Primary Structure and Differential Gene Expression of Three Membrane Forms of Guanylyl Cyclase Found in the Eye of the Teleost Oryzias latipes*

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Three cDNAs (Olgc3, Olgc4, and Olgc5) encoding membrane guanylyl cyclases were isolated from a medaka (Oryzias latipes) eye cDNA library. An open reading frame for Olgc3 predicted a protein of 1057 amino acids, and those for Olgc4 and Olgc5, 1134 and 1151, respectively. These proteins consist of an apparent signal peptide (21 residues for Olgc3, 50 residues for Olgc4, and 48 residues for Olgc5) and a single transmembrane domain that divides the protein into an amino-terminal extracellular domain and a carboxyl-terminal intracellular domain that further divides into a kinase-like domain and a cyclase catalytic domain. Phylogenetic analysis with amino acid sequences of Olgc3, Olgc4, and Olgc5, as well as those of other membrane guanylyl cyclases, indicated that Olgc3, Olgc4, and Olgc5 are members of the sensory organ-specific guanylyl cyclase family. Reverse transcription-polymerase chain reaction and Northern blot analyses demonstrated that Olgc3, Olgc4, and Olgc5 transcripts are present in the eye, which contains more cGMP than the other organs. In addition to being expressed in the eye, Olgc3 transcripts are also present in the brain, heart, liver, pancreas, and ovary, while Olgc4 is present in the liver and Olgc5 in the heart. Reverse transcription-polymerase chain reaction analysis with RNA from unfertilized eggs and embryos showed that Olgc3 and Olgc5 are expressed both maternally and zygotically, while Olgc4 is expressed only zygotically, and that the zygotic expression of these three genes is differentially activated. These results suggest a structural and functional diversity of sensory organ-specific guanylyl cyclases in vertebrates.

Cyclic GMP (cGMP) is a ubiquitous second messenger in intracellular signaling cascades and responsible for a wide variety of physiological responses. Guanylyl cyclases (GCs) compose a small family of proteins that catalyze the conversion of GTP to cGMP (1, 2). The cyclases are grouped into two major forms, those found on the plasma membrane (membrane GCs) and those in the cytoplasm (soluble GCs). The soluble GC is a heme-containing heterodimeric protein that is activated by binding of nitric oxide (3, 4). Analysis of cDNA clones for soluble GC subunits has shown that each subunit contains a cyclase catalytic domain which is also conserved in the membrane GCs (5–8).

The membrane GCs consist of a single polypeptide with about 150–200 kDa. In mammals, cDNA clones for six membrane GCs (GC-A, GC-B, GC-C, GC-D, GC-E, and GC-F) have been isolated and characterized (9–14). The predicted proteins contain an extracellular region, a kinase-like domain, and a cyclase catalytic domain. GC-A and GC-B are activated by binding of natriuretic peptides to their extracellular domain (2, 9, 10, 15, 16), and GC-C activity is stimulated by extracellular binding of heat-stable enterotoxin and guanylin (17–19).

Sensory neural tissues such as olfactory neurons and retina express specific isoforms of membrane GC. GC-D is specifically expressed in a subpopulation of olfactory sensory neurons (13). Two retina-specific GCs (RetGC-1 and RetGC-2) have been isolated and characterized from human retina (20, 21). These GCs are expressed mostly in the photoreceptor cells, and their expression patterns are indistinguishable. Two membrane GCs, GC-E and GC-F, have also been isolated from a rat eye cDNA library (14). GC-E and GC-F are thought to be orthologs of human RetGC-1 and RetGC-2. Rat GC-E and GC-F show different expression patterns. GC-E is expressed in the eye and pineal gland, whereas the expression of GC-F is confined to the eye (14). Contrary to GC-A, GC-B, and GC-C, no extracellular ligand has been reported for olfactory tissue- and eye-specific membrane GCs. Instead, the existence of two activators, GCAP-1 and GCAP-2, for retina-specific membrane GCs has been reported in bovine rod outer segment membranes (21–23). Both activators are a Ca2+–binding protein. GCAP-2 activates RetGC-1 and RetGC-2, whereas GCAP-1 activates RetGC-1 only. RetGC-1 is activated through binding of GCAP-2 to the intracellular domain, and the removal of the extracellular and transmembrane domains from RetGC-1 does not significantly alter the activation by GCAP-2 (24).

In Drosophila, some membrane GC transcripts are present in the unfertilized egg, along with embryos in the early developmental stages (25, 26). This suggests that the cGMP signaling pathway mediated by a membrane GC is involved in the early development of Drosophila. Despite rapidly accumulating information concerning the molecular nature of membrane GCs, there are few reports related to the expression of membrane GCs during vertebrate development or to the molecular phylogenetic relationship among membrane GCs. In this study,
to understand the developmental role and diversity of membrane GCs in vertebrates, we begin the characterization of membrane GCs in medaka fish (Oryzias latipes), a species allowing for both classical and molecular genetic analyses.

In this first report, we discuss the isolation and characterization of cDNA clones encoding three different membrane GCs expressed in the medaka eye, and the phylogenetic relationship of these membrane GCs. We also report that transcripts of two of the three medaka membrane GCs are present as maternal messages, and that each of these membrane GCs shows distinct expression patterns during embryogenesis and adulthood.

**EXPERIMENTAL PROCEDURES**

**Animals and Embryos**—Mature adults of the orange-red variety of medaka O. latipes were purchased from a dealer. They were kept in indoor tanks under artificial reproductive conditions (10 h dark, 14 h light: 27 °C) and fed on TetraMin flakes (TetraWerke, Germany). Naturally spawned and fertilized eggs were collected and the embryos cultured in distilled water containing 0.6 ppm methylene blue at 27 °C. The developmental stage was expressed in days and the day of fertilization was referred to as day 0. Hatching occurs usually at day 10.

**Determination of cGMP Contents in Adult Organs and Embryos**—Adult organs (eye, brain, heart, liver, intestine, and gall bladder) were obtained separately from five different medaka fishes. Five eggs or embryos were collected at various stages (immature oocyte, unfertilized eggs, fertilized eggs, 26 h, 34 h, 54 h, 4 days 5 h, 5 days 12 h, and hatched fry). Samples were homogenized with cold 6% trichloroacetic acid on ice to give a 10% homogenate. The homogenate was centrifuged at 5000 rpm for 15 min and the resultant supernatant fluid was extracted four times with 5 ml of ethyl ether saturated with distilled water to remove trichloroacetic acid. An aliquot (1 ml) was lyophilized and the residue suspended in 1 ml of assay buffer (Amerham). After acetylation of the sample and standard, cGMP concentrations were determined by enzyme-linked immunosass according to the manufacturer’s protocol. The probe was isolated and hybridized to the cDNA inserts under the same condition as the first PCR. A 267-bp cDNA fragment amplified in the third PCR was purified, subcloned into the plasmid vector pBluescript II KS+ (Stratagene), and used as a probe to screen the cDNA library.

The probe was labeled with [32P]dCTP using the Random Primer DNA labeling Kit, Version 2 (Takara Shuzo Co., Ltd.). 1.2 × 109 cpm was plated, and the membranes were prehybridized for 2 h at 50 °C in 6 × SSC (1 × SSC: 0.15 mM NaCl and 0.015 mM sodium citrate, pH 7.0) containing 0.1% SDS. 5 × Denhardt’s solution, 200 μg/ml denatured salmon sperm DNA, and 30% formamide. Radioactive probe (106 cpm/ml) was added to the prehybridization solution and incubated at 50 °C for 2 h. The membrane was rinsed briefly in 0.5 × SSC containing 0.1% SDS, washed in 1 × SSC containing 0.1% SDS for 30 min at 55 °C, and then in 0.5 × SSC containing 0.1% SDS for 30 min at 55 °C. Eight positive clones 2A02, O2A2, and O5G1 were obtained after the secondary screening, and the cDNA inserts were excised in vitro into pBluescript SK+ (Stratagene) according to the manufacturer’s protocol. The sequences of cDNA inserts were determined as described above. 2A02 and O2A2 had the same sequence, encoding GC, and the encoded protein was named OlGC4 from O. latipes guanylyl cyclase. O5G1 clone encoded another GC, and it was named OlGC5. Subsequent screening of the eye cDNA library with the 267-bp cDNA fragment and the most 5′ portion (678 bp) of O5G1 clone as probes resulted in isolation of another 11 clones. One of these, O5G2, encoded a GC that is distinct from either OlGC3 or OlGC4, and this was named OlGC6.

**5′-Rapid Amplification of cDNA Ends (5′-RACE)**—To obtain a full-length cDNA sequence of OlGC3, OlGC4, or OlGC5, a 5′ portion of the cDNA was amplified by the 5′–RACE method (31) using the 5′-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Life Technologies, Inc.). One μg of total RNA was reverse-transcribed with a gene-specific antisense oligonucleotide primer (GSP1). The cDNA was then labeled with dCTP using terminal deoxynucleotidyl transferase, and amplified by PCR with the Abridged Anchor Primer (Life Technologies, Inc.) and another gene-specific antisense oligonucleotide primer (GSP2). Two different PCR conditions were used: for OlGC3, denaturation at 94 °C for 2 min followed by 30 amplification cycles (94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and a final extension at 72 °C for 7 min; for OlGC4 and OlGC5, 15 amplification cycles (94 °C for 1 min and 72 °C for 1 min) followed by another 35 amplification cycles (94 °C for 1 min, 62 °C for 30 s, and 72 °C for 1 min) and a final extension of 72 °C for 7 min. The PCR products were cloned into pBluescript II KS+ and sequenced. The gene-specific primers used were: OlGC3, nt positions 1268–1287 (GSP1) and 1227–1246 (GSP2); OlGC4, 2788–2807 (GSP1) and 2753–2772 (GSP2); OlGC5, 2902–2921 (GSP1) and 2800–2809 (GSP2) or 2902–2921 (GSP1) and 2902–2915 (GSP2) or 2902–1071 (GSP2). Each 5′-RACE product overlapped in 25–127 bp with the 5′ end of the clone that had been isolated, and the sequence was identical in the overlapping region (Fig. 2). To confirm further that the 5′-RACE product of OlGC3 corresponds to the authentic transcript, cDNA containing the overlapping region was amplified by RT-PCR with a pair of primers corresponding to nucleotide positions 1024–1043 and 1380–1400, cloned into pBluescript II KS- and sequenced.

**Molecular Phylogenetic Analysis—**The amino acid sequences of OlGC3, OlGC4, and OlGC5 were aligned with those of 10 vertebrate and invertebrate membrane GCs with the aid of the Clustal W program (32) and the sequence editor SeqPup (D. Gilbert, Indiana University). GenBank/EMBL/DDJB accession numbers for the GC sequences are: S55279, bovine RetGC (33); S03348, rat GC-A (9); S33305, rat GC-B (10); A63929, rat GC-C (11); L57293, rat GC-D (12); S05915, rat GC-E (14); B55519, rat GC-F (14); S04459, human GC-A (16); S05514, human GC-B (15); A40940, human GC-C (34); M92432, human RetGC-1 (20); L37378, human RetGC-2 (21); D21101, sea urchin (Hemicentrotus pulcherrimus) spermatozoa GC (HpGC; 35); A33535, sea urchin (Strongylocentrotus purpuratus) spermatogenic GC (SpGC; 36); A36568, mouse GC-A (37); Z74374, guinea pig GC-C; D17513, pig GC-C (38); S46578, Xenopus laevis GC-A (39); L57701, Drosophila melanogaster GC-1 (25); and A56114, D. melanogaster GC-2 (26). Phylogenetic trees were constructed with the aligned sequences by

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2 M. Kruhoffer, Y. Cetin, U. Kaempf, and W. G. Forssmann, unpublished data.
R. T. MacFarland, unpublished data.
the maximum parsimony algorithm in the PROTPARS program of PHYLYP (version 3.572) (40) and the neighbor-joining algorithm (41) in the Clustal W program (32). For the neighbor-joining analysis, evolutionary distances were estimated using Kimura’s (42) empirical method for protein distances.

RT-PCR—Three μg of total RNA was used as the template to synthesize the first strand cDNA using an oligo(dT) primer according to the manufacturer’s protocol (SuperScript Preamplification System for First Strand cDNA Synthesis, Life Technologies, Inc.). cDNA fragments containing 3′ coding and noncoding regions of OlGC3, OlGC4, and OlGC5 were amplified by PCR from the first strand cDNA. A pair of primers specific for the medaka cytoplasmic actin gene OlCA1 was used to amplify the OlCA1 cDNA fragment as an internal control. The primer pairs used were: OlGC3, 5′-CTGTGACAACAGCTCTACTC-3′ (complimentary to nt 3800–3819) and 5′-GTGCCCTGTTTGGTCCTA-3′ (complimentary to nt 3349–3368); OlGC4, 5′-CCGTCAACACTGCCTCTCGT-3′ (identical to nt 883–902) and 5′-GTGCCCTGTTTGGTCCTA-3′ (complimentary to nt 1175–1194). The PCR reaction buffer (Takara Shuzo Co., Ltd.) and 10 μM of each primer. PCR was performed under the following conditions: 5 min of denaturation at 95 °C, 30 cycles of denaturation (30 s at 95 °C), annealing (30 s at 66 °C), and extension (1 min at 72 °C), and elongation reaction for 15 min at 72 °C at the final cycle. The same reaction was carried out without reverse transcriptase as a control experiment for false-positive PCR amplification of contaminating genomic DNA. One-sixth of the PCR products was separated on a 3% NuSieve, 1% SeaKem GTG-agarose (FMC Corp.) gel. The gel was soaked with ethidium bromide solution (10 μg/ml) and photographed under UV irradiation. The DNA fragments were transferred onto a nylon membrane (Hybond-N, Amersham) using 0.4 M NaCl as the transferring solution, and the membrane was washed with 2 × SSC. The membrane was then prehybridized in 6 × SSPE containing 5 × Denhardt’s solution, 0.5% SDS, 50 μg/ml salmon sperm DNA, and 50% formamide for 1 h at 42 °C and hybridized in the same buffer containing a 106 cpm/ml denatured probe at 42 °C for 16 h. The membrane was washed three times with 2 × SSC at 65 °C for 15 min and with 0.1 × SSC containing 0.1% SDS at 65 °C for 15 min and then exposed to a Kodak X-AR film with intensifying screens at −80 °C for 1–24 h. The size of DNA was estimated using 1-kb ladder (Life Technologies, Inc.) bands as standards.

RESULTS
cGMP Contents in Various Adult Organs and Embryos—cGMP contents in various adult organs and embryos were determined by enzyme-linked immunoassay. Among adult organs examined, cGMP was most abundant in the eye (Fig. 1A). The intestine and gall bladder contained more cGMP than the brain, heart, or liver (Fig. 1A). Fig. 1B shows changes of the cGMP contents during embryogenesis. Embryonic cGMP contents increased with embryo development, reaching its maximum level at the hatched fry stage. A dramatic increase was observed between day 4 and day 5. This increase may reflect eye development, since the eye contains more cGMP than any other organs.
isolated are shown in Figs. 3, 4, and 5, respectively. The 944-bp cDNA consists of a 57-bp 5'-UTR with a poly(A) tail, a 3453-bp open reading frame, and an 814-bp 3'-UTR of OlGC3, OlGC4, and OlGC5. The mature protein of these medaka membrane GCs comprises a large extracellular domain (residues 1-413 for OlGC3, 1-425 for OlGC4, and 1-466 for OlGC5), a single membrane-spanning domain (residues 414-438 for OlGC3, 426-440 for OlGC4, and 467-491 for OlGC5), a protein kinase-like domain (residues 492-769 for OlGC3, 480-761 for OlGC4, and 536-815 for OlGC5), and a cyclase catalytic domain (residues 782-1015 for OlGC3, 754-1010 for OlGC4, and 808-1064 for OlGC5) (Figs. 3, 4, and 5).

Comparison and Phylogenetic Analysis of Amino Acid Sequences of Various Membrane GCs—To investigate structural and evolutionary relationships among OlGC3, OlGC4, OlGC5, and other membrane GCs, their amino acid sequences were compared, and phylogenetic analyses were performed based on the amino acid sequences of catalytic domain of various GCs by the neighbor-joining method (Ref. 41; data not shown). In the phylogenetic tree, three major clusters of vertebrate membrane GCs were found: (i) natriuretic peptide receptors, (ii) enterotoxin receptors, and (iii) sensory organ-specific GCs. Invertebrate GCs, including those of sea urchins and Drosophila, were shown to be distantly related to all of the vertebrate membrane GCs. The medaka fish GCs, OlGC3, OlGC4, and OlGC5, isolated from the eye were grouped with mammalian sensory organ-specific GCs such as rat GC-D, GC-E, and GC-F, suggesting that, together with mammalian sensory organ-specific GCs, OlGC3, OlGC4, and OlGC5 form a family of membrane GCs that are expressed specifically in the sensory organs. Among the sensory organ-specific GC subfamily, the medaka fish GCs have unique sequences. OlGC3 and OlGC5 are closer to each other than to other members of the subfamily, and OlGC4 is moderately related to the rat olfactory neuron-specific GCs.
specific GC-D. All mammalian eye-specific GCs were grouped together in the tree based on amino acid sequences of the cyclase catalytic domain.

To understand further the structural and evolutionary relationships among members of the sensory organ-specific GC subfamily, amino acid sequences of extracellular domains were compared and subjected to phylogenetic analysis. Fig. 6A shows an alignment of amino acid sequences of the extracellular domains. Amino acid sequences are less conserved in the extracellular domains than in the cyclase catalytic and protein kinase-like domains. However, the relative positions of some amino acids are highly conserved among the extracellular domains of sensory organ-specific membrane GCs including OlGC3, OlGC4, and OlGC5 (Fig. 6A). In particular, six cysteine residues are conserved within the extracellular domain (Fig. 6A).

A phylogenetic tree was constructed with amino acid sequences of the extracellular domains of various membrane GCs (Fig. 6B). All the sensory organ-specific GCs are grouped on a branch and are separated from the sea urchin GC and mammalian GC-C, further confirming a close relationship among the sensory organ-specific GCs including OlGC3, OlGC4, and OlGC5. The phylogenetic tree of the extracellular domains reveals a branching pattern different from that of the cyclase catalytic domains within the clade of sensory organ-specific GCs. OlGC3 and OlGC5 are closer to each other than to other membrane GCs, as in the case of the catalytic domains, while OlGC4 is grouped with human RetGC-2 and rat GC-F. OlGC3 and OlGC5 are then clustered with a clade containing rat GC-D, human and bovine RetGC-1, and rat GC-E (Fig. 6B).

Expression of OlGC3, OlGC4, and OlGC5 Genes in Adult Organs—

The nucleotide and deduced amino acid sequences of OlGC3 cDNA. The deduced amino acid sequence is indicated by single-letter code. The signal peptide sequence is indicated by lowercase letters. The amino acids are numbered relative to the predicted signal cleavage site (+1). The predicted transmembrane domain is boxed, and the putative polyadenylation signal is underlined.
adult tissues was examined by RT-PCR using oligonucleotide primers specific for each *OlGC* gene (Fig. 7). Expression of all three *OlGC* genes was evident in the eye. *OlGC3* expression was also detected in the brain, heart, liver, pancreas, and ovary, among which the brain and ovary showed an expression as strong as that of the eye. In addition to expression in the eye, *OlGC4* transcripts were also detected in the liver, and *OlGC5* was expressed in the heart.

Northern blot hybridization using a cDNA probe encoding the extracellular domain of each *OlGC* further demonstrated that the *OlGC3*, *OlGC4*, and *OlGC5* genes are expressed in the eye (Fig. 7, B and C). The size of the major transcripts for *OlGC3*, *OlGC4*, and *OlGC5* was shown to be 3.5, 8.8, and 6.2 kb, respectively (Fig. 7B). In addition to the major band of 3.5 kb, a weaker signal of 4.3 kb was detected for *OlGC3*. Two weaker bands of 7.2 and 5.3 kb were also detected for *OlGC4* (Fig. 7B).

Northern blot analysis failed to detect *OlGC4* and *OlGC5* transcripts in the brain, liver, and ovary, whereas faint signals were detected in the brain and ovary but not in the liver for *OlGC3* (Fig. 7C).

**Expression of *OlGC3*, *OlGC4*, and *OlGC5* Genes during Embryogenesis—**

Temporal expression patterns of *OlGC3*, *OlGC4*, and *OlGC5* during embryogenesis were further analyzed. The results showed a complex expression pattern for each gene during development.

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**Fig. 4.** The nucleotide and deduced amino acid sequences of *OlGC4* cDNA. The deduced amino acid sequence is indicated by *single-letter code*. The signal peptide sequence is indicated by *lowercase letters*. The amino acids are numbered relative to the predicted signal cleavage site (1). The predicted transmembrane domain is *boxed*, and the putative polyadenylation signal is *underlined*. Medaka Fish Guanylyl Cyclases 23412
and OlGC5 during embryogenesis of medaka fish were examined by RT-PCR analysis with RNA prepared from embryos at various developmental stages (Fig. 8A). OlGC3 and OlGC4 transcripts were present in embryos at early cleavage stages (day 0) and decreased at day 1. Expression of OlGC3 and OlGC5 was detected again from day 2, and the signal became stronger. FIG. 5. The nucleotide and deduced amino acid sequences of OlGC5 cDNA. The deduced amino acid sequence is indicated by single-letter code. The signal peptide sequence is indicated by lowercase letters. The amino acids are numbered relative to the predicted signal cleavage site (1). The predicted transmembrane domain is boxed, and the putative polyadenylation signal is underlined.
FIG. 6. Alignment (A) and phylogenetic tree (B) of the amino acid sequences of the extracellular domain of sensory organ-specific membrane GCs. A, amino acid sequences of OlGC3, OlGC4, and OlGC5 were compared with those of bovine RetGC-1 (BORETGC-1), rat GC-D (RATGC-D), rat GC-E (RATGC-E), rat GC-F (RATGC-F), human RetGC-1 (HURETGC-1), and human RetGC-2 (HURETGC-2). The identical amino acids in all proteins are indicated by asterisks below the sequences. Six conserved cysteine residues are indicated by open boxes. B, amino acid sequences of the extracellular domain (412 residues) were subjected to phylogenetic analysis. A phylogenetic tree was constructed by the maximum parsimony method. The sea urchin sperm guanylyl cyclase (HpGC) was included as the outgroup. Sources, references, and accession numbers for the GC sequences are described under “Experimental Procedures.”
more distinct during later embryogenesis. On the other hand, no OlGC4 expression was observed in early cleavage stage embryos. The OlGC4 transcripts were first detected at day 4, and then more strongly as the development proceeded.

To determine whether OlGC3 and OlGC5 are transcribed in

FIG. 7. Expression of OlGC3, OlGC4, and OlGC5 transcripts in adult medaka fish. A, detection of transcripts of OlGC3, OlGC4, and OlGC5 by RT-PCR in various adult organs. Transcripts of the medaka cytoplasmic actin gene OlCA1 were amplified as an internal control. RT+ and RT− represent amplification with and without reverse transcriptase, respectively. The PCR products were hybridized with a 32P-labeled cDNA probe for OlGC3, OlGC4, OlGC5, or OlCA1. B, Northern blot analysis of poly(A)+ RNA from the eye for OlGC3, OlGC4, and OlGC5 mRNA. The same blot containing 2 μg of poly(A)+ RNA was hybridized with a 32P-labeled cDNA probe for OlGC3, OlGC4, or OlGC5. Radioactive bands were indicated by arrowheads. The positions and sizes of RNA markers are shown on the right. An asterisk indicates a nonspecific band detected in all four tissues examined (brain, eye, liver, and ovary; data not shown) with the OlGC5 probe. C, Northern blot analysis of total RNA from the brain, eye, liver, and ovary. The same blot containing 30 μg of total RNA was hybridized with a 32P-labeled cDNA probe for OlGC3, OlGC4, OlGC5, or OlCA1. Northern blot analysis of poly(A)+ RNA gave the same results (data not shown).

FIG. 8. RT-PCR analysis of OlGC3, OlGC4, and OlGC5 transcripts during embryogenesis. A, detection of OlGC3, OlGC4, and OlGC5 transcripts in embryos at various developmental stages from day 0 (early cleavage stage) to day 9 (hatching stage). B, detection of OlGC3, OlGC4, and OlGC5 transcripts in unfertilized eggs. UF, unfertilized eggs; Day 2, embryos cultured for 2 days after fertilization; Day 8, embryos cultured for 8 days after fertilization. Transcripts of the medaka cytoplasmic actin gene OlCA1 were amplified as an internal control. RT+ and RT− represent amplification with and without reverse transcriptase, respectively. The PCR products were hybridized with a 32P-labeled cDNA probe for OlGC3, OlGC4, OlGC5, or OlCA1.
ogon. These results suggest that OlGC3 and OlGC5 genes are expressed both maternally and zygotically, while OlGC4 is expressed only zygotically. Our results also suggest that expression of the three OlGC genes is differentially regulated during fish development.

DISCUSSION

In this paper we report the characterization and expression patterns of three genes, OlGC3, OlGC4, and OlGC5, each encoding a different GC in the medaka fish. OlGC3, OlGC4, and OlGC5 are predominantly expressed in the eye, which contains more cGMP than any other organ, suggesting the important role(s) of these GCs in the phototransduction pathway. Hayashi and Yamazaki (49) reported multiple forms of photoreceptor GCs in toad, frog, and bovine rods. In mammals, two forms of membrane GC have been identified in the eye (14, 20, 21). Our study demonstrated that at least three related membrane GCs encoded by different genes are present in the fish eye. The presence of multiple forms of membrane GC in the eye seems to be common among a wide variety of vertebrates. Since the amino acid sequences of OlGC3, OlGC4, and OlGC5 are related but discrete, the enzyme activity of these GCs may be regulated via different pathways. Alternatively, these membrane GCs might show different cellular and/or subcellular distribution patterns in the eye.

The size of the major transcripts for OlGC3, OlGC4, and OlGC5 is 3.5, 8.8, and 6.2 kb, respectively. Although the transcripts of OlGC4 and OlGC5 are larger than those for most known membrane GCs (about 4.0 kb or smaller), the transcript lengths of 8.5 and 11 kb have been reported for the rat eye-specific GCs, GC-E and GC-F, respectively (14). The increase in size seems to be attributable to large 3′- and 5′-UTR sequences (14). Relatively large transcript size may thus be a common feature among vertebrate eye membrane GCs.

In addition to the major band of 3.5 kb, a weaker signal of 4.3 kb was detected for OlGC3. Similarly, two weaker bands of 7.2 and 5.3 kb were detected for OlGC4. The OlGC3 transcripts of different size may be attributable to transcripts with differently sized 3′-UTR, because we isolated a cDNA clone with a sequence identical to that of OlGC3 except for the size of 3′-UTR. It is uncertain whether the OlGC4 transcripts of different size are due to differently sized UTR. The size of cDNA sequences for OlGC3, OlGC4, and OlGC5 determined in the present study is 4110, 4651, and 5839 bp, respectively. These cDNA sequences probably correspond to transcripts of 4.3, 5.3, and 6.2 kb detected in the Northern blot analysis, respectively. The OlGC3 cDNA clone with smaller 3′-UTR may correspond to the 3.5-kb transcripts of OlGC3.

The phylogenetic analysis of intracellular catalytic domains revealed that there are three major groups of membrane GCs in vertebrates: (i) natriuretic peptide receptors, (ii) enterotoxin receptors, and (iii) sensory organ-specific GCs. OlGC3, OlGC4, and OlGC5 are grouped with the sensory organ-specific GCs. These three groups probably represent three distinct subfamilies of membrane GCs that are defined by families of ligands or activators as well as by localization. The phylogenetic tree of extracellular domains also suggests a close relationship among sensory organ-specific GCs, including OlGC3, OlGC4, and OlGC5. Within the sensory organ-specific GC subfamily, relationships among members were different between the phylogenetic trees of intracellular catalytic and extracellular domains. This may reflect different functional constraints on the structure of extracellular and intracellular domains.

The relative positions of some amino acids, including six cysteine residues, are highly conserved within the extracellular domains of the sensory organ-specific GCs. Although no extracellular ligands have been reported for the sensory organ-specific GCs, the conservation of these residues suggests a functional importance of the extracellular domains. These conserved cysteine residues may be involved in intramolecular disulfide bond formation and/or oligomerization of the proteins.

The RT-PCR analysis showed that OlGC3, OlGC4, and OlGC5 genes are also expressed in a number of organs other than the eye and that the expression patterns in adults are different among the three genes. In contrast, previous studies on mammalian eye- and olfactory neuron-specific GCs revealed that their expression is confined to sensory organs and related tissue (pineal gland) (13, 14, 20, 21). The difference between the reported expression patterns of medaka GCs and mammalian sensory organ GCs might be attributable to differences between fish and mammals or simply to differences in detection methods. Alternatively, since the sequences of OlGC3, OlGC4, and OlGC5 are relatively diverged from those of any known mammalian sensory organ GCs, there may be unidentified members of the mammalian sensory organ GC subfamily that exhibit sequences and expression patterns similar to those of OlGC3, OlGC4, or OlGC5. The presence of transcripts of OlGC3, OlGC4, and OlGC5 in various adult organs suggests that members of sensory organ GC subfamily play previously unrecognized roles in these organs. Since the Northern blot analysis failed to detect OlGC3 and OlGC4 mRNA in the liver and only faint signal for OlGC3 was detected in the brain and ovary, however, these organs may express much smaller amounts of the OlGC3 and OlGC4 transcripts than does the eye.

There have been few reports on the expression patterns of sensory organ GC genes during vertebrate development. Our results demonstrated that the transcripts of OlGC3 and OlGC5 are present in unfertilized eggs as maternal messages, suggesting that cGMP signaling pathways mediated by these membrane GCs are involved in the early development of fish. Since the OlGC3 transcripts are evident in unfertilized eggs as well as in the adult ovary, OlGC3 may play an important role during oogenesis. In Drosophila, membrane GCs are expressed during oogenesis and early development (25, 26). cGMP signaling pathways mediated by membrane GCs regulating oogenesis and early development may have been conserved between vertebrates and invertebrates. The RT-PCR analysis revealed that the three medaka GC genes are differentially activated during embryogenesis. The differing expression patterns of medaka GC genes suggest that these GCs have distinct roles during development. Two activators for eye-specific membrane GCs have been reported in bovine rod outer segment (21–23). Similar activators may regulate the activity of the medaka GCs during early development and in adult organs other than the eye.

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