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Molecular Characterization of Putative Calcitonin Gene-Related Peptide Receptors and Expression of Calcitonin Gene-Related Peptide and its Receptor in the Early Development of Flounder, *Paralichthys olivaceus*

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Abstract: Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide that has been identified in teleosts and mammals. Calcitonin receptor-like receptor (CLR) forms the CGRP receptor. In teleost fish, CGRP plays a role in vasodilation and is associated with osmotic regulation. However, the embryonic function of CGRP remains unclear. Therefore, we have investigated CGRP function related to early development in the flounder, *Paralichthys olivaceus*. We first identified two CLRs from the flounder genome database. According to molecular phylogenetic analysis, two CLRs are classified in the group formed by teleost CLR1 and 2. They represented high amino acid sequence identity among mefugu CLR1-3 and human CLR. We also identified another CLR/CTR candidate from the flounder genome. Molecular phylogenetic analysis revealed that this gene was located at the clade formed by vertebrate CTRs that are CLR paralogs. The deduced protein of the gene also had four hormone-binding domains, which are the typical feature of teleost CTR, and was completely different from the CLR of teleosts, including flounder CLRs. These data strongly suggest that flounder possess two putative CGRP receptors. Finally, we performed the expression analysis of flounder CGRP and two CLRs in the flounder embryo. Both expressions of CGRP mRNA and two CLR mRNAs were detected from 96 hours post fertilization (hpf). These results demonstrate that CGRP has some functions at 96 hpf. At that stage, embryos have already hatched and the circulatory system functions, whereas digestive organs still continue to develop. Taken together, these findings suggest that the CGRP peptide may play a role in the circulatory system, as in adult fish, or it may be associated with the development of the digestive tract.

Keywords: Calcitonin gene-related peptide, Calcitonin receptor-like receptor, Calcitonin receptor, Flounder

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide. The CGRP gene encodes both CGRP and the calcitonin (CT) peptide in the different exons (Amara *et al.*, 1982; Rosenfeld *et al.*, 1983). In mammals, CGRP and CT mRNA are expressed in the central and peripheral nervous systems and thyroid calcitonin cells (C cells), respectively, by alternative splicing (Amara *et al.*, 1985; Rosenfeld *et al.*, 1983). CGRP genes and this type of post-transcriptional process are also observed in teleosts (Takei *et al.*, 2010).

Mammalian CGRP plays a role in vasodilation (Brain *et al*., 1985; Brain and Grant, 2004). It is also associated with pain transmission because of CGRP synthesis in the sensory nerve (Winston *et al.*, 2005). Notably, the CGRP peptide of the trigeminal nerve has garnered attention as a cause of migraines (Hay and Walker 2017; Edvinsson *et al.*, 2018). The teleost CGRP peptide also represents vasodilatory function (Kagstrom and Holmgren, 1998; Shahbazi *et al.*, 2009). In rainbow trout, adrenaline-inducing precontracted small diameter gut arteries were relaxed by treatment with chicken CGRP. This reaction was antagonized by the N-terminal truncated $CGRP_{8–37}$ (Kagstrom and Holmgren, 1998). Cod CGRP induced dose-dependent relaxation of celiac arteries precontracted with adrenaline in Atlantic cod *Gadus morhua* (Shahbazi *et al.*, 2009). Interestingly, CGRP is implicated in fluid homeostasis (Suzuki *et al.*, 2002; Lafont *et al.*, 2006). In flounder, CGRP mRNA was expressed in the gill in seawater conditions. Transferring flounder from seawater to freshwater decreased the expression of CGRP mRNA to an undetectable level (Suzuki *et al.*, 2002). Moreover, an increase in the plasma CGRP level was induced by the transfer from freshwater to seawater in the eel, *Anguilla anguilla* (Lafont *et al.*, 2006). Although the physiological function of adult teleosts has been evaluated, but the function of CGRP in early or larval development remains unclear in both mammals and teleosts.

The canonical CGRP receptor comprises the CT receptor-like receptor (CLR) (Hay and Walker, 2017; Hay *et al.*, 2018). On the other hand, the CT receptor (CTR) is paralogous to CLR. CLR and CTR belong to the secretin family G protein-coupled receptor (GPCR). They contain seven transmembrane domains and hormone-binding domains (HBDs) that are responsible for binding to the ligand peptide (Parthier *et al.*, 2009). Both receptors show close phylogenetic relationships in vertebrates (Martins *et al*., 2014). Therefore, discrimination between CLR and CTR is required to clarify the CGRP receptor.

We previously cloned and determined the nucleotide sequence of a CGRP gene in the flounder, *Paralichthys olivaceus* (Suzuki *et al.*, 2001). The flounder CGRP gene encoded both CGRP and the CT peptide in different exons as in another teleost. In addition, we also identified the nucleotide sequence of a putative CLR-type gene and CTR-type gene from a flounder gill cDNA library and clarified their tissue distribution (Suzuki N *et al.*, 2000). Although the deduced amino acid sequences of both receptors were similar to that of vertebrate CLR, the CTR-type receptor resembled vertebrate CTR because of its expression in the vertebral bone (Suzuki N *et al.*, 2000). However, there was no evidence that the CLR-type and CTR-type genes are vertebrate CLR and CTR orthologs,

respectively. Therefore, the CGRP receptor has yet to be determined.

In the present study, we comprehensively annotated CLR and CTR from the flounder genome. First, candidates of two CLRs were extracted from the flounder genome database using two previously unannotated sequences as queries. Moreover, a CTR candidate also was detected from the flounder genome. To identify the CGRP receptor candidate, flounder CLR and CTR were classified by molecular phylogenetic analysis and molecular characteristic analysis. To gain clues as to the CGRP function of early flounder development, we finally performed an expression analysis of flounder CGRP and CLRs in flounder embryos.

Materials and Methods

Experimental Materials

Fertilized eggs of the flounder, *Paralichthys olivaceus*, were maintained in a 50 L tank supplied with running seawater (17 ± 1 C) circulating at a flow rate of 500 ml/min. Embryos were collected at 0 (immediately after fertilization), 24, 48, 72, 96, 120, and 144 hours post fertilization (hpf). Embryos were frozen at -80 C for reverse transcription-polymerase chain reaction (RT-PCR).

Data mining of flounder CLR and CTR

Candidates of flounder CLR were searched by TBLASTN algorithms from RefSeq RNA in the Japanese flounder genome database (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&BLAST_SPEC=OGP__8255__73699&LINK_LOC=blasttab) (Shao *et al*., 2017). The query amino acid sequence was selected from our previous data (NCBI accession numbers BAA92817.1 and BAA92817.1). Amino acid sequences obtained by the data mining were used for molecular phylogenetic analysis and sequence comparison.

Molecular phylogenetic analysis of flounder CLR/CTR

We performed molecular phylogenetic analysis with modifications of the previous study (Sekiguchi *et al*., 2016). Multiple alignments of amino acid sequences were generated by using the Muscle program of MEGA 7.0.26 software (Kumar *et al*., 2016). Next, we manually removed the gap and constructed unrooted molecular phylogenetic trees using the neighbor-joining method with MEGA 7.0.26 software. The bootstrap value was calculated by 500 pseudo-replications using MEGA 7.0.26 software. We performed molecular phylogenetic analysis of CLR/CTR. The accession numbers of the CLR and CTR are as follows: cattle CLR, NP_001095577.1; elephant shark CLR, XP_007888311; flounder CLR1, BAA92817; flounder CLR2, Q9IB86; human CLR, NP_005786; medaka CLR1, XP_020560071; mefugu CLR1, BAE45312; mefugu CLR2, BAE45313; mefugu CLR3, BAE45314; mouse CLR, NP_061252;pig CLR, Q867C1; red stingray CLR-like, BAL63062; tropical clawed frog CLR, Q28DX2; zebrafish CLR1, NP_001004010; cattle CTR, NP_001069737.2; chicken CTR, XP_425985; elephant shark CTR, XP_007903520; flounder CTR, XP_019945977; green anole CTR, XP_008110735.1; human CTR, NP_001733; medaka CTR, XP_011479129; mefugu CTR, BAE76018; mouse CTR, AAI19273; pig CTR, AAA31023.1; red stingray CTR, BAL63063; tropical clawed frog CTR, XP_002934645.2; vase tunicate CTR-like, BAI63096; and zebrafish CTR, XP_003200679.2.

Comparison of the amino acid sequence of flounder CLR or CTR with those of teleost and mammalian CLR or CTR

Multiple alignments of CLR or CTR were generated by the T-Coffee program using the web server (Poirot *et al*., 2003), followed by using the ESPrint3.0 server (http://espript.ibcp.fr/ESPript/ESPript/index.php).

RT-PCR analysis

Total RNAs were extracted from the embryos using the total RNA isolation kit (Nippon Gene, Tokyo, Japan). RT-PCR was performed in accordance with the method of Suzuki *et al*. (1997). The gene-specific primers (5': GGATGCAACACATCCACGTGTGTG; 3': TGCTCA CACAGGGCCGTGACGCTT) are designed from CGRP nucleotide sequences (Suzuki *et al*., 2001). The conditions for PCR amplification of CGRP cDNA (144 bp) included 45 cycles of denaturation for 0.5 min at 96 C, annealing for 1 min at 60 C, and extension for 2 min at 72 C, followed by a single cycle at 72 C for 15 min. The primer sets of CLR1 (5': TGCCAACGCAACACGACG; 3': AGCTCACGGA GTCGTTGT) and CLR2 (5': GAGGGACGG GTGTCCTCTGAAATCT; 3': TTTTAAGATGGA GGCGTCTGCCTCGT) were designed based on the nucleotide sequences of CLR1 and CLR2, respectively (Suzuki N *et al*., 2000). PCR conditions for the amplification of CLR1 cDNA using a 5' and 3' primer set included 30 cycles of denaturation for 0.5 min at 96 C, annealing for 1 min at 57 C, and extension for 2 min at 72 C, followed by a single cycle at 72 C for 15 min. The conditions for PCR amplification of CLR2 cDNA using a 5' and 3' primer set included 35 cycles of denaturation for 0.5 min at 96 C, annealing for 1 min at 60 C, and extension for 2 min at 72 C, followed by a single cycle at 72 C for 15 min. Elongation factor 1-α (EF1-α) cDNA was also amplified using a set of primers (5': TGCTG CAAGCTTCAACGCCC; 3': TTGATGACACCGACA GCCAC) designed to produce a flounder EF1-α fragment of 308 bp (Suzuki *et al*., 1999). The parameters of PCR were 25 cycles at 96 C for 0.5 min, 60 C for 1 min, and 72 C for 2 min, followed by a single cycle at 72 C for 15 min. The PCR products were analyzed on 2.5% NuSieve GTG agarose gel (FMC Bioproducts, Philadelphia, PA) and stained with ethidium bromide.

Results and Discussion

CGRP acts as a vasodilatory neural peptide in teleost fish (Kagstrom and Holmgren, 1998; Shahbazi *et al*., 2009). The physiological function of CGRP in adult teleost fish has been characterized, whereas its embryonic or larval function remains unclear.

We have investigated the role of teleost CGRP using the Japanese flounder, *Paralichthys olivaceus*. We have already identified a gene encoding flounder CGRP (Suzuki *et al*., 2001). Moreover, we previously cloned and deposited two flounder CLR/CTR candidates in the NCBI database (accession numbers BAA92816.1 and BAA92817.1) (Suzuki N *et al*., 2000). However, the numbers of CLRs/CTRs in the flounder genome and the molecular characteristics of CLR/CTR candidates have not yet been elucidated. Therefore, in this study we performed a comprehensive search of CLRs/CTRs of the flounder genome. The TBLASTN search was performed to extract putative CLR or CTR. The amino acid sequence of two CLR/CTR candidates, BAA92816.1 and BAA92817.1, were used as query sequences. A genomic

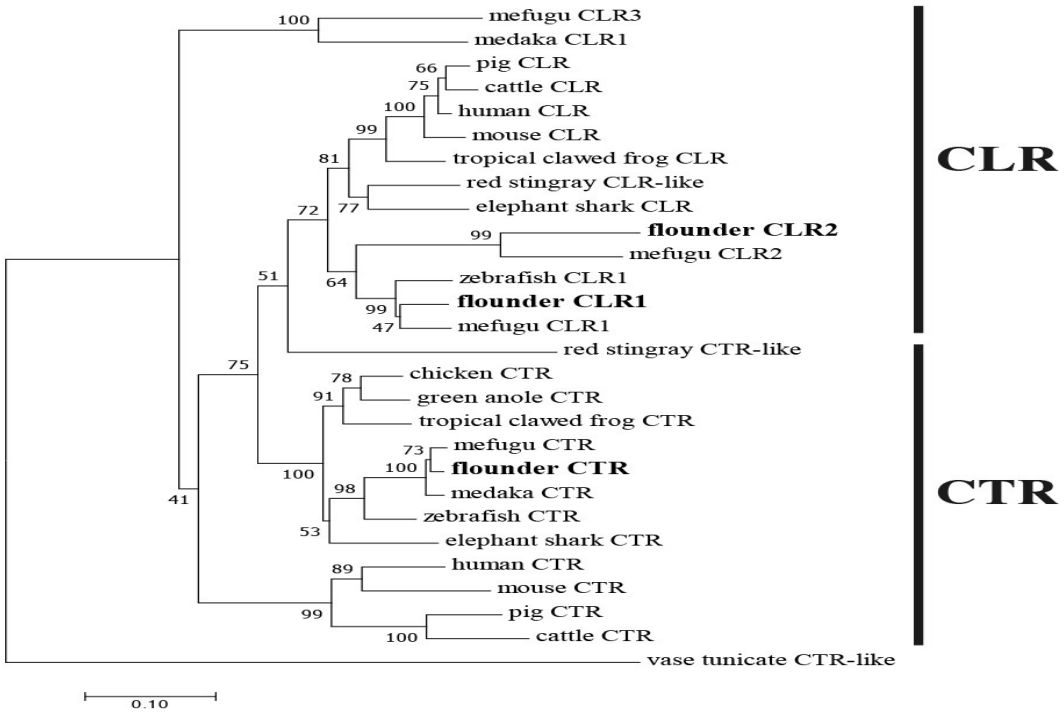

Fig. 1: Molecular phylogenetic tree of vertebrate CLR/CTR generated by the neighbor-joining method. The bootstrap values were calculated by 500 pseudoreplications and depicted beside each branch. The scale bar represents an evolutionary distance of 0.1 amino acid substitutions per protein.

search of BAA92816.1 results in a 100% match of the mRNA sequence that is *Paralichthys olivaceus* calcitonin receptor-like receptor (calcrl), transcript variant X2 (Sequence ID: XM_020099598.1, Protein ID: XP_019955157.1). On the other hand, TBLASTN analysis of BAA92817.1 produced the best hit sequence that is *Paralichthys olivaceus* calcitonin gene-related peptide type 1 receptor-like, transcript variant X1, mRNA (Sequence ID: LOC109645530, Protein ID: XP_019966707.1) with 100% identity. A BLAST search clarified the existence of the other candidate (NCBI protein accession number XP_019945977). To clarify whether these candidates are authentic members of CLR or CTR, we conducted molecular phylogenetic analysis (Fig. 1). Flounder CLR candidates XP_019955157.1 and XP_019966707.1 were located at the same clade with mefugu CLR1 and mefugu CLR2, respectively (Fig. 1). Therefore, we designated XP_019955157.1 and XP_019966707.1 as flounder CLR1 and CLR2, respectively. The other candidate, XP_019945977, was grouped in the clade including mefugu CTR, medaka CTR, and zebrafish CTR with high bootstrap value (Fig. 1). Additionally, XP_019945977

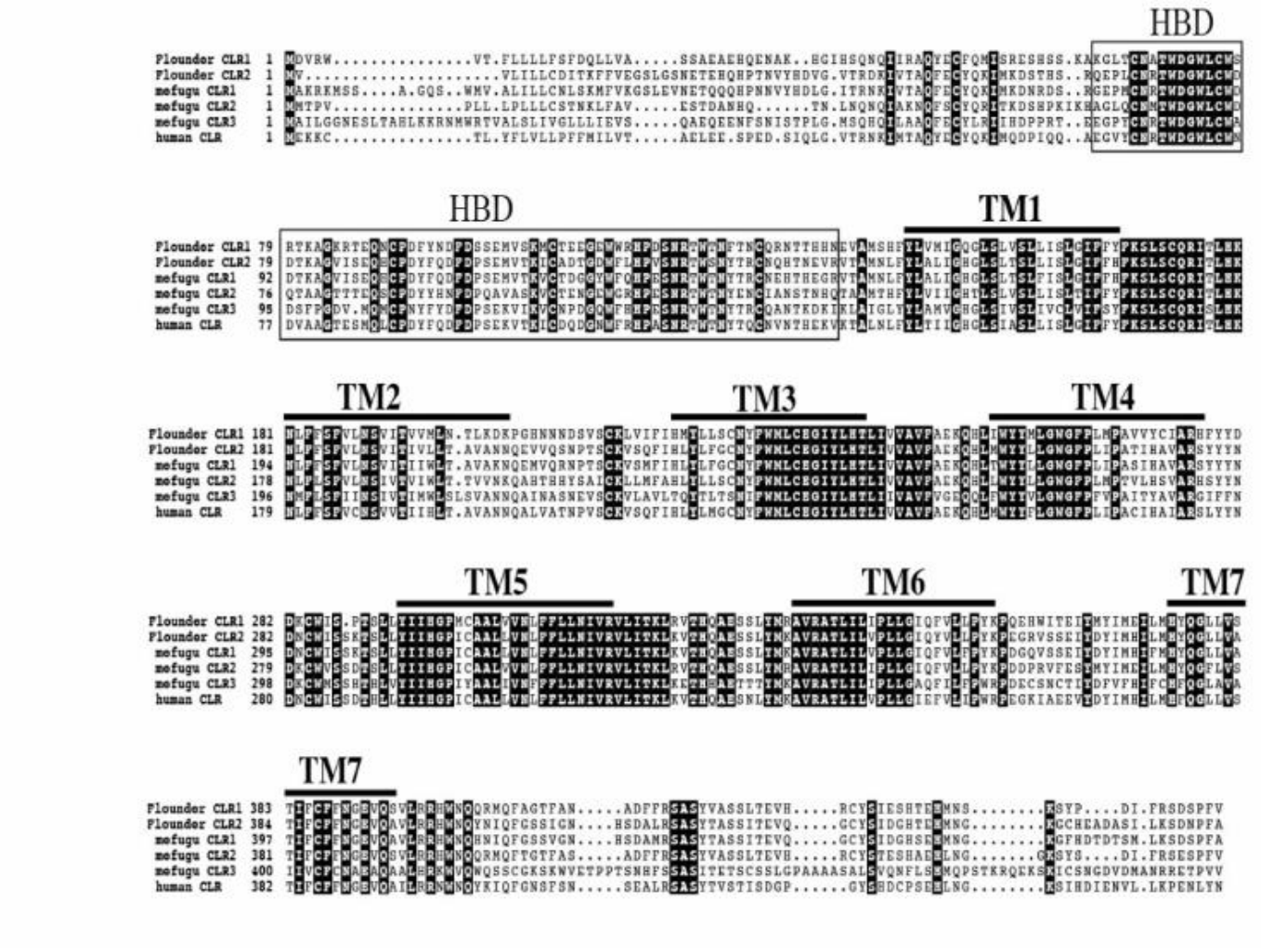

Fig. 2: Multiple alignments of flounder CLR1, flounder CLR2, mefugu CLR1, mefugu CLR2, mefugu CLR3, and human CLR. Identical amino acids of all members are depicted in black boxes. The transmembrane domain (TM) and hormone-binding domain (HBD) were predicted by Nag *et al.* (2007). Putative TMs 1 to 7 are depicted by the top bar. HBDs are within the open box. Accession numbers are as follows: flounder CLR1, BAA92817; flounder CLR2, Q9IB86; mefugu CLR1, BAE45312; mefugu CLR2, BAE45313; mefugu CLR3, BAE45314; and human CLR, NP_005786

was also included in the clade formed by CTRs of teleost fish, cartilaginous fish, amphibians, and reptiles with significant bootstrap value (Fig. 1). Thus, we refer to XP_019945977 as flounder CTR.

We compared the amino acid sequence of flounder CLR with mefugu CLRs and human CLR. CLR is a member of the secretin family of GPCR, which possesses seven transmembrane domains and an N-terminal extracellular HBD that is involved in the ligand binding (Parthier *et al.*, 2009). Two flounder CLRs possessed seven transmembrane domains and one HBD in two flounder CLRs (Fig. 2). These characteristics were observed in human CLR and three mefugu CLRs (Fig. 2). On the other hand, the amino acid sequence of flounder CTR was compared with that of mefugu CTR, medaka CTR, and human CTR (Fig. 3). CTR is paralogous to CLR and also belongs to the secretin family of GPCR. Of interest, teleost CTR possesses multiple HBDs (Nag *et al.*,

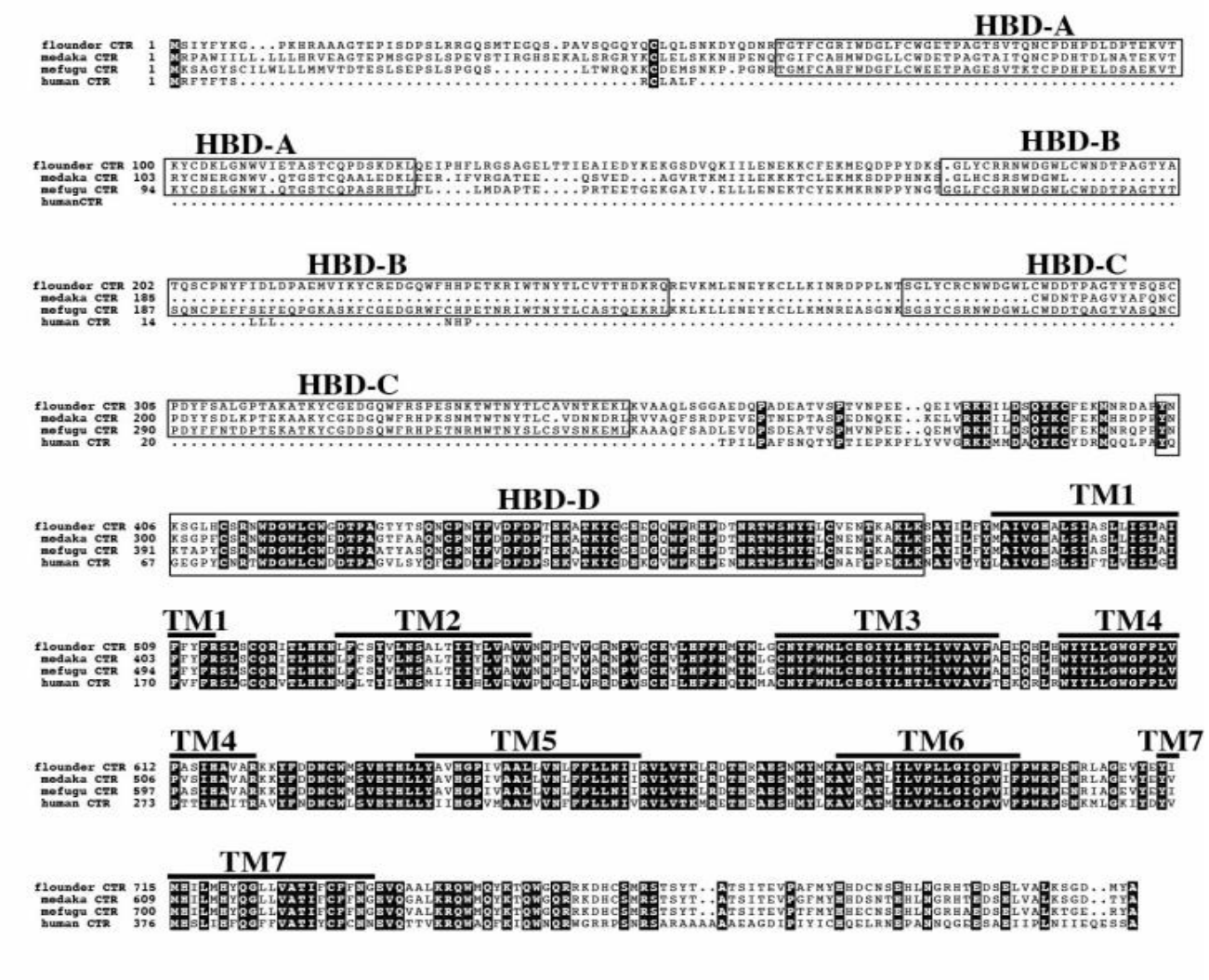

Fig. 3: Multiple alignments of flounder CTR, medaka CTR, mefugu CTR, and human CTR. Identical amino acid residues of all members are depicted in black boxes. The TM domain and HBD were predicted by Nag *et al*. (2007). TM1 to 7 are indicated by the top bar. Four HBDs are represented by HBD-A, HBD-B, HBD-C, and HBD-D. Accession numbers are as follows: flounder CTR (XP_019945977), medaka CTR (XP_011479129), mefugu CTR (BAE76018), and human CTR (NP_001733).

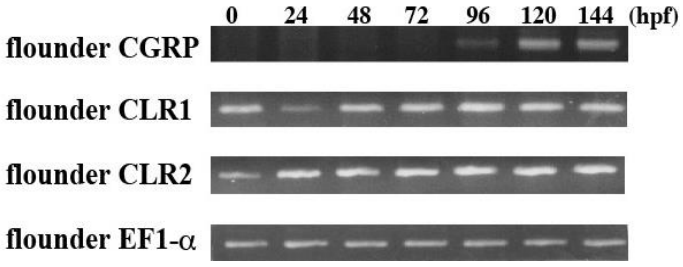

Fig. 4: Expressions of flounder CGRP, CLR1, CLR2, and EF1-α mRNAs in embryos. Sample was collected at 0 (immediately after fertilization), 24, 48, 72, 96, 120, 144 hours post fertilization.

2007). Mefugu CTR and medaka CTR have four and three HBDs, respectively (Fig. 3). In flounder CTR, we detected seven transmembrane domains and four HBDs, suggesting that flounder CTR possesses characteristics typical of teleost CTR (Fig. 3). These results suggest that flounder CTR is a genuine teleost CTR. Collectively, we identified two CLRs and one CTR in the flounder genome. This finding is in good agreement with the fact that teleosts possess multiple CLRs and a single CTR. For instance, mefugu possesses three CLR genes and one CTR gene (Nag *et al*., 2006).

In the previous study, we regarded CLR1 as a CTR-type gene because the gene was expressed in the vertebral bone such as mammalian CTR (Suzuki N *et al*., 2000). After our previous study, it was reported that mammalian CLR is localized in mature osteoblasts (Schinke *et al*., 2004; Villa *et al*., 2006), and CGRP also plays a role in bone metabolism (Schinke *et al*., 2004; Villa *et al*., 2006). These issues suggest that flounder CGRP has physiological function similar to that of bone via CLR1. The previous study also demonstrated the different tissue distribution patterns of two CLRs (Suzuki N *et al*., 2000). CLR1 mRNA was expressed in the gill, brain, vertebral bone, fin, liver, kidney, heart, intestine, and gonad; the CLR2 gene was transcribed in the gill, brain, heart, intestine, and gonad (Suzuki N *et al*., 2000). These findings suggest that CGRP functions in various tissues and plays multiple roles using two receptors.

To gain clues as to the embryonic function of CGRP, we finally performed RT-PCR analysis of flounder CGRP and CLRs in the early developmental stage. Flounder CGRP mRNA was expressed at 96, 120, and 144 hpf.

The expression of flounder CLR1 and 2 were detected from the unfertilized eggs to 144 hpf. The larva is already hatched, and the formation of the cartilaginous skeletons of the pharyngeal arch is finished by 96 hpf (Suzuki T *et al*., 2000). At this stage, the larva already has a function of circulatory system, and the digestive tract continues to develop (Kurokawa and Suzuki, 1996). These facts suggest that flounder CGRP plays a role in vasodilation, as in adult fish, or in the development of the digestive tract. CLRs were expressed earlier than CGRP. It is assumed that CLR is not functional until 96 hpf. Further study to determine the localization of CGRP and CLRs would be a prerequisite for clarifying the role of CGRP in flounder embryos.

Conclusion

We annotated two CLRs from the genome information of the Japanese flounder. Molecular characterization and molecular phylogenetic analysis certified that two CLRs are genuine members of teleost CLR but not of CTR. Moreover, both CGRP and two CLR mRNAs were expressed from 96 hpf. These findings will contribute to future studies of the role of CGRP in teleost embryos and larvae.

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References

Amara SG, Jonas V, Rosenfeld MG, Ong ES and Evans RM. (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. Nature 298: 240-244.

Amara SG, Arriza JL, Leff SE, Swanson LW, Evans RM and Rosenfeld MG. (1985) Expression in brain of a messenger RNA encoding a novel neuropeptide homologous to calcitonin gene-related peptide. Science 229: 1094-1097.

Brain SD, Williams TJ, Tippins JR, Morris HR and MacIntyre I. (1985) Calcitonin gene-related peptide is a potent vasodilator. Nature 313: 54-56.

Brain SD and Grant AD. (2004) Vascular actions of calcitonin gene-related peptide and adrenomedullin. Physiol. Rev. 84: 903-934.

Edvinsson L, Haanes KA, Warfvinge K and Krause DN. (2018) CGRP as the target of new migraine therapies - successful translation from bench to clinic. Nat. Rev. Neurol. 14: 338-350.

Hay DL and Walker CS. (2017) CGRP and its receptors. Headache 57: 625-636.

Hay DL, Garelja ML, Poyner DR and Walker CS. (2018) Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br. J. Pharmacol. 175: 3-17.

Kagstrom J and Holmgren S. (1998) Calcitonin gene-related peptide (CGRP), but not tachykinins, causes relaxation of small arteries from the rainbow trout gut. Peptides 19: 577-584.

Kumar S, Stecher G and Tamura K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33: 1870-1874.

Kurokawa T and Suzuki T. (1996) Formation of the diffuse pancreas and the development of digestive enzyme synthesis in larvae of the Japanese flounder *Paralichthys olivaceus.* Aquaculture 141: 267-276.

Lafont AG, Fitzpatrick T, Cliff Rankin J, Dufour S and Fouchereau-Peron M. (2006) Possible role of calcitonin gene-related peptide in osmoregulation via the endocrine control of the gill in a teleost, the eel, *Anguilla anguilla*. Peptides 27: 812-819.

Martins R, Vieira FA and Power DM. (2014) Calcitonin receptor family evolution and fishing for function using in silico promoter analysis. Gen. Comp. Endocrinol. 209: 61-73.

Nag K, Kato A, Nakada T, Hoshijima K, Mistry AC, Takei Y and Hirose S. (2006) Molecular and functional characterization of adrenomedullin receptors in pufferfish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 290: 467-478.

Nag K, Kato A, Sultana N, Ogoshi M, Takei Y and Hirose S. (2007) Fish calcitonin receptor has novel features. Gen. Comp. Endocrinol. 154: 48-58.

Parthier C, Reedtz-Runge S, Rudolph R and Stubbs MT. (2009) Passing the baton in class B GPCRs: Peptide hormone activation via helix induction? Trends Biochem. Sci. 34:303-310.

Poirot O, O'Toole E and Notredame C. (2003) Tcoffee@igs: A web server for computing, evaluating and combining multiple sequence alignments. Nucleic Acids Res. 31: 3503-3506.

Rosenfeld MG, Mermod JJ, Amara SG, Swanson LW, Sawchenko PE, Rivier J, Vale WW and Evans RM. (1983) Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. Nature 304: 129-135.

Schinke T, Liese S, Priemel M, Haberland M, Schilling AF, Catala-Lehnen P, Blicharski D, Rueger JM, Gagel RF, Emeson RB and Amling M. (2004) Decreased bone formation and osteopenia in mice lacking alpha-calcitonin gene-related peptide. J. Bone Miner. Res. 19: 2049-2056.

Sekiguchi T, Kuwasako K, Ogasawara M, Takahashi H, Matsubara S, Osugi T, Muramatsu I, Sasayama Y, Suzuki N and Satake H. (2016) Evidence for conservation of the calcitonin superfamily and activity-regulating mechanisms in the basal chordate *Branchiostoma floridae*: Insights into the molecular and functional evolution in chordates. J. Biol. Chem. 291: 2345-2356.

Shahbazi F, Holmgren S and Jensen J. (2009) Cod CGRP and tachykinins in coeliac artery innervation of the Atlantic cod, *Gadus morhua*: Presence and vasoactivity. Fish Physiol. Biochem. 35: 369-376.

Shao C, Bao B, Xie Z, Chen X, Li B, Jia X, Yao Q, Orti G, Li W, Li X, Hamre K, Xu J, Wang L, Chen F, Tian Y, Schreiber AM, Wang N, Wei F, Zhang J, Dong Z, Gao L, Gai J, Sakamoto T, Mo S, Chen W, Shi Q, Li H, Xiu Y, Li Y, Xu W, Shi Z, Zhang G, Power DM, Wang Q, Schartl M and Chen S. (2017) The genome and transcriptome of Japanese flounder provide insights into flatfish asymmetry. Nat. Genet. 49: 119-124.

Suzuki N, Eguchi C, Hirai T and Sasayama Y. (1997) Nucleotide sequences of reptile calcitonins: Their high homology to chicken calcitonin. Zool. Sci. 14: 833-836.

Suzuki N, Suzuki T and Kurokawa T. (2000) Cloning of a calcitonin gene-related peptide receptor and a novel calcitonin receptor-like receptor from the gill of flounder, *Paralichthys olivaceus*. Gene 244: 81-88.

Suzuki N, Suzuki T and Kurokawa T. (2001) Cloning of a calcitonin gene-related peptide from genomic DNA and its mRNA expression in flounder, *Paralichthys olivaceus*. Peptides 22: 1435-1438.

Suzuki N, Suzuki T and Kurokawa T. (2002) Possible involvement of calcitonin gene-related peptide in seawater adaptation of flounder: Expression analysis of its receptor mRNA in the gill. Fisheries Sci. 68: 425-429.

Suzuki T, Kurokawa T and McVey DC. (1999) Sequence and expression analyses of cholecystokinin (CCK) precursor cDNA in the Japanese flounder (*Paralichthys olivaceus*). Fish Physiol. Biochem. 21: 73-80.

Suzuki T, Srivastava AS and Kurokawa T. (2000) Experimental induction of jaw, gill and pectoral fin malformations in Japanese flounder, *Paralichthys olivaceus*, larvae. Aquaculture 185: 175-187.

Takei Y, Ogoshi M, Wong MK and Nobata S. (2010) Molecular and functional evolution of the adrenomedullin family in vertebrates: What do fish studies tell us? In: "The calcitonin gene-related peptide family". Springer, pp 1-21.

Villa I, Mrak E, Rubinacci A, Ravasi F and Guidobono F. (2006) CGRP inhibits osteoprotegerin production in human osteoblast-like cells via cAMP/PKA-dependent pathway. Am. J. Physiol. Cell Physiol. 291: 529-537.

Winston JH, He ZJ, Shenoy M, Xiao SY and Pasricha PJ. (2005) Molecular and behavioral changes in nociception in a novel rat model of chronic pancreatitis for the study of pain. Pain 117: 214-222.