Cell-surface expression, progesterin binding, and rapid nongenomic signaling of zebrafish membrane progesterin receptors α and β in transfected cells

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Abstract

Recently, a unique family of membrane progesterin receptors (mPR α , mPR β , and mPR γ) was identified, which may be responsible for mediating rapid, nongenomic actions of progestins in a variety of target tissues. In this study, the mPR α and mPR β isoforms from zebrafish were shown to be rapidly and specifically activated by the maturation-inducing steroid (MIS) of this species, 4-pregnen-17,20 β -diol-3-one (17,20 β -DHP). The zebrafish mPR α and a previously uncharacterized mPR β isoform were stably expressed in nuclear progesterone receptor-deficient mammalian breast cancer cells, MDA-MB-231. Expression and surface localization of the receptors were verified by flow cytometry, biotin surface labeling, and Western blotting. Plasma membrane proteins from mPR α - or mPR β -transfected cells showed high affinity (mPR α , K_d 7 nM; mPR β , K_d 12 nM), saturable, displaceable, single-binding sites specific for 17,20 β -DHP, whereas negligible specific 17,20 β -DHP binding was observed in nontransfected cells. Progesterin treatment caused significant activation of mitogen-activated protein kinase (MAPK) within 5 min in cells transfected with

either of the receptors as measured by western blotting and flow cytometry. The rank order of the potencies of several progestins in activating MAPK via mPR α and mPR β was the same (17,20 β -DHP > progesterone > 4-pregnen-17,20 β ,21-triol-3-one). Interestingly, the MIS in zebrafish, 17,20 β -DHP, was also the most potent inhibitor, among the progestins tested, of adenylyl cyclase activity in cells transfected with either of the receptors. This progesterin significantly decreased cAMP levels in both mPR α - and mPR β -transfected cells in a dose-responsive and time-dependent manner. In addition, signaling of the zebrafish mPR α was blocked by pertussis toxin, implying activation of a G_i protein, while sensitivity to pertussis or cholera toxin was not shown with mPR β -mediated signaling, possibly indicating that this receptor activates a different pertussis toxin-insensitive G protein. The results of this study suggest that zebrafish mPR α and mPR β signal similarly upon progesterin binding resulting in rapid activation of MAPK and downregulation of adenylyl cyclase activity.

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Introduction

Steroids have been known to exert nonclassical steroid actions, not involving genomic mechanisms, for over 30 years (Pietras & Szego 1975, Kostellow *et al.* 1980). All major classes of steroids have been shown to exert rapid nongenomic actions (Norman *et al.* 2004). Some specific nongenomic actions of progestins identified thus far include induction of the acrosomal reaction in sperm (Blackmore *et al.* 1990, Sabeur *et al.* 1996, Baldi *et al.* 1998, Luconi *et al.* 2004), an increase in sperm motility (Thomas *et al.* 2005), modulation of gonadotropin-releasing hormone discharge in the brain (Majewska *et al.* 1986, Calogero *et al.* 1998, Sim *et al.* 2001), and induction of oocyte maturation in fish and amphibian species (Kostellow *et al.* 1980, Patiño & Thomas

1990a, Ferrell 1999). Moreover, steroid-binding moieties with the characteristics of progesterin receptors have been detected by radioreceptor assays on plasma membranes prepared from several of these progesterin target tissues (Patiño & Thomas 1990b, Blackmore & Lattanzio 1991, Falkenstein *et al.* 1999). Although these studies have provided strong evidence for the existence of rapid, nongenomic progesterin actions, and specific membrane receptors through which they can act, many details of this nonclassical steroid mechanism remain unclear. For example, the precise molecular structures of the progesterin membrane receptors and their mechanisms of action are unresolved (Losel *et al.* 2003, Norman *et al.* 2004).

Recently, a novel family of membrane progesterin receptors (mPR α , mPR β , and mPR γ) has been identified, which may be responsible for mediating some of these rapid nongenomic

progesterone actions (Zhu *et al.* 2003a, 2003b). The mPRs are well conserved across a broad range of vertebrate species from fish to humans, and have similar structures and progesterone-binding activities (Zhu *et al.* 2003a, 2003b). Recombinant mPR α proteins from seatrout, as well as mPR α , mPR β , and mPR γ proteins from human and mouse have been shown to specifically bind progesterone (Zhu *et al.* 2003a, 2003b). In addition, the mPRs have G protein coupled receptor (GPCR)-like structures, including seven transmembrane domains, N-terminal glycosylation sites, and conserved cysteine residues for disulfide bonding. Despite their similarities to GPCRs, the mPRs have been grouped in a unique receptor class called the progesterone and adiponectin receptor (PAQR) family, which is based on seven transmembrane domains and an uncharacterized UPF0073 motif (Fernandes *et al.* 2005, Tang *et al.* 2005). It is generally accepted that all GPCRs have seven transmembrane domains with the N-terminus on the outside of the cell and C-terminus on the inside of the cell. However, Tang *et al.* (2005) have proposed an extracellular C-terminus topology of the PAQR superfamily based on a computer prediction. As a result of this unique receptor classification and topology prediction, it is difficult to assess whether all members of the receptor family signal and function through typical GPCR pathways.

The spotted seatrout mPR α was the first mPR to be characterized, and evidence was obtained for its involvement in oocyte maturation in this species (Zhu *et al.* 2003a). The seatrout mPR α is localized on the oocyte membrane, its expression in late stage oocytes increases prior to oocyte maturation, and is hormonally upregulated by gonadotropin during oocyte 'priming' (Zhu *et al.* 2003a). Similar findings have been obtained with the goldfish mPR α , including oocyte membrane localization, inhibition of oocyte maturation by antisense microinjections, and a correlation between receptor protein levels and the ability of the maturation-inducing steroid (MIS) to induce oocyte maturation (Tokumoto *et al.* 2006).

The signaling of seatrout mPR α is also consistent with previous reports on the intracellular-signaling pathways activated by progesterone during oocyte maturation in fish (Nagahama 1997, Thomas *et al.* 2002, Pace & Thomas 2005). Activation of recombinant seatrout mPR α in transfected human breast cancer cells (MDA-MB-231) by the known MIS of this species, 4-pregnen-17,20 β 3,21-triol-3-one (20 β -S), causes stimulation of the mitogen-activated protein kinase (MAPK) cascade and inhibition of cAMP production through a pertussis toxin-sensitive, inhibitory G-protein (G_i) pathway (Zhu *et al.* 2003a). These findings support the hypothesis that mPR α acts as an intermediary in MIS induction of oocyte maturation in teleost fish. Recent results in human myometrial cells have shown that both mPR α and mPR β are involved in downregulation of adenylyl cyclase activity through a pertussis toxin-sensitive G_i pathway (Karteris *et al.* 2006). However, only mPR α appears to mediate progesterone phosphorylation of the myosin light chain through

activation of p38MAPK and downregulate expression of the nuclear receptor co-activator, SRC2, in these myometrial cells (Karteris *et al.* 2006). Therefore, the role of mPR β in nongenomic signaling and its physiological functions still remain to be elucidated.

Zebrafish may be an excellent model to compare the functions and signaling mechanisms of different mPRs, since mPR α and mPR β are co-expressed in the ovary and brain (Kazeto *et al.* 2005, Kohli *et al.* 2005). However, it is not known if mPR β can mediate similar signaling as the mPR α in zebrafish and has similar functions. In this study, we examine the ability of various steroids to bind to the previously uncharacterized zebrafish mPR α and mPR β receptors and rapidly activate the MAPK and cAMP pathways in stably transfected, nuclear progesterone receptor-deficient human cell lines. Our findings show that progesterone binds to the mPR α and mPR β specifically, and among the progesterone tested, the MIS in zebrafish causes the greatest activation of MAPK and cAMP pathways through both mPR α and mPR β , suggesting these receptors have similar roles in mediating rapid progesterone nongenomic actions.

Materials and Methods

Plasmid constructs

Zebrafish mPR α and mPR β cDNAs, obtained previously (Zhu *et al.* 2003b), were inserted into mammalian expression vectors containing a CMV promoter and two C-terminal tags, V5 and His. The coding region of the mPR α was amplified by PCR with primers for removal of the stop codon and containing BamHI and EcoRI enzyme-cutting sites for insertion into the mammalian expression vector pcDNA3.1/V5-His A vector (Invitrogen). The primers used to amplify mPR α cDNA were 5'-AATGGATCCTCACCATGG-3' (forward) and 5'-CCACGACATGAATTCCTG-3' (reverse). The mPR β -coding region was amplified with primers for removal of the stop codon and the CACC TOPO recognition site in the pcDNA TOPO vector (Invitrogen). The primers used to amplify mPR β cDNA were 5'-CAC-CATGTCAAGTGGAGT-3' (forward) and 5'-TCA-GTCTTTTTCCTCACCTG-3' (reverse). The PCR products for both receptors were purified by electrophoresis on a 1% agarose gel, extracted with a gel prep kit (Qiagen), ligated with vector, and transformed into chemically competent Top 10 *Escherichia coli* cells following the manufacturer's instructions (Invitrogen). Transfected *E. coli* were spread on ampicillin-coated plates and grown overnight. Resistant colonies were then selected, regrown overnight, and the plasmid was purified by a MiniElute gel extraction kit (Qiagen). Constructs were verified for proper sequence by DNA sequencing using the BigDye terminator mix. The verified plasmid stocks were grown overnight and purified by alkaline lysis and PEG precipitation as described in Current Protocols in Molecular Biology (Ausubel *et al.* 2000).

Cell culture and transfection

Human MDA-MB-231 breast carcinoma cells (American Type Culture Collection, Manassas, VA, USA) were used to express the recombinant mPR α and mPR β . The cells were cultured and maintained at 37 °C with 5% CO₂ in Dulbecco's modified Eagle's medium/F-12 media without phenol red (Sigma) containing 10% fetal bovine serum (Gibco). Media were changed every 2 days and cells were split among three plates when 90% confluent. Purified vector constructs of mPR α and mPR β were then transfected into the cells using Lipofectamine reagent (Invitrogen) following the manufacturer's instructions. Two days after transfection, plasmid-expressing cells were selected using 1000 μ g/ml G418 (Research Products International, Mt Prospect, IL, USA). Resistant colonies were then isolated and propagated with 500 μ g/ml G418 in order to produce stably transfected cell lines.

Western blotting

Expression of mPR α and mPR β in the stably transfected cell lines was confirmed by Western blotting using a previously developed polyclonal antibody developed for the N-terminal of seatrout mPR α (F1N2; [Zhu et al. 2003a](#)), which cross-reacts with both zebrafish mPR α and mPR β cells. The corresponding N-terminal region of zebrafish mPR α (variable residues, VSDVPWVFRESHIITGYR β) shares 84% identity with the peptide antigen (VSDVPWVFRERHILTGYRQ) used for developing seatrout mPR α N-terminal antibodies, whereas the corresponding N-terminal region of zebrafish mPR β (ASEVPSLFREPYILSGYR β) shares 58% identity with the peptide. Samples were collected by scraping the cells at 4 °C into PBS containing 0.1% protease inhibitor cocktail (Sigma). Ovary samples were collected by cervical dislocation followed by immediate removal and transfer of the ovary into PBS. Samples were sonicated with ten short bursts on a sonicator (Sonic Dismembrator, Fisher Scientific, Pittsburgh, PA, USA), the homogenate was centrifuged for 10 min at 20 000 g , and the resulting pellets were resuspended in lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% NP-40 (Calbiochem, San Diego, CA, USA), and 0.1% protease inhibitor cocktail (Sigma), and incubated for 30 min at 4 °C in order to solubilize membrane proteins. Samples were then spun for 10 min at 20 000 g to remove undissolved material. The remaining supernatant was mixed with equal amounts of 2 $\times$ SDS sample buffer (0.125 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol), boiled for 5 min and then cooled on ice.

For western-blot analysis, 20 μ g (MAPK assays) and 50 μ g (mPR expression assays) total protein estimated by the Bradford assay (Bio-Rad), were loaded onto a SDS/12% PAGE gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris, 100 mM NaCl, 0.1% Tween 20 (pH 7.4)) for

1 h. The membrane was further incubated with the mPR antibody (1:5000 dilution) or extracellular signal-related kinase (ERK) and phosphorylated ERK antibodies overnight (Cell Signaling, Beverly, MA, USA), followed by four 5-min TBST washes, incubated for 1 h with horseradish peroxidase conjugated to goat anti-rabbit antibody (1:2000 dilution; Cell Signaling), and finally washed three times for 5 min with TBST. The blots were then developed using Super Signal West Extended Dura Substrate (Pierce, Rockford, IL, USA) and visualized using a Fluor Chem 8900 imaging station (Alpha Innotech, San Leandro, CA, USA).

Cell surface biotinylation and pull-down assay

Surface expression of the receptor was also verified by biotin labeling of surface proteins with EZ-Link Sulfo-NHS-LC-Biotin (Pierce), followed by immunoprecipitation with immobilized streptavidin (Pierce) and western blotting with the seatrout mPR α -specific antibody. Cells were grown for at least 48 h until they were 90% confluent in 25 cm² cell culture-treated flasks and then washed three times with PBS (pH 8). The cells were then incubated with 1 mg/ml sulfo-NHS-LC biotin (Pierce) for 30 min at 4 °C. The reaction was quenched by washing the cells twice with 10 ml ice-cold PBS containing 50 mM Tris-HCl (pH 7.5). Cell samples were removed by the addition of lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1% NP-40 (Calbiochem), and 0.1% protease inhibitor cocktail (Sigma) directly to the plates. The samples were solubilized for 30 min at 4 °C with shaking and then centrifuged for 10 min at 20 000 g to remove undissolved material. The biotinylated proteins were purified by incubating supernatants with 200 μ l immobilized streptavidin-agarose slurry (Pierce) at 4 °C overnight with continuous mixing. Beads were then washed three times with 1 ml 0.1% wash buffer containing 0.1% NP40, 150 mM NaCl, and 20 mM Tris-HCl (pH 7.5), and then biotinylated proteins were eluted by incubating the slurry for 1 h at 55 °C in 100 μ l 2 $\times$ SDS sample buffer. Final samples were spun down, extracted from the resin and then analyzed by western blotting as described previously.

Flow cytometry

Expression of the receptors was further verified by flow cytometry using a fluorescein-5-isothiocyanate (FITC)-conjugated V5 antibody (Invitrogen) or His (Cell Signaling) and mPR primary antibody with a FITC-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA). Approximately 1×10^6 cells were collected by scraping into 2 ml PBS and then spun down at 1350 r.p.m. for all the washing steps. The cells were decanted, permeabilized for 15 min on ice with 90% methanol for staining with V5 and His antibodies, or left on ice for mPR antibody staining. Following two washes in PBS, the harvested cells were washed with blocking solution consisting of 0.5% BSA in PBS. The cells were then incubated with unlabeled

primary antibodies (His or mPR, 1:200 dilution in blocking solution) or FITC-conjugated V5 antibodies for 30 min at 4 °C in the dark. Those cells that reacted with the unlabeled primary antibodies were further incubated with FITC-conjugated anti-rabbit antibodies (1:500 dilution) following two washes with blocking solution. These samples were resuspended in 100 μ l PBS for analysis on the flow cytometer following two washes in blocking solution. Analysis of 10 000 events was performed on a FACScan flow cytometer using CELLQuest software (BD Biosciences, Palo Alto, CA, USA). For expression analysis, a gate (a minimum fluorescence intensity) was set above 95% of the untransfected control cell population. The percentage of recombinant cells expressing fluorescence intensity above the gate was reported after subtracting 5% from the total.

Receptor-binding assays

[1,2,6,7-³H]17 α -Hydroxyprogesterone (85 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA) and enzymatically converted to 4-pregnen-17,20 β -diol-3-one (17,20 β -DHP) as described previously (Scott *et al.* 1982). Plasma membrane fractions of MDA-MB-231 cells were obtained following established procedures with few modifications (Patiño and Thomas 1990b, Zhu *et al.* 2003a). The cells were washed with assay buffer and then sonicated for 15 s, followed by a 1000 *g* centrifugation for 7 min to remove any nuclear and heavy mitochondrial material. The resulting supernatant was centrifuged at 20 000 *g* for 20 min to obtain the plasma membrane fraction. Progestin receptor binding in the membrane fractions was characterized as described previously (Patiño & Thomas 1990b). One set of tubes contained radiolabeled 17,20 β -DHP alone (total binding), another set also contained cold progestin competitor at a 1000-fold greater concentration to measure nonspecific binding. For competition assays, a third set of tubes contained radiolabeled steroids and various concentrations of the steroid competitors ranging from 100 pM–10 μ M (dissolved in 1–5 μ l ethanol (EtOH)), which do not affect ligand binding in the receptor assay. After a 30-min incubation at 4 °C with the membrane fractions, the reaction was stopped by filtration (Whatman GF/B filters, presoaked in assay buffer; Fisher Scientific, Pittsburgh, PA, USA). The filters were washed twice with 25 ml assay buffer and the bound radioactivity was measured by scintillation counting. The displacement of the radiolabeled 17,20 β -DHP binding by the steroid competitors was expressed as a percentage of the maximum specific binding of the 17,20 β -DHP for the mPRs.

Immunofluorescence staining of mPR in zebrafish oocytes

Oocytes were collected from adult zebrafish, separated by size, fixed in Bouin's fixative (70% v/v Picric acid, 23% v/v of 40% aqueous formaldehyde, and 4–7% v/v glacial acetic acid) for 24 h at 4 °C and stored in 100% methanol at –20 °C until processed for immunohistochemistry. Oocytes were then dehydrated for 5 min with 75, 90, 100% EtOH, 100% xylene,

and then mounted in paraffin wax cassettes. Paraffin sections were cut at 6 μ m and placed on Superfrost glass slides (Fisher Scientific). The oocyte sections were then deparaffinized by 5-min washes with 100% xylene twice, 100, 95, 70% EtOH, and twice with PBS.

Blocking was carried out at room temperature for 1 h in PBS containing 2% BSA and 1% goat normal serum. The slides were then incubated overnight at 4 °C with mPR antibody (1:400 dilution) in PBS containing 2% BSA, washed three times with PBS and then incubated for 2 h at room temperature with FITC-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch; 1:500 dilution) in PBS with 2% BSA and 1% goat normal serum. The slides were then washed three times with PBS, treated with 70% EtOH for 5 min, equilibrated with PBS, and then mounted using an antifade reagent (1% 1,4-diazabicyclo[2,2,2]octane; Sigma), in 90% glycerinaldehyde, phosphate buffered) and observed under a fluorescence microscope (Olympus BX-40 fluorescence microscope).

MAPK activation and ligand screening

MAPK activation was measured by both western blotting and flow cytometry using a phospho-specific p44/p42 antibody (1:2000 dilution for western-blots and 1:200 dilution for flow cytometry; Cell Signaling). Cells were split at least 48 h prior to testing to allow mPR protein expression to recover and then serum starved for 72 h to reduce basal MAPK activity. Activation was measured after 5-min exposure to various steroids (Steraloids, Newport, RI, USA) added directly to the media. Steroids were dissolved in EtOH, so an equivalent amount of EtOH was added in the control group. Epidermal growth factor was used as a positive control of activation. Cells were then either immediately lysed in 1 $\times$ SDS sample buffer for western analysis or fixed with 2% paraformaldehyde dissolved in PBS for 10 min for flow cytometry analysis, both using the same phospho-specific p44/p42 antibody. Following western blotting with phospho-specific antibody, the membrane blots were stripped for 30 min at 50 °C in stripping buffer (2% SDS, 62.5 mM Tris (pH 6.8), 100 mM β -mercaptoethanol) and washed five times in TBST. Then, the membrane blots were reblotted with total MAPK antibody in order to confirm equal protein loading. For MAPK flow cytometry analysis, the following modifications were made to the flow cytometry methods described previously. The cells were washed two more times with PBS before scraping from the plate. Then, the cells were permeabilized and incubated with the phospho-specific MAPK primary antibody (1:250), and a FITC-conjugated goat anti-rabbit secondary antibody (1:500 dilution; Jackson ImmunoResearch).

cAMP measurements

The levels of cAMP in transfected cells were measured using a nonradioactive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA). Cells were prepared by splitting at least 48 h prior to testing to allow mPR protein expression to

recover and then serum starved for 18 h to reduce the background levels of steroids in the media. For pertussis toxin pretreatment, cells were incubated overnight with 200 ng/ml pertussis toxin added to the serum-free media. For cholera toxin treatment, cells were incubated with 1 μ g/ml cholera toxin 2 h before treatment. Cells were then stimulated with 1 μ M forskolin for 15 min followed by steroid treatment for 15 min. Samples were then placed on ice, the media removed, 66% EtOH added, and then samples were immediately frozen at -80°C . Samples were thawed and cAMP was measured in aliquots of supernatant according to the manufacturer's instructions. Data were analyzed by a spreadsheet program provided by Cayman Chemical, which calculated cAMP content in picomoles per milliliter. For each sample, the amount of protein was measured by the Bradford assay and then standardized to express the final value as picomoles cAMP per microgram protein.

Statistical analysis

All experiments were repeated at least three times. Western-blots were analyzed for fluorescent intensity by Alpha Innotech analysis software and expressed as the mean-fold change (MFC) in band intensity $\pm$ S.E.M. Data were analyzed using the GraphPad Prism Program (San Diego, CA, USA) to calculate a one-way ANOVA. A Newman-Keuls multiple comparison test was used to determine specific differences between means when determined as significant by ANOVA. A P value <0.05 was considered statistically significant.

Results

Western-blot analysis of zebrafish ovarian membrane proteins using the seatrout mPR α antibody showed a single immunoreactive band at ~ 40 kDa (Fig. 1). The membrane localization of the receptor in zebrafish oocytes was further confirmed by immunohistochemistry using the same mPR α antibody (Fig. 2), in agreement with previous results showing membrane localization of the mPR α in spotted seatrout oocytes.

In order to examine the signaling pathways activated through the zebrafish mPR α and mPR β , the receptors were stably expressed in a human breast cancer cell line not responsive to progestins, MDA-MB-231 cells. The cell line has no nuclear progesterone receptor (Horwitz *et al.* 1978) or rapid progestin response (see supplementary data in the online version of Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol190/issue2/>), therefore, any response induced in the transfected cells can be directly related to the presence of the recombinant mPRs and independent of the nuclear progestin receptor. The membrane expression of mPR α and mPR β was first verified by western blotting of 50 μ g total protein extracted from cell membranes (Fig. 1A). A distinct band at ~ 45 kDa was present in the membrane proteins extracted from mPR α - or mPR β -transfected cell lines, whereas the band was absent in the nontransfected control cells (Fig. 1A). The slightly higher molecular mass (45 kDa) of recombinant mPR α or mPR β

Figure 1 Western-blot analysis of zebrafish ovary and transfected membrane progestin receptor (mPR α and mPR β) cells using antibody against seatrout mPR α . A unique band at 40 kDa was seen for the ovarian membrane sample corresponding to the mPR protein and bands at 45 kDa corresponding to the recombinant mPR α and mPR β receptors from 50 μ g total proteins (A) extracted from zebrafish ovarian membrane or MDA-MB-231 cell membranes respectively, and plasma membrane proteins (B) following biotin-surface labeling and immunoprecipitation of cell-surface proteins.

compared with the native protein expressed in the zebrafish ovary was due to the added tags that had approximately 5 kDa. The cell surface localization of the receptor was further confirmed by biotinylation and a streptavidin pull-down assay. Only one unique 45 kDa band corresponding to the recombinant molecular size of tagged mPR α and mPR β was observed in the streptavidin-immunoprecipitated plasma membrane proteins using the seatrout mPR α antibody (Fig. 1B). These results indicate that the recombinant receptors are localized on the surface of the cell (Fig. 1B), which is consistent with the cell-surface expression of the wild-type mPR proteins in the zebrafish oocytes (Fig. 2). To further verify receptor expression in transfected cells, flow cytometry was used to measure the presence of C-terminal receptor tag impermeabilized cells with fluorescently linked V5 or His antibodies, or the N-terminal of the receptor with the mPR antibody in nonpermeabilized cells (Fig. 3). Repeated measurements with each antibody showed at least 16–24% of the mPR α -transfected cells expressed the receptor and at least 54–64% of the cells transfected with mPR β -expressed mPR β (Fig. 3). Significant staining with the N-terminal mPR antibody in nonpermeabilized cells also implies surface expression of the receptors. Low levels of receptor expression in the stable cell lines may account for less than 100% measured expression by flow cytometry.

Specific progestin binding was measured in plasma membranes prepared from mPR α - and mPR β -transfected cells, whereas significantly lower amounts of progestin binding were detected in nontransfected cell membranes (Fig. 4). Saturation analysis showed progestin binding to the cell membranes of transfected cells is saturable and of limited capacity (mPR α , $B_{\max} = 0.04$ nM; mPR β , $B_{\max} = 0.07$ nM; Fig. 5). Scatchard analysis showed the presence of a single class of high-affinity-binding sites (mPR α , $K_d = 7$ nM; mPR β , $K_d = 12$ nM; Fig. 5) in the cell

Figure 2 Immunofluorescent localization of mPR in zebrafish oocytes. (A) An oocyte reacted with mPR antibody under normal light. (B) Positive fluorescence reaction (GFP) was observed on the ovarian membrane of the same oocyte under GFP fluorescent light. Insert, mPR was observed on the oocyte membrane at a high magnification. (C) An oocyte reacted with secondary antibody only under normal light. (D) No GFP was observed in the same oocyte under GFP fluorescent light. Bar=100 μ m.

Figure 3 Flow cytometry analysis of mPR α and mPR β in stably transfected cells using His or mPR primary antibody with FITC-conjugated secondary antibody or FITC-conjugated V5 antibody. Cells were permeabilized for His and V5 staining of C-terminal receptor tag and nonpermeabilized for staining with the N-terminal mPR antibody. Results, expressed as percentage of mPR α - and mPR β -transfected cells, show greater fluorescent intensity than a gated control population. Horizontal bar in the figure represents a gate set above 95% of the untransfected control population. The number in the figure indicates the percentage of recombinant cells under the gate after 5% were subtracted.

Figure 4 Specific [^3H]4-pregnen-17,20 β -diol-3-one (17,20 β -DHP) binding assessed by a single point radioreceptor assay (total binding tubes: 4 nM [^3H]17,20 β -DHP, nonspecific-binding tubes: + 4 μM 17,20 β -DHP) to the plasma membranes of nontransfected MBA-MD-231 cells (231), zebrafish mPR α -transfected (tr-mPR α) or zebrafish mPR β -transfected (tr-mPR β) cells. * $P < 0.05$ compared with controls. Values are means $\pm$ S.E.M. from three independent assays with different batches of cells.

Figure 5 Saturation analyses and Scatchard plots of specific [^3H]4-pregnen-17,20 β -diol-3-one (17,20 β -DHP) binding to the plasma membranes of mPR α - and mPR β -transfected cells. Representative plots are shown. Nearly identical results were obtained in three independent assays with different batches of cells.

Figure 6 Competition curves of steroid binding expressed as a percentage of maximum specific [^3H]4-pregnen-17,20 β -diol-3-one (17,20 β -DHP) binding to plasma membranes prepared from mPR α - and mPR β -transfected cells ([^3H]17,20 β -DHP: 4 nM). ■, 17,20 β -DHP; ◇, progesterone; ○, 20 β -S; ▼, testosterone; ▲, estradiol-17 β ; ◆, cortisol. Values are means from three independent assays with different batches of cells.

membranes. Steroid competition studies showed that binding is highly specific for 17,20 β -DHP in membranes prepared from both mPR α - and mPR β -transfected cells (Fig. 6). The relative binding affinities of steroids to the recombinant mPR α and mPR β were very similar. Progesterone (P4) and 20 β -S have binding affinities approximately 10% that of 17,20 β -DHP, whereas testosterone and estradiol-17 β only displaced [^3H]17,20 β -DHP binding from the receptors at 100-fold higher concentrations, and cortisol showed no displacement at any of the concentrations tested (Fig. 6).

The degree of MAPK activation in the transfected cell lines through the mPRs in response to various steroids, including progesterone (P4), 17,20 β -DHP, 20 β -S, 4-pregnen-20 β -ol-3-one (20 β -hydroxyprogesterone, 20HP), 4-pregnen-17 α -ol-3-one (17-hydroxyprogesterone, 17HP), cortisol (Cort), 11-desoxycortisol (11DC), testosterone (Test), and estrogen (E2), was measured. Two independent methods – flow cytometry and western blotting – were used to measure the amount of MAPK activation by means of a phospho-specific MAPK antibody. Western blotting showed a significant increase ($P < 0.05$) MAPK activation in mPR-transfected cells after only

5 min of stimulation with 1 μ M P4 along with the known MISs in fish, 17,20 β -DHP, and 20 β -S (Fig. 7). MAPK activation was expressed as an average MFC in western-blot band intensity in active p-ERK over untreated samples. Interestingly, the known zebrafish MIS, 17,20 β -DHP, was the most significant ($P < 0.05$)

steroid activator of MAPK in both mPR α - (3.48 ± 0.26 MFC)- and mPR β - (3.62 ± 0.18 MFC) transfected lines. Although testosterone (mPR α , 2.55 ± 0.34 MFC; mPR β , 2.40 ± 0.31 MFC) also caused lower, but significant ($P < 0.05$) MAPK activation in the transfected cell lines, this effect was also

Figure 7 MAPK pathway activation in mPR α - and mPR β -transfected cells after 5 min stimulation with 1 μ M of various steroids – progesterone (P4), 4-pregnen-17,20 β diol-3-one (17,20 β -DHP), 20 β -S, 4-pregnen-20 β -ol-3-one (20HP), 4-pregnen-17-ol-3,20-dione (17HP), cortisol (Cort), 11-desoxycortisol (11DC), testosterone (Test), and estradiol-17 β (E2). Representative western-blot for active phosphorylated p-ERK and total ERK (top). Mean-fold change (MFC) in active p-ERK over untreated samples, expressed as the mean $\pm$ s.e.m. for at least four separate experiments (bottom). P4 as well as the fish maturation-inducing progestins, 17,20 β -DHP and 20 β -S, show significant MAPK activation over control treatments. 17,20 β -DHP showed the most significant activation of the steroids tested. Epidermal growth factor (EGF) was used as a positive control of MAPK activation and the vehicle for steroid delivery, EtOH, was used as a negative control. Bars (mean $\pm$ s.e.m.) with different letters are significantly different from one another ($P < 0.05$). UN, untreated control.

Figure 8 MAPK activation in mPR α - and mPR β -transfected cells after 5 min stimulation with various steroids at various concentrations (1 nM–1 μ M) – progesterone (P4), 4-pregnen-17,20 β -diol-3-one (17,20 β -DHP), 4-pregnen-17,20 β ,21-triol-3-one (20 β -S), and estradiol-17 β (E2). MFC in active p-ERK over untreated samples, expressed as an average $\pm$ S.E.M. for at least four separate experiments (* P < 0.05). Un, untreated control.

seen in the control cells (1.85 ± 0.23 MFC), implying that the response was not specific for the transfected receptors (see Fig. 1 in Supplementary data). Furthermore, the degree of MAPK activation in response to P4, 17,20 β -DHP, and 20 β -S was dose dependent (Fig. 8). Significant MAPK activation in mPR α -transfected cells was observed after treatment with 17 α ,20 β -DHP or P4 at a concentration of 10 and 100 nM respectively, whereas, tenfold lower concentrations of 17,20 β -DHP (1 nM) or P4 (10 nM) were able to elicit significant MAPK activation in mPR β -transfected cells. Significant activation by 20 β -S was observed in mPR α - and mPR β -transfected cells only at 1 μ M concentrations.

A second method, flow cytometry, was used in order to verify progesterin induction of MAPK activity in the transfected cells. An increase in median fluorescent intensity (MFI; median fluorescent intensity of treated/median fluorescent intensity of untreated) corresponding to the amount of MAPK activation was observed in both mPR α - and mPR β -transfected cells in response to 1 μ M P4 and 17,20 β -DHP activation for 5 min (Fig. 9). Once again, the zebrafish MIS, 17,20 β -DHP showed consistently higher amounts of MAPK induction than the other progestins in both the mPR α and mPR β cell lines.

The effect of various steroids on cAMP concentrations, a measure of adenyl cyclase activity, in the transfected cell lines was also investigated. Cells were first pretreated for

15 min with 1 μ M forskolin, an activator of adenylate cyclase, in order to better examine a significant decrease in cAMP by the steroids of interest. The transfected cells were then treated with 1 μ M of the steroids for 15 min. The zebrafish MIS, 17,20 β -DHP, caused a significant decrease in total cAMP levels in both mPR α - (29% decrease) and mPR β - (27% decrease) transfected cells (Fig. 10). A significant decrease in cAMP levels was also observed after E2 treatments (mPR α , 19%; mPR β , 25%), but this was not specific for the transfected receptor cell lines and was observed in the untransfected control cells (16%) as well (see Fig. 2 in Supplementary data). Furthermore, the 17,20 β -DHP-mediated decrease in cAMP levels was shown to be concentration-dependent (Fig. 11) and time-dependent (Fig. 12) for both receptors. Maximal inhibition occurred with 1 μ M 17,20 β -DHP after 15 min of activation, although a significant response was seen after only 5-min stimulation with as little as 1 nM 17,20 β -DHP. Interestingly, cAMP levels in both cell lines increased again after 30 min, which is possibly due to desensitization, internalization, or down-regulation of the receptors and should be investigated further.

In order to confirm possible G $_i$ protein coupling, cAMP measurements in response to 17,20 β -DHP were repeated after overnight incubation with 200 ng/ml pertussis toxin, a specific G $_i$ inhibitor. Pertussis toxin blocked the 17,20 β -DHP-mediated reduction of cAMP concentrations in mPR α -transfected cells,

Figure 9 MAPK activation in mPR α - and mPR β -transfected cells after 5 min stimulation with 1 μ M progestins (solid line) compared with untreated mPR-transfected cells (solid filled line) and negative control of secondary antibody only (dotted line) as detected by flow cytometry. The mPR α -transfected cells showed a 1.64 ± 0.06 MFI for progesterone stimulation (A) and a 1.79 ± 0.08 MFI for 17,20 β -DHP stimulation (B). The mPR β -transfected cells showed a 1.41 ± 0.04 MFI for progesterone stimulation (C) and a 1.61 ± 0.06 change for 17,20 β -DHP stimulation (D). Activation was measured for 10 000 cells (MFI; median fluorescent intensity of treated/median fluorescent intensity of untreated, mean MFI $\pm$ S.E.M., $n=3$).

Figure 10 cAMP levels in mPR α - and mPR β -transfected cells after 15 min stimulation with 1 μ M various steroids – progesterone (P4), 4-pregnene-17 α ,20 β diol-3-one (17,20 β -DHP), 4-pregnen-17,20 β ,21-triol-3-one (20 β -S), 4-pregnen-20 β -ol-3-one (20HP), 4-pregnen-17-ol-3,20-dione (17HP), cortisol (Cort), 11-desoxycortisol (11DC), testosterone (Test), and estrogen (E2). Samples were pretreated with 1 μ M forskolin (Fskln) for 15 min prior to the addition of steroid (mean $\pm$ S.E.M., $n=6$, * $P<0.05$). Un, untreated control.

implying G $_i$ coupling of the receptor (Fig. 13). However, pertussis toxin caused no inhibition of mPR β -mediated reduction of cAMP levels, implying possible coupling to a different member of the G-protein superfamily (Fields & Casey 1997). In order to examine the possibility that mPR β is a G $_s$ coupled receptor, the experiments were repeated in the presence of 1 μ g/ml cholera toxin, a G $_s$ activation enhancer. Cholera toxin caused no change in cAMP levels after addition of 17,20 β -DHP in both mPR α - and mPR β -expressing cell lines, implying no coupling to a G $_s$ protein (see Fig. 4 in Supplementary data).

Discussion

The research presented in this study lays the groundwork for future research to characterize the distinct roles of zebrafish mPR α and mPR β in mediating the nongenomic functions of

Figure 11 cAMP inhibition by various doses of 17,20 β -DHP for 15 min in mPR α - and mPR β -transfected cells. Samples were pretreated with 1 μ M forskolin (Fskln) for 15 min prior to addition of steroid (mean $\pm$ S.E.M., $n=4$, * $P<0.05$). Un, untreated control.

progestins in this species by identifying the signaling pathways and ligand specificity of zebrafish mPR α and the previously uncharacterized mPR β . In previous research, the seatrout mPR α was shown to rapidly mediate nongenomic signaling through activation of MAPK and inhibition of cAMP by a G $_i$ -coupled pathway (Zhu *et al.* 2003a). In addition, findings with seatrout mPR α , and more recently with the mPR α from goldfish, support the hypothesis that mPR α acts as an intermediary in MIS induction of oocyte maturation in teleost fish (Zhu *et al.* 2003a, Tokumoto *et al.* 2006). The present research in the zebrafish further suggests that mPR α signaling is conserved and is most significantly activated in response to the MIS through a G $_i$ inhibitory protein. Furthermore, the zebrafish mPR β was activated in a manner similar to mPR α in response to the MIS, implying a possible similar role in mediating nongenomic progestin actions.

Cell-surface localization, specific mPR-binding activity, and potential nongenomic-signaling pathways initiated by progestins acting through zebrafish mPR α and mPR β were identified by stably transfecting the nonprogestin responsive and nuclear progestin receptor-deficient MDA-MB-231 cell line with the receptors. Clear evidence was obtained for expression and cell-surface localization of the recombinant receptors by three independent methods, western blotting, flow cytometry, and biotin-surface labeling. Moreover,

Figure 12 cAMP inhibition of mPRα- and mPRβ-transfected cells at various time points after addition 1 μM 17,20β-DHP. Samples were pretreated with 1 μM forskolin (Fskln) for 15 min prior to addition of steroid (mean ± s.e.m., n = 3, *P < 0.05). Un, untreated control.

expression of both zebrafish mPR proteins was associated with marked increases in specific membrane-bound 17,20β-DHP. These binding moieties have characteristics typical of progestin membrane receptors. High affinity, saturable, displaceable, single-binding sites specific for 17,20β-DHP, and other progestins were identified in plasma membrane preparations from both mPRα- and mPRβ-transfected cells. In addition, a comprehensive series of experiments showed rapid activation of signaling pathways via mPRα and mPRβ after progestin treatment, indicating that the two mPR proteins can function as receptors in the transfected cells to transduce progestin signaling. Progesterone, together with the known MISs in fish, 17,20β-DHP and 20β-S, significantly increased MAPK activity in receptor-transfected cells within 5 min. The zebrafish MIS, 17,20β-DHP, caused activation of MAPK for both receptors at lower concentrations (1–10 nM) compared with P4 (10–100 nM) and 20β-S (1 μM). Furthermore, cAMP levels in both receptor-transfected cell lines were significantly decreased in a concentration- and time-dependent manner in response to physiologically relevant concentrations of 17,20β-DHP. Although circulating levels of 17,20β-DHP have not yet

Figure 13 Effects of pertussis toxin on cAMP levels in mPRα- and mPRβ-transfected cells after 15 min stimulation with 1 μM 17,20β-DHP. Samples were pretreated as indicated with 1 μM forskolin (Fskln) for 15 min and 200 ng/ml active pertussis toxin (PTX) or inactive pertussis toxin (inPTX) for 12 h prior to the addition of steroid (mean ± s.e.m., n = 3, *P < 0.05).

been measured in zebrafish due to their small size, MIS concentrations (1–10 nM) that were effective in significantly decreasing cAMP levels and activating MAPK in this experiment are within the range found in the plasma of a variety of fish species during oocyte maturation and ovulation (Kobayashi *et al.* 1987, Truscott *et al.* 1992, King *et al.* 1994). The finding that the MIS, 17,20β-DHP, is the preferred ligand for the zebrafish mPRs is important for establishing their ovarian functions in the presence of a variety of similar progestin derivatives in the ovary. The observation that both the untransfected MDA-MB-231 cells and receptor-transfected cells showed significant increases in MAPK in response to testosterone, and a significant decrease in cAMP levels in response to estrogen, raises the possibility that membrane receptors for these steroids are expressed in this cell line. However, no rapid signaling in response to progestins or corticosteroids was observed (see

Supplementary data) in nontransfected cells. Taken together, the results provide clear evidence that both zebrafish mPR α and mPR β can mediate rapid nongenomic actions of progestins and that this can occur independent of the nuclear progestin receptor since it is not present in this cell model (Horwitz *et al.* 1978).

Previous results showing co-localization of mPR α and mPR β in the brain and reproductive tissues of zebrafish, and inhibition of oocyte maturation in this species by microinjection of antisense oligos to mPR α and/or mPR β microinjection, support the suggestion that the receptors share common functions (Zhu *et al.* 2003a, Thomas *et al.* 2004, Kazeto *et al.* 2005). Zebrafish mPR α and mPR β may have a similar involvement in oocyte maturation to that proposed for the seatrout mPR α and goldfish mPR α as intermediaries in MIS induction of this process. The seatrout mPR α is localized in the oocyte plasma membrane, its hormonal regulation and pattern of expression are consistent with its involvement in oocyte maturation, and the signal transduction pathway initiated through the receptor is the same as that identified during MIS induction of oocyte maturation in seatrout and other fish species (Nagahama 1997, Thomas *et al.* 2002, Zhu *et al.* 2003a, Pace & Thomas 2005). In the zebrafish, mPR α and mPR β have been co-localized primarily in the ovaries, the receptors have been shown to be upregulated prior to oocyte maturation and antisense microinjections for both receptors have been shown to block MIS-induced oocyte maturation (Zhu *et al.* 2003a, Thomas *et al.* 2004, Kazeto *et al.* 2005, Kohli *et al.* 2005). In the present study, the zebrafish MIS shows the greatest activation of MAPK and inhibition of cAMP, signaling that is consistent with previous studies of fish oocyte maturation, through both zebrafish mPR α and mPR β . The mPRs have also been localized primarily on the plasma membrane in zebrafish oocytes, in agreement with the predicted localization of the MIS receptor.

The zebrafish is an excellent model for further examination of the actions of mPR α and mPR β in oocyte maturation and early development, because it spawns daily, responds to a known MIS, develops rapidly, and their embryos survive externally. However, additional studies are required to demonstrate direct roles for each of the receptors in mediating MIS induction of oocyte maturation *in vivo*. Detailed information is also needed on the progestin-mediated signaling pathways involved in induction of oocyte maturation in this species, on the pattern of daily changes in receptor expression and functionality, and possible interactions between mPR α and mPR β . A better understanding of the functions mediated by the mPRs in zebrafish has the potential to significantly advance our understanding of progestin-mediated nongenomic signaling in vertebrate reproductive tissues. For example, mPR α and mPR β have been localized in mammalian ovaries, where they may have similar roles in progestin signaling (Zhu *et al.* 2003b, Cai & Stucco 2005).

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