Recombinant Production and Properties of Binding of the Full Set of Mouse Secreted Phospholipases A 2 to the Mouse M-Type Receptor †

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Recombinant Production and Properties of Binding of the Full Set of Mouse Secreted Phospholipases A_2 to the Mouse M-Type Receptor†

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ABSTRACT: To date, 12 secreted phospholipases A_2 (sPLA$_2$s) have been identified in the mouse species and divided into three structural collections (I/II/V/X, III, and XII). On the basis of their different molecular properties and tissue distributions, each sPLA$_2$ is likely to exert distinct functions by acting as an enzyme or ligand for specific soluble proteins or receptors, among which the M-type receptor is the best-characterized target. Here, we present the properties of binding of the full set of mouse sPLA$_2$s to the mouse M-type receptor. All enzymes have been produced in *Escherichia coli* or insect cells, and their properties of binding to the cloned and native M-type receptor have been determined. sPLA$_2$s IB, IIA, IIE, IIF, and X are high-affinity ligands ($K_{0.5}$ = 0.3−3 nM); sPLA$_2$s IIC and V are low-affinity ligands ($K_{0.5}$ = 30−75 nM), and sPLA$_2$s IID, III, XIIA, and XIIB bind only very weakly or do not bind to the M-type receptor ($K_{0.5}$ > 100 nM). Three exogenous parvoviral group XIII PLA$_2$s and two fungal group XIV sPLA$_2$s do not bind to the receptor. Together, these results indicate that the mouse M-type receptor is selective for only a subset of mouse sPLA$_2$s from the group I/II/V/X structural collection. Binding of mouse sPLA$_2$s to a recombinant soluble mouse M-type receptor leads in all cases to inhibition of enzymatic activity, and the extent of deglycosylation of the receptor decreases yet does not abolish sPLA$_2$ binding. The physiological meaning of binding of sPLA$_2$ to the M-type receptor is discussed on the basis of our current knowledge of sPLA$_2$ functions.

Secreted phospholipases A_2 (sPLA$_2$s)[1] form a diverse group of enzymes that are widespread in Nature. Numerous members have been identified for decades in insect and snake venoms (*1*, *2*). More recently, sPLA$_2$s have been found in mammals, but also in plants, bacteria, fungi, and viruses (*3*−*8*). All of these enzymes catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position to release free fatty acids and lysophospholipids, and they share a common set of structural features, including a relatively low molecular mass (14−19 kDa), a compact structure with several disulfides, and a conserved Ca^{2+}-dependent catalytic mechanism.

Mammalian sPLA$_2$s now comprise up to 12 different members that belong to three main structural collections (*3*, *5*, *9*). sPLA$_2$s IB, IIA, IIC, IID, IIE, IIF, V, and X and otoconin-90 are the nine members of the group I/II/V/X collection. The atypical group III sPLA$_2$, the group XIIA sPLA$_2$, and the sPLA$_2$-like group XIIB protein are members of group III and XII collections. Despite the important knowledge accumulated at molecular and cellular levels (*3*, *10*−*13*), the in vivo biological functions of most mammalian sPLA$_2$s remain to be elucidated. The group IB, IIA, V, and X sPLA$_2$s, which were the first enzymes to be identified (*14*−*17*), are likely to play a role in lipid digestion (*18*), host defense (*19*), or production of lipid mediators during normal and pathological conditions, including inflammatory diseases and cancer (*20*−*25*). Interestingly, these four sPLA$_2$s are the most catalytically active mammalian enzymes (*10*), and many of their currently attributed biological roles involve catalytic activity. However, during the search for biological roles for these four sPLA$_2$s and studies on their mechanism of action, it has been found that some of their biological functions may be related to specific binding to various

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[1] Abbreviations: sPLA$_2$, secreted phospholipase A_2 [a comprehensive abbreviation system for the various mouse sPLA$_2$s is used; each sPLA$_2$ is abbreviated with the lowercase letter m for mouse species followed by uppercase letters identifying the sPLA$_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX, GXIIA, and GXIIB)]; bvPLA$_2$, bee venom PLA$_2$; OS$_1$, *Oxyuranus scutellatus scutellatus* toxin 1; mMR, mouse M-type receptor; CTLD, C-type lectin-like carbohydrate recognition domain; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SELDI-TOF, surface-enhanced laser desorption ionization time-of-flight; ACN, acetonitrile.

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soluble or membrane-bound proteins, including the M-type receptor and heparan sulfate proteoglycans (*25*−*31*). These observations and those independently obtained for venom $sPLA_2$s have led to the proposal that mammalian $sPLA_2$s may be bifunctional proteins that act as either enzymes, ligands, or both (*25*, *32*). Conversely, almost nothing is known about the in vivo biological functions of the more recently identified group IID, IIE, IIF, III, XIIA, and XIIB $sPLA_2$s (*3*, *5*, *33*, *34*). Since these $sPLA_2$s have much lower enzymatic activities than group IB, IIA, V, and X $sPLA_2$s (*5*, *10*) and display specific tissue distribution patterns, it is possible that they have nonredundant functions which would not depend on their catalytic activity.

Venom $sPLA_2$s can be neurotoxic, myotoxic, anticoagulant, and proinflammatory (*2*, *35*, *36*) and can exert various pharmacological effects with therapeutic potential (*36*−*39*). Their toxic and pharmacological effects were found to be dependent on or independent of enzymatic activity, leading to the early proposal that $sPLA_2$s may act not only as enzymes but also as ligands for cellular targets distinct from phospholipids (*40*). The presence of catalytically inactive snake venom $sPLA_2$s which are myotoxic, proinflammatory, antibacterial, and anti-HIV or able to trigger apoptosis and cell proliferation further supports nonenzymatic functions of venom $sPLA_2$s (*38*, *41*−*43*). Consequently, a collection of mammalian $sPLA_2$ binding proteins has been identified for several snake venom $sPLA_2$s. These proteins include the N- and M-type receptors (*32*), calmodulin and 14-3-3 proteins (*44*), pentraxins and associated proteins (*45*), crocalbin (*46*), pulmonary surfactant proteins (*47*), KDR VEGF receptor 2 (*48*), and factor Xa (*49*). Different types of $sPLA_2$ inhibitory proteins have also been found in the blood of venomous and nonvenomous snakes, and these inhibitors belong to superfamilies of proteins, including members in mammals (*50*). Such natural $sPLA_2$ inhibitors have been proposed to protect snakes from the leakage of their own venom $sPLA_2$s in the circulatory system.

Among the $sPLA_2$ binding proteins mentioned above, the M-type receptor is the best-characterized target, yet many of its molecular and functional properties are still unclear. This receptor was first identified as a 180 kDa protein in rabbit skeletal muscle cells (M-type stands for muscle-type) using the snake venom $sPLA_2$s OS_1 and OS_2 (*51*). Later studies indicated that the receptor is expressed in several tissues, including lung, kidney, spleen, and colon, from different mammalian species (*29*, *32*). The M-type receptor is structurally similar to the macrophage mannose receptor, the DEC-205 receptor, and the endo-180 receptor, which all belong to a particular subgroup within the C-type lectin superfamily (*52*−*54*). It is a type I membrane glycoprotein comprising a single transmembrane domain, a short cytoplasmic tail, and a very large extracellular region made up of an N-terminal cysteine-rich domain, a fibronectin-like type II domain, and a tandem repeat of eight distinct C-type lectin-like carbohydrate recognition domains (CTLDs). A series of structure−function studies have indicated that the CTLD5 domain of the M-type receptor is likely to be one key element in $sPLA_2$ binding, that $sPLA_2$ residues from the Ca^{2+} loop and interfacial binding domain are involved in binding to the receptor, and that binding of $sPLA_2$ to the receptor leads to inhibition of enzymatic activity (*29*, *32*).

The biological roles of the M-type receptor are still unclear. The receptor has been proposed to mediate several in vitro cellular effects of group IB or IIA $sPLA_2$s on cell proliferation, cell migration, eicosanoid release, or activation of various signaling pathways (*29*, *55*−*57*). More definitive evidence of the implication of the receptor is, however, still required. Nonetheless, M-type receptor-deficient mice are partially resistant to lipopolysaccharide-induced lethality, and levels of tumor necrosis factor-α and interleukin-1β are reduced in these mice after lipopolysaccharide challenge, suggesting a pro-inflammatory role of the receptor (*58*). On the other hand, on the basis of the fact that the membrane-bound M-type receptor has potent endocytic properties and rapidly internalizes and degrades $sPLA_2$s, the M-type receptor was proposed to play a role in the clearance of $sPLA_2$ (*29*, *32*). In vitro studies on transfected cells or cells endogenously expressing the receptor clearly supported this view (*59*, *60*). However, in vivo studies using receptor-deficient mice were inconclusive (*58*). Finally, the presence of soluble forms of the M-type receptor that can block enzymatic activity upon $sPLA_2$ binding (*29*, *32*) was another indication that the receptor may serve to counteract $sPLA_2$ action.

The identification of the natural ligands of the M-type receptor is another key question which is still incompletely answered. The pancreatic group IB $sPLA_2$, but not the inflammatory-type group IIA $sPLA_2$, was proposed as a first endogenous ligand of the receptor (*61*). The apparent controversial results which were subsequently observed in the interaction of group IB and group IIA $sPLA_2$s with the receptor (*62*−*64*) were clarified by Cupillard et al. (*65*). It was found that there is a strict species dependence of the $sPLA_2$−receptor interaction that depends on the animal species for both the M-type receptor and $sPLA_2$. Results from these studies indicated that mouse group IB (mGIB) and mouse IIA (mGIIA) $sPLA_2$s are two endogenous ligands of the mouse M-type receptor (*65*). Conversely, rat group IB $sPLA_2$, but not the rat group IIA enzyme, appears as an endogenous ligand of the rat M-type receptor (*62*), and human group IB and IIA $sPLA_2$s are not high-affinity ligands of the human M-type receptor (*64*). More recently, mouse group X (mGX) $sPLA_2$ was also found to be an endogenous ligand of the mouse receptor (*66*).

Considerable molecular knowledge about the full set of mouse $sPLA_2$s regarding their primary structure (*3*, *5*, *16*, *67*, *68*), enzymatic properties (*10*), antibacterial properties (*11*), potential role in lipid mediator release (*10*, *12*, *24*, *66*), tissue distribution (*69*−*71*), and roles in pathological conditions such as colorectal cancer (*72*, *73*), lipid digestion (*18*), atherosclerosis (*74*), lung injury (*75*), and host defense (*19*) has accumulated. In this study, we have produced the full set of mouse $sPLA_2$s (except otoconin-90) as pure recombinant proteins using *Escherichia coli* or insect cells as expression hosts and have characterized their properties for binding to the mouse M-type receptor. The binding properties of exogenous $sPLA_2$s from parvoviruses (group XIII) and fungi (group XIV) were also analyzed. Our results show that only a subset of $sPLA_2$s from the group I/II/V/X collection can bind to this receptor. $sPLA_2$s IB, IIA, IIE, IIF, and X are very high-affinity ligands for the mouse M-type receptor, while $sPLA_2$s IIC and V bind with weaker affinities. Conversely, $sPLA_2$s IID, III, and XIIA and the group XIIB $sPLA_2$-like protein, as well as exogenous $sPLA_2$ enzymes

from fungi and parvoviruses, bind only very weakly or do not bind to the receptor. In all cases, binding of $sPLA_2$ to the receptor leads to inhibition of enzymatic activity, and deglycosylation of the receptor decreases, yet does not abolish $sPLA_2$ binding. The possible physiological implications of these binding properties are discussed in relation with our current understanding of $sPLA_2$ functions.

EXPERIMENTAL PROCEDURES

Preparation of the Full Set of Recombinant Mouse sPLA$_2$s. All the procedures described below were developed with the aim of producing $sPLA_2$s as wild-type, mature, nonfusion, and properly folded proteins. Toward this goal, most mouse $sPLA_2$s were produced by in vitro refolding of inclusion body protein obtained from *E. coli*. In some cases, recombinant production was performed in baculovirus (*65*) or *Drosophila* S2 insect cell systems (*10*) because these systems gave a high yield (mGIB or mGIIA), permitted the production of N-glycosylated $sPLA_2$s (mGIIC and mGIII), or allowed us to obtain folded $sPLA_2$s when no refolding conditions from the *E. coli* inclusion body could be found (mGIIC and mGIII). In most cases, mouse $sPLA_2$s were purified to homogeneity by a combination of ion exchange and reverse phase chromatography, quantified by the OD_{280} using their calculated molar absorption coefficients, and analyzed by SDS−polyacrylamide gel electrophoresis and MALDI-TOF mass spectrometry. MALDI-TOF analysis of $sPLA_2$s was carried out on an Applied Biosystems voyager DE-PRO mass spectrometer in linear mode with sinapinic acid as a matrix using external or internal calibration (Figure 1). $sPLA_2$ activity was followed using a labeled *E. coli* membranes as a substrate (*65*). The cDNAs encoding $sPLA_2$s IB, IIA, IID, IIE, IIF, X, XIIA, and XIIB were those previously cloned in the laboratory (*5* and references cited therein). The cDNAs encoding mGIIC and mGV $sPLA_2$s were amplified by PCR and found to be identical to the original sequences (*16*), except for a P86L mutation in mGIIC mature protein. The cDNA encoding mGIII $sPLA_2$ was obtained by PCR cloning from mouse testis cDNA and found to be identical to GenBank entry AK033572.

Production in E. coli. Inclusion bodies for mouse $sPLA_2$s IIA, IID, IIE, IIF, X, XIIA, and XIIB were produced using the pAB3 vector encoding a truncated 8.4 kDa glutathione *S*-transferase protein followed by a factor Xa protease cleavage site fused to the N-terminus of mature $sPLA_2$ (*76*). The inclusion body for mGV $sPLA_2$ was produced using the pET21a expression plasmid in which the sequence of mature mGV $sPLA_2$ is fused to the methionine initiator. N-Terminal sequence analysis of the purified mGV inclusion body indicated that the initiator methionine was removed by the *E. coli* aminopeptidase in ~70% of the protein. Inclusion bodies were obtained after transformation of BL21 DE3 *E. coli* with appropriate plasmids and overnight induction of a 2 L culture (OD_{600} = 0.8) in Terrific Broth with 0.5 mM IPTG. Inclusion bodies were extracted, denatured, reduced, and sulfonated as described previously (*76*) and then processed for refolding, trypsin digestion, and HPLC purification as detailed below.

Recombinant mGIIA, mGIID, and mGIIE produced from *E. coli* were refolded essentially as originally described for mGIID (*76*). The sulfonated protein (100 mg) was dissolved in 500 mL of 6 M guanidine-HCl and 50 mM Tris (pH 8.0) at room temperature by stirring. The protein solution was dialyzed against 8 L of prechilled 0.6 M guanidine-HCl, 50 mM Tris-HCl (pH 8.0), 5 mM freshly added cysteine, and 5 mM EDTA at 4 °C for 2−3 days. The dialysis bag was transferred directly to 8 L of prechilled factor Xa buffer for mGIIA and mGIIE [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM $CaCl_2$] or factor Xa buffer with lauryl sulfobetaine for mGIID [factor Xa buffer with 5 mM dodecyldimethyl(3-sulfopropyl)ammonium hydroxide]. The buffer was changed twice after dialysis for 6−12 h. Insoluble material was removed by centrifugation at 15000*g* for 20 min at 4 °C. For mGIIA, the protein was digested with factor Xa (300 units) overnight at room temperature. After filtration through a syringe filter (Uniflo-25, 0.2 μm, low protein binding, Schleicher & Schuell model 02330), the solution was pumped at 3 mL/min directly onto the C18 reverse phase HPLC column (Vydac TP1010, 1 cm × 10 cm, protein/peptide column). After loading had been carried out, elution was performed at 3 mL/min using a water/acetonitrile (ACN) gradient in 0.1% TFA (from 0 to 30% over 40 min and then from 30 to 60% over 90 min). mGIIA eluted at ~31% ACN and was recovered as a pure protein after lyophilization. For mGIID, the fusion protein was cleaved with trypsin (1/40 by weight, Sigma catalog no. T8642), and cleavage was followed by monitoring $sPLA_2$ activity every 30 min over 3 h. The trypsinized sample was filtered as described above and pumped at 4 mL/min onto a 5 mL heparin−Sepharose HiTrap column (Amersham Biosciences) attached to an HPLC pump and equilibrated in 20 mM Tris-HCl (pH 8.0). After loading had been carried out, the column was washed with 20 mM Tris (pH 8.0) for 10 min and eluted using a gradient of NaCl [from 0 to 1 M in 20 mM Tris-HCl (pH 8.0) over 30 min]. The mGIID protein eluted at ~20 min and was loaded onto the C18 reverse phase HPLC column as described above. Elution was performed using a gradient of ACN in 0.1% TFA (from 10 to 30% over 20 min and then from 30 to 60% over 110 min). mGIID eluted at ~33% ACN and was recovered as a pure protein after lyophilization. For mGIIE, the protein was treated with trypsin as described above, filtered, and immediately pumped onto the C18 reverse phase HPLC column. Elution was performed using a gradient of ACN in 0.1% TFA (from 10 to 30% over 20 min and then from 30 to 45% over 110 min). mGIIE eluted at ~34% ACN and was recovered as a pure protein after lyophilization.

Recombinant mGIIF was sulfonated and refolded using a slightly different procedure as follows. Inclusion bodies (100 mg) were solubilized in 80 mL of 8 M urea, 0.1 M NH_4Cl, 50 mM Tris-HCl (pH 8.0), and 0.3 M Na_2SO_3 and sulfonated by addition of 20 mL of Thannhauser reagent for 30 min at 56 °C. After dialysis for 24 h against 4 L of 1% acetic acid at 4 °C, the precipitated sulfonated protein was resuspended in 500 mL of 8 M urea, 50 mM Tris-HCl (pH 8.0), 5 mM $CaCl_2$, 0.1 M NH_4Cl, and 5 mM L-methionine and refolded by dialysis at 4 °C for 3 days using a 8 kDa membrane tubing (SpectraPor, diameter of 76 mm) against 8 L of 1.6 M urea, 50 mM Tris-HCl (pH 8.0), 0.1 M NH_4Cl, 5 mM $CaCl_2$, 5 mM L-methionine, and 8 mM L-cysteine. The dialyzed solution (560 mL) was centrifuged at 10000*g* for 30 min to remove aggregates and subjected to digestion by trypsin (1/300 ratio by weight, Sigma T-1005) for 90 min at room

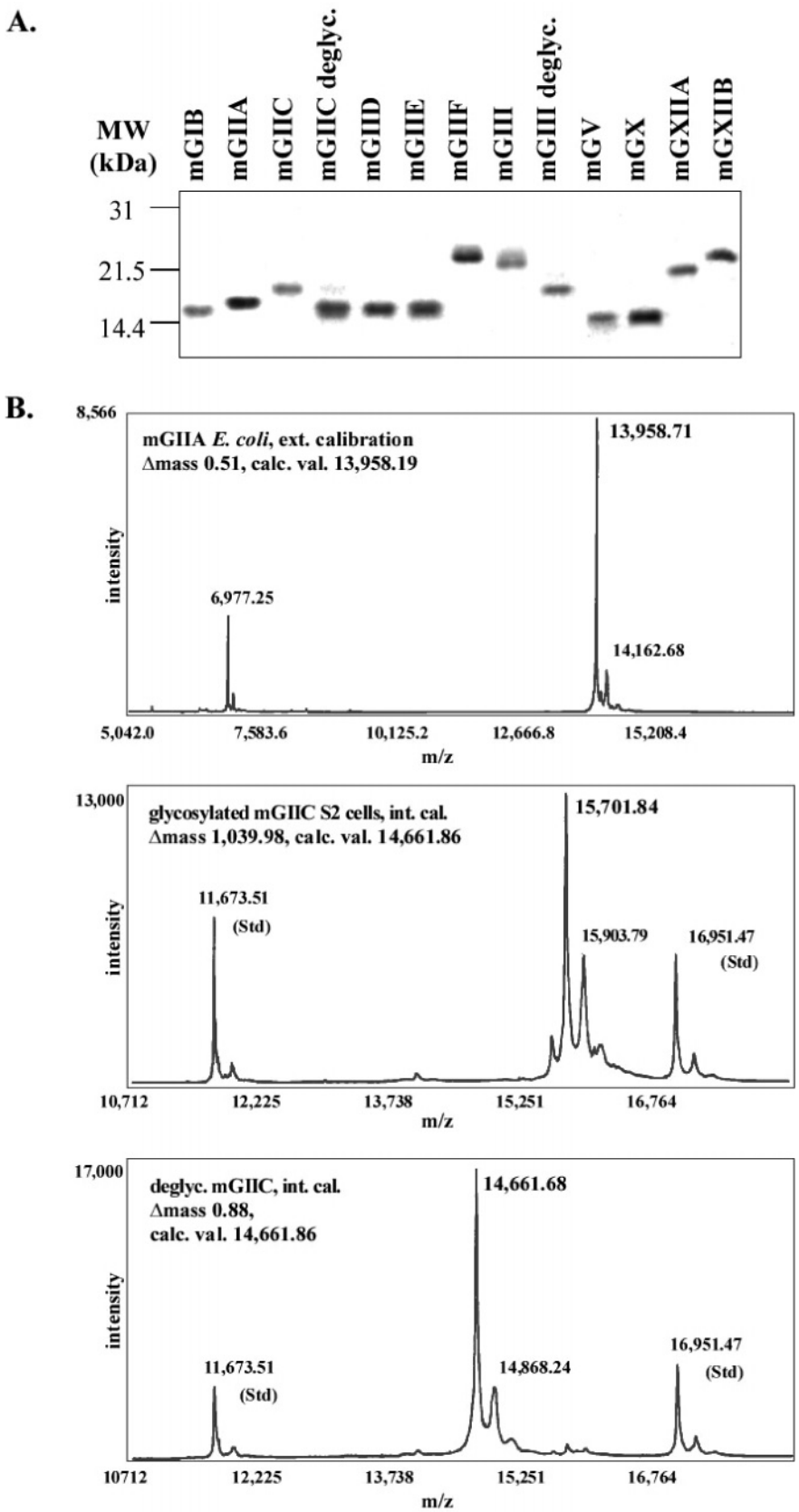

FIGURE 1: Analysis of the recombinant mouse $sPLA_2$s by SDS−PAGE and mass spectrometry. (A) One microgram of $sPLA_2$ was loaded on a 14% SDS−polyacrylamide gel under reducing conditions and stained with Coomassie brilliant blue. mGIIC and mGIII $sPLA_2$s were loaded before and after deglycosylation by *N*-glycosidase F. (B) Examples of mass spectrometry analyses with external or internal calibration for mGIIA produced in *E. coli* and mGIIC produced as an N-glycosylated protein in *Drosophila* S2 cells.

temperature. The cleaved mGIIF protein was concentrated by ultrafiltration using an Amicon YM-10 cell membrane, buffer exchanged against 10% ACN and 0.1% TFA, filtered, and then directly loaded onto a C5 semipreparative reverse phase HPLC column (Supelco, 1 cm × 25 cm, 5 μm, 300 Å). The elution was performed at 3 mL/min using a gradient

of ACN in 0.1% TFA (10% for 5 min, from 10 to 30% over 20 min, and then from 30 to 35% over 100 min). The mGIIF-Cys monomer (i.e., recombinant mGIIF with its C-terminal cysteine disulfide linked to the cysteine amino acid from the refolding buffer) eluted at 32% ACN and was recovered as a pure protein after lyophilization.

Recombinant mGV and mGX were refolded by the rapid dilution method as follows. L-Methionine (5 mM) was added throughout the procedure to prevent oxidation of mGX $sPLA_2$. The sulfonated fusion protein was dissolved at 10 mg/mL in 7 M guanidine-HCl and 50 mM Tris-HCl (pH 8.0) by being stirred for 2 h at room temperature. The sample was centrifuged at 4 °C and 12 000 rpm for 20 min to remove undissolved protein. A 4 mL portion of protein solution was added dropwise (~1 drop/s) to 2 L of refolding buffer [for mGV, 50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 10 mM $CaCl_2$, 5 mM freshly added cysteine, and 30% ACN; for mGX, 50 mM Tris-HCl (pH 8.0), 0.9 M guanidine-HCl, 0.8 M NaCl, 10 mM $CaCl_2$, 5 mM freshly added cysteine, and 5 mM lauryl sulfobetaine] in an Erlenmeyer flask with rapid stirring (at 4 °C for mGV and at room temperature for mGX). After addition of protein, the sample was stirred for an additional 2−3 min and then allowed to sit at 4 °C (mGV) or room temperature (mGX) without stirring until the increase in enzymatic activity reached a plateau (~2−3 days). After addition of 5 mM lauryl sulfobetaine (for mGV), the protein solution was concentrated at room temperature in an ultrafiltration cell (YM-10 membrane, Amicon) to a final volume of 40−50 mL. The concentrated protein solution was dialyzed against factor Xa buffer at 4 °C (three cycles, 40 volumes each). After cleavage of the mGX fusion protein with trypsin (1/300 ratio by weight, Sigma T-1005), the protein solution was acidified to pH 3.0 with 1% acetic acid in 30% ACN, filtered on 0.45 μm hydrophilic PVDF membrane filters (Millipore model SLHVBZ5NZ), and loaded onto a Spherogel TSK SP-5PW column (10 μm, 7.5 mm × 75 mm, TosoHaas) equilibrated in 1% acetic acid and 50% ACN. The column was eluted at 1 mL/min using a linear gradient of NaCl (from 0 to 0.5 M over 100 min) in a 50% ACN/25 mM ammonium acetate mixture. The shallow gradient of NaCl allowed separation of mature mGV $sPLA_2$ from mGV bearing the methionine initiator (due to incomplete cleavage by the *E. coli* aminopeptidase). Fractions containing $sPLA_2$ activity were pooled, lyophilized, resuspended in 20% ACN and 0.1% TFA, and applied to a reverse phase HPLC column (C18, 5 μm, 1 cm × 25 cm, Macherey Nagel Nucleodur). Elution was performed at 4 mL/min using a shallow water/ACN gradient in 0.1% TFA (from 20 to 50% over 120 min). Mature mGV and mGX $sPLA_2$s eluted at ~33 and ~35% ACN, respectively, and were recovered as pure proteins after lyophilization.

Recombinant mGXIIA and mGXIIB were produced as previously described (*5*).

Production in Insect Cells. Recombinant mGIB $sPLA_2$ was produced using the *Sf*9/baculovirus system as described previously (*65*). Recombinant mGIIA, mGIIC, mGIID, and the mGIII $sPLA_2$ domain were produced in *Drosophila* S2 cells as for hGIID $sPLA_2$ (*10*) with some modifications as follows. mGIIA was produced in *Drosophila* cells as an alternative to the *E. coli* expression system described above with similar yields. mGIID was produced in insect cells to check for its possible glycosylation at the putative N-glycosylation site (*76*). For expression of mature mGIIA, mature mGIIC, and the mGIII $sPLA_2$ domain (amino acid residues 146−257 from GenBank entry AK033572), the corresponding cDNAs were subcloned by PCR in frame with the human group IIA signal peptide sequence into *Drosophila* expression vector pS2neo (*77*). For expression of mGIID, the full-length cDNA coding sequence (*76*) was directly inserted into the pS2neo vector. *Drosophila* S2 cells were grown at 24−27 °C in HyQ SFX-insect medium (Hyclone) or *Drosophila* SFM medium (InvitroGen) supplemented with 1% fetal bovine serum and antibiotic−antimycotic cocktail from Invitrogen (Gibco catalog no. 15240-062). Cells were transfected using the calcium phosphate method, and stably transfected cells were selected for at least 3 weeks using 2 mg/mL (±)-G418 neomycin sulfate. For large-scale production, transfected cells were seeded into 500 mL of complete medium in 2 L spinner culture flasks. Cells were induced at a density of $3-4 \times 10^6$ cells/mL with 500 μM $CuSO_4$ for up to 7 days, after which cells were pelleted and the medium containing $sPLA_2$ activity was collected. For mGIIA and mGIID, cell-associated $sPLA_2$ activity was extracted by treating cells with PBS and 2 M KCl (0.1 volume of cell culture volume) for 1 h at 4 °C with gentle agitation. Treated cells were spun down, and the supernatant was combined with the $sPLA_2$-containing medium described above. No cell-associated $sPLA_2$ activity was found for mGIIC or the mGIII $sPLA_2$ domain. Purification of mGIIA and mGIID $sPLA_2$s was performed as for hGIID (*10*). None of these last two proteins were found to be glycosylated.

To purify mGIIC, 2 L batches of cell medium were diluted to 4 L with 1% acetic acid and loaded onto 500 mL of Sephadex SPC-25 gel (Amersham Biosciences) which had been equilibrated with 1% acetic acid and poured into a 500 mL glass-sintered funnel. The gel was washed with 1 L of 1% acetic acid and 1 L of 1% acetic acid containing 400 mM ammonium acetate. Bound proteins were eluted with 4 L of 1% acetic acid containing 1.5 M ammonium acetate. Fractions of 500 mL were collected and assayed for $sPLA_2$ activity using the *E. coli* assay (*65*). $sPLA_2$-containing fractions were concentrated to 30 mL by ultrafiltration using an Amicon stirred cell concentrator with a YM-10 membrane, and the buffer was exchanged with 1% acetic acid containing 10% ACN. The resulting fraction was filtered on 0.45 μm hydrophilic PVDF membrane filters (Millipore model SLHVBZ5NZ) and loaded onto a Spherogel TSK SP-5PW column (10 μm, 21 mm × 150 mm, TosoHaas) equilibrated in 1% acetic acid with 200 mM ammonium acetate and 10% ACN. The column was eluted at 8 mL/min using a linear gradient of ammonium acetate (from 0.2 to 2 M, at pH 6.8, over 90 min) in 10% ACN. Fractions containing $sPLA_2$ activity were pooled, lyophilized, resuspended in 10% ACN and 0.1% TFA, and applied to a reverse phase HPLC column (C18, 5 μm, 1 cm × 25 cm, Beckman ultrasphere). Elution was performed at 4 mL/min using a water/ACN gradient in 0.1% TFA (from 10 to 20% over 10 min and from 20 to 40% over 60 min). mGIIC eluted at ~39% ACN and was recovered as a pure protein after lyophilization.

To purify the mGIII $sPLA_2$ domain, 2 L batches of cell medium were diluted with 2 L of water, adjusted to pH 7.0, and loaded at room temperature onto 300 mL of Sepharose fast flow Q-XL gel (Amersham Biosciences) poured into a 500 mL glass-sintered funnel and pre-equilibrated with 20

Table 1: Expression System, Yield, and Molecular Mass of Recombinant Mouse $sPLA_2$s[a]

$sPLA_2$	expression system	yield of production (mg/L)	calcd molecular mass (Da)	measured molecular mass (Da)
mGIB	*Sf*9/baculovirus	30	14 074.82	14 074.79
mGIIA	*E. coli*/pAB_3 fusion protein	1	13 958.19	13 957.71
mGIIC	S2 cells	0.15	14 661.87[b]	14 661.68
mGIID	*E. coli*/pAB_3 fusion protein	2	14 252.41	14 252.53
mGIIE	*E. coli*/pAB_3 fusion protein	0.8	14 066.08	14 065.92
mGIIF	*E. coli*/pAB_3 fusion protein	2	16 925.10 (+Cys)[c]	16 925.19
mGIII	S2 cells	0.07	15 908.86[b]	15 908.38
mGV	*E. coli*/pET21a	20	13 790.82	13 790.40
mGX	*E. coli*/pAB_3 fusion protein	10	13 854.87	13 855.70
mGXIIA	*E. coli*/pAB_3 fusion protein	1	18 724.32	18 724.29
mGXIIB	*E. coli*/pAB_3 fusion protein	2	19 402.04	19 402.93

[a] The proteins were analyzed by MALDI-TOF mass spectrometry to confirm that all the disulfide bonds are formed and that proteins have not been covalently modified during purification. [b] After deglycosylation with *N*-glycosidase F (see Experimental Procedures). [c] The odd cysteine of mGIIF at position 137 makes a disulfide bond with L-cysteine from the refolding buffer.

mM Tris-HCl (pH 8.0). The gel was washed with 1 L of 20 mM Tris-HCl (pH 8.0) and then with 1 L of 20 mM Tris-HCl (pH 8.0) and 0.5 M NaCl. The mGIII protein did not elute under these conditions. Bound proteins were eluted stepwise with 1 L of 1% acetic acid, 1 L of a 1% acetic acid/20% ACN/0.5 M NaCl mixture, and finally with 1 L of 1% acetic acid containing 80% ACN. $sPLA_2$-containing fractions were pooled, evaporated with a rotary evaporator to remove ACN, and then concentrated to 30 mL by ultrafiltration using an Amicon stirred cell concentrator with a YM-10 membrane, and the buffer was exchanged with 20 mM Tris-HCl (pH 8.0) containing 30% ACN. The $sPLA_2$ sample was then loaded onto a Spherogel TSK DEAE-5PW column (10 μm, 7.5 mm $\times$ 75 mm, TosoHaas) equilibrated in 20 mM Tris-HCl (pH 8.0) and 30% ACN. The column was eluted at 1 mL/min using a linear gradient of NaCl (from 0 to 1 M over 70 min) in 20 mM Tris-HCl (pH 8.0) and 30% ACN. Fractions containing $sPLA_2$ activity were pooled, diluted to 20% ACN and 0.1% TFA, and applied to a reverse phase HPLC column (C8 Symmetry Shield, 5 μm, 0.46 cm $\times$ 25 cm, Waters). Elution was performed at 1 mL/min using a water/ACN gradient in 0.1% TFA (from 20 to 30% over 20 min and from 30 to 40% over 100 min). The mGIII $sPLA_2$ domain eluted as a broad peak at ~37% ACN and was recovered as a pure protein after lyophilization.

As previously found for hGIID (*10*), both mGIIC and mGIII $sPLA_2$s were glycosylated. mGIIC $sPLA_2$ was produced as a homogeneously glycosylated protein with a molecular mass of 15 700 Da corresponding to a sugar moiety of 1038 Da (Figure 1). The recombinant mGIII $sPLA_2$ domain was found to be heterogeneously glycosylated (not shown) with measured molecular masses of 17 989.91 (major peak), 18 192.01 (minor peak), and 18 315.74 Da (minor peak). The Δmass between the major peak and the calculated sequence of the mGIII $sPLA_2$ domain suggests that the protein is glycosylated at two sites, each with a sugar moiety of 1038 Da. N-Terminal sequencing and analysis of $sPLA_2$ trypsin digests by MALDI-TOF mass spectrometry showed that the glycosylation occurred only on Asn-72 in mGIIC while it occurs on both Asn-18 and Asn-131 in the mGIII $sPLA_2$ domain (not shown). To determine the molecular mass of the protein moiety (Table 1), deglycosylation experiments were performed by overnight treatment of 3 μg of purified $sPLA_2$ at 20 °C with 0.5 unit of *N*-glycosidase F (Roche catalog no. 1365169) in 40 μL of 20 mM NaH_2PO_4 (pH 6.5) and protease inhibitor cocktail (Roche catalog no. 1836153). MALDI-TOF mass spectrometry analysis was performed after reverse phase, solid phase extraction with a C18 ZipTip (Millipore).

Nonmammalian PLA_2s. Purified recombinant group XIII PLA_2s from human parvovirus B19 (B19), porcine parvovirus (PPV-VP1up), and adeno-associated virus type 2 (AAV2-VP1up) were produced as previously described (*78*). Purified group XIV $sPLA_2$s from *Tuber borchii* (TbSP1) and *Helicosporium* (p15) were kindly provided by S. Ottonello (University of Parma, Parma, Italy) (*7*).

Recombinant Expression of the Soluble Mouse M-Type Receptor and Production of Rabbit Polyclonal Antibodies. A cDNA encoding a soluble secreted mouse M-type receptor (amino acids 1−1395) was generated by PCR using the full-length mouse M-type receptor as a template (GenBank entry NM_008867) and subcloned into the pCineo vector from Promega. The cDNA was entirely sequenced and stably transfected into HEK293 cells (American Type Cell Collection) by the calcium/phosphate procedure. Fifty percent confluent cells were transfected on day 1, trypsinized and replated the next day, and selected for 2−3 weeks with 2 mg/mL (±)-G418. Forty-eight individual resistant colonies were isolated and amplified, and culture medium from confluent cells was assayed for [^{125}I]OS_1 binding. The colonies giving the highest level of [^{125}I]OS_1 binding were assayed for expression of the receptor by Western blotting (*79*), further amplified, and frozen. Selected clones were used for large-scale production in roller bottles. Typically, 2 L roller bottles were seeded with 5×10^7 cells in 200 mL of DMEM supplemented with 10% fetal calf serum and 2 mg/mL G418. Cells were grown to confluence (~5 days); the conditioned medium was collected and replaced with fresh medium, and cells were maintained in culture for an additional 5 days. The collected medium was pooled and processed for purification of the soluble mouse M-type receptor. Batches (2 L) of conditioned medium were filtered under vacuum through a 10 cm bed of Sephadex G-50 poured into a glass-sintered funnel and then pumped onto an OS_2-Affigel-10 affinity column (60 mL), which was prepared as previously described (*51*). The column was washed with 300 mL of 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 2 mM $CaCl_2$, then washed with 300 mL of 20 mM Tris-HCl (pH 7.5), 140 mM NaCl, and 2 mM EDTA, and finally eluted with 50 mM sodium acetate (pH 4.5) with 140 mM NaCl and protease inhibitors (Roche catalog no. 1836153). Fractions (75 mL) were collected in tubes containing 25 mL of

1 M Tris-HCl (pH 8.0), analyzed for [^{125}I]OS_1 binding, pooled, and concentrated to 4 mL by ultrafiltration using an Amicon stirred cell concentrator equipped with a YM-30 membrane. The buffer was exchanged with 100 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA, and the resulting receptor preparation was lyophilized. Approximately 1 mg of purified soluble M-type receptor could be obtained from 2 L of cell supernatant. The receptor preparation was analyzed for protein concentration by OD_{280} using the calculated molar absorption coefficient and for purity with a SDS−PAGE gel. The molecular mass of the receptor was determined by SELDI-TOF (surface-enhanced laser desorption ionization time-of-flight) using the SELDI ProteinChip system (PBS-1, Ciphergen Biosystems, Inc., Fremont, CA). The receptor (5 μg) was loaded on a reverse phase ProteinChip (H4), washed with 10% ACN and 0.1% TFA, and cocrystallized using sinapinic acid as a matrix. For deglycosylation, 20 μg of receptor was treated overnight with *N*-glycosidase F (Roche catalog no. 1365169), *O*-glycosidase (Roche catalog no. 11347101100), and neuraminidase (Roche catalog no. 11729720) in 20 mM Na_2HPO_4 (pH 6.5) in the presence of protease inhibitor cocktail (Roche catalog no. 1836153). Rabbit antiserum against the mouse M-type receptor was obtained by immunization with the purified native receptor as previously done for mouse $sPLA_2$s (*69*).

Receptor Binding Studies and Preparation of Cell Membranes. Crude microsomal membranes from transfected COS cells expressing the mouse or rabbit membrane-bound M-type receptors or from NIH 3T3 cells expressing endogenous membrane-bound mouse receptor were obtained as described previously (*65*). Scatchard plot experiments and competition binding assays were performed under equilibrium conditions using as a ligand [^{125}I]OS_1 labeled to a specific activity of 3500 cpm/fmol as described previously (*51*). Briefly, membrane-bound or soluble M-type receptor, [^{125}I]OS_1, and unlabeled mouse $sPLA_2$ competitors 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 receptor and filtered after 1 h through APFC glass fiber filters (Millipore catalog no. 58585) presoaked in 0.5% polyethyleneimine (for cell membranes) or through APFF glass fiber filters (Millipore catalog no. 15050) presoaked in 5% polyethyleneimine (for the soluble M-type receptor). The $K_{0.5}$ value is defined as the concentration of $sPLA_2$ competitor that inhibits 50% of the specific binding.

Inhibition of Enzymatic Activity by the Recombinant Soluble M-Type Receptor. Mouse $sPLA_2$s were preincubated with various concentrations of purified soluble mouse M-type receptor in 100 μL of $sPLA_2$ activity buffer [0.1 M Tris (pH 8.0), 10 mM $CaCl_2$, and 0.1% bovine serum albumin] for 15 min at room temperature. The residual enzymatic activity was then measured at room temperature for 40 min by addition of 100 000 dpm of [^{3}H]oleate-radiolabeled *E. coli* membranes in 50 μL of $sPLA_2$ activity buffer (*65*). Reactions were stopped by addition of 300 μL of stop buffer [0.1 M EDTA (pH 8.0) and 0.1% fatty acid-free bovine serum albumin]; mixtures were centrifuged at 10000*g* for 5 min, and the supernatants containing released [^{3}H]oleate were counted.

RESULTS

Recombinant Expression and Structural Characterization of the Full Set of Mouse $sPLA_2$s. Over the past two decades, many venom and mammalian $sPLA_2$s have been produced in significant amounts by different methods, including expression in *E. coli* (*10*, *36*, *80*−*83*), mammalian cells (*65*, *66*, *84*), insect cells or larvae (*10*, *65*, *85*), or yeast cells (*86*, *87*) and chemical synthesis (*88*, *89*). The highest expression levels were usually obtained from *E. coli*, provided that efficient in vitro refolding conditions could be found. Here, we have successfully produced the full set of mouse $sPLA_2$s in *E. coli* or insect cells and characterized the final products by mass spectrometry to verify that all proteins are produced as mature, functional, and nonmodified forms. Except for mGXIIB which is catalytically inactive, all the $sPLA_2$s were found to be enzymatically active, and their interfacial enzymatic properties were previously described in detail (*10*). When assayed on *E. coli* membranes, the mGIII $sPLA_2$ domain was found to be as active as mGIIF or mGX (the detailed enzymatic properties of this particular $sPLA_2$ will be published elsewhere).

For most mouse $sPLA_2$s, we could obtain expression from *E. coli* with final yields ranging from 0.8 to 20 mg/L of cell culture (Table 1). Such yields were obtained after optimization of refolding conditions by either dialysis or rapid dilution methods from inclusion body protein (see Experimental Procedures). All but one *E. coli* $sPLA_2$ were produced using the pAB3 vector that encodes the $sPLA_2$ fused to the N-terminal portion of glutathione *S*-transferase with a factor Xa cleavage site preceding the mature $sPLA_2$ protein (*76*). This vector allows strong protein expression even when the authentic cDNA sequence of mouse $sPLA_2$s is directly inserted, while the factor Xa protease site allows efficient cleavage with factor Xa or trypsin which is cost-effective. Since the mature protein sequence of mGV $sPLA_2$ starts with a glycine residue, the protein was expressed as a nonfusion protein. In this case, ~70% of the methionine initiator was removed by the *E. coli* aminopeptidase, and the mature refolded protein without methionine was purified by optimized conditions of HPLC purification (see Experimental Procedures). For mGIIC and mGIII $sPLA_2$s, no refolding conditions could be found, and thus, the recombinant enzymes were obtained from *Drosophila* S2 cells. Although the yield of production in *Drosophila* S2 cells was low for these two $sPLA_2$s (Table 1), yields of 1−2 mg/L were obtained in this system for mGIIA and mGIID $sPLA_2$s (not shown). Finally, mGIB $sPLA_2$ could be produced with a very high yield of 30 mg/L using the *Sf*9/baculovirus expression system (*E. coli* expression was thus not attempted for mGIB), showing that insect cell systems can be efficient for the production of some $sPLA_2$s. One reason for a low expression yield in insect cells could be the $sPLA_2$ toxicity for the host cell.

Our goal was to produce pure and properly folded mature mouse $sPLA_2$s devoid of N-terminal extensions or amino acid mutations. As shown in Figure 1A, the HPLC-purified proteins migrated as single bands of the expected size on a Laemmli SDS−PAGE gel. To establish that the five to eight disulfide bonds of the different mouse $sPLA_2$s were formed, we measured the molecular masses of the recombinant proteins by MALDI-TOF mass spectrometry with external

Table 2: Properties for Binding of the Full Set of Mouse $sPLA_2$s to Mouse and Rabbit M-Type Receptors[a]

	$K_{0.5}$ (nM)				
	membrane-bound recombinant mouse receptor				
$sPLA_2$	$CaCl_2$	EDTA	membrane-bound endogenous mouse receptor	soluble recombinant mouse receptor	membrane-bound recombinant rabbit receptor
venom $sPLA_2$s					
OS_1	0.2	0.2	0.1	0.1	0.08
$bvPLA_2$	>300	>300	>300	>300	>300
mouse $sPLA_2$s					
mGIB	2	0.7	1	1.1	~1000
mGIIA	2.4	1.9	12	3	0.2
mGIIC	82	nd[b]	31	48	5.6
deglycosylated mGIIC	150	nd[b]	nd[b]	nd[b]	4.5
mGIID	>300	>300	>300	>300	15
mGIIE	1	0.58	0.7	1.5	0.3
mGIIF	0.35	0.6	0.25	0.45	0.1
mGIII	~300[c]	~300[c]	~300[c]	~300[c]	4.5
deglycosylated mGIII	≥300	nd[b]	nd[b]	nd[b]	6
mGV	71	74	60	40	1.4
mGX	2.9	2.2	0.9	1.2	0.15
mGXIIA	>300	>300	>300	>300	>300
mGXIIB	>300	>300	>300	>300	>300

[a] The relative affinities ($K_{0.5}$ values) of venom and mouse $sPLA_2$s were measured by competition binding assays between $[^{125}I]OS_1$ and unlabeled $sPLA_2$s for binding to the different forms of the M-type receptor in the presence of 2 mM $CaCl_2$. For the membrane-bound recombinant receptor, the affinities were also determined in the presence of 2 mM EDTA instead of 2 mM $CaCl_2$. $K_{0.5}$ values are representative of at least three independent experiments with standard errors of the mean of <50%. [b] Not determined. [c] The maximal concentration that was assayed was 300 nM. This concentration inhibited 40−60% of the $[^{125}I]OS_1$ binding depending on the receptor preparation.

or internal calibration (see Figure 1B for examples). For all $sPLA_2$s, the measured molecular mass differed from the calculated mass by less than 1 Da, clearly establishing that all the disulfide bonds are formed and also that other residues have not been chemically oxidized or modified in other ways during the refolding and purification procedure. Despite the lower yield of $sPLA_2$ production observed on average in insect cells, *Drosophila* S2 cells allowed the production of $sPLA_2$ proteins in glycosylated forms. In silico scanning of all mouse $sPLA_2$s using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/), which accurately predicts protein glycosylation, indicated that only mGIIC, mGIIF, and mGIII $sPLA_2$s may be N-glycosylated, while none of the $sPLA_2$s were predicted to be O-glycosylated (not shown). Structural analyses of recombinant mGIIC and mGIII $sPLA_2$s produced in *Drosophila* S2 cells by mass spectrometry and N-terminal sequencing indicated that mGIIC is indeed glycosylated at Asn-72, whereas the mGIII $sPLA_2$ domain is glycosylated at both Asn-18 and Asn-131. The major glycan chain (~1038 Da) found in mGIIC $sPLA_2$ (Figure 1) and mGIII $sPLA_2$ domain (not shown) is likely to consist of six sugar residues. The same glycan motif was previously found in other $sPLA_2$s endogenously or exogenously expressed in insect cells, including $bvPLA_2$ and hGIID $sPLA_2$ (*10*, *90*). Because a putative N-glycosylation site was previously found on Asn-80 (*76*), we also expressed mGIID in *Drosophila* S2 cells and found that this $sPLA_2$ is not glycosylated (not shown). mGIIF $sPLA_2$ was predicted to be glycosylated on Asn-72, but this protein was not expressed in sufficient amount from *Drosophila* S2 cells to determine if this site is indeed glycosylated. When refolded from *E. coli*, mature full-length mGIIF was predominantly found as a monomeric protein in which the odd cysteine in the unique C-terminal extension forms a disulfide bond with free L-cysteine from the refolding buffer (Table 1).

Properties for Binding of the Full Set of Mouse $sPLA_2$s to the Mouse M-Type Receptor by Competition Assay with Iodinated OS_1. The properties for binding of the full set of mouse $sPLA_2$s to the mouse M-type receptor were analyzed by competition binding assays between the mouse $sPLA_2$s and iodinated OS_1, the very high-affinity and specific snake venom $sPLA_2$ ligand initially used to identify the M-type receptor in various species (*51*, *65*). The affinities were determined in parallel on the cloned membrane-bound receptor, the endogenous membrane-bound receptor, and a recombinant soluble secreted receptor produced in HEK293 cells (see below). On all receptor preparations, OS_1 binds with a very high affinity between 0.08 and 0.2 nM (Table 2). The specificity of OS_1 binding was confirmed by the absence of competition by $bvPLA_2$ (Table 2), which does not bind to different M-type receptor species (*65*). Of the 11 $sPLA_2$s that were assayed, mGIB, mGIIA, mGIIE, mGIIF, and mGX were revealed as high-affinity ligands ($K_{0.5}$ values of 0.3−12 nM), while mGIIC and mGV appeared to be moderate-affinity ligands ($K_{0.5}$ values of 31−74 nM). mGIII exhibited partial inhibition at the highest concentration that was assayed (300 nM), and mGIID, mGXIIA, and mGXIIB were unable to bind to the different mouse receptor preparations ($K_{0.5}$ values of >300 nM). Similar binding profiles were obtained for the different mouse receptors (Figure 2 and Table 2). Because all $sPLA_2$s bind Ca^{2+} and because the M-type receptor belongs to the C-type (Ca^{2+}-dependent) lectin superfamily, it was of interest to analyze the effect of Ca^{2+} on the affinities of the mouse $sPLA_2$s for the M-type receptor. The binding profile was similar with and without free Ca^{2+}, yet slightly higher affinities were observed in the absence of Ca^{2+} for some $sPLA_2$s. Deglycosylation of mGIIC and mGIII moderately lowers their level of binding to the mouse receptor, suggesting that the sugar moiety may contribute a little in the $sPLA_2$−receptor interaction. Together, these results indicate that the mouse M-type receptor is selective for some mouse $sPLA_2$s of the I/II/V/X structural collection (*3*).

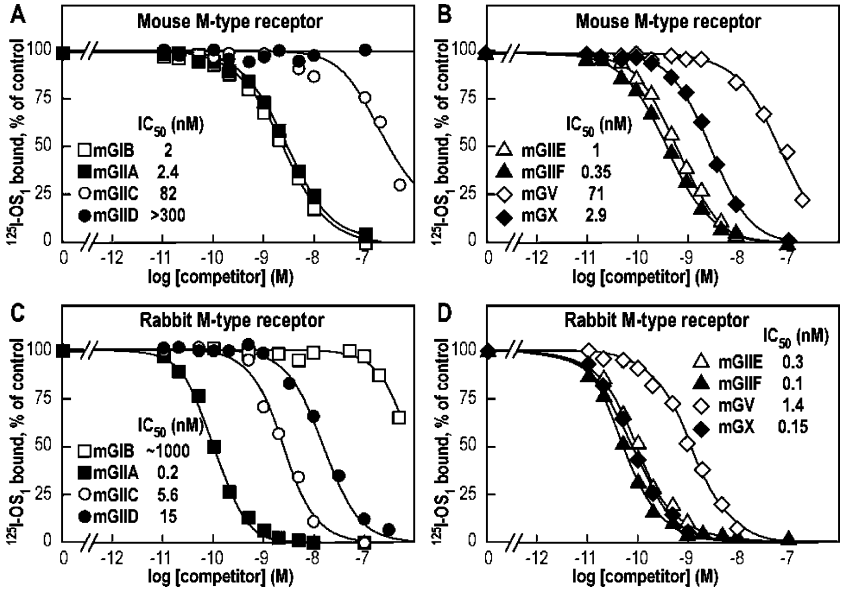

FIGURE 2: Properties for binding of mouse $sPLA_2$s from the I/II/V/X structural collection to the cloned mouse and rabbit membrane-bound M-type receptors. Competition binding experiments between [^{125}I]OS_1 and unlabeled recombinant mouse $sPLA_2$s for binding to the mouse (A and B) and rabbit (C and D) M-type receptor. Microsomal membranes expressing the mouse M-type receptor (50 μg of total protein/mL) or the rabbit M-type receptor (10 μg of total protein/mL) were incubated with [^{125}I]OS_1 and various concentrations of unlabeled $sPLA_2$s. Results are expressed as the percentage of [^{125}I]OS_1 specific binding measured in the absence of competitor. The membrane concentration was adjusted to obtain a specific binding of 10−20% of the total radiolabeled ligand added (60 pM). The level of nonspecific binding was measured in the presence of 100 nM unlabeled OS_1 and was below 10% of the total level of binding.

Properties for Binding of the Full Set of Mouse $sPLA_2$s to the Rabbit M-Type Receptor. Because of the particular binding characteristics of the rabbit M-type receptor (*65*), it was of interest to analyze and compare the binding properties of the different mouse $sPLA_2$s on this receptor. As expected, the binding profile on the rabbit M-type receptor was quite different from that of the mouse receptor, and the affinities were in general higher on the rabbit receptor (Table 2 and Figure 2). Indeed, the rabbit receptor was found to bind all but one mouse $sPLA_2$ that belongs to the I/II/V/X structural collection with high affinities between 0.1 and 5.6 nM. Only mGIB was found to be a low-affinity ligand. On the other hand, like for the mouse receptor, both mGXIIA and mGXIIB were unable to bind to the rabbit receptor. Interestingly, mGIII was found to bind to the rabbit receptor with a fairly high affinity, and this binding was independent of the presence of the glycan motifs since deglycosylated mGIII still binds to the receptor. The binding of mGIIC to the rabbit receptor also did not depend on the glycan motif of mGIIC since both glycosylated and deglycosylated mGIIC could bind to the receptor. Importantly, the fact that mGIIC, mGIID, mGIII, and mGV do bind to the rabbit receptor with high affinities indicates that their poor binding or their absence of binding to the mouse receptor is not due to unproper folding.

Properties for Binding of Mouse $sPLA_2$s to the Mouse M-Type Receptor Measured by Inhibition of Enzymatic Activity. Previous studies have shown that binding of $sPLA_2$ to the M-type receptor leads to inhibition of enzymatic activity (*29*, *64*) and that a soluble secreted form of the receptor can be produced by alternative splicing or shedding from the membrane-bound receptor (*29*, *64*). To further establish which mouse $sPLA_2$s bind to the mouse M-type receptor and to determine if $sPLA_2$ binding leads to inhibition of enzymatic activity for all enzymes, we produced and purified a recombinant soluble secreted mouse M-type

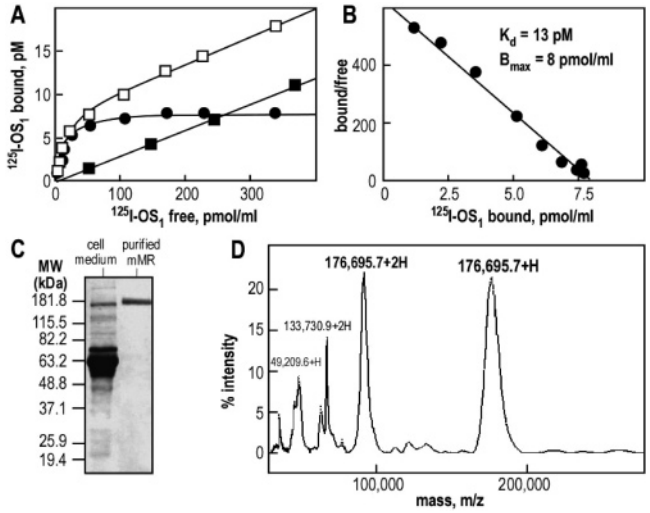

FIGURE 3: Production of a recombinant soluble mouse M-type receptor in HEK293 cells. (A and B) Saturation curves and Scatchard plot analysis of binding of [^{125}I]OS_1 to 7-day-conditioned medium of a HEK293 cell clone stably expressing the soluble mouse M-type receptor. Cell medium (10 μL) was incubated with various concentrations of [^{125}I]OS_1 in the absence (□) or presence (■) of 100 nM unlabeled OS_1. The specific binding (●) which represents the difference between total (□) and nonspecific binding (■) is shown. (C) SDS−PAGE analysis of the purified receptor (1 μg) on a 6.5% gel. (D) Molecular mass of the purified receptor determined by SELDI-TOF mass spectrometry analysis.

receptor comprising the full-length extracellular region of the receptor and matching the structure of the soluble receptor previously identified in humans (*64*). A subclone of HEK293 cells stably transfected with the mouse M-type receptor cDNA construct (see Experimental Procedures) secretes large amounts of a functional soluble M-type receptor with an apparent molecular mass of ~180 kDa (Figure 3). The secreted receptor has a very high affinity of 13 pM for iodinated OS_1 and is present in cell medium at a concentra-

Table 3: Inhibition of $sPLA_2$ Enzymatic Activity by the Soluble Recombinant Mouse M-Type Receptor[a]

$sPLA_2$	[$sPLA_2$] (nM)	IC_{50} (nM)
venom $sPLA_2$s		
OS_1	20	9
$bvPLA_2$	0.002	>200
mouse $sPLA_2$s		
mGIB	0.05	10
mGIIA	3	30
mGIIC	20	70
mGIID	30	>300
mGIIE	10	30
mGIIF	1	2
mGIII	1	~200
mGV	1	~150
mGX	1	2

[a] The final concentration of $sPLA_2$ used in enzymatic assays is given. The IC_{50} value is the concentration of the purified soluble recombinant mouse M-type receptor that inhibits 50% of enzymatic activity. Results are representative of at least three experiments with standard errors of the mean of <60%.

tion as high as 8 pmol/mL, corresponding to ~1.4 μg of receptor/mL (Figure 3A,B). Competition binding experiments with this receptor showed that the binding properties of the soluble receptor for all mouse $sPLA_2$s are similar to those of the membrane-bound receptor (Table 2). After a single purification step on an affinity column with snake venom $sPLA_2$ OS_2, the receptor migrates as a single protein on a SDS−PAGE gel (Figure 3C), and SELDI-TOF mass spectrometry showed that the receptor is pure and has a molecular mass of ~177 kDa (Figure 3D).

We next analyzed the inhibitory effects of the recombinant purified receptor on the enzymatic activity of the different mouse $sPLA_2$s (Table 3 and Figure 4). Because of their very low $sPLA_2$ enzymatic activity or its absence (*5*), mGXIIA and mGXIIB could not be analyzed. For the other $sPLA_2$s, the inhibition was evaluated using the lowest enzyme concentrations that give a significant level of enzymatic activity. Table 3 shows that these concentrations vary importantly between mouse $sPLA_2$s and lead for mGIIE, mGIIF, and mGX to pseudostoichiometric conditions that are expected to give shifted affinity values, as compared to those measured by competition binding assays (Table 2). When these particular assay conditions are taken into account, a fairly good relationship could be observed between the enzymatic activity inhibition profile and the binding affinity for the mouse receptor, indicating that all $sPLA_2$s that are capable of high-affinity binding to the soluble receptor (Table 2 and Figure 2) are inhibited at relatively low concentrations of the soluble receptor (Table 3 and Figure 4). As expected, the enzymatic activity of OS_1, but not $bvPLA_2$, was also inhibited.

Glycosylation of the Soluble M-Type Receptor Is Important for $sPLA_2$ Binding. Deglycosylation of the purified mouse soluble receptor with a mixture of *N*-glycosidase F, *O*-glycosidase, and neuraminidase leads to a receptor with an apparent molecular mass on a SDS−PAGE gel of 148 kDa (Figure 5A), which is close to the calculated molecular mass of the protein deduced from the cDNA sequence (156 752 Da), indicating that the receptor has been fully deglycosylated. When the receptor was analyzed for its inhibitory effect on the enzymatic activity of OS_1, mGIB, and mGIIF $sPLA_2$s, which all bind to the mouse receptor with high affinities, a complete loss of inhibition (OS_1) or an 8−17-fold lower affinity (for mGIB and mGIIF) was measured. Together, these results indicate that glycosylation of the mouse receptor contributes to different extents to the interaction with $sPLA_2$s.

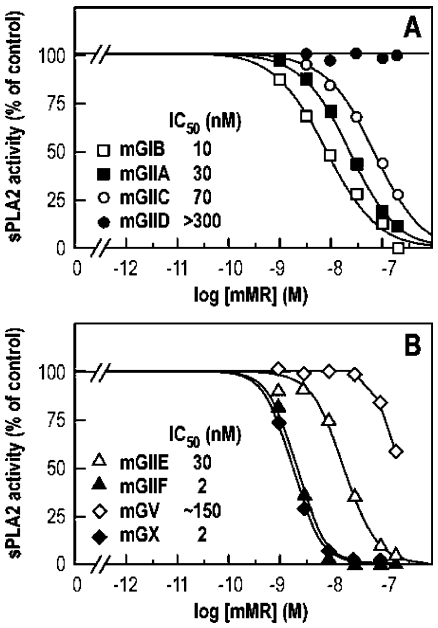

FIGURE 4: Inhibition of enzymatic activity of mouse $sPLA_2$s by the soluble recombinant mouse M-type receptor. The different mouse $sPLA_2$s at concentrations as indicated in Table 3 were preincubated for 15 min with various concentrations of soluble receptor, after which labeled *E. coli* membranes were added to measure $sPLA_2$ activity. One hundred percent $sPLA_2$ activity corresponded to the activity measured in the absence of a receptor for each $sPLA_2$. Zero percent represents the nonspecific hydrolysis of labeled phospholipids measured without $sPLA_2$ which was less than 5% of total radioactivity.

The Mouse M-Type Receptor Does Not Bind Exogenous Viral Group XIII and Fungal XIV PLA_2s. We finally determined whether the mouse membrane-bound receptor may bind the structurally distinct and exogenous group XIII and group XIV PLA_2s from parvovirus and fungi. As indicated in Table 4, PLA_2 proteins from three different parvoviruses and two species of fungus could not compete with iodinated OS_1 for binding to the receptor, further showing that the M-type receptor is specific for only a subset of $sPLA_2$s from the group I/II/V/X collection.

DISCUSSION

The major current challenge in the $sPLA_2$ field is elucidation of the biological functions and mechanisms of action of each of the 11 human or 12 mouse $sPLA_2$ proteins (*1*, *3*, *5*, *12*, *25*). A great deal of knowledge about the structure, enzymatic properties, and in vitro cellular actions of the different $sPLA_2$s, more especially on the "old $sPLA_2$ members" which include group IB, IIA, V, and X $sPLA_2$s, has accumulated. Their biochemical properties and their specific tissue distribution have been used to tentatively infer some of their physiological and/or pathophysiological roles, which have been mostly based on their enzymatic capability. Additionally, many $sPLA_2$ inhibitors have been generated against some of the old $sPLA_2$ members and used to probe their in vivo functions (especially inflammation). However, the specificity of these inhibitors with regard to the different $sPLA_2$ members is questionable and needs to be reevaluated

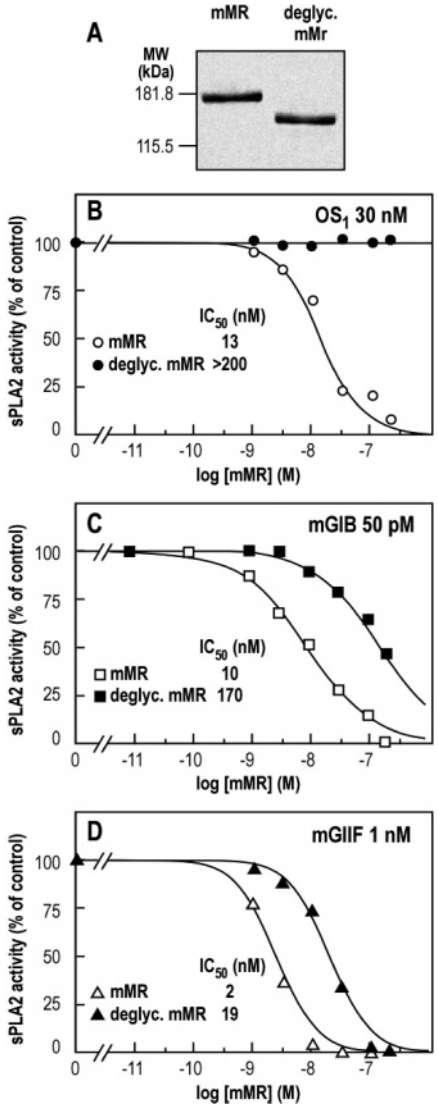

FIGURE 5: Inhibition of enzymatic activity of OS_1, mGIB, and mGIIF by the soluble recombinant mouse M-type receptor after deglycosylation. (A) The soluble recombinant mouse receptor was deglycosylated with a cocktail of *N*-glycosidase F, *O*-glycosidase, and neuraminidase and analyzed on a 6.5% SDS−PAGE gel. The gel was Coomassie-stained. More than 99% of the receptor appears to be deglycosylated. (B−D) Inhibition profiles of the different $sPLA_2$s by the glycosylated (mMR) or deglycosylated receptor. Conditions for assays were as indicated in Figure 4.

Table 4: Viral and Fungal PLA_2s Do Not Bind to the Cloned Membrane-Bound Mouse M-Type Receptor

PLA_2	concentration (nM)	[^{125}I]OS_1 specifically bound (% of control)
adeno-associated virus type 2 PLA_2 (Aav2)	100	101
human parvovirus B19 PLA_2 (B19)	100	86
	500	93
porcine parvovirus PLA_2 (PPV)	100	94
	500	98
T. borchii $sPLA_2$ (TbSP1)	100	80
	1000	74
Helicosporium $sPLA_2$ (p15)	100	99
	500	100

more systematically (*10*, *91*). Furthermore, despite the identification of null mice for mGIIA a decade ago (*92*), the biological role of this $sPLA_2$ remains puzzling, and its functional relevance with regard to the expected human or rat group IIA orthologs, which in these species correspond to genuine "inflammatory-type" $sPLA_2$, is largely questionable (*65*, *76*). Finally, it has only been recently that null mice for mGIB and mGV have been used to unravel in vivo biological roles of $sPLA_2$ (*18*, *24*, *93*).

At the molecular level, it is still largely unclear whether mammalian $sPLA_2$s exert their biological roles as enzymes for different phospholipid substrates, ligands by mechanisms involving binding to proteins, or both (*3*, *25*, *29*, *32*). Among $sPLA_2$s, it is now fairly clear that group IIA, V, and X $sPLA_2$s can function as enzymes in diverse cellular settings to participate in lipid mediator release in a $cPLA_2$-dependent or $cPLA_2$-independent manner (*4*, *10*, *12*, *13*, *24*). However, the weak enzymatic activity or its absence in some "new $sPLA_2$ members", including IID, IIE, XIIA, and XIIB $sPLA_2$s (*5*, *10*), supports nonenzymatic biological functions for these "$sPLA_2$-like" proteins (*33*). Many structure−function studies on the old $sPLA_2$ members also suggest that some of their biological actions are independent of enzymatic activity (*26*−*31*, *49*, *55*, *79*, *94*, *95*).

Among $sPLA_2$ binding proteins, the best known target is the M-type receptor (*32*). Despite the large amount of knowledge at the molecular level that has accumulated (*29*, *32*) and the generation of null mice (*58*), the biological role of the M-type receptor with regard to $sPLA_2$ function remains obscure. In previous studies, group IB, IIA, and X $sPLA_2$s have been identified as natural ligands of the receptor, but the pharmacological profile appears to be highly dependent on the receptor species (*29*, *64*, *65*, *96*). These previous studies have also shown that proenzymes for IB and X $sPLA_2$s do not bind to the M-type receptor (*29*, *96*, *97*).

In the mouse species, group IB, IIA, and X $sPLA_2$s were shown to bind with nanomolar affinities to the M-type receptor (*65*, *96*), and we now provide the binding data for all $sPLA_2$ members. Analysis of the full set of mouse $sPLA_2$s shows that up to seven $sPLA_2$s can bind to the mouse M-type receptor with affinities in the nanomolar range and thus can be considered as natural ligands of this receptor. The order of potency is as follows: mGIIF > mGIB, mGIIE, and mGX > mGIIA > mGIIC and mGV. On the other hand, mGIID, mGXIIA, and mGXIIB were unable to bind to the receptor, and mGIII exhibited poor binding. Our findings raise a number of questions regarding the structure−function relationship between the $sPLA_2$ and the M-type receptor, especially about how they interact molecularly and what could be the functional impact of this $sPLA_2$−receptor interaction, on both $sPLA_2$ biological functions and M-type receptor biological functions.

Molecular Features of the $sPLA_2$−M-Type Receptor Interaction. On the $sPLA_2$ side, previous studies have shown that residues of the interfacial binding site and Ca^{2+} loop of group IB $sPLA_2$ are critical elements for the interaction with the M-type receptor (*97*). Because the Ca^{2+} loop of mouse $sPLA_2$s from the group I/II/V/X collection is highly conserved (*98*), it is likely that the different binding properties of mouse $sPLA_2$s are due to amino acid differences in the interfacial binding surface. Detailed structure−function studies will help to determine which residues of each $sPLA_2$ are critical for the interaction with the M-type receptor. For example, it would be interesting to know why the closely

related mGIIA, mGIID, and mGIIE have different binding properties, and why mGIID does not bind to the mouse receptor but does bind to the rabbit receptor. Although mGIII $sPLA_2$ does not bind well to the mouse M-type receptor, we observed that this $sPLA_2$ has a high affinity for the rabbit M-type receptor. This result was particularly surprising since the structurally homologous $bvPLA_2$ does not bind to the rabbit M-type receptor (*51*). Importantly, this result indicates that some M-type receptor species can accommodate in their $sPLA_2$ binding site both group I/II/V/X $sPLA_2$s and group III $sPLA_2$s. However, the $sPLA_2$ binding site appears to be selective for only a subset of $sPLA_2$s from the two different structural groups. Interestingly, some $sPLA_2$ inhibitors from snake blood are also able to bind $sPLA_2$s from group I/II/V/X and group III structural collections (*50*, *99*). Since the M-type receptor has lectin-like properties (*65*) and since some $sPLA_2$s are glycosylated (refs *10* and *66* and this work), we found it was interesting to analyze whether $sPLA_2$s can be glycosylated in certain recombinant expression systems and, if so, how glycosylation may affect the interaction with the receptor. It was previously found that mouse group IB, IIA, and X $sPLA_2$s are not glycosylated when produced in insect or mammalian cells, and thus, their high-affinity interaction with the receptor does not require glycosylation (*65*, *66*). Here, we found that mGIIE, mGIIF, and mGV, which were produced in *E. coli* as nonglycosylated proteins, are also high-affinity ligands for the receptor, indicating that glycosylation is dispensable. It should be noted that of these three latter $sPLA_2$s, only mGIIF is likely to be glycosylated when produced by mammalian cells (F. Surrel et al., unpublished data). Finally, although mGIIC and mGIII $sPLA_2$s are glycosylated, their interaction with the receptor was also independent of their sugar moiety. Together, these results suggest that $sPLA_2$ glycosylation does not play a major role in the $sPLA_2$−receptor interaction.

On the receptor side, previous studies have suggested that the receptor binding domain for the venom $sPLA_2$s OS_1 and OS_2 or the mammalian group IB consists of C-type lectin-like carbohydrate recognition domains 3−5 (CTLDs 3−5) for the bovine receptor or CTLDs 4−6 for the rabbit receptor (*29*, *32*). This binding site is likely to be the same for all $sPLA_2$s currently known to bind to the receptor and including those identified in this study, since their binding properties were all measured by competition assays with iodinated OS_1. All CTLDs 3−6 are predicted to be N-glycosylated (*29*, *32*), and treatment of the receptor with *N*-glycosidase F was reported to abolish the binding of porcine group IB $sPLA_2$ (*100*). Our results using the recombinant mouse soluble receptor indicate that deglycosylation of the receptor differentially affects the $sPLA_2$ interaction and leads to either a decreased level of interaction or abolished interaction, suggesting that N-linked carbohydrates of the receptor participate in part in $sPLA_2$ binding. The situation appears different for the rabbit M-type receptor. Indeed, we could produce a soluble form of the rabbit receptor comprising CTLDs 4−6 in baculovirus insect cells, and deglycosylation of the purified truncated soluble receptor only marginally reduced the level of binding of radiolabeled OS_1 as measured by a binding overlay (data not shown). It is not yet clear whether N-linked carbohydrates of the full-length soluble mouse M-type receptor directly participate to the interaction or if deglycosylation of the receptor induces a large conformational change in the receptor that would affect its affinity for the $sPLA_2$. In support of this hypothesis, several models of three-dimensional conformation and large conformational changes induced by pH have been proposed for the mannose receptor, for Endo180, and for FcRY, the overall structural organization of which is similar to that of the M-type receptor (*101*−*103*). Interestingly, a recent study on the structurally related $sPLA_2$ inhibitor PLIα from snake blood which consists of three CTLDs indicates that the $sPLA_2$ binding site is located at the helical neck region connecting the CTLDs (*50*). Cocrystallization of the $sPLA_2$−receptor complex is required to definitely address where and how the $sPLA_2$ interacts with the M-type receptor.

Finally, the fact that the M-type receptor belongs to the superfamily of C-type lectins, has a highly complex extracellular domain, and is similar to the mannose receptor which is known to interact with several types of ligands and to participate in innate immunity (*53*, *54*) suggests that the M-type receptor may have multiple ligands, including other types of endogenous or exogenous PLA_2s from different pathogens. Our current data indicate that the mouse receptor does not bind three different types of group XIII PLA_2s from parvoviruses and two different types of group XIV $sPLA_2$s from filamentous fungi (Table 4). It would be interesting to test whether the acidic low-molecular mass Ca^{2+}-independent lysosomal PLA_2 can bind to the M-type receptor. Indeed, this particular PLA_2 has recently been found to bind to pulmonary surfactant protein A which is homologous to the M-type receptor and belongs to the C-type lectin family (*104*). On the other hand, it also remains to be determined if some mouse $sPLA_2$s can bind to the structural homologues of the M-type receptor, i.e., the mannose receptor, Endo180, and DEC-205 (*53*, *54*). An interaction of several mouse $sPLA_2$s with mouse surfactant protein A has already been demonstrated (*75*).

Functional Implications of the $sPLA_2$−M-Type Receptor Interaction. A survey of the tissue distribution of the mouse M-type receptor and of the different mouse $sPLA_2$s able to bind to the receptor found in the litterature (*65*, *98*, *105*) or based on the EST expression profile found at UniGene (http://www.ncbi.nlm.nih.gov.gate1.inist.fr/UniGene/) clearly indicates that one or several $sPLA_2$s are colocalized with the receptor in many different tissues. This suggests that interaction between the M-type receptor and one or several mouse $sPLA_2$s is very likely to occur in vivo in different physiological or physiopathological settings. On the basis of EST counts and northern blot analysis, high expression levels of the receptor were found in bladder, mouth, eye, female genital organs, liver, ear, spleen, thymus, kidney, lung, and gastrointestinal tract, including small intestine and colon. In one or several of these tissues, a significant expression of mGIB, mGIIA, mGIIC, mGIIE, mGIIF, mGV, and mGX $sPLA_2$s was found.

On the basis of our current results and previous data (*32*, *106*) showing that binding of $sPLA_2$ to the receptor leads for all $sPLA_2$s to inhibition of enzymatic activity, one role of the M-type receptor, in particular of the soluble secreted form, would be to control the level of circulating $sPLA_2$ enzymatic activity in extracellular fluids, including blood (*32*, *106*). This idea fits well with the fact that except for mGIIE, all the $sPLA_2$s that bind to the M-type receptor have high specific activities on several types of phospholipids (*10*). This

view also fits well with the presence of snake blood $sPLA_2$ inhibitors that inhibit the enzymatic activity of venom $sPLA_2$s from the same species. Interestingly, such inhibitors are also present in the blood of nonvenomous snakes like pythons, suggesting that these inhibitors may be present also for the control of the biological functions of endogenous snake $sPLA_2$s distinct from those present in venom glands (*107*). Even more interesting is the fact that some snake blood $sPLA_2$ inhibitors like BaMIP can bind not only catalytically active Asp-49 venom $sPLA_2$s but also catalytically inactive Lys-49 venom $sPLA_2$s and thereby prevent the myotoxic activity of these Lys-49 variants (*99*). This result suggests that binding of mouse $sPLA_2$s to the M-type receptor may serve not only to block their enzymatic functions but also to block their nonenzymatic functions. This may be particularly true for mGIIE which has a very low catalytic activity and binds tightly to the receptor. Binding of mouse $sPLA_2$s to the membrane-bound receptor would not only block enzymatic activity but also serve to internalize and degrade $sPLA_2$s by translocation to lysosomes (*59*, *60*). However, it has been shown that mGX $sPLA_2$ releases similar amounts of arachidonic acid from spleen cells expressing the membrane-bound M-type receptor or not expressing it (*66*). Alternatively, the internalization of $sPLA_2$s by the M-type receptor may also lead to translocation of the $sPLA_2$ to the nucleus (*108*) or other compartments, including phagosomes (*93*).

Another possibility would be that the interaction of the different mouse $sPLA_2$s with the M-type receptor triggers cellular signaling events that contribute to $sPLA_2$ biological effects. The M-type receptor has been proposed to be involved in many in vitro effects of group IB $sPLA_2$ (*29*, *57*, *94*), but the demonstration of its true contribution still awaits more conclusive evidence, including the use of cells from receptor null mice or the use of antagonists of the receptor. Nothing is known about the signaling events and immediate protein partners that are triggered by receptor activation, but the use of all mouse $sPLA_2$s able to bind to the receptor plus the fact that they have dramatically different enzymatic activities may help to elucidate the enigmatic biological functions of this receptor. The most convincing evidence for a signaling role of the M-type receptor was obtained using null mice for the receptor, revealing that the receptor would play a role in the production of proinflammatory cytokines during the progression of endotoxic shock induced by lipopolysaccharides (*58*). Although mGIB $sPLA_2$ was proposed to be involved in this mechanism, our results suggest that this $sPLA_2$ and/or several other mouse $sPLA_2$s that bind to the receptor may play a role.

In summary, we have provided novel data that indicate that in the mouse species, up to seven $sPLA_2$s from the I/II/V/X structural collection are endogenous ligands of the M-type receptor. We already know that this situation will not be similar in humans because group IB and IIA $sPLA_2$s do not bind to the cloned recombinant human M-type receptor (*64*).

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