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Molecular cloning and characterization of a new RGS protein of Medaka

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Abstract

We identified eight genes of putative RGS proteins in skin of Medaka fish using PCR amplification with degenerate primers for the RGS domain of known RGS proteins. Then, we cloned a full-length cDNA for a new RGS protein. This RGS protein was similar to human RGS3 within the RGS domain, but other parts were unique among known RGS proteins. RT-PCR analysis demonstrated that this Medaka RGS3-like protein (MeRGS3L) is mainly expressed in skin and heart. When coexpressed in *Xenopus* oocytes, MeRGS3L accelerated the turning-on and -off of Gi/o-mediated modulation of GIRK channels without apparent desensitization in the presence of ligand. MeRGS3L also decreased the response of Gq signaling upon activation of m1 muscarinic receptor. This new RGS protein may play important roles in regulation of melanophore responses in Medaka skin.

Keywords: G protein; Receptor; RGS; Desensitization; Medaka

1. Introduction

In the poikilothermal vertebrates, a rapid color change is caused by the motile responses of chromatophores in the skin. The responses of fish melanophores are controlled mainly by the sympathetic nervous system, and the neurotransmitter, norepinephrine (NE), stimulates the pigment-aggregation response by attenuating cAMP-PKA signaling via alpha2-adrenoceptors. This signaling process involves pertussis toxin-sensitive heterotrimeric G proteins. In addition, a variety of the G proteins are thought to be involved in the signal transduction of other hormones which also induce motile responses of melanophores (reviewed by Fujii and Oshima, 1986; Fujii, 1993). In Medaka *Oryzias latipes*, it was previously reported that three subtypes of Gαi and two subtypes of Gαs, which exhibited significant similarity with mammalian Gαi and Gαs proteins, are present by RT-PCR amplification method (Oba et al., 1997). Further, using Medaka, it was shown that the sensitivity of melanophores to exogenous NE and melanin-concentrating hormone (MCH) was downregulated or upregulated during long-term adaptation of the fish to a black or white background, respectively (Sugimoto, 1993). NE and MCH also influence melanophore morphology and apoptosis during long-term background adaptation (Sugimoto, 2002). Therefore, to elucidate the regulation mechanism of the sensitivity is critical to our understanding of melanophore biology.

Abbreviations: RGS, regulators of G protein signaling; G protein, heterotrimeric guanine nucleotide-binding proteins; RT-PCR, reverse transcriptase-PCR; MeRGS3L, Medaka RGS3-like protein; GIRK, G protein-gated inwardly rectifying K^+ channel.

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Recently, a new family of RGS (regulators of G protein signaling) proteins that modulate the heterotrimeric G protein signaling was discovered (Dohlman and Thorner, 1997; Berman and Gilman, 1998). They share a homologous domain, the RGS domain, which functions as a GTPase activating protein for the α-subunits of heterotrimeric G proteins. They are considered as key elements that reduce the signal generated through G-protein-coupled receptors. RGS proteins may play important roles in the regulation of melanophore sensitivity during background adaptation of fish.

To identify RGS proteins involved in the regulation of melanophore responses, here, we searched RGS proteins expressed in Medaka skin by the degenerate primer PCR method. Eight putative RGS proteins (MeRGSI–VIII) were identified. For further characterization of Medaka RGS proteins, we isolated a full-length cDNA clone of MeRGS3L and investigated biochemical and physiological properties.

2. Materials and methods

2.1. PCR identification of Medaka RGS proteins

Poly(A) RNA was isolated from skin tissues of Medaka (*Oryzias latipes,* HNI strain) using QuickPrep Micro mRNA Purification Kit (Amersham Bioscience). Then, we performed RT-PCR using degenerate primers corresponding to the RGS domain of known RGS proteins as previously described (Koelle and Horvitz, 1996). Cloning of the PCR products into pGEM-T vector (Promega) and subsequent sequence analysis were carried out.

2.2. Cloning of MeRGS3L cDNA

The PCR-amplified DNA fragment of MeRGSII was used to screen a Medaka cDNA library prepared from Medaka skin tissues using SMART cDNA Construction Kit (BD Biosciences Clontech). The longest cDNA clone was sequenced on both strands.

2.3. RT-PCR assay

Total RNA was isolated from various tissues of Medaka and subjected to reverse transcription with random primers. The reverse-transcribed cDNA was used as a template of PCR. The primers used for MeRGS3L were the following: 5′ -CTC TAG AGG ATC CAT GCC AAG CTT AGT TGC ATC ACC-3′ , 5′ -CCT CTA GAG TCC TGG AGG GTT TTA CGT CTT TAC-3′ . After 35 cycle of the reaction, PCR products were analyzed on 0.9% agarose gels.

2.4. Immunoprecipitation

Cell transfection and immunoprecipitation were performed as previously described (Masuho et al., 2004). Briefly, we expressed MeRGS3L as a chimeric protein with GFP at the carboxyl terminus in HEK293T cells. Active rat Gαo and human Gαq were coexpressed. By amplification with the polymerase chain reaction, cDNA fragments for MeRGS3L was isolated. This was fused in-frame to GFP in pEGFP-N1 (BD Biosciences Clontech). The resultant plasmid cDNA and the cDNA for QL mutants of Gαo or Gαq were transiently transfected into HEK293T cells using Effectene (Qiagen). At 48 h after transfection, cell lysate was prepared by sonication of cells in Tx buffer (50 mM Tris–HCl, pH 7.5, 300 mM NaCl, 1% Triton X-100, and 0.1 mM PMSF). Then, immunoprecipitation was performed using anti-GFP antibody and Protein G-agarose.

2.5. Two electrode voltage clamp

Functional expression in *Xenopus* oocytes and electrophysiological analysis under a two-electrode voltage clamp were done as described previously (Saitoh et al., 2002). In the experiments of Fig. 4a and b, Gαi-coupled responses were recorded at −80 mV by co-expressing the m2 muscarinic receptor and GIRK1/2 channels. The bath solution contained 90 mM KCl, 3 mM $MgCl_2$, and 10 mM Hepes (pH 7.32). In the experiments of Fig. 4c and d, m1 muscarinic receptor was coexpressed, and Gαq/11-coupled responses were monitored as an increase in the current amplitude through the endogenous Ca^{2+}-activated Cl^- channel in *Xenopus* oocytes. The current was recorded by applying depolarizing step pulses to +60 mV for 200 ms repeatedly from the holding potential of −80 mV every 2 s. The bath solution contained 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 3 mM $MgCl_2$, and 15 mM Hepes (pH 7.6).

3. Results

3.1. Eight Medaka genes encoding putative RGS proteins

All known RGS proteins share a conserved RGS domain of about 120 amino acids. Total RNA was isolated from Medaka skin, and the reverse-transcribed cDNA was used as a template of PCR. To identify RGS proteins involved in the regulation of melanophore responses, we carried out PCR amplification with degenerate primers corresponding to the RGS domain. DNA fragment of about 250 bp was amplified (Fig. 1a). The amplified DNA was cloned into a pGEM-T vector, resulting in the isolation of 58 clones. Based on sequencing analysis, these clones for Medaka RGS proteins were classified into eight groups and designated as MeRGSI-VIII. The alignment of the deduced sequence of these eight MeRGS proteins is in Fig. 1b. Within the PCR-amplified region, MeRGSI was similar to mouse RGS2 (54% identity), MeRGSII was also close to mouse RGS2 (55% identity), MeRGSIII was highly homologous to human RGS3 (83% identity), MeRGSIV was similar to mouse RGS3 (54% identity), MeRGSV was homologous to