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Functional expression and characterization of the *C. elegans* G-protein-coupled FLP-2 Receptor (T19F4.1) in mammalian cells and yeast

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ABSTRACT

Profound neuropeptide diversity characterizes the nematode nervous system, but it has proven challenging to match neuropeptide G protein-coupled receptors (GPCR) with their cognate ligands in heterologous systems. We have expressed the *Caenorhabditis elegans* GPCR encoded in the locus T19F4.1, previously matched with FMRFamide-like peptides encoded on the *flp*-2 precursor gene, in mammalian cells and in the yeast *Saccharomyces cerevisiae*. Pharmacological characterization revealed that the receptor is potently activated by *flp*-2 peptides in CHO cells (~10 nM EC_{50}) and in yeast (~100 nM EC_{50}), signaling through a $G_q\alpha$ pathway in each system. The yeast GPCR expression system provides a robust assay for screening for agonists of the *flp*-2 receptor and is the target of an ongoing high-throughput screening exercise.

1. Introduction

FMRFamide-like peptides (FLPs) are important neurotransmitters and neuromodulators in invertebrates, including parasitic helminths, arthropods and other organisms (Price and Greenberg, 1989; Greenberg and Price, 1992; Walker, 1992; Halton et al., 1994; Shaw et al., 1996; Brownlee et al., 2000; Maule et al., 2002). At present, nearly 80 distinct FLPs encoded on 34 *flp*s (precursor genes) have been predicted from the *Caenorhabditis elegans* genome (McVeigh et al., 2005, 2006; Husson et al., 2007; Li and Kim, 2008, 2010; Walker et al., 2009). Despite extensive bioinformatic searches, all family members may not have been identified. In addition to FLPs, 48 *C. elegans* neuropeptide-like protein (*nlp*) genes have been identified, defining almost 140 total putative neuropeptides in this classification (Nathoo et al., 2001; McVeigh et al., 2006, 2008; Husson et al., 2007; Li and Kim, 2008, 2010), as well as 40 precursor genes encoding insulin-like peptides (Li and Kim, 2008, 2010).

Approximately 60 genes encoding putative neuropeptide G protein-coupled receptors (GPCRs) have been annotated in the *C. elegans* genome (Bargmann, 1998; Keating et al., 2003). The number of putative *flp*- and *nlp*-encoded neuropeptides is much higher than the number of predicted neuropeptide GPCRs, implying that a single receptor is likely to be activated by multiple peptides. In *C. elegans* (Nathoo et al., 2001; McVeigh et al., 2005, 2006; Husson et al., 2007; Li and Kim, 2008, 2010; McVeigh et al., 2008; Walker et al., 2009) and other invertebrates (Bellés et al., 1999; Fujisawa et al., 1999; Furukawa et al., 2001), multiple forms of related peptides are typically encoded on one precursor protein gene and these peptide families can recognize the same GPCR, as has been shown for the *Drosophila* allatostatin type A receptor (Larsen et al., 2001; Lenz et al., 2001) and as appears to be the case for several FLP families analyzed in *Ascaris suum* physiological experiments (see McVeigh et al., 2006). Since the publication of the *C. elegans* genome in 1998 (Consortium, 1998), a relatively small number of orphan *C. elegans* neuropeptide GPCRs has been matched with a cognate ligand (see Lowery et al., 2003; McVeigh et al., 2006; Husson et al., 2007). Although many orphan vertebrate GPCRs have been paired with endogenous ligands using approaches employing heterologous receptor expression systems and reverse pharmacology (Civelli et al., 2001), this approach has not been uniformly straightforward for nematode neuropeptidergic GPCRs.

Abbreviations: flp, FMRFamide-like protein gene; FLP, FMRFamide-like peptide; FLP2-R, receptor for peptides encoded on *flp-2*; FLIPR, fluorescence imaging plate reader; GPCR, G protein-coupled receptor; *nlp*, neuropeptide-like protein gene.

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As part of a large-scale project devoted to identifying peptide-receptor matches in *C. elegans* (Greenwood et al., 2005; Woods et al., 2010), we identified the GPCR annotated as T19F4.1 as a receptor for peptides encoded on the *flp-2* precursor gene (Lowery et al., 2003). This finding was also reported by another group (Mertens et al., 2005). We report here further characterization of the pharmacology of this putative *C. elegans* GPCR with its cognate ligands following expression in mammalian cells and in the yeast *Saccharomyces cerevisiae*, which has proven useful for the heterologous expression of GPCRs and for high-throughput screening for small molecule ligands of these receptors (Broach and Thorner, 1996; Pausch, 1997; Ladds et al., 2005; Minic et al., 2005; Wang et al., 2006; Dowell and Brown, 2009; Geary, 2012; Geary and Ubalijoro, 2012). This GPCR has been termed the FLP-2 receptor (FLP-2R), because the receptor activating peptides are encoded on the *C. elegans* precursor gene *flp-2* (Mertens et al., 2005).

2. Materials and methods

2.1. Materials

Synthetic peptides were made at Auspep Pty. Ltd. (Parkville, Australia) and Sheldon Biotechnology Center (McGill University). Chinese hamster ovary cell line CHO-10001A (CHO cells), cell culture media, transfection and assay reagents were as described previously (Larsen et al., 2001; Kubiak et al., 2002). U-73122 (a phospholipase C inhibitor) was obtained from the Pfizer compound collection (Bleasdale et al., 1990). Yeast strains and vectors (Wang et al., 2006) were obtained under license from Cadus Corp.

2.2. Cloning and plasmid preparation

Molecular biological techniques followed either manufacturer's recommendations or general protocols. A variety of PCR primers were designed using the coding sequence for locus T19F4.1 as predicted in Wormpep (release 13). Bioinformatic analyses of the *C. elegans* genome using previously cloned *C. elegans* FLP GPCRs (Kubiak et al., 2002, 2003) had identified this gene as a candidate neuropeptide GPCR (not shown). The only significant modification to the amplicon was the addition of an optimized translational initiation sequence immediately preceding the authentic initiation codon ("GCC GCC") (Kozak, 1987). Using cDNA prepared from *C. elegans* strain N2 with sense and antisense primers deduced from the *C. elegans* genome, PCR products encompassing the complete open reading frame of T19F4.1 were cloned directly into the eukaryotic expression vector pCR3.1 (Invitrogen, Carlsbad, CA). Nucleotide sequence analysis by standard protocols revealed the presence of 3 distinct clones, each in multiple copies (Fig. 1). The longest of these was chosen for further analysis; this clone was designated the Ce50b (*flp-2*R)/pCR3.1 plasmid and the cDNA it encodes is identical to T19F4.1b (Lowery et al., 2003; Mertens et al., 2005).

2.3. Cell culture, cell transfection and intracellular Ca^{2+} mobilization assay

CHO cells were cultured and transfected essentially as described previously (Kubiak et al., 2002, 2003) with some modifications. The *flp-2*R/pCR3.1 plasmid (5 μg DNA/10 cm plate) was used for transfection with the LipofectAmine PLUS™ (Invitrogen, Carlsbad, CA) method. The assay for functional expression employed a 96-well fluorescence imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA) essentially as described previously (Kubiak et al., 2002, 2003), exposing the transformed cells to ~200 invertebrate neuropeptides (Kubiak et al., 2002). In temperature shift experiments, transfected cells were split and plated in 96-well FLIPR plates 24 h post transfection, followed by an additional 24 h incubation at 37 °C and then a 28 °C incubation for 24 h prior to peptide testing. In some experiments, the phospholipase C inhibitor U-73122 was added in HBBS/HEPES/probenecid, 0.1% DMSO, 10 μM final concentration, 10 min before the addition of peptide.

2.4. Expression in yeast

The ORF from plasmid *flp-2*R/pCR3.1 was subcloned into the yeast vector Cp4258, which carries a LEU2 selectable marker (see Wang et al., 2006) following PCR amplification using 5′- and 3′- terminal primers that generated an *Nco*I and an *Xba*I restriction site, respectively, which were the cloning sites used in the vector. The resultant *flp-2*R-Cp4258 plasmid was transformed into *Escherichia coli* for propagation using ampicillin selection and then transformed into a collection of strains of *S. cerevisiae* based on CY13193 (MATα P_{FUS1}-HIS3 far1Δ1442 gpa1Δ1163 *his3 leu2 lys2 trp1 ura3 sst2Δ2* ste14::trp1::LYS2 ste18γ6-3841 ste3Δ1156 tbt1-1) as described previously (Wang et al., 2006; Kimber et al., 2009). Each strain contains an integrated modified copy of the yeast Gα-subunit gene (GPA1) that incorporates the terminal pentapeptide sequences from the mammalian G_i-, G_{12}-, G_{13}-, G_z-, G_q- and G_s-α proteins, in addition to others based on *C. elegans* G-α proteins (*gpa-1*, *gpa-2, gpa-7, gpa-9, gpa-12* and *gpa-16*), to facilitate the interaction between this protein and heterologous GPCRs. It should be noted that the C-terminal pentapeptide is identical in *C. elegans* and mammals for G_q (*egl-30*) and G_s (*gsa-1*). The yeast strains express HIS3 under control of the FUS1 promoter when an agonist activates the heterologously expressed GPCR. Thus, activation of FLP-2R by its peptide ligand leads to the correction of the histidine auxotrophy, enabling growth in histidine-deficient medium.

Strains were cultured in Complete Minimal (CM) dropout medium (Sigma–Aldrich, St. Louis, MO) and transformed using the lithium acetate method (Gietz et al., 1995). Positive transformants were selected in plates with CM medium lacking leucine (CM/Leu^-) and used for receptor activation assays in CM liquid medium lacking leucine and histidine (CM/Leu^-/His^-), supplemented with 0.05 M MOPS, pH 6.8. Briefly, a colony was grown overnight in CM/Leu^- at 250 rpm and 30 °C, cells were harvested by centrifugation at 4000*g* for 1 min and rinsed thoroughly in CM/Leu^-/His^- to eliminate histidine. Cell density was established by absorbance at 600 nm and 3000 cells/well in CM/Leu^-/His^- were seeded a 96-well plate to incubate with serial dilutions of flp-2 peptides. After 44 h incubation at 30 °C, cell growth was measured by Alamar Blue fluorescence after 2–4 h of incubation with 20 μl Alamar Blue (Sigma–Aldrich) at 30 °C for color development (Klein et al., 1997). Fluorescence was measured in a Synergy H4 Hybrid Multi-Mode Microplate Reader (BioTek) set at 560 nm excitation/590 nm emission. Experiments were performed three times in three replicates using as controls untransformed strains and transformants in presence of histidine.

2.5. Data analysis

Concentration–response curves for CHO cell and yeast experiments were analyzed by nonlinear regression using Prism (GraphPad Software, Inc. San Diego, CA) based on each treatment run in triplicate and expressed as mean values ±95% confidence intervals (C.I.).