

Novel Mammalian Group XII Secreted Phospholipase A₂ Lacking Enzymatic Activity^{†,‡}

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ABSTRACT: An increasing number of mammalian secreted phospholipases A₂ (sPLA₂s) has been identified over the past few years. Here, we report the identification and recombinant expression of a novel sPLA₂-like protein in mouse and human species that has been called group XIIIB (GXIIIB). The mature protein has a molecular mass of 19.7 kDa and structural features similar to those of the previously identified GXII sPLA₂, now called GXIIA. Strikingly, the GXIIIB sPLA₂ has a mutation in the active site, replacing the canonical histidine by a leucine, suggesting that this sPLA₂ is catalytically inactive. Recombinant expression of human (hGXIIIB) and mouse (mGXIIIB) sPLA₂s in *Escherichia coli* indicates that GXIIIB sPLA₂s display no measurable lipolytic activity on various types of phospholipid substrates. Furthermore, these sPLA₂-like proteins display relatively weak affinity to phospholipid vesicles. Binding experiments indicate that these proteins are also unable to bind to the well-known M-type sPLA₂ receptor. The RNA tissue distribution of GXIIIB sPLA₂s is distinct from that of other sPLA₂s including the homologous GXIIA. Strong expression was observed in liver, small intestine, and kidney in both human and mouse species. Interestingly, the expression of the novel sPLA₂ is dramatically decreased in human tumors from the same tissues. The absence of enzymatic activity suggests that the GXIIIB sPLA₂-like proteins probably exert their biological roles by acting as ligands for as yet unidentified receptors.

Phospholipases A₂ (PLA₂s) are enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids (1–4). Over the past few years, it has been realized that PLA₂s constitute in humans a superfamily of enzymes comprising at least a set of 10 distinct intracellular enzymes and another set of 10 different extracellular, i.e., secreted PLA₂s (sPLA₂s). This is in marked contrast with the phospholipase C and D families that only comprise intracellular forms (5–8). The sPLA₂ family can be further subdivided into three main structural collections (1, 4, 9, 10). sPLA₂s IB, IIA, IIC, IID, IIE, IIF, V, X, and otoconin-95 belong to the I/II/V/X sPLA₂ collection, while sPLA₂s III and XII are unique members of the group III and XII sPLA₂ collections. All of the sPLA₂s have no sequence homology with the intracellular PLA₂s but share among each other a number of common properties including a relatively low molecular mass of 14–19 kDa (except for GIII sPLA₂), a large number of disulfides, and a similar Ca²⁺-dependent catalytic mechanism (3).

Collectively, mammalian PLA₂s are proposed to play a key role in various biological processes, including homeostasis of cellular membranes, lipid digestion, host defense, and production of potent lipid mediators such as eicosanoids and lysophospholipids, which in turn exert a wide variety of functions under normal and pathological conditions (1, 2, 4, 11, 12). Studies with knock-out mice and specific inhibitors have clearly demonstrated that the cytosolic PLA₂ IVA plays a major role in the production of lipid mediators under both normal and disease conditions (12–21). On the other hand, the specific biological functions of the different sPLA₂s are still unclear, despite a number of studies suggesting that sPLA₂s contribute to host defense and lipid mediator release, especially in inflammation and its associated diseases and in various cancers (1, 4, 11, 22–28). Clearly, the different mammalian sPLA₂s are not isoforms because they all share a low level of sequence identity (as low as 15%) and have specific tissue distribution patterns, suggesting distinct biological roles for each of them (29–31). Furthermore, the recent analysis of their enzymatic properties has revealed dramatic differences in terms of their specific activities and substrate selectivity (32), again arguing for distinct functions. Finally, the identification of specific sPLA₂ receptors in mammals has opened the possibility that sPLA₂s function not only as enzymes but also independently of their catalytic activity by acting as ligands, as is the case for hormones and cytokines (23, 33).

Besides mammals, sPLA₂s are also present in many other genomes, indicating that some of the sPLA₂ functions may have been conserved throughout evolution. Indeed, several

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[‡] The nucleotide sequences reported in this paper have been deposited in the GenBank/EBI Data Bank with accession numbers AY358032 (hGXIIIB) and AY358033 (mGXIIIB).

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sPLA₂s have been identified in other vertebrates including reptiles and fish, in invertebrates including *Drosophila* and worms, as well as in plants, viruses, and procaryotes (1, 2, 34, 35). sPLA₂s are also particularly abundant in snake venoms, and up to 15 distinct sPLA₂s can be found in a single snake venom (36–39). More than 400 sPLA₂ sequences have now been reported from snake, insect, and molluscan venoms. Besides digestion, these sPLA₂s serve toxic functions that are not directly correlated with their catalytic activity. Interestingly, several natural sPLA₂ variants with deletions or point mutations in the active site have been described in snake venoms (37, 39). Remarkably, such sPLA₂s are still toxic, though they are devoid of sPLA₂ activity.

In the past few years, a systematic screening of the nucleic acid databases generated by the genome projects using all known sPLA₂ structures has allowed us to identify and clone several novel mammalian sPLA₂s (9, 29, 30, 40–42). Within our continuous search for novel mammalian sPLA₂s, we now report the molecular characterization of the mammalian group XIIB sPLA₂. Strikingly, this enzyme represents the first typical mammalian sPLA₂ that is expressed in many tissues under normal and pathological conditions and that is clearly catalytically inactive because of a single point mutation in the active site and altered phospholipid-binding properties.

EXPERIMENTAL PROCEDURES

Molecular Cloning of hGXIIIB and mGXIIIB sPLA₂s. Searching for sPLA₂ homologues in genomic databases using the tBLASTn program and known venom and mammalian sPLA₂ sequences as queries resulted in the identification of novel GenBank entries from different species (for example, AI657758, AI958157, AI530426, BF196453, and BY708023). Among these entries, three human and mouse entries (AF349540, AF339053, and AF339738) were reported to code for a putative novel sPLA₂ that was tentatively submitted as group XIII sPLA₂. However, because of its typical group XII structural properties, this sPLA₂ will be referred hereafter as GXIIIB sPLA₂. To isolate by RT-PCR¹ the full-length open reading frame coding for this novel sPLA₂ in mouse and humans, we first designed two sets of forward and reverse primers flanking the coding sequences. These primers contained appropriate restriction sites for the subsequent cloning into the mammalian expression vector pRc/CMVneo (Invitrogen). Forward and reverse mouse primers were 5'-TTT GCG GCC GCC ATG AAG CTG CTC TGC GGC TTC T-3' and 5'-AAA TCT AGA ATT CAT AGC TCT TCT TTC TCC TCC T-3'. Forward and reverse human primers were 5'-TTT GCG GCC GCC ATG AAG CTG GCC AGT GGC AAC T-3' and 5'-AAA TCT AGA ATT CAT AAC TCT TCC TTC TCC TCC T-3'. The

expected PCR fragment coding for mGXIIIB was amplified from pooled cDNA from mouse embryos, small intestine, liver, colon, and lymph node tissues (BD Clontech), while that coding for hGXIIIB was amplified from human liver cDNA (BD Clontech). PCR was performed for 35 cycles of amplification (94 °C/30 s, 60 °C/30 s, 72 °C/60 s) using a proofreading Taq/Pwo DNA polymerase mixture (Hybaid). The PCR fragments were digested with *NotI* and *XbaI* and ligated into the mammalian expression vector pRc/CMVneo, and several clones were entirely sequenced. All of the mouse clones were found to be identical to the open reading frame of the submitted mouse sequence GenBank AF339738. On the other hand, two sets of human clones differing at nucleotide position 464 (relative to the open reading frame) were found. The first set contains an open reading frame of 585 nucleotides that is identical to the submitted human sequence GenBank AF349540. The second set contains an open reading frame of 588 nucleotides identical to the first set, but with a three nucleotide insertion coding for an extra alanine residue, that matches the second submitted human sequence GenBank AF339053 (see also Figure 1). We also designed two other sets of mouse and human primers flanking the longest consensus nucleotide sequences obtained by alignment of the various ESTs and other sequences found in the nucleotide databases. These primers were used for PCR experiments as above to isolate these longer sequences. The DNA fragments corresponding to these consensus nucleotide sequences could be amplified from human kidney and mouse embryo cDNA and were entirely sequenced on both strands. These sequences have been deposited in GenBank with the accession numbers AY358032 (hGXIIIB) and AY358033 (mGXIIIB).

Molecular Cloning of mGXIIA. A forward primer (5'-TTT GCG GCC GCC ATG GTG ACT CCG CGG CGG CCC GCG CCC-3') and a reverse primer (5'-AAA TCT AGA GAA TTC ATA GAT CTG TTT TTT CTT CAT AAC-3') flanking the open reading frame of mGXIIA (10) and containing appropriate restriction sites were used to amplify by RT-PCR the sequence coding for mGXIIA. The expected fragment of 579 nucleotides was amplified from mouse liver cDNA (BD Clontech) and subcloned into the pRc/CMVneo vector as described above. The sequence was found to be identical to that published by Ho et al. (10).

Recombinant Expression of mGXIIA, mGXIIIB, and hGXIIIB sPLA₂s in *Escherichia coli*. Mature GXIIA and GXIIIB sPLA₂s (i.e., without their signal peptide) were produced in *E. coli* as C-terminal fusion proteins with a truncated glutathione S-transferase (8.7 kDa) encoded by the pAB3 vector (41). This vector has previously been used in our laboratories to produce various sPLA₂s in *E. coli* (32) and was originally derived from the pGEX-2T vector (43). To subclone the sPLA₂s into the pAB3 vector, PCR reactions were performed as above using the pRc/CMVneo constructs as templates, the above reverse primers and forward primers containing a *Bam*HI site, and a factor Xa cleavage site (Ile-Glu-Gly-Arg) followed by the predicted N-terminal mature sequences of mGXIIA, mGXIIIB, and hGXIIIB (mGXIIA, 5'-TTT GGA TCC ATC GAA GGT CGT CAG GAA CAG GAC CAG ACC ACC GAC-3'; mGXIIIB, 5'-TTT GGA TCC ATC GAA GGT CGT GAC CCC AGC CCC AAG GAA GAG-3'; hGXIIIB, 5'-TTT GGA TCC ATC GAA GGT CGT CAG AGC GAC ACG AGC CCT GAC A-3'). The PCR

¹ Abbreviations: EST, expressed sequence tag; RT-PCR, reverse transcription–polymerase chain reaction; MALDI-TOF, matrix-assisted laser desorption/ionization–time of flight; DoetPC and DoetPS, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and -phosphoserine, respectively. A comprehensive abbreviation system for the various secreted phospholipases A₂ (sPLA₂s) is used: each sPLA₂ is abbreviated with a lowercase letter indicating the sPLA₂ species (h and m for human and mouse) that is followed by capital characters identifying the sPLA₂ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIIL, GV, GX, GXIIA, and GXIIB for group IB, IIA, IIC, IID, IIE, IIF, IIL, V, X, XIIA, and XIIB, respectively).

products were digested with *Bam*HI and *Hind*III and subcloned in-frame with the truncated GST protein in the pAB3 vector. All the constructs were entirely sequenced.

Protein expression was performed in *E. coli* BL21 host cells grown in Terrific broth containing ampicillin (100 μ g/mL). Cells were grown in 2 L flasks to OD₆₀₀ ~0.8 and induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside overnight at 37 °C. Cells from a 600 mL culture were pelleted, solubilized in 40 mL of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1% Triton X-100, and 1% sodium deoxycholate) for 1 h at 4 °C, and homogenized by two passages in a French press (SLM Aminco, maximum pressure 1500 psi). Inclusion bodies were collected by centrifugation at 10000g for 20 min and washed twice with lysis buffer without detergent. The resulting pellet was solubilized in 40 mL of 50 mM Tris-HCl, pH 8.0, 8 M urea, 0.1 M NH₄Cl, and 0.3 M Na₂SO₃. The solubilized protein was sulfonated by addition of 0.2 volume of Thannhauser's reagent (44) for 1 h at room temperature. After overnight dialysis at 4 °C against 8 L of 1% acetic acid with two buffer changes, the precipitated proteins were pelleted and resuspended at 0.2 mg/mL in 500 mL of 50 mM Tris-HCl, pH 8.0, 8 M urea, and 0.1 M NH₄Cl. The denatured sPLA₂ was refolded by dialysis against 8 L of 50 mM Tris-HCl, pH 8.0, 2 M urea, 0.1 M NH₄Cl, 5 mM CaCl₂, 5 mM L-cysteine, and 5 mM L-methionine for 2–4 days at 4 °C. The refolded mixture was filtered through a layer of Sephadex G-50 to remove large aggregates and subjected to extensive digestion at room temperature with 400 units of factor Xa (Amersham Biosciences). The time course of cleavage (up to 3 days) was followed by sPLA₂ activity (mGXIIA) or MALDI-TOF mass spectrometry (hGXIIIB and mGXIIIB). The protein solution was concentrated to 20 mL by ultrafiltration using an Amicon stirred cell concentrator with a YM-10 membrane, and the buffer was then exchanged to 1% acetic acid (10% acetonitrile was added for GXIIIB sPLA₂s). The solution was filtered and loaded onto a Spherogel TSK SP-5PW column (10 μ m, 7.5 \times 75 mm, Tosoh Biosep) equilibrated in 1% acetic acid (and 10% acetonitrile for GXIIIB sPLA₂s). The column was eluted at 1 mL/min using a linear gradient of ammonium acetate (0–2 M, pH 6.8, over 100 min) in 10% acetonitrile. The fractions containing cleaved sPLA₂ were pooled, lyophilized, resuspended in 10% acetonitrile and 0.1% TFA, and applied to a reverse-phase HPLC column (C18 Nucleosil 4.6 \times 250 mm, 300 Å, 5 μ m, Macherey-Nagel). Elution was performed at 1 mL/min using a gradient of acetonitrile in 0.1% TFA (10–30% acetonitrile over 20 min, then 30–35% acetonitrile over 25 min, and 35–60% acetonitrile over 30 min). mGXIIA, mGXIIIB, and hGXIIIB proteins appear more than 99% pure when analyzed by SDS–polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry. MALDI-TOF analysis of sPLA₂s was carried out on an Applied Biosystems voyager DE-Pro spectrometer under linear mode and using sinapinic acid as a matrix. Analysis of pure proteins was performed with internal calibration using appropriate protein standards (see Figure 3).

Enzymatic Activity of Group XII sPLA₂s and Interfacial Binding to Vesicles. Enzymatic activity of GXII sPLA₂s was analyzed using [³H]oleate-radiolabeled *E. coli* membranes (40), pyrene–phosphatidylglycerol assays (32), and the fatty

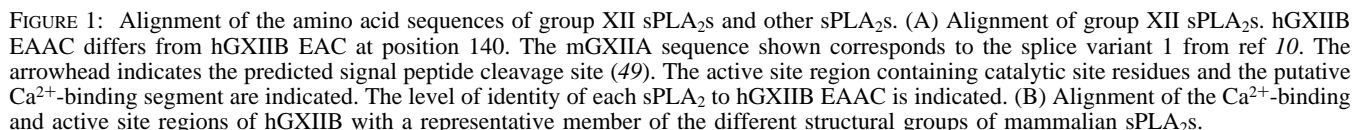
acid binding protein assay using dioleoylphosphatidylcholine vesicles as substrate (45). *E. coli* enzymatic activity assays were performed at room temperature for 1 h in 100 μ L of sPLA₂ activity buffer (0.1 M Tris-HCl, pH 8.0, 10 mM CaCl₂, 0.1% bovine serum albumin) containing 100000 dpm of [³H]oleate-radiolabeled *E. coli* membranes. Reactions were stopped by addition of 300 μ L of stop buffer (0.1 M EDTA, pH 8.0, 0.1% fatty acid-free bovine serum albumin) and centrifuged at 10000g for 5 min, and the supernatants containing released [³H]oleate were counted.

Binding studies with GXII sPLA₂s on sucrose-loaded unilamellar vesicles of diether phospholipids were performed as described (45). Binding assays were carried out in 5 mM MOPS, pH 7.4, 0.1 M KCl, and 2 mM CaCl₂ at room temperature using the centrifugation method in which the amount of sPLA₂ remaining in the supernatant above pelleted vesicles is quantified by either enzymatic assay (GXIIA) or protein quantification by Bradford assay (GXIIIB). Binding experiments with GXIIA sPLA₂s were carried out with 0.5 μ g of pure enzyme in 100 μ L of binding buffer, whereas those with GXIIIB sPLA₂s were carried out with 400 μ L of binding buffer and 2 μ g of pure proteins.

M-Type Receptor Binding Studies. Competition binding assays between unlabeled GXIIIB sPLA₂s and iodinated OS₁ to recombinant mouse M-type receptor expressed in COS cells were performed as described (46). For direct binding experiments with iodinated mGXIIIB, mGXIIIB was iodinated with lactoperoxidase (47) and purified by reverse-phase HPLC as above, and the labeled sPLA₂ was analyzed for its binding properties to recombinant mouse M-type receptor. Briefly, membranes from COS cells containing mouse M-type receptor, labeled sPLA₂ ligand (OS₁ or mGXIIIB), and unlabeled sPLA₂s were incubated at 20 °C in 1 mL of binding buffer (140 mM NaCl, 0.1 mM CaCl₂, 20 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin). Incubations were initiated by addition of membranes and filtered after 60 min through GF/C glass fiber filters presoaked in 0.5% poly(ethylenimine).

Analysis of the Tissue Distribution of Mouse Group XII sPLA₂s. A mouse northern blot (BD Clontech, catalog no. 7762-1) and a mouse RNA master blot (BD Clontech, catalog no. 7771-1) were successively hybridized with ³²P-labeled mGXIIIB and mGXIIA antisense riboprobes in ULTRAHyb hybridization buffer (Ambion, catalog no. 8670) for 18 h at 70 °C. High-sensitivity strippable riboprobes corresponding to the sequence of mature sPLA₂s were synthesized using the Strip-EZ RNA Ambion kit (catalog no. 1360) and purified using quick spin columns for radiolabeled RNA purification (Roche Applied Sciences). Blots were washed to a final stringency of 0.1 \times 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 hybridization, blots were stripped as specified in the Strip-EZ RNA Ambion kit, checked for efficient destripping, and hybridized with the next sPLA₂ riboprobe. The absence of cross-hybridization with other mouse sPLA₂s was confirmed by parallel hybridization of Southern blots containing the 11 full-length mouse sPLA₂ cDNAs under identical conditions (not shown).

Analysis of the Tissue Distribution of Human Group XII sPLA₂s and Cancer Profiling Array. A human northern blot (BD Clontech, catalog no. 7780-1) and a Cancer Profiling



During the identification of hGXIIA, we noticed that genomic databases contain several ESTs coding for a

zebrafish group XII sPLA₂ (GenBank AI657758, AI397414, AI878221, among others) with a leucine in place of histidine in the active site (9). This sPLA₂ had a low sequence identity with hGXIIA, much lower than that observed between hGXIIA and mGXIIA or even with GXIIA from *Xenopus laevis* (9), suggesting the existence of a possible second group XII sPLA₂ member (hereafter called GXIIB sPLA₂). Within our continuous searching in databases, we then found several GenBank releases for ESTs coding for partial sequences of human and mouse forms of this sPLA₂ (GenBank BF196453 and AI530426, for example). More recently, we found the releases for a putative human and mouse sPLA₂ that was tentatively called group XIII (GenBank AF349540, AF339053, and AF339738) and that corresponds in fact to the above group XIIB sPLA₂. The human and mouse protein sequences deduced from all of the nucleic sequences were found to be highly homologous to the previously identified protein sequence obtained from the alignment of the zebrafish ESTs, but they were much less homologous to mouse and human group XIIA sPLA₂s (9, 10). Because these new protein sequences had obvious structural features of group XIIA sPLA₂s, we called them group XIIB sPLA₂s (Figure 1). Using all of the above sequence information available, a consensus nucleic acid sequence of 1069 nucleotides containing 67 nucleotides of the 5' noncoding sequence, an open reading frame of 588 nucleotides, and 414 nucleotides of the 3' noncoding

sequence was compiled for hGXIIIB sPLA₂. Similarly, a nucleic acid sequence of 1078 nucleotides with 5' and 3' noncoding sequences of 115 and 375 nucleotides and an open reading frame of 588 nucleotides was obtained for mGXIIIB.

To experimentally verify the identity of these sequences and isolate the cDNAs coding for hGXIIIB and mGXIIIB, we first designed specific primers flanking the complete open reading frames and used these primers in RT-PCR experiments on various human and mouse cDNAs. The expected cDNAs were amplified from human liver cDNA and pooled cDNAs from various mouse tissues (see Experimental Procedures). Sequencing of the PCR fragments revealed the correct sequences for both hGXIIIB and mGXIIIB. However, a number of hGXIIIB clones were found to contain a deletion of three nucleotides coding for an alanine at nucleotide position 464 (amino acid position 140 in Figure 1). Further analysis of the databases revealed that the genes for hGXIIIB and mGXIIIB sPLA₂s consist of at least 4 exons and 5 introns spanning about 20 kilobase pairs. The alanine deletion above was found to occur at an intron–exon junction, suggesting that this deletion results from uncorrect splicing of the primary RNA transcript. We also designed primers flanking the longest consensus sequences obtained above for hGXIIIB and mGXIIIB. The corresponding DNA fragments could be amplified from human kidney and mouse embryo and were entirely sequenced on both strands. These sequences have been deposited in GenBank with the accession numbers AY358032 (hGXIIIB) and AY358033 (mGXIIIB).

The genes for hGXIIIB and mGXIIIB were found to be located on human and mouse chromosome 10, i.e., on a chromosome distinct from those where hGXIIA (chromosome 4) and mGXIIA (chromosome 3) have been identified (9). Finally, searching the nucleic acid databases for orthologues of GXIIIB sPLA₂ in different species revealed that this sPLA₂ is present in various mammals (rat, ox, hog), birds (chicken), fishes (zebrafish, salmon), and amphibians (frog). No evidence for an orthologue of GXIIIB was found in invertebrates including *Drosophila melanogaster* and *Caenorhabditis elegans* and in plants.

Structural Features of Group XIIIB sPLA₂s. Similar to other sPLA₂s, the open reading frame of GXIIIB sPLA₂s begins with a canonical signal peptide of 19 residues (Figure 1), as predicted from Nielsen et al. (49). This signal peptide appears to be shorter than those of GXIIA sPLA₂s (Figure 1). The mature hGXIIIB (with the EAAC sequence) and mGXIIIB proteins consist of 176 residues with calculated molecular masses of 19727.36 and 19402.04 Da. An acidic pI value of 5.16 was calculated for both proteins. No putative sites of N-glycosylation were found in the sequences.

Sequence comparison revealed that the level of identity between hGXIIIB and mGXIIIB is very high (90%), as previously observed between hGXIIA and mGXIIA [94% (9)]. Interestingly, this level of interspecies identity is much higher than that found for the different sPLA₂s from the I/II/V/X collection (29, 30). On the other hand, only about 40% identity is found between GXIIIB and GXIIA for both mouse and human sPLA₂s, clearly indicating that these sPLA₂s are distinct. Fifty-seven amino acids are identical between the two proteins. Of these residues, 14 are cysteines and several are found within the Ca²⁺-binding segment and the active site. Most importantly, the two proteins differ in the active site, with the presence of a leucine in GXIIIB instead of the

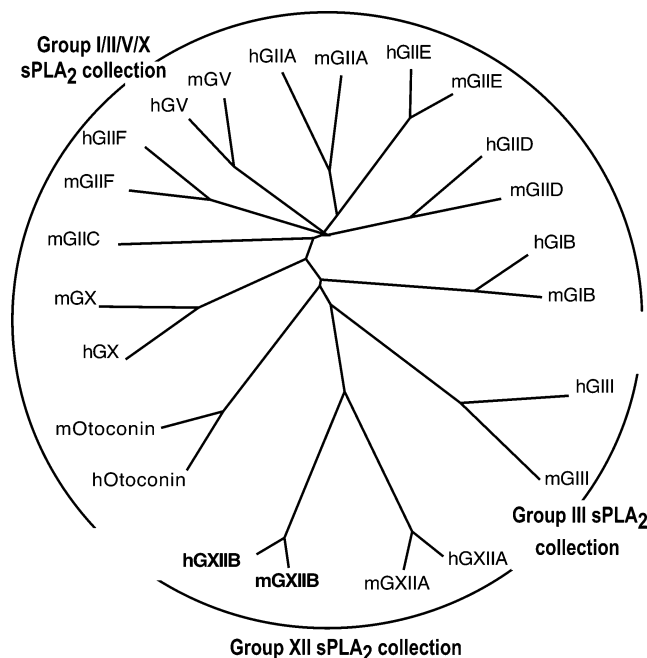


FIGURE 2: Sequence identity dendrogram of mammalian sPLA₂s. Sequences of mature sPLA₂ proteins or sPLA₂ domains of otoconin-95 and group III sPLA₂s were aligned using Clustal W, and the dendrogram was generated with Treeview. The sPLA₂ sequences have been retrieved from GenBank accession numbers.

canonical histidine found in GXIIA and all other sPLA₂s (1, 2), suggesting that GXIIIB sPLA₂s might be catalytically inactive enzymes (see below). As shown in Figure 1B, human GXIIIB sPLA₂ has a low sequence homology with the other groups of human sPLA₂s, and this homology is limited to the Ca²⁺-binding segment and the active site. Finally, on the basis of the dendrogram shown in Figure 2, it is clear that GXIIIB sPLA₂s belong to the group XII sPLA₂ collection (1, 9).

Recombinant Expression of Group XIIIB sPLA₂s. To determine whether the novel mGXIIIB and hGXIIIB (EAC and EAAC) sPLA₂s are catalytically active, we first transiently transfected HEK293 cells (41) with the mammalian expression vector pRc/CMVneo constructs containing the full-length human and mouse GXIIIB cDNAs (see Experimental Procedures). No sPLA₂ activity could be detected with the sensitive sPLA₂ assay using radiolabeled *E. coli* membranes in cell supernatant or lysed cells between 1 and 5 days after transfection (not shown). In comparison, weak, but significant sPLA₂ activity could be measured when cells were transfected with the pRc/CMVneo constructs for hGXIIA and mGXIIA (not shown). We therefore expressed the GXIIIB proteins in *E. coli* using the pAB3 expression system that has been previously used in our laboratory to produce several mammalian sPLA₂s including hGXIIA (9, 32, 41). Human and mouse group XIIIA sPLA₂s were also produced in parallel. With this expression system, GXII sPLA₂s are produced as insoluble fusion proteins with the ~8 kDa N-terminal fragment of glutathione *S*-transferase, and a factor Xa protease site is located before the N-terminal residue of mature sPLA₂s. After in vitro refolding of inclusion bodies, the fusion proteins are cleaved with factor Xa and purified to homogeneity by cation-exchange and reverse-phase HPLC. The purified sPLA₂s migrate as pure proteins of the expected size on a Laemmli SDS gel (Figure

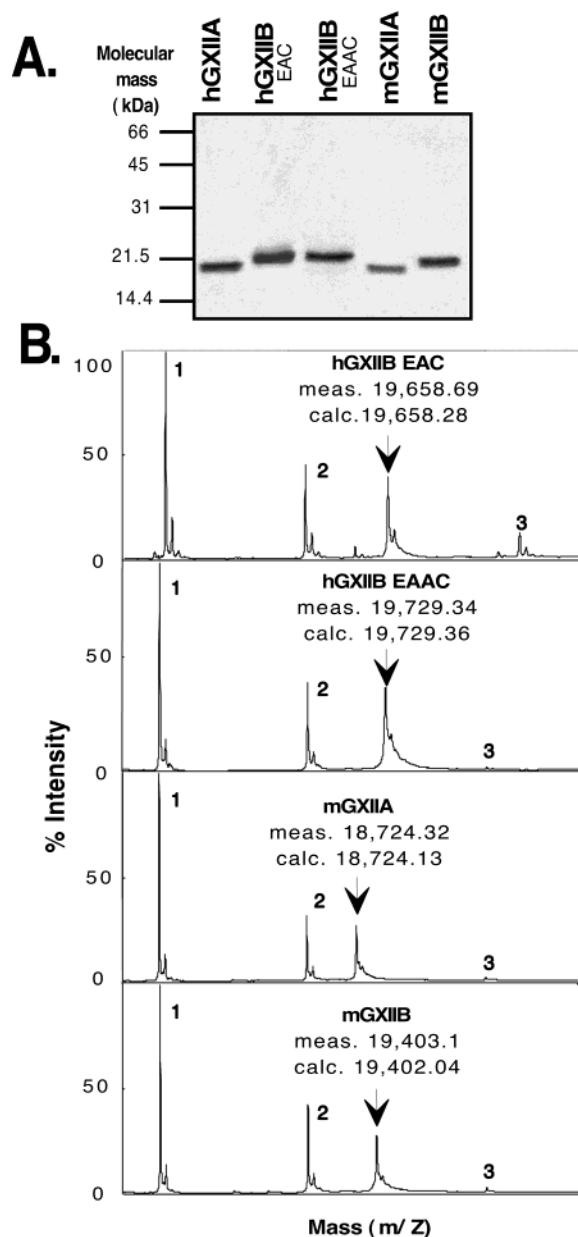


FIGURE 3: Analysis of recombinant group XII sPLA₂s by SDS-PAGE and MALDI-TOF mass spectrometry. (A) One microgram of purified recombinant GXII sPLA₂s was loaded on a 14% SDS-polyacrylamide gel under reducing conditions and stained with Coomassie brilliant blue. (B) Three picomoles of purified recombinant proteins was analyzed by MALDI-TOF mass spectrometry using internal calibration. Horse heart cytochrome *c* (MM = 12361.12 Da, peak 1), horse heart apomyoglobin (MM = 16952.5 Da, peak 2), and bovine trypsinogen (MM = 23981.98 Da, peak 3) were used as internal standards. Measured and calculated molecular masses of each sPLA₂ are indicated.

3A). Indeed, hGXIIA and mGXIIA migrate slightly faster than hGXIIIB and mGXIIIB sPLA₂s, in agreement with their calculated molecular masses (Figure 3B). To establish that all of the disulfide bonds are formed in the refolded sPLA₂s, we determined the molecular masses of the purified sPLA₂s by MALDI-TOF mass spectrometry with internal calibration (Figure 3B). For all sPLA₂s, we measured molecular masses which are different from the calculated molecular masses by less than 1 amu. These data established that the disulfide bonds are formed in the refolded sPLA₂s and that the proteins have not been modified during their production.

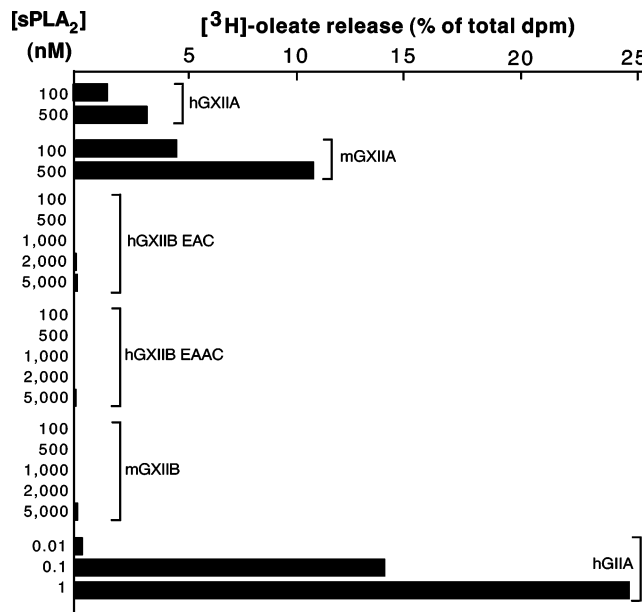


FIGURE 4: Comparison of the catalytic activity of group XIIB and XIIB sPLA₂s. The enzymatic activity of recombinant sPLA₂s was measured using labeled *E. coli* membranes as described in Experimental Procedures. sPLA₂s were incubated at the indicated concentrations for 1 h at room temperature with 100000 dpm of radiolabeled membranes. Values are expressed as the percentage of [³H]oleic acid specifically released by sPLA₂s. The average amount of [³H]oleic acid released in the absence of sPLA₂ was 6400 dpm. No significant sPLA₂ catalytic activity was measured for GXIIIB sPLA₂s. Recombinant hGIIA and hGXIIA were prepared as described (32). Note that hGIIA is about 1000-fold more active than GXIIA sPLA₂s.

Enzymatic Properties of Recombinant Group XIIB sPLA₂s.

We first analyzed the enzymatic activity of the refolded GXIIIB sPLA₂s with the radiolabeled *E. coli* assay (40). At concentrations as high as 5 μ M, no significant sPLA₂ activity could be observed for mGXIIIB and the two forms of hGXIIIB proteins, indicating that GXIIIB sPLA₂s are indeed catalytically inactive (Figure 4). Using the same assay conditions, hGXIIA and mGXIIA were able to release a significant amount of radiolabeled [³H]oleic acid at lower concentrations of 100 and 500 nM (Figure 4), despite the fact that these enzymes have been reported to already have very low specific activities (9, 32). For comparison, we found that, in the same assay conditions, 1 nM human group IIA sPLA₂ (hGIIA) could hydrolyze up to 25% of the substrate. The three GXIIIB proteins were also found to be inactive when anionic vesicles of pyrene-phosphatidylglycerol (32) or dioleoylphosphatidylcholine vesicles (45) were used (not shown).

We also analyzed the binding properties of GXIIIB sPLA₂s to phospholipid vesicles composed of 70% DoetPC and 30% DoetPS (diether, nonhydrolyzable phospholipids). These types of vesicles are known to tightly bind various sPLA₂s (32). With or without added Ca²⁺ in the binding assays, very low dissociation constants (*K_d*) of about 3 and 5 mM were measured for hGXIIIB and mGXIIIB sPLA₂s (not shown). For comparison, a *K_d* value of 0.3 mM was found for hGXIIA. The very weak, but still significant binding of GXIIIB sPLA₂s to the phospholipid vesicles, however, indicates that the proteins are most probably properly folded. Together, the low binding to phospholipids and the natural His-to-Leu

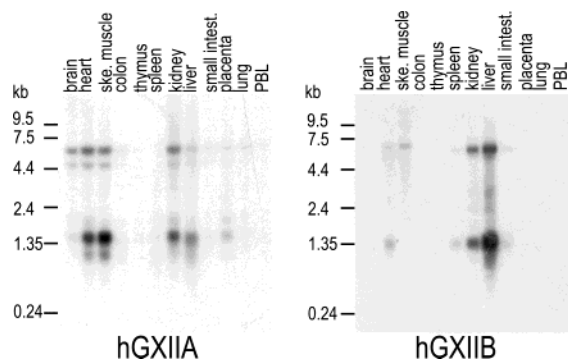


FIGURE 5: Tissue distribution of hGXII sPLA₂s. A human multiple tissue northern blot [2 μ g of poly(A⁺) mRNA per lane] was first hybridized at high stringency with a ³²P-labeled hGXIIIB sPLA₂ riboprobe as described in Experimental Procedures. Abbreviations: ske. muscle, skeletal muscle; small intest., small intestine; PBL, peripheral blood leukocytes. The same blot was stripped and then hybridized at high stringency with the hGXIIA sPLA₂ riboprobe. Filters were exposed for 7 days at -70°C .

active site mutation clearly explain why GXIIB sPLA₂s are not enzymatically active.

Binding Properties of Group XIIB sPLA₂s to the Recombinant Mouse M-Type Receptor Expressed in COS Cells. Previous studies have indicated that several mouse sPLA₂s are endogenous ligands of the mouse M-type receptor (23, 46, 50). We therefore addressed whether mGXIIIB and hGXIIIB could also act as ligands for this receptor. Competition binding assays with iodinated OS1, which is a specific ligand for the mouse M-type receptor (51), indicated that mGXIIIB and hGXIIIB do not bind to this receptor at concentrations as high as 100 nM (not shown). Because it was possible that GXIIB sPLA₂s bind to other domains within the large extracellular domain of the M-type receptor (33), while they do not compete for the OS1 binding site, we radiolabeled mGXIIIB sPLA₂ and performed direct binding assays on COS cell membranes containing large amounts of the mouse M-type receptor. No specific binding was observed in these assays, suggesting that mGXIIIB is not an endogenous ligand for the mouse M-type receptor.

Tissue Distribution of Group XIIB sPLA₂s. The tissue distribution of hGXIIIB sPLA₂ was analyzed by hybridization at high stringency to a northern blot containing various human adult tissues (Figure 5). The same northern blot was then stripped and hybridized with hGXIIA to allow for a direct comparison of the tissue distribution. Transcripts for hGXIIIB of about 1.4 and 6.5 kb are detected by decreasing abundance in liver, kidney, skeletal muscle, and heart. This tissue distribution appears to be different from that of hGXIIA, although some overlap could be observed. Indeed, the strongest level of expression for hGXIIA is found in skeletal muscle, followed by heart, kidney, and liver. A weak expression is also observed in brain and placenta, whereas no signal is observed for hGXIIIB. Transcripts of similar sizes are observed for both sPLA₂s, which might be explained at least in part by the similar exon–intron structure of their respective genes. The northern blot tissue distribution of hGXIIIB is in accordance with that estimated from the sources of the ESTs found in databases. Indeed, several ESTs were obtained from human liver and kidney.

The tissue distribution of mGXIIIB sPLA₂ was analyzed by hybridization to a mouse northern and a RNA dot blot

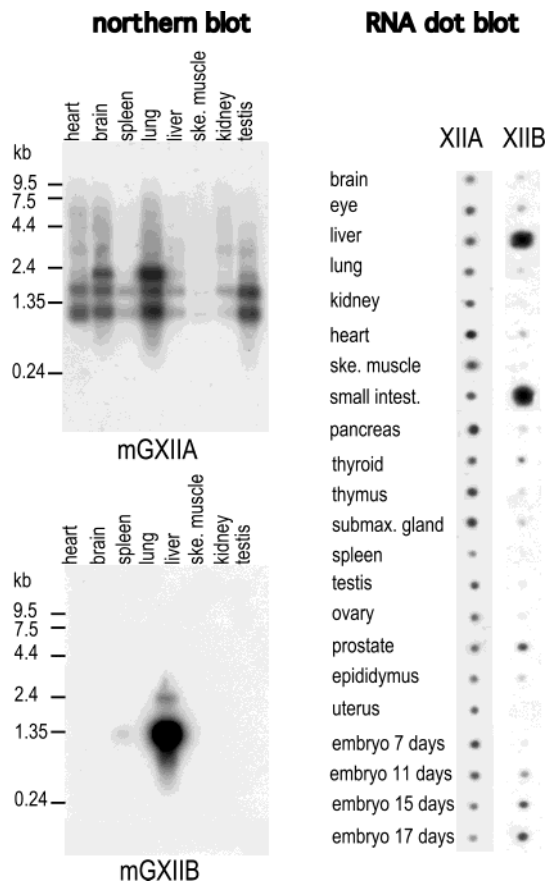


FIGURE 6: Tissue distribution of mGXII sPLA₂s. A mouse multiple tissue northern blot containing 2 μ g of poly(A⁺) mRNA per lane and a master blot containing 100–500 ng of poly(A⁺) RNA from mouse tissues were first hybridized at high stringency with a ³²P-labeled mGXIIIB riboprobe as described in Experimental Procedures. Abbreviations: ske. muscle, skeletal muscle; small intest., small intestine; PBL, peripheral blood leukocytes; submax. gland, submaxillary gland. The same blots were stripped and then hybridized at high stringency with the mGXIIA sPLA₂ riboprobe. Filters were exposed for 7 days at -70°C .

and then compared to that of mGXIIA hybridized to the same blots. A very strong expression of mGXIIIB is observed in adult liver with a major transcript of about 1.3 kb and a minor one at 2.4 kb. Analysis of the RNA dot blot reveals that mGXIIIB is also expressed at very high levels in small intestine and at lower levels in prostate, thyroid, and eye, among other tissues. A significant level of expression is also found during embryogenesis, and the signal is increasing upon development. This tissue distribution is in accordance with the identification of many ESTs from small intestine, liver, and embryos. On the other hand, mGXIIA is expressed from three transcripts, with two of them being similar in size with those of mGXIIIB. The tissue distribution of mGXIIA is quite different, with a rather ubiquitous expression pattern in most, if not all, mouse tissues and highest expression levels in lung, heart, pancreas, and submaxillary glands. The wide expression pattern of mGXIIA is in accordance with that previously described by RT-PCR (10). Interestingly, the expression pattern of the two sPLA₂s during embryogenesis appears to be opposite (Figure 6).

All together, these results indicate that GXIIA and GXIIB sPLA₂s have different expression patterns in both mouse and human species, though some tissues express both sPLA₂s. Furthermore, as for other sPLA₂s (29–31, 48), a distinct,

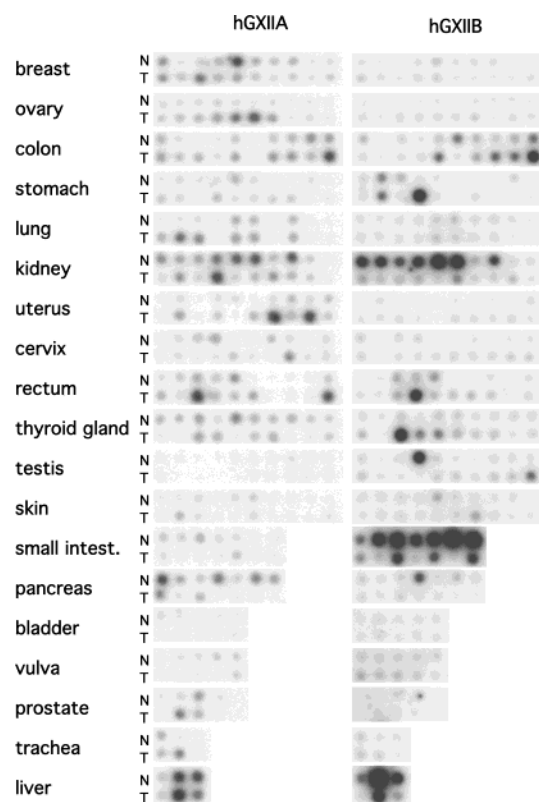


FIGURE 7: Cancer profiling array of hGXII sPLA₂s. A cancer profiling array II from Clontech containing 160 tumors (T) and their matched normal tissues (N) from individual patients was first hybridized with a ³²P-labeled hGXIIIB riboprobe as described in Experimental Procedures. Abbreviation: small intest., small intestine. The same blot was stripped and then hybridized with the hGXIIA sPLA₂ riboprobe. Filters were exposed for 6 days at -70°C .

yet overlapping tissue distribution is found for both sPLA₂s when mouse species is compared to human species. The mGXIIA sPLA₂ was previously found to be preferentially expressed in Th2 cells, suggesting that this sPLA₂ may be involved in the production of eicosanoids in these cells (10). It remains to be analyzed whether mGXIIIB is also expressed in Th2 or Th1 cells.

hGXIIIB sPLA₂ as a Cancer Marker. Several reports have shown that sPLA₂s including IIA, IID, and X are increased in human and mouse tumors (28, 52–57, 58, 59), where they may play a role in providing arachidonic acid for subsequent prostaglandin E₂ release. Alternatively, sPLA₂s such as IIA may exert protective effects against progression of colorectal and gastric cancers, as suggested for mouse group IIA (26, 27) and very recently for human group IIA (28). Besides sPLA₂s, a decrease in the expression of the cPLA₂ group IV was also observed in both mouse and human tumors (59, 60, 61). It was therefore of interest to determine the expression status of hGXIIA and hGXIIIB sPLA₂s in various human tumors and cancer cell lines. Figure 7 shows the expression patterns of the two group XII sPLA₂s in 160 tumors and their matched normal tissues from individual patients. A strong level of expression for hGXIIIB is observed in the normal kidney, small intestine, and liver of several patients. However, the level of expression is dramatically decreased in nearly all of the corresponding tumors, indicating that hGXIIIB is significantly downregulated during tumorigenesis in these tissues (Figure 7). In several cases, a

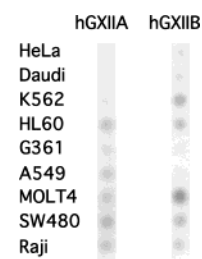


FIGURE 8: Expression of hGXII sPLA₂s in various human cancer cell lines. The cancer profiling array II from Clontech containing cDNA from the indicated cell lines was first hybridized with a ³²P-labeled hGXIIIB riboprobe as described in Experimental Procedures. The same blot was stripped and then hybridized with the hGXIIA sPLA₂ riboprobe. Filters were exposed for 6 days at -70°C .

decrease in hGXIIA and hGXIIIB expression in testis and pancreas is also observed. On the other hand, a higher level of expression of hGXIIIB is observed in some patients with tumors of the colon, stomach, rectum, and thyroid gland, although the significance of these data remains to be established, given the scatter in the expression data across the different patient samples. A detailed analysis of the hGXIIIB promoter may reveal some clues to explain the tissue-specific expression patterns observed in these studies. Interestingly, hGXIIIB expression was recently found to be significantly elevated in hepatitis C virus-associated hepatocellular carcinomas (62). Conversely, no dramatic upregulation or downregulation of the expression of hGXIIA could be observed in a whole set of tumor samples from the same tissue (Figure 7). However, as for hGXIIIB, an increased or decreased expression was observed in the different tissues for some patients.

hGXIIIB was also found to be expressed in a number of human cancer cell lines (Figure 8) including HL-60 (promyelocytic leukemia), Raji (B cell Burkitt's lymphoma), MOLT-4 (T cell lymphoblastic leukemia), A549 (lung carcinoma), and SW480 (colorectal adenocarcinoma). In contrast, we found either the absence of expression or only very low levels of hGXIIIB in the following cancer cell lines: HeLa (adenocarcinoma), Daudi (B cell Burkitt's lymphoma), K-562 (myelogenous leukemia), and G-361 (skin melanoma). A dissimilar pattern of expression was found for hGXIIA, with the highest levels of expression observed in MOLT-4, SW480, HL-60, and K-562 cancer cell lines (Figure 8).

Concluding Remarks. This paper describes the molecular characterization of a novel human and mouse group XIIIB sPLA₂ that belongs to the recently identified group XII collection (9, 10). Interestingly, this sPLA₂ is present only in higher order organisms and is fairly highly conserved among different species, suggesting conserved and important functions. Most importantly, we demonstrated that this sPLA₂ is the first mammalian sPLA₂ of about 20 kDa that is completely devoid of enzymatic activity because of a mutation in the active site and very poor phospholipid-binding properties. In some regards, this protein can be considered as one mammalian "equivalent" of the various inactive snake venom sPLA₂-like proteins (37, 39, 63). Interestingly, the lipase family also comprises naturally occurring inactive forms (64–66), whose functions have not yet been determined. The tissue expression pattern of the GXIIB sPLA₂ is different from that of all other sPLA₂s including the homologous GXIIA sPLA₂, suggesting that this

sPLA₂-like protein has a function distinct from those of other sPLA₂ members. Its expression pattern is also different from that of otoconin-95, the atypical catalytically inactive sPLA₂-like protein that has been exclusively detected, to date, only in inner ears (67, 68). Because the expression of hGXIIb sPLA₂ was found to be dramatically decreased in tumors of several tissues, it is tempting to speculate that the protein may act as a gene suppressor controlling tumor progression. Interestingly, the expression of the CC-10 protein (also called uteroglobin), which has been reported to act as an anti-inflammatory and antitumoral agent and possibly as a sPLA₂ inhibitor, is also known to be dramatically decreased in several tumors and cancer cell lines (69–75). The regulation of the expression of the GXIIb sPLA₂ during inflammatory conditions and in associated diseases will also be interesting to analyze in future studies. Finally, because of the absence of enzymatic activity, it is likely that the GXIIb sPLA₂ exerts its function(s) by acting as a ligand through binding to specific soluble or membrane proteins that remain to be identified (1, 33). So far, we found that the GXIIb sPLA₂ does not bind to the well-known M-type receptor, but other sPLA₂ targets specific for this sPLA₂ remain to be discovered. The identification of such targets will certainly help to reveal the *in vivo* function of this particular sPLA₂.

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