Purification of the goldfish membrane progestin receptor α (mPRα) expressed in yeast Pichia pastoris

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ABSTRACT

Membrane progestin receptors (mPRs) are key mediators of rapid, nongenomic actions of progestins on plasma membranes. We established a procedure for the expression and purification of recombinant goldfish mPRα using the methylotropic yeast Pichia pastoris. In P. pastoris, the recombinant protein, which carried C-terminal histidine and c-Myc tags, was expressed in an active form as the receptor for maturation-inducing steroids of fish. Expressed proteins were bound reversibly with a high affinity (K_d = 9.4 nM) at a single binding site that could be saturated. After solubilization of mPRα with n-dodecyl-β-D-maltoside (DDM) from yeast membranes, the recombinant protein was purified using three different columns: first it was affinity-purified over nickel-nitrilotriacetic acid (Ni-NTA), then bound to a cellulose resin with free amino groups and finally to a column with affinity for the c-Myc epitope. The identity of the purified protein was verified by MALDI-TOF/MS analysis and its capacity to bind progestin remained. Expression and purification of mPRα protein in its functional form will enable the screening of ligands and the determination of its three dimensional structure.

Progestins are important steroids regulating final maturation at reproductive tissues in male and female vertebrates. They can be divided into natural and synthetic types. Natural type is found in humans and certain animals (32). Natural progestin in human is progesterone. In the medical fields, a variety of synthetic progestins are used. In teleost fishes, two distinct progestins have been identified as naturally occurring maturation inducing steroids (MIS), which control oocyte maturation. 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) was identified in amago salmon (Oncorhynchus rhodurus) and 17a,20β,21-trihydroxy-4-pregnen-3-one (20β-S) was identified in Atlantic croaker (Micropogonias undulatus) and spotted seatrout (Cynoscion nebulosus) (22). The structural differences between human progestin, progesterone, and fish progestins are that fish progestins have multiple hydroxyl groups on the side chain at positions 17, 20 and 21. In fish, MIS is produced by the follicular envelope in response to luteinizing hormone (LH) from the pituitary (15). In salmon, LH stimulates a number of signaling cascades leading to the production of 17α-hydroxyprogesterone (17α-HP) from progesterone and 17α-hydroxyprogrenolone in the theca cells. Then, 17α-HP is converted to 17,20β-DHP by 20β-hydroxysteroid dehydrogenase (20β-HSD) in the granulosa cells (18, 22). The membrane progestin receptor (mPR) mediates the nongenomic actions of progestins on the plasma membrane. It was identified in teleost, a ray-finned fish, at first and subsequently identified in other vertebrates including human (44, 45). Research into the
functions of mPR have revealed that this receptor mediates the action of steroids that play a role in, for example, oocyte maturation in fish (13, 21, 35, 37, 42). Genome wide phylogenetic analyses have shown that mPRs can be classified into a new protein family, the progestin and adipoQ receptor (PAQR) family and they have three subtypes named mPRα, β and γ (corresponding to PAQR7, 8 and 5, respectively) (33, 36). The mRNAs of mPRs in human show differential expression in reproductive tissues such as the ovary, testis, placenta, uterus and nonreproductive tissues such as brain, kidney and intestinal tissues, suggesting they mediate different nongenomic actions of progestins (35). In goldfish, mPRs are also expressed in reproductive tissues, brain, kidney, eye, gill and spleen. The wide tissue distribution of mPRs suggests mPRs mediate the multiple progestin actions in a wide variety of target tissues (37). Recently, based on the analyses of proteins expressed in yeast, mPRδ and ε (PAQR6 and 9) were proposed to form novel mPR subtypes (31). Also human orthologs of them, when expressed in human breast cancer cells, were found to have progestin binding activity (24). Therefore, five mPR subtypes have been described at present.

Rapid nongenomic effects of progestin and mPR expression have been investigated in a wide variety of tissues of fishes and mammals. In addition to mPRs, there are two receptor candidates for mediating progestin signaling from the cell surface: nuclear progestin receptors (nPRs) and progestin receptor membrane components (PGRMCs). Since their tissue-specific expression overlaps, it seems likely that their functions do as well. Thus, physiological roles of receptors mediating progestin actions might be complicated and difficult to unravel (12, 17, 46). For example, in addition to its function as a nuclear transcription factor, nPR modulates cell signaling pathways by activating c-Src and downstream MAPK outside the nucleus (5, 27). In Xenopus oocytes, like mPRβ, nPR was shown to mediate the nongenomic action of progesterin during oocyte maturation, suggesting a potential interaction between mPRs and nPRs (46). Also, transactivation of nPRs by the activation of mPRs was demonstrated in human myometrium (16). Although the details about the signaling pathway through mPRs still remain unclear, several pathways different from those involving nPRs have been suggested (8, 11, 47).

We cloned and characterized four mPR subtypes, mPRα, β, γ-1 and γ-2 from goldfish (37, 40). Microinjection of antisense oligonucleotides targeting mPRα and β blocked the maturation of goldfish oo-
ture, the analysis of intermolecular interactions, and the production of monoclonal antibodies.

MATERIALS AND METHODS

*P. pastoris* strains expressing goldfish mPRα. For expression in *P. pastoris*, the cDNA for goldfish mPRα, isolated from a mammalian expression vector (38), was fused to the pre-pro secretion signal of α-factor from *S. cerevisiae* in the *P. pastoris* expression vector pPICZaC (Invitrogen). The ORF region of the resulting expression cassette (Fig. 1A) was verified by DNA sequencing.

Three *P. pastoris* strains GS115, KM71H and X-33 (Invitrogen), were transformed by electroporation with the mPRα-expression construct as described in the manual of the EasySelect *Pichia* Expression Kit (Invitrogen). Prior to transformation, the plasmid was linearized by PmeI. Electroporation was performed at 1500 V, 25 μF and 800 Ω using a Gene Pulser (Bio-Rad).

Recombinant clones were selected on YPD plates (1% yeast extract, 2% peptone, 2% dextrase, 2% agar) containing 100 μg/mL Zeocin and genomic integration of the mPRα-expression construct was verified by PCR using Ex Taq polymerase (Takara Bio, Siga, Japan) and primers (5’ AOX1; 5’-GACTGGTTCCAATTGACAAAGC and 3’ AOX; 5’-GCRAATGGCATCTGACATCC) that bind to the AOX1 promoter and terminator regions flanking the ORF (Fig. 1A). Several Zeocin-resistant clones were tested for the production of recombinant protein. Clones with the best expression out of ten were kept and stored on MD plates (1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin, 2% dextrase, 1.5% agar) at 4°C.

Expression of goldfish mPRα in *P. pastoris*. In order to achieve optimal expression of goldfish mPPα in *P. pastoris*, we tested the protein production in three different strains, GS115, KM71H and X-33, and varied the conditions of growth. Transformants of X-33 showed the highest level of mPRα expression (data not shown), which could be optimized by growing these cells in buffered media (see Materials and Methods) and induce expression at 20°C for 24 h. For the production of the mPRα receptor, cells were pre-cultured in 30 mL BMGY medium (1% yeast extract, 2% bactopeptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 4 × 10⁻⁵% biotin, 1% glycerol) in 200 mL baffled flasks at 30°C using a shaking incubator (250 rpm) for 1 day. Then, 4 mL of the preculture was used to inoculate 100 mL BMGY medium in a 500 mL baffled flask which was incubated at 30°C for 1 day. For induction of mPRα-expression by methanol, cells were harvested by centrifugation, and taken up in 1 L of BMMY medium (1% yeast extract, 2% bactopeptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base without amino acids, 4 × 10⁻⁵% biotin, 0.5% methanol) to an OD₆₀₀ of 1.0–3.0. The medium was placed in a 2 L baffled flask and incubated at 20°C for one day, after which the cells were pelleted at 3,000 × g for 5 min, frozen with liquid nitrogen and stored at −80°C until use.

Membrane preparation and solubilization of membrane proteins. Frozen cell pellets were thawed and resuspended in ice-cold breaking buffer (50 mM sodium phosphate, 1 mM PMSF, 1 mM EDTA, 5% glycerol, pH 7.4) containing 0.1% digitonin (Sigma Aldrich) with an equal volume of zirconium beads. Cells were broken with six rounds of vigorous shaking for 5 min using a Micro Homogenizing System MS-100 (TOMY Seiko) followed by 5 min incubation on ice. Intact cells and cell debris were separated from the fraction containing the membranes by a low speed centrifugation step (1,000 × g, 4°C, 5 min), after which the pellet was resuspended in ice-cold breaking buffer for another round of cell-breakage. In all, the supernatants of three subsequent rounds of beat-beating were combined and the membrane fraction was recovered by centrifugation at 20,000 × g, 4°C, for 20 min. This fraction was used for the measurement of receptor-activity by a binding assay (see below) or formed the starting material from which membrane proteins were solubilized by resuspending the membrane pellet in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM Imidazole, 1 mM PMSF, 10% Glycerol) to which a detergent was added. As an initial test, eight detergents (MEGA 10, FOS-choline (FOS), n-octyl-β-D-thioglucoside (OTG), Tween-20, Tween-80, n-dodecyl-β-D-maltoside (DDM), 4,4'-Dithiodibutyric acid (DDA), n-octyl-β-D-glucoside (OG)) were, at various final concentrations of 0.01 or 0.1%, incubated with a membrane preparation for 20 min at room temperature, after which the solubilized fractions were separated from the insoluble material by centrifugation (20,000 × g, 4°C, 20 min) and analyzed by Western blot analysis using anti-His-tag antibodies. For the preparation of solubilized proteins that had to be further purified, the most effective detergent, DDM, was added to the membrane samples to a final concentration of 0.1%. After an incubation at 4°C for 30 min insoluble material was removed by centrifug-
concentration (20,000 \times g, 4°C, 20 min) and the supernatant containing the solubilized proteins was frozen with liquid nitrogen and stored at −80°C until further use.

**Radiolabeled ligand binding assays.** For ligand binding assays, frozen pellets of membrane fractions were resuspended in HEAD buffer (25 mM HEPES, 10 mM NaCl, 1 mM Dithiothreitol, 1 mM EDTA, pH 7.6) containing 0.1% digitonin, or, when testing purified recombinant protein, pellets were taken up in HEAD buffer without digitonin. Progestin receptor binding was measured following procedures established previously (38). For saturation analyses and Scatchard plots 17α-Hydroxyprogesterone (Sigma Aldrich) was enzymatically converted to radiolabeled 17,20β-DHP by 3α,20β-hydroxysteroid dehydrogenase (Sigma) as described previously (29). Various concentrations (3–12 nM) of [1\(^{\alpha}\)]H-17,20β-DHP (specific activity, 40 Ci/mmol) were added to the assay tubes with or without 100-fold molar excess cold 17,20β-DHP for the measurement of the number of nonspecific and total interactions, respectively. Binding reactions were done at 4°C for 30 min and stopped by filtration over Whatman GF/B filters, that had been presoaked in wash buffer (25 mM HEPES, 10 mM NaCl, 1 mM EDTA, pH 7.4) containing 2.5% Tween 80 only when analyzing membrane fractions. The filters were washed three times with 5 mL of wash buffer at 4°C and bound radioactivity was measured by scintillation counting. Binding reactions with purified protein samples were done in the presence of BSA (final concentration 10 mg/mL) and with 1.5 nM [1\(^{\alpha}\)]H-17,20β-DHP in the presence or absence of 1 μM 17,20β-DHP. Linear and nonlinear regression analyses for all receptor binding assays and calculations of K_d and binding capacity (Bmax) were done using GraphPad Prism for Macintosh (version 4.0c; www.graphpad.com). The results were shown on Scatchard plots.

**Purification of mPRα.** To purify the recombinant mPR, the solubilised proteins were thawed on ice and loaded onto a 25 mL nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) column (φ2.6 × 20 cm) that had been equilibrated with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 mM PMSF, 0.01% DDM, pH 8.0). The proteins were eluted with a 500 mL gradient of 10–250 mM imidazole in buffer A. The fractions that contained recombinant mPRα as revealed by western analysis (using anti-His-tag antibodies) were collected and concentrated with a Centriprep YM-3 spin column (Millipore), which was also used to replace the buffer to 50 mM Tris-HCl, pH 8.0 containing 0.01% DDM and 1 mM PMSF (buffer B). In the second purification step, the sample was loaded onto a 5 mL Cellfine Amino (JNC, Tokyo, Japan) column (φ1.6 × 10 cm) that had been equilibrated with buffer B. The column was washed with 15 mL buffer B and eluted with a 100 mL gradient of 0–0.5 M NaCl in buffer B. The fractions that contained recombinant mPRα as shown by western analysis using anti-His-tag antibodies were collected and concentrated with a Centriprep YM-3 spin column, and the buffer was replaced with PBS buffer before the sample was purified with anti-c-Myc-tag beads (part of the “c-Myc tagged protein mild purification kit”; MBL, Nagoya, Japan). Recombinant mPRα was eluted from the beads by washing with PBS buffer containing 1 mg/mL c-Myc-epitope peptides. The purified protein was analyzed by SDS-PAGE and visualized by silver staining using the 2D-Silver Stain II kit (Daiichi Pure Chemicals, Tokyo, Japan).

**SDS-PAGE and Western blot analysis.** Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel under denaturing conditions by the method of Laemmli, and transferred to Immobilon membranes (Millipore). Membranes were processed using the SNAP i.d. protein detection system (Millipore) and blocked in 5% non-fat powdered milk in 20 mM Tris-buffered saline, pH 7.6 (TBS) containing 0.1% Tween 20 (TTBS) for 1–2 h at room temperature, and then incubated with primary antibodies (500-fold diluted in TBS buffer), i.e. polyclonal anti-His-tag antibody or anti-Myc-tag antibody (MBL, Nagoya, Japan), and with anti-rabbit antibody conjugated with peroxidase (Invitrogen) as the secondary antibody (2,000-fold diluted in TBS buffer). Proteins were visualized by enhanced chemiluminescence using an ECL detection kit (PerkinElmer). Signals were digitized using a CCD camera system, the Luminescent Image Analyzer LAS-4000 mini (Fujifilm).

**Kex2 digest.** Purified proteins were digested by Kex2 protease. Proteins were incubated in buffer containing 200 mM Bistris, 1 mM CaCl_2, 0.01% TritonX-100, 0.5% dimethyl sulfoxide, pH 7.0 with Kex2 (final concentration 0.1 mg/mL; Abcam, Cambridge, MA) in a total volume of 20 μL at 30°C for 1 h. Digested mPRα protein was detected by anti-His-tag antibodies.
MALDI-TOF/MS analysis. After tryptic digestion of SDS-PAGE gel-slices containing purified recombinant protein (stained with CBBR), peptides were recovered and purified using a ZipTip (Millipore) and eluted in 2 μL of a solution containing 5 mg/mL of CHCA (Bruker Daltonics), 60% acetonitrile, and 0.1% TFA. The samples were loaded onto a 384-well plate, that was double layered with CHCA dissolved in acetone, and air-dried. The peptide mass spectrum was obtained on a MALDI-TOF/MS Autoflex (Bruker Daltonics, Billerica, USA) in positive ion mode. All MALDI-TOF/MS spectra were externally calibrated using a molecular weight standard mixture (Bruker Daltonics). The peptide fingerprint was analyzed with MASCOT software (Matrix Science, London, UK) searching against peptides from ray-finned fishes or fungi in the NCBInr database, using the following parameters: trypsin digest (zero miss cleavage), cysteines modified by carbamidomethylation, and mass tolerance 0.4 Da. The identification was based on probability-based MOWSE scores.

Statistical analysis. All experiments were repeated three times. One-way analysis of variance (ANOVA) was calculated using GraphPad Prism (San Diego, CA). A P value < 0.05 was considered statistically significant.

RESULTS

Expression of recombinant goldfish mPRα
In order to purify goldfish mPRα using the yeast P. pastoris, the cDNA of goldfish mPRα was fused to the pre-pro secretion signal of the α-factor from S. cerevisiae in an expression cassette (Fig. 1A) that was integrated into the host yeast genome by homologous recombination (Materials and Methods). Insertion of the cassette was verified by PCR using primers that amplified both the inserted gene-fusion as well as the endogenous P. pastoris gene, AOX1 (Fig. 1B), whose promoter and terminator regions control transcription of heterologous mPRα gene-fusion (Fig. 1A).

Expression of the fusion-protein, that also carried an epitope for c-Myc and a histidine tag at its C-terminal end, was induced by the presence of 0.5% methanol in the medium. When the synthesis of recombinant protein was analyzed by western blotting using cell membranes prepared from P. pastoris cells carrying the expression cassette, two immunoreactive bands of around 50 kDa were detected using anti-His-tag antibodies (Fig. 1C). The apparent molecular weights were consistent with molecular masses predicted for mPRα with and without the pre-pro α-factor signal peptide, 54 kDa and 44 kDa, respectively. These two bands were major bands, only detected after induction by methanol (data not shown). On the same blot, a 50 kDa protein was detected in the membrane fraction isolated from the P. pastoris host strain that did not have the mPRα expression cassette (Fig. 1C), but this protein did not co-purify with mPRα (see below). Thus, we concluded that these two bands were goldfish mPPα proteins expressed from the integrated cDNA.

Specific [3H]-17,20β-DHP binding to plasma membranes from mPRα-expressing P. pastoris
For the detection of progestin (17,20β-DHP) binding activity of expressed recombinant mPRα protein present in the P. pastoris cell membranes, digitonin was present during preparation of the cell-membranes (see Materials and Methods) as this glycoside is known to facilitate receptor-access for this steroid (26). As has been previously reported for Pi- chia cells that had to be permeabilized (1), we found that a final concentration of 0.1% digitonin was optimal to facilitate steroid binding as measured by a filter-binding assay established previously (38) (Materials and Methods) whereas cholate did not (Fig. 2A). After treatment with 0.1% digitonin, specific 17,20β-DHP binding activity was detected in plasma membranes that were prepared from P. pas toris cells expressing the goldfish mPRα but not in membranes from untransformed host cells (Fig. 2B). Saturation analysis showed that progestin binding to the cell membranes of mPRα-expressing cells was saturable and of a limited capacity (Bmax = 0.024 nM). Scatchard analysis indicated the presence of a single class of high-affinity binding sites (Kd = 9.4 nM) in these cell membranes (Fig. 2C). Therefore, these results suggested that heterologously produced, recombinant goldfish mPRα is active.

Receptor solubilization
In order to obtain pure mPRα that could be used for structural studies and biochemical assays, the protein has to be solubilized and separated from the membrane fractions. We found that out of eight detergents tested, 0.1% n-Dodecyl-β-D-maltoside (DDM) solubilized the largest amount of mPRα (Fig. 3), which was used to prepare the protein fraction. The recombinant receptor could be purified from this fraction by column chromatography.
Purification of solubilized mPRα by column chromatography

Recombinant mPRα was prepared from large-scale cultures grown in buffered medium containing 0.5% methanol from which membrane proteins had been isolated by solubilization with 0.1% DDM as described above and in the Materials and Methods. Recombinant mPRα was purified from the fraction of solubilized membrane proteins by three steps of column chromatography. Proteins were eluted from the columns by linear salt-gradients and the recovered fractions were assessed by immunoblotting using anti-His-tag antibodies. As a first purification step, the DDM-solubilized membrane proteins were passed over a Ni-NTA column, from which two bands that cross-reacted with anti-His-tag antibodies were eluted after inclusion of over 10 mM imidazole in the buffer (Fig. 5A). The bulk of the protein, which also included the endogenous 50 kDa protein that could be detected with anti-His-tag antibodies (Fig. 1C), washed off the column at lower imidazole concentrations (Fig. 5A). As the putative recombinant mPRα protein eluted over multiple fractions, the protein had to be concentrated and the imidazole...
Purification of recombinant mPRα

Record ligand-binding activity of the membrane fractions (Fig. 2). Specific \[^3H\]-17,20β-DHP binding activity was detected for the purified proteins (Fig. 6C). The purified proteins were cleaved by Kex2 protease (Fig. 6D). Immunoblotting with anti-His-tag antibodies showed a decrease in intensity of 54 kDa band while the intensity of 44 kDa band was increased. This result suggested that recombinant mPRα with pre-pro α-factor signal peptide was cleaved at the Kex2 cleavage site (Fig. 1A) and supported that these two bands were recombinant goldfish mPRα proteins.

Characterization of purified recombinant mPRα

Steroid binding activity of the purified proteins was measured by a similar filter-binding assay as used to record ligand-binding activity of the membrane fractions (Fig. 2). Specific \[^3H\]-17,20β-DHP binding activity was detected for the purified proteins (Fig. 6C). The purified proteins were cleaved by Kex2 protease (Fig. 6D). Immunoblotting with anti-His-tag antibodies showed a decrease in intensity of 54 kDa band while the intensity of 44 kDa band was increased. This result suggested that recombinant mPRα with pre-pro α-factor signal peptide was cleaved at the Kex2 cleavage site (Fig. 1A) and supported that these two bands were recombinant goldfish mPRα proteins.

The identity of the two purified proteins with apparent molecular weights that were close to those predicted for mPRα with (54 kDa) or without (44 kDa) the pre-pro α-factor peptide, was established by MALDI-TOF/MS analysis. The 54 kDa protein was
Proteins, heterologously expressed in \textit{P. pastoris}, can either remain intracellular or be targeted for secretion by use of the secretion signal sequence of \( \alpha \)-factor from \textit{S. cerevisiae} (7, 28). We identified two proteins that co-purified with the plasma-membrane that carried the epitopes attached to the C-terminus of recombinant mPR\( \alpha \). The larger form of these proteins turned out to be goldfish mPR\( \alpha \) that still had the \( \alpha \)-factor signal peptide attached, suggesting that during the intracellular transport the removal of this sequence did not occur very efficiently. When we tried to compare the expression levels of goldfish mPR\( \alpha \) when expressed with or without \( \alpha \)-factor sequence fused to its N-terminus, we found that only the version targeted to the cell membrane by means of the pre-pro peptide of \( \alpha \)-factor was detectable, suggesting that the receptor needs a membrane environment for stability. No mPR\( \alpha \) was found in the medium, thus the recombinant receptor was not secreted. Accordingly, steroid confirmed to be goldfish mPR\( \alpha \) containing the signal sequence of \( \alpha \)-factor as peptide mass fingerprint analysis resulted in 6 matched peptides and 18\% of sequence coverage for goldfish mPR\( \alpha \), and a peptide for the N-terminus of pre-pro \( \alpha \)-factor (Fig. 7). Peptide mass analysis of fragments derived from the 44 kDa band revealed that these matched those of the goldfish mPR\( \alpha \) peptides from the 54 kDa band but no fragments matching \( \alpha \)-factor were retrieved. Therefore, we concluded that the 54 kDa band represents the unprocessed form of the \( \alpha \)-factor-mPR\( \alpha \) fusion protein while the 44 kDa band is recombinant mPR\( \alpha \) from which the signal sequence has been cleaved off, probably during intra-cellular transport to the plasma membrane.

**DISCUSSION**

In this study, engineered goldfish mPR\( \alpha \) was expressed in yeast \textit{P. pastoris} and subsequently purified. Proteins, heterologously expressed in \textit{P. pastoris}, can either remain intracellular or be targeted for secretion by use of the secretion signal sequence of \( \alpha \)-factor from \textit{S. cerevisiae} (7, 28). We identified two proteins that co-purified with the plasma-membrane that carried the epitopes attached to the C-terminus of recombinant mPR\( \alpha \). The larger form of these proteins turned out to be goldfish mPR\( \alpha \) that still had the \( \alpha \)-factor signal peptide attached, suggesting that during the intra-cellular transport the removal of this sequence did not occur very efficiently. When we tried to compare the expression levels of goldfish mPR\( \alpha \) when expressed with or without \( \alpha \)-factor sequence fused to its N-terminus, we found that only the version targeted to the cell membrane by means of the pre-pro peptide of \( \alpha \)-factor was detectable, suggesting that the receptor needs a membrane environment for stability. No mPR\( \alpha \) was found in the medium, thus the recombinant receptor was not secreted. Accordingly, steroid
In order to purify recombinant mPRα from cell-membranes, the seven-transmembrane receptor had to be solubilized. For this, DDM was used, as has been described before (4) and found to be the only detergent that was effective (Fig. 3). When the solubilized membrane proteins were fractionated on a Ni-NTA column, the His-tagged recombinant mPRα was separated from the bulk of the proteins, although it eluted in all fractions upon increasing the imidazole concentration above 10 mM. To recover binding activity could be detected in membranes prepared from *P. pastoris* expressing recombinant mPRα but only after treatment with digitonin. For ligand binding assays with bovine membrane fractions, digitonin has been found to enhance the binding of progesterone (26), while progestin binding to a membrane fraction isolated from human sperm could only be detected in the presence of digitonin (2). Digitonin supposedly forms a complex with progesterone-like steroids and is thought to support steroid binding by interaction with the surrounding hydrophobic residues (26).

In order to purify recombinant mPRα from cell-membranes, the seven-transmembrane receptor had to be solubilized. For this, DDM was used, as has been described before (4) and found to be the only detergent that was effective (Fig. 3). When the solubilized membrane proteins were fractionated on a Ni-NTA column, the His-tagged recombinant mPRα was separated from the bulk of the proteins, although it eluted in all fractions upon increasing the imidazole concentration above 10 mM. To recover
the receptor from these fractions, we found a cellulose resin with free amino groups that retained mPRα, but purification was not very specific. Therefore, a third step of affinity chromatography, beads having the ability to bind the protein via its C-terminal c-Myc tag, was required and yielded very pure mPRα (Fig. 6B) which was able to bind progesterin (Fig. 6C). The purified protein was confirmed as goldfish mPRα protein by MALDI-TOF/MS analysis, which also revealed that the signal sequence of α-factor was still attached to the higher-molecular weight form of the isolated receptor. Thus, we succeeded in expressing and purifying a recombinant goldfish mPR which was active for the first time.

The potential cellular roles of progesterin binding to mPRs have been investigated widely, and it was found these interactions triggered major events during reproduction, breast cancer cell physiology and neuroendocrine responses. During the reproductive cycle in mammals, fish and frogs mPRs, like mPRα, mediate the induction of oocyte maturation and sperm motility, which could involve the activation of a G-protein, upon binding of progestins (34, 37, 41, 45). An immunological response linked to reproduction is the up-regulation of the leukemia inhibitory factor by progesterone, which is essential for the implantation and development of the embryo and inhibits several macrophage functions (25). Progesterone, possibly via interaction with mPRα, mPRβ or mPRγ, suppresses the proliferation of human T cells, which might attack the fetus during pregnancy, by regulating changes in the [Ca2+]i and pHi inside the T cells (6). Furthermore, progesterone might modulate the activation of bovine T lymphocytes and their proliferation in the corpus luteum by binding to mPRs (23).

Progesterone interactions with mPRα are associated with changes in breast cancer cells. An increase in the levels of mRNAs coding for mPRs within breast cancer cells has been observed (8), which fits recent findings that show how a cell-signaling cascade in breast cancer cells originates from progesterone binding to mPRα in the cell membrane (47). Other, recent data suggest that, as part of the cellular response to the binding of progesterone to mPRα, apoptosis is inhibited in breast cancer cells (8–10).

In the membranes of neuroendocrine cells, mPRs appear to interact with G proteins and have a role in the release of hormones and maintain oocyte maturation by regulating gonadotropin releasing hormone (GnRH) secretion (24). Progesterone is known to have neuroprotective effects, which possibly is mediated by mPRα. Interestingly, this receptor is only present in neurons in basal condition, while it is induced in glial cells after traumatic brain injury (20).
Since mPRs are potential intermediaries of the various cellular responses to progesterone described above, they are expected to be targets for new drugs that would address fertility problems, suppress cancer or fit in therapies for the treatment of encephalitis and brain injury. The establishment of a procedure by which large amounts of pure, functional mPR proteins can be produced will facilitate the screening of ligands for mPRs. We previously reported that the binding of endocrine disrupting chemicals to mPRs could induce or inhibit the maturation of fish oocytes (39). The recombinant protein purified in this study could be used in a high throughput assay to identify such endocrine disrupting chemicals by their affinity for membrane-receptors.

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