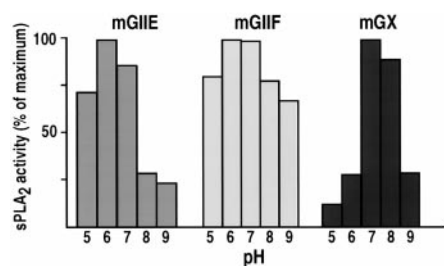

FIG. 4. **pH dependence of mGIIE, mGIIF, and mGX sPLA$_2$s.** pH rate profiles for the hydrolysis of [^{3}H]DPPG/POPG by mGIIE, mGIIF, and mGX sPLA$_2$s. Results are presented as percentage of maximal background-corrected dpm values measured in a 30-min reaction at the indicated pH containing 20 μl of COS cell supernatant and 30 μl of buffer. Maximal background-corrected values were 20,601, 8,081, and 1,207 for mGIIE, mGIIF, and mGX sPLA$_2$s, respectively.

species. So far, human orthologs of these two sPLA$_2$s have not been found in the EST data bases, possibly because of low expression of these enzymes in human tissues. Indeed, numerous ESTs have been identified for hGIB and hGIIA sPLA$_2$s, and both enzymes are widespread in human tissues (9). On the other hand, a few ESTs for hGV and hGX sPLA$_2$s have been found, in agreement with their relatively lower levels of expression (9).

The presence of a diversity of sPLA$_2$s, which all have a specific tissue distribution, raises the question of the respective biological functions of these enzymes. Because all of them are catalytically active enzymes, their function can be to regulate the release of lipid mediators in different tissues and cells, acting on various phospholipid substrates, extracellularly or within different cellular compartments, and under physiological or pathological conditions (5, 32, 33). However, the identification of sPLA$_2$ receptors with venom sPLA$_2$s, which can have mammalian sPLA$_2$s as endogenous ligands, suggests that mammalian sPLA$_2$s not only function as enzymes but also as ligands (6). Furthermore, role of sPLA$_2$s in host defense against various invading organisms including bacteria also must be considered (35–37). Further work is clearly needed to understand the biological functions of the different members of this growing family of sPLA$_2$s.

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