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Short communication

Overexpression of relaxin family peptide receptor 3 in *Escherichia coli* and characterization of its ligand binding properties

Meng-Jun Hu[1], Jia-Hui Wang[1], Xiao-Xia Shao, Ya-Li Liu, Zeng-Guang Xu, Zhan-Yun Guo*

Research Center for Translational Medicine at East Hospital, School of Life Sciences and Technology, Tongji University, Shanghai, China

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ABSTRACT

Relaxin family peptide receptor 3 (RXFP3) is an A-class G protein-coupled receptor. It is implicated in the regulation of food intake and stress response upon activation by the neuropeptide relaxin-3. So far, preparation of enough functional RXFP3 for structural analysis is still challenging due to its integral membrane protein nature. In the present study, we overexpressed an N-terminally secretory maltose-binding protein (sMBP)-fused shortened human RXFP3 in prokaryotic host *Escherichia coli*. A small fraction of the sMBP-RXFP3 fusion protein could be integrated into the *E. coli* cell membrane and this fraction retained ligand binding function although the measured binding parameters were different from those of sMBP-RXFP3 overexpressed in mammalian cells due to different cell membrane lipids of the different host cells. Our present work paved the way for production of functional RXFP3 using the low-cost *E. coli* expression system.

1. Introduction

Relaxin family peptide receptor 3 (RXFP3) is an A-class G protein-coupled receptor (GPCR). Upon activation by the neuropeptide relaxin-3 (also known as INSL7), RXFP3 is implicated in the regulation of food intake, stress response, arousal, and exploratory behaviors [1–4]. As a GPCR, RXFP3 has seven predicted transmembrane domains (TMDs) with an extracellular N-terminus and an intracellular C-terminus. Recent studies have disclosed an essential WxxExxxD motif at the extracellular end of TMD2 of RXFP3. This motif forms electrostatic and hydrophobic interactions with the positively charged B-chain Arg residues (B12Arg, B16Arg, and B26Arg) and the large aromatic B27Trp residue of relaxin-3 [5–7]. A negatively charged aspartate residue in the middle of TMD2 is essential for transduction of the extracellular ligand-binding information to the intracellular region of RXFP3 to initiate downstream signaling [8].

So far, preparation of enough functional RXFP3 for structural analysis is still challenging due to its integral membrane protein nature. In the present work, we attempted to overexpress RXFP3 using the low-cost *Escherichia coli* expression system. In previous studies, fusion of a secretory maltose-binding protein (sMBP) at the N-terminus can improve the expression of some GPCRs in *E. coli* [9–11]. Thus, we fused the sMBP partner to the N-terminus of a shortened human RXFP3 in which 33 N-terminal residues and 40 C-terminal residues were removed. A previous study has demonstrated that removal of 33 N-terminal residues has no detriments to the ligand-binding function of RXFP3 [5]. We expected that the C-terminal fragment was also irrelevant to the ligand-binding function of RXFP3 due to its intracellular location. Thus, we shortened the flexible N-terminus and C-terminus of human RXFP3 in order to improve its expression in *E. coli*. Our present work demonstrated that functional sMBP-RXFP3 fusion protein could be overexpressed in *E. coli*, although the *E. coli*-expressed receptor displayed different ligand binding properties compared with that expressed in mammalian cells due to different cell membrane lipids of the different host cells.

2. Experimental methods

2.1. Generation of the expression constructs for sMBP-RXFP3

The coding sequence of the shortened human RXFP3 was PCR amplified using our pcDNA6/RXFP3 construct as the template. After cleavage with the restriction enzymes NdeI and EcoRI, the amplified DNA fragment was ligated into the *E. coli* expression vector pMal-p5X (New England Biolabs, Ipswich, MA, USA) that was pretreated with the same restriction enzymes, resulting in the *E. coli* expression construct pMal-p5X/RXFP3, which encodes a shortened RXFP3 carrying an sMBP-fusion partner at the N-terminus. Coding region of the *E. coli*-expressed sMBP-RXFP3 was confirmed by DNA sequencing, and its nucleotide and amino acid sequences are shown in Supplementary Fig.

* Corresponding author.
E-mail address: zhan-yun.guo@tongji.edu.cn (Z.-Y. Guo).
[1] These authors contributed equally to this work.

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S1.

A DNA fragment encoding the signal peptide (29 amino acids) of human interleukin-6 was generated by annealing four synthetic oligonucleotide primers. The synthetic DNA fragment with sticky ends was then ligated into a modified pcDNA6 vector pretreated with the restriction enzymes HindIII and BamHI, resulting in the intermediate construct pcDNA6/sIL6. For generation of a mammalian cell expression construct for sMBP-RXFP3, the coding region of the mature MBP-RXFP3 (without the *E. coli* signal peptide of sMBP) was PCR amplified using the pMal-p5X/RXFP3 construct as the template. After cleavage with the restriction enzymes ApaI and AgeI, the amplified DNA fragment was ligated into the pcDNA6/sIL6 construct pretreated with the same restriction enzymes, resulting in the mammalian cell expression construct pcDNA6/sMBP-RXFP3, which encodes a shortened RXFP3 carrying an sMBP-fusion partner at the N-terminus. Coding region of the mammalian cell-expressed sMBP-RXFP3 was confirmed by DNA sequencing, and its nucleotide and amino acid sequences are shown in Supplementary Fig. S2.

2.2. Overexpression of sMBP-RXFP3 in E. coli and western blotting analysis

The expression construct pMal-p5X/RXFP3 was transformed into NEB Express *E. coli* cells (New England Biolabs) and the transformants were cultured in liquid LB medium at 37 °C to $OD_{600nm} \approx 1.0$. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM and the *E. coli* cells were continuously cultured at 20 °C for ~20 h. Subsequently, the *E. coli* cells were collected by centrifugation (5000*g*, 5 min), resuspended in lysis buffer (20 mM phosphate, pH7.4, 0.5 M NaCl), and lysed by French press. The cell lysate was treated with DNase on ice for 1 h and then centrifuged (8000*g*, 30 min). Thereafter, the supernatant was ultracentrifuged (100 000*g*, 30 min). Fractions of the centrifugation and ultracentrifugation were analyzed by SDS-PAGE and western blotting using a monoclonal antibody against MBP (New England Biolabs).

2.3. Preparation of peptides and bioluminescent tracers for ligand binding assays

The chimeric relaxin family peptides R3/I5 and R3(BΔ23–27)R/I5 were prepared according to our previous procedures [7]. The mature peptides were purified to homogeneity by high performance liquid chromatography (HPLC) using a C18 reverse-phase column (Zorbax 300SB-C18, 4.6 mm × 250 mm, from Agilent Technologies, Santa Clara, CA, USA) and confirmed by mass spectrometry. The NanoLuc-based bioluminescent tracers, R3/I5-Luc and R3(BΔ23–27)R/I5-Luc, were prepared according to our previous procedure [7].

2.4. Ligand binding assays for the E. coli-expressed sMBP-RXFP3

The *E. coli* cells overexpressing sMBP-RXFP3 were collected from the culture broth by centrifugation (5000*g*, 5 min), resuspended in 1/10 vol of phosphate-buffered saline (PBS), and then treated with 0.35 mg/ml lysozyme and 10 mM ethylenediaminetetraacetic acid (EDTA) at 37 °C for 20 min. Subsequently, the digested *E. coli* cells were collected by centrifugation (10 000*g*, 10 min), washed once by PBS, and resuspended in 1/3 vol of binding solution [serum-free DMEM medium plus 1% bovine serum albumin (BSA)]. To conduct ligand binding assays, the *E. coli* suspension was transferred to 1.5 ml eppendorf tubes (50 μl/tube), and then mixed with the sample solution (100 μl/tube). For saturation binding assays, the sample solution contains varied concentrations of tracer; for competition binding assays, the sample solution contains a constant concentration of tracer and varied concentrations of competitor. The nonspecific binding data were obtained by competition with 1.0 μM of R3/I5 or R3(BΔ23–27)R/I5. After incubation at 20 °C for 1–2 h, the binding solution was removed after centrifugation (10 000*g*, 3 min), and the cell pellet was washed twice with ice-cold PBS (200 μl/tube for each wash). Finally, the *E. coli* cells were resuspended in PBS (100 μl/tube) and transferred to a white opaque 96-well plate (50 μl/well). After addition of the diluted substrate (50 μl/well, diluted in lysis buffer from Promega, Madison, WI, USA), bioluminescence was immediately measured on a Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA) using the luminescence mode. The measured bioluminescence data were expressed as mean ± standard error (SE) ($n = 3$) and fitted with a one-site binding model using the SigmaPlot10.0 software.

2.5. Ligand binding assays for the HEK293T cell-expressed sMBP-RXFP3

Human embryonic kidney (HEK) 293T cells were seeded into 35 mm dishes, grown to ~80% confluence, and transfected with the expression construct pcDNA6/sMBP-RXFP3 using the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the user's manual. The next day, the transfected cells were trypsinized, seeded into 60 mm or 100 mm dishes, and continuously cultured at 37 °C for 24–36 h. Thereafter, the cells were detached by addition of 1.0 mM of EDTA solution (in PBS), collected by centrifugation (1000*g*, 2 min), washed by PBS, and resuspended in binding solution (serum-free DMEM medium plus 1% BSA). To conduct ligand binding assays, the cell suspension was transferred to a 96-well filtration plate (50 μl/well), and mixed with sample solution (100 μl/well). For saturation binding assays, the sample solution contains varied concentrations of tracer; for competition binding assays, the sample solution contains a constant concentration of tracer and varied concentrations of competitor. The nonspecific binding data were obtained by competition with 1.0 μM of R3/I5 or R3(BΔ23–27)R/I5. After incubation at 20 °C for 1–2 h, the binding solution was removed by centrifugation (1000*g*, 3 min), and cells were washed twice with ice-cold PBS (200 μl/well for each wash) through centrifugation (1000*g*, 3 min). Finally, the HEK293T cells were resuspended in PBS (100 μl/well) and transferred to a white opaque 96-well plate (50 μl/well). After addition of the diluted substrate (50 μl/well, diluted in lysis buffer from Promega), bioluminescence was measured on a Spectramax M5 plate reader (Molecular Devices) using the luminescence mode. The measured bioluminescence data were expressed as mean ± SE ($n = 3$) and fitted with a one-site binding model using the SigmaPlot10.0 software.

3. Results and discussion

3.1. Overexpression of sMBP-RXFP3 in E. coli

After the transformed *E. coli* cells were induced by IPTG, a major band with an apparent molecular weight of ~85 kDa was detected by Western blotting using antibody against the MBP fusion partner (Fig. 1A, lane 3). Its size was consistent with the expected value (86.7 kDa) of sMBP-RXFP3, suggesting expression of the fusion receptor in *E. coli*. However, most of sMBP-RXFP3 was present in the cell pellet after the *E. coli* cells were lysed and centrifuged (Fig. 2A, lane 4), suggesting that most of the fusion receptor formed inclusion bodies despite presence of the highly soluble MBP fusion partner. Meanwhile, a small percentage of sMBP-RXFP3 was also present in the centrifugation supernatant (Fig. 1A, lane 5), suggesting this fraction might be the folded fusion receptor. After this fraction underwent ultracentrifugation, the fusion receptor was only present in the pellet (Fig. 2A, lane 6), suggesting that all sMBP-RXFP3s in this fraction were integrated into the *E. coli* cell membrane. Thus, cell membrane-integrated sMBP-RXFP3 could be obtained through overexpression in *E. coli*, despite the yield was still quite low at the present stage.

3.2. Ligand binding properties of the E. coli-expressed sMBP-RXFP3

Before further improvement of its expression yield, we needed to know whether the *E. coli*-expressed sMBP-RXFP3 was functional. Thus,