Molecular and functional characterization of adrenomedullin receptors in pufferfish

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Nag, Kakon, Akira Kato, Tsutomu Nakada, Kazuyuki Hoshijima, Abinash Chandra Mistry, Yoshiio Takei, and Shigehisa Hirose. Molecular and functional characterization of adrenomedullin receptors in pufferfish. Am J Physiol Regul Integr Comp Physiol 290: R467–R478, 2006. First published September 29, 2005; doi:10.1152/ajpregu.00507.2005.—The receptors for the calcitonin gene-related peptide (CGRP)/adrenomedullin (AM) family peptides were characterized in the mefugu Takifugu obscurus, a euryhaline fugu species very close to Takifugu rubripes, which has as many as five adrenomedullin (AM) genes (AM1–5). CGRP and AM share a G protein-coupled core receptor called calcitonin receptor-like receptor (CLR), and the specificity of the CLR is determined by the interaction with receptor activity-modifying proteins (RAMPs). Through database mining, three CLRs (CLR1–3) and five RAMPs (RAMP1–5) were identified, and all of them were cloned by RT-PCR and characterized by functional expression in COS7 cells in every possible combination of CLR-RAMP. The following combinations generated cAMP in response to physiological concentrations of CGRP, AM1 (an ortholog of mammalian AM), AM2, and AM5: CLR1-RAMP1/4 (CGRP), CLR1-RAMP2/3/5 (AM1), CLR2-RAMP2 (AM1), CLR1-RAMP3 (AM2), and CLR1-RAMP3 (AM5). Their expressions were found by Northern blot analysis to be tissue specific and salinity dependent. For example, CLR1-RAMP5 and CLR1-RAMP2 are expressed specifically in the gill and kidney, respectively, suggesting their involvement in osmoregulation. Furthermore, relatively high levels of CLRs and RAMPs were found in the spleen and ovary, suggesting roles in the immune and female reproductive systems. Immunohistochemistry revealed that AM receptors of the following types are expressed in the locations, indicated in brackets, of the mefugu gill and kidney: CLR1-RAMP5 (interlamellar vessels), CLR2-RAMP2 (pillar cells), and CLR1-RAMP2 (apical side of renal proximal tubule cells).

ADRENOMEDULLIN (AM) is a multifunctional peptide hormone discovered in an extract of human pheochromocytoma by using rat platelet cAMP activation assay (17). It is a member of the calcitonin gene-related peptide (CGRP) superfamily consisting of calcitonin (CT), αCGRP, or CGRP-I; βCGRP, or CGRP-II; adrenomedullin (AM), and amylin (1, 2, 12). Despite low homology in their peptide sequence, they are structurally similar, having a six-membered (7 for CT) intramolecular disulfide ring structure near the NH2 terminus followed by a potential amphipathic α-helical midregion, and an amidated COOH terminus. They exert their physiological functions through two members of class B, G protein-coupled receptor (GPCR), namely 1) CT receptor (CTR) and 2) CT receptor-like receptor (CLR) (29) that is associated with a new class of single transmembrane protein termed receptor activity-modifying protein (RAMP) (20). To date, three RAMP family members, RAMP1–3, have been cloned and shown to define the specificity of CLR for CGRP and AM (18, 23, 32). When CLR associates with RAMP1, it forms a functional CGRP-I receptor, whereas its association with RAMP2 or RAMP3 yields a functional AM receptor. Although CTR itself works as the CT receptor, it can also interact with any of the three RAMPs to form amylin receptors (32).

Recently, five AM family peptides have been identified in the pufferfish Takifugu rubripes or torafugu and named AM1–5 (26). Also in mammals, a second member, AM2 or intermedin (30, 37, 38), has been identified and shown to be a nonselective ligand for the receptor complexes of CLR and RAMP1–3. The complexity of the fish AM system, as represented by the presence of 5 AMs and the fact that the AM system has not been systematically characterized in any of the fish species, led us to characterize the CLR-RAMP receptor complexes of the mefugu Takifugu obscurus, which is a close relative of the torafugu T. rubripes and is a euryhaline fugu species that can adapt to both seawater and freshwater (16, 40–42). In this study, by combining database mining and actual cDNA cloning, we found 5 RAMP genes (RAMP1–5) and 3 CLR genes (CLR1–3) in the mefugu genome, determined their expression patterns in freshwater and seawater, clarified ligand specificities of all possible combinations of RAMP1–5 and CLR1–3, and finally visualized AM receptor complexes on gill and kidney sections of the mefugu T. obscurus by immunohistochemistry using specific antisera raised against the mefugu proteins. The results indicated that CLR1-RAMP5 and CLR2-RAMP2 are densely localized in the endothelial cells of the interlamellar vessels and pillar cells of the gill, respectively, suggesting the role of AM1 in the regulation of branchial circulation in fish. In the kidney, CLR1-RAMP2 is expressed on the apical side of renal proximal tubule cells and significant amounts of AM1 were detected in the glomerular filtrate. Because urinary AM1 concentration is much higher in seawater fish, filtered AM1 was detected in the glomerular filtrate. Therefore, AM1 may be involved in urine concentration.

MATERIALS AND METHODS

Polyptides and Oligonucleotides

Polyptides of human CGRP-1, cel CT, and fugu AM1, AM2, and AM5 were synthesized by Peptide Institute (Osaka, Japan). Synthetic

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oligonucleotides were purchased from Sigma Genosys (Tokyo, Japan) and their uses and sequences are listed in Supplemental Tables 1S and 2S [Supplemental tables and figures may be found at ajpregu.physiology.org/cgi/content/full/00507.2005/DC1].

Animals

Mefugu *T. obscurs*us (190–350 g) were imported from Bionics, Seoul, South Korea and torafugu *T. rubripes* (180–290 g) were purchased from a local dealer. They were maintained in a 150–2,000-liter aquarium with adequate aeration and filtration at 18°C to 20°C. For salinity adaptation, five mefugu in each tank were kept in seawater or freshwater for 2 wk. Five torafugu were also acclimated in seawater or in 14% (vol/vol) seawater as brackish water for 1 wk. To isolate various tissues from the adapted fish, they were killed after being anesthetized by immersion in 0.1% (wt/vol) tricane methanesulfonate. The animal protocols and procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Institute of Technology and conform to the American Physiological Society’s guiding principles in the care and use of laboratory animals.

RNA Isolation and cDNA Cloning

Various tissues were isolated and pooled from two males and two females except the ovary. Total RNA was extracted from the tissues by Isogen according to the manufacturer’s manual (Nippon Gene, Toyama, Japan). The individual RNA samples were quantified in diethyl pyrocarbonate-treated water by spectrophotometer and stored at -40°C as ethanol precipitates. Complementary DNA of mefugu RAMP and CLR genes were amplified from total RNA by RT-PCR with gene-specific primers mentioned in Supplemental Table 1S.

The full-length of the cDNA were obtained by rapid amplification of cDNA ends (RACE) using First Choice RLM-RACE kit (Ambion), according to the manufacturer’s instructions; see Supplemental Table 1S for a list of primers.

The amplified fragments were cloned into pZero-2 vector (Invitrogen, Carlsbad, CA), and their sequences were confirmed by Thermosequenase cycle sequencing kit (USB, Cleveland, OH) and LI-COR model-4000L automated sequencer. To express the mRAMPs and mfCLRs in cultured mammalian cells, the coding region of each gene was amplified from first-stranded mefugu cDNA with the gene-specific primers (listed in Supplemental Table 2S) conjugated with restriction enzyme site and cloned into pcDNA3 (Invitrogen).

cAMP Accumulation Assay

Green monkey kidney cell line COS-7 and Henrietta Lacks’ (HeLa) cells were cultured in DMEM (Sigma, St. Louis, MO) supplemented with 10% FBS (Life Technologies, Gaithersburg, MD) and 1% penicillin-streptomycin (Invitrogen) at 37°C under a humidified atmosphere of 95% air and 5% CO₂. Expression plasmids were transfected into the cells at 90% confluency in 24-well plates using lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction and dialyzed against saline (0.9% wt/vol NaCl) at 4°C. Polyclonal antibodies to the purified proteins were also obtained by using a part of CLR1 (amino acid residues 407–478) was cloned into bacterial expression vector pRSET A (Invitrogen). These plasmids were transformed into *Escherichia coli* BL21 strain (Codon Plus). When the cells grew in Luria-Bertani broth containing 100 μg/ml ampicillin to an A₆₀₀ of 0.55–0.60 at 37°C, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside to final concentration of 1 mM for 6 h. The cells were harvested by centrifugation at 2,000 g for 5 min, and the cell pellets were washed once with ice-cold PBS, pH 7.4, and resuspended in wash buffer (50 mM Na₃PO₄, 300 mM NaCl, pH 7.0). After disrupting the cells by freeze-thaw and subsequently by sonication, the lysates were centrifuged at 12,000 g for 20 min, and the insoluble fractions were recovered as the pellet. In pilot experiments, all of the desired recombinant proteins were found in the insoluble fractions. The insoluble fractions were solubilized in the wash buffer containing 8 M urea. The urea-solubilized recombinant proteins were purified by BD TALON metal affinity resin (BD Biosciences) according to the manufacturer’s instruction and dialyzed against saline (0.9% wt/vol NaCl) at 4°C. Polyclonal antibodies to the purified proteins were raised in Japanese White rabbits, as described previously (21).

Specificities of the antisera were established by Western blotting with protein extracts from COS7 cells expressing FLAG-tagged mRAMPs or HA-tagged mfCLRs exogenously (Supplemental Fig. 3). The fragments corresponding to the coding sequences of mRAMPs or mfCLRs were subcloned into the pcDNA3 vector that was modified to express the tagged proteins (tag on the COOH terminus). COS7 cells were cultured in DMEM (Sigma) containing 10% FBS (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). The cells were transfected with the mRAMPs-FLAG, mfCLRs-HA, C- or mock plasmid using lipofectamine 2000 (Invitrogen), according to the manufacturer’s instruction. At 48 h after transfection, the cells were washed three times with PBS, and membrane proteins were collected. The samples were boiled with 1% vol/vol β-mercaptoethanol for 5 min. separated by SDS-PAGE using a 12% and 10% polyacrylamide gel for RAMPs and CLRs, respectively, and electroblotted onto a PVDF membrane. Nonspecific binding was blocked with 5% nonfat skimmed milk in Tris-buffered saline-Tween (TBS-
Fig. 1. Structural features of mefugu receptor activity-modifying proteins (RAMPs). A: alignment of amino acid sequences of mefugu RAMP and members of RAMP family of human (hRAMP) and mouse (mRAMP). Accession numbers are hRAMP1, AAP23298; hRAMP2, AAH27975; hRAMP3, AAP23300; mRAMP1, NP_058590; mRAMP2, AAF21038; and mRAMP3, NP_062384. The alignment was constructed with GENETYX-MAC computer program. The amino acid sequences were deduced from the cloned mfRAMP cDNAs, whose sequence and tissue source information have been deposited in the DNA Data Bank of Japan/European Molecular Biology Laboratories/GenBank database under the following accession numbers: mfRAMP1 (AB219765), mfRAMP2a (AB219766), mfRAMP2b (AB219767), mfRAMP3 (AB219768), mfRAMP4 (AB219770), and mfRAMP5 (AB219771). The amino acid residues that are conserved among RAMP family are shaded, and the completely conserved ones are marked on top of the alignment: *, cysteine; @, tryptophan; #, tyrosine; $, proline-asparagine pair; %, histidine; and &, phenylalanine. Solid line labeled as TM indicates a transmembrane region predicted from the Kyte-Doolittle hydropathy plot in B. B: prediction of a transmembrane region and schematic representations of mfRAMPs. Topological analyses by Kyte-Doolittle hydropathy plot predicted a presequence (Pre) on the NH2 terminus and a transmembrane region (TM) on the COOH terminus of all of the mfRAMPs. On the schematic representation, the total numbers of amino acid residues are denoted by bold font and characteristic sites are marked with regular font: Anchor symbol andpsi (Ψ) represent signal sequence cleavage site and N-linked glycosylation sites, respectively. Putative phosphorylation sites are indicated by an asterisk (*) and triangle (△) for PKC and PKA, respectively. Extracellular dotted stretches on mfRAMP2a/2b and mfRAMP5 were used as an antigen.
Tyro100 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature. The membranes were incubated with 3% FBS preabsorbed anti-mfRAMPs, anti-mfCLR, or respective preimmune serum at 1:7,000 dilution or tag-specific antibodies (mouse anti-FLAG monoclonal antibody, Sigma, 1:7,000 and rat anti-HA monoclonal antibody, Sigma, 1:5,000) for two overnight at 4°C. After washing with TBS-T, the membranes were reacted with horseradish peroxidase-conjugated secondary antibodies (donkey anti-rabbit IgG, goat anti-mouse IgG, or donkey anti-rat IgG) at 1:30,000 dilution for 1 h at room temperature. The bound secondary antibody was visualized by enhanced chemiluminescence detection using ECL-Plus reagent (GE Healthcare) according to the manufacturer’s instructions. Identical staining patterns were obtained with specific antisera and tag-specific antibodies, demonstrating the specificity of the antisera to mfRAMPs and mfCLRs.

Membrane proteins (20 µg of each sample in 0.5% SDS) of the transfected COS7 cells were boiled in 50 mM sodium phosphate buffer, pH 7.2, containing 0.1 M β-mercaptoethanol for 3 min and cooled to room temperature. Nonidet P-40 was added to the sample at a final concentration of 1% vol/vol and digested with 2 µl of endoglucosidase F (Roche) for overnight at 37°C. Samples were boiled for 3 min with Laemmli buffer and subjected to Western blot analysis. The sizes of the tagged mfCLRs and mfRAMPs, determined by Western blot analysis, are consistent with those predicted from the coding sequences of their cDNAs.

Fluorescence Immunohistochemistry

Seawater-adapted mefugu and torafugu were perfused with ice-cold PBS, pH 7.4, for 2 min through intra-arterial infusion to remove blood and were subsequently fixed by perfusion with 2% wt/vol paraformaldehyde for 10 min. The fixed kidneys and gills were excised and equilibrated within 15% wt/vol sucrose solution at 4°C overnight. The tissues were once soaked in 30% wt/vol sucrose solution for 6 h and then briefly soaked in 30% sucrose solution containing 50% vol/vol optimum cutting temperature (OCT) compound, frozen in the OCT compound in cryostat holder, and sliced with 0.6 µm thickness in cryostat at −25°C. A series of sections were mounted on 3-aminopropyltriethoxysilane-coated glass slides (Matsunami Glass, Tokyo, Japan) and air-dried for 1 h. The sections were first incubated in PBS with 0.1% vol/vol Triton X-100 (Acros Organics, Giel, Belgium) for 10 min and washed gently with PBS and incubated within 3% vol/vol normal goat serum for 2 h at room temperature. After the blocking, they were incubated within the goat serum containing the diluted antisera for 14 h at 4°C: 1:500 dilution for anti-mfRAMP2a and the preimmune serum, or 1:1,000 dilution for other antisera and the preimmune sera. Then, they were rinsed with PBS and incubated for 2 h at room temperature in cocktail mix consisting of Alexa Fluor 488-conjugated anti-rabbit IgG (1 µg/ml; Molecular Probes, Carlsbad, CA) and tetramethylrhodamine isothiocyanate (TRITC)-phalloidin (0.1 µg/ml; Sigma) with Hoechst 33342 (100 ng/ml; Molecular Probes) for the kidney sections or not for the gill sections. After washing three times in PBS, they were embedded in fluorescence mounting medium (Fluromount-G, Southern Biotechnology Associates Birmingham, AL). The fluorescence was detected by fluorescence microscope (Olympus, Model-IX70). The photographs were taken by a Princeton Instruments-cooled CCD camera (MicroMax 5 MHz, Roper Scientific) and interpreted by MetaMorph software (Universal Imaging).

Quantification of AM1 Activity in Urine

Approximately 500 µl of urine were collected from the urinary bladder of seawater- or brackish water-adapted torafugu using a needle and syringe, and stored immediately at −80°C. To measure AM1 activity in the urine, the samples were diluted with a volume of DMEM containing 1 mM IBMX and incubated with HeLa cells, in which pcDNA-CLR1 and pcDNA3-RAMP2a were transiently transfected. Then, the amount of accumulated cAMP in the cells were measured, plotted on the standard curve, as described above, and compared with those induced by a series of fugu AM1 with known concentrations.

Synteny Analysis

Synteny of neighboring genes of the RAMP1 and RAMP2 genes was investigated by comparing physical mapping data of the relevant scaffolds, namely, scaffold 117 and scaffold 43 of the torafugu genome database [Joint Genome Institute, U.S. Department of Energy, (JGI), http://genome.jgi-psf.org/fugu6] with those of the human chromosomes 2 and 17, respectively, as the human RAMP1 gene is located on chromosome 2 and the RAMP2 gene is on chromosome 17. Human chromosomal information was collected using Map Viewer (http://www.ncbi.nlm.nih.gov/mapview/).

RESULTS

Identification and Molecular Properties of Mefugu RAMPs

A database search revealed the presence of five members of the RAMP family in the torafugu genome (JGI; http://genome.jgi-psf.org/fugu6): RAMP1–5. On the basis of these sequences, we designed PCR primers for amplifying the corresponding sequences of mefugu transcripts (mRAMP1–5, mf for mefugu). The nucleotide sequences of their cDNAs were determined by RT-PCR and RLM-RACE using mRNA preparations of the mefugu kidney, heart, and gill, which yielded the amino acid sequences of the protein products (Fig. 1A), the exon-intron organization of the corresponding genes (Supplemental Fig. 1S), and the presence of variant forms deriving from a single gene by alternative usage of different promoters and alternative splicing (mRAMP2a and mRAMP2b; mRAMP3a and mRAMP3b) (Supplemental Fig. 1S). Like mammalian RAMPs, mfRAMPs are predicted to have a single transmembrane span in their COOH terminal region (Fig. 1B). The amino acid sequences of RAMPs are diverse among the family members and also among the species, but they share several invariant residues, including four conserved cysteine residues (*), a tryptophan residue (Ψ), a tyrosine residue (φ), a proline-asparagine pair (ð), a histidine residue (Δ), a phenyl-
alanine residue (#), and an aspartic acid-proline/alanine-proline triplet (A) in the extracellular NH₂ terminal region, which may be considered as signature residues of RAMPs (Fig. 1A).

**Identification and Molecular Properties of Mefugu CLR**

Similarly, three CLR family members, mfCLR1–3, were identified by database mining, followed by cDNA cloning (Supplemental Fig. 1S) and their amino acid sequences were deduced from the cDNA sequences (Fig. 2A). Short variant forms of mfCLR1 and mfCLR3 were also found that are generated by alternative splicing or differential usage of dual promoters, as depicted in Supplemental Fig. 1S. The database search revealed the presence of another member of the CLR family on the fugu scaffold 77, which was tentatively
termed mfCLR4 but not further characterized here since it turned out to be the CT receptor. Amino acid sequences are relatively highly conserved among the family members and species (Fig. 2A).

Hydropathy analysis showed that the precursors of mfCLR1–3 consist of a hydrophobic presequence, an NH2 terminal extracellular domain, seven transmembrane spans, and a COOH terminal cytoplasmic tail (Fig. 2B). In the case of mfCLR3, an extra short hydrophobic segment is present as indicated by H9251 in Fig. 2B. A hormone-binding domain is predicted to localize in the NH2 terminal extracellular domain of each mfCLR by a motif search using Simple Molecular Architecture Research Tool (SMART; http://dylan.embl-heidelberg.de/) (indicated by a solid thick line, Fig. 2C). This hormone-binding domain is present in a similar location of many secretin family members of the GPCR superfamily, including the CT receptor and CLRs.

Phylogenetic Relationships of mfRAMP1–5 and mfCLR1–3

Fig. 3A shows a phylogenetic tree of the members of the RAMP family, including mfRAMP1–5, which are divided into three groups. The tree also suggests that the mfRAMP4 and mfRAMP5 genes, whose counterparts are not present in mammals, were generated by duplications of the mfRAMP1 and mfRAMP2 genes, respectively. The orthologous nature of 1) the torafugu and human genes for RAMP1 and 2) those for RAMP2 is established by synteny mapping, which involves alignment of two genomes from different species to detect orthologous regions (Supplemental Fig. 2S). For the synteny analysis, the sequence information from the torafugu (T. rubripes) genome project was used since the genome sequence is not yet available for mefugu (T. obscurus).

Phylogenetic analysis of CLR family members indicated separate clustering of 1) amphibian/avian/mammalian CLR, 2) fish CLR1, 3) fish CLR2, and 4) mfCLR3, which is most closely related to the CTR family (Fig. 3B). An orthologous relationship has been established between the mfCLR4 and human CTR by synteny mapping; mfCLR4 was therefore renamed mfCTR (not characterized in the present study). Concerning synteny mapping around the CLR genes, the pufferfish genome database does not contain enough information to allow such an analysis.

Functional Characterization of Receptor Complexes

To determine the functional receptors for fugu AM1, AM2, and AM5, which are representative ligands of the three major classes of fugu adrenomedullins (26), we expressed subtypes of mefugu RAMPs and CLRs alone or in combinations in COS7 cells, confirmed their cell surface expression, and measured 125I-labeled-AM-binding activities and cAMP responses. In addition to the native forms, COOH terminally tagged forms of mfRAMP1–5 and mfCLR1–3 were also expressed for assessing the expression levels and cell surface expression by immunofluorescence staining using antisera specific for FLAG and HA tags. These tagged constructs were useful for monitoring their expression in COS7 cells and later confirmed to be

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**Fig. 3.** Phylogenetic trees among the RAMP and CLR families. The phylogenetic trees among RAMP (A) and CLR (B) families were constructed using Clustal W computer program. Mefugu genes are indicated as bold letters. Scale bar represents genetic distance of 0.1 amino acid substitutions per site. A: accession numbers are rat RAMP1 (AAG09434), mouse RAMP1 (NP_058590), human RAMP1 (AAP23298), pig RAMP1 (NP_999364), chicken A (XP_421873), medaka A (BJS01607), rainbow trout A (BX69063), zebrabith A (AAH76448), Xenopus A (DN006113), little skate (CO051467), mouse RAMP2 (AAF21038), rat RAMP2 (AAG09435), human RAMP2 (A0H27975), pig RAMP2 (NP_999247), chicken B (XP_418145), Xenopus B (BF426698), salmon A (CB501818), zebrafish B (CD759275), medaka B (BJS03602), mouse RAMP3 (NP_062384), rat RAMP3 (AAG09436), human RAMP3 (AAP23300), chicken C (XP_418881), pig RAMP3 (AAP39794), salmon B (CK863721), and rainbow trout B (BX860628). B: mouse CLR (NP_061252), rat CLR (NP_068694), human CLR (AAQ91352), pig CLR (NP_999256), chicken (XP_421850), Xenopus (AAN43289), Tritonodon A (CAF89940), halibut CQR (BAA92816), salmon CLR (CAD48406), zebrabith (AAH80226), Tritonodon B (CA01331), halibut CLR (BAA92817), bullfrog CLR (BAC77166), guinea pig CLR (AAM5886), human CTR (AAR06949), rat CTR (A37430), and mouse CTR (AAK56132).
active to the same extent as the native ones in mediating the cAMP response (data not shown).

Radioreceptor assay using 125I-labeled AM1, AM2, and AM5 revealed that iodination of the pufferfish AMs results in complete loss of binding activity, suggesting that the iodination target, namely the COOH terminal tyrosine residue of AMs, is directly involved in receptor binding. We therefore concentrated on using cAMP assay for determining functional combinations of RAMPs and CLRs.

When assayed for intracellular cAMP accumulation, COS7 cells transfected with either mfRAMPs or mfCLRs alone exhibited no response to fugu AM1, AM2, and AM5, human CGRP-I, and eel CT. We next examined all possible combinations of mfRAMPs and mfCLRs, a total of 18 combinations [6 × 3; 6 RAMPs (1, 2a, 2b, 3, 4, 5) and 3 CLRs]. Positive combinations of the cotransfection and their responses to AMs and related peptides are illustrated in Fig. 4. Active combinations that elicited cAMP response at physiological concentrations (∼10−9 M) of AMs were as follows: CLR1-RAMP2a/2b/3/5 for AM1 (Fig. 4A), CLR1-RAMP3 for AM2 and AM5 (Fig. 4B and C), CLR2-RAMP2a/2b for AM1 (Fig. 4D), and CLR1-RAMP1/4 for calcitonin gene-related peptide (CGRP-I, Fig. 4E). These results suggest, as schematically summarized in Fig. 4G, that the fugu AM receptors are formed by association of CLR1/2 and RAMP2/3/5 and, like the mammalian CGRP receptors, the fugu CGRP-I receptor is a complex of CLR1 and RAMP1/4 (see Fig. 3A for a close relationship between RAMP1 and RAMP4).

Tissue Distribution and Salinity-Dependent Expression of Mefugu RAMPs and CLRs

Total RNA preparations from the kidney, heart, spleen, liver, skin, ovary, gill, intestine, and muscle of seawater or freshwater mefugu were subjected to Northern blot analysis (Figs. 5 and 6). The messages for the CGRP sensitivity-conferring components, namely mfRAMP1 and mfRAMP4, were found to be expressed in a complementary or mutually exclusive pattern (Fig. 5A); for example, mfRAMP4 mRNA is very highly expressed in the muscle and moderately in the heart and skin, whereas mfRAMP1...
mRNA is widely expressed in the other tissues examined except the liver. All RAMP family members except RAMP4 are relatively abundantly expressed in the spleen. Among the RAMPs that define the selectivity of the mefugu AM receptors, RAMP2a and RAMP5 are relatively highly expressed in the osmoregulatory organs such as the gill and kidney; abundant expression of RAMP5 mRNA in the gill is noteworthy. RAMP2a is also highly expressed in the heart of seawater mefugu.

All of the three mfCLR genes were found expressed ubiquitously in a salinity-dependent pattern (Figs. 5B and 6). In the ovary, a shorter form of mfCLR2 mRNA was highly expressed in seawater, whereas its expression disappeared completely in freshwater. cDNA cloning and sequencing revealed that the short form arises from an alternative use of polyadenylation signals (AATTAA and AATAAA; accession number, AB232050) and its protein product is the same as that encoded by the long one.

Immunohistochemical Localization of AM Receptors in the Mefugu Gill and Kidney

Among the mefugu RAMPs and CLRs cloned above, we raised antisera against the components of the AM receptors (i.e., RAMP2a, RAMP5, CLR1, and CLR2) relatively highly expressed in the gill and kidney. Specificities of the antisera were established by Western blot analysis combined with epitope tagging as described in MATERIALS AND METHODS (Supplemental Fig. 3S).

For immunohistochemistry, we chose here the osmoregulatory organs, gill and kidney, among the five tissues (gill, kidney, heart, spleen, and ovary), because results from the Northern blot analysis suggested that they contained high levels of adrenomedullin receptors. Immunostaining of serial sections of mefugu gill with the four antisera revealed that 1) CLR1 and RAMP5 colocalize in interlamellar vessels (ILV; Fig. 7A, b and c) and 2) CLR2 and RAMP2a, in pillar cells (Fig. 7A, f and g). ILVs were not stained with anti-CLR2 and anti-RAMP2a, and pillar cells were negative for anti-CLR1 and anti-RAMP5 (data not shown). These results indicate that the CLR1-RAMP5 and CLR2-RAMP2a combinations are working as AM receptors in the ILV and pillar cells, respectively.

In the kidney, apical sides of the proximal tubules were strongly stained with anti-CLR1 and anti-RAMP2a (Fig. 7B). Phalloidin-positive actin bundles were used as a marker for the renal proximal tubules to distinguish the proximal and distal tubules (Fig. 7B, m). Glomerular capillaries were also moderately stained with the same antisera. The result suggests that the renal AM receptor is a complex of CLR1 and RAMP2a that is specific for AM1 (Fig. 4, A–C and G).

Identical immunohistochemical results were obtained using torafugu gill and kidney sections (data not shown), indicating that a similar system is also working in torafugu.

AM1 Levels in Fugu Urine

The localization of the CLR1-RAMP2a AM receptor on the apical membrane of the proximal tubule cells suggests that the receptor responds to the ligand (AM1) in the glomerular filtrate. Therefore, we determined whether fugu urine contains AMs by a bioassay. As torafugu and mefugu gave almost identical staining patterns on immunohistochemistry, we used easily available torafugu for urine collection. We first screened cultured cells (HeLa, COS7, HEK293, MDCK, and C2C12) for a cell line appropriate for such an assay by measuring their cAMP response to the urine samples, namely, a cell line that gave little response before cotransfection with CLR1 and
RAMP2a expression plasmids, but, after the transfection, it gave a large cAMP response. HeLa cells were the only cell line that satisfies the criterion among the cell lines examined; a standard curve is shown in Fig. 8. The AM concentration in seawater torafugu urine measured by this method was much higher \( \frac{21.2}{2.5} \mu M, n = 5 \) than that of brackish-water fish \( \frac{1.2}{0.2} \mu M, n = 3 \) (Fig. 8, inset).

**DISCUSSION**

Compared to the mammalian CGRP/AM systems, the fish CGRP/AM systems appear to be complex since five AMs have been identified in the tiger pufferfish *T. rubripes* (26) and also in the zebrafish *Danio rerio* (26). In the present study, in an attempt to clarify their receptor systems, we cloned six RAMPs and three CLRs from mefugu, a euryhaline fugu, and determined active combinations of RAMPs and CLRs and their tissue distributions (for a comprehensive summary, see Supplemental Fig. 4S). Until this study, RAMPs were cloned only from a few closely related species (for a review, see Ref. 32), allowing no useful comparative sequence analysis. The amino acid sequences of the RAMP family members of the mefugu *T. obscurus*, determined here, were useful for establishing the signature motif of RAMPs (Fig. 1).

**AM2-Specific Receptor in Fish**

In mammals, a high-affinity receptor selective for AM2/intermedin has not been identified yet; only low levels of cAMP response were observed with physiological concentrations of the ligand in all combinations of RAMP1–3 and CLR (5, 30). In mefugu, we demonstrated that the mfRAMP3-CLR combination serves as a high-affinity AM2 receptor, suggesting that a similar combination may serve as the physiological AM2 receptor also in mammals. The low affinity previously observed for mammalian RAMP3-CLR may be due to their incomplete posttranslational modification in cultured cells (SK-N-MC, L6, Rat2, and 293T cells) used for the assay.

**CGRP Receptor in Fish Muscle**

Like the mammalian system, RAMP1-CLR1 acted as a CGRP receptor. In addition, the combination of RAMP4-CLR1 was also demonstrated to act as a CGRP receptor, which appears to be specific for fish since the ortholog for the RAMP4 gene is not present in the mammalian genomes. The evolution of the RAMP4 gene in fish species and its limited expression in the muscles are noteworthy in the context that fish muscles are different from those of mammals in their development and growth. The increase in muscle mass occurs

**Fig. 6. Relative message levels of mfRAMP1–5 (A–F) and mfCLR1–3 (G–I) in various tissues of mefugu. Total RNA was prepared from seawater (SW; solid columns) and freshwater (FW; open columns) mefugu and subjected to Northern blot analysis, in which intensities of hybridization signals were quantified using National Institutes of Health software ImageJ (version 1.34s). Two sets each of freshwater and seawater mefugu RNA preparations were analyzed.**
through generation of new fibers (hyperplasia) and their expansion (hypertrophy). Postnatal muscle growth in mammals is largely contributed by hypertrophy. In contrast, in most teleost fish, muscle growth in postembryonic life is attributed to continuous hyperplastic and hypertrophic growth (31). The CGRP receptor of the RAMP4-CLR1 type, specific for fish muscle, may be somehow related to the unique mechanisms of postembryonic muscle growth of fish.

**AM Receptors in the Gill**

Colocalization of RAMP5 and CLR1 in the endothelium of ILV of the gill suggests that the AM receptor of the RAMP5-CLR1 type is expressed in ILV and plays important roles there. ILV is a ladder-like vascular network that traverses the filamental epithelium in between and parallel to the lamellae from afferent to efferent filamental artery (27). They can be identified and distinguished from nearby nutritional vessels by their thin vascular wall (9, 27) (Fig. 7A, d). In some species ILVs run as a pair (28) and, in the present study, pairing tendency was also found in mefugu. ILV has been proposed to be physiologically similar to the mammalian lymphatic duct (9). Considering the vasodilatory action of AM1 established in mammals, the mefugu AM receptor system appears to be involved in the regulation of blood flow through ILV. This role, however, may not be the major one since the ILVs...
expressing the RAMP5-CLR1 AM receptor are not surrounded by phalloidin-positive smooth muscle cell layer (Fig. 7A, d). Other possible roles may include 1) formation and maintenance of the ILV network as suggested by the angiogenic effects of AM demonstrated by Fernandez-Sauze et al. (10) using human umbilical vein endothelial cells and 2) stabilization of endothelial barrier function, which reduces microcirculatory disturbances typically seen in sepsis or septic shock. Consistent with the latter role, plasma AM levels have been shown to be elevated during septic conditions and injury (1, 11), and evidence is accumulating that AM stabilizes circulatory homeostasis in systemic inflammatory response (3).

There is growing evidence that pillar cells are contractile in nature and their contraction is regulated by endothelin factors such as endothelin, a powerful vasoconstrictor peptide (36). The pillar cell localization of a second AM1 receptor, composed of mfCLR2 and mfRAMP2a (Fig. 7A, f–h), suggests that the AM system is involved in the control of lamellar microcirculation through its relaxation effect on pillar cells whose contraction and dilation give rise to large changes in the lamellar blood flow. Furthermore, localization of the CLR2-RAMP2a AM receptor in the central trunk region but not in the flange of the bobbin-like pillar cells (Fig. 7A, h) supports the above speculation. Although the fish gill and the mammalian lung are structurally different, it is interesting, from the functional point of view, to note that AM has been demonstrated to be a powerful pulmonary vasodilator in mammals (6, 13).

Targeted disruption of the mouse AM1 gene indicated that the gene product is indispensable for the lymphatic and vascular morphogenesis during embryonic development, and its null mutation results in embryonic lethality (for a review, see Refs. 24 and 33). Shindo et al. (35) developed knockout mice lacking the precursor sequence that encodes AM1 and the proadrenomedullin NH2 terminal 20 peptide (PAMP) and found poor formation of large vessels in the yolk sac and placenta of AM−/−/PAMP−/− embryos and attributed lethality to massive hemorrhage probably as a result of deformed vascular endothelial cells and an absence of the normal three-layer structure of the basement membrane. Caron and Smithies (4) developed similar knockout mice and reported that AM−/−/PAMP−/− embryos die at midgestation with extreme hydrops fetalis and cardiovascular abnormalities, including overdeveloped ventricular trabeculae and underdeveloped arterial walls. Although AM−/− knockout mice are also embryonic lethal, no hydrops fetalis and hemorrhage were found (34) suggesting more severe embryonic phenotype of AM−/−/PAMP−/− compared with AM−/− knockout mice. Recently, AM has been demonstrated to possess novel angiogenic properties mediated by its ability to enhance vascular endothelial growth factor expression and Akt activity (15). It is, therefore, likely that the AM systems identified in the gill are involved not only in the regulation of vascular tone but also in the formation and maintenance of complex lamellar and interlamellar vasculatures.

**AM Receptor in the Kidney**

In the kidney of mefgu, CLR1 and RAMP2a were found to be coexpressed in the glomerular capillaries and proximal tubule cells, suggesting that a third AM receptor (CLR1-RAMP2a) is densely localized in the specific regions of the kidney and plays important regulatory roles in glomerular filtration and tubular reabsorption processes. What was totally unexpected was the apical membrane localization of the receptor in the proximal tubule cells (Fig. 7B). This apical location means that the receptor system responds to the luminal ligand contained in the glomerular filtrate. We, therefore, confirmed the presence of AM1 in urinary samples in levels sufficient to evoke a cAMP response. These observations indicate that AM1 contained in glomerular filtrates is not just for secretion but can act as a hormone; in this context, the observation by Hino et al. (14) is noteworthy: that renal glomerular podocytes secrete AM1 and the secretion was stimulated with H2O2, hypoxia, puromycin aminonucleoside, albumin overload, and TNF-α. In mammals, it has also been demonstrated that urinary AM levels are significantly higher than those in plasma, especially the levels of the amidated mature form (25), and that AM administration significantly increases renal blood flow, glomerular filtration rate, Na+ excretion, and urine flow (7, 8, 19, 39). A similar mechanism, involving the receptor facing the tubular luminal fluid, may therefore be working also in the mammalian kidneys.

**Perspectives**

Tissue distribution of mfRamps and mfCLRs suggests multifunctionality of the AM/CGRP systems in mefgu, including roles in muscle development and growth, gill circulation, renal filtration and reabsorption, immune and inflammatory response, neural activities, and reproduction. Among them, although not addressed here, the ovarian AM receptor and its possible role in reproduction may deserve comments since its component CLR2 is very highly expressed in the mefgu ovary in seawater but not in freshwater (Fig. 5B). The mefgu T. obscurus is anadromous; most of the growth takes place in the sea, but they spawn in brackish and fresh water. It is therefore tempting to speculate that the AM system is preventing a final step(s) of oocyte maturation or ovulation, while mefgu is in seawater and its disappearance in freshwater sets a stage for spawning.

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