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# Spinophilin inhibits the binding of RGS8 to M1-mAChR but enhances the regulatory function of RGS8

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ABSTRACT

We showed previously that RGS8 directly binds to the third intracellular loop (i3L) of the M1 muscarinic acetylcholine receptor using the sequence MPRR at the N-terminus of RGS8 and specifically inhibits signal transduction. Here, we identified spinophilin (SPL) as an RGS8-interacting protein. We found that the SPL-binding site of RGS8 is the MPRR sequence, and the M1 receptor and SPL compete for binding to RGS8. However, we also observed that the expression of SPL significantly enhances the inhibitory function of RGS8, and that SPL can bind to the M1 receptor, demonstrating the indirect binding of RGS8 to the M1 receptor through SPL for an efficient regulatory function.



RGS (regulators of G-protein signaling) proteins act as GTPase-activating proteins (GAPs) toward Gα proteins [1,2]. RGS proteins negatively regulate G-protein signaling and sometimes also function to accelerate the kinetics of G-protein-coupled receptor (GPCR) systems [3,4]. The B/R4 subfamily of RGS is relatively small and consists of a conserved N-terminal cationic amphipathic α-helix and the conserved RGS domain [1,2,5]. Most of the members of this family can interact with Gi/o and Gq class Gα proteins *in vitro* and inhibit Gi/o and Gq-mediated signaling in mammalian cells [1,6]. However, further studies showed that several B/R4 members can distinguish between receptor pathways coupled to the same G-protein [7–9]. As a molecular mechanism for receptor-specificity, it has been demonstrated that some B/R4 RGS proteins can directly interact with the third intracellular loops (i3L) of certain GPCRs to regulate their function [10–12]. Furthermore, a recent study identified spinophilin (SPL) as a scaffold that binds both the i3L of GPCRs and B/R4 RGS proteins to regulate receptor-mediated signaling [13,14]. Indirect receptor recognition by B/R4 RGS protein has been also demonstrated to contribute to the receptor-specificity of B/R4 RGS proteins. However, it is not still clear how the direct receptor recognition relates to the indirect receptor recognition, the extent to which each receptor recognition event contributes to receptor-specificity, and whether other new proteins are also involved in the indirect receptor recognition.

We previously identified RGS8, which belongs to the B/R4 subfamily and binds specifically to Gαo and Gαi3 but not to Gαq [3]. We also showed that RGS8 decreased the response upon activation of substance P or M1 muscarinic acetylcholine receptor (mAChR) but did not remarkably inhibit signaling from M3-mAChR. Thus, RGS8 suppressed Gq signaling depending on the receptor type despite its weak binding to Gαq protein [9]. Next, we studied how RGS8 attenuates Gq signaling in a receptor type-specific manner, and found that RGS8 binds strongly to i3L of M1-mAChR and weakly to i3L of M3-mAChR. By mutagenesis analysis, we clarified that the interaction between RGS8 and M1-mAChR requires the sequence of 6–9aa of the N-terminus of RGS8 (MPRR) and that the two arginine residues (R8, R9) are the key amino acids. We further observed that an RGS8 mutant with lower binding ability to M1-mAChR showed a reduced inhibitory function on Gq signaling through M1-mAChR, demonstrating that the direct interaction between RGS8 and certain receptors is one of the mechanisms for receptor type-specific regulation by RGS8 [12].

To examine the possibility of an indirect receptor recognition system of RGS8 and to investigate the relationship between direct and indirect receptor recognition, we carried out a two-hybrid screen for RGS8-interacting proteins. We also identified SPL as a

*Abbreviations:* RGS, regulators of G-protein signaling; G-protein, heterotrimeric guanine nucleotide-binding proteins; GPCR, G-protein-coupled receptor; MACHR, muscarinic acetylcholine receptor; i3L, third intracellular loop; BRET, bioluminescence energy transfer; GST, glutathione-S-transferase; SPL, spinophilin

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binding partner of RGS8 from the cerebellum. Then, we focused on the binding sites of RGS8 for M1-mAChR and SPL, analyzed the binding properties among these three proteins, and studied the functional effect of SPL expression on the regulation of M1 signaling by RGS8.

## Materials and methods

*DNA constructs.* All constructs were prepared by a polymerase chain reaction (PCR) strategy. Expression constructs for recombinant proteins of wild type, deletion mutants, the N9-RGS8S chimera, and point mutants (RGS8R8A, RGS8R9A, RGS8R8A/R9A) of RGS8 with N-terminal hexahistidine tags were described previously [3,12]. For RGS8 recombinant protein with a C-terminal hexahistidine tag (RGS8-His), the PCR fragment containing the coding sequence of RGS8 was cloned into the *Nde* I-*Not* I sites of pET-20b (Novagen).

For the bacterial expression of a fusion of glutathione-S-transferase (GST) and the i3L domain of M1-mAChR, the cDNA of M1i3L was subcloned into pGEX-2T (GE Healthcare; kindly provided by Dr. Levey, Emory University) [10,15]. For the GST-spinophilin construct (GST-SPL), the cDNA fragment encoding spinophilin (amino acids 391–545) was excised from a pGADT7 clone, which was isolated by yeast two-hybrid screening, as an *EcoR* I-*Xho* I fragment and subcloned into the *EcoR* I-*Xho* I sites of pGEX-4T1. The cDNA fragment containing the receptor-binding region (amino acids 100–480, [16,17]) was further isolated from a rat SPL cDNA (kindly provided by Dr. Shmuel Muallem, Univ. of Texas Southwestern Medical Center) by PCR-amplification, and was cloned into the *EcoR* I-*Sal* I sites of pGEX-4T1 (GST-SPLRB).

For heterologous expression in *Xenopus* oocytes, the RGS8 cDNA was cloned into pGEMHE vector, which includes 5′ and 3′ non-coding sequences of the *Xenopus* β-globin gene and enables a high expression level of the protein in oocytes. The construct was used for cRNA transcription as described previously [9,18]. The coding sequence of SPL with a myc-tag was amplified by PCR from the expression vector of myc-tagged full-length SPL and also subcloned into pGEMHE.

*Yeast two-hybrid screens.* The coding sequence of RGS8 was amplified by PCR, cloned into the pGBKT7 vector, and used to screen a mouse cerebellum cDNA library as suggested by the manufacturer (Clontech). Positive clones were analyzed by sequencing.

*GST pull-down assay.* The assays for the interaction between GST-SPL and His-RGS8 proteins were performed as described previously [10,12]. Briefly, GST (4 μg) or GST-SPL (8 μg) were mixed with His-RGS8 proteins (4 μg) in buffer B containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 20% glycerol, 1.5 mM $MgCl_2$, 0.5% Triton X-100, 1 mM DTT, 1 mM PMSF, and complete mini EDTA-free (Roche) and incubated. After further incubation with glutathione-beads, the beads were washed with buffer B. Bead-bound proteins were subjected to Western blot analysis with an anti-RGS8 antibody as described previously [12]. The assay for the interaction between GST-SPLRB and full-length M1-mAChR was carried out as follows. N-terminal 3×HA-tagged M1 receptor protein was produced using human M1-mAChR cDNA (UMR cDNA Resource Center) as a template in coupled *in vitro* transcription/translation reactions (TNT T7 Quick system, Promega). GST (20 μg) or GST-SPLRB (80 μg) was mixed with 20 μl of TNT reaction product in buffer B and a GST pull-down assay was carried out as described above. M1 receptor was detected using an anti-HA antibody (Roche).

*Pull-down assay with $Ni^{2+}$-NTA beads.* RGS8-His, GST, GST-M1i3L, and GST-SPL were used for the pull-down assay with $Ni^{2+}$-NTA beads. These proteins (20 μg each) were mixed in various combinations in buffer C containing 50 mM NaCl, 10 mM HEPES pH 7.5, 0.25% Triton X-100, 1.5 mM $MgCl_2$, 10 mM Imidazole-HCl, 1 mM DTT, and 0.1 mM PMSF and incubated. After further incubation with $Ni^{2+}$-beads, the beads were washed with buffer C. The proteins bound to $Ni^{2+}$-beads were analyzed by Western blot with an anti-GST antibody (GE Healthcare).

*Two-electrode voltage clamp.* Functional expression in *Xenopus* oocytes and electrophysiological analysis using a two-electrode voltage clamp were performed as described previously [9]. Oocytes of the functional expression experiments were frozen after electrophysiological analysis and sonicated in PBS containing protease inhibitor. The homogenate was centrifuged at 3000 rpm at 4 °C for 10 min, and the resultant supernatant was used as a whole protein extract of oocytes. To compare the expression levels of RGS8 protein in oocytes, Western blotting of whole protein extracts was performed using an anti-RGS8 antibody as described above.

## Results and discussion

### *Identification of spinophilin as a binding partner of RGS8*

To examine the possibility of the indirect recognition of G-protein-coupled receptors (GPCRs) by RGS8, we searched for RGS8-binding proteins linking to GPCRs. By yeast two-hybrid screening of a mouse cerebellum library using RGS8 as the bait, twenty positive clones were isolated. Sequencing analysis revealed that one strong positive clone encoded the middle part (391–545 aa) of the scaffold protein spinophilin (SPL) [19]. To further examine whether RGS8 can actually bind to SPL, a GST fusion protein containing 391–545 aa of SPL (GST-SPL) was generated and used for pull-down assays with recombinant RGS8 protein with an N-terminal hexahistidine tag (His-RGS8). The result indicated that RGS8 binds to SPL (Fig. 1A, lane 1). On the other hand, RGS8S, which is a splice variant with 7 unique N-terminal amino acids instead of amino acids 1–9 of RGS8 [9], showed a quite weak binding ability to SPL (Fig. 1A, lane 5).

### *The spinophilin-binding site of RGS8 corresponds to the receptor-binding site*

Since the difference between two RGS8 variants is only present in the N-terminus, the N-terminus of RGS8 was considered to be important for the interaction with SPL. To determine the SPL-binding site of RGS8, we performed pull-down assays of GST-SPL using a series of N-terminal deletion mutants of RGS8 (Fig. 1A). We observed that the binding of His-ΔN5RGS8 to SPL was same as His-RGS8 but those of His-ΔN9RGS8 and His-ΔN15RGS8 were dramatically decreased. In addition, to test whether the N-terminal 9 aa of RGS8 can promote binding of RGS8S, we used a His-N9-RGS8S chimera, which was constructed by adding the first 9 amino acids of RGS8 to RGS8S. This chimera bound to SPL more efficiently than His-RGS8S and its binding was similar to that of His-RGS8. Thus, it was shown that the N-terminal 9 aa of RGS8 are essential for the interaction between RGS8 and SPL. It was also suggested that key amino acids that are responsible for the interaction between RGS8 and SPL may exist within 6–9 aa of RGS8. In the previous study, we found that the interaction between RGS8 and M1-mAChR requires the sequence of 6–9 aa at the N-terminus of RGS8 (MPRR), and the two arginine residues (R8, R9) are the key amino acids for the RGS8-M1 receptor interaction [12]. Therefore, we performed pull-down experiments using point mutants of RGS8 in which either or both of the two arginine residues were replaced with alanine (His-RGS8R8A, His-RGS8R9A, and His-RGS8R8A/R9A, respectively). As shown in Fig. 1B, we observed that the single point mutations (R8A or R9A) resulted in slightly decreased binding, whereas the double point mutation resulted in a

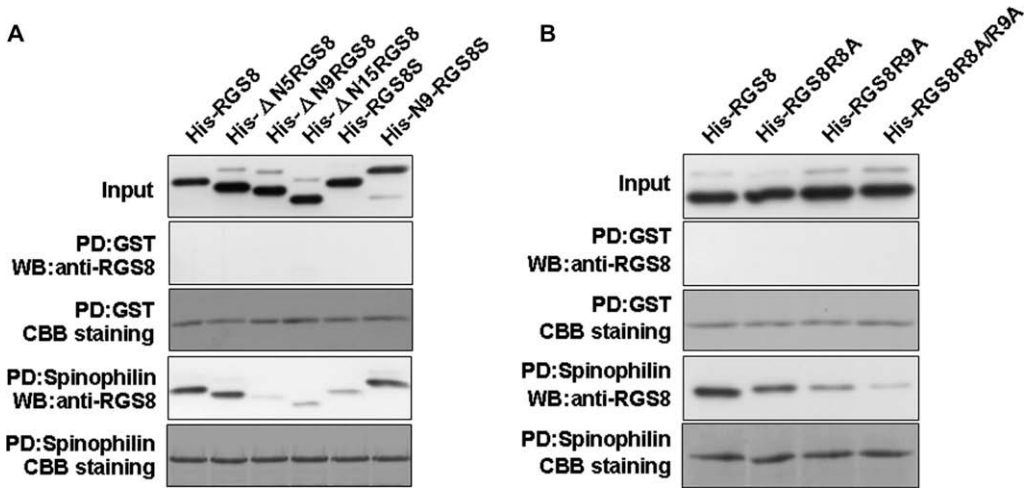


**Fig. 1.** Spinophilin binding site on RGS8 is the MPRR of N-terminus. GST alone (4 μg) or GST fusion protein containing 391–545 aa of spinophilin (8 μg) were incubated with His-RGS8, His-RGS8S, or their mutants (4 μg each) and recovered using glutathione-beads. The beads were washed and bead-bound proteins (PD) were eluted with SDS sample buffer. A, we generated deletion mutants of RGS8 lacking the N-terminal amino acids as indicated. The N9-RGS8S chimera was constructed by fusing the first 9 amino acids of RGS8 to the full-length RGS8S sequence at the N-terminus. Bound RGS8 proteins were analyzed by Western blot with an anti-RGS8 antibody (WB: anti-RGS8). The SDS–PAGE gel of the same set of fractions was also subjected to CBB staining as a loading control (CBB staining). Only the band of GST alone or GST fusion protein (27 or 44 kDa, respectively) is shown. Results were repeated twice and similar results were obtained. B, GST pull-down assays with His-RGS8 or its point mutants (4 μg) were performed. The R8A and R9A mutants were created by substituting Arg-8 or Arg-9 of RGS8 with Ala, respectively. The R8A/R9A mutant was obtained by substituting both Arg-8 and Arg-9 with Ala. Bound RGS8 proteins were analyzed by Western blotting with an anti-RGS8 antibody (WB: anti-RGS8). An SDS–PAGE gel of the same set of fractions was subjected to CBB staining as a loading control. The experiments were repeated twice and similar results were obtained.

remarkable decrease in binding. Thus, it was demonstrated that RGS8 binds to SPL through the sequence of 6–9 aa of the N-terminus of RGS8, and the two arginine residues there are key amino acids. These results imply that the binding site of RGS8 for SPL is located at the same position as the M1 receptor-binding site.

*Spinophilin competes with M1-mAChR for the binding to RGS8*

Since the binding site of RGS8 for SPL and M1-mAChR was shown to be the same, we investigated whether SPL competes with M1 receptor for binding to RGS8.

We first performed pull-down experiments with three recombinant proteins. We generated a recombinant protein of RGS8 with a C-terminal hexahistidine tag (RGS8-His). We used proteins of RGS8-His, GST-M1i3L, and GST-SPL (391–545 aa) for the pull-down assay with $Ni^{2+}$-NTA beads (Fig. 2). GST-SPL (391–545 aa) contains only a short part of the sequence required for binding to the i3L of several GPCRs (151–444 aa, [16]). When RGS8-His was incubated with GST-SPL, $Ni^{2+}$-beads precipitated RGS8-His with GST-SPL. This co-precipitation of GST-SPL with RGS8-His was significantly reduced in the presence of GST-M1i3L (reduced to 62%, estimated by densitometry). No reduction was observed with the GST-only control. When RGS8-His was mixed with GST-M1i3L, co-precipitation of M1i3L with RGS8-His was observed using $Ni^{2+}$-beads. The addition of GST-SPL decreased the binding of M1i3L to RGS8-His to 20%. To further confirm this competition of SPL and M1-mAChR for RGS8, we analyzed how the expression of SPL affects the association between M1-mAChR and RGS8 in cells by BRET assay [20,21] (see Supplementary Information). Results supported the same conclusion. Thus, the results from these pull-down and BRET experiments demonstrated clearly that M1-mAChR and SPL compete for binding to RGS8.

*Spinophilin enhances the regulatory function of RGS8 on the M1-mAChR*

We previously reported that the direct interaction between the N-terminus of RGS8 and M1-mAChR confers receptor-selective inhibitory functions on Gq signaling by RGS8. In this study, we indicated that SPL expression reduces the association of M1-mAChR with RGS8. Therefore, we studied how SPL modifies the function of RGS8 in M1 receptor-mediated signaling by monitoring the increase of $Ca^{2+}$-activated $Cl^-$ current caused by intracellular $Ca^{2+}$ in *Xenopus* oocytes (Fig. 3). As shown previously, when RGS8 was expressed with M1-mAChR, RGS8 suppressed Gq signaling from M1-mAChR. Then, SPL and RGS8 were expressed with M1-mAChR. Unexpectedly, the inhibitory function was significantly enhanced. The single expression of SPL had no significant effects on M1-mAChR signaling. When Western blot analysis with anti-

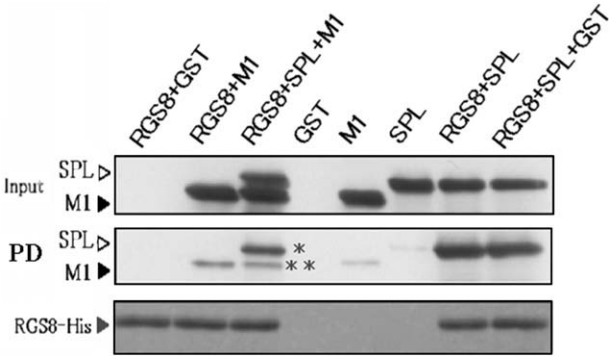


**Fig. 2.** Competitive binding of spinophilin and M1-mAChR to RGS8. The recombinant proteins of RGS8 with C-terminal hexahistidine tags (RGS8), GST, GST fusion protein containing the third intracellular loop of M1-mAchR (M1), and GST fusion protein containing 391–545 aa of spinophilin (SPL) were used for pull-down assays with $Ni^{2+}$-NTA beads. These proteins (20 μg each) were mixed in various combinations (SPL and M1 are shown in top panel as Input) and the proteins bound to $Ni^{2+}$-beads (PD) were analyzed. Western blot analysis with an anti-GST antibody was performed (top and middle panels). The SDS–PAGE gel of the same set of pull-down fractions was also subjected to CBB staining as a loading control (bottom). The experiments were repeated twice and similar results were obtained. The intensities of the bands of the pull-down fractions quantified by Densitograph (Atto) were compared. The precipitation of SPL with RGS8 was reduced in the presence of M1 to 62% (*). The precipitation of M1 with RGS8 decreased to 20% with SPL (**).

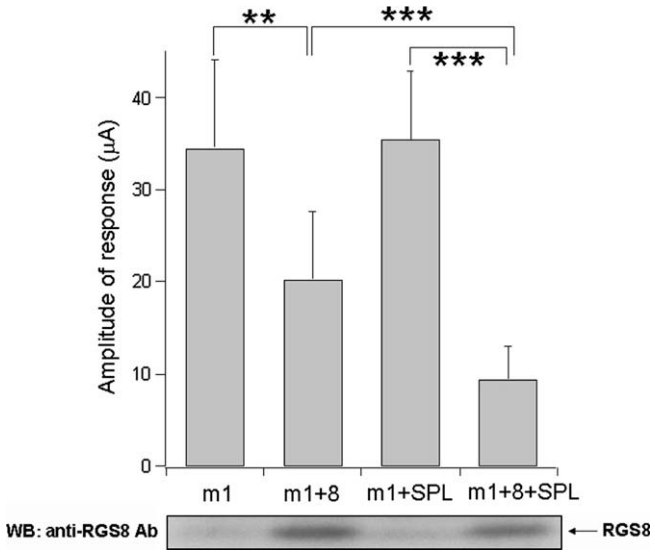


**Fig. 3.** Spinophilin enhances the regulatory function of RGS8 in M1 receptor-mediated signaling. In the same batch of *Xenopus* oocytes, M1-mAChR was expressed alone, with RGS8, with spinophilin, or with RGS8 and spinophilin by injecting mixtures of cRNA. The responses upon stimulation of M1-mAChR by 10 μM ACh were monitored as an increase in the amplitude of the $Ca^{2+}$-$Cl^-$ current at +60 mV. The peak amplitudes of the responses were compared ($n = 10$). The mean ± SD of each group were plotted. Differences were judged to be statistically significant by Student's unpaired *t*-test ($^{**}P < 0.01$; $^{***}P < 0.001$). Similar results were observed in two independent sets of experiments. After electrophysiological analysis, the whole protein fraction of the oocytes was extracted and Western blotting was performed using an RGS8 antibody (bottom).

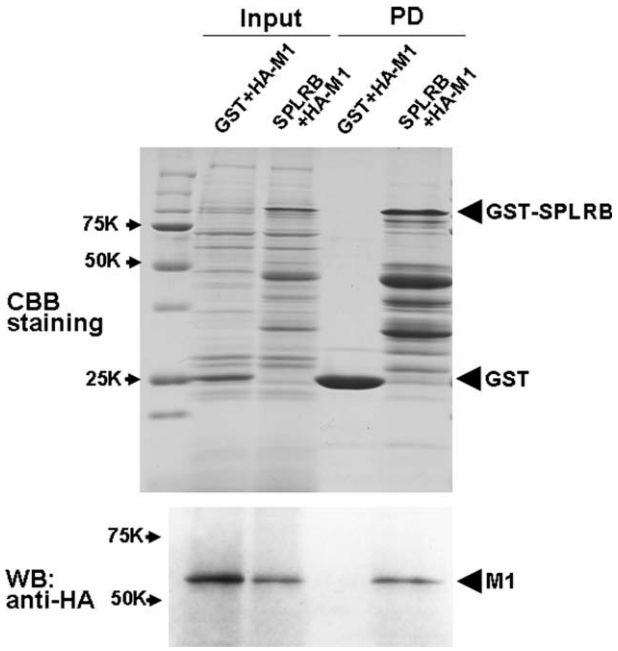


**Fig. 4.** Spinophilin can bind to M1-mAChR. GST fusion protein containing the receptor-binding region (amino acids 100–480) of SPL was prepared (GST-SPLRB). GST (20 μg) or GST-SPLRB (80 μg) was incubated with the N-terminal 3×HA-tagged M1 receptor protein, which was generated by *in vitro* transcription/translation reactions, and recovered by glutathione-beads. The beads were washed and bead-bound proteins (PD) were eluted with SDS sample buffer. Bound proteins were analyzed by Western blotting with an anti-HA antibody (WB: anti-HA). The SDS–PAGE gel of the same set of fractions was also subjected to CBB staining (CBB staining). The experiments were repeated twice and similar results were obtained.

RGS8 antibody was performed, similar levels of expression of RGS8 were confirmed in oocytes injected with RGS8 cRNA. These results raised the possibility that SPL removes direct binding of RGS8 from the M1 receptor and that a new complex M1-SPL-RGS8 is formed to efficiently regulate G-protein signaling.

*SPL can bind to M1-mAChR*

After the removal of RGS8 binding from the M1 receptor by SPL, it is possible that the SPL-RGS8 complex may associate with the M1 receptor using the receptor-binding region of SPL to form a complex of M1-SPL-RGS8. To address this possibility, we generated a GST fusion protein containing the receptor-binding region (amino acids 100–480, [16,17]) of SPL (GST-SPLRB). This GST-SPLRB fusion protein was incubated with the full-length M1 receptor protein and pull-down assays were performed. As shown in Fig. 4, GST-SPLRB could precipitate the M1 receptor, indicating the binding ability of SPL to M1-mAChR. Therefore, it is strongly suggested that RGS8 can interact indirectly but efficiently with M1-mAChR through SPL by the formation of an M1-SPL-RGS8 complex to specifically regulate G-protein signaling.

In this study, to understand the mechanism of receptor type-specific regulation by RGS8, we carried out a two-hybrid screen for RGS8-interacting proteins. We identified a cDNA clone encoding the middle part (391–545 aa) of spinophilin (SPL) from the cerebellum. By *in vitro* binding assays using deletion mutants of RGS8, we found that the SPL-binding site is located in the MPRR residues of the N-terminus of RGS8. This N-terminal sequence exactly corresponds to the M1-mAChR binding site on RGS8 [12]. We then observed that the M1 receptor and SPL 391–545 aa compete for the binding site on RGS8 with pull-down experiments and further confirmed this competition by BRET assays. Therefore, we examined how SPL modifies the regulatory function of RGS8 in M1 signaling, and found that the expression of SPL significantly enhances the inhibitory function of RGS8. Finally, we also found that SPL can bind to the M1 receptor, demonstrating that SPL removes the direct binding of RGS8 from M1 to form the M1-SPL-RGS8 complex for the efficient regulation of G-protein signaling. As a result, the selectivity of receptor signaling controlled by RGS8 depends on the receptor-binding properties of SPL. Although a clarification of the binding properties to GPCRs and SPL of other B/R4 RGS proteins is required, this regulatory system of receptor recognition might be general for the B/R4 RGS proteins.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.09.096.

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