Identification of the Binding Domain for Secretory Phospholipases A2 on Their M-type 180-kDa Membrane Receptor*

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The rabbit muscle (M)-type receptor for secretory phospholipases A2 (sPLA2s) has a large extracellular domain of 1394 amino acids, composed of an N-terminal cysteine-rich domain, a fibronectin-like type II domain, and eight carbohydrate recognition domains (CRDs). It is thought to mediate some of the physiological effects of mammalian sPLA2s, including vascular smooth muscle contraction and cell proliferation, and is able to internalize sPLA2s. Here, we show by site-directed mutagenesis that OS1, a snake venom sPLA2, binds to the receptor via its CRDs and that deletion of CRD 5 completely abolishes the binding of sPLA2s. Moreover, a receptor lacking all CRDs but CRD 5 was still able to bind OS1 although with a lower affinity. Deletion of CRDs 4 and 6, surrounding the CRD 5, slightly reduced the affinity for OS1, thus suggesting that these CRDs are also involved in the binding of OS1. The M-type sPLA2 receptor and the macrophage mannose receptor are homologous and are predicted to share the same tertiary structure. p-Aminophenyl-a-d-mannopyranoside bovine serum albumin, a known ligand of the macrophage mannose receptor, binds to the M-type sPLA2 receptor essentially via CRDs 3-6.

Secretory phospholipases A2 (sPLA2s) are structurally homologous enzymes that have been isolated from a large number of biological sources, including mammalian tissues as well as insect and snake venoms (1-3). At least six different sPLA2s have been found in mammalian tissues. The pancreatic sPLA2 and the inflammatory sPLA2 are well characterized enzymes, while the others have only been purified (4) or cloned (5-7) recently. The pancreatic sPLA2 has long been thought to act only as a digestive enzyme (8). However, the presence of this sPLA2 in several non-digestive tissues has been demonstrated (9-11), and it is now thought to play a role in airway and vascular smooth muscle contraction (12, 13) as well as in cell proliferation (14). The inflammatory sPLA2 has been purified and cloned from several sources (15, 16) and is believed to play a central role in inflammatory processes (reviewed in Refs. 17-20). It is secreted by a large number of cell types in which its expression is strongly up-regulated by inflammatory cytokines. Its concentration in various extracellular fluids is dramatically increased in several inflammatory diseases. Moreover, this enzyme has potent proinflammatory activities (21).

sPLA2s are also found in abundance in snake and bee venoms. Besides their role in prey digestion, the snake venom sPLA2s can have neurotoxic, myotoxic, anticoagulant, and proinflammatory effects (22-25). High affinity receptors for these enzymes have been characterized (26-29). They are apparently involved in their biological effects. A first type of sPLA2 receptors called N-type sPLA2 receptors (for neuronal) has been initially identified in rat brain (26) and then in other tissues (28, 29) using OS2, a novel neurotoxic sPLA2, purified from the Taipan snake (Oxyuranus scutellatus scutellatus) venom (26). It recognizes several other neurotoxic sPLA2s with high affinity while non-neurotoxic venom sPLA2s display much lower affinities. A second type of sPLA2 receptor called M-type sPLA2 receptor has been initially characterized in rabbit skeletal muscle (27), using OS2 and OS3, another sPLA2 purified from the Taipan snake venom (26). Very interestingly, this receptor binds the porcine pancreatic sPLA2 as well as the human inflammatory sPLA2 with high affinities (30), suggesting that these endogenous sPLA2s might be its physiological ligands. M-type sPLA2 receptors were subsequently characterized in fibroblasts and other tissues using the porcine pancreatic sPLA2 as a ligand (14, 31).

More recently, the M-type sPLA2 receptor has been cloned in rabbit, bovine, and human species (30, 32-34). The cloned receptors are homologous to the macrophage mannose receptor, a protein involved in the endocytosis of glycoconjugates and microorganisms (35, 36), as well as to DEC-205, a protein recently cloned in dendritic cells and involved in the presentation of antigens (37). Interestingly, all of these proteins, which may constitute a new family of receptors, are predicted to share the same structural organization, i.e. a large extracellular region composed of an N-terminal cysteine-rich domain, a fibronectin-like type II domain, eight (30, 35) or ten (37) repeats of a carbohydrate recognition domain (CRD), followed by a unique transmembrane domain and a short intracellular C-terminal domain. This latter domain contains a consensus sequence for the internalization of ligand-receptor complexes and is thought to confer to these receptors their endocytic properties (30, 31, 33, 37, 38).

The purpose of this paper is to identify by site-directed mutagenesis techniques the specific domain in the large extracellular part of the rabbit M-type sPLA2 receptor (1394 residues) that is responsible for the binding of sPLA2s.

EXPERIMENTAL PROCEDURES

Materials—OS1 and OS2 were purified as described previously (26) from the venom of O. scutellatus scutellatus. p-Aminophenyl-a-d-mannopyranoside-BSA (mannose-BSA, A4664) was purchased from Sigma.

Mutagenesis of the Rabbit M-type sPLA2 Receptor—M-type sPLA2 receptor mutants were obtained by the methyl-dCTP method (39) using

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1 The abbreviations used are: sPLA2, secretory phospholipase A2; CRD, carbohydrate recognition domain; mannose-BSA, p-aminophenyl-a-d-mannopyranoside bovine serum albumin; M-type, muscle-type; PBS, phosphate-buffered saline.
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**RESULTS AND DISCUSSION**

Design and Expression of the Rabbit M-type sPLA₂ Receptor Mutants—Ten mutants of the M-type sPLA₂ receptor have been designed for determination of the domain involved in the binding of sPLA₂ to this receptor (Fig. 1). To prevent possible misfolding of expressed proteins, the boundaries of deleted portions were always placed at junction sites between predicted domains. Western blot experiments were performed to check that all of these mutants were correctly expressed in COS cells. The native receptor and all of the mutant receptors were visible as two bands of slightly different molecular weights (Fig. 2). These two bands probably result from differences in the glycosylation level of proteins when overexpressed in COS cells. Apparent molecular weights of native and mutated M-type sPLA₂ receptors are consistent with their calculated molecular weights, indicating that all mutants are efficiently translated. No protein band was detected in control mock-transfected cells. Indirect immunostaining experiments were also performed to demonstrate that the wild-type and mutated receptors are targeted to the plasma membrane. Representative results are shown in Fig. 3. A strong labeling was observed at the cell surface for the native receptor and the various mutant receptors, indicating that all of these proteins are normally processed. The low level of staining, which is visible inside the cells, is probably due to receptors engaged in translational or posttranslational processes. Taken together, these results suggest that all these mutants are properly expressed in a way similar to that of the wild-type M-type sPLA₂ receptor.

Identification of the Binding Domain for ¹²⁵I-sOS₁ on the
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**Table I**

| Receptor | \(K_d\) value | \(B_{\text{max}}\) value |
| --- | --- | --- |
| WT | 64 ± 11 | 4.10 ± 0.30 |
| ΔNF | 70 ± 9 | 5.30 ± 0.70 |
| Δ12 | 135 ± 13 | 0.99 ± 0.09 |
| Δ34 | 174 ± 7 | 1.35 ± 0.06 |
| Δ14 | 390 ± 40 | 0.65 ± 0.05 |
| Δ6 | 620 ± 15 | 0.53 ± 0.03 |
| Δ68 | 760 ± 240 | 4.00 ± 0.90 |
| ΔX-5 | 2600 ± 300 | 0.72 ± 0.02 |
| Δ5 | NM | NM |
| Δ56 | NM | NM |
| Δ58 | NM | NM |

indicating that all mutants are expressed at similar levels as compared with the wild-type M-type sPLA₂ receptor (Fig. 4 and Table I). Analysis of the affinities (\(K_d\) values) shows that \(^{125}\text{I}-\text{OS}_1\) binds to the ΔNF mutant with almost the same affinity as compared with the wild-type receptor. These data clearly indicate that the N-terminal cysteine-rich and the fibronectin-like type II domains are not involved in \(^{125}\text{I}-\text{OS}_1\) binding. In agreement with this conclusion, a similar deletion of these two domains in the bovine M-type sPLA₂ receptor did not modify the binding properties of porcine pancreatic sPLA₂ (32).

Deletion of CRDs 1–4 (Δ12, Δ34, and Δ14 mutants) only results in 2- to 6-fold reduced affinities for \(^{125}\text{I}-\text{OS}_1\) (Table I). This suggests that the sequence comprising CRDs 1–4 is involved but is not essential for \(^{125}\text{I}-\text{OS}_1\) binding to the receptor.

Deletion of CRDs 6–8 (Δ6 and Δ68 mutants) also leads to lower affinities of \(^{125}\text{I}-\text{OS}_1\) for the receptor (Table I). These deletions decreased the affinity for \(^{125}\text{I}-\text{OS}_1\) by 10- and 12-fold, respectively. These changes indicate that the CRDs 6–8 sequence is not crucially involved in \(^{125}\text{I}-\text{OS}_1\) binding, although CRD 6 is likely to play a role in \(^{125}\text{I}-\text{OS}_1\) binding.

A more dramatic effect was observed in different mutants lacking CRD 5 (Δ58, Δ56, and Δ5, see Table I), demonstrating that this CRD is directly involved in \(^{125}\text{I}-\text{OS}_1\) binding. All these mutants have completely lost their ability to bind \(^{125}\text{I}-\text{OS}_1\). It was then interesting to test whether CRD 5 expressed alone was able to bind \(^{125}\text{I}-\text{OS}_1\). The ΔX-5 construct, in which all CRDs but CRD 5 have been deleted, was still able to bind \(^{125}\text{I}-\text{OS}_1\), although with a 40-fold reduced affinity (Table I).

Taken together, these data show that the main domain of the extracellular region of the rabbit M-type sPLA₂ receptor involved in \(^{125}\text{I}-\text{OS}_1\) binding is CRD 5, while CRDs 6 and 8 provide further interactions to increase the affinity of \(^{125}\text{I}-\text{OS}_1\).

**CRDs Involved in Mannose-BSA Binding**—The interaction of the M-type sPLA₂ receptor with sPLA₂s is clearly a protein-protein interaction (30). However, besides its sPLA₂ binding property, this receptor is also able to recognize sugars such as mannose and galactose (30). Indeed, based on the similarity between the rabbit M-type sPLA₂ receptor and the macrophage mannose receptor, we previously observed by competition experiments against sPLA₂ binding that the M-type receptor also binds mannose-BSA (30). Mannose-BSA is a typical ligand of the mannose receptor (42) and is known to bind to this receptor mainly via CRDs 4 and 5 (43). It was thus interesting to determine which CRDs on the M-type sPLA₂ receptor were involved in mannose-BSA binding.

The \(K_{0.5}\) values obtained for the inhibition of \(^{125}\text{I}-\text{OS}_1\) bind-
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The affinity of mannose-BSA for the ΔΔ and ΔΔ8 mutants of the N-terminal cysteine-rich region of the fibronectin-like type II domain and CRDs 1–2 are not involved in the binding of mannose-BSA to the M-type sPLA₂ receptor. Conversely, mannose-BSA was not able to inhibit 

\[ \Delta \] binding on the ΔΔ1, ΔΔ3, and ΔΔ5 mutant forms of the M-type receptor. These results are in accordance with those establishing the importance of CRD 4 for the binding of mannose-BSA to the macrophage mannose receptor (42). Since 

\[ \Delta \] \[ \Delta \] had no measurable affinity on mutated receptors lacking CRD 5, it was not possible to determine the importance of this latter CRD in the binding of mannose-BSA to the M-types sPLA₂ receptor.

The affinity of mannose-BSA for the ΔΔ and ΔΔ8 mutants of the M-type sPLA₂ receptor was decreased 13- and 19-fold, respectively, as compared to its affinity toward the wild-type receptor (Table II). This suggests that CRDs 6–8 are not crucially involved in the binding of mannose-BSA to the M-type sPLA₂ receptor but that these latter CRDs contribute to the binding of mannose-BSA. The involvement of these CRDs in the binding of a glycoprotein such as invertase and in the binding of mannan has already been described in the case of the macrophage mannose receptor (43, 44). The CRD 4 structure of the macrophage mannose receptor is sufficient by itself to bind invertase and mannan, but it does it with a weak affinity and needs the presence of the CRDs 5–8 to bind with an affinity identical to that of the native receptor (43, 44).

Thus, as for the macrophage mannose receptor, the CRD 4 structure of the M-type sPLA₂ receptor is involved in the binding of glycosylated ligands, but the CRD 5–8 structure is required to confer a full affinity. Although the same CRDs appear to be involved in the binding of mannose-BSA, several lines of evidence suggest that the amino acid residues within these CRDs, which are implicated in the binding process, are different in the two receptors. First, these two receptors, although predicted to share a similar overall structural organization, only display a low identity (28%) at the amino acid level (30).

The percentage of identity between CRDs in identical positions in M-type sPLA₂ receptors from different species are given for CRDs 3 to 6.

\[ \text{Table II} \]

| CRD | Human | Bovine | Mouse | Rat |
|-----|-------|--------|-------|-----|
| 3   | 87    | 92     | 82    | 86  |
| 4   | 82    | 90     | 83    | 81  |
| 5   | 75    | 83     | 69    | 77  |
| 6   | 79    | 82     | 69    | 77  |

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^2^ J.-P. Nicolas, G. Lambeau, and M. Lazdunski, unpublished observations.
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