The calcitonin (CT)/CT gene-related peptide (CGRP) family is conserved in vertebrates. The activities of this peptide family are regulated by a combination of two receptors, namely the calcitonin receptor (CTR) and the CTR-like receptor (CLR), and three receptor activity-modifying proteins (RAMPs). Furthermore, RAMPs act as escort proteins by translocating CLR to the cell membrane. Recently, CT/CGRP family peptides have been identified or inferred in several invertebrates. However, the molecular characteristics and relevant functions of the CTR/CLR and RAMPs in invertebrates remain unclear. In this study, we identified three CT/CGRP family peptides (Bf-CTFPs), one CTR/CLR-like receptor (Bf-CTFP-R), and three receptor activity-modifying proteins (Bf-RAMP-LPs) in the basal chordate amphioxus (Branchiostoma floridae). The Bf-CTFPs were shown to possess an N-terminal circular region typical of the CT/CGRP family and a C-terminal Pro-NH₂. The Bf-CTFP genes were expressed in the central nervous system and in endocrine cells of the midgut, indicating that Bf-CTFPs serve as brain and/or gut peptides. Cell surface expression of the Bf-CTFP-R was enhanced by co-expression with each Bf-RAMP-LP. Furthermore, Bf-CTFPs activated Bf-CTFP-R/Bf-RAMP-LP complexes, resulting in cAMP accumulation. These results confirmed that Bf-RAMP-LPs, like vertebrate RAMPs, are prerequisites for the function and translocation of the Bf-CTFP-R. The relative potencies of the three peptides at each receptor were similar. Bf-CTFP2 was a potent ligand at all receptors in cAMP assays. Bf-RAMP-LP effects on ligand potency order were distinct to vertebrate CGRP/adrenergulin/amylin receptors. To the best of our knowledge, this is the first molecular and functional characterization of an authentic invertebrate CT/CGRP family receptor and RAMPs.

Calcitonin (CT),² CT gene-related peptide (CGRP), adrenomedullin, amylin, and CT receptor-stimulating peptide (CRSP) belong to the CT/CGRP family in vertebrates. These peptides reciprocally display low sequence similarity and participate in distinct biological functions; CT, CGRP, adrenomedullin, amylin, and CRSP play major roles in bone metabolism, vasodilation, blood pressure decrease, glucose metabolism, and food intake behavior, respectively (1–4). On the other hand, all of the CT/CGRP family peptides harbor a circular structure that is formed by a disulfide bridge between two cysteine residues in the N-terminal region.

In vertebrates, CT/CGRP family peptides bind to two paralogous G protein-coupled receptors: CT receptor (CTR) and CTR-like receptor (CLR) (5). The ligand selectivity of CT/CGRP family peptides is regulated through a combination of CTR/CLR and three receptor activity-modifying proteins (RAMPs). For example, CGRP shows the highest affinity and potency for the CLR-RAMP1 heterodimer, whereas CLR-RAMP2 and -3 heterodimers are most responsive to adrenomedullin in humans (6, 7). RAMPs also play a crucial role in the translocation of the CLR to the plasma membrane, because the CLR remains in the endoplasmic reticulum without association with RAMP (6–8).

The molecular characterization or inference of CT/CGRP family peptides in deuterostome invertebrates, as well as in non-mammalian vertebrates, has been extensively documented (9–12). The first deuterostome invertebrate CT/CGRP family peptide, Ci-CT, was characterized in an ascidian, Ciona intestinalis (12). Furthermore, genes of CT/CGRP-like peptides have been predicted in the genome and cDNA sequences of...
Amphioxus Calcitonin Family Peptides and Their Receptors

deuterostome invertebrates, including the sea urchin, sea cucumber, and acorn worm (9–11). Nevertheless, neither molecular nor functional characterization of a receptor or RAMP has been carried out in any deuterostome invertebrate. Accordingly, the regulatory mechanisms underlying ligand selectivity and translocation of deuterostome invertebrate CT/CGRP family peptide receptors assisted by RAMPs remain to be investigated.

The amphioxus, Branchiostoma floridae belongs to the Cephalochordata, which constitutes the chordate phylum with vertebrates and urochordates (13). Comparative genomic studies have revealed that the gene structure and chromosome composition are largely conserved between B. floridae and vertebrates (13–15). Thus, B. floridae is believed to be an appropriate model for research into the molecular features, functionalities, and evolutionary aspects of CT/CGRP family peptides, receptors, and RAMPs in chordates. Here, we describe the molecular and functional characterization of three CT/CGRP family peptides (Bf-CTFPs), one CTR/CLR-like receptor (Bf-CTR), and three novel RAMP-like proteins (Bf-RAMP-LPs) of B. floridae. Our findings provide new insights into the original functions of a CT/CGRP family receptor and a RAMP in a common ancestor in chordates. To the best of our knowledge, we are the first to identify an authentic CT/CGRP family peptide receptor and RAMPs and to elucidate the regulatory functions of RAMPs that are unique as well as those in common with their vertebrate counterparts.

Experimental Procedures

Animals—Adults of B. floridae were obtained from Tampa Bay, Florida.

In Silico Search for CT/CGRP Superfamily Peptides, Receptors, and RAMPs in the Branchiostoma Genome—To obtain orthologous peptides of the CT/CGRP family, we performed tblastn searches against the B. floridae genome database (available at the JGI Genome Portal Web site), using the amino acid sequence of Takifugu CGRP (CAC81277) as a query and an E-value cut-off of <10,000. To examine whether the gene models extracted were indeed candidates for CT/CGRP superfamily genes, we manually searched for characteristics typical of the CT/CGRP superfamily peptides, namely the presence of two Cys residues flanking four or five amino acids, two dibasic processing sites, and an amidation signal. Furthermore, to obtain all paralogous peptide genes, extracted CT/CGRP family peptide candidates were used as queries for tblastn as described above.

To identify homologous genes of CTR/CLR in amphioxus, we conducted a tblastn search using human CTR (NP_001733) as a query, with a cut-off value of 0.001. We confirmed the similarity of the amphioxus candidates with CTR/CLR by performing a tblastn search against the NCBI Nucleotide collection (nr/nt) database. We extracted paralogous genes of amphioxus RAMP by performing a tblastn search with amphioxus RAMP candidates as query sequences; we confirmed these genes by using tblastn analysis as described above.

Cloning of the Bf-CTFP-R and Bf-RAMP-LPs—Cloning of the Bf-CTFP-R and Bf-RAMP-LPs was performed as described previously (12). Total RNA (1 μg) from adult amphioxus (whole body) was reverse-transcribed to template cDNA at 55 °C for 60 min by using the oligo(dT) anchor primer and SuperScriptIII RNase H-reverse transcriptase (Invitrogen). We obtained partial sequences of Bf-CTFP-R and Bf-RAMP-LPs by using PCR with the primers listed in supplemental Table S1.

In addition, we determined the transcriptional start sites of Bf-CTFP-R and Bf-RAMP-LPs by using the 5′-RACE system for rapid amplification of cDNA ends, version 2.0 (Invitrogen). Template cDNA was synthesized using each gene-specific complementary primer and was amplified using the anchor primer and each first gene-specific complementary primer. The second PCR products were obtained by amplification of the first PCR products using the same anchor primer and each second gene-specific complementary primer and were then subcloned. All of the primers used in the 5′-RACE analysis are listed in supplemental Table S1. The 3′-UTRs of Bf-CTFP-R and Bf-RAMP-LPs were obtained by using the 3′-RACE system for rapid amplification of cDNA Ends (Invitrogen). The first PCR was performed using the anchor primer and each gene-specific primer. The second PCR was also performed using the anchor primer and each gene-specific primer. The second PCR products were subcloned. All of the subcloned inserts were sequenced on an ABI PRISM™ 3130 Genetic Analyzer with a Big-Dye Sequencing Kit version 3.1 (Applied Biosystems, Carlsbad, CA), using universal primers (SP6 and T7).

Prediction of the Secondary Structure of Bf-RAMP-LPs—The secondary structures of Bf-RAMP-LPs were predicted on the basis of position-specific scoring matrices using the Web server PSIPREDICT version 3.3 (16).

Phylogenetic Analysis of Bf-CTFP-R and Bf-RAMP-LPs—Phylogenetic analysis was performed as described previously (17). The amino acid sequence of Bf-CTFP-R was aligned with the corresponding amino acid sequences of CTR, CLR, and CRHR using the CLUSTAL program (18), and the alignment was checked manually. After removal of the gaps, the alignments were used to construct phylogenetic trees. The trees were constructed using the neighbor-joining method in MEGA4.1 (19, 20). Phylogenetic analysis of Bf-RAMP-LPs was performed as described above. The names and accession numbers of the genes used in the phylogenetic analysis are shown in supplemental Table S2.

In Situ Hybridization—Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3 cDNA fragments were amplified from the adult amphioxus cDNA library by using PCR with a pair of gene-specific primers summarized in supplemental Table S1 and were subcloned into pBlueScriptII SK(+) plasmids. The cDNA clones of Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3 were used to synthesize a digoxigenin-labeled RNA probe using a digoxigenin RNA labeling kit (Roche Applied Science). Whole-mount in situ hybridization of the adult amphioxus was performed as described previously (21).

Peptide Synthesis—Bf-CTFPs were synthesized by using a solid-phase synthesizer (Applied Biosystems 430A) with the FastMoc method, according to the manufacturer’s instructions.
In brief, 0.25 mmol of Fmoc (N-(9-fluorenyl)methoxycarbonyl)-NH-SAL-REG resin and 1 mmol of amino acid were used in the reaction. An intramolecular disulfide bond was formed by oxidation of the resultant Bf-CTFPs with K$_3$(Fe(CN)$_6$) as reported previously (22). Higher than 95% purity and appropriate molecular weight were confirmed by HPLC (GILSON 321 HPLC Pump) and mass spectrometry (Bruker UltraflexIII), respectively.

Construction of Expression Vectors—The ORF sequences of Bf-CTFP-R and the three Bf-RAMP-LPs were amplified using PCR with the primers listed in supplemental Table S1 and were subcloned into pENTER/D-TOPO (Invitrogen). In addition, pcDNA6.2-Bf-CTFP-R, pcDNA6.2-Bf-RAMP-LP1, pcDNA6.2-Bf-RAMP-LP2, and pcDNA6.2-Bf-RAMP-LP3 were constructed by using an LR recombination reaction between the pENTR constructs described above and the destination vector pcDNA6.2/V5-DEST (Invitrogen), pIRES1-V5-Bf-CTFP-R, pIRES-V5-Bf-RAMP-LP1, pIRES-V5-Bf-RAMP-LP2, and pIRES-V5-Bf-RAMP-LP3 were constructed by subcloning the coding sequence of Bf-CTFP-R and Bf-RAMP-LPs into pIRES/Neo-V5.

Fluorescence-activated Cell Sorting (FACS) Analysis of Bf-CTFP-R and Bf-RAMP-LPs—The green monkey kidney cell line COS-7 was maintained in DMEM supplemented with 10% FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B at 37 °C under a humidified atmosphere of 95% air and 5% CO$_2$. For experimentation, cells were seeded into 24-well plates; upon reaching 70–80% confluence, the cells were transiently transfected with the indicated cDNAs by using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. Expression vector constructs for Bf-CTFP-R and each Bf-RAMP-LP were cotransfected in equal amounts. As a control, cells were transfected with empty vector (mock). All experiments were performed 48 h after transfection. Evaluation of cell surface expression of the indicated V5-tagged constructs was performed using an EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA), as reported previously (23). This experiment was independently performed five times with duplicate samples.

cAMP Accumulation Assay—COS-7 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C under a humidified atmosphere of 5% CO$_2$. Expression plasmids were transfected into the cells in 25-cm$^2$ flasks by using Lipofectamine 2000 (Invitrogen). The cells were harvested in 96-well plates 24 h after transfection. After 48 h of incubation for 10 min at room temperature in Krebs-Ringer bicarbonate buffer with 10 mm glucose (pH 7.4) containing 1 mm 3-isobutyl-1-methylxanthine (Sigma-Aldrich), the cells were exposed to each of the synthesized Bf-CTFPs at 37 °C for 15 min. The cells were lysed by shaking for 10 min and were then analyzed with the CatchPoint cyclic AMP fluorescent assay kit (Molecular Devices, Toronto, Canada). The cAMP level was measured as described previously (24). This assay was independently performed three times with duplicate samples.

Calcium Mobilization Assay—The calcium mobilization assay was performed as described previously (17). In brief, the transfection procedure is the same as that used in the cAMP accumulation assay. Forty-eight hours after transfection into HEK293MSR cells, ligand-induced intracellular calcium ion accumulation was measured by using the FLIPR Calcium 5 assay kit (Molecular Devices). Fluorescence evoked by calcium accumulation was detected by using a FlexStation II automated apparatus (Molecular Devices) at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. This assay was independently performed three times with duplicate samples.

Statistical Analysis—The results were expressed as the mean ± S.E. The FACS, cAMP, and calcium ion data were analyzed using Prism version 6 (GraphPad Software Inc., La Jolla, CA). The cAMP and calcium ion data were fitted to a concentration-response curve, and the pEC$_{50}$, $E_{max}$, and Hill slope were calculated. Differences among multiple groups were evaluated by using one-way analysis of variance as appropriate; $p < 0.05$ was considered significant. Post hoc testing was conducted by using Dunnett’s test for comparison with the controls.

Results

Identification of Bf-CTFPs—We initially searched the genome database of B. floridae by using tblastn with Takifugu CGRP (accession number CAC81277) as a query sequence. We retrieved three gene models, namely fgenesh2_pg.scaffold_167000051, fgenesh2_pg.scaffold_167000044, and fgenesh2_pg.scaffold_167000049, as possible candidates. We confirmed the possible candidate of the CT/CGRP superfamily peptides, which possess four or five amino acids between two Cys residues, a dibasic processing site, and an amidation signal. Furthermore, we searched the Branchiostoma genome database for paralogous CT/CGRP family peptides using the amphioxus candidates as query sequences. After removing overlapping gene models, we detected three gene models, namely fgenesh2_pg.scaffold_167000043, fgenesh2_pg.scaffold_167000044, and fgenesh2_pg.scaffold_167000051. Partial sequences of these genes, including the complete open reading frame, were cloned from adult amphioxus cDNA by using RT-PCR. The deduced 138-, 128-, and 154-amino acid sequences encoded a different CT/CGRP-like peptide, followed by a Gly amidation signal at the C terminus and dibasic endoproteolytic sites at both termini (Fig. 1A). The predicted CT/CGRP-like peptides were composed of 31, 33, and 33 amino acids. These peptides possessed an N-terminal circular region containing four amino acids flanked by cysteines (Fig. 1), which is reminiscent of vertebrate CGRP, adrenomedullin, and amylin (Fig. 1B) (3, 25–27). In addition, the peptides contained a C-terminal Pro-NH$_2$, which is characteristic of vertebrate CT (Fig. 1B) (27). Taken together, these results implied that CT/CGRP family peptides are conserved in amphioxus; thus, we designated these three peptides as B. floridae CT family peptide (Bf-CTFP1), Bf-CTFP2, and Bf-CTFP3 (Fig. 1).

Localization of Bf-CTFP mRNA in Adult B. floridae—To examine the localization of Bf-CTFP mRNA, we performed in situ hybridization in adult B. floridae. We found that Bf-CTFP1 gene expression was localized in neurons in the rostral part of the central nervous system (Fig. 2A). In addition, we showed that the transcript of Bf-CTFP2 was expressed in neurons in the
Amphioxus Calcitonin Family Peptides and Their Receptors

A

FIGURE 1. Alignment of the amino acid sequences of Bf-CTFPs. A, comparison of partial amino acid sequences of three Bf-CTFP precursors. Identical and similar amino acid sequences among the three peptides are indicated by black and gray boxes, respectively. The predicted 5′-processing residue is indicated by an overline. The predicted 3'-amidation site is indicated by a dotted overline. A disulfide bridge between two Cys residues is indicated by a line. The GenBank/EBI Data Bank accession numbers of Bf-CTFP1–3 are AB985775, AB985776, and AB985777, respectively. B, multiple alignments of mature peptides of vertebrate CT/CRP and Bf-CTFPs. Identical and similar amino acid sequences among at least five members are indicated by black and gray boxes, respectively. A Pro-amide conserved between Bf-CTFPs and vertebrate Cts is highlighted by an asterisk.

rostral part of the central nervous system (Fig. 2B) and in endocrine cells in the midgut (Fig. 2, C and D). Bf-CTFP2 mRNA was expressed in morphologically typical endocrine cells in the invertebrate gut epithelium (Fig. 2D) (28, 29). We detected expression of Bf-CTFP3 in the rostral part of the central nervous system and in epithelial cells in the hindgut (Fig. 2, E and F). These results suggested that the Bf-CTFPs function as brain and/or gut peptides and that they have different biological roles in adult amphioxus.

Molecular Characterization of the Bf-CTFP-R—To identify the CTR/CLR family receptor in amphioxus, we searched the genome database of B. floridæ using the tblastn algorithm, with the human CTR protein sequence as a query. We identified the entry e_gw.397.20 as a candidate. In addition, we determined the full-length sequence of the cdNA (2321 base pairs) by applying a combination of RT-PCR, 5′-RACE, and 3′-RACE to the adult B. floridæ cdNA library. The deduced 341-amino acid sequence contained a hormone-binding domain, which is typical of the secretin GPCR family (including CTR and CLR), and seven transmembrane domains (TM1–TM7) (Fig. 3A). Sequence alignment demonstrated that this protein showed 61.8 and 64.3% sequence similarity with the human CTR and CLR, respectively (Fig. 3A). Furthermore, this receptor possesses four N-glycosylation sites that are well conserved among mammalian and human CLRs (Fig. 3A). Molecular phylogenetic analysis revealed that this protein was grouped in the same clade as that formed by the chordate CTR and CLR with high bootstrap support (Fig. 3B). Taken together, these results indicated that amphioxus possesses the only CTR/CLR orthologous receptor that is a candidate Bf-CTFP receptor, Bf-CTFP-R.

Molecular Characterization of Bf-RAMP-LPs—Next, we performed a blast search of the B. floridæ genome database with Takifugu RAMP1 (BAE45305) as a query sequence. We obtained two gene model entries, namely fgenes2_pg.scaffold_571000001 and fgenes2_pg.scaffold_372000009, as possible candidates. In addition, we searched paralogs by using tblastn analysis with the candidate sequences as queries. We obtained four non-overlapping gene model entries, fgenes2_pg.scaffold_571000001, fgenes2_pg.scaffold_372000005, fgenes2_pg.scaffold_397000019, and fgenes2_pg.scaffold_372000009, as possible candidates. We identified three full-length sequences of amphioxus RAMP candidates from the adult B. floridæ cdNA library by using RT-PCR and RACE. The deduced 103-, 194-, and 189-amino acid sequences were shown to harbor a transmembrane domain and four Cys residues that are conserved in vertebrate RAMPs (Fig. 4A). Thus, we designated these three RAMP-like proteins as Bf-RAMP-LP1, Bf-RAMP-LP2, and Bf-RAMP-LP3, respectively. Alignment of the RAMPs indicated that all three Bf-RAMP-LPs show low amino acid sequence similarity (15.4–39.3%) with human RAMPs and that Bf-RAMP-LP2 and -3 possess a longer extracellular domain than the human RAMPs (Fig. 4A). However, secondary structure prediction conducted using PSIPREDICT version 3.3 indicated conservation of three α-helical structures at a similar position between the three Bf-RAMP-LPs and their human counterparts (Fig. 4A).

A molecular phylogenetic tree of chordate RAMPs revealed that these amphioxus proteins belong to a group different from the vertebrate RAMPs, suggesting that Bf-RAMP-LPs are orthologous to vertebrate RAMPs and that they were triplicated in a B. floridæ-specific lineage (Fig. 4B).

Cell Surface Expression of Bf-CTFP-R Co-expressed with Bf-RAMP-LPs—Given the biological role of mammalian RAMPs in assisting the translocation of the CLR to the plasma membrane, we postulated that Bf-RAMP-LPs also enhance translocation of Bf-CTFP-R to the plasma membrane, where it is involved in signaling. To examine this hypothesis, we assessed the translocation of V5-tagged Bf-CTFP-R (V5-Bf-CTFP-R) to the cell surface in the presence or absence of Bf-RAMP-LP by using flow cytometry. When the empty vector was transfected into COS-7 cells (mock), surface binding of the FITC-labeled V5 antibody was within the 2% detection limit of flow cytometry (Fig. 5). In the absence of wild-type Bf-RAMP-LPs, ~14% of V5-Bf-CTFP-R-expressing cells were observed (Fig. 5, A–C). This phenomenon has been previously observed in COS-7 cells expressing mouse CLR alone (30, 31). When wild-type Bf-RAMP-LP1, Bf-RAMP-LP2, or Bf-RAMP-LP3 was co-transfected with V5-CTFP-R, we detected 28.6, 28.8, and 21.7% of FITC-labeled cells, respectively. These results confirmed that co-transfection of Bf-RAMP-LP1–3 with V5-CTFP-R increased their cell surface expression by 2.0-, 2.1-, and 1.6-fold, respectively (Fig. 5, A–C). Next, we estimated the cell surface translocation of V5-tagged Bf-RAMP-LPs (V5-Bf-RAMP-LPs). In contrast to expression of V5-Bf-CTFP-R alone, we observed only ~5.8% of FITC-labeled cells without the wild-type Bf-CTFP-R (Fig. 5, D–F). Furthermore, in the presence of the wild-type Bf-CTFP-R, we detected 18, 26, and 22.6% of FITC-labeled cells, respectively. Thus, co-transfection of V5-Bf-RAMP-LP1, V5-Bf-RAMP-LP2, and V5-Bf-RAMP-LP3 with the wild-type Bf-CTFP-R increased the cell surface expression frequency by 3.1-, 4.5-, and 3.9-fold, respectively (Fig. 5, D–F).

Thus, the flow cytometric data indicated that the interaction of Bf-CTFP-R with Bf-RAMP-LP is required for translocation.
of Bf-CTFP-R to the cell surface and for its receptor function; furthermore, the role of RAMP as a transporter of CLR is conserved in amphioxus.

Ligand-Receptor Interaction of Bf-CTFPs and Bf-CTFP-R-Bf-RAMP-LPs—To evaluate the molecular function of Bf-CTFP-R and Bf-RAMP-LPs, we examined cAMP accumulation using COS-7 cells, which express no functional endogenous RAMPs (32). No Bf-CTFP-induced cAMP accumulation was detected in COS-7 cells transfected with an empty or a Bf-CTFP-R-expressing vector (Fig. 6A), confirming that Bf-CTFP-R on its own fails to serve as a functional receptor.

We then co-transfected COS-7 cells with Bf-CTFP-R and each Bf-RAMP-LP and evaluated Bf-CTFP-induced cAMP accumulation. Mammalian CLR that is associated with RAMP reportedly elicits cAMP production in response to CGRP or adrenomedullin (8, 33, 34). In COS-7 cells, co-expression of Bf-CTFP-R and Bf-RAMP-LP1, Bf-RAMP-LP2, or Bf-RAMP-LP3, three Bf-CTFPs, elicited significant cAMP production in a concentration-dependent manner (Fig. 6B–D). The pEC50 and Emax values for Bf-CTFPs were calculated and summarized in Table 1.

Comparison of the pEC50 values of Bf-CTFPs within each Bf-CTFP-R-Bf-RAMP-LP complex demonstrated a unique order of potency that was dependent on the Bf-RAMP-LP: Bf-CTFP2 > Bf-CTFP3 = Bf-CTFP1 in Bf-CTFP-R-Bf-RAMP-LP1; Bf-CTFP2 = Bf-CTFP3 > Bf-CTFP1 in Bf-CTFP-R-Bf-RAMP-LP2; and Bf-CTFP2 > Bf-CTFP3 = Bf-CTFP1 in Bf-CTFP-R-Bf-RAMP-LP3 (Table 1). The potency of Bf-CTFP2 (pEC50 = –10.21) in Bf-CTFP-R-Bf-RAMP-LP1 was higher than that of the remaining two peptides within the Bf-CTFP-R-RAMP-LP1 complex or the other complexes. These results revealed that Bf-CTFP2 was the most potent ligand in all Bf-CTFP-R-Bf-RAMP-LP complexes. Furthermore, the potencies of the three peptides were strongly dependent on co-expressed Bf-RAMP-LPs, and the order was roughly RAMP-LP1 > RAMP-LP3 > RAMP-LP2.

The efficacies (Emax) of the three peptides were not significantly different when Bf-CTFP-R was co-expressed with Bf-RAMP-LP1 or Bf-RAMP-LP3; however, maximal cAMP production varied in Bf-CTFP-R-Bf-RAMP-LP2 (Table 1). Fig. 6C demonstrates that the concentration-response curves for three peptides were shallow, and a large deviation from unity was estimated by the Hill coefficient (0.637, 0.453, and 0.597 for Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3, respectively). These findings possibly reflect multiple interactions between peptides and Bf-CTFP-R-Bf-RAMP-LP2, which may be associated with different efficacies of the three peptides. However, further study is necessary in a persistent as opposed to a transient expression system.

In addition to cAMP, vertebrate RAMPs are known to regulate the intracellular signal profiles formed by second messengers, such as Ca2+ (35). To address this issue in amphioxus, we analyzed Ca2+ accumulation. In cells that expressed Bf-CTFP-R only, Ca2+ accumulation was not induced by Bf-CTFPs (Fig. 7A). However, Bf-CTFPs increased intracellular Ca2+ in the cells that co-expressed Bf-CTFP-R and Bf-RAMP-LPs (Fig. 7B–D, and Table 2). Bf-CTFP2 was most effective in inducing Ca2+ accumulation within all Bf-CTFP-R-Bf-RAMP-LP complexes; however, the effective concentrations of the three peptides were much higher than those of cAMP (Table 2).

We also examined the effects of vertebrate CT/CGRP family peptides. Human CGRP and salmon CT failed to evoke any cAMP or Ca2+ responses within all Bf-CTFP-R and Bf-RAMP-LP complexes (data not shown).

**FIGURE 2. In situ hybridization analysis of Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3 mRNA in adult amphioxus.** Localization of the Bf-CTFP1 (A) and Bf-CTFP2 transcripts (B) in the rostral part of amphioxus is indicated by yellow arrowheads. In the transverse section of the middle part of the digestive tract, expression of Bf-CTFP2 mRNA is indicated by yellow arrowheads (C). A magnification of C is shown in D. Localization of Bf-CTFP3 mRNA in the rostral part of amphioxus (E) and in the final part of the digestive tract (F) is indicated by yellow arrowheads and yellow circles, respectively. Scale bars, 100 μm.
Amphioxus Calcitonin Family Peptides and Their Receptors

Figure 3. Molecular characterization of Bf-CTFP-R. A, alignment of the amino acid sequences of chordate CTR and CLR. Amino acid sequences conserved among at least four members are indicated by black boxes. The predicted hormone-binding domains and seven transmembrane domains (TM1–TM7) are depicted by open boxes and underlines, respectively. Putative N-glycosylation sites are highlighted by dotted overbars. GenBankTM/EBI Data Bank accession number of Bf-CTFP-R is AB979721.B, phylogenetic tree of CTR and CLR in metazoans, constructed by using the neighbor-joining method. The number beside each branch indicates the percentage of times that the node was supported in 1000 bootstrap pseudorepli- cations. Human CRHR1 was used as an outgroup. For simplicity, the unrooted tree is shown as a rooted tree. Bf-CTFP-R is indicated in boldface type and is boxed. The scale bar indicates an evolutionary distance of 0.1 amino acid substitutions/protein.
Amphioxus Calcitonin Family Peptides and Their Receptors

**Discussion**

In the present study, we examined all of the CT/CGRP family peptides and RAMPs and a receptor in the most primitive chordate, *B. floridae*. Due to its phylogenetic position as a basal chordate and the availability of its genome (13–15), *B. floridae* represents a valuable tool for research into the molecular and functional evolution of the endocrine and neuroendocrine systems in chordates. To our knowledge, this is the first study to report the complete molecular and functional characterization of invertebrate CT/CGRP family peptides and their receptor and associated proteins.

**CT/CGRP Family Peptides in Deuterostome Invertebrates**—In the present study, we identified three CT/CGRP family peptides, designated Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3, in *B. floridae*. These peptides possess an N-terminal circular region containing four amino acids flanked by two cysteines.
Ampelis californica, a non-chordate invertebrate deuterostome (9, 10). These peptides are reminiscent of vertebrate CTs in terms of the amino acid length of the N-terminal circular structure and the C-terminal amidated Pro (9–12). However, an N-terminal extension flanked by a circular domain, which is typical of CGRP, adrenomedullin, amylin, and CRSP, has also been observed in CT-like peptides of echinoderms and hemichordates (9–11). Altogether, these findings suggest that comparison of CT/CGRP family peptide sequences is insufficient to elucidate the evolutionary process of the CT/CGRP family in ancestral invertebrate deuterostomes, including amphioxus. Thus, investigation of the functional relationship between the CT/CGRP family peptides, their receptor, and RAMPs is required.

**FIGURE 5.** Flow cytometric analysis of the cell surface translocation of V5-tagged Bf-CTFP-R and Bf-RAMP-LPs. Shown is the frequency of V5-immunopositive cells co-expressing V5-tagged Bf-CTFP-R with each wild-type Bf-RAMP-LP (A–C) and each V5-tagged Bf-RAMP-LP with wild-type Bf-CTFP-R (D–F). The bars denote the mean ± S.E. (error bars) (n = 10). *p < 0.05.

**FIGURE 6.** cAMP accumulation assay in the cells co-transfected with Bf-CTFP-R and Bf-RAMP-LPs. A, concentration-response curves for Bf-CTFP1–3 in the cells expressing Bf-CTFP-R alone. B–D, Bf-CTFP-R/Bf-RAMP-LP1-expressing cells (B), Bf-CTFP-R/Bf-RAMP-LP2-expressing cells (C), and Bf-CTFP-R/Bf-RAMP-LP3-expressing cells (D). Error bars, S.E. (n = 6).
TABLE 1
Comparison of Bf-CTFP potencies (pEC₅₀) calculated from the cAMP second messenger change in the COS7 cells expressing Bf-CTFP-R-Bf-RAMP-LPs.

| Peptides | Bf-CTFP-R-Bf-RAMP-LP1 | Bf-CTFP-R-Bf-RAMP-LP2 | Bf-CTFP-R-Bf-RAMP-LP3 |
|----------|------------------------|------------------------|------------------------|
|          | pEC₅₀  | EC₅₀  | E_max  | pEC₅₀  | EC₅₀  | E_max  | pEC₅₀  | EC₅₀  | E_max  |
| Bf-CTFP1 | -7.29 ± 0.07±b  | 5.13E ± 8  | 70.84 ± 2.10  | -5.93 ± 0.40  | 1.17E ± 6  | 71.23 ± 15.69  | -6.68 ± 0.07  | 2.09E ± 7  | 56.44 ± 2.12  |
| Bf-CTFP2 | -10.21 ± 0.14±b  | 6.17E ± 11  | 69.41 ± 1.94  | -7.54 ± 0.23  | 2.88E ± 8  | 136.1 ± 10.83b  | -8.97 ± 0.07b  | 1.07E ± 9  | 66.63 ± 1.31b  |
| Bf-CTFP3 | -8.04 ± 0.08±b  | 9.12E ± 9  | 71.18 ± 1.84  | -7.43 ± 0.14  | 3.72E ± 8  | 97.93 ± 5.21b  | -7.10 ± 0.08  | 7.94E ± 8  | 59.29 ± 2.20 |

*Significantly different (p < 0.05) between three Bf-RAMP-LP-Bf-CTFP-R complexes.
*Significantly different (p < 0.05) between three Bf-RAMP-LP within each complex of Bf-CTFP-R.
Significantly different (p < 0.05) from C3TP1 within each complex of Bf-RAMP-LP and Bf-CTFP-R.

**Distinct Tissue Distribution of Gene Expression of CT/CGRP Family Peptides in Invertebrate Chordates: An Emerging Enigma**—We detected the expression of Bf-CTFP1, Bf-CTFP2, and Bf-CTFP3 genes in neurons in the central nervous system (Fig. 2, A, B, and E). In vertebrates, CT/CGRP family peptides play pivotal roles in the central nervous system (39, 40). For example, the transcript of CGRP is expressed in the central and peripheral nervous systems by alternative splicing of the CGRP-α gene, and it is involved in olfaction, gustation, memory, and learning in vertebrates (40). CRSP is associated with food intake, thermogenesis, and behavior via CTR expression in the central nervous system (4).

These findings support the view that Bf-CTFPs function as neurotransmitters and/or neuromodulators in a neuropeptidergic fashion in amphioxus. Furthermore, Bf-CTFP2 mRNA was expressed in endocrine cells of the midgut and in epithelial cells of the hindgut (Fig. 2, C, D, and F). CT-immunoreactive cells have been observed in the goldfish intestine; the number of these cells increased with food consumption, suggesting that CT responds to nutrients and controls the digestive process (41). In rats, adrenomedullin is expressed in intestinal enterochromaffin cells and stimulates insulin secretion (42). These findings further support the view that Bf-CTFPs are postulated to be gut hormones.

In contrast to Bf-CTFPs, Ci-CT, the Ciona CT/CGRP family peptide, is widely expressed in several non-neuronal organs. In vertebrates, CT/CGRP family peptides exhibit neuronal and non-neuronal expression. We postulate three possible roles for an original CT/CGRP family peptide in chordate ancestors. First, an original CT/CGRP family peptide, such as Bf-CTFP2 or Bf-CTFP3, might have functioned exclusively in the central nervous systems and gut tissues in chordate ancestors; hence, vertebrates and ascidians might have acquired novel hormonal functions, whereas the neural function might have been lost in ascidian-specific lineages. Second, a CT/CGRP family peptide of an ancestral chordate (e.g. Ci-CT) might have served as a non-neural hormonal factor. In this case, the hormonal functions of the CT/CGRP family peptide in tissues other than the gut might have been abolished in amphioxus lineages, but not in vertebrate lineages, whereas its biological role as a brain peptide might have been acquired in amphioxus and vertebrate lineages. Finally, a CT/CGRP family peptide might have played multiple roles as a non-neural hormone and a peptide in ancestral chordates, as seen in vertebrates. In this case, specialization of neuropeptidic (for all Bf-CTFPs) and gut hormonal (for Bf-CTFP2 and Bf-CTFP3) functions might have occurred in amphioxus. Further investigation of the physiological function of Bf-CTFPs in amphioxus may provide insights into the functional evolution and diversification of the chordate CT/CGRP family peptides.

**Evolutionary Aspects of Bf-CTFP-R and Bf-RAMP-LPs**—Additionally, we conducted a molecular and functional characterization of a receptor for the Bf-CTFPs, Bf-CTFP-R, and its associated proteins Bf-RAMP-LP1, Bf-RAMP-LP2, and Bf-RAMP-LP3. Sequence comparison of Bf-CTFP-R showed high similarity with human CTR and CLR (Fig. 3A). Molecular phylogenetic analysis of CTR/CLR demonstrated that Bf-CTFP-R is derived from a common ancestor of vertebrate CTR and CLR (Fig. 3B). The N-terminal region of the Bf-CTFP-R possesses a hormone-binding site and three N-glycosylation sites, which are responsible for ligand binding and cell surface translocation in the vertebrate CTR/CLR, suggesting that this is a conserved functional domain.

Our previous genome database search in Ciona revealed that Ci-CT-R is a single CTR/CLR ortholog (12). These data suggest that a single orthologous gene of CTR/CLR existed in a common ancestor of chordates and that this gene was duplicated to CTR and CLR in the vertebrate lineage. Bf-RAMP-LPs possessed four Cys residues and a transmembrane domain, which are crucial for the biochemical functions of vertebrate RAMPs (43–46). Furthermore, prediction of the secondary structure of Bf-RAMP-LPs demonstrated that, similar to vertebrate RAMPs, they possess three α-helical regions (Fig. 4A). The molecular phylogenetic tree of the chordate RAMPs indicated that Bf-RAMP-LPs are categorized in a group different from the vertebrate RAMPs, suggesting that these proteins are orthologous to vertebrate RAMPs and that they were triplicated in a B. floridiae-specific lineage (Fig. 4B).

Unlike in amphioxus, a homology search against a genome database of another invertebrate chordate, C. intestinalis, did not reveal the existence of a RAMP-like gene (12). Our molecular characterization of Bf-RAMP-LPs provides evidence that three α-helical regions linked by two disulfide bonds are well conserved in all RAMPs of vertebrates (46) and amphioxus (Fig. 4A) but that the sequence homology of RAMPs is low among chordates. These findings imply that a Ciona RAMP-like gene, if one exists, may show a similar secondary structure to vertebrate RAMPs, regardless of the low similarity of the primary amino acid sequence with vertebrate RAMPs. Alternatively, RAMP might have been lost in the Ciona lineage, given that Ciona has lost a number of genes and has undergone chromosome rearrangements during the evolu-
Amphioxus Calcitonin Family Peptides and Their Receptors

Effect of Bf-CTFPs on Bf-CTFP-R-Bf-RAMP-LP Complexes and Functional Evolution of the CTR/CLR-RAMP System—The molecular function of mammalian CTR/CLR has been extensively investigated (32, 48). In an attempt to elucidate the function of CTR/CLR in protochordates, we previously analyzed the accumulation of cAMP in Ciona. However, we obtained no functional data for Ciona CTR (Ci-CT-R), because a Ci-CT-R expression system could not be established (12); therefore, the functions of protochordate CTR/CLR and the evolutionary origin of chordate CTR/CLR remained unclear. The present investigation provides clear evidence that Bf-CTFPs are authentic ligands for Bf-CTFP-R and that activation of Bf-CTFP-R requires co-expression with Bf-RAMP-LPs. To the best of our knowledge, this is the first functional characterization of invertebrate CTR/CLR.

In vertebrate CTR, RAMP is not essential for ligand recognition and signal transduction, whereas the functions of CLR depend on RAMP (6, 7). Thus, the modes of action of the Bf-CTFP-R are in congruence with those of vertebrate CLRs and strongly suggest that Bf-CTFP-R is probably a CLR-type receptor and that a CT/CGRP family receptor of a chordate ancestor is more closely related to CLRs than to CTRs (6).

Three Bf-CTFPs elicited cAMP accumulation and an increase in Ca²⁺ except for the markedly low Ca²⁺ response at the Bf-CTFP-R-Bf-RAMP-LP3 complex. However, the potencies varied among the peptides and between different receptor-RAMP-LP complexes. Bf-CTFP2 was the most potent ligand among the three peptides, and the highest responsivity was observed in cAMP production via the Bf-RAMP-LP1 complex. The present findings suggest that Bf-CTFP2 is a physiologically dominant peptide that works effectively through the Bf-CTFP-R-Bf-RAMP-LP1 complex in amphioxus. Thus, Bf-RAMP-LPs are responsible for both cell surface translocation of the receptor and ligand selectivity. This is consistent with the characteristics of vertebrate RAMP (48), suggesting that both RAMP functions are conserved in chordates.

However, the present study indicates a difference in CTR/CLR-RAMP phenotype between vertebrates and amphioxus. In vertebrates, RAMP modifies CLR sensitivity to CGRP and adenomedullin, resulting in distinct phenotypes (6, 7). However, this phenotype conversion was not observed in amphioxus, because the relative rank of potency for all three authentic peptides was similar in all Bf-CTFR-Bf-RAMP-LP complexes. These findings imply that RAMP of vertebrates and amphioxus undergo distinct evolutionary processes to regulate receptor phenotype.

In protostomes, the diuretic hormone 31 (DH31) is thought to belong to the CT/CGRP superfamily (49). DH31 receptors are thought to be orthologs of vertebrate CTR/CLRs, denoted as “GPCRCAL.” Analyses of molecular characteristics have been reported for Drosophila melanogaster, Rhodnius prolixus, and Bombyx mori (50–52). In Drosophila, co-expression of mammalian RAMPs with GPCRCAL1 or CG17415 induces cAMP accumulation, suggesting that GPCRCAL possesses CLR-like features. However, as far as we are aware, no endogenous RAMPs have been identified in Drosophila. On the other hand, RAMP-independent receptor activity has been observed in R. prolixus and B. mori. Thus, the molecular function of

![Diagram](image_url)

**FIGURE 7.** Ca²⁺ accumulation assay of in the cells co-transfected with the Bf-CTFP-R and Bf-RAMP-LPs. A, concentration-response curves for Bf-CTFP1-3 in the cells expressing Bf-CTFP-R alone. B-D, Bf-CTFP-R-Bf-RAMP-LP1-expressing cells (B), Bf-CTFP-R-Bf-RAMP-LP2-expressing cells (C), and Bf-CTFP-R-Bf-RAMP-LP3-expressing cells (D). Maximal value of Ca²⁺ accumulation in each experiment was defined as 100%. Error bars, S.E. (n ≥ 6).
GPCCRCAL remains unclear. Further studies of GPCRCAL function will provide deeper insight into the evolutionary processes of CTR/CLR and RAMP in metazoans.

In conclusion, we have identified novel CT/CGRP family peptides, the CLR-like receptor, and RAMP-like proteins in the amphioxus, *B. flordiae*. Furthermore, we are the first to elucidate the molecular function of the invertebrate CLR-like receptor and RAMPs. On the basis of our findings, we propose that the original biological role of RAMP is the transportation of a CLR-type receptor to the cell surface; this role confers the receptor with response activity and selectivity to the cognate CT/CGRP family peptides. Our findings provide a valuable new insight into the molecular and functional evolution of the chordate CT/CGRPergic system, which is concertedely regulated by the CT/CGRP family peptides, their receptors, and activity-modifying proteins.

Author Contributions—T. S. and H. S. designed the study and wrote the paper. K. K. performed the translocation assay of Bf-CTFP-R and Bf-RAMP-LPs using FACS. M. O. and H. T. analyzed Bf-CTFP mRNA localization using *in situ* hybridization. T. S., Y. S., and N. S. performed the molecular cloning and molecular characterization of Bf-CTFPs, Bf-CTFP-R, and Bf-RAMP-LPs. T. S., S. M., and T. O. performed the ligand-receptor assay using a mammalian cell line. I. M. performed the pharmacological analysis of the ligand-receptor assay data.

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## Table 2

Comparison of Bf-CTFP potencies (pEC₅₀) calculated from the calcium second messenger in the HEK293MSR cells expressing Bf-CTFP-R/Bf-RAMP-LPs

| Peptides       | Bf-CTFP-R/Bf-RAMP-LP1 | Bf-CTFP-R/Bf-RAMP-LP2 | Bf-CTFP-R/Bf-RAMP-LP3 |
|----------------|-----------------------|-----------------------|-----------------------|
|                | pEC₅₀ | EC₅₀ | Eₚ₅₀ | pEC₅₀ | EC₅₀ | Eₚ₅₀ | pEC₅₀ | EC₅₀ | Eₚ₅₀ |
| Bf-CTFP1       | 6.27  | 5.37E-7 | 66.86 | 5.14  | —    | —    | 6.49  | 3.24E-7 | 38.82 |
| Bf-CTFP2       | 6.99  | 1.02E-7 | 97.68 | 6.12  | 6.70 | 2.00E-7 | 92.97 | 5.71  | —    |
| Bf-CTFP3       | 6.46  | 3.47E-7 | 65.15 | 4.07  | 6.20 | 6.31E-7 | 70.75 | 4.03  | —    |

*Note: EC₅₀ values are significantly different (p < 0.05) from Bf-CTFP1 and Bf-CTFP3 within each complex of Bf-RAMP-LP and Bf-CTFP-R.*
Amphioxus Calcitonin Family Peptides and Their Receptors

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2356 JOURNAL OF BIOLOGICAL CHEMISTRY

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