Characterization of membrane progestin receptor α (mPRα) of the medaka and role in the induction of oocyte maturation

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ABSTRACT

Oocyte maturation in medaka is induced by the maturation-inducing hormone (MIH) via its membrane receptor. The most likely candidates for the membrane receptor are membrane progestin receptors (mPRs). In order to characterize the mPRα subtype of medaka, a human cell line expressing the mPRα gene of medaka was established and its steroid binding property was assessed. The α subtype exhibited high binding affinity for 17,20β-DHP, the MIH in medaka. Treatment with a morpholino antisense oligonucleotide to mPRα blocked oocyte maturation in vivo. These results suggest that the medaka mPRα protein acts as an intermediary during MIH-induced oocyte maturation in medaka in a manner similar to that described previously for fish species.
od of 14 h light and 10 h dark. This orderly spawning allows us to collect oocytes at predictable stages, which makes medaka an ideal model for the study of hormonal regulation of oocyte growth and maturation. 17α, 20β-dihydroxy-4-pregnen-3-one (17,20β-DHP) was shown to be a major naturally occurring MIH in medaka (1). Furthermore, a systematic strategy for screening for deficiencies in genes of interest has been established in medaka fish (14). From this screening system, we have selected three strains in which a point mutation was induced in the coding sequence of mPRα. We are currently continuing with back-crossing with wild-type fish to establish strains to analyze the phenotype of these mutants. We also established mutant strains for mPR subtypes (17). These reverse genetic analyses should elucidate the central roles of mPRs in the near future (18). Recently, mRNA expression levels of mPRs in medaka tissues and follicles were reported (2). Although the results demonstrated that mRNA expression levels of mPRs do not change during the course of induction of oocyte maturation and ovulation, expression analysis of the mPRα gene in cultured human cells suggested that mPRα is responsible for the decrease of intracellular cAMP levels induced by 17,20β-DHP as goldfish mPRα (7).

The present study was conducted to characterize the mPRα subtype in medaka and investigate its role in induction of oocyte maturation in this species. The results suggest that medaka mPRα possesses high affinity binding activity to 17,20β-DHP and is involved in induction of oocyte maturation.

MATERIALS AND METHODS

Fish and chemicals. Mature female medaka were purchased from a local supplier and maintained in outdoor tanks during summer time (from June to September) and fish under daily spawning conditions were selected and used for experiments. Ovari-an donor fish were humanely sacrificed following procedures approved by Shizuoka University Animal Care Committee. 17,20β-DHP, buffers, and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). [1,2,6,7 3H]-17α-hydroxyprogesterone (85 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ) and enzymatically converted to radiolabeled 17,20β-DHP by 3α, 20β-hydroxysteroid dehydrogenase (Sigma, St Louis, MO) as described previously (10).

Plasmid construct of medaka mPRα and transfection into cultured cells. The coding region of medaka mPRα was amplified by PCR using genome DNA as a template with primers for removal of the stop codon and containing EcoRI and BamHI enzyme cutting sites for insertion into the mammalian expression vector, pIRE2 DsRed (Discosoma sp. red fluorescent protein)-Express2 (Clontech, Mountain View, CA). The primers used to amplify mPRα cDNA were 5’-CGGAATTCATGGCAACGGTTGTGATGGAA-3’ (forward) and 5’-GCGGATCCTCAC TCCTCTTTGGTGAGTGGTAC-3’ (reverse). Thirty five cycles were performed using a cycle of 30 s at 94°C, 30 s at 67°C and 60 s at 72°C, followed by a final 10-min extension at 72°C. PCR products were purified by electrophoresis on a 1% agarose gel, extracted with a gel prep kit (Qiagen, Valencia, CA), ligated with vector, and transformed into chemically competent XL1-Blue E. coli cells. Transfected E. coli were spread on kanamycin-coated plates and grown overnight. Resistant colonies were then selected, regrown overnight and the plasmid was purified. Constructs were verified by DNA sequencing. Data was analyzed with GENETYX (Genetyx Co., Tokyo, Japan).

Prepared constructs were transfected into human MDA-MB-231 breast carcinoma cells (American Type Culture Collection) as described previously for mPRα (25). Cells were cultured and maintained at 37°C with 5% CO2 in DMEM media (Sigma, Saint Louis, MO) containing 5% FBS (Gibco, Carlsbad, CA) and 100 μg/mL gentamycin (Invitrogen, Carlsbad, CA). Media were changed every 3 days and cells were split among three plates when they became 90% confluent. Each mPR construct was then transfected into cells using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. Two days after transfection, plasmid-expressing cells were selected using 500 μg/mL G418 (Research Products International, Mt. Prospect, IL). Resistant colonies were then isolated and propagated with 250 μg/mL G418 in order to produce stably transfected cell lines.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from cultured cells using pre-made reagents for RNA extraction (ISOGEN; Nippon Gene, Tokyo, Japan) in accordance with the manufacturer’s protocol. RT-PCR was carried out using Ready-to-Go RT-PCR Beads (Amersham Biosciences) in accordance with the manufacturer’s instructions. The primers used to amplify mPRα cDNA were 5’-CGGAATTCATGGCAACGGTTGTGATGGAA-3’ (forward) and 5’-GCGGATCCTCAC TCCTCTTTGGTGAGTGGTAC-3’ (reverse).
Characterization of medaka mPRα

GATCCTCAGTGTTCGAGGTGTCGTGGAGT-3’ (reverse). PCR conditions involved incubation for 30 s at 94°C, 30 s at 67°C and 60 s at 72°C for a total of 35 cycles. pIRES2 was used as a positive control with gene-specific primers 5’-GGCTCAAGGTGTACGTGA-3’ (forward) and 5’-GGTGATGTCCTCGTTGTGGG-3’ (reverse). PCR conditions involved incubation for 30 s at 95°C, 30 s at 68°C and 30 s at 72°C for 35 cycles.

Preparation of membrane fractions from cultured cells. Plasma membrane fractions from cell lines expressing mPRs were prepared and suspended in HAED buffer (HEPES, 25 mM; NaCl, 10 mM; EDTA, 1 mM; dithiothreitol, 1 mM; pH 7.6 at 4°C) as described earlier (22).

Membrane binding assays. Progestin receptor binding in membrane fractions from cultured cells was measured following procedures established previously (22). Competition studies: One set of tubes contained 2 nM [3H]-17,20β-DHP alone (total binding) and another set also contained cold progestin competitor at a 100-fold greater concentration to measure nonspecific binding. After 30 min incubation at 4°C with the membrane fractions, reactions were stopped by filtration (Whatman GF/B filters, presoaked in HAED buffer containing 2.5% Tween 80). Filters were washed three times with 5 mL of wash buffer (HEPES, 25 mM; NaCl, 10 mM; EDTA, 1 mM; pH 7.4 at 4°C) and bound radioactivity was measured by scintillation counting. Displacement of radiolabeled 17,20β-DHP binding by steroids or diethylstilbestrol (DES) was expressed as a percentage of the maximum specific binding of 17,20β-DHP to the membrane fractions.

Saturation analyses and Scatchard plots: Various concentrations (0.5–32 nM) of [3H]-17,20β-DHP (specific activity, 40 Ci/mmol) were added to the assay tubes with (nonspecific) or without (total) 100-fold molar excess of cold 17,20β-DHP. Linear and nonlinear regression analyses for all receptor binding assays and calculations of Kd and binding capacity (Bmax) were conducted using GraphPad Prism for Macintosh (version 6.0d; Graph Pad Software, San Diego, CA). Results are shown on Scatchard plots (9).

Injection of medaka with Vivo-Morpholino antisense oligonucleotides. Vivo-Morpholino antisense oligonucleotide for medaka mPRα or standard control morpholino antisense oligonucleotide (50 μL of 0.2 mM) were injected into the medaka abdominal cavity at 9–10 AM. The day after injection, medaka were sacrificed by severing the neural connection and blood supply to the head. Ovaries were excised from the fish and placed in Iwamatsu Ringer’s solution (4). The percentage of oocytes that had completed maturation as assessed by germinal vesicle breakdown was examined under a binocular microscope (SMZ645; Nikon, Tokyo, Japan). The remaining follicle-enclosed oocytes (five oocytes/treatment) were processed for mPR protein analysis by Western blotting.

Production of polyclonal antibodies. Polyclonal antibodies were generated by a commercial vendor (Eurofins Genomics, Tokyo, Japan) in rabbits against three injections of synthetic 14-mer peptides derived from the C-terminal domain of medaka mPRα (RRPLYERLHGDALAH) conjugated to keyhole limpet hemocyanin (Fig. 1).

SDS-PAGE and Western blot analysis. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel under denaturing conditions using the method of Laemmli (5), and transferred to Immobilon membranes (Millipore, Billerica, MA). Membranes were blocked in 5% non-fat powdered milk in 20 mM Tris buffered saline, pH 7.6 (TBS) containing 0.1% Tween 20 overnight at 4°C, and then incubated with primary antibodies (1,000-fold dilution in TBS buffer) for 1 h at room temperature. Immunocomplexes were visualized using an ECL detection kit (Amersham Biosciences), by a method based on a chemiluminescent reaction mediated by peroxidase conjugated to a secondary antibody. Signals were digitized using a CCD camera system (Luminescent Image Analyzer LAS-4000 mini; FUJIFILM, Japan).

Statistical analysis. Summary data are presented as mean ± S.D. Student’s t-test was used to determine the statistical significance of the obtained data, which were considered significant at P < 0.05(*).

RESULTS

Characterization of cDNA clones for medaka mPRα
We cloned and characterized cDNA for medaka mPRα based on gemone sequence. The cDNA for medaka mPRα encoded a protein of 354 amino acid residues with predicted molecular mass of 41030 daltons (GenBank accession No. AB111911) (Fig. 1). Some structural motifs were identified: an N-glyco-
Specific binding of MIH to plasma membranes prepared from mPRα-transfected cells

In order to examine the binding activity of medaka mPRα, cDNA was stably expressed in a human breast cancer cell line, MDA-MB-231 (22). To obtain stably expressed cells, mPRα-expressing cells were cloned by the aid of fluorescence of DsRed that was included in the same plasmid and simultaneously but independently expressed with inserted cDNA. Expression of mPR mRNAs was verified by RT-PCR of total RNA extracted from cultured cells (Fig. 2A). A polyclonal antibody was raised against a synthetic

\[ N\{P\}S/T\{P\}, \text{and a leucine zipper pattern} \ (L\{6\}L\{6\}L\{6\}L) \text{was conserved in fish mPRs. Computer analyses of deduced amino acid sequences by SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html)(3) predicted that medaka mPRα protein has seven-transmembrane domains, and properties characteristic of G protein-coupled membrane receptors. The predicted overall structure of medaka mPRα with a large 3rd extracellular loop was similar to that of goldfish mPRα and β (17).} \]
peptide of medaka mPRα (residues 306–319; Fig. 1 underline). An immunoreactive band of around 40 kDa was detected by Western blot analysis of cell membranes prepared from cells transfected with cDNA using polyclonal antibodies (Fig. 2B). Insertion and expression of proteins in vectors were also confirmed by observation of fluorescence (Fig. 2C).

Specific progestin binding was measured in plasma membranes prepared from medaka mPRα-transfected cells, whereas significantly lower amounts of 17,20β-DHP binding was detected in empty pRES2 vector-transfected cell membranes (Fig. 3A). Saturation analysis showed that progestin binding to the cell membranes of transfected cells was saturable and of limited capacity (Bmax = 0.02 nM, Fig. 3B). Scatchard analysis showed the presence of a single class of high-affinity binding sites (Kd = 5.42 nM, Fig. 3B) in the cell membranes.

**Steroid and DES binding characteristics of mPRs**

Steroid competition studies showed that binding is highly specific for 17,20β-DHP in membranes prepared from cells transfected with medaka mPRα (Fig. 3C). Progesterone (P4) and testosterone (T) were relatively weak competitors for [3H]-17,20β-DHP binding in medaka mPRα (Table 1). Diethyl-
S. R. Roy et al.

suggest that medaka mPRα is involved in the induction of oocyte maturation in medaka.

**DISCUSSION**

In the present study, cDNA encoding mPRα was cloned from medaka and expressed in cultured cells. Medaka mPRα shares high sequence identity with mPRα of other fish species (77–93%) and relatively low homology (54–56%) with their mammalian counterparts (Fig. 1). Computer analysis predicted that protein structures of the mPRs, exhibiting seven-transmembrane domains, were the same as that of mPRα reported previously (16). An N-glycosylation site (N[P]S/T{P}) was also conserved within this region among mPRs (24). This conserved domain is a potential candidate for the ligand binding domain or other critical function of mPR proteins.

Smith et al. described consensus sequences among the PAQR family of proteins (11). These sequences suggest that medaka mPRα is involved in the induction of oocyte maturation in medaka.

**Inhibition of oocyte maturation by mPRα Vivo-Morpholino antisense oligonucleotides**

In order to investigate the involvement of medaka mPRα in oocyte maturation, we injected Vivo-Morpholino antisense oligonucleotides for medaka mPRα into the abdominal cavity of medaka. In the next day after injection, rate of oocyte maturation was compared between antisense injected group and control group. Also protein levels of mPRα in plasma membrane from antisense injected group and control group were analyzed by western blot. The mean percentage of matured oocytes was significantly lower in the mPRα morpholino antisense group (50%) than in the control group (85%) (Fig. 4A, P < 0.05). The mPRα antisense oligonucleotide caused reductions in mPRα protein levels in the plasma membrane fraction from ovary (Fig. 4B). These results suggest that medaka mPRα is involved in the induction of oocyte maturation in medaka.
Characterization of medaka mPRα

Previously, we have shown that oocyte maturation in zebrafish and goldfish can be induced by an endocrine disrupting chemical, diethylstilbestrol (DES) (19). In addition, we showed that DES binds to recombinant mPRα proteins expressed on the cell membranes of stably-transfected cultured cells, suggesting that DES induces oocyte maturation via interactions with mPRα and β (20, 22). In the present study, we established cell lines expressing medaka mPRα and determined the binding affinity for DES. Our results demonstrated that medaka mPRα possesses a lower affinity for DES, which correlates with the results showing that DES did not induce oocyte maturation in medaka (unpublished results).

It may be explained that the reason why medaka oocytes did not respond to DES was due to low affinity of medaka mPRα for DES. This idea supports that mPRα is a receptor for induction of oocyte maturation.

Direct evidence of a role for medaka mPRα in the induction of oocyte maturation in medaka was obtained from knockdown experiments using Vivo-Morpholino, consistent with previous studies in zebrafish and goldfish (15). Vivo-Morpholinos antisense oligonucleotides are the knockdown, exon-skipping or miRNA blocking reagent of choice for in vivo experiments. A Vivo-Morpholino is comprised of a Morpholino oligo with a unique covalently linked delivery moiety, which is comprised of an arginine rich delivery peptides (the guanidinium group) with improved stability. Efficiently localized delivery into the cell without injection can be achieved by putting the Vivo-Morpholino directly into the area of interest.

We also applied Vivo-Morpholino to analyze the mPRα protein expression and oocyte maturation. Vivo-Morpholino antisense oligonucleotide was injected into the medaka abdominal cavity and subsequent oocyte maturation in vivo was assessed (mean ± SD, n = 5; *, P < 0.05). Proceeding of meiotic cell division (oocyte maturation) was assessed by observation of germinal vesicle (nuclear of oocytes) breakdown (GVBD).

Fig. 4 Effects of medaka mPRα Vivo-Morpholino antisense oligonucleotide injection on mPRα protein expression and oocyte maturation. (A) Vivo-Morpholino antisense oligonucleotide was injected into the medaka abdominal cavity and subsequent oocyte maturation in vivo was assessed (mean ± SD, n = 5; *, P < 0.05). Proceeding of meiotic cell division (oocyte maturation) was assessed by observation of germinal vesicle (nuclear of oocytes) breakdown (GVBD). (B) Expression of medaka mPRα protein detected by Western Blot analysis after the following overnight treatment: control, injected with standard control Vivo-Morpholino; Vivo-Morpholino, antisense oligonucleotide for medaka mPRα.

Table 1 Rank order of binding affinities of steroids and DES to plasma membranes prepared from MDA-MB-231 cells transfected with medaka mPRα

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nM)</th>
<th>RBA</th>
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<tbody>
<tr>
<td>17,20β-DHP</td>
<td>6.4</td>
<td>100.0</td>
</tr>
<tr>
<td>Progesterone</td>
<td>92.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Testosterone</td>
<td>584.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Estradiol-17β</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DES</td>
<td>2626.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Each value represents the mean of 3 separate competitive binding assays. IC50 is the competitor concentration that causes 50% displacement of [3H]17,20β-DHP binding. RBA represents relative binding affinity (%) compared to that of 17,20β-DHP. ND, not determined, displacement less than 50% at 10−7 M.

were conserved in medaka mPRα protein (P{X}GYR{X}E{2}N{3}H). A sequence highly related to the leucine zipper pattern (L{6}L{6}L{6}L) (12–33 residues) exists within the N-terminal extracellular region of medaka mPRα, as in goldfish mPRα. It is possible that these sequences could contribute to the formation of homodimers or heterodimers between mPRα and mPRβ.

The binding affinity of mPRα expressed in cultured cells to 17,20β-DHP was similar to zebrafish and goldfish mPRs (21, 22, 24). These results support that 17,20β-DHP is a MIH in these species. Previously, we have shown that oocyte maturation in zebrafish and goldfish can be induced by an endocrine disrupting chemical, diethylstilbestrol (DES) (19). In addition, we showed that DES binds to recombinant mPRα proteins expressed on the cell membranes of stably-transfected cultured cells, suggesting that DES induces oocyte maturation via interactions with mPRα and β (20, 22). In the present study, we established cell lines expressing medaka mPRα and determined the binding affinity for DES. Our results demonstrated that medaka mPRα possesses a lower affinity for DES, which correlates with the results showing that DES did not induce oocyte maturation in medaka (unpublished results).

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in vivo effects of knockdown on mPRα protein levels. As in previous in vitro knockdown experiments for mPRα and mPRβ in goldfish, it was demonstrated that knockdown with an mPRα Vivo-Morpholino antisense oligonucleotide prevented oocyte maturation in medaka in vivo. By injection of morpholino into the abdominal cavity at timing before the LH surge, expression levels of mPRs protein decreased by the effect of morpholino and accordingly oocyte maturation was prevented. As previously reported, it is suggested that de novo synthesis of mPRα is needed for the development of oocyte maturation competency in medaka.

In this study, we demonstrated that medaka mPRα is involved in induction of oocyte maturation. The result would be a base for phenotype analysis of mutant medaka fishes of mPR genes.

Acknowledgments

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