Both Group IB and Group IIA Secreted Phospholipases A\(_2\) Are Natural Ligands of the Mouse 180-kDa M-type Receptor*

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Snake venom and mammalian secreted phospholipases A\(_2\) (sPLA\(_2\))s have been associated with toxic (neurotoxicity, myotoxicity, etc.), pathological (inflammation, cancer, etc.), and physiological (proliferation, contraction, secretion, etc.) processes. Specific membrane receptors (M and N types) for sPLA\(_2\)s have been initially identified with snake venom sPLA\(_2\)s as ligands, and the M-type 180-kDa receptor was cloned from different animal species. This paper addresses the problem of the endogenous ligands of the M-type receptor. Recombinant group IB and group IIA sPLA\(_2\)s from human and mouse species have been prepared and analyzed for their binding properties to M-type receptors from different animal species. Both mouse group IB and group IIA sPLA\(_2\)s are high affinity ligands (in the 1–10 nM range) for the mouse M-type receptor. These two sPLA\(_2\)s are expressed in the mouse tissues where the M-type receptor is also expressed, making it likely that both types of sPLA\(_2\)s are physiological ligands of the mouse M-type receptor. This conclusion does not hold for human group IB and IIA sPLA\(_2\)s and the cloned human M-type receptor. The two mouse sPLA\(_2\)s have relatively high affinities for the mouse M-type receptor, but they can have much lower affinities for receptors from other animal species, indicating that species specificity exists for sPLA\(_2\) binding to M-type receptors. Caution should thus be exerted in avoiding mixing sPLA\(_2\)s, cells, or tissues from different animal species in studies of the biological roles of mammalian sPLA\(_2\)s associated with an action through their membrane receptors.

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Secreted phospholipases A\(_2\) (PLA\(_2\))s,\(^1\) phosphatidate 2 acylhydrolase, EC 3.1.1.4) form a growing family of Ca\(^{2+}\)-dependent enzymes that release free fatty acids and lysophospholipids from glycerophospholipids (1–4). To date, five different sPLA\(_2\)s referred to as group IB, IIA, IIC, V, and X sPLA\(_2\)s have been characterized in mammals. The main common properties of these sPLA\(_2\)s are their relatively low molecular mass (13–16 kDa), the presence of many disulfide bridges in their structure, and a low selectivity for phospholipids with different polar head groups and fatty acid chains (5).

Group IB sPLA\(_2\) is known as the pancreatic-type sPLA\(_2\). It was originally found in large amounts in the pancreas and then proposed to function in the digestion of dietary lipids (6). Later, this enzyme was identified and cloned in other tissues such as lung, spleen, kidney, and ovary (3, 7), and it has now been proposed to be involved in various physiological and pathophysiological responses such as cell proliferation (8), cell contraction (9, 10), lipid mediator release (11), acute lung injury (12), and endotoxic shock (13).

Group IIA sPLA\(_2\) is also referred to as the inflammatory-type sPLA\(_2\), since it is highly expressed in the plasma and synovial fluids of patients with various inflammatory diseases such as rheumatoid arthritis, acute pancreatitis, Crohn's disease, and endotoxic shock (3, 14–16) as well as in various cancers (17, 18). The group IIA sPLA\(_2\) has been shown to participate in the production of lipid mediators of inflammation (3, 19, 20) and in the destruction of pathogenic microorganisms (21). Recent data using group IIA sPLA\(_2\)-deficient mice have suggested, however, that this sPLA\(_2\) may not play a pivotal role in the progression and/or pathogenesis of inflammatory processes, at least in the mouse (22, 23). The mouse group IIA sPLA\(_2\) (mGIIA)\(^2\) has also been proposed to have a role in cell proliferation (24) and more recently to act as a tumor suppressor gene in a mouse model of colorectal cancer (25, 26).

Much less is known about the regulation and the biological roles of the more recently cloned group IIC, V, and X sPLA\(_2\)s. Group IIC sPLA\(_2\) has been cloned in rat and mouse (27) but appears to be a nonfunctional pseudogene in humans (28). Group V sPLA\(_2\) is highly expressed in heart (29) and is detected in murine macrophages and mastocytes, where it is proposed to play an important role in lipid mediator production in place of the group II sPLA\(_2\) (22, 23). Group X sPLA\(_2\) was recently cloned in human and has structural features that resemble those of group IB and group IIA sPLA\(_2\)s (30). It is expressed in the immune system, suggesting possible roles related to inflammation or immunity.

Snake and insect venoms also contain a large diversity of sPLA\(_2\)s (31, 32). Most venom sPLA\(_2\)s are potent toxins that exert many effects including neurotoxicity and myotoxicity (31,

\(^1\) A comprehensive abbreviation system for the various mammalian sPLA\(_2\)s is used. Each sPLA\(_2\) is abbreviated with a first lowercase letter indicating the sPLA\(_2\) species (b, h, n, p, r, and rb representing bovine, human, mouse, porcine, rat, and rabbit species, respectively) that is followed by capital letters identifying the sPLA\(_2\) group (GIB, GIHA, GIIC, GV, and GX representing group IB, IIA, IIC, V, and X sPLA\(_2\), respectively).

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The nucleotide sequence(s) reported in this paper has (have) been submitted to the GenBank\(^{TM}\) EBIf Data Bank with accession number(s) AF097637.

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\(^{a}\) The abbreviations used are: sPLA\(_2\), secreted phospholipase A\(_2\); O. scutellatus sPLA\(_2\)-1; OS, O. scutellatus sPLA\(_2\)-2; bvPLA\(_2\), bee venom sPLA\(_2\); BSA, bovine serum albumin; CRD, carbohydrate recognition domain; HPLC, high performance liquid chromatography; LPS, lipopolysaccharide.
Two main types of high affinity sPLA₂ receptors have been identified using venom sPLA₂, including OS₂ and OS₃, purified from Taipan snake venom (33). N-type sPLA₂ receptors were first identified in rat brain membranes (34). These receptors have high affinities for neurotoxic sPLA₂ such as OS₂ and the bee venom sPLA₂ (bvPLA₂) but not for nontoxic sPLA₂ such as OS₁, suggesting that N-type receptors contribute to the neurotoxic effects (34–36). M-type sPLA₂ receptors were first identified in skeletal muscle cells (37) but are also expressed in other tissues (33). They consist of a single 180-kDa subunit and recognize with high affinity OS₁ and OS₂ but not the neurotoxic bvPLA₂. The M-type receptor was later also identified by using as ligand the group IB sPLA₂, and was then proposed to have an essential role in the various biological effects produced by the group IB sPLA₂ (8, 38). The M-type receptor has now been cloned in different species (39–42) and found to belong to a novel family of membrane receptors comprising the macrophage mannose receptor, the dendritic cell receptor, and the endothelial lectin receptor (43, 44). The protein domains involved in the sPLA₂-receptor binding have now been elucidated (38, 45, 46). Knock-out mice for the M-type receptor have now been generated (13). The real endogenous ligands of the M-type receptor, however, remain elusive. Available data on the binding properties of mammalian sPLA₂ to the M-type receptor appear contradictory, depending on the animal origin of both the sPLA₂ and the M-type receptor. For instance, rat and bovine M-type receptors can bind both porcine (pGIB) or rat (rGIB) group IB sPLA₂ with a high affinity (KD values of ~1 nM) but do not associate with rat (rGIIA) or rabbit (rbGIIA) group IIA sPLA₂, suggesting that group IB sPLA₂, but not group IIA sPLA₂, are the physiological ligands of the M-type receptor (38). On the contrary, the rabbit M-type receptor associates with high affinity with both pGIB and human group IIA sPLA₂ (hGIIA) with KD values of 1–10 nM, suggesting that both types of sPLA₂ may be the natural ligands of the M-type receptor (39). Finally, the cloned human M-type receptor binds with very weak affinities group IB and group IIA sPLA₂, suggesting that neither of these two sPLA₂ is physiological ligands of this receptor (40).

In an effort to clarify this situation, we have prepared various native and recombinant group IB and group IIA sPLA₂ and analyzed their binding properties to M-type receptors of different animal species. We found that mouse group IB sPLA₂ and mouse group IIA sPLA₂ are recognized by the mouse M-type receptor, indicating that both types of sPLA₂ are probably true endogenous ligands of the M-type receptor in mouse. We also provided evidence that this binding may occur in vivo, since sPLA₂ and the M-type receptor are co-expressed in several mouse tissues.

**EXPERIMENTAL PROCEDURES**

**Preparation of Native sPLA₂—Oxysuranus scutellatus scutellatus sPLA₂ (OS₂) and OS₁, and bvPLA₂ were purified as described previously (34). pGIB was purchased from Boehringer Mannheim and further purified on a Spherogel TSK SP-5PW HPLC column (10 μm, 75 × 7.5 mm, 3.3 ml) equilibrated in acetic acid 1% (v/v) and eluted using a linear gradient of ammonium acetate (0–2M, pH 6.8 in 70 min). Human pancreatic group IB sPLA₂ was then eluted stepwise with 1% acetic acid containing 350 mM ammonium acetate. sPLA₂ containing fractions were lyophilized and applied to a C18 Beckman reverse phase HPLC column (10 × 250 mm, 19.6 ml, 5 μm, 100 A). Elution was performed using an acetone-triltrifluoroacetic acid linear gradient in 0.1% trifluoroacetic acid, 10–60% acetonitrile for 40 min at a flow rate of 0.5 ml/min. The sPLA₂ containing sPLA₂ was then purified by size-exclusion chromatography on the Spheroel TSK SP-5PW HPLC column as indicated above for the purification of pGIB. The sPLA₂ peak was finally applied on a C18 Nucleosil reverse phase HPLC column (4.6 × 250 mm, 4.2 ml, 5 μm, 300 Å) that was eluted using an acetone-triltrifluoroacetic acid linear gradient in 0.1% trifluoroacetic acid, 15–25% acetonitrile for 10 min followed by 25–45% acetonitrile for 10 min at 1 ml/min. The final yield for the production of mGIB was 1.5 mg of purified sPLA₂/liter of cell medium.

**Preparation of Recombinant Human Group IIA sPLA₂—AV12 fibroblast recombinant hGIIA (47) was provided by Dr. Ruth Kramer (Lilly). Recombinant hGIIA from insect Tn5 cells was prepared as above for mGIB and using the full-length hGIIA cDNA (48). Membrane-bound hGIIA sPLA₂ activity (3) was extracted from pelleted baculovirus-infected Tn5 cells for 30 min with 100 ml of phosphate-buffered saline containing 10 μM EDTA and 0.1% Triton X-100. The sPLA₂-containing sPLA₂ was then applied to the Spherogel TSK SP-5PW HPLC column and C18 Nucleosil column as indicated above for the purification of pGIB. The sPLA₂ peak was finally applied on a C18 Nucleosil reverse phase HPLC column (4.6 × 250 mm, 4.2 ml, 5 μm, 300 Å) that was eluted using an acetone-triltrifluoroacetic acid linear gradient in 0.1% trifluoroacetic acid, 15–25% acetonitrile for 10 min followed by 25–45% acetonitrile for 10 min at 1 ml/min. The final yield for the production of recombinant mGIIA in Tn5 cells was about 0.1 mg of purified sPLA₂/liter of cell medium.**

**Preparation of Native and Recombinant Mouse Group IIA sPLA₂—** Native mGIIA was acid-extracted from BALB/c mouse intestinal mGIIA. Recombinant mGIIA from baculovirus-infected Tn5 cells was prepared as above for hGIIA. To produce recombinant mGIIA, the following procedure was used for cell extraction of recombinant mGIIA as described above for hGIIA. The Bio-Gel P100 sPLA₂ fractions were further purified on a TSK SP-5PW column and C18 Nucleosil large pore column. The final yield for the production of hGIIA was about 0.3 mg of purified sPLA₂/liter of cell medium.

**Cloning and Preparation of Recombinant Mouse Group IB sPLA₂—** A search for homology in genome data bases with sPLA₂ protein sequences led to the identification of an I.M.A.G.E. Consortium cDNA clone (identification number 315430, 5; GenBank accession no. W12659) from the mouse with a strong similarity to known pancreatic group IB sPLA₂. The obtained cDNA clone was sequenced and found to contain a cDNA insert of 555 base pairs that encodes for the full-length mouse group IB sPLA₂ (mGIB) cDNA. The full-length cDNA including the prepropeptide sequence was subcloned into the baculovirus transfer vector pVL 1392 and transfected into Spodoptera frugiperda cells (Sf9; ATCC CRL 1711) using the BaculoGold™ transfection kit (Pharmingen). After two rounds of virus amplification into Sf9 cells, Trichoplusia ni High Five insect cells (Tan) were used for the production of recombinant sPLA₂, since preliminary experiments have shown a 3-fold higher yield of sPLA₂ production compared with Sf9 cells. Furthermore, preliminary experiments indicated that infection of insect Sf9 baculovirus mediated with fraction of serum from bovine immune serum resulted in the secretion of a mixture of proenzyme and mature forms of mGIB, since the sPLA₂ activity of cell supernatant can be increased by the addition of trypsin, while fully activated mGIB was recovered from supernatant of cells that were cultivated in protein-free Insect-Xpress medium (BioWhittaker) (not shown). Large scale sPLA₂ preparations were thus performed with Tan5-infected cells (2.10⁶ cells/ml) grown in spinner culture bottles in protein-free Insect-Xpress medium for 5 days. Cell-free supernatants of infected cells (1 liter) were diluted twice in 1% (v/v) acetic acid and incubated batchwise for 2 h, at 4 °C, and under continuous agitation with 150 ml of SP Sephacel C-25 gel (Amersham Pharmacia Biotech), which had been preequilibrated with 1% acetic acid. The gel was washed with 1% acetic acid and 1% acetic acid containing 100 mM ammonium acetate. Bound proteins were then eluted stepwise with 1% acetic acid containing 350 mM ammonium acetate. sPLA₂ containing fractions were lyophilized and applied to a C18 Beckman reverse phase HPLC column (10 × 250 mm, 19.6 ml, 5 μm, 100 Å). Elution was performed using an acetone-tritrifluoroacetic acid linear gradient in 0.1% trifluoroacetic acid, 10–60% acetonitrile for 40 min at a flow rate of 0.5 ml/min. The sPLA₂ containing sPLA₂ was then purified by size-exclusion chromatography on the Spherogel TSK SP-5PW HPLC column as indicated above for the purification of pGIB. The sPLA₂ peak was finally applied on a C18 Nucleosil reverse phase HPLC column (4.6 × 250 mm, 4.2 ml, 5 μm, 300 Å) that was eluted using an acetone-tritrifluoroacetic acid linear gradient in 0.1% trifluoroacetic acid, 15–25% acetonitrile for 10 min followed by 25–45% acetonitrile for 10 min at 1 ml/min. The final yield for the production of mGIB was 1.5 mg of purified sPLA₂/liter of cell medium.
**RESULTS**

**Preparation of Native and Recombinant Mammalian sPLA$_2$s**—Native or recombinant group IB and group IIA sPLA$_2$s from different species have been prepared in order to analyze their binding properties to M-type receptors. The human pancreatic group IB sPLA$_2$ (hGIB) was purified to homogeneity as described previously (54). Recombinant expression of the mouse M-type receptor was performed in COS cells after cloning of its full-length cDNA from NIH 3T3 cells according to published sequence (41) and transfection into COS cells as for the human M-type receptor. All binding experiments were performed under equilibrium binding conditions using as ligand $^{125}$I-OS1, labeled to a specific activity of 3000–3500 cpm/fmol as described by Lambeau et al. (37). Briefly, membranes, $^{125}$I-OS1, and competitors were incubated at 20 °C in 0.5 or 1 ml of buffer (140 mM NaCl, 0.1 mM CaCl$_2$, 20 mM Tris, pH 7.4, and 0.1% BSA). Incubations were started by the addition of membranes and filtered after 90 min of incubation through GF/C glass fiber filters presoaked in 0.5% polyethyleneimine. Cross-linking experiments were performed with 50 μM suberic acid bis-N-hydroxysuccinimide ester (Sigma) as described previously (37, 52).

**Northern Blot Analysis**—A homemade and a commercial mouse Northern blot (CLONTECH Laboratories, Inc., catalog no. 7762-1) containing RNAs from various adult mouse BALB/c tissues were first probed with the random primed $^{32}$P-labeled full-length mGIIA cDNA in 50% formamide, 5× SSPE (0.9× NaCl, 50 mM sodium phosphate, pH 7.4, 5× EDTA), 5× Denhardt’s solution, 0.1% SDS, 20 mM sodium phosphate, pH 6.5, and 250 μg/ml denatured salmon sperm DNA at 50 °C for 18 h. Blots were washed to a final stringency of 0.1× SSC (30 mM NaCl, 3 mM trisodium citrate, pH 7.0) with 0.1% SDS at 55 °C and exposed to Biomax MS Kodak films with an HE intensifying screen (Amersham Pharmacia Biotech). Northern blots were then stripped, checked for dehybridization, and hybridized under the same conditions as above with the entire coding sequence of mGIB. The integrity and relative quantities of RNAs were checked with the manufacturer’s mouse $\beta$-actin probe (not shown).

**Binding Properties of Group IB and Group IIA sPLA$_2$s to M-type Receptors from Different Species**—$^{125}$I-OS1, a specific and very high affinity ligand of the M-type receptor (33), was used to analyze the binding properties of the various group IB and group IIA sPLA$_2$s to M-type receptors from different species. Fig. 2 and Table I show the results obtained from competition binding experiments between labeled OS$_1$ and unlabeled sPLA$_2$s to M-type receptors from mouse, rat, rabbit, human, and bovine species. OS$_1$ has a similar and very high affinity (100–200 pM) for all receptors except for the human receptor, for which the observed $K_{0.5}$ value is only 4 nM (Table I). A common pharmacological property of the different M-type receptors is that $^{125}$I-OS$_1$, binding is not inhibited by the neurotoxic bvPLA$_2$, which was previously found to be a specific sPLA$_2$ ligand for rat and rabbit N-type sPLA$_2$ receptors (33, 34, 37).

The binding profiles of pGIB and hGIB to the various M-type receptors are very similar. Both sPLA$_2$s display an affinity in the nanomolar range for mouse, rat, and bovine receptors, have a 50–100-fold weaker affinity for the rabbit receptor, and bind very weakly to the human receptor (Table I). These results are in accordance with previous data obtained with pGIB and hGIB on mouse, rat, and bovine M-type receptors (38). The binding profile of mGIB is slightly different from that of pGIB and hGIB as it binds with high affinity to mouse and rabbit receptors but with an ~10-fold lower affinity to the rat receptor. However, as for pGIB and hGIB (40), mGIB is a very low affinity ligand of the human M-type receptor. These data indicate that the pancreatic-type sPLA$_2$ mGIB is probably a natu-
Both native and recombinant mGIIA sPLA2s were found with high affinities to the rabbit M-type receptor. Similar to hGIIA proteins appear properly folded, since they can bind ligand for the human receptor. However, the recombinant together, these results suggest that hGIIA is not a physiological ligand for the M-type receptor in the mouse.

Altogether, both mGIB and mGIIA appear as ligands of the mouse M-type receptor endogenously expressed in NIH 3T3 cells (Fig. 2). To confirm this view, we investigated the binding properties of these sPLA2s to recombinant mouse M-type receptor expressed in transfected COS cells. The recombinant receptor was found to have the expected binding properties for venom sPLA2s, i.e. a high affinity for OS1 (Kd = 0.3 nM) and no measurable affinity for bvPLA2, suggesting that this receptor is successfully expressed in COS cells (Fig. 3). Furthermore, labeled OS1 was found unable to bind to mock-transfected cells (not shown), indicating that OS1 binds specifically to the mouse M-type receptor. Fig. 3 shows that mGIB and mGIIA bind to the recombinant mouse M-type receptor with affinities similar to those observed in OS1 competition assays on NIH 3T3 membranes (Fig. 2), clearly indicating that these two sPLA2s can bind to the cloned mouse M-type receptor.

Since the M-type receptor was found to share similarities with the macrophage mannose receptor that belongs to the C-type lectin superfamily, and since the rabbit M-type receptor was previously found to bind with nanomolar affinities various glycoconjugates of BSA (39) (i.e. to display lectin-like properties), it was of interest to analyze the binding properties of the different M-type receptors for the various glycoconjugated derivatives of BSA. We observed that only the rabbit receptor has a high affinity for the various BSA glycoconjugates, whereas the other receptors displayed either a much weaker affinity (human and mouse) or even no measurable affinity (rat), suggesting that lectin properties of the M-type receptor (such as binding of glycosylated BSA) have not been conserved between mammalian species and therefore would not be physiologically relevant. This view is in agreement with a previous observation that mannosylated BSA is unable to bind to the bovine M-type receptor (42).

**Tissue Distribution of the Mouse M-type Receptor, mGIB, and mGIIA**—The above binding data indicate that both mGIB and mGIIA bind to the mouse M-type receptor and therefore may be physiological ligands in the mouse. To strengthen this view, the tissue distribution of the M-type receptor was analyzed in BALB/c mice and then compared with that of mGIB and mGIIA, with the idea that a colocalization of the receptor with the macrophage mannose receptor that belongs to the C-type lectin superfamily (41). Most notably, the highest amounts of receptor were found in heart and liver (41). Lung, colon, kidney, and salivary glands were found to contain the highest amounts of receptor, while binding sites for OS1 were absent in brain membranes. The maximal number of binding sites in the various tissues remains low as compared with that observed in the mouse fibroblast cell line NIH 3T3 (Table II), which was used as a source of mouse M-type receptor for the competition binding assays (Fig. 2). The tissue distribution of the M-type receptor protein shown here using 125I-OS1 binding is in agreement with the previous analysis of the M-type receptor transcript in mouse (41). Most notably, the highest amounts of transcripts were also found in lung and kidney, while lower levels were observed in heart and liver (41).

The tissue distribution of mGIB and mGIIA was carried out...
by successively probing BALB/c mouse tissue Northern blots at high stringency with the two different cDNAs (Fig. 5). Transcripts of 0.8 kb coding for mGIB were detected at very high levels in pancreas and at lower levels in liver, lung, and spleen. No transcript was detected in other tissues such as intestine, heart, brain, skeletal muscle, kidney, and testis. This tissue distribution is in accordance with that previously described in mice (41) and is similar to the distribution observed in humans, with the exception of liver where no transcript was detected (7, 30). Also in agreement with previous data (56, 57), we found that very high amounts of the mGIIA transcript are present in intestine but not in other analyzed tissues with the exception of liver, where a very weak expression is observed. Taken together, these data indicate that different mouse tissues express both the mouse M-type receptor and mGIB or mGIIA.

Both mGIB and mGIIA sPLA₂s Bind to the 180-kDa M-type Receptor Expressed in Mouse Colon—To finally confirm that the binding properties of mGIB and mGIIA observed on mouse M-type receptor endogenously expressed in the fibroblast NIH 3T3 cell line (Fig. 2) or transiently expressed in COS cells (Fig. 3) are similar in normal mouse tissues, we performed competition binding experiments as well as cross-linking experiments with mGIB and mGIIA on mouse colon membranes (Fig. 6). The \( K_{d,5} \) values determined for mGIB (\( K_{d,5} = 1.3 \) nM) and mGIIA (\( K_{d,5} = 10 \) nM) appeared very similar to those measured

### Table I

| Competitor                  | Bevine | Human | Mouse | Rabbit | Rat |
|-----------------------------|--------|-------|-------|--------|-----|
| Venom sPLA₂                 | 0.1    | 4     | 0.1   | 0.1    | 0.2 |
| OS₁                         | >1000  | >1000 | >1000 | >1000  | >1000|
| bvPLA₂                      |        |       |       |        |     |
| Group IB sPLA₂              |        |       |       |        |     |
| pGIB                        | 0.6    | >200  | 0.2   | 30     | 0.3 |
| hGIB                        | 2.8    | >200  | 1.2   | 78     | 8   |
| mGIB                        | ND     | >1000 | 1.3   | 0.5    | 12  |
| Group IIA sPLA₂             |        |       |       |        |     |
| hGIIA                       |        |       |       |        |     |
| Av12 cells                  | >200   | >200  | ~400  | 2      | >200|
| Tn5 cells                   | >200   | >200  | ~400  | 4.9    | >200|
| mGIIA                       |        |       |       |        |     |
| Intestinal                  | 31     | >500  | 12    | 1.4    | 125 |
| 293 cells                   | 25     | >500  | 13    | 1.6    | 115 |
| Tn5 cells                   | 16     | >500  | 7     | 0.8    | 98  |
| BSA glycoconjugates         |        |       |       |        |     |
| Mannose BSA                 | ND     | ~450  | ~1000 | 25     | >1000|
| N-Acetylglucosamine BSA     | ND     | ~350  | ~2000 | 34     | >1000|
| Galactose BSA               | ND     | ~450  | ~1500 | 35     | >1000|

\( \text{ND, not determined.} \)

**Fig. 3.** Binding properties of sPLA₂s to the recombinant mouse M-type receptor expressed in COS cells. Membranes (10 μg of protein/ml) of COS cells transfected with the full-length mouse M-type receptor were incubated in the presence of \( ^{125}\text{I-OS₁} \) (25 pM) and various concentrations of OS₁ or recombinant mGIB and mGIIA from Sf9 baculovirus-infected cells. All results are expressed as percentages of the specific binding measured in the absence of unlabeled sPLA₂s. 100% corresponds to a \( ^{125}\text{I-OS₁} \) specific binding of 1.6 pM. The nonspecific binding was determined in the presence of 30 nM unlabeled OS₁ and was below 6% of the total binding.

**Fig. 4.** Equilibrium binding of \( ^{125}\text{I-OS₁} \) to mouse colon membranes. A, membranes (140 μg/ml) were incubated with increasing concentrations of \( ^{125}\text{I-OS₁} \) in the absence (○) or presence (■) of 30 nM unlabeled OS₁. Specific binding (●) represents the difference between total binding (○) and nonspecific binding (■). B, Scatchard plot analysis of the specific binding (●). M-type receptor endogenously expressed in the fibroblast NIH 3T3 cell line (Fig. 2) or transiently expressed in COS cells (Fig. 3) are similar in normal mouse tissues, we performed competition binding experiments as well as cross-linking experiments with mGIB and mGIIA on mouse colon membranes (Fig. 6). The \( K_{d,5} \) values determined for mGIB (\( K_{d,5} = 1.3 \) nM) and mGIIA (\( K_{d,5} = 10 \) nM) appeared very similar to those measured...
**TABLE II**

**Tissue distribution of the mouse M-type receptor**

The different membrane preparations were incubated with increasing concentrations of $^{125}$I-OS$_1$ as indicated in Fig. 1. Data are mean values from at least two independent sets of experiments on different membrane preparations.

| Membrane preparation | $B_{\text{max}}$ (fmol/mg protein) | $K_d$ (pM) |
|----------------------|-----------------------------------|------------|
| Liver                | 4                                 | 18         |
| Kidney               | 17                                | 46         |
| Lung                 | 27                                | 27         |
| Spleen               | 8                                 | 52         |
| Salivary glands      | 14                                | 20         |
| Small intestine      | 5                                 | 55         |
| Colon                | 22                                | 55         |
| Heart                | 5                                 | 14         |
| Pancreas             | 5                                 | 19         |
| Brain                | ND$^*$                            |            |
| NIH 3T3 cells        | 118                               | 46         |

$^*$ Not detectable.

**Fig. 5.** High stringency Northern blot analysis of mGIB and mGIIA expression in various BALB/c mouse tissues. Northern blots containing 2 µg of poly(A)$^+$ mRNA/lane (B) from various adult mouse tissues were successively hybridized at high stringency with $^{32}$P-labeled probes for mGIIA sPLA$_2$ and mGIB sPLA$_2$ as described under “Experimental Procedures.” sk. muscle, skeletal muscle. Filters were exposed for 3 days using Biomax MS Kodak films with an HE intensifying screen.

**DISCUSSION**

The 180-kDa M-type receptor was initially identified using snake venom sPLA$_2$s such as OS$_1$ (37). A first clue to the physiological function of the M-type receptor was provided when it was observed that the mammalian pancreatic group IB sPLA$_2$, but not the inflammatory group IIA sPLA$_2$, was a ligand of this M-type receptor with a $K_d$ value of 1 nM (8). However, other binding experiments to the rabbit M-type receptor suggested that both group IB and group IIA sPLA$_2$s may be natural endogenous ligands of this receptor (39). On the other hand, the human M-type receptor was found to have very weak affinities for both group IB and group IIA sPLA$_2$s (40). The situation was thus clearly confusing, and the physiological relevance of these binding data was difficult to evaluate because sPLA$_2$s and M-type receptors from different animal species were used in many of these binding experiments (38, 39).

This paper now presents data showing that mGIB and mGIIA sPLA$_2$s are high affinity ligands of the mouse M-type receptor (Fig. 2) and therefore would be natural candidates to act as endogenous ligands of this receptor. This view is strengthened by the colocalization in mice of the two sPLA$_2$s and of the M-type receptor. In particular, both mGIIA and the M-type receptor are expressed in small intestine and colon (Table II, Fig. 5, and Ref. 56). Although the affinity of mGIIA for the M-type receptor is not very high ($K_d$ close to 10 nM), this binding is likely to occur in small intestine and colon, because huge amounts of mGIIA transcript and protein are found in these tissues (49, 56, 57). The presence of mGIB and mGIIA sPLA$_2$s in mouse serum or platelets is not well documented. However, since both group IB and group IIA sPLA$_2$ activities were detected in serum from other animal species (14), it is likely that these sPLA$_2$s are also present in mouse serum and then may reach M-type receptors that are far away from cells producing sPLA$_2$s.

The view that both group IB and group IIA sPLA$_2$s would operate as endogenous ligands of the M-type receptor in the mouse would not apply to humans, since both types of human sPLA$_2$s were found unable to bind to the human M-type receptor (Ref. 40 and Table I). Another situation is found in the rat, since the rat M-type receptor binds rGIB but not rGIIA (38). The rabbit M-type receptor is unique in binding all the different mammalian group IB and group IIA sPLA$_2$s analyzed so far (Table I). It is also the only one that has lectin-like binding properties (Table I). Altogether, the available data indicate that group IB sPLA$_2$s could behave as an endogenous ligand of M-type receptors at least in mouse, rat, and porcine (35) species. On the other hand, the group IIA sPLA$_2$ appears to serve as an endogenous ligand of the M-type receptor in mice but not in rats (38) and humans (40). It is then possible that rGIIA and hGIIA have their own receptors, distinct from the M-type receptor. Finally, whether the more recently characterized group IIC, group V, and group X sPLA$_2$s are endogenous ligands of M-type receptors remains to be determined.
The CRD5 domain of the M-type receptor is centrally involved in sPLA₂ binding (46). It is therefore likely that the different binding properties of the M-type receptor in various animal species have to do with differences between these receptors in the CRD5 domain. Interestingly, we previously observed that, of the eight CRDs of the M-type receptor, the CRD5 domain is the least conserved among rabbit, mouse, bovine, and human receptors (46). On the other hand, we have also previously demonstrated that residues close to or within the Ca²⁺-binding loop domain of sPLA₂ are involved in binding to the M-type receptor (45). We particularly suggested that, besides glycine 30 and aspartate 49, which are perfectly conserved in sPLA₂s and which are essential for binding, the identity of the residues at position 31 and possibly 34, which greatly varies in sPLA₂ (59), may determine whether binding to the M-type receptor is possible or not. It is noteworthy that this view fits well with the fact that mGIIA binds with high affinity to the mouse M-type receptor, while rGIIA and hGIIA do not bind to this receptor (Table I and Ref. 38). Indeed, the Ca²⁺-binding loop domain of these three sPLA₂ is perfectly conserved except at positions 31 and 34, where rGIIA and hGIIA, but not mGIIA, have the same residues (3, 48, 57). This observation, however, does not eliminate the possibility that residues located elsewhere in the sPLA₂ structure also contribute to the large differences in binding properties.

The physiological reason why mGIIA binds to the mouse M-type receptor while rGIIA and hGIIA do not bind to the respective rat and human M-type receptors is hard to understand. A tempting hypothesis would be that mGIIA is not the ortholog of rGIIA and hGIIA, i.e. that mGIIA has physiological functions that are distinct from those of rGIIA and hGIIA. In addition to differences in binding properties, several other lines of evidence would support this hypothesis. First, mGIIA, rGIIA, and hGIIA display relatively low levels of sequence identity compared with those observed between group IB sPLA₂. mGIIA has only 76 and 67% of identity with rGIIA and hGIIA, while mGIB has 89 and 81% of identity with rGIB and hGIB, respectively. Second, both hGIIA and rGIIA are expressed in many different tissues and cells (3, 30), while the tissue distribution of mGIIA is thus far essentially restricted to intestine (where it is expressed at a very high level), with a low expression level in liver (Fig. 5) and the skin of newborn mice (56). Furthermore, in hGIIA transgenic mice established with the complete hGIIA gene, the tissue distribution of hGIIA resembles that of hGIIA in humans, where it is expressed in many organs (60), in marked contrast with the endogenous expression of mGIIA (Fig. 5). In these transgenic mice, hGIIA was not expressed in the intestinal Paneth cells (60), whereas Paneth cells in wild-type mice are known to contain huge amounts of mGIIA (56, 61, 62). Since the transgenic mice were established with a transgene comprising a reasonably large 5' noncoding sequence of 1.6 kilobase pairs that contains transcriptional regulatory elements of the hGIIA gene (63, 64), and since one would expect a conservation of the transcriptional regulatory elements between mGIIA and hGIIA, the difference observed between the endogenous expression of mGIIA and that of hGIIA in transgenic mice suggests that the transcriptional regulatory elements of the mGIIA and hGIIA genes are distinct and therefore supports the idea that mGIIA and hGIIA are not true orthologs. The third indication that mGIIA may not be the ortholog of rGIIA and hGIIA comes from physiological considerations. While there are many studies showing that expression of rGIIA and hGIIA is dramatically increased by proinflammatory cytokines, in many human inflammatory diseases, and in various rat models of inflammatory diseases (3), there is no clear evidence for an increased expression of mGIIA in inflammatory conditions in mouse cells or tissues. Thus far, only two studies have shown that the expression of mGIIA could be increased in intestine after injection of lipopolysaccharide (LPS) (65, 66), but this increase was only modest as compared with the induction observed in LPS-treated rats (3, 67). Finally, while many studies have shown that rGIIA and hGIIA play a role in lipid mediator release (3, 68), mGIIA-deficient mice were found to have a normal inflammatory response that is mediated by mouse group V sPLA₂ (22, 23), and while mGIIA has been proposed as a genetic modifier of colon tumorigenesis (25, 26), the numerous attempts to demonstrate a similar role for hGIIA in human colorectal cancer were unsuccessful (69–75).

The M-type receptor has been associated with a myriad of biological roles such as cell proliferation, cell contraction, cell migration, hormone release, and lipid mediator release (38, 76). These effects are currently believed to be mediated by group IB sPLA₂ but not by group IIA sPLA₂. However, since group IIA sPLA₂ and cells from different animal species have been used in these previous studies, and since we now know that animal specificity is important for the M-type receptor interaction, some of these previous data may require reevaluation. For example, the mitogenic effect of pGIB on mouse fibroblasts was believed to indicate a specific action of group IB sPLA₂ through binding to the mouse M-type receptor, since rGIIA and rbGIIA were found unable to bind to this receptor (8). Since mGIIA now appears as a ligand of the mouse M-type receptor, this suggests that group IIA sPLA₂ may also have mitogenic effects on these mouse fibroblasts that may be linked to the mouse M-type receptor. The recent targeted disruption of the M-type receptor gene in the mouse suggests that the M-type receptor plays a critical role in inflammatory processes induced by LPS and leading to endotoxic shock (13). M-type receptor-deficient mice have a longer survival time than wild-type mice after challenge with LPS and are also resistant to the lethal effects of pGIB after sensitization with sublethal dose of LPS. The results of the present study indicating that mGIB is a natural ligand of the mouse M-type receptor fit well with the view that group IB sPLA₂ would play a role in processes leading to endotoxic shock after LPS challenge through binding to the mouse M-type receptor (13). mGIIA, which now appears as a second natural ligand of the mouse M-type receptor, is certainly not implicated in this resistance to endotoxic shock, since M-type receptor knock-out mice are also naturally deficient for mGIIA (13, 25, 65). Since several other mouse sPLA₂ have now been identified (among them the mouse group V that has been shown to play a role in the release of lipid mediators of inflammation in place of mGIIA (22, 23)), it will be important to analyze whether they can also act as natural ligands of the M-type receptor and whether they are involved in the inflammatory processes leading to endotoxic shock.

Besides a possible role of mGIIA in the production of inflammatory lipid mediators (20), mGIIA has been proposed to have bactericidal properties to protect the small intestine crypts from microbial invasion (62), and mice lacking mGIIA show an altered response to microbial infection (77). Whether the M-type receptor may contribute to these effects remains to be analyzed. mGIIA was originally discovered as an intestinal protein called enhancing factor that increases the binding of the epidermal growth factor and synergizes with this latter to stimulate cell proliferation (24). The potential role of the M-type receptor in these effects would be worth analyzing. More recently, mGIIA was identified as a genetic modifier of tumor formation in a mouse model of colorectal cancer (25, 26). Mice carrying a deficient mGIIA gene were found to develop more
intestinal adenomas than mice expressing a functional mGIIA (25). In addition, transgenic mice overexpressing mGIIA become resistant to intestinal tumorigenesis (26). While the mechanism by which mGIIA confers protection against adenoma formation is presently unknown (26), the colocalization of the M-type receptor with mGIIA in colon may suggest an implication of the M-type receptor in the resistance to intestinal tumorigenesis conferred by mGIIA.

In conclusion, this work has shown that both mGIB and mGIIA are probably physiological ligands of the mouse M-type receptor. This observation may be important to the understanding of the physiological and pathological roles of these sPLA2s in various processes such as cell proliferation, inflammation, and cancer. Furthermore, comparison of the binding properties of mouse and human sPLA2s to receptors from different animal species has indicated that sPLA2 binding to M-type receptors is species-specific. Interestingly, species specificity of binding was previously observed for cytokines such as IL-1. This observation may be important to the understanding of the molecular mechanism of tumorigenesis conferred by mGIIA.

In addition, transgenic mice overexpressing mGIIA have been shown to become resistant to intestinal tumorigenesis (26). While the molecular mechanism of tumorigenesis conferred by mGIIA is presently unknown, the colocalization of mGIIA with the M-type receptor in colon may suggest an implication of the M-type receptor in the resistance to intestinal tumorigenesis.

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REFERENCES

1. Dennis, E. A. (1994) J. Biol. Chem. 269, 13057–13060
2. Tischfield, J. A. (1997) J. Biol. Chem. 272, 17247–17250
3. Murakami, M., Nakatani, Y., Atemu, G., Inoue, K., and Kudo, I. (1997) Crit. Rev. Immunol. 17, 225–283
4. Dennis, E. A. (1997) Trends Bio. Sci. 22, 1–2
5. Gelh, M. H., Hanel, A. M., and Berg, O. G. (1995) Annu. Rev. Biochem. 64, 653–688
6. De Haas, G., Postema, N. M., Nieuwenhuizen, W., and Van Deenen, L. L. M. (1996) Biochim. Biophys. Acta 1303, 103–117
7. Seilhearn, J. J., Randall, T. L., Yamanaka, M., and Johnson, L. K. (1988) DNA 5, 519–527
8. Arita, H., Hanasaki, K., Nakano, T., Oka, S., Teraoka, H., and Matsumoto, K. J. Biol. Chem. 266, 19139–19141
9. Nakajima, M., Hanasaki, K., Ueda, M., and Arita, H. (1992) FEBS Lett. 309, 261–264
10. Sommers, C. D., Bobbitt, J. L., Bemis, K. G., and Snyder, D. W. (1992) Eur. J. Pharmacol. 216, 87–96
11. Kishino, J., Ohara, O., Nomura, K., Kramer, R. M., and Arita, H. (1994) J. Biol. Chem. 269, 5092–5098
12. Rae, D., Beechey-Newman, N., Burditt, L., Sumar, N., and Heron-Taylor, J. (1996) Stand. J. Gastroenterol. Suppl. 219, 24–27
13. Hanasaki, K., Yokota, Y., Ishizaki, J., Itoh, T., and Arita, H. (1991) J. Biol. Chem. 266, 5279–5287
14. Nevalainen, T. J. (1993) Clin. Chem. 39, 2453–2459
15. Vadas, P., Browning, J., Edelson, J., and Pruzanski, W. (1993) J. Lipid Mediat. 8, 1–30
16. Mukherkar, A. B., Miele, G., and Padattabhiraman, N. (1994) Biochem. Pharmacol. 48, 1–10
17. Abe, T., Sakamoto, K., Kamohara, H., Hirono, Y., Kuyawara, N., and Ogawa, M. (1997) Int. J. Cancer. 74, 245–250
18. Murata, K., Egami, H., Kiyohara, H., Ohashi, S., Kurizaki, T., and Ogawa, M. (1993) Br. J. Cancer 68, 103–111
19. Furca, O., Simon, M. F., Viode, C., Ragusa, N., Lehalde, F., Ragag, A., Fourbaye, B., Suard, M., and Chap, H. (1995) Cell 60, 919–927
20. Murakami, M., Shimbara, S., Kambe, T., Kawaata, H., Winstead, M. V., Tischfield, J. A., and Kudo, I. (1998) J. Biol. Chem. 273, 14411–14417
21. Weinrauch, Y., Abud, C., Liang, N. S., Lovery, S. F., and Weiss, J. (1998) J. Clin. Invest. 102, 633–638
22. Reddy, S. T., Winstead, M. V., Tischfield, J. A., and Herschman, H. R. (1997) J. Biol. Chem. 272, 13591–13596
23. Balboa, M. A., Balsinde, J., Winstead, M. V., Tischfield, J. A., and Dennis, E. (1996) J. Biol. Chem. 271, 32381–32384
24. Mulherkar, R., and Deo, M. G. (1986) J. Cell. Physiol. 127, 183–188
72. Dobbie, Z., Heinimann, K., Bishop, D. T., Muller, H., and Scott, R. J. (1997) *Hum. Genet.* 99, 653–657
73. Haluska, F. G., Thiele, C., Goldstein, A., Tsao, H., Benoit, E. P., and Housman, D. (1997) *Int. J. Cancer.* 72, 337–339
74. Minami, T., Kanayama, S., Tojo, H., Akedo, I., and Matsuzawa, Y. (1997) *J. Gastroenterol.* 32, 431–432
75. Kennedy, B. P., Soravia, C., Moffat, J., Xia, L., Hiruki, T., Collins, S., Gallinger, S., and Bapat, B. (1998) *Cancer Res.* 58, 500–503
76. Kundu, G. C., and Mukherjee, A. B. (1997) *J. Biol. Chem.* 272, 2346–2353
77. Wang, T. C., Goldenring, J. R., Dangler, C., Ito, S., Mueller, A., Jeon, W. K., Koh, T. J., and Fox, J. G. (1998) *Gastroenterology* 114, 675–689
78. Lewis, M., Tartaglia, L. A., Lee, A., Bennett, G. L., Rice, G. C., Wong, G. H., Chen, E. Y., and Goeddel, D. V. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 2830–2834
79. Brouckaert, P., Libert, C., Everaerdt, B., Takahashi, N., Cauwels, A., and Fiers, W. (1993) *Immunobiology* 187, 317–329
80. Thieme, T. R., Hefeneider, S. H., Wagner, C. R., and Burger, D. R. (1987) *J. Immunol.* 139, 1173–1178