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Cloning and Recombinant Expression of a Novel Mouse-secreted Phospholipase A_2*

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Secreted phospholipases A_2 (sPLA$_2$s) form a class of structurally related enzymes that are involved in a variety of physiological and pathological effects including inflammation and associated diseases, cell proliferation, cell adhesion, and cancer, and are now known to bind to specific membrane receptors. Here, we report the cloning and expression of a novel sPLA$_2$ isolated from mouse thymus. Based on its structural features, this sPLA$_2$ is most similar to the previously cloned mouse group IIA sPLA$_2$ (mGIIA sPLA$_2$). As for mGIIA sPLA$_2$, the novel sPLA$_2$ is made up of 125 amino acids with 14 cysteines, is basic (pI = 8.71) and its gene has been mapped to mouse chromosome 4. However, the novel sPLA$_2$ has only 48% identity with mGIIA and displays similar levels of identity with the other mouse group IIC and V sPLA$_2$s, indicating that the novel sPLA$_2$ is not an isoform of mGIIA sPLA$_2$. This novel sPLA$_2$ has thus been called mouse group IID (mGIID) sPLA$_2$. In further contrast with mGIIA, which is found mainly in intestine, transcripts coding for mGIID sPLA$_2$ are found in several tissues including pancreas, spleen, thymus, skin, lung, and ovary, suggesting distinct functions for the two enzymes. Recombinant expression of mGIID sPLA$_2$ in *Escherichia coli* indicates that the cloned sPLA$_2$ is an active enzyme that has much lower specific activity than mGIIA and displays a distinct specificity for binding to various phospholipid vesicles. Finally, recombinant mGIID sPLA$_2$ did not bind to the mouse M-type sPLA$_2$ receptor, while mGIIA was previously found to bind to this receptor with high affinity.

Phospholipases A_2 (PLA$_2$,[1] phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4) are a family of enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position, producing free fatty acids and lysophospholipids, and the list of members is expanding (1–5). Several mammalian intracellular and secreted PLA$_2$s (sPLA$_2$s) have been characterized and classified as different groups (5). Intracellular PLA$_2$s comprise the well known Ca^{2+}-sensitive arachidonoyl-selective 85-kDa cPLA$_2$ (6) and a number of Ca^{2+}-independent PLA$_2$s (7). Over the last decade, five different secreted PLA$_2$s have been identified and classified as five distinct groups (4, 5). Main common characteristics of these sPLA$_2$s are a relatively low molecular mass (13–16 kDa), the presence of many disulfide bridges, a broad selectivity for phospholipids with different polar head groups and fatty acid chains, and an absolute catalytic requirement for millimolar concentrations of Ca^{2+} (1, 8). Group IB sPLA$_2$ is known as pancreatic-type sPLA$_2$ because of its initial purification from pancreatic juice (9). Subsequently, this sPLA$_2$ was detected in other tissues including lung, spleen, kidney and ovary (10), and its involvement in various physiological and pathophysiological responses such as cell proliferation, cell contraction, lipid mediator release, acute lung injury, and endotoxic shock has been proposed (11–13). 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 (3, 14). This sPLA$_2$ is a potent mediator of inflammation (3, 14) and a potent bactericidal agent (15–17). It is also expressed at high levels in various gastrointestinal cancers (18, 19). More recently, it has been proposed that mouse group IIA (mGIIA)[2] sPLA$_2$ serves as a tumor suppressor gene in colorectal cancer (20, 21). Much less is known about the regulation and biological roles of group IIC, V, and X sPLA$_2$s. Rat and mouse group IIC sPLA$_2$s have been cloned (22), but this sPLA$_2$ appears to be a non-functional pseudogene in humans (23). Group V sPLA$_2$ is highly expressed in heart (24) and has been recently detected in murine macrophages and mastocytes, where it plays a role in lipid mediator production (25, 26). Group X sPLA$_2$ has been cloned in humans and displays distinct structural features (4). It is mainly expressed in tissues

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF124374.

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[1] The abbreviations used are: PLA$_2$, phospholipase A_2; sPLA$_2$, secreted phospholipase A_2; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RACE-PCR, rapid amplification of cDNA ends by polymerase chain reaction; GST, glutathione *S-t*ransferase; DOPM, 1,2-dioleoyl-*sn*-glycerol-3-phosphomethanol; DPPC, [^{3}H]DPPC and [^{3}H]DPPG, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoglycerol (mixture of stereo isomers) ([^{3}H] in the 9,10 positions of the *sn*-2 chain); DTPM, 1,2-ditetradecyl-*sn*-glycero-3-phosphomethanol; [^{14}C]PAPE, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphoethanolamine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; [^{14}C]SAPC, 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; EST, expressed sequence tag; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

[2] A comprehensive abbreviation system for the various mammalian sPLA$_2$s was used: each sPLA$_2$ was abbreviated with a lowercase letter indicating the sPLA$_2$ species (m, h, and r for mouse, human, and rat, respectively), followed by uppercase letters identifying the sPLA$_2$ group (GIB, GIIA, GIIC, GIID, GV, and GX for group IB, IIA, IIC, V, and X sPLA$_2$s, respectively).

and cells of the immune system, suggesting a role related to inflammation and/or immunity (4).

Besides their roles as enzymes, some sPLA$_2$s have been shown to bind to specific membrane receptors (27). To date, two main types of high affinity sPLA$_2$ receptors have been identified initially using venom sPLA$_2$s as ligands. N-type receptors are highly expressed in brain membranes and display high affinities for neurotoxic venom sPLA$_2$s but not for nontoxic venom sPLA$_2$s, suggesting that these receptors play a role in the neurotoxic effects of sPLA$_2$s (28, 29). The physiological role(s) and the endogenous ligands of N-type receptors remain to be discovered. M-type receptors were identified in skeletal muscle (30), and have now been cloned from different animal species (11, 27). These receptors may be involved in various biological effects of pancreatic group IB sPLA$_2$ (11, 13), and the recent targeted disruption of the M-type receptor gene has indicated a role of this receptor in the inflammatory processes leading to endotoxic shock (12). Finally, studies with mammalian sPLA$_2$s have shown that M-type receptors can be physiological targets for group IB and/or group IIA sPLA$_2$s, depending on the animal species (11, 27, 31).

In light of the growing molecular diversity of mammalian sPLA$_2$s, we have searched for novel sPLA$_2$s in data bases and found a novel mouse sPLA$_2$. This enzyme displays all the structural features of mammalian group IIA sPLA$_2$s (7 disulfides and a C-terminal extension), and displays the best identity score (48%) with the previously cloned mGIIA sPLA$_2$, also known as enhancing factor (32, 33). However, the novel sPLA$_2$ has similar levels of identity with mouse group IIC (43%) and group V (47%) sPLA$_2$s, indicating that the novel sPLA$_2$ is not significantly more related to mGIIA sPLA$_2$ than to these two other sPLA$_2$s and thus is not an isoform of mGIIA sPLA$_2$. For these reasons, the novel sPLA$_2$ is designated hereafter as mouse group IID (mGIID) sPLA$_2$.

EXPERIMENTAL PROCEDURES

Molecular Cloning of mGIID sPLA$_2$—Search for sPLA$_2$ homologs in gene data bases stored at the National Center for Biotechnology by using the tBLASTn sequence alignment program (34) resulted in the identification of an expressed sequence tag (EST) (IMAGE Consortium clone identification 1225779 5′, GenBank accession number AA762051) that was derived from mouse thymus and that encodes a partial sequence of a novel sPLA$_2$. The 501-nucleotide EST sequence was then used to clone the entire cDNA sequence coding for this novel sPLA$_2$ by 5′ RACE-PCR experiments. These experiments were performed as follows: 10 μg of total mouse thymus RNA were reverse-transcribed using oligo(dT) primers (Promega) and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). Second strand DNA synthesis was carried out using RNase H and DNA polymerase I for 2 h at 16 °C. After blunt-ending with T4 DNA polymerase, double-strand cDNA was precipitated and ligated to adaptors containing sequences for the universal primers Sp6 and KS and *Sal*I and *Eco*RI restriction sites. A first PCR reaction using KS primer and a specific sPLA$_2$ reverse primer corresponding to nucleotides 211–234 (Fig. 1) was followed by a second amplification using the same KS primer and a second specific reverse primer corresponding to nucleotides 89–111 (Fig. 1). PCR conditions were: 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, 35 cycles. PCR products were subcloned into the pGEM-T easy vector (Promega) and screened using a ^{32}P-labeled primer corresponding to nucleotides 59–87 (Fig. 1). Positive clones were analyzed by restriction and sequenced by using an automatic sequencer (Applied Biosystems model 377).

Chromosomal Localization of the mGIID sPLA$_2$ Gene—The CV panel of mouse X Chinese hamster somatic cell hybrids (35) was used for the chromosomal assignment of mGIID sPLA$_2$. For that purpose, a genomic fragment of 5.5 kilobase pairs was isolated by PCR and partially sequenced, and a set of specific primers was designed. A forward primer within an intron (5′-AAAGATTAGGTGGCTGGAACAACCA-3′) and an antisense primer within the exon coding for the active site region (5′-CATCCATCGATCTTCAGGTGGGCA-3′) were found to amplify a fragment of 230 nucleotides from a mouse CBA/H DNA template, while a product of 250 nucleotides was amplified with hamster V79TOR DNA as template. PCR reactions were performed in 25 μl containing 50 ng of DNA template, 0.5 μg of each primer, 1.5 mM $MgCl_2$, and 0.25 units of *Taq* polymerase (Eurobio, France). PCR conditions were: 94 °C for 2 min, followed by 5 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by 25 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. PCR products were analyzed on a 2% agarose gel.

Analysis of the Tissue Distribution of mGIID sPLA$_2$—Two Northern blots (Origene, catalog no. MB-1002 and MB-1012) and a mouse RNA Master blot (CLONTECH, catalog no. 7771-1) were probed with a randomly primed ^{32}P-labeled mGIID cDNA fragment (nucleotides 58–436 in Fig. 1) in 50% formamide, 5× SSPE (0.9 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM EDTA), 5× Denhardt's solution, 0.1% SDS, 20 mM sodium phosphate, pH 6.5, and 250 μg/ml denatured salmon sperm DNA for 18 h at 42 °C and 50 °C, respectively. The membranes were washed to a final stringency of 0.2× SSC (30 mM NaCl, 3 mM trisodium citrate, pH 7.0) in 0.1% SDS at 60 °C and exposed to Kodak Biomax MS films with a Transcreen-HE intensifying screen.

Recombinant Expression of mGIID sPLA$_2$ in COS Cells—The full-length cDNA coding for mGIID sPLA$_2$ (nucleotides 1–435 in Fig. 1) was subcloned into the expression vector pCI-neo (Promega) and transfected into COS cells as described (4). Five days after transfection, cell medium was collected and analyzed for sPLA$_2$ activity or loaded on a heparin-agarose column (Sigma) to concentrate sPLA$_2$ activity. The column was washed with 0.1 M NaCl, eluted stepwise with 1 M NaCl, and eluted fractions were assayed for sPLA$_2$ activity using [^{3}H]oleate-labeled *E. coli* membranes (36).

Recombinant Expression of mGIID sPLA$_2$ in E. coli—A PCR fragment coding for a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the mGIID mature protein was prepared with *Pwo* DNA polymerase (Roche Molecular Biochemicals) and subcloned in frame with a truncated glutathione *S*-transferase (~10 kDa) encoded by the modified pGEX-2T vector (pAB_3), which was previously used for the expression of porcine pancreatic sPLA$_2$ (37). Recombinant expression of the fusion protein was performed in *E. coli* BL21 host cells grown in 1 liter of Terrific broth containing ampicillin (100 μg/ml). Cells were grown to an OD_{600} ~0.8, and induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM) for 4 h at 37 °C. Cells were pelleted and resuspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, and 1% deoxycholate) for 1 h at 4 °C. The suspension was homogenized with a French press (SLM Aminco). Inclusion bodies were collected by centrifugation at 10,000 × *g* for 20 min and washed four times with lysis buffer without detergents. The resulting pellet was solubilized in 100 ml of 6 M guanidine-HCl, 0.3 M Na_2SO_3, 20 mM borate, pH 7.4, and proteins were sulfonated by addition of 0.05 volume of Thannhauser reagent (38) for 1 h at room temperature. After overnight dialysis at 4 °C against 5 liters of 1% acetic acid, the precipitated protein was pelleted and resuspended at 0.2 mg/ml protein in 6 M guanidine-HCl, 20 mM borate, pH 8.0. The denatured sPLA$_2$ (200 ml) was refolded by dialysis against 8 liters of 0.9 M guanidine-HCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 5 mM cysteine for 24 h at 4 °C. The refolded protein was then dialyzed against 8 liters of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$. The fusion protein solution was clarified by centrifugation and finally subjected to overnight digestion at 20 °C with 40 units of factor Xa (Amersham Pharmacia Biotech). The mixture was loaded at 2 ml/min onto a 5-ml Hi-Trap heparin-Sepharose column (Amersham Pharmacia Biotech) equilibrated with buffer A (100 mM NaCl, 20 mM Tris-HCl, pH 7.4). The column was washed with buffer A until OD_{280} dropped to zero and then eluted with a linear gradient of NaCl (0.1–1 M NaCl, in 50 min at 1 ml/min). Fractions containing sPLA$_2$ activity were pooled and directly loaded on a Nucleosil™ C18 reverse phase HPLC column (4.6 × 250 mm, 4.2 ml, 300 Å, 5 μm). Elution was performed at 1 ml/min using water/acetonitrile with 0.1% trifluoroacetic acid (10–30% acetonitrile over 20 min, followed by 30–60% acetonitrile over 110 min). N-terminal sequences were determined by automated Edman degradation with an Applied Biosystems Sequencer model A473. Ion spray mass spectrometry was performed on a simple-quadrupole mass spectrometer equipped with an ion-spray source and using polypropylene glycol for calibration.

Interfacial Kinetic and Phospholipid Binding Studies—Small unilamellar vesicles of DOPM, POPC, POPE, POPG, and POPS (all from Avanti Polar Lipids) were prepared by sonication as described (39). Large unilamellar POPC vesicles were prepared by extrusion (40). *N*-Dansyl-1,2-dihexadecyl-phosphatidylethanolamine and DTPM were prepared as described (41). Initial velocities for the hydrolysis of these vesicles by mGIIA, mGIID, and hGIIA sPLA$_2$s were monitored using a fluorescent fatty acid displacement assay (42). Reactions contained 40 μM phospholipid, 10 μg of rat liver fatty acid binding protein, 1 μM

11-(dansylamino)-undecanoic acid (Molecular Probes, Eugene, OR) in 1 ml of 100 mM Tris-HCl, pH 8.0, 2.5 mM $CaCl_2$ at 30 °C. mGIIA (2–100 ng, prepared as described in Ref. 31), mGIID (1 μg), or hGIIA (2 ng to 1 μg, prepared as described in Ref. 43) sPLA$_2$s was added to start the reaction in a stirred fluorescence cuvette with excitation at 350 nm and emission at 500 nm. For each type of phospholipids, assays were calibrated by measuring the fluorescence change following the addition of a known amount of oleic acid to reaction mixtures containing all components except enzyme. Competitive substrate specificity studies to examine the phospholipid headgroup preferences of mGIIA, mGIID, and hGIIA sPLA$_2$s (43) were carried out with the dual radiolabel method (44) using pairs of competing and radiolabeled substrates present as minor components in sonicated DOPM vesicles. Reaction mixtures contained 40 μM DOPM containing ~120,000 cpm of ^{3}H-phospholipid and ~20,000 cpm ^{14}C-phospholipid in 100 μl of the same buffer as was used in the kinetic studies described above. Lipids were mixed in chloroform, solvent was removed with a stream of N_2 and then *in vacuo* for 30 min, and buffer was added followed by sonication. Mixtures were incubated at 30 °C for 30 min with sufficient enzyme to hydrolyze 10–20% of the preferred radiolabeled substrate (10–20 ng of mGIIA or hGIIA sPLA$_2$s, ~1 μg of mGIID sPLA$_2$). Reactions were quenched, and liberated fatty acids were prepared for scintillation counting as described (45) to determine the relative $k_{cat}*/K_m*$ values (44). The sources of radiolabeled phospholipids for these experiments are as follows: [^{3}H]DPPC (89 Ci/mmol, NEN Life Science Products), [^{3}H]DPPG (400 Ci/mol, prepared from [^{3}H]DPPC by head group exchange with cabbage phospholipase D from Sigma as described in Ref. 46), [^{14}C]PAPE (53 Ci/mol, NEN Life Science Products), [^{14}C]SAPC (53 Ci/mol, Amersham Pharmacia Biotech). Interfacial binding of sPLA$_2$s to anionic vesicles was monitored using energy transfer measured with a spectrofluorimeter as described (47). The release of fatty acids from live RAW 264.7 cells (400,000 cells/ml) treated exogenously with sPLA$_2$s was measured with the fatty acid binding protein assay as described (48). Specific activities were calculated from the rates measured in the presence of two or three different amounts of enzyme under conditions where the rate was proportional to the amount of enzyme (5 ng to 6 μg, depending on the sPLA$_2$). Cobra venom (*Naja naja*) sPLA$_2$ was from Sigma.

Binding Studies with Mouse M-type Receptor—Competition binding assays with recombinant mouse M-type receptor expressed in COS cells were performed as described using ^{125}I-OS$_1$ as labeled sPLA$_2$ ligand (31). Briefly, membranes containing M-type receptor, ^{125}I-OS$_1$, and unlabeled sPLA$_2$s were incubated at 20 °C in 0.5 ml of binding buffer (140 mM NaCl, 0.1 mM $CaCl_2$, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin). Incubations were started by addition of membranes and filtered after 60 min through GF/C glass fiber filters presoaked in 0.5% polyethyleneimine.

RESULTS AND DISCUSSION

Molecular Cloning of mGIID sPLA$_2$ and Chromosomal Localization—Protein sequences of various sPLA$_2$s were used to search for novel sPLA$_2$s in gene data bases by using the tBLASTn sequence alignment program (34). This resulted in the identification of an EST of 501 nucleotides (Fig. 1) that was derived from a mouse thymus cDNA library, and that displayed high homology to sPLA$_2$s. We thus postulated that this EST was a partial copy of a mRNA coding for a novel low molecular mass sPLA$_2$. This thymus EST sequence was then used to search in data bases for other related EST sequences. A second EST derived from a mouse mammary gland cDNA library was identified and found to have identity to the 3′-end of the thymus EST sequence. On the other hand, no related sequence was found on human EST data bases. Since none of the mouse EST sequences were found to encode for the full-length sPLA$_2$, PCR experiments were performed to clone the entire cDNA sequence. Primers were first designed from the thymus EST sequence and used in PCR experiments on different mouse tissue cDNAs. DNA products of the expected size were obtained from mouse BALB/c thymus cDNAs and found to have the same sequence as the original EST sequence (Fig. 1). 5′ RACE-PCR experiments were then performed on the same cDNAs to obtain the full-length cDNA (see "Experimental Procedures"). Screening of the amplified products with a specific oligonucleotide probe resulted in the identification of a DNA fragment of

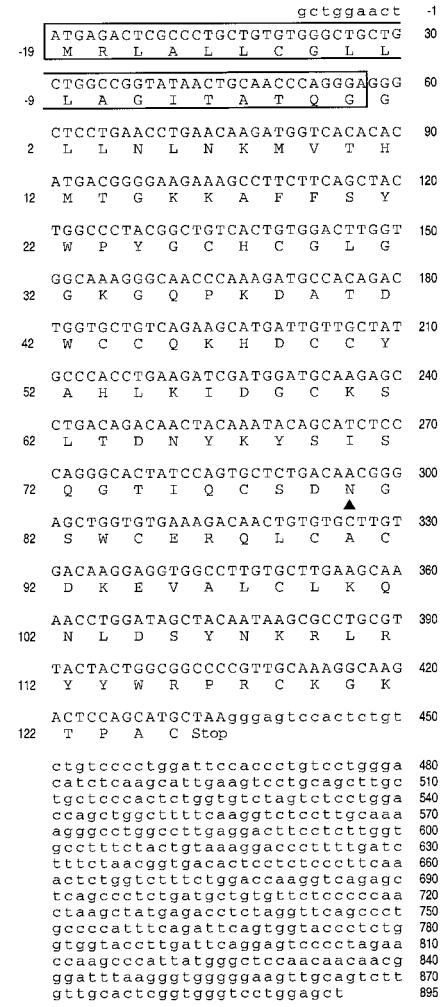

FIG. 1. **Nucleotide and amino acid sequences of mGIID sPLA$_2$.** The predicted signal peptide segment is *boxed*. The putative *N*-glycosylation site is marked with a *triangle*. The two EST sequences identified from thymus and mammary gland cDNA libraries (IMAGE. Consortium Clone identification 1225779 5′ and 875198 5′) corresponded to nucleotides 34–534 and 443–895, respectively.

128 nucleotides that is identical to the EST sequence in its 3′-end and contains in its 5′-end sequence all the expected features of a sPLA$_2$ including a signal peptide sequence preceded by an initiator methionine. Based on this sequence, a new set of primers was designed to amplify the full-length sPLA$_2$ cDNA from mouse BALB/c thymus cDNAs. A DNA fragment of the expected size (435 nucleotides) was amplified, and its sequence was found to be identical to the thymus EST sequence and the 5′ RACE-PCR product and to encode for the full-length sPLA$_2$ (Fig. 1). Furthermore, an identical PCR product was obtained from mouse 129/Sj intestine cDNAs. This latter result also indicates that the novel sPLA$_2$ is not mutated in 129/Sj mice, in contrast to mGIIA sPLA$_2$ (20, 49). The final cDNA sequence resulting from the alignment of the amplified PCR products and of the two EST sequences is made of 904 nucleotides with an open reading frame of 435 nucleotides (Fig. 1).

The open reading frame of the novel sPLA$_2$ encodes for a signal peptide of 19 amino acids, followed by a mature protein of 125 amino acids (calculated molecular mass of ~14.3 kDa) containing a putative *N*-glycosylation site (Fig. 1). The calculated isoelectric point of the mature protein is 8.71, thus making the novel mouse sPLA$_2$ a slightly less basic protein than mGIIA sPLA$_2$ (pI 9.22). The protein sequence contains all the

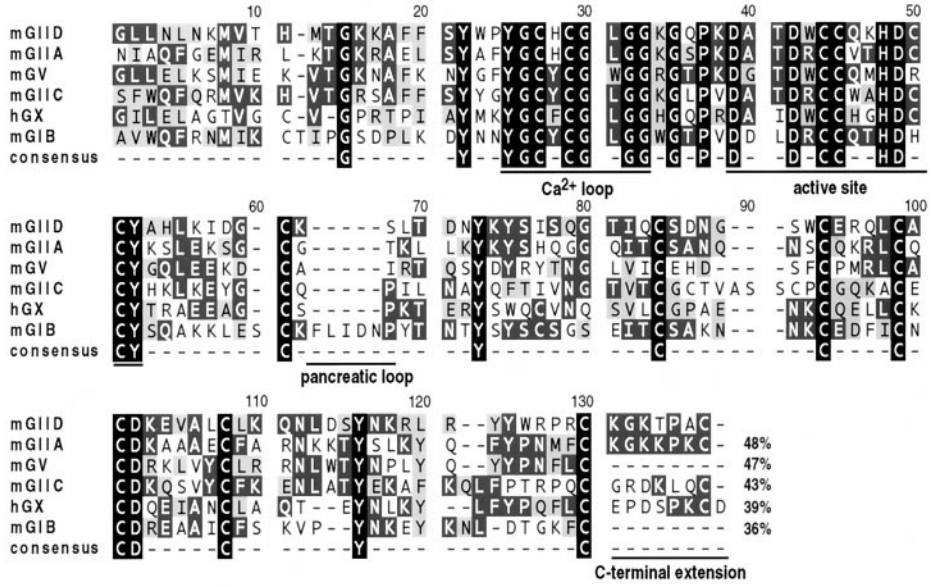

FIG. 2. **Alignment of the amino acid sequence of mGIID sPLA$_2$ with other mouse sPLA$_2$s and hGX sPLA$_2$.** Sequences of mature sPLA$_2$ proteins are shown: mGIIA sPLA$_2$ (33), mGV sPLA$_2$ (23), mGIIC sPLA$_2$ (22), hGX sPLA$_2$ (4), and mGIB sPLA$_2$ (31). The level of identity between mGIID sPLA$_2$ and other sPLA$_2$s is indicated.

amino acids that are conserved in active sPLA$_2$s, indicating that the newly cloned sPLA$_2$ could be catalytically active. Fig. 2 shows an alignment of the amino acid sequence of the cloned sPLA$_2$ with that of mammalian sPLA$_2$s belonging to various groups. Based on the structural features of the novel sPLA$_2$ and on the updated classifications of sPLA$_2$s (2, 4, 5, 27), the cloned sPLA$_2$ clearly belongs as a group IIA enzyme. Indeed, it displays all the structural features of group IIA sPLA$_2$s, including 7 disulfides and the C-terminal extension terminating with a cysteine linked to cysteine 50 that is specifically found in group II sPLA$_2$s. On the other hand, the cloned sPLA$_2$ does not contain the specific features of group I (specific disulfide at cysteines 11–77, pancreatic or elapid loops, no C-terminal extension), group IIB (6 disulfides, C-terminal extension), group IIC (8 disulfides, 4 amino acid insertion at positions 90–94, C-terminal extension), group V (6 disulfides, no C-terminal extension), or group X sPLA$_2$s (8 disulfides including those specific of group I and group II enzymes, prepropeptide sequence, C-terminal extension). The cloned sPLA$_2$ is, however, only 48% identical to mGIIA, indicating that this sPLA$_2$ is not an isoform of mGIIA sPLA$_2$, but a second member of group IIA sPLA$_2$s in mice. This is in marked contrast to the previous identification of the two close isoforms of porcine group IB sPLA$_2$ that differ only at four amino acid positions (50). The level of identity of the cloned sPLA$_2$ to mGIIC (47%) and mGV (43%) sPLA$_2$s is in fact similar to that to mGIIA sPLA$_2$ (48%), further indicating that the novel sPLA$_2$ is not an isoform of mGIIA sPLA$_2$. Finally, the novel sPLA$_2$ displays slightly lower levels of identity to mGIB (39%) and hGX (36%) sPLA$_2$s. All together, these data indicate that the cloned sPLA$_2$ displays typical structural features of group IIA sPLA$_2$s, but is not closely related to the other mouse sPLA$_2$s including mGIIA sPLA$_2$. This novel sPLA$_2$ has thus been called mouse group IID (mGIID) sPLA$_2$.

The chromosomal localization of the mGIID sPLA$_2$ gene was determined by PCR screening of mouse X hamster somatic cell hybrids with a specific set of primers derived from the mGIID sPLA$_2$ gene. This set of primers was found to amplify a fragment of 230 nucleotides from mouse genomic DNA (CBA/H), and gave a fragment of 250 nucleotides with genomic hamster DNA (V79TOR) as template. Out of the 24 hybrid lines tested, 9 gave a PCR product of the expected size. The chromosomal content of these hybrids as well as the results of PCR amplification are shown in Table I. The discordance ratio for each chromosome was calculated, and the lowest value (0/24) is consistent with the mapping of the mGIID sPLA$_2$ gene to mouse chromosome 4. Interestingly, the genes for mGIIA, mGIIC, and mGV sPLA$_2$s have all been mapped in the distal part of chromosome 4 (23). Whether the mGIID sPLA$_2$ gene is localized in a gene cluster with these three sPLA$_2$s remains to be determined.

Transcription Pattern of mGIID sPLA$_2$—The tissue distribution of mGIID sPLA$_2$ was analyzed by hybridization at high stringency to mouse Northern blots (Fig. 3*A*) and RNA Master blot (Fig. 3*B*). These experiments indicate the presence of two transcripts of 1.2 and 2.2 kb, which are abundant in spleen and thymus and present at lower levels in lung and skin. No hybridization was observed in several other tissues including brain, heart, liver, and testis, while a signal was barely detectable in small intestine (Fig. 3*A*). Screening of a mouse RNA Master blot was then used to extend the analysis of the tissue distribution of mGIID sPLA$_2$ and to more accurately determine the relative expression levels of the mGIID transcripts (Fig. 3*B*), since poly(A)$^+$ loadings on this commercial Master blot have been accurately quantified with several housekeeping genes. With this Master blot, highest levels of mGIID transcripts were found in pancreas and spleen. Decreasing levels were observed in thymus, skeletal muscle, lung, ovary, small intestine, thyroid, eye, and epididymus. As for Northern blots, no signal was detected in brain, liver, kidney, heart, and testis. Finally, no expression of mGIID sPLA$_2$ was found in submaxillary gland, in uterus, and in embryos at different stages of development (Fig. 3*B*).

The tissue distribution pattern of mGIID sPLA$_2$ appears to be distinct from that of other mouse sPLA$_2$s, suggesting that mGIID sPLA$_2$ could have specific function(s). In particular, its tissue distribution is very different from that of mGIIA sPLA$_2$, which is expressed at very high levels in intestine and is not detected in several other tissues (31, 32). Another difference between these two sPLA$_2$s is the presence of a functional mGIID sPLA$_2$ in the mouse 129/Sj strain. Indeed, our results indicate that a full-length mGIID cDNA that encodes for a functional sPLA$_2$ can be amplified from mouse 129/Sj intestine cDNA (see above). In contrast, the mouse 129/Sj strain mGIIA sPLA$_2$ gene contains a frameshift mutation (20, 49). The pattern of expression of mGIID sPLA$_2$ is also different from those of mGIIC and mGV sPLA$_2$s. Indeed, these latter two sPLA$_2$s are mainly expressed in testis (22) and heart (24), and mGIID

TABLE I

Chromosomal assignment of mGIID sPLA$_2$ with a mouse/hamster somatic cell hybrid panel

The table shows the mouse chromosomal content of each hybrid cell line: + and − indicate presence or absence of the chromosome, respectively; (+) indicates that the chromosome is present in only a portion of the cells; +/− indicates that inconsistent results have been observed in PCR experiments. The presence or absence of the PCR product of 230 nucleotides specific for the mGIID sPLA$_2$ gene is indicated in the mGIID column. Discordance ratio for each chromosome is given underneath.

Hybrid	Mouse Chromosome																					mGIID
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X	Y	
CV1	+	−	−	−	−	−	−	(+)	−	−	−	−	−	+	−	+	−	+	−	+	+	−
CV1/1/1	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	−	+	−	−
CV1/3/1/1	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	−	−	−	+	−	−
CV3	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	+	+	+	−
CV3/1/1/1	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	+	−	−	+	+	+	−
CV3/2/1/1	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−	+	−	−	−	+	+	−
CV3/3/1/1	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	+	+	−	+	+	+	−
CV4	+	+	−	+	−	+	−	−	−	−	−	−	+	+	+	−	+	−	−	+	+	+
CV4/1/1/1	+	+	−	+	−	+	−	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+
CV4/2/1/1	+	−	−	+	−	+	−	−	−	−	−	−	−	+	+	−	+	−	−	+	+	+
CV7	−	+	−	+	−	+	+	−	−	−	−	−	+	+	−	+	+	−	−	+	+	+
CV7/1/1/1	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−	−	+	+	−
CV7/2/1/1	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	+	+	−
CV7/3/1/1	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	−
CV8	+	+	−	+	−	+	+	+	−	+	−	+	+	+	+	−	−	−	+	+	+	+
CV8/1/1/1	+	+	−	+	−	+	+	+	−	+	−	+	+	+	+	−	+	−	+	+	+	+
CV8/2/1/1	−	+	−	+	−	+	+	+	−	+	−	+	+	+	+	−	−	−	−	+	−	+
CV10	−	−	+	−	−	+	+	+	−	−	−	−	−	+	−	+/−	−	+	−	+	+	−
CV10/1/1/1	−	−	−	−	−	+	+	+	−	−	−	−	+	+	−	+	+	+	−	+	+	−
CV10/2/1/1	−	−	+	−	−	+	+	−	−	−	−	−	+	+	−	+	+	+	−	+	+	−
CV10/3/1/1	−	−	−	−	−	+	+	−	−	−	−	−	+	+	−	+	+	+	−	+	+	−
CV10/3/1/1R	−	−	−	−	−	−	−	−	−	−	−	−	+	+	−	+	−	+	−	−	+	−
MOV11/2/4/1	+/−	−	+	+	+	+	+	−	−	+	−	+	+	−	+	−	−	+	+	+	−	+
MOV11/3/1/2	+	(+)	+	+	+	+	+	−	+	−	−	+	+	−	+	−	+	+	+	+	−	+
Discordance	6	2	12	0	7	9	13	9	8	5	9	4	12	16	1	21	9	13	8	14	16	

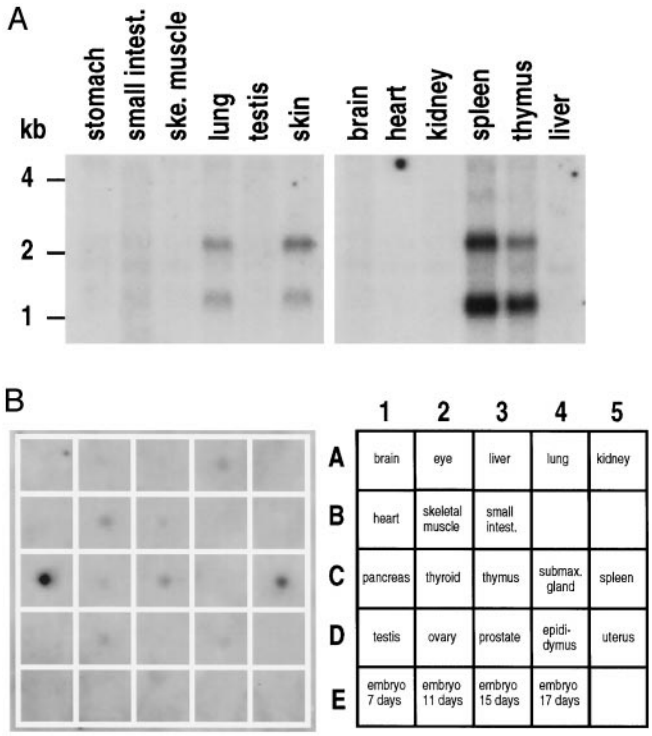

FIG. 3. **Northern blot and Master blot analysis of the tissue distribution of mGIID sPLA$_2$.** Commercial Northern blots containing 2 μg of poly(A)$^+$ RNA (*panel A*) and a Master blot (*Panel B*) containing 100–500 ng of poly(A)$^+$ RNA from mouse tissues were hybridized at high stringency with a ^{32}P-labeled mGIID sPLA$_2$ probe as described under "Experimental Procedures." *ske. muscle*, skeletal muscle; *small intest.*, small intestine; *submax. gland*, submaxillary gland. Filters were exposed for 7 days.

sPLA$_2$ is not expressed in these tissues (Fig. 3). In fact, the tissue distribution of mGIID sPLA$_2$ is somewhat similar to that of mGIB sPLA$_2$, which is expressed in very large amounts in pancreas, and found at lower levels in lung and spleen (31). However, mGIB sPLA$_2$, but not mGIID sPLA$_2$, was found to be expressed at high levels in liver (31). In further contrast to mGIID sPLA$_2$, hGIB sPLA$_2$ is detected in prostate, testis, and lung and liver from fetal tissues (4). Finally, while group IB sPLA$_2$ has been shown to be secreted by both exocrine and endocrine pancreatic cells (9, 51, 52), the type of pancreatic cells that secrete mGIID sPLA$_2$ is unknown.

Recombinant Expression of mGIID sPLA$_2$—As a first step to express mGIID sPLA$_2$, we transfected its full-length cDNA into COS cells and analyzed sPLA$_2$ activity in cell medium at different times after transfection. Only very low sPLA$_2$ activity was detected in cell supernatants using radiolabeled *E. coli* membranes as a substrate (data not shown), suggesting that mGIID sPLA$_2$ is expressed at low levels and/or has low specific activity. Furthermore, higher sPLA$_2$ activity was not detected after cell surface extraction with 1 M KCl or after cell lysis (data not shown). These results are in marked contrast with the transfection of the mGIIA sPLA$_2$ cDNA that leads to a relatively high sPLA$_2$ activity in the cell medium (data not shown). We also tried transfection with a plasmid containing a chimera cDNA construct consisting of the hGIIA sPLA$_2$ signal peptide followed by the mGIID mature protein, as was previously performed for group IIC sPLA$_2$ (22). This construct did not, however, lead to higher expression of sPLA$_2$ activity in COS cell supernatants, although such constructs produced efficient expression of several other sPLA$_2$s (data not shown). Because mammalian group IIA sPLA$_2$s are known to display high affinity for heparin (3), we also tried to concentrate mGIID sPLA$_2$ contained in COS cell supernatants on a heparin column to more readily detect the mGIID sPLA$_2$ activity. We found that mGIID sPLA$_2$ activity can be bound to heparin column and eluted with high salt buffer, indicating that mGIID sPLA$_2$, as for mGIIA (31), group V (53), and hGIIA (48) sPLA$_2$s, binds to heparin. The sPLA$_2$ activity recovered from the heparin column eluates was higher than that measured in crude cell supernatants and no sPLA$_2$ activity was detected when mock-trans-

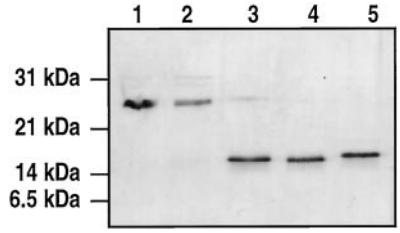

FIG. 4. **SDS-PAGE analysis of recombinant *E. coli* mGIID sPLA$_2$.** Protein samples obtained at different steps of the refolding procedure were loaded on a 14% SDS-polyacrylamide gel under reducing conditions and silver-stained. *Lane 1*, inclusion bodies (400 ng); *lane 2*, refolded fusion protein (100 ng); *lane 3*, sPLA$_2$ fractions after heparin-Sepharose purification; *lane 4*, HPLC-purified recombinant mGIID sPLA$_2$ (100 ng); *lane 5*, recombinant mGIIA sPLA$_2$ (100 ng). Markers were from Promega.

fected cell culture medium was applied to the column. The sPLA$_2$ activity measured in column eluates was, however, too weak for a detailed analysis of the enzymatic properties of mGIID sPLA$_2$.

Based on these initial studies, an attempt was made to produce recombinant mGIID sPLA$_2$ in *E. coli* as a truncated glutathione *S*-transferase (GST) fusion protein. For this purpose, the mGIID sPLA$_2$ cDNA was inserted into the modified pGEX-2T-derived expression vector PAB$_3$, in which the C-terminal region of GST has been deleted (37). In addition, a factor Xa cleavage site was inserted between the truncated GST (~8 kDa) and the mature form of the sPLA$_2$ by PCR. The chimera construct was confirmed by sequencing and used to transform *E. coli* BL 21 cells for protein expression. When BL21 cells were induced with isopropyl-1-thio-β-D-galactopyranoside, a major protein of ~24 kDa was detected in bacterial extracts (Fig. 4). Yields of about 150 mg of fusion protein/liter of culture were obtained, but as for many other sPLA$_2$s expressed in *E. coli*, the mGIID fusion protein was found in inclusion bodies. Extensive washing of inclusion bodies provided an efficient purification step, and the fusion protein represented about 70% of the total protein at this stage (Fig. 4). Inclusion body protein was solubilized in strong denaturant, reduced, and sulfonated. The fusion protein was refolded by dialysis against 0.9 M guanidine hydrochloride, during which a large portion of protein precipitated, even when dialysis was performed at low protein concentration. After centrifugation to remove insoluble material and dialysis against factor Xa cleavage buffer, the fusion protein was cleaved with factor Xa protease. sPLA$_2$ activity and protein content were determined at different steps of the refolding procedure and a large increase in sPLA$_2$ activity was observed after factor Xa cleavage (data not shown). Accordingly, a band of ~16 kDa corresponding to the cleaved sPLA$_2$ was detected by gel analysis, while most of the fusion protein was cleaved (Fig. 4). The active sPLA$_2$ was purified to homogeneity in a two-step procedure using heparin chromatography and reverse phase HPLC. Both remaining fusion protein and cleaved sPLA$_2$ were found to elute as a broad peak at 500–600 mM NaCl from the heparin column (data not shown). Fractions containing sPLA$_2$ activity were then loaded on a C18 reverse phase column, and the cleaved sPLA$_2$ protein was eluted as a sharp peak with 33% acetonitrile, giving protein of >99% purity, as shown by SDS-PAGE analysis (Fig. 4). The electrophoretic mobility of mGIID sPLA$_2$ is slightly faster than that of mGIIA sPLA$_2$ (Fig. 4). N-terminal sequencing of the first 15 amino acids indicated that the mGIID fusion protein had been cleaved by factor Xa at the expected site. In addition, electrospray mass spectrometry indicated that the recombinant mGIID sPLA$_2$ has a molecular mass of 14,252, which is identical to that predicted from its cDNA with seven disulfides

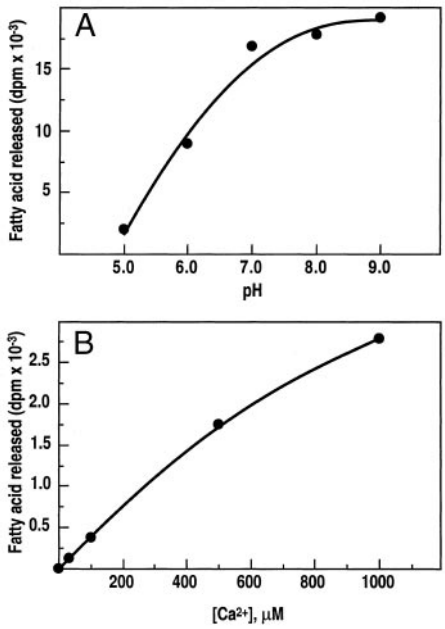

FIG. 5. **pH and Ca^{2+} dependence of mGIID sPLA$_2$.** pH dependence (*panel A*) of recombinant mGIID enzymatic activity was measured with 1 μg of enzyme and 18 μM [^{3}H]DPPG in POPG (50 Ci/mol based on total lipid) in 50 μl of 50 mM buffers of various pH values (acetate, 5; MES, 6; HEPES, 7 and 8; glycine, 9). Amount of fatty acid released (dpm) was measured after 15 min at 37 °C. Ca^{2+} dependence (*panel B*) of recombinant mGIID enzymatic activity was measured as for the pH dependence except that substrate was 1, 2-dioleoyl-*sn*-glycero-3-phosphocholine containing with [^{3}H]DPPC (18 μM lipid, 50 Ci/mol based on total lipid) and the reaction buffer was 50 μl of 100 mM Tris-HCl, pH 8.0. The *solid line* shows the fit of the data to the standard binding equation with a dissociation constant of 1.4 ± 0.2 mM. No enzymatic activity was detected using buffered free Ca^{2+} in the 0–2 μM range.

formed. Finally, analysis of the purified protein using Ellman's reagent indicated the absence of free thiol groups (data not shown). All together, these results suggest that mGIID sPLA$_2$ has been successfully refolded and recombinantly expressed. The overall yield of production of recombinant mGIID sPLA$_2$ was about 1 mg of protein/liter of culture.

Enzymatic Properties of Recombinant mGIID sPLA$_2$—Like other sPLA$_2$s (8, 9), the enzymatic activity of mGIID sPLA$_2$ was found to decrease as the pH drops below 7, presumably due to protonation of the active site histidine (Fig. 5*A*). mGIID sPLA$_2$ required millimolar concentrations of Ca^{2+} for full activity (Fig. 5*B*). As a first step toward the characterization of the substrate preferences of mGIID sPLA$_2$, we measured the initial velocities for the hydrolysis of small sonicated unilamellar vesicles composed of different pure phospholipid molecular species (Table II). Studies with mGIIA and hGIIA sPLA$_2$s were also carried out for comparative purposes. The specific activities of mGIID sPLA$_2$ acting on the different phospholipid substrates are considerably lower than those for mGIIA and hGIIA sPLA$_2$s. This observation is in good agreement with our transfection experiments on COS cells, where a very low sPLA$_2$ activity was measured for mGIID sPLA$_2$ compared with mGIIA sPLA$_2$ (see above). mGIIA and hGIIA sPLA$_2$s are >100-fold more active on anionic POPG and DOPM vesicles than on zwitterionic POPC or DOPC vesicles. In contrast, the specific activities of mGIID sPLA$_2$ on POPG, DOPM, and POPC vesicles are comparable. With all three sPLA$_2$s, POPS vesicles are much less preferred (7–120-fold) compared with POPG (or DOPG) and DOPM vesicles, showing that not all anionic phospholipid vesicles are good substrates. POPE vesicles serve as a relatively poor substrate for mGIIA and mGIID sPLA$_2$s. Finally, both mouse enzymes show a small (~2-fold) preference

TABLE II
Specific activities of mGIIA, mGIID, and hGIIA sPLA$_2$s acting on phospholipid vesicles and on RAW 264.7 cells

Phospholipid[a]	Specific activity		
	mGIIA sPLA$_2$	mGIID sPLA$_2$	hGIIA sPLA$_2$
		μmol/min/mg	
POPC[a]	1.8 ± 0.2[c]	0.062 ± 0.02	0.25 (DOPC)[d]
POPC[b]	3.2 ± 0.5	0.162 ± 0.03	
POPE[a]	1.6 ± 0.2	<0.003	
POPG[a]	240 ± 30	0.2 ± 0.03	~250 (DOPG)[d]
DOPM[a]	320 ± 30	0.2 ± 0.04	670
POPS[a]	2.7 ± 0.2	<0.003	38
RAW 264.7 cells	1.5 ± 0.2	0.032 ± 0.01	0.036 ± 0.01

[a] All vesicles are small unilamellar prepared by sonication except those indicated by footnote *b*.
[b] Large unilamellar vesicles prepared by extrusion.
[c] Numbers are the average ± standard deviation of duplicate analyses.
[d] From Ref. 65 using vesicles of the phospholipid indicated in parentheses.

for large unilamellar POPC vesicles over small sonicated vesicles.

sPLA$_2$s bind to the phospholipid interface via an interfacial binding surface, and a phospholipid molecule in the interface then binds to the active site of the vesicle-bound enzyme (8). Thus, the specific activity of a sPLA$_2$ acting on phospholipid vesicles reflects the affinity of the enzyme in the aqueous phase for the membrane interface, the intrinsic specificity of the active site of the vesicle-bound sPLA$_2$ for various phospholipids, or a combination of both factors (44). The best way to determine the intrinsic specificity of interface-bound sPLA$_2$ is to use a vesicle composed mainly of a phospholipid to which the enzyme binds tightly (DOPM) and to dope the vesicles with small amounts of competing substrates (one labeled with ^{3}H and the other with ^{14}C in this case). The vesicle-bound enzyme "chooses" among the two competing and radiolabeled substrates according to its relative intrinsic specificity constants (k_{cat}*/K_m*), where the asterisks donate kinetic constants for the interface-bound enzyme (44). Previous studies have shown that hGIIA sPLA$_2$ binds several orders of magnitude more weakly to phosphatidylcholine vesicles than to phosphatidylmethanol and phosphatidylglycerol vesicles (42, 54, 55). However, hGIIA sPLA$_2$ displays only a 5.5-fold preference for [^{3}H]DPPG *versus* [^{14}C]SAPC when it is tightly bound to DOPM vesicles (Table III). This shows that the >1,000-fold preference of hGIIA sPLA$_2$ for pure POPG *versus* pure POPC vesicles (Table II) is mostly due to a difference in aqueous phase-to-vesicle binding. mGIIA sPLA$_2$ behaves similarly to hGIIA sPLA$_2$ in this regard. It is 180-fold more active on pure POPG vesicles than on pure POPC vesicles, and yet when bound to DOPM, [^{3}H]DPPG is preferred 11-fold over [^{14}C]SAPC. On the other hand, mGIID sPLA$_2$ behaves very differently. Since it displays only 3-fold intrinsic specificity for [^{3}H]DPPG *versus* [^{14}C]SAPC, the fact that its specific activity on pure POPG vesicles is only 3-fold larger than on POPC vesicles shows that this enzyme is unique in being able to bind to phosphatidylcholine and phosphatidylglycerol vesicles with similar affinity.

The relatively low specific activity of mGIID sPLA$_2$ on all substrates may be due to poor interfacial binding. However, as shown in Fig. 6, this is not the case. Binding of mGIID sPLA$_2$ to lipid vesicles was studied by monitoring energy transfer in a spectrofluorimeter due to close proximity of enzyme tryptophan residues to the fluorescent phospholipid *N*-dansyl-1,2-dihexadecyl phosphatidylethanolamine present at 3 mol% in vesicles of the non-hydrolyzable phospholipid DTPM (56). Since native hGIIA sPLA$_2$ does not contain tryptophan, the V3W mutant of hGIIA sPLA$_2$ was used as a positive control for these binding studies (57). Addition of increasing amounts of mGIID and hGIIA (V3W) sPLA$_2$s to a fixed amount of anionic vesicles (5 μM) leads to an increase in energy transfer until the vesicles become saturated with enzyme. Such binding occurs in the absence of calcium, as is the case for other secreted sPLA$_2$s (8). With both enzymes, saturation occurs when the ratio of outer vesicle layer phospholipid to enzyme is ~12. Other sPLA$_2$s show saturation at a lipid/protein ratio of ~15–40 (47, 54, 56). These results show not only that mGIID sPLA$_2$, like hGIIA (V3W) sPLA$_2$, binds tightly to anionic phosphatidylmethanol vesicles but that most, if not all, of the recombinant mGIID sPLA$_2$ is folded into a conformation that binds tightly to vesicles. Thus, the relatively low specific activity of mGIID sPLA$_2$ among sPLA$_2$s is due to intrinsically low catalytic efficiency of this enzyme acting on the phospholipids tested in this study.

We also monitored the release of fatty acids from a suspension of live RAW 264.7 macrophages treated exogenously with sPLA$_2$s (Table II). The results show that mGIID and hGIIA sPLA$_2$s have comparable specific activities, which are ~40-fold lower than that of mGIIA sPLA$_2$. Cobra venom sPLA$_2$ (*Naja naja*) is much more active (41 ± 5 μmol/min/mg) than all three mammalian enzymes (data not shown). As reported previously (48, 57), the low activity of hGIIA sPLA$_2$ compared with cobra venom sPLA$_2$ acting exogenously on RAW 264.7 cell membranes probably reflects the fact that the mammalian enzyme binds poorly to phosphatidylcholine vesicles and that the outer layer of mammalian cell membranes is rich in zwitterionic phospholipids (phosphatidylcholine and sphingomyelin). On the other hand, mGIID sPLA$_2$ binds well to phosphatidylcholine vesicles, and the fact that its specific activity on cells is comparable to that of hGIIA sPLA$_2$ is probably due to its low catalytic efficiency. mGIIA sPLA$_2$, like cobra venom sPLA$_2$, has a high catalytic turnover number, but only the venom enzyme binds tightly to phosphatidylcholine vesicles, which presumably explains the turnover numbers reported with RAW 264.7

TABLE III
Relative specificity constants of sPLA$_2$s

Phospholipid pair[a]	(k_{cat}*/K_M*)^{3}H-substrate/(k_{cat}*/K_M*)^{14}C-substrate		
	mGIIA sPLA$_2$	mGIID sPLA$_2$	hGIIA sPLA$_2$
[^{3}H]DPPC *vs.* [^{14}C]SAPC	1.1 ± 0.2[b]	1.6 ± 0.1	1.8 ± 0.2
[^{3}H]DPPC *vs.* [^{14}C]PAPE	1.9 ± 0.2	1.9 ± 0.2	1.0 ± 0.1
[^{3}H]DPPG *vs.* [^{14}C]SAPC	11.2 ± 0.3	3.3 ± 0.1	5.5 ± 0.2

[a] All radiolabeled substrates are present as minor components in sonicated DOPM vesicles.
[b] Numbers are the average ± standard deviation of duplicate analyses.

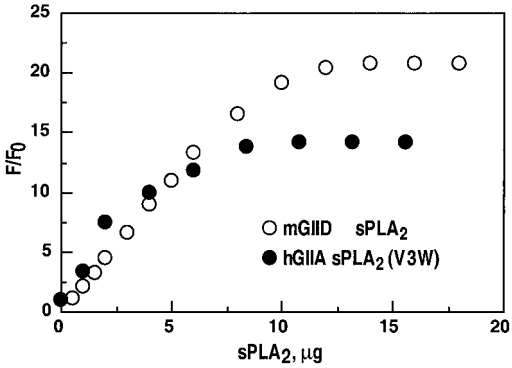

FIG. 6. **Interfacial binding of sPLA$_2$s.** The indicated amount of sPLA$_2$ was added to a fixed amount (5 μM) of DTPM sonicated vesicles containing 3 mol% *N*-dansyl-1,2-dihexadecyl phosphatidylethanolamine in 1.3 ml of 100 mM Tris-HCl, pH 8.0, 2 mM EGTA, 2 mM EDTA at 30 °C. Excitation was at 280 nm, and emission was monitored at 500 nm. *F* is the emission in the presence of all components, and F_o is the emission in the presence of buffer plus vesicles but no enzyme.

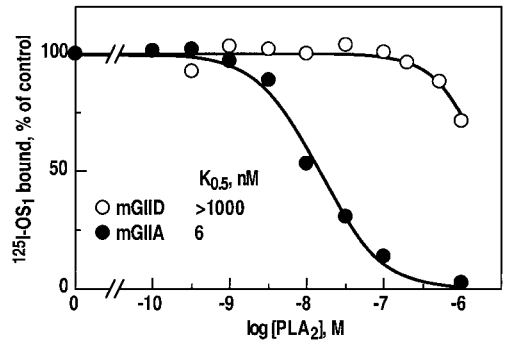

FIG. 7. **Binding properties of mGIIA and mGIID sPLA$_2$s to the recombinant mouse M-type receptor.** Membranes (10 μg of protein/ml) of COS cells transfected with the full-length mouse M-type receptor were incubated in the presence of ^{125}I-OS$_1$ (25 pM) and various concentrations of recombinant mGIIA or mGIID sPLA$_2$s. All results are expressed as percentage of the specific binding measured in the absence of unlabeled sPLA$_2$. 100% corresponds to a ^{125}I-OS$_1$ specific binding of 1.3 pM. Nonspecific binding was determined in the presence of 30 nM unlabeled OS$_1$ and was less than 20% of total binding.

cells (Table II).

The molecular basis for the much higher affinity of mGIID sPLA$_2$ *versus* mGIIA and hGIIA sPLA$_2$s for phosphatidylcholine vesicles remains to be established. It may be noted that mGIID sPLA$_2$ has four tryptophans. The likelihood that these residues form part of the interfacial binding surface was assessed by overlaying the sequence of this enzyme onto the conserved protein backbone structure of other sPLA$_2$s. Trp-22 is almost certainly on the interfacial binding surface since it lies at the opening of the active site slot. Trp-114 lies on the same face of the protein as Trp-22 but further from the active site slot and so, it may be part of the interfacial binding surface. Trp-42 and Trp-83 are clearly not involved in membrane binding. Multiple tryptophans are a common feature of the interfacial binding site of cobra venom sPLA$_2$, which binds tightly to phosphatidylcholine vesicles (58). Addition of a single tryptophan residue to the interfacial binding site (V3W) of hGIIA sPLA$_2$ promotes catalytically productive binding to phosphatidylcholine interfaces by more than two orders of magnitude (57). Finally, hGV sPLA$_2$ binds tightly to phosphatidylcholine vesicles (59), and mutation of tryptophan 31 on its interfacial binding site to alanine dramatically reduces interfacial binding (60). Finally, data in Table III show that mGIIA, mGIID, and hGIIA sPLA$_2$s display little (<2-fold) intrinsic specificity for *sn*-2 palmitoyl *versus* arachidonyl fatty acyl chains ([^{3}H]DPPC *versus* [^{14}C]SAPC) and for phosphatidylcholine *versus* phosphatidylethanolamine ([^{3}H]DPPC *versus* [^{14}C]PAPE).

Binding Properties of mGIID sPLA$_2$ to the Recombinant Mouse M-type Receptor Expressed in COS Cells—Previous studies have indicated that mGIIA sPLA$_2$ behaves as a high affinity endogenous ligand of the mouse M-type receptor (31). It was therefore of interest to analyze whether mGIID sPLA$_2$ may also be an endogenous ligand of the mouse M-type receptor. Fig. 7 shows the results obtained from competition binding experiments to the mouse M-type receptor between ^{125}I-OS$_1$ and unlabeled mGIIA or mGIID sPLA$_2$s. In contrast to mGIIA sPLA$_2$, which binds to this receptor with high affinity ($K_{0.5}$ = 6 nM), mGIID sPLA$_2$ does not bind with high affinity. The recombinant mGIID sPLA$_2$ was, however, able to bind to the rabbit M-type receptor with a relatively high affinity ($K_{0.5}$ = 20 nM) (data not shown), indicating that the inability of mGIID sPLA$_2$ to bind to the mouse M-type receptor is not due to a misfolding of the recombinant enzyme. Whether mGIID sPLA$_2$ can bind to other mouse receptors remains to be analyzed.

Concluding Remarks—The discovery of a second mouse group IIA sPLA$_2$ in mice raises the possibility that mGIIA sPLA$_2$, also known as enhancing factor (32, 33), may not be the mouse ortholog of hGIIA sPLA$_2$, *i.e.* that mGIIA and hGIIA sPLA$_2$s do not have similar functions in mice and humans. Based on sequence similarity, mGIIA, but not mGIID, appears to be the mouse ortholog of hGIIA sPLA$_2$. Indeed, hGIIA sPLA$_2$ has 67% identity with mGIIA sPLA$_2$, while it has only 48% with mGIID sPLA$_2$. However, mGIIA and hGIIA sPLA$_2$s have a number of distinct biological properties (31). First, mGIIA and hGIIA sPLA$_2$s have very different tissue distributions (3, 4, 31). Second, hGIIA sPLA$_2$ is strongly induced in inflammation and associated diseases (3, 14), while there is no strong evidence for induction of mGIIA expression upon inflammatory challenges (61). Third, expression of hGIIA sPLA$_2$ in transgenic mice carrying the hGIIA gene does not fit with the endogenous expression of mGIIA (62). Fourth, while both mGIIA and hGIIA sPLA$_2$s have antibacterial properties (15–17), only mGIIA sPLA$_2$ is likely to play a role as a genetic modifier of colorectal cancer (20, 31, 49, 63, 64). Fifth, mGIIA sPLA$_2$ binds to the mouse M-type receptor, but hGIIA sPLA$_2$ does not bind to the human M-type receptor (31). On the other hand, as for hGIIA sPLA$_2$, mGIID sPLA$_2$ is expressed in many tissues (Fig. 3) and does not bind to its homologous mouse M-type receptor (Fig. 7). In future work, it will be particularly interesting to analyze the regulation of mGIID sPLA$_2$ expression upon inflammatory challenges, and also to determine whether mGIID sPLA$_2$ has antibacterial or antitumoral properties. Finally, it is also intriguing that mGIIA sPLA$_2$ has only 67% identity with hGIIA sPLA$_2$, while mouse and human group IB sPLA$_2$s have 81% identity. Taken together, these data raise the possibility that the real mouse ortholog of hGIIA sPLA$_2$ has not yet been found. Further work is also needed to determine whether a gene corresponding to mGIID sPLA$_2$ exists in humans.

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