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Improved expression and purification of sigma 1 receptor fused to maltose binding protein by alteration of linker sequence

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Abstract

Sigma 1 receptor (S1R) is a eukaryotic membrane protein that functions as an inter-organelle signaling modulator and chaperone. Here we report an improved expression of S1R in *Escherichia coli* as a fusion to maltose binding protein (MBP) and a high-yield purification. Variants with linking amino acid sequences consisting of 0 to 5 alanine residues between MBP and S1R were created and tested in several *E. coli* expression strains in order to determine the best combination of construct and host for production of active MBP-S1R. Among the linker variations, the protein containing a 4-Ala linker exhibited superior expression characteristics (MBP-4A-S1R); this construct was most productively paired with *Escherichia coli* B834-pRARE2 and a chemically defined growth and expression medium. A 3-step purification was developed, including extraction from the *E. coli* membrane fraction using a mixture of Triton X-100 and *n*-dodecyl-beta-D-maltopyranoside identified by screening constrainted by retention of binding function, and purification by amylose affinity and gel filtration chromatographies. This procedure yields ~3.5 mg of purified fusion protein per L of bacterial culture medium. Purified MBP-4A-S1R showed a 175-fold purification from the starting cellular lysate with respect to specific ligand binding activity, and is stable during concentration and freeze-thaw cycling.

Keywords

sigma 1 receptor; maltose binding protein; fusion protein; *Escherichia coli*; selenomethionine labeling; integral membrane protein

Introduction

Sigma 1 receptor (S1R) is a 223 amino acid eukaryotic membrane protein found in the ER membrane of tissues of the endocrine, immune, and nervous systems. S1R interacts with progesterone and testosterone, and a diverse set of compounds including cocaine amphetamines, haloperidol, pentazocine, ditolylguanidine, hallucinogens and others [1–3]. S1R is implicated in inter-organelle signaling associated with neurological disorders and stroke, regulation of calcium homeostasis in mitochondria, sterol hormone synthesis and the etiology of addiction [4–10]. Knockout mice, which are otherwise viable, exhibit a variety

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of changes in psychological responses to stimuli, implicating S1R in pain response, learning, and psychoses [11–13]. S1R also interacts with other membrane proteins like acid-sensing channel and Nav1.5 voltage-gated Na^+ channel, potentially providing modulator or chaperone-like properties [14,15].

S1R is a member of the ERG2_Sigma1 family (PFAM PF04622), which has a single domain architecture; greater than 100 homologous sequences have been identified in different eukaryotes. The family also includes fungal sterol binding proteins [16]. The primary sequence is highly conserved among different mammals, with >90% identity over 223 residues in human, chimpanzee, mouse, cow, rat, Mongolian gerbil and others [16]. Alternative splicing may produce transcript variants encoding distinct isoforms whose functions are not yet established. Although there are no three-dimensional structures known for this family, a membrane topology model for S1R has been assembled from biochemical and biophysical studies [17, 18]. S1R is thus predicted to be an α-helical membrane protein with two potential transmembrane domains [19].

S1R genes have been cloned from rodents and humans, and the protein has been expressed in *Escherichia coli*, *Saccharomyces cerevisiae* and CHO cells [2, 20–23]. The average yield of purified functional protein, whether from previous recombinant systems or from natural tissues, is low: e.g., 0.2 mg/L from *E. coli* culture and 0.2 mg from microsomes prepared from guinea pig liver.

Maltose binding protein (MBP) has been fused to membrane proteins in order to promote their expression, purification, and formation of crystals [24–30]. Moreover, periplasmic export of MBP has been shown to facilitate incorporation of appended membrane protein domains into the bacterial membrane [31–33]. Correspondingly, previous work showed that an MBP-S1R fusion containing a linker sequence for the Factor Xa protease recognition sequence could be expressed in *E. coli* BL21(DE3) and a functional form could be obtained, albeit in low yield, 0.6 mg of fusion protein per L of culture medium [2]. Our new results show that the *E. coli* strain used for expression and the linker region between MBP and S1R play important roles in production of the active form of the receptor. Arising from the improved expression, improved detergent extraction and improved purification protocols for MBP-S1R have been developed, and results from these are reported.

Materials and Methods

Reagents

All reagents were ACS grade unless otherwise specified. All buffers were prepared from deionized and distilled water (18MOhm) and filtered through a 0.8 μm filter.

Cloning

The guinea pig S1R gene is summarized as Uniprot Q60492. A plasmid encoding guinea pig S1R fused to periplasm-exportable maltose binding protein with a linker including a tobacco etch virus protease recognition site (MBP-TEV-S1R) was used as the template for PCR reactions. This plasmid was derived from MBP-Xa-S1R [1, 2]. The TEV protease linker between MBP and S1R was mutagenized to contain zero to five Ala residues by use of PIPE cloning [34, 35]. PCR was done using Pfu-UltraII polymerase (Stratagene, La Jolla, CA) and the primers listed in Table 1. All oligonucleotides were purchased from IDT (Coralville, IA). When the PCR reaction was completed, the template was destroyed by Dpn1 digestion [35]. The Dpn1-digested PCR product was purified using a kit (Qiagen, Valencia, CA) and the eluted DNA was used to transform *E. coli* 10G chemically competent cells (Lucigen, Middleton, WI). Plasmids were isolated from transformants and DNA sequencing was used to identify those containing the correct linker and coding region.

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Expression strains

The progenitor MBP-TEV-S1R plasmid and new plasmids with variations in the linker region were transformed into the following *Escherichia coli* strains: BL21(DE3); B834-pRARE2; BL21(DE3)-RILP; C41(DE3)-pRARE2; and C43(DE3)-pRARE2. Chemically competent BL21(DE3) and B834 were from Novagen (Merck KGaA, Darmstadt, Germany), BL21(DE3)-RILP was from Stratagene, and C41(DE3) and C43(DE3) were from Lucigen. The rare codon supplementation plasmid pRARE2 was isolated from Rosetta 2 cells (Novagen) and then transformed into the appropriate strains [36]. All culture media were supplemented with 200 μg/mL of ampicillin; media used to grow strains transformed with the rare codon supplementation plasmids (pRARE2, RILP) were supplemented with 34 μg/mL of chloramphenicol.

Expression protocol

Starting inocula were grown in 3 mL of MDAG non-inducing medium at 37°C until the OD_{600} reached 0.2–0.3; the 3 mL culture was transferred to 100 mL of MDAG non-inducing medium [37]. The growth was continued at 25°C overnight with shaking. The scale-up of protein expression was carried out in selenomethionine-labeling medium 5SM having a modified sugar composition to permit IPTG induction (0.8% glycerol, 0.265% glucose, [37]). Polyethylene terephthalate 2-L soda bottles containing 480 mL of 5SM medium were inoculated with 20 mL of the overnight [38, 39]. The bottle cultures were grown at 37°C until the OD_{600} reached ~2. IPTG was then added to give a final concentration of 0.2 mM. The expression was continued at 25°C for ~20 h. Cells were harvested by centrifugation and stored at −80°C. A 0.1 g aliquot of cell paste was kept for expression analysis by denaturing electrophoresis.

Detergent solubilization screening

A 0.2 g aliquot of cell paste was re-suspended in 35 mL of 20 mM NaH_2PO_4, pH 7.5, containing 100 mM NaCl, 1 μM protease inhibitor E-64 (Sigma-Aldrich, Saint Louis, MO) and 0.3 mM (tris(2-carboxyethyl)phosphine). The cell suspension was sonicated on ice for 15 min using a Misonix 3000 sonicator (Farmingdale, NY) equipped with a micro tip horn (pulse on time of 2 s, pulse off time of 1 s) with output set to 7.0. The cell sonicate was centrifuged for 1 h at 75,000*g* between 4 and 8°C (Beckman-Coulter rotor JA- 25.50) and the resulting membrane fraction (pellet) was re-suspended in solubilization buffer containing 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 1 mM 2-mercaptoethanol to give a final volume of 800 μL. The total protein content was measured by reducing agent compatible bichronic assay (BCA, ThermoFisher Scientific, Rockford, IL). The BCA assay was modified from the original protocol as follows. Instead of diluting with sample buffer, all standards and dilutions were prepared with deionized water. The values calculated for buffers were subtracted from the values obtained for sample dilutions.

A detergent screen was prepared to test the efficiency of combining Triton X-100 (5% or 10% w/v) with a second detergent for extraction of MBP-TEV-S1R. The following second detergents were included: *n*-decylphosphocholine (FC-10), *n*-dodecylphosphocholine (FC-12), lauryldimethylamine N-oxide (LDAO), 3-[(chloramidopropyl)-dimethylammonio]-1-propansulfonate (CHAPS), *n*-dodecyl-beta-D-maltopyranoside (DDM), sodium cholate, and *n*-octyl-beta-D-glucopyranoside (β-OG). Triton X-100 and sodium cholate were from Sigma, CHAPS was AppliChem GmbH (Darmstadt, Germany), and all other detergents from Affymetrix-Anatrace (Santa Clara, CA). Properties and applications of detergents used in this solubilization screen are discussed in detail in by Linke [40] and others [41,42,43]. A detergent master plate sufficient for 5 screening reactions was prepared in a 96 well plate. A 45 μL aliquot was transferred from the master plate to a corresponding well in an assay microplate; a control well was given buffer instead

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of detergent. A 5-μL aliquot of the re-suspended membrane fraction was then added to each well. The assay plate was covered and incubated at 4°C overnight or at room temperature for 3 h. After incubation, the assay microplate was centrifuged for 1 h at 5,500 rpm (Beckman-Coulter rotor JS-5.9) at 10°C. The supernatant (containing the solubilized protein) was transferred to a new microplate, while the pellet in the original plate was re-suspended in 50 μL of solubilization buffer. Aliquots (10 μL) of the solubilized (S) and non-solubilized fractions (P-pellet) were separated by SDS-PAGE. The solubilization efficiency was calculated by visually comparing the corresponding pellet (P) and solubilized (S) lanes. When greater than 50% of the protein was present in the S fraction, the detergent combination and concentration was considered acceptable for further investigation of specific ligand binding activity as described below. The final detergent composition, which optimizes extraction and purification efficiency and specific ligand binding activity, was set at 6.9 mg of Triton X-100 and 6.2 mg DDM per mg of protein in the *E. coli* membrane fraction. This ratio was successfully applied to all extractions of S1R linker constructs from *E. coli* membrane fraction reported herein.

Extraction from Escherichia coli membranes

Escherichia coli cell paste from a 1 L growth was suspended in 25 mL of 20 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 1 mM 2-mercaptoethanol, and 1 mM of EDTA per gram of cells. The cell suspension was supplemented to contain 1 μM of protease inhibitor E-64 (Sigma) and 0.25 mM phenylmethyl sulfonyl fluoride (Sigma). The cell suspension was placed on ice/water/KCl slurry to prevent overheating and sonicated in 75 mL batches using a Misonix 3000 sonicator equipped with a 1 cm probe for 15 min total processing time with a pulse sequence of 2 s on, 1 s off and output set to 7.0. The total cell lysate was centrifuged for 1 h at 75,000*g* between 4 and 8°C (Beckman-Coulter rotor JA- 25.50) to separate the membrane fraction from soluble proteins. The pelleted membrane fraction was re-suspended in 3 mL of the above buffer per initial gram of cell paste and then analyzed for total protein content using the modified reducing agent compatible BCA reaction. Membrane protein solubilization was initiated by directly adding 6.2 mg of DDM and 6.9 mg of Triton X-100 per mg of total protein in the re-suspended membrane fraction. The extraction was performed for 3 h with gentle stirring at 4°C and then the solution was centrifuged for 1 h at 75,000*g* (Beckman-Coulter rotor JA- 25.50). The supernatant, containing the solubilized protein, was collected and diluted with buffer to decrease the Triton X-100 concentration to 1.0% (w/v).

Purification

A 2.2 cm diameter column containing 10 mL of amylose resin (New England BioLabs, Ipswich, MA) was equilibrated in 20 mM Tris-HCl, pH 8.0, containing 300 mM NaCl, 1 mM 2-mercaptoethanol, 1 mM of EDTA and 1% (w/v) Triton X-100. The supernatant containing the fusion protein was loaded onto the column at a flow rate of 2 mL/min at 4°C. After loading, the column was washed with 10 column volumes of equilibration buffer and then with 3 column volumes of equilibration buffer lacking EDTA. The fusion protein was eluted with 10 column volumes of the EDTA-free buffer containing 10 mM maltose; 5 mL fractions were collected. The purity of fractions obtained from the amylose column was determined by 4–20% gradient SDS-PAGE (BioRad, Hercules, CA). Protein concentration was estimated using the SDS-PAGE based stain-free technology (BioRad) or the reducing agent compatible bichronic assay (ThermoFisher Scientific). Following analysis of purity and protein concentration, the appropriate fractions were pooled and Triton X-100 was added to a final concentration of 0.031% (w/v). The pooled fractions were concentrated using 50-kDa molecular weight cut off centrifugal concentrator to a protein concentration of ~5–10 mg/mL. Preparative gel filtration was performed using a 25 mL Superdex 200 10/300 GL column (GE Lifesciences, Pittsburgh, PA) and an AKTA purifier. The column was

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equilibrated in 10 mM HEPES, pH 7.2, containing 150 mM NaCl, 0.3 mM TCEP, and 0.018% (w/v) DDM. The concentrated protein sample was injected in 500 μL aliquots and fractions were collected. The collected fractions were analyzed for purity and protein concentration and appropriate fractions were combined and concentrated as described above using 100-kDa molecular weight cut off centrifugal concentrator.

Ligand binding assays

Ligand binding assays were performed in 20 mM Tris, pH 8.0, containing 0.1% (w/v) Triton X-100 according to published protocols with the following modifications [2, 44, 45]. Assays were performed in 100-μL total reaction volume in a 48-well block format. Each assay contained 80 ng of total protein and each reaction (total binding and non-specific binding) was carried out in triplicate. The final concentration of $[H^3]$-(+)-pentazocine (specific activity 36 Ci/mmol, Perkin-Elmer, Waltham, MA) in both total and non-specific binding reactions was 100 nM. Haloperidol (Tocris, Bristol, UK) was used for masking in the non-specific binding reaction at final concentration of 10 μM. The incubation with ligands was performed for 90 min at 32°C, followed by filtration on a glass fiber filter (Whatman GF/B, Piscataway, NJ) in a Brandel cell harvester (Gaithersburg, MD). The glass filter was washed with 50 mM Tris, pH 8.0 and individual filters were transferred into vials containing 3 mL of scintillation cocktail (Ultima Gold, Perkin-Elmer). The level of radioactivity was measured the following day, employing a liquid scintillation counter (Packard model 1600 CA, Perkin Elmer). Raw count data were normalized to nmol of S1R in the assay and plotted as the percentage of specific binding activity of the original control sample, MBP-TEV-S1R. MBP purified in the same buffer and detergent conditions as the fusion protein was also assayed for ligand binding activity using the method described above. Less than 0.2% binding activity was detected.

Results

Expression construct design

We investigated whether the linker present in MBP-TEV-S1R (Table 2) could be replaced with shorter linkers in order to improve the expression and handling properties. Thus constructs with linkers consisting of one to five Ala residues and another construct that contained no additional amino acids between the MBP and S1R domains were produced by PIPE cloning [34, 35]. Because of the length of the TEV protease recognition site, and uncertainty regarding how this sequence would influence the secretion, we did not include this sequence in these constructs. The abbreviations for these constructs, their molecular weights, and the primary sequences of their linker regions are shown in Table 2.

Expression of MBP-TEV-S1R

In preliminary tests, we found that use of modified selenomethionine-labeling medium coupled with induction using a low concentration of IPTG (0.2 mM) gave a higher yield of purified control protein MBP-TEV-S1R (~3 mg/L) than the previously published protocol starting with growth in Luria Bertani medium (2 mg/L) [37, 2]. Consequently, this medium and induction method was used in all subsequent experiments.

Extraction from E. coli membranes

Previous studies showed that Triton X-100 was useful for stabilizing the active form of S1R [2]. However, since less than 50% of the total MBP-S1R fusion was extracted from *E. coli* membranes using Triton X-100 alone, we examined mixtures of Triton X-100 and one additional detergent for their extraction capability. Fig. 1 shows the composition of the screen, which assessed the ability of combinations of Triton X-100 (5% or 10% w/v) and a

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second detergent (FC-10, FC-12, CHAPS, LDAO, β-OG, DDM, and sodium cholate) to solubilize MBP-TEV-S1R. Several combinations of detergents were successful in solubilizing MBP-TEV-S1R. Indeed, the *yellow* highlighted combinations of detergents gave ~50% solubilization efficiency, whereas the *green* highlighted combinations gave greater than 90% recovery. Moreover, the specific ligand binding activity was also satisfactory for MBP-TEV-S1R extracted with the Triton X-100/DDM and Triton X-100/FC-10 combinations. For subsequent experiments, DDM was preferred over FC-10 because of the lower critical micelle concentration (0.009% versus 0.35% w/v, respectively), the lower cost, and the potential for better behavior in crystallization trials. The optimization of the Triton X-100/DDM mixture provided by this screening procedure gave an ~4-fold decrease in the amount of detergent used relative to earlier purifications while improving the efficiency of extraction and also retaining the specific ligand binding activity.

Influence of linker

Fig. 2 compares the results of purification of short linker constructs after expression in *E. coli* strains BL21(DE3) and B834-pRARE2. On average, better yields of protein were obtained from B834-pRARE2. Fig. 2A shows that the MBP-3A-S1R and MBP-4A-S1R gave roughly double the yield after amylose affinity chromatography as compared to MBP-TEV-S1R (10 and 12 mg/L versus 5 mg/L, respectively). Moreover, Fig. 2B shows that the specific pentazocine binding activities of MBP-3A-S1R and MBP-4A-S1R expressed in B834-pRARE2 were higher than MBP-TEV-S1R expressed in BL21(DE3). While the specific binding of MBP-1A-S1R expressed in BL21(DE3) was comparable to (or slightly better) relative to other active constructs, the yield (6 mg/L) was significantly less than for either MBP-3A-S1R (10 mg/L) or MBP-4A-S1R (12 mg/L), so further studies of MBP-1A-S1R were not undertaken.

MBP-3A-S1R and MBP-4A-S1R were also tested for expression in C41(DE3)-pRARE2 and C43(DE3)-pRARE2, two *E. coli* strains that were developed for overexpression of problematic proteins including integral membrane proteins [46, 47]. Fig. 3A shows that the yield from these two specialized strains was roughly equivalent to that obtained from B834-pRARE2. However, Fig. 3B shows that B834-pRARE2 gave the highest specific ligand binding activity of all tested strains.

Purification of MBP-S1R

Fig. 4 provides denaturing PAGE images of the purification of MBP-4A-S1R, while Table 3 summarizes a purification starting with 4 L of bacterial culture (~23 g of wet cell paste). Cell lysis, centrifugation and optimized two-detergent extraction from the bacterial membrane removed ~75% of the original cellular protein and also gave ~5-fold increase in the specific ligand binding activity. Amylose affinity chromatography gave another ~30-fold increase in specific ligand binding activity, and the protein obtained at this stage was greater than 90% pure based on visual inspection of the denaturing PAGE images. Gel filtration provided a further, modest increase in specific ligand binding activity. The protein-detergent complex was estimated to have molecular mass of ~470 kDa based on retention times observed during calibrated gel filtration.

The purified protein could be concentrated using centrifugation, and concentrations greater than 10 mg/mL could be routinely obtained in the identified buffer and detergent composition. Overall, MBP-4A-S1R was purified by 175-fold from the cell lysate. The purified protein was stable to freezing and storage at –80°C as indicated by minimal changes in specific ligand binding activity relative to original assay results. Furthermore, after 5 months of storage at 4°C, MBP-4A-S1R retained ~98% of the original specific ligand binding activity.

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Discussion

Here we have presented an improved procedure for expressing and purifying MBP-S1R in a form that is suitable for additional research on biological function, biophysical characterizations, and potentially structure determination. Three key improvements in the methodology for MBP-S1R are summarized here.

Both the yield of fusion protein and the specific ligand binding activity were best in the rare codon supplemented strain *E. coli* B834-pRARE2. This strain has been successfully used in the Center for Eukaryotic Structural Genomics as part of the NIH-funded Protein Structure Initiative since 2003. We previously showed this strain can be effectively combined with a customized growth and expression medium that permits autoinduction if desired and high-level incorporation of selenomethionine for structure determination [38, 48]. This combination of host strain and medium was successfully applied to MBP-S1R. Rare codon supplementation in strain BL21(DE3)-RILP did not improve the average yields of active S1R fusion protein. Moreover, although the codon supplemented specialty strains C41(DE3)-pRARE2 and C43(DE3)-pRARE2 did give levels of protein expression roughly comparable to that observed with B834-pRARE2, these former strains gave significantly lower yield of active S1R fusion protein.

We found that shortening the linker between the MBP and S1R domains had a strong influence on the expression level and the specific ligand binding activity of the resulting fusion protein. The best case, based on the combination of high specific ligand binding activity and yield of purified protein, MBP-4A-S1R, had the two domains separated by only 4 Ala residues. Shorter linkers steadily decreased the yield of purified protein.

Detergent screening showed that active MBP-4A-S1R could be efficiently extracted from *E. coli* membranes by a combination of Triton X-100 and DDM. The detergent mixture decreased the total amount of detergent needed, and improved the extraction to near quantitative level while also retaining the specific ligand binding activity. The subsequent two-step chromatographic purification yielded purified protein that also retained high specific ligand binding activity. These improvements are of great advantage, as they may facilitate future studies on this enigmatic membrane protein.

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Highlights

- Yield of active MBP-S1R depends on *E. coli* expression host and domain linker length
- *Escherichia coli* B834-pRARE2 and a 4-Ala linker gave the highest yield of active protein
- The fusion protein can be purified in high yield in a mixture of Triton-X100 and DDM
- The purified fusion protein has high specific ligand binding activity

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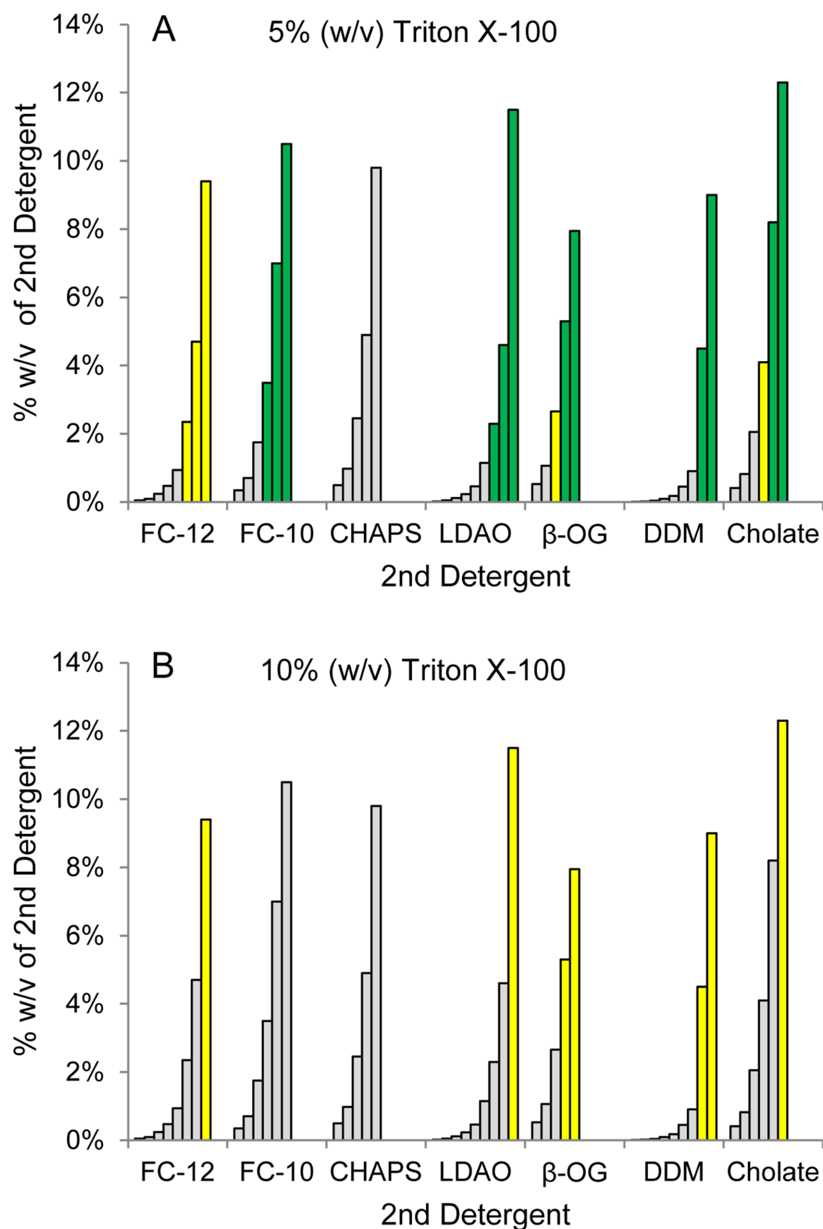

Fig. 1.

Detergent mixtures used to extract MBP-S1R variants from *Escherichia coli* membranes. In A, 5% Triton X-100 (w/v) was mixed with various amounts of a second detergent; 10% Triton X-100 (w/v) was used in B. Bars with *yellow* color indicate at least 50% extraction; bars with *green* color indicate 90% or greater extraction; *gray* bars indicate less than 50% extraction.

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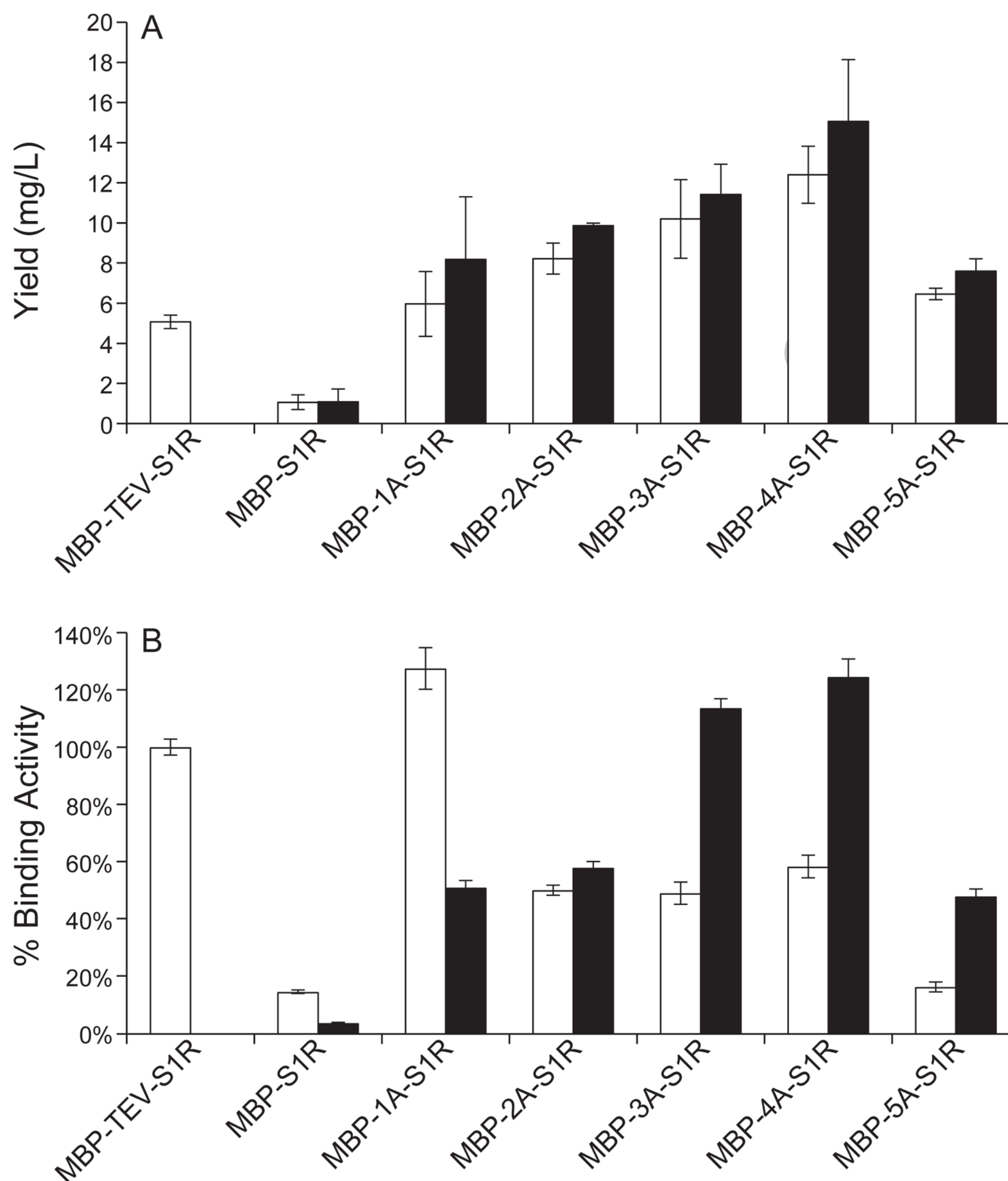

Fig. 2.

Protein yields and specific ligand binding activity determined for MBP-S1R variants with different linker regions. A, average protein yields from amylose affinity purification of MBP-SR1 variants expressed in *E. coli* strains BL21(DE3) (white bar) and B834-pRARE2 (black bar) compared to the yield of MBP-TEV-S1R expressed in BL21(DE3) (n = 3; error bars represent 1σ deviation). B, average specific binding activities (cpm/nmol) presented as a percentage of the specific ligand binding activity observed for MBP-TEV-S1R. MBP-4A-S1R and MBP-3A-S1R expressed in B834-pRARE2 gave the highest yield of the active protein, and so were used in subsequent studies.

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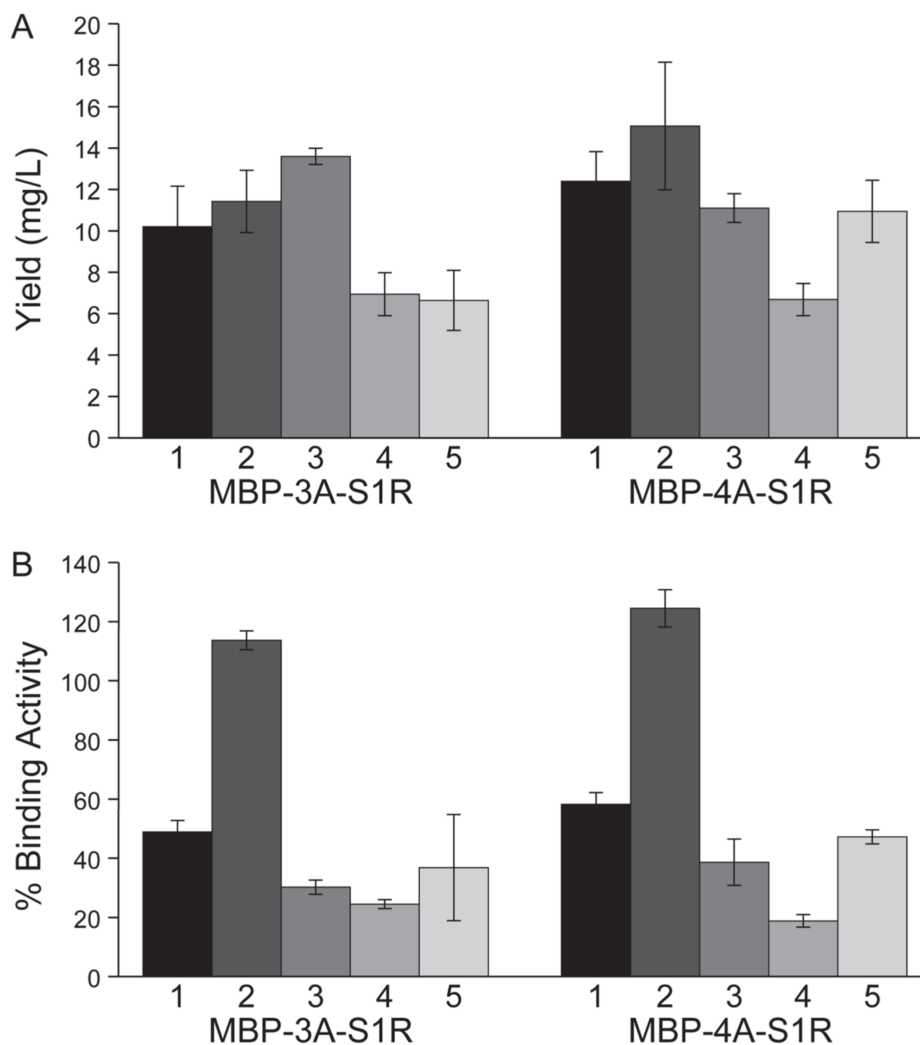

Fig. 3.

Expression of MBP-3A-S1R and MBP-4A-S1R in *E. coli* strains BL21(DE3) (1), B834-pRARE2 (2), C41(DE3)-pRARE2 (3), BL21(DE3)-RILP (4) and C43(DE3)-pRARE2 (5). After expression and purification by amylose affinity, the average yields and ligand binding activity were determined. A, yields of the purified MBP-S1R variants (n = 3; error bars represent 1σ deviation). B, specific ligand binding activities of the purified MBP-S1R variants presented as a percentage of the binding activity of MBP-TEV-S1R.

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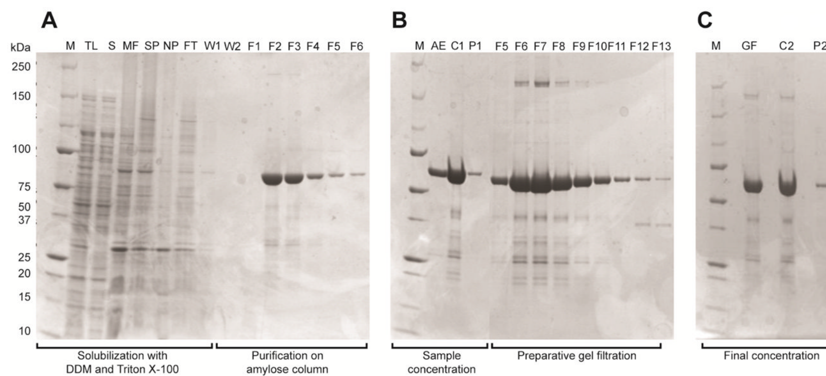

Fig. 4.

Denaturing PAGE analysis of the purification of MBP-4A-S1R. The *lanes* are annotated as follows: M, markers; TL, total lysate; S, soluble fraction; MF, membrane fraction; SP, solubilized membrane proteins; NP, non-solubilized membrane proteins; FT, flow-through of the amylose affinity column; W1, first column wash; W2, second column wash; F1–F6, fractions eluted from the amylose affinity column; AE, pooled fractions after elution from the amylose affinity column; C1, concentrated sample from amylose affinity chromatography; P1, precipitate formed during concentration; F5–F13, fractions eluted from the gel filtration column; GF, pooled fractions from gel filtration chromatography; C2, concentrated sample from gel filtration; P2, precipitate formed during concentration. A, extraction of MBP-4A-S1R from *E. coli* membranes and results of amylose affinity chromatography. Fractions F2–F5 were pooled and concentrated. B, Superdex 200 10/300 gel filtration chromatography. Fractions F5–F10 were pooled and concentrated. C, MBP-4A-S1R was purified to greater than 90% purity by visual inspection.

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Table 1

PCR primers used in this work[a]

Primer name	Nucleotide sequence (5′-3′)	Length
gpS1R-F	ATGCAGTGGGCCGTGGGCCGGCGATG	26
gpS1R-A5R	GCCCACGGCCCACTGCATagctgcagctgcagcAGTCTGCGCGTCTTTCAGGGCTTC	57
gpS1R-A4R	GCCCACGGCCCACTGCATtgcagctgcagcAGTCTGCGCGTCTTTCAGGGCTTC	54
gpS1R-A3R	GCCCACGGCCCACTGCATagctgcagcAGTCTGCGCGTCTTTCAGGGCTTC	51
gpS1R-A2R	GCCCACGGCCCACTGCATtgcagcAGTCTGCGCGTCTTTCAGGGCTTC	48
gpS1R-A1R	GCCCACGGCCCACTGCATagcAGTCTGCGCGTCTTTCAGGGCTTC	45
gpS1R-NLR	GCCCACGGCCCACTGCATAGTCTGCGCGTCTTTCAGGGCTTC	42

[a]Each MBP-S1R variant was created using gpS1R-F and the appropriate reverse primer. Nucleotides shown in lower case encode the linker between MBP and S1R.

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Table 2

Properties of MBP-S1R variants with a modified linker sequence

Variant	Residues	MW (Da)	Sequence
MBP-TEV-S1R	623	69240	MBP-NSSSNNNNNNNNNNLGIENLYFQSGSAT-S1R
MBP-5A-S1R[a]	600	66598	MBP-AAAAA-S1R
MBP-4A-S1R	599	66527	MBP-AAAA-S1R
MBP-3A-S1R	598	66456	MBP-AAA-S1R
MBP-2A-S1R	597	66385	MBP-AA-S1R
MBP-1A-S1R	596	66313	MBP-A-S1R
MBP-S1R	595	66242	MBP-S1R

[a]MBP-S1R variant having a 5 Ala residues in the linker between MBP and S1R. The other variants are named in a corresponding manner.

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Table 3

Summary of the purification of MBP-4A-S1R

	Volume	Protein concentration	Total protein	Total binding activity	Specific binding[b]	Fold purification
	mL	**mg/mL**	**mg[a]**	**cpm x 10^9**	**(cpm/mg) x 10^6**	
Lysate[a]	330	9.4	3102	4.4	1.4	1
Membrane fraction	75	9.7	728	5.6	7.7	5
Amylose affinity	43	0.6	26	5.8	225	158
Gel filtration	1	13.7	14	3.4	248	175[c]

[a]Prepared from 4 L of *E. coli* B834-pRARE2 culture, 23.3 g wet cells.

[b]Normalized to mg of total protein instead of nmol of MBP-4A-S1R.

[c]The 175-fold purification of MBP-4A-S1R was calculated based on increase in specific binding activity for the ligand [H^3]-(+)-pentazocine.