FMRFamide-Like Peptides Encoded on the *flp-18* Precursor Gene Activate Two Isoforms of the Orphan *Caenorhabditis elegans* G-Protein-Coupled Receptor Y58G8A.4 Heterologously Expressed in Mammalian Cells

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ABSTRACT:

Two alternatively spliced variants of an orphan Caenorhabditis elegans *G-protein-coupled receptors (GPCRs; Y58G8A.4a and Y58G8A.4b) were cloned and functionally expressed in Chinese hamster ovary (CHO) cells. The Y58G8A.4a and Y58G8A.4b proteins (397 and 433 amino acid residues, respectively) differ both in amino acid sequence and length of the C-terminal tail of the receptor. A calcium mobilization assay was used as a read-out for receptor function. Both receptors were activated, with nanomolar potencies, by putative peptides encoded by the flp-18 precursor gene, leading to their designation as FLP-18R1a (Y58G8A.4a) and FLP-18R1b (Y58G8A.4b). Three* Ascaris suum *neuropeptides AF3, AF4, and AF20 all sharing the same FLP-18 C-terminal signature, -PGVLRF-NH_2, were also potent agonists. In contrast to other previously reported C. elegans GPCRs expressed in mammalian cells, both FLP-18R1 variants were fully functional at 37°C. However, a 37 to 28°C temperature shift improved their activity, an effect that was more pronounced for FLP-18R1a. Despite differences in the C-terminus, the region implicated in distinct G-protein recognition for many other GPCRs, the same signaling pathways were observed for both Y58G8A.4 isoforms expressed in CHO cells. Gq protein coupling seems to be the main but not the exclusive signaling pathway, because pretreatment of cells with U-73122, a phospholipase inhibitor, attenuated but did not completely abolish the Ca^{2+} signal. A weak Gs-mediated receptor activation was also detected as reflected in an agonist-triggered concentration-dependent cAMP increase. The matching of the FLP-18 peptides with their receptor(s) allows for the evaluation of the pharmacology of this system in the worm in vivo.* © *2007 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 90: 339–348, 2008.*

Keywords: *C. elegans*; *flp-18*; orphan GPCR; Y58G8A.4; calcium signaling

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INTRODUCTION

FMRFamide-like peptides (FLPs also known as FaRPs) are the largest and most diverse family of neuropeptides known.[1–3] *Caenorhabditis elegans* contains a very large and diverse family of neuropeptide genes. From at least 109 genes encoding over 250 potential neuropeptides identified in this nematode, more than 70 FLPs have been predicted and/or identified. Many have been found to play roles in vital functions such as locomotion, reproduction, and social behavior (for review see Ref. [3] and references within). Many of the *C. elegans* peptides are identical or highly homologous to those isolated or predicted in parasitic nematodes, including *Ascaris suum*, *Haemonchus contortus*, *Ancylostoma caninum*, *Heterodera glycines*, and *Meloidogyne arenaria*, suggesting that the function of these peptides is similar across species.[4,5]

Neuropeptide–receptor matching is needed to gain a better understanding of the biological function of these systems in situ. If that function is found essential for the maintenance of the parasite in the host, the receptor defined high-throughput screens could be established for finding nonpeptidic small molecules, either receptor agonists or antagonists, as potential antiparasitic drug candidates.[6–9]

Despite intense efforts in many laboratories over the last decade, most of the *C. elegans* neuropeptide G-protein-coupled receptors (GPCRs) remain orphans, as only a few have been matched with their native ligands.[10–16] Functional expression of *C. elegans* neuropeptide GPCRs in mammalian expression systems has been challenging and problematic, mainly due to the apparently poor compatibility of mammalian G-proteins and other accessory proteins with heterelogously expressed nematode receptors.

In this report, we describe functional annotation of two spliced variants of the orphan *C. elegans* G-protein-coupled receptor Y58G8A.4 as receptors for peptides predicted to be encoded on the *flp-18* precursor gene, designated FLP-18R1a (Y58G8A.4a) and FLP-18R1b (Y58G8A.4b).

EXPERIMENTAL

Materials

The synthetic peptides used in the study were purchased from Auspep Pty. (Parkville, Australia). The mutant Chinese hamster ovary cell line CHO-10001A (referred to hereafter as CHO cells), cell culture media, transfection, and various assay reagents were as previously described.[11,12,17,18] U-73122 (phospholipase C inhibitor) and its inactive analog U-73343 were obtained from the Upjohn/Pharmacia/Pfizer compound collection.[19]

Cloning and Plasmid Preparation

Molecular biological techniques followed either manufacturer's recommendations or general protocols.[20] Initial attempts to clone the cDNA corresponding to Y58G8a.a using RT-PCR and PCR primers based on the predicted sequence (Wormpep release 23) were unsuccessful (not shown). Further experimentation using various combinations of the flanking primers and sense/antisense internal primers suggested that the 3′ end of the open reading frame (ORF) was incorrectly predicted. PCR amplification using a *C. elegans* strain N2 mixed stage cDNA library (Strategene, La Jolla, CA) along with an internal sense primer in combination with a vector primer gave two PCR products that corresponded to intact Y58G8a.a ORFs. PCR primers were designed based on the new 3′ ends and were used to amplify the intact ORFs. PCR products were cloned into the eukaryotic expression vector pCR3.1 (Invitrogen, Carlsbad, CA), the only modification being the incorporation of a Kozak consensus sequence (GCCGCC) before the initiator methionine (ATG) to optimize translational initiation.[21] The DNA sequence of the clones was verified before transfection. The sequences are now correctly described in GenBank entry AC006808, hypothetical proteins Y58G8A.4a and Y58G8A.4b (referred to as FLP-18R1a and FLP-18R1b, respectively, in this report). The ORF for each variant was cloned directly into pCR3.1 to yield *flp-18*-R1a/pCR3.1 and *flp-18*-R1b /pCR3.1 plasmids.

Phylogenetic Analysis

The amino acid sequences of the ~60 predicted Family A (rhodopsin-like) *C. elegans* peptidergic GPCRs were obtained through Wormpep. For all of the sequences noted in the subgroup shown in Figure 1, amino acid sequences deduced from cloned full-length cDNAs were used in the alignment (unpublished observation). A group of ~250 additional vertebrate and invertebrate GPCRs, chosen to cover the spectrum of related peptidergic GPCRs, along with a smaller group (~30) of representative aminergic GPCRs, were aligned using ClustalX,[22] followed by manual editing. ClustalX was then used to generate a neighbor-joining phylogenetic tree. The subgroup shown in Figure 2 is excerpted from that larger tree. For purposes of this publication, only the *C. elegans* members of the subgroup are shown. The graphic shown in Figure 2 was generated using Treeview.[23]

Cell Cultures, Transfection, Stable Cell Line Generation and Membrane Preparation

CHO cells were cultured and transfected as described earlier.[11,12,17,18] The *flp-18*R1a/pCR3.1 or *flp-18*R1b /pCR3.1 plasmids (5 μg DNA/10 cm plate) were used for transfections. The cells were harvested 24 h after transfection and membranes were prepared as described.[11,12] In cases where a 37 to 28°C shift was implemented, media were supplemented with 0.1*M* HEPES pH 7.4 and the transfected cells were first incubated at 37°C for 24 h posttransfection and then moved to a humidified 28°C/3% CO_2 incubator for an additional 16–24 h before harvesting for membrane preparation or receptor signaling experiments.

To generate stable cell lines expressing Y58G8A.4a/FLP-18R1a or Y58G8A.4b/FLP-18R1b, the transfected CHO cells were cultured in complete DMEM media at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 h and then split 1:50, 1:100, and 1:200 into complete DMEM media additionally supplemented with geneticin (G418) initially at 1.0 mg/ml and then after 10 days at 0.5 mg/ml. Individual colonies were selected, grown, and propagated in the complete DMEM media supplemented with 0.5 mg/ml G418.

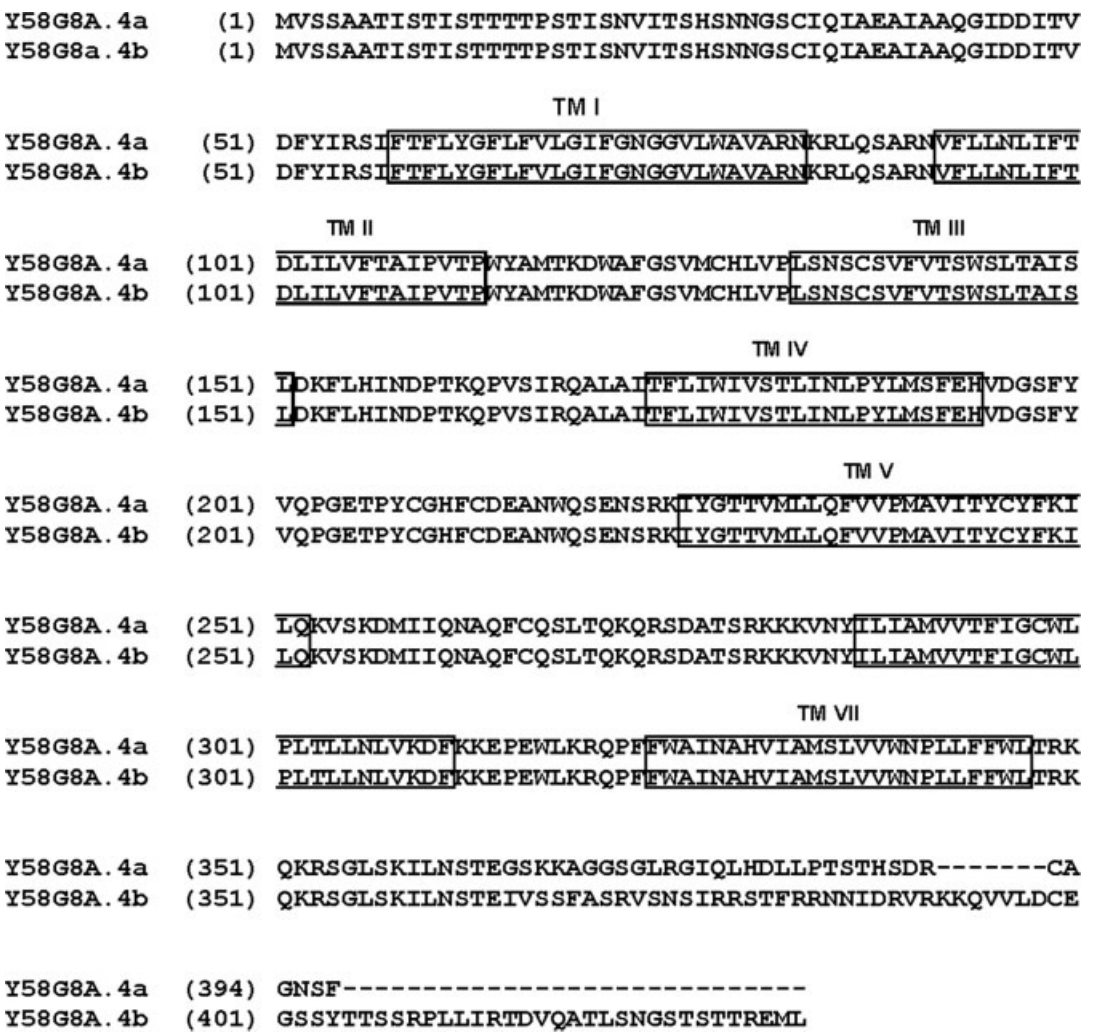

FIGURE 1 Alignment of amino acid sequences of the two isoforms of the Y58G8A.4 receptor, Y58G8A.4a (FLP-18R1a) and Y58G8A.4b (FLP-18R1b). Predicted transmembrane domains are boxed.

Radioligand Preparation and Receptor Binding Via Scintillation Proximity Assay

[^{125}I-Y°]-DVPGVLRF-NH_2 was prepared by radio-iodiation of [Y°]-DVPGVLRF-NH_2 using the chloramine T procedure. The crude products were separated by RP-HPLC on a Vydac C18, 0.45 × 15 cm column using a 0.1*M* ammonium acetate pH 6.5/acetonitrile system. The radioactive fractions with retention time corresponding to that of the cold [3-mono-iodoY°]-DVPGVLRF-NH_2 standard were collected into vials containing capture buffer (0.1*M* Na_2HPO_4, pH 8.0, with 0.5% BSA, 0.1% Triton 100X, and 0.05% Tween-20). Transfected cell membranes (prepared from clonal cells cultured with a 37 to 28°C shift) were incubated for 30 min at room temperature with occasional shaking with wheat germ agglutinin-coated scintillation proximity assay (SPA) beads (Amersham Pharmacia Biotech, Piscataway, NJ) in assay buffer (20 m*M* HEPES, 10 m*M* $MgCl_2$, pH 7.4, 200 μg membrane protein/20 mg beads/ml). After centrifugation at 1200*g* for 10 min the beads were resuspended in the assay buffer. In saturation binding experiments, each well in 96-well SPA plates (Wallac, Turku, Finland) received: 60 μl assay buffer, various concentrations of [^{125}I-Y°]-DVPGVLRF-NH_2 diluted into assay buffer (20 μl) and 20 μl reconstituted membrane-coated SPA beads suspension (1.5 μg membranes/150 mg beads) to result in a total volume of 100 μl/well. Nonspecific binding (NSB) was determined in the presence of 10 μM DVPGVLRF-NH_2 at each radioligand concentration. Plates were counted on a Wallac 1450 Microbeta counter continuously and the 2 h counts, corrected for NSB, were used for K_d and B_{max} determinations. Competitive displacement methods were similar to those used for determination of the saturation curve except that a fixed [^{125}I-Y°]-DVPGVLRF-NH_2 concentration was used added in 10 μl assay buffer (40 p*M* final), followed by the addition of increasing peptide concentrations, also in 10 μl assay buffer. Each treatment was run in duplicate. Total binding was determined in the absence of any peptides and ~10% of the label was added with 80% specific binding at the highest peptide concentrations used (10 μM).

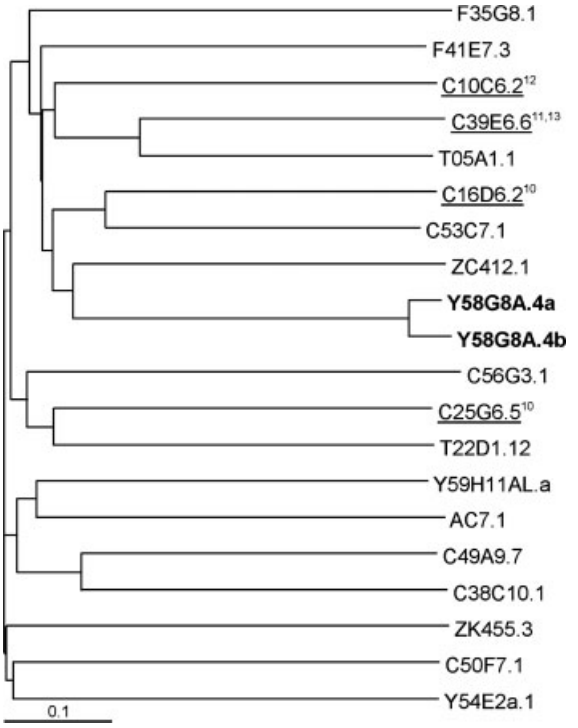

FIGURE 2 Phylogenetic analysis of the *C. elegans* GPCRs. The two isoforms of the FLP-18 receptor described in this report, Y58G8A.4a and Y58G8A.4b are shown in bold. Other deorphanized receptors for which ligands have been identified are underlined (see text for more details).

cAMP Assay

Clonal cells were plated in 96-well plates (1.5×10^4 cells/well) and incubated at 37°C overnight. Before cAMP experiments, the media were aspirated, replaced with plain DMEM (without serum), and the cells were equilibrated for 1 h at 37°C. The cells were then challenged with peptides, serially diluted in DMEM, for 20 min at 37°C followed by cell lysis and cAMP determination using a competitive ELISA method according to the manufacturer's protocol (Applied Biosystems, Bedford, MA).[24]

Calcium Mobilization Assay

Assays were run using a 96-well fluorescence imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA) essentially as described earlier[12,18] either with clonal cell lines or cells transiently transfected with the *flp-18*R1a/pCR3.1 or *flp-18*R1b/pCR3.1 plasmids and plated at 2×10^4 cells/well 24 h after transfection. In experiments incorporating a 37 to 28°C temperature shift, cells plated in black-walled 96-well plates were first incubated at 37°C for 24 h and then at 28°C for 16–24 h before testing in complete media supplemented with 10 m*M* HEPES. Cell loading was with 4 μM FLUO3 (Molecular Devices) in the presence of 2.5 m*M* probenecid.[12,18] In experiments with U-73122 (phospholipase C inhibitor), or its inactive analog U-73343, these compounds were added 10 min before peptide challenge (at a final concentration of 10 μM) in 10 m*M* HBSS/10 m*M* HEPES/2.5 m*M* probenecid containing 0.1% DMSO. In yet another experiment, plated cells were incubated with pertussis toxin (PTX, 100 ng/ml) overnight before peptide challenge.

Data Analysis

Data analysis was performed using GraphPad software (GraphPad, San Diego, CA). Dose–response curves (Ca^{2+}, cAMP, and binding data) were analyzed using nonlinear regression analysis. In competition binding experiments, binding constants (K_i) were calculated from the equation: $K_i = (IC_{50})/(1 + F/K_d)$ where F is the concentration of [^{125}I] ligand and K_d is the dissociation constant of the ligand. Each treatment was run in triplicate, unless otherwise indicated, values were expressed as means ± SEM.

RESULTS

Cloning of Y58G8A.4a and Y58G8A.4b and Phylogenetic Analysis

PCR amplification using a *C. elegans* cDNA library as template, using an internal sense primer in combination with a vector primer, yielded two PCR products that corresponded to intact Y58G8A.4 ORFs. PCR primers designed on the correct 3′ends were then used to amplify the intact ORFs. The deduced proteins encoded by the ORFs of splice variants Y58G8A.4a and Y58G8A.4b are 397 and 433 amino acid long, respectively (Figure 2). Both proteins are predicted to have seven transmembrane domains, three extracellular and three intracellular loops along with a C-terminal tail, a topology consistent with the structure of G-protein-coupled receptors. The first 364 amino acids are identical for both variants, while the Y58G8A.4b protein is 36 residues longer and has some different amino acids in the C-terminal 365–397 segment when compared with the shorter splice variant Y58G8A.4a (Figure 1). The Y58G8A.4 receptors are most closely related to three orphan *C. elegans* GPCRs ZC412.1, C53C7.1, and C56G3.1 as well as to the previously matched GPCRs C10C6.2 (a FLP-15 receptor[12]), C39E6.6 (also known as NPR-1[13] or a FLP-21/AF9 receptor[11]), and C16D6.2, a second FLP-18 receptor deorphanized by our group,[10] which will be described in a separate report.

Matching of Both Isoforms of Y58G8A.4 with FLP-18 Peptides and Other -VPGVLRF-NH_2 Peptides

Receptor–ligand matching was detected by functionally expressing both Y58G8A.4a and Y58G8A.4b in CHO cells (transient transfections) using a 37–28°C cooling step, as described for two previously deorphanized *C. elegans* GPCRs.[11,12] A collection of over 150 synthetic peptides representing a variety of *C. elegans* and other invertebrate FMRFamide-related peptides (based on both known and predicted sequences) was used as a source of potential activating ligands.[11,12] A Ca^{2+} mobilization assay in an FLIPR format was performed in a 96-well plates as a readout for receptor activation in response to a challenge with individual pep-

Table I Calcium Mobilization in Response to *C. elegans* FLP-18 Peptides and Other FMRF-Amides in CHO Cells Stably Expressing FLP-18R1a or FLP-18R1b Receptors*

Peptide	Species of Origin	FLP-18R1b (Long Form)		FLP-18R1a (Short Form)	
		EC_{50} (nM)	95% CL (nM)	EC_{50} (nM)	95% CL (nM)
DVPGVLRF-NH_2	*C. elegans/flp*-18	45.9	32.1–65.8	32.3	24.6–43.2
KSVPGVLRF-NH_2	*C. elegans/flp*-18	48.3	35.3–65.9	28.3	29.6–38.8
SEVPGVLRF-NH_2	*C. elegans/flp*-18	58.4	38.6–88.4	25.9	16.0–41.9
SVPGVLRF-NH_2	*C. elegans/flp*-18	43.3	30.4 –61.7	14.1	8.3–24.0
DFDGAMPGVLRF-NH_2	*C. elegans/flp*-18	124.0	81.2 –168.5	117.2	84.3–162.9
EIPGVLRF-NH_2	*C. elegans/flp*-18	44.4	32.8–60.15	13.3	7.6–23.1
AVPGVLRF-NH_2	*A. suum* (AF3)	13.1	10.1–16.9	7.6	5.6–10.4
GDVPGVLRF-NH_2	*A. suum* (AF4)	37.2	21.8–63.3	13.5	8.1–22.4
GMPGVLRF-NH_2	*A. suum* (AF20)	63.1	45.1–88.1	44.6	33.1–60.2
GLGPRPLRF-NH_2	*A. suum* (AF9) *or C. elegans/fl-p21*	254.1	174.2–370.5	267.0	179.9–396.1
SPLGTMRF-NH_2	*C. elegans/flp*-3	942.6	708.6–1254	886.3	621.6–1264
SAEPFGTMRF-NH_2	*C. elegans/flp*-3	1539	1035–2287	2092	1396–3133
SADDSAPFGTMRF-NH_2	*C. elegans/flp*-3	2880	1794–4623	2398	1659–3466
EDGNAPFGTMRF-NH_2	*C. elegans/flp*-3	1598	993.1–2571	2437	1776–3343
SQPNFLRF-NH_2	*C. elegans/flp*-1	4746	2260–9967	1684	1267–2238

* Cells were cultured at 37°C.

tides, tested at 5 μM. The strongest signal was detected for peptides with a C-terminal -VPGVLRF-NH_2 motif, which included not only the *C. elegans* FLP-18 peptides but also three *A. suum* FMRF-amides, GDVPGVLRF-NH_2 (AF4), AVPGVLRF-NH_2 (AF3), and MPGVLRF-NH_2 (AF20).

Hits from the initial 5 μM screen were later tested in a full-concentration response format with clonal cell lines. Data shown in Table I are EC_{50} values determined by FLIPR using CHO cells stably expressing either Y58G8A.4a or Y58G8A.4b and cultured at 37°C, after it was found that both receptors are fully functional at this temperature (see below). EC_{50} values for the *C. elegans* FLP-18 peptides and *A. suum* peptides with the common FLP-18 C-terminal motif -VPGVLRF-NH_2 were in the nanomolar range for activation of both receptor isoforms. The *A. suum* AF3 peptide, AVPGVLRF-NH_2, was the most potent agonist for both receptor variants (EC_{50} 7.6–13.1 nM). The least potent peptide in the FLP-18 series was DFDGAMPGVLRF-NH_2 (EC_{50} 117.2–124.0 nM), which has the longest N-terminal extension (Table I). The cognate ligand for the *C. elegans* NPR-1 receptor,[11,13] GLGPRPLRF-NH_2 (also known as AF9 or FLP-21 peptide), with a slightly different C-terminal motif than the FLP-18 peptides also activated both FLP-18R1a and FLP-18R1b, although with a considerably lower potency (EC_{50} 254–267 nM).

With regard to other *C. elegans* peptides, most of them did not titrate down below 5 μM, except for one FLP-1 peptide (SQPNFLRF-NH_2, EC_{50} values 1.7–4.7 μM) and also four FLP-3 peptides (Table I). The latter activated both receptor isoforms with the potency in the 0.9–2.9 μM range (Table I). Based on these results, it is evident that the most potent peptides by far are those encoded on *flp-18*, and that is why we believe that these peptides are the endogenous ligands for the Y58G8A.4/FLP-18 receptor.

Temperature-Dependent Functional Expression of Y58G8A.4a and Y58G8A.4b

Temperature-dependent functional activity of FLP-18R1a and FLP-18R1b was tested by using a single FLP-18 peptide, DVPGVLRF-NH_2, in cells stably expressing the individual receptor variants. The potency of this agonist was determined and compared for its ability to mobilize calcium in cells cultured either at 37 or 28°C. As can be seen in Figure 3B, signaling through the FLP-18R1b receptor was about 2.6 more efficient at 28°C than at 37°C (EC_{50} values and 95% confidence limits of 17.8 nM/12.8–24.9 nM and 46.0 nM/31.3–67.7 nM, respectively). Signaling through the shorter receptor isoform, FLP-18R1a, was even more responsive to the temperature downshift, which is reflected in both about fourfold higher potency (EC_{50} values and 95% confidence limits: 5.6 nM/3.01–10.4 nM at 28°C and 23.3 nM/7.6–71.9 nM, at 37°C) and a 1.7 higher maximal response at 28°C (Figure 3A). Since both receptors were fully functional at 37°C, some experiments were performed solely at this temperature for expedience.

Receptor Binding

Membranes prepared from cells stably expressing the FLP-18R1a or FLP-18R1b proteins were used in the receptor binding experiments. The cells were subjected to a 37 to 28°C

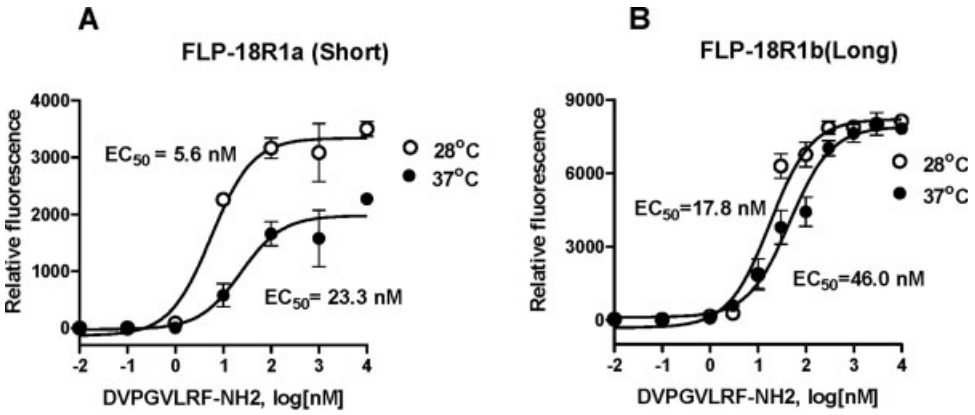

FIGURE 3 Ca^{2+} mobilization as monitored by FLIPR. Receptor signaling in cells stably transfected with *flp-18R1a* (panel A) or *flp-18R1b* DNA (panel B) and cultured either at 37 or 28°C in response to increasing concentrations of DVPGVLRF-NH_2.

temperature shift and incubation at 28°C for 16–24 h before cell harvesting for membrane preparation.

In a saturation binding experiment, [^{125}I-Y°]-DVP GVLRF-NH_2 was bound with a similar high affinity to both receptors FLP-18R1a and FLP-18R1b (K_d values of 0.16 and 0.25 n*M*, respectively). The shorter receptor isoform FLP-18R1a was expressed in the plasma membrane with a higher receptor density than the longer variant FLP-18R1b (B_{max} values of 1.12 and 0.67 pmol/mg membrane protein, respectively; Figure 4A).

In competition binding experiments, DVPGVLRF-NH_2 potently competed with the radioactive probe at both receptors with K_i values of 3.8 and 2.5 n*M* for FLP-18R1a and FLP-18R1b, respectively (Figure 4B).

Signaling Pathways

Strong calcium signaling observed for both receptor isoforms implied GPCR coupling to Gq protein and signaling via phospholipase C. However, calcium responses can also result from a GPCR binding to Gs protein and activating cAMP-dependent calcium channels as well as from an activated trimeric Gi protein(s) yielding a dissociated G$\beta\gamma$ subunit, which can trigger an increase in cellular calcium through a separate pathway.

To investigate the nature of FLP-18R1a and FLP-18R1b signaling, clonal cells were grown at 28°C, preincubated with 10 μM phospholipase inhibitor U-73122 or its inactive analog U-73343[19] before a challenge with increasing concentrations of DVPGVLRF-NH_2. Signaling of both receptors was attenuated, but not completely abolished by U-73122 but was essentially unaffected by U-73343 (Figures 5A and 5B). After the U-73122 treatment, the EC_{50} value for DVPGVLRF-NH_2 changed from 11.3 to 239.2 n*M* (a 21-fold increase) at FLP-18R1a and from 8.6 to 426.6 n*M* (ca. 50-fold increase) at FLP-18R1b (Figures 5A and 5B).

In a separate experiment to test possible Gi protein involvement in FLP-18R1a and FLP-18R1b signaling, clonal cells were grown at 37°C and pretreated overnight with PTX to uncouple the receptors from Gi before measuring calcium responses to increasing concentrations of DVPGVLRF-NH_2. PTX pretreatment resulted in more active receptors and an EC_{50} shift to lower values. The EC_{50} value for DVPGVLRF-NH_2 decreased from 32.4 to 1.5 n*M* for FLP-18R1a (Figure 6A) and from 56.1 to 1.0 n*M* for FLP-18R1b (Figure 6B).

cAMP experiments were carried out with clonal cells grown at 37°C. Exposure to DVPGVLRF-NH_2 resulted in weak (about 15% cAMP increase over the basal) but concentration-dependent cAMP responses (EC_{50} values of 75.3 and 240 n*M* for FLP-18R1a and FLP-18R1b, Figures 7A and 7B, respectively). It is quite possible that if the cAMP assays were run with cells cultured at 28°C, the agonist responses could have been stronger.

DISCUSSION

Described in this report is the cloning and functional annotation of two alternatively spliced variants of the orphan *C. elegans* GPCR Y58G8A.4 as receptors activated by peptides encoded by the *flp-18* precursor gene, designated FLP-18R1a (Y58G8A.4a) and FLP-18R1b (Y58G8A.4b). TheY58G8A.4a and Y58G8A.4b proteins consist of 397 and 433 amino acids residues, respectively, and differ both in amino acid sequence and length in the C-terminal tail. The C-terminal regions Y58G8A.4a/FLP-18R1a and Y58G8A.4b/FLP-18R1b contain, respectively, seven and eight possible serine phosphorylation (only two of them are the same, Ser354 and Ser 357) and fewer predicted threonine phosphorylation sites. There is

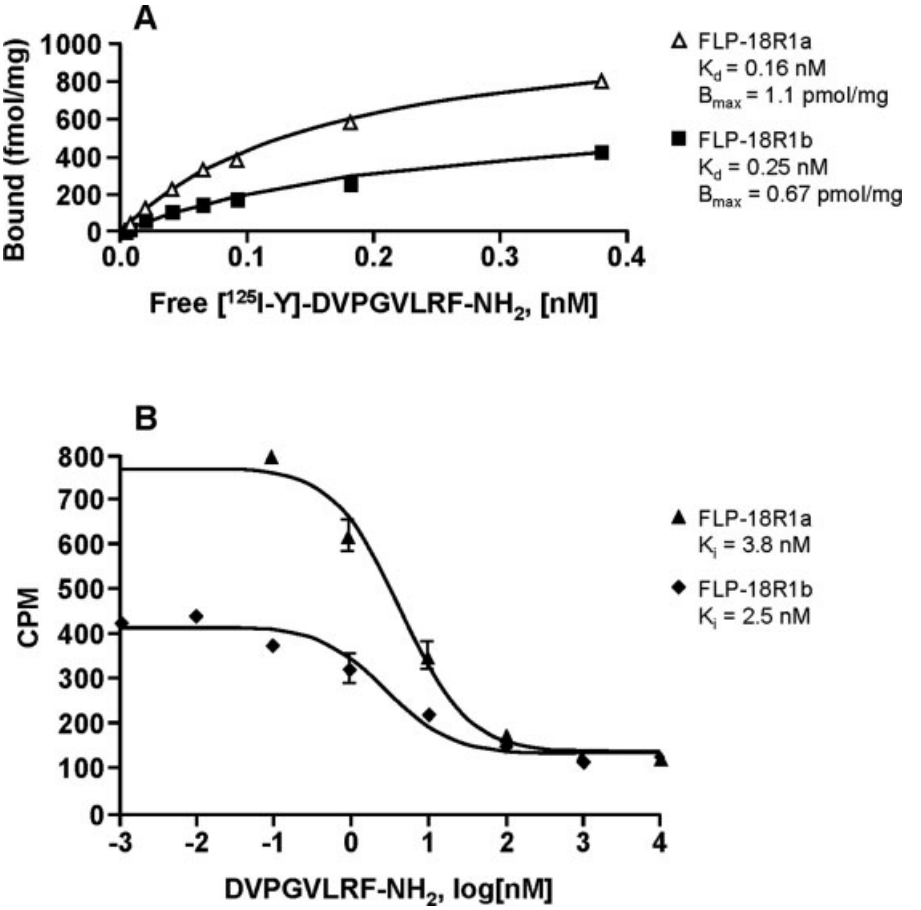

FIGURE 4 Receptor binding using membranes expressing the FLP-18R1a or FLP-18R1b receptors prepared from clonal cells cultured at 28°C. (A) Direct binding of increasing concentrations of [^{125}I-Y^{o}]-DVPGVLRF-NH$_2$. (B) Competition binding experiment (see Experimental section for details).

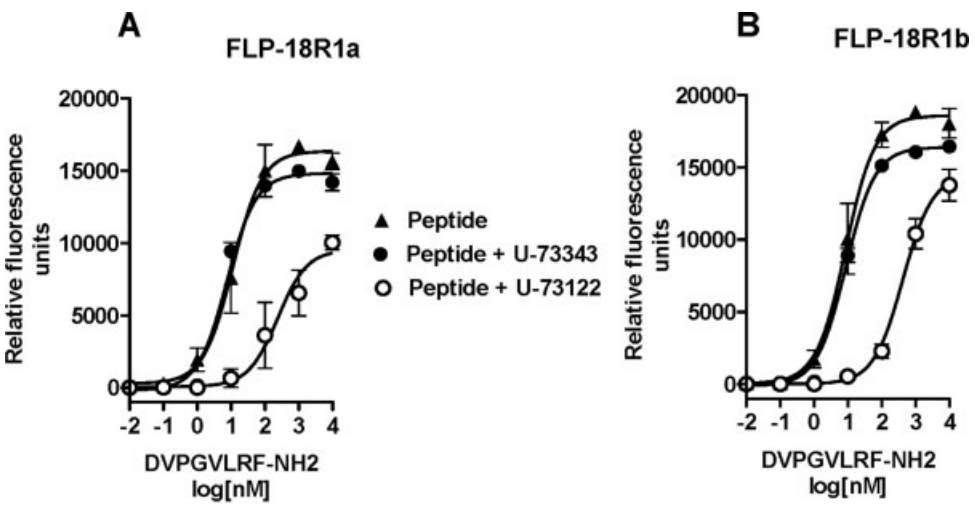

FIGURE 5 Effects of a phospholipase inhibitor U-73122 and its inactive analog U-73343 on DVPGVLRF-NH$_2$-mediated Ca^{2+} mobilization in cells stably expressing FLP-18R1a (panel A) or FLP-18R1b (panel B) as monitored by FLIPR. Cells were incubated overnight at 28°C before the experiment.

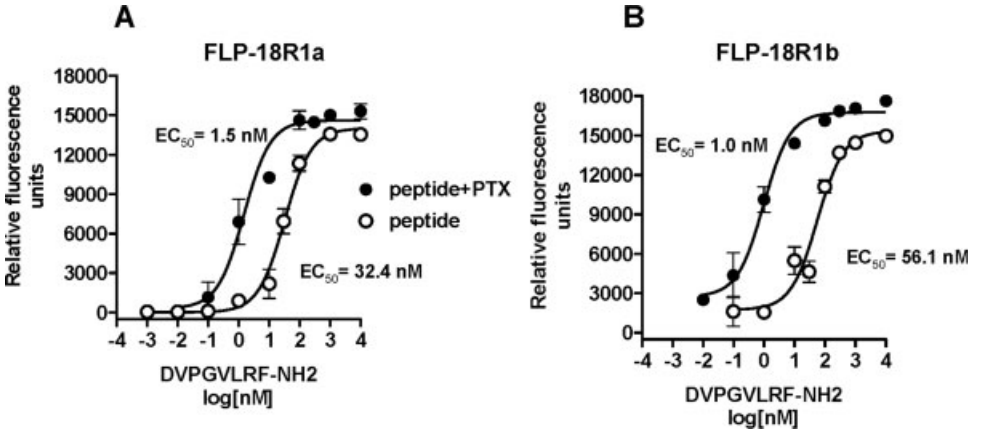

FIGURE 6 Effects of PTX on DVPGVLRF-NH_2-mediated Ca^{2+} mobilization at 37°C in cells stably expressing FLP-18R1a (panel A) or FLP-18R1b (panel B) as monitored by FLIPR.

only one predicted Tyr phosphorylation site in the C-terminus of Y58G8A.4b/FLP-18R1b (Tyr404) and none in Y58G8A.4a/FLP-18R1a (www.cbs.dtu.dk/services/NetPhos/).

Activation of both receptor variants in CHO cells by FLP-18 peptides resulted in potent, concentration-dependent intracellular Ca^{2+} mobilization. Three *A. suum* neuropeptides AF3, AF4, and AF20 that share the FLP-18 peptides C-terminal motif, -PGVLRF-NH_2, were also potent agonists. It is worth noting that the FLP-18 peptides reported here represent only putative sequences predicted from the *flp-18* precursor gene at the time of our study.[25] Since then, most of the FLP-18 peptides listed in Table I have been isolated and sequenced.[3,26–28] Recently, SYFDEKKSVPGVLRF-NH_2 was isolated[3,27] and EMPGVLRF-NH_2 was both predicted and isolated[3,27]; neither was included in the current study. When we tested KSVPGVLRF-NH_2 (a predicted sequence[25]) and SVPGVLRF-NH_2 (the isolated peptide[26–28]), both peptides were equally potent to activate FLP-18R1b but SVPGVLRF-NH_2 was two fold more potent at the FLP-18R1a receptor (Table I). It is interesting to note that the shorter peptides were more potent than longer peptides and that the most potent was not a *C. elegans* but an *A. suum* peptide, AVPGVLRF-NH_2 (EC_{50} 7.6–13.1 n*M*). The longest *C. elegans* FLP-18 peptide DFDGAMPGVLRF-NH_2 was the least potent (EC_{50} 117.2–124 n*M*). It is also interesting to note that GLGPRPLRF-NH_2 (AF9 or FLP-21 peptide) identified as a cognate ligand for the *C. elegans* NPR-1 receptor[11,13] also activated FLP-181Ra and FLP-18R1b, although with a considerably lower potency (EC_{50} 254–267 n*M*). It is worth noting that one FLP-1 peptide and also peptides encoded on *flp-3* activated both isoforms of the FLP-18 receptor; however, with the EC_{50} in the submicromolar to micromolar range it is likely that these peptides are not endogenous ligands for this receptor.

Several FLP-18 peptides were reported to activate NPR-1/FLP-21-R when this receptor was expressed in oocytes or *C.elegans* pharyngeal tissue.[13] These findings suggest a promiscuous nature for the NPR-1 and FLP-18 receptors,

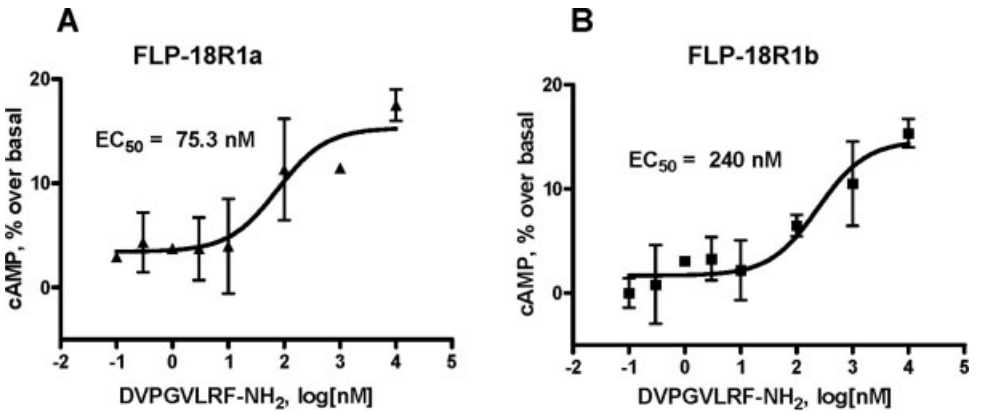

FIGURE 7 DVPGVLRF-NH_2-mediated cAMP release at 37°C in cells stably expressing FLP-18R1a (panel A) or FLP-18R1b (panel B).

which seem to be capable of recognizing multiple FLP ligands produced by different *flp* genes or conserved FLPs from other invertebrates. There are now many examples of the potentially enormous complexity of peptide actions in *C. elegans*, in which a given receptor can bind to multiple FLPs encoded by distinct genes and a single FLP can bind to multiple receptors.[3,26]

Despite different C-termini, the region implicated in distinct G-protein recognition for many other GPCRs, the same signaling pathways were observed for both Y58G8A.4 isoforms expressed in CHO cells. Gq protein coupling seems to be the main but not exclusive signaling pathway since cells pretreated with U-73122, a phospholipase inhibitor, attenuated but did not completely abolish the Ca^{2+} signal. A much less pronounced Gs-mediated pathway was also detected by an agonist-triggered concentration-dependent increase in cAMP. Somewhat unexpectedly, PTX pretreatment of cells stably expressing either FLP-18R1a or FLP-18R1b resulted in more active receptors. Concentration-dependent response curves for DVPGVLRF-NH_2-mediated Ca^{2+} mobilization were left-shifted. This result may not reflect a direct contribution of Gi to Ca^{2+} signaling via the released G$\beta\gamma$ subunit but rather an increased contribution from the receptor-Gs coupling to open more cAMP-gated Ca^{2+} channels resulting from the Gi inactivation by PTX. Possible contributions from the receptor—Gs couplings were confirmed by cAMP responses to increased concentrations of an FLP-18 peptide. However, this effect was rather small and might be due to the fact that the experiments with Gq signalling were carried out after the 37 to 28°C temperature shift whereas those on Gs and Gi were carried out at 37°C. It is therefore possible that the higher temperature could have significantly diminished the signaling efficiency such that the contribution of Gi/Gs appears artificially low.

At present it is not known what the preferred signaling pathways of the FLP-18R1 receptors are in their native environment in *C. elegans*. As shown by Rogers et al.,[13] NPR-1 expressed in *Xenopus laevis* oocytes coupled to Go/Gi activation resulting in opening of inwardly rectifying K^+ channels. The NPR-1 coupling to Gi was also demonstrated in CHO cells.[11] However, when the same NPR-1 receptor was ectopically expressed in the *C. elegans* pharynx, agonist-mediated signaling appeared to be mediated through Gq.[13] This result underscores the context-dependent pharmacology of GPCRs, which must be taken into consideration when interpreting data from heterologously expressed systems.

In contrast to some earlier deorphanized *C. elegans* neuropeptide GPCRs,[11,12] the two FLP-18 receptor variants were fully functional at 37°C when expressed in CHO cells, but their activity was increased by cell incubation at 28°C. The functional expression of the FLP2 receptor at 37°C in CHO cells was also reported by Mertens et al.[14] Therefore, a 37 to 28°C temperature shift is absolutely necessary for functional expression of some, but not all, nematode GPCRs in mammalian cells.

As reported previously, we were unable to successfully generate stable CHO cell lines for the NPR-1/FLP-21-R[11] or FLP-15[12] receptors. In contrast, this was not a problem for both FLP-18R1a and FLP-18R1b. Likewise, Mertens et al.[14] obtained stable clonal cell lines for the FLP2 receptor in CHO cells and for another *C. elegans* GPCR, C26F1.6, heterologously expressed in HEK293 cells.[29] To date, biological roles for neither the FLP-18 receptors nor the *flp-18* precursor have been defined in *C. elegans*; matching the FLP-18 peptides with their receptors facilitates the experimental investigation of this system. However, an RNAi approach has shown that silencing of the *flp-18* ortholog in the potato cyst nematode, *Globodera pallida*, resulted in a distinctly aberrant behavioral phenotype, which indicates a key role of the FLP-18 peptides in motor function.[30] Physiological effects of PGVLRFamides have been observed in other nematodes. For instance, peptides with this motif were excitatory on *A. suum* somatic and ovijector muscle tissue.[31–33] Threshold effects were observed at $\sim$1 n*M* for at least two of these peptides, though a comprehensive structure–activity relationship has not been established. They also reduced cAMP levels in somatic muscle tissue.[33] These data suggest that the FLP-18 receptors are intriguing targets for anthelmintic discovery.

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