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Steroid and G Protein Binding Characteristics of the Seatrout and Human Progestin Membrane Receptor α Subtypes and Their Evolutionary Origins

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A novel progestin receptor (mPR) with seven-transmembrane domains was recently discovered in spotted seatrout and homologous genes were identified in other vertebrates. We show that cDNAs for the mPR α subtypes from spotted seatrout (st-mPRα) and humans (hu-mPRα) encode progestin receptors that display many functional characteristics of G protein-coupled receptors. Flow cytometry and immunocytochemical staining of whole MDA-MB-231 cells stably transfected with the mPRαs using antibodies directed against their N-terminal regions show the receptors are localized on the plasma membrane and suggest the N-terminal domain is extracellular. Both recombinant st-mPRα and hu-mPRα display high affinity (Kd 4.2–7.8 nM), limited capacity (Bmax 0.03–0.32 nM), and displaceable membrane binding specific for progestins. Progestins activate a pertussis toxin-sensitive inhibitory G protein (G_i) to down-regulate membrane-bound adenylyl cyclase activity in both st-mPRα- and hu-mPRα-transfected cells. Co-immunoprecipitation experiments demonstrate the receptors are directly coupled to the G_i protein. Similar to G protein-coupled receptors, dissociation of the receptor/G protein complex results in a decrease in ligand binding to the mPRαs and mutation of the C-terminal, and third intracellular loop of st-mPRα causes loss of ligand-dependent G protein activation. Phylogenetic analysis indicates the mPRs are members of a progesterone and adipoQ receptor (PAQR) subfamily that is only present in chordates, whereas other PAQRs also occur in invertebrates and plants. Progesterone and adipoQ receptors are related to the hemolysin3 family and have origins in the *Eubacteria*. Thus, mPRs arose from *Eubacteria* independently from members of the GPCR superfamily, which arose from *Archeabacteria*, suggesting convergent evolution of seven-transmembrane hormone receptors coupled to G proteins. (*Endocrinology* 148: 705–718, 2007)

ALTHOUGH THE IMPORTANCE of rapid (*i.e.* nonclassical) steroid actions initiated at the cell surface through binding to steroid membrane receptors has become more widely accepted within the past few years, details of the initial steroid-mediated events, including the identities of the steroid membrane receptors and their mechanisms of action, remain unclear and are surrounded by controversy (1–3). There is clear evidence that a variety of receptor proteins are involved in initiating these nonclassical steroid actions in different cell models, including nuclear steroid receptors or nuclear steroid receptor-like forms (1, 2, 4), receptors for other ligands that also bind steroids (2, 5), and unidentified receptors with different characteristics from those of any known receptors (2, 6). Recently, a novel cDNA was discovered in spotted seatrout ovaries that has several characteristics of the progestin membrane receptor (mPR) mediating progestin induction of oocyte maturation in this species by a nongenomic mechanism (7). The seatrout cDNA (st-mPRα) encodes a 40 kDa protein, which has seven transmembrane domains, and receptor activation alters pertussis toxin-sensitive adenylyl cyclase activity, both of which suggest st-mPRα is a G protein-coupled receptor (GPCR) or GPCR-like protein (7). More than 20 closely related genes have been cloned from other vertebrate species, including three mPR subtypes in humans, named α, β, and γ, which show high levels of expression in human reproductive, brain, and kidney tissues, respectively (8). The identification of a new class of putative steroid receptors, unrelated to nuclear steroid receptors, but instead related to GPCRs, provides a plausible explanation of how steroids can initiate rapid hormonal responses in target cells by activating receptors on the cell surface. There has been broad recognition of the potential significance of these findings (1, 9, 10) and also an extensive research effort to determine the distribution, hormonal regulation, and biological roles of the mPRs in various vertebrate models (11–16). However, critical information is still lacking on several key features of mPRs essential for clearly establishing this proposed alternative model of steroid action and for understanding its likely evolutionary origins.

The st-mPRα protein has been localized to the plasma membrane of seatrout oocytes (7), but progestin binding and activation of signal transduction pathways in the plasma membranes of cells transfected with the st-mPRα and human mPRs remain to be demonstrated. To date, progestin binding has only

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Abbreviations: GPCR, G protein-coupled receptor; HLY3, hemolysin 3; hu-mPRα, human membrane progestin receptor α; MMD, monocyte to macrophage differentiation protein; mPR, membrane progestin receptor; nPR, nuclear progestin receptor; st-mPRα, spotted seatrout membrane progestin receptor α; PAQR, progesterone and adipoQ receptors; RBA, relative binding affinity.

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been shown to the soluble recombinant mPR proteins produced in a bacterial expression system (7, 8). Moreover, the binding characteristics of st-mPRα in the plasma membrane and its physiological role are still uncertain, because the principal teleost progestin hormones that induce oocyte maturation do not show any binding affinity for this soluble recombinant protein (7). Practically no information is currently available for the human counterpart, hu-mPRα, including whether its recombinant protein is expressed in plasma membranes and binds progestins specifically, whether it transduces signals in target cells by activating G proteins, and its orientation in the plasma membrane. Several phylogenetic analyses have grouped the mPRs with adiponectin receptors as members of a progesterone and adipoQ receptor (PAQR) family (17–19), which have an opposite orientation in the plasma membrane to GPCRs with an intracellular N terminal (20), and it has been proposed that all PAQRs, including mPRs, do not activate G proteins (18). In addition, an intracellular location, rather than a plasma membrane one of mammalian mPRαs, has been observed in certain eukaryotic expression systems (15, 16). Although our previous structural and functional analyses suggest st-mPRα may be a GPCR, clear evidence that the receptor activates a G protein is directly coupled to it and has the characteristics of a GPCR is lacking. Finally, the phylogenetic relationship of the mPRs to GPCRs is unknown. Therefore, in the present study, we investigated the localization of the recombinant seatrout and human mPRα proteins, steroid binding, and activation of second messengers in the plasma membranes of st-mPRα and hu-mPRα-transfected human breast cancer cells (MDA-MB-231 cells), which do not express the nuclear progestin receptor (21). Activation of G proteins, their identities, as well as direct receptor/G protein coupling were also examined after progestin treatment. A preliminary investigation of the functional domains of the st-mPRα protein for G protein coupling and their similarity to GPCRs was conducted with st-mPRα mutants with a truncated C-terminal and modified intracellular loop three domains. Finally, phylogenetic analyses of mPRs and other PAQRs were performed to reveal their likely origins. Collectively, the results show that the mPRαs are membrane-bound specific progestin receptors that activate G proteins and function as GPCRs but have a different ancestral origin to members of the GPCR superfamily.

Materials and Methods

Chemicals

The steroids progesterone, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S), 17,20β-dihydroxy-4-pregnen-3-one, 17-hydroxyprogesterone, 20β-hydroxyprogesterone, cortisol, estradiol-17β, testosterone, 11-deoxycorticosterone were purchased from Steraloids (Newport, RI). The synthetic progestin R5020 was purchased from Amersham (Piscataway, NJ). The synthetic antiprogestin RU486 was purchased from Sigma-Aldrich (St. Louis, MO). The other synthetic and natural progestins were obtained from Organon (Oss, The Netherlands). [2,4,6,7-^{3}H]-11-deoxycortisol, activity 50 Ci/mmol was obtained from American Radiolabeled Chemicals (ARC, St. Louis, MO) and [2,4,6,7-^{3}H]-progesterone ([^{3}H-P4]), approximately 102 Ci/mmol, was purchased from Amersham. 20β-hydroxysteroid dehydrogenase and all other chemicals, buffers, and media were purchased from Sigma-Aldrich unless otherwise noted.

Culture of MDA-MB-231 cells stably expressing mPRαs

MDA-MB-231 cells stably transfected with the st-mPRα, obtained as described previously (7), or hu-mPRα (described below) were cultured in DMEM/Ham's F-12 medium supplemented with 10% charcoal-stripped fetal bovine serum (FBS) and 100 μg/ml of gentamicin. Charcoal-stripped FBS was prepared by incubating FBS with 0.5% activated charcoal and 0.05% dextran T-70 for 30 min at 55 C. The charcoal particles then were removed by centrifugation at 4 C for 20 min at 4500 $\times$ *g*. The stripped serum was sterile filtered and stored in aliquots at $-$80 C until use. The transfected cells were selectively maintained with 500 μg/ml geneticin with changes of medium every 1–2 days. The cells reached 80% confluence after 3 days in culture. One day before the experiments, fresh medium without phenol red and supplemented with 5% charcoal-stripped FBS was added to the cell cultures. A 150-mm culture dish with a monolayer culture typically contained approximately 2×10^8 cells and yielded approximately 0.6 mg of cell membrane protein. Cells were subsequently collected with a cell scraper and washed twice before experimentation.

Expression of hu-mPRα and st-mPRα mutants in MDA-MB-231 cells

The procedures described previously for PCR amplification of the mPRα cDNA, its insertion into an expression vector, and transfection in human MDA-MB-231 breast carcinoma cells (American Type Culture Collection, Manassas, VA) (7) were followed with few modifications for stable expression of hu-mPRα and transient transfection of st-mPRα mutants (see supplemental Fig. 1, A and B, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The coding regions of hu-mPRα and st-mPRα mutants were amplified by PCR from full-length cDNA plasmid clones (2 min denaturation at 94 C; 5 PCR cycles with denaturation at 94 C for 1 min, annealing at 50 C for 1 min, and polymerization at 72 C for 2 min followed by 25 cycles under the same conditions except annealing, which was conducted at 55 C) and the PCR products were purified by electrophoresis using a low-melting agarose and a QIAquick Gel Extraction Kit as described previously (8) before ligation into a PBK-CMV expression vector (Stratagene, La Jolla, CA). The correct insertion was confirmed by DNA sequencing. Cells were transfected with hu-mPRα, st-mPRα, st-mPRα mutant cDNAs, or vectors containing reversed mPRα inserts using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's suggestions. Transient transfection experiments with st-mPRα mutants were conducted with cells grown to confluence for 2–3 d in media containing 10% charcoal-stripped FBS, whereas experiments with stably transfected hu-mPRα cells were conducted after continued selection with geneticin (500 μg/ml) for several (8–10) weeks.

Confirmation of correct expression of mPRα mRNAs in transfected cells

RT-PCR was performed periodically during the course of the functional studies as described previously followed by sequencing to confirm continued successful expression of the entire coding regions of st-mPRα and hu-mPRα in stably transfected cells (reverse transcriptase reactions without the addition of reverse transcriptase were used as controls to verify lack of genomic DNA contamination). Two overlapping DNA fragments from RT-PCR of hu-mPRα and st-mPRα obtained from the transfected cell lines [primers: hu-mPRα 1, sense 5′-GCT CCC TGC CCA GGC CCA CA-3′ (before start codon), antisense: 5′-GCC AGC AGA AAG AAG ACC AC-3′; 2, sense: 5′-TCT TTG TGG AGA CCG TGG AC-3′, antisense 5′-TCC CTA CCA GAT GCC ATC CC-3′ (after stop codon); st-mPRα 1, sense: 5′-TAC CGT CTA CAA GTT TGC C-3′ (before start codon), antisense: 5′-GTG AGC AGC AGC CAA AGC AAG-3′; 2, sense 5′-CGC CAT AGA GAA AGA GTG G-3′ antisense, 5′-AGT CAC TGT CAC AAA CTT CAT T-3′ (after stop codon)] were cloned into a pGEM vector with a TA cloning system (Promega, Madison, WI). The plasmids containing the mPRαs were subsequently sequenced with SP6 and T7 primers from both ends. The results confirmed that the transfected mPRαs were correctly transcribed.

Membrane preparation and solubilization

Plasma membrane fractions of transfected MDA-MB-231 cells were obtained following procedures described previously (7, 22) with few modifications. The cell suspension was washed once with assay buffer and then sonicated for 15 sec followed by a 1000 $\times$ *g* centrifugation for

7 min to remove any nuclear and heavy mitochondrial material (nuclear fraction). The resulting supernatant was centrifuged at 20,000 × *g* for 20 min to obtain the plasma membrane fraction. The remaining supernatant was centrifuged at 100,000 × *g* for 60 min to obtain the microsomal and the cytosolic fractions. The plasma membrane was further purified for some studies by centrifuging the membrane pellet one to three times with a sucrose pad (1.2 M sucrose) at 6500 × *g* for 45 min (7).

mPRα binding assays

The general procedures for measuring binding of radioactive steroid ligand to plasma membranes (22, 23) was used to measure binding of [2,4,6,7-^{3}H]-17,20β,21-trihydroxy-4-pregnen-3-one ([^{3}H]-20β-S,41.9 Ci/mmol) to the recombinant st-mPRα and [2,4,6,7-^{3}H]-progesterone ([^{3}H]-P4,102.1 Ci/mmol) to the recombinant hu-mPRα in the presence or absence of steroid competitors. [^{3}H]-20β-S was converted from [^{3}H]-11deoxycortisol as described in (24). One set of tubes contained radiolabeled progestin alone (total binding); another set also contained nonradiolabeled progestin at concentrations 60- to 100-fold greater than the Kd of the receptor (450–750 nM) to measure nonspecific binding (NSB). For competition assays, a third set of tubes contained the radiolabeled progestins and 4–6 different concentrations of the steroid (range 1–10 μM) competitors (dissolved in 1–5 μl ethanol, which does not affect ligand binding in the receptor assays). 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). The filters were washed twice with 25 ml assay buffer and bound radioactivity was measured by scintillation counting. The displacement of the radiolabeled steroid binding by the steroid competitors was expressed as a percentage of the maximum specific binding of the steroid for its receptor. Progestin binding to membranes pretreated with nonradiolabeled GTPγS (25–50 μM) or activated and inactive pertussis toxin (0.5 μg/ml) was performed as described previously (25). The same filtration assay protocol was used to measure specific [^{3}H]-P4 binding to microsomal and nuclear fractions of MDA cells transfected with hu-mPRα (>65% of the proteins in these subcellular fractions are retained on the glass-fiber filters), whereas dextran-coated charcoal was used to separate bound from free [^{3}H]-P4 in a soluble radioreceptor assay for cytosolic fractions as described previously (7).

Western blot analysis and immunocytochemistry

Polyclonal antibodies generated by a commercial vendor (Sigma-Genosys, Woodlands, TX) in rabbits against six injections of synthetic 15-mer peptides derived from the N-terminal domains of st-mPRα (YRQPDQSWRYYFLTL) and hu-mPRα (TVDRAEVPPLFWKPC) and a 11-mer peptide from C-terminal domain of hu-mPRα (RPIYEPLHTHW) conjugated to keyhole limpet hemocyanin were used for immunodetection of the mPRαs (see supplemental Fig. 1, A and B). Plasma membrane fractions were resuspended and solubilized in gel loading buffer containing sodium dodecyl sulfate (SDS) for electrophoresis. Ten micrograms of solubilized plasma membrane proteins were resolved in 12% SDS-polyacrylamide gel electrophoresis gels and transferred to a nitrocellulose membrane for Western blot analysis. The membranes were incubated for 1 h with blocking solution (5% nonfat milk in TBST buffer: 50 mM Tris/100 mM NaCl/0.1% Tween 20, pH 7.4) before incubation with the mPRα antibodies overnight at 4 C. The specificity of the immunoreactions was evaluated by blocking them with the peptide antigens (2–3 ng/μl mPRα antiserum diluted 1:20 in PBS buffer and preincubated at room temperature with the antibodies for 1.5–2 h). The next day, the membranes were washed with TBST buffer and incubated for 1 h at room temperature with horseradish peroxidase conjugated to goat antirabbit antibody (Cell Signaling, Beverly, MA). The blots were washed three times for 15 min with TBST buffer and treated with enhanced chemiluminescence (Pierce) and exposed to x-ray film.

Transfected cells were grown on coverslips for immunocytochemical analysis. The cells were rinsed twice with PBS and fixed with 2% paraformaldehyde and 0.25% glutaraldehyde in PBS for 15 min at 4 C. Cells were permeabilized by adding 0.5% Triton X-100 to the fixative. The cells were then rinsed briefly with PBS, incubated with 13 mM $NaBH_4$ in PBS for 10 min at 4 C to reduce autofluorescence, followed by three 5-min washes in PBS. The cells were subsequently blocked in 2% BSA in PBS for 1.5 h at 4 C followed by three 5-min washes in PBS. The cells were then incubated with the st-mPRα or hu-mPRα primary antibodies (dilution 1:1000) described previously in 2% BSA overnight at 4 C followed by three 5-min washes in PBS. Antisera were preabsorbed with peptide (0.02 mg peptide/1 ml antibody) overnight at 4 C for peptide block controls. The cells were then incubated with AlexaFluor 488 goat antirabbit secondary antibody (Molecular Probes, Carlsbad, CA; dilution 1:2000) followed by three 5-min washes in PBS. The coverslips were wet-mounted to slides using 80% glycerol in PBS and the presence of fluorescent-labeled mPRα proteins in the cells visualized using a Nikon C1 confocal microscope.

Adenylyl cyclase activity in transfected MDA-MB-231 cells

The production of cAMP by isolated plasma membranes over 30 min at 25 C was measured as an estimation of adenylyl cyclase activity in response to treatment with progestins (20–100 nM) in the presence or absence of activated (30 min incubation with 50 mM DTT) or heat-inactivated (15 min incubation at 100 C) pertussis toxin (0.5 μg/ml). cAMP concentrations in the membranes were measured by enzyme immunoassay using a kit following the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA).

[^{35}S]GTPγ-S binding to cell membranes

Activation of G proteins was determined by measuring an increase in specific binding of [^{35}S]GTPγ-S to plasma membranes as described previously (6, 23). Membranes (~10 μg protein) were incubated for 30 min at 25 C in the presence of 20 nM progestins with 10 μM GDP and 0.5 nM [^{35}S]GTPγ-S (~12,000 cpm, 1 Ci/mol; Amersham) in the absence (total binding) or presence of 100 μM GTPγ-S (nonspecific binding). Bound [^{35}S]GTPγ-S was separated from free by filtering the incubation mixture through Whatman GF/B glass fiber filters followed by several washes.

Immunoprecipitation of [^{35}S]GTPγ-S-labeled G protein α-subunits

Immunoprecipitation of the G protein α-subunits was performed as described previously (23, 26). Plasma membranes (~20 μg protein) of the transfected cells were incubated for 30 min at 25 C in 250 μl buffer containing 4 nM [^{35}S]GTPγ-S, 10 μM GDP, and protease inhibitors with 1 μM progestin and stopped by the addition of ice-cold buffer containing 100 μM GDP and 100 μM unlabeled GTPγ-S. The samples were subsequently centrifuged and the pellet resuspended in immunoprecipitation buffer containing Triton X-100, SDS, and protease inhibitors. Specific antisera to the α-subunits of G proteins (G_i, G_o, and G_s, 1:300; Santa Cruz Biotechnology, Santa Cruz, CA) were added to the mixture and incubated at 4 C with gentle shaking for 6 h. Protein A-Sepharose beads were added and after an overnight incubation the immunoprecipitates were collected by centrifugation, washed, boiled in SDS, and the radioactivity in the immunoprecipitated [^{35}S]GTPγ-S-labeled G protein α-subunits counted.

Coimmunoprecipitation of G protein αi-subunit with mPRαs

Transfected cells were treated with 100 nM progestin (20β-S for recombinant st-mPRα and progesterone for hu-mPRα) for 10 min or untreated (controls) followed by two washes with PBS at 4 C. Triethanolamine buffer (50 mM triethanolamine, 25 mM KCl, 5 mM $MgCl_2$, 0.25 M sucrose, 0.1% protease inhibitor cocktail; Sigma-Aldrich; pH 7.5) was added and the cells were frozen at −80 C until analyzed. Plasma membranes were prepared as described previously and resuspended in immunoprecipitation buffer (0.1 mM EDTA, 1% Triton X-100 in Ca^{2+}- and Mg^{2+}-free PBS, pH 7.5) to a final volume of 300 μl (2 mg/ml membrane protein). The membrane suspension was incubated overnight at 4 C with 1:100 of goat anti-Gi and -Go antibody and control goat IgG (Santa Cruz Biotechnology, Inc.). Plasma membranes were then incubated for an additional 2 h at 4 C with 20 μl protein-A agarose beads (Santa Cruz Biotechnology, Inc.) added in the immunoprecipitation buffer. Beads were washed twice with 1 ml immunoprecipitation buffer, and immunoprecipitates were eluted by boiling for 10 min in SDS sample buffer. Samples were run on a 10% Tris-glycine SDS-polyacrylamide gel, and

proteins were transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk in a buffer of 50 mM Tris, 100 mM NaCl, and 0.1% Tween 20 (pH 7.4) for 1 h, and then incubated at 4 C overnight with the st-mPRα or hu-mPRα antibodies (1:2500). The membrane was then washed three times with Tris-buffered saline and then incubated for 1 h at room temperature with horseradish peroxidase conjugated goat antirabbit (hu-mPRα) or mouse (st-mPRα) antibodies (Cell Signaling), and visualized by treatment with enhanced chemiluminescence substrate (SuperSignal kit; Pierce, Rockford, IL).

Flow cytometry

Cells were carefully removed from the culture plates with a scraper and washed several times in PBS followed by low speed centrifugation to remove any cellular debris and damaged cells. Before conducting flow cytometry, the integrity of the cell membranes and their impermeability was confirmed by incubating them with clathrin antibody. Cells were either pretreated with 90% methanol for 15 min on ice and then blocked in the 1% BSA in PBS (permeabilized group) or directly incubated in blocking solution (nonpermeabilized group). N-terminal and C-terminal hu-mPRα antibodies (~1:1000) were added to the cell suspension in blocking solution and incubated at room temperature for 1 h followed by two washes in PBS. Alexa Fluor 488 goat antirabbit IgG antibody (Alexa 488; Molecular Probes) in blocking solution was added to the cell suspension and incubated for 30 min at room temperature in the dark followed by two washes with blocking solution. The cells were resuspended in 500 μl PBS and analyzed within 24 h on a flow cytometer (Becton and Dickinson FACSCallbur). Data were analyzed with CellQuest Pro software (BD Biosciences, San Jose, CA).

Phylogenetic analyses

The majority of the sequences used to construct the phylogeny of the PAQR/hemolysin 3 (HLY3) family was derived from the SMART database (27) corresponding to the Pfam model HLY3 (291 sequences) including all species. The Hidden Markow Model was used to screen for additional PAQR protein sequences in the complete eukaryotic genome sequences using HMMSearch (version 2.3.2c; part of HMMer package by S. Eddy, Janelia Farm Research Campus, Howard Hughes Medical Institute Laboratory). These complete proteomes were obtained from the Ensembl database (www.ensembl.org). The *Ciona intestinalis* protein sequences were obtained from the Joint Genome institute (http://genome.jgi-psf.org/Cioin2/Cioin2.home.html). The total set of protein sequences (397) was then aligned using ClustalW1.83 (28). The alignment was manually curated to remove redundancy (fragments, duplicates, chimaeras). All the remaining sequences (281) were aligned again and used for construction of a phylogeny using the Neighbor-Joining method and subsequently visualized using Treeview 1.6 (29).

Results

Steroid binding characteristics of recombinant seatrout and human mPRαs

Expression of st-mPRα and hu-mPRα mRNAs in MDA-MB-231 cells and proteins in the plasma membranes was confirmed after stable transfection of their cDNAs (Figs. 1A and 2A). Weak endogenous expression of hu-mPRα mRNA was also detected in untransfected MDA-MB-231 cells after 35 cycles of RT-PCR (Fig. 2A). Plasma membranes of st-mPRα-transfected cells showed a 6.5-fold increase in specific 20β-S binding compared with untransfected cells in single-point receptor binding assays ($P < 0.001$, Fig. 1B). Saturation analysis and Scatchard plotting indicated the presence of a high-affinity (Kd 7.58 ± 0.93 nM, n = 3), limited-capacity (Bmax 0.026 nM), saturable, single, specific binding site for 20β-S (Fig. 1C). Dissociation of [^{3}H]-20β-S from the receptor was demonstrated in the presence of excess unlabeled steroid (Fig. 1D). Moreover, the rates of dissociation and association were rapid and reached 50% binding within 5 and 2 min, respectively (Fig. 1D). Competitive binding assays revealed that the major seatrout progestin hormone, 20β-S, and the tetrapod hormone, progesterone, bound with high affinity to the receptor with IC_{50} values of 47.5 nM and 185 nM, respectively (Fig. 1, E and F). Receptor binding was specific for progestins. Testosterone had a 28-fold lower affinity than 20β-S (IC_{50}: 1374 nM), whereas 200-fold higher concentrations of estradiol-17β and cortisol (10 μM) than the IC_{50} of 20β-S did not cause any displacement of [^{3}H]-20β-S from the receptor. Interestingly, the synthetic progestin R5020 and antiprogestin RU486, which have relatively high binding affinities for the mammalian nuclear progesterone receptor, failed to bind to the recombinant seatrout mPRα at concentrations up to 10 μM (Fig. 1F).

The steroid binding characteristics of the recombinant human receptor (hu-mPRα) protein are similar to those of st-mPRα. Transfection of the MDA-MB-231 cells with hu-mPRα resulted in a 2.5-fold increase in specific progesterone binding (Fig. 2B, $P < 0.05$). A high-affinity (Kd 4.17 nM), limited-capacity (Bmax 0.32 nM), single, specific progesterone binding site was detected by saturation analysis and Scatchard plots (Fig. 2C). Progesterone ([^{3}H]-P4) was readily displaced from the receptor, and ligand association and dissociation were rapid, reaching 50% binding in approximately 5 min, which is typical of steroid membrane receptors (Fig. 2D). Binding to the hu-mPRα protein was specific primarily for progestins; several androgens displayed moderate affinity for the receptor, whereas no binding of estradiol-17β and cortisol to the receptor was detected at concentrations up to 1 μM (Fig. 2E). Progesterone showed the highest affinity for the receptor among the more than 30 steroidal compounds tested with an IC_{50} of 87.3 nM. The relative binding affinities (RBA) of the progestins, norprogesterone and pregna-4,9(11)-diene-3,20-dione, were 51.8 and 50.9% that of progesterone. The RBAs of the remaining progestins and 11-deoxycorticosteroids tested were less than 50% (Table 1). The two teleost progestin hormones, 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) and 17,20β-dihydroxy-4-pregnen-3-one, had low affinities with RBAs less than 1%. Similar to the results with st-mPRα, RU486 and R5020 were poor competitors for hu-mPRα. R5020 had low binding affinity for the receptor with an IC_{50} of 2 μM and a RBA of 4.1%, whereas RU486 caused less than 50% displacement of P4 from the receptor at the highest concentration tested, 10 μM (Fig. 2F). Interestingly, testosterone also bound to hu-mPRα (IC_{50} 390 nM) with an RBA 22.4% that of progesterone, somewhat higher than its affinity for the st-mPRα. Several other androgens, dihydrotestosterone, and a methyl ether testosterone derivative were also moderately effective competitors of progesterone binding (Table 1).

Activation of G proteins and second messengers

Treatment of plasma membranes of st-mPRα-transfected cells, but not untransfected cells, with 100 nM 20β-S caused a decrease in adenylyl cyclase activity (Fig. 3A). The progestin-induced decrease in adenylyl cyclase activity was blocked by prior treatment with pertussis toxin, a specific inhibitor of activation of inhibitory G proteins, $G_{i/o}$ (Fig. 3A). Progesterone (20 nM) caused a similar decrease in adenylyl

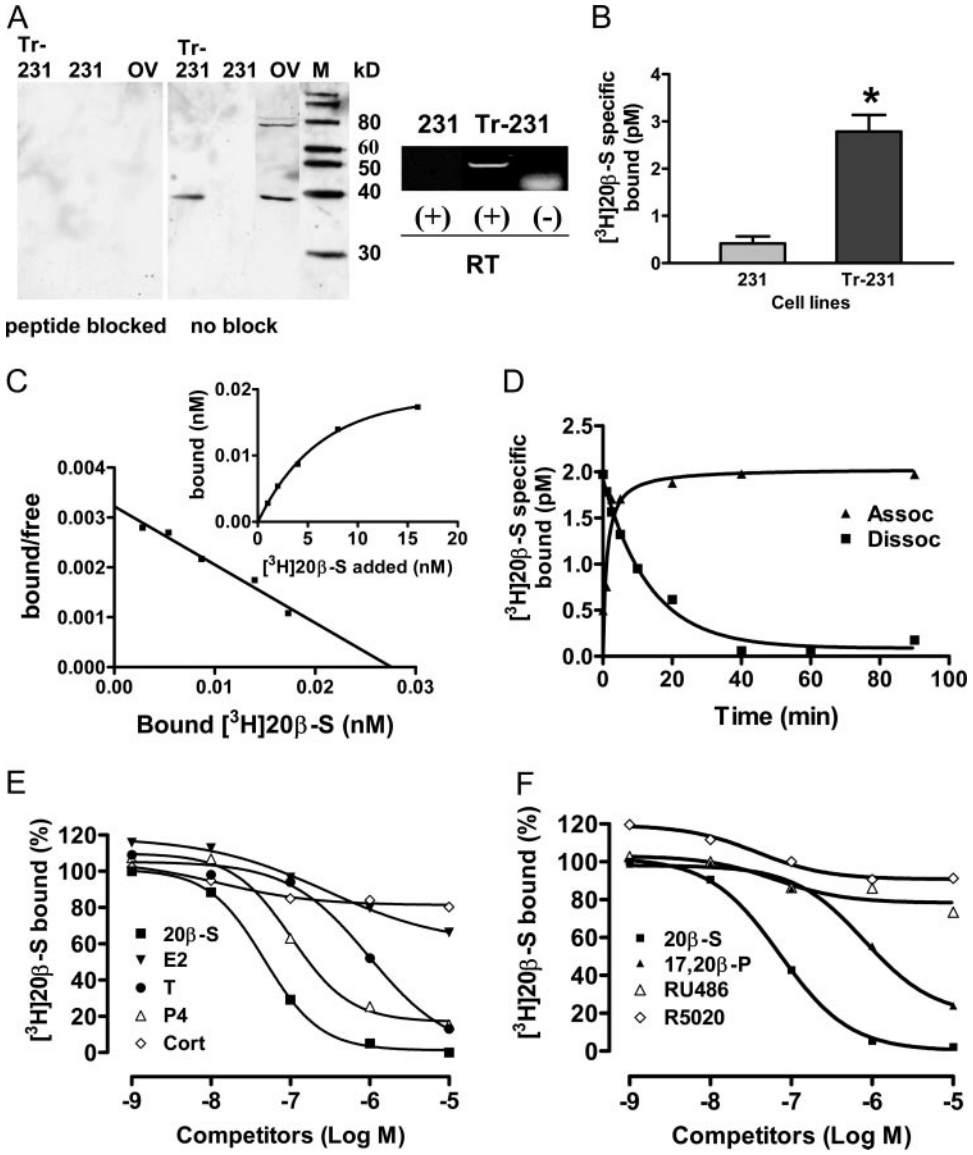

FIG. 1. Progestin binding to plasma membranes of MDA-MB-231 cells stably transfected with st-mPRα. A, Detection of st-mPRα protein in transfected (Tr-231) cell membranes by Western blot analysis and st-mPRα mRNA in cells by RT-PCR [(+)RT]. 231, Untransfected cells; OV, seatrout ovarian membranes; M, molecular weight protein standards; (-)RT, lacking reverse transcriptase; peptide blocked, blocked with peptide antigen. B, Specific [^{3}H]-20β-S binding to transfected cell membranes in a single point assay (see key in A) (n = 6,*, $P < 0.05$, Student's t test). C, Representative saturation curve and Scatchard plot of specific [^{3}H]-20β-S binding to plasma membranes of transfected cells. D, Time course of association (Assoc) and dissociation (Dissoc) of [^{3}H]-20β-S binding. E and F, Competition curves for steroid (E) and progestin (F) binding expressed as a percentage of maximum specific 20β-S binding. E2, Estradiol-17β; T, testosterone; P4, progesterone; cort, cortisol; 17,20β-P, 17,20β-dihydroxy-4-pregnen-3-one; RU486, mifepristone; R5020, promegestone.

cyclase activity in membranes of cells transfected with hu-mPRα, whereas cortisol, which showed no binding affinity for hu-mPRα, was ineffective (Fig. 3B). These progestin-induced decreases in adenylyl cyclase activities in the st-mPRα- and hu-mPRα-transfected cells were dose-dependent (see supplemental Fig. 1, C and D). Possible activation of G proteins was investigated using a radiolabeled nonhydrolyzable form of GTP, [^{35}S]GTPγ-S. Treatment of st-mPRα- and hu-mPRα-transfected cell membranes, but not untransfected ones, with progestins (st-mPRα: 20 nM 20β-S; hu-mPRα: 20 nM progesterone) in the presence of [^{35}S]GTPγ-S caused significant increases in specific GTPγ-S binding to the cell membranes, indicating ligand-dependent activation of G proteins (Fig. 3, C and D). G protein activation of hu-mPRα was specific for progestin ligands, because cortisol and R5020, which do not bind to the receptor, were ineffective (Fig. 3D), and was dose-dependent (see supplemental Fig. 1D). The identities of the G protein(s) activated on progestin binding to st-mPRα and hu-mPRα were determined by immunoprecipitation of the activated G protein α-subunits bound to [^{35}S]GTPγ-S with specific G protein α-subunit antibodies. A polyclonal rabbit antibody directed against the α-subunit of inhibitory G proteins (G_i) precipitated approximately 90% of the total radioactive GTPγ-S activated through st-mPRα (Fig. 3E) and approximately 75% of the total GTPγ-S activated via hu-mPRα (Fig. 3G), whereas little

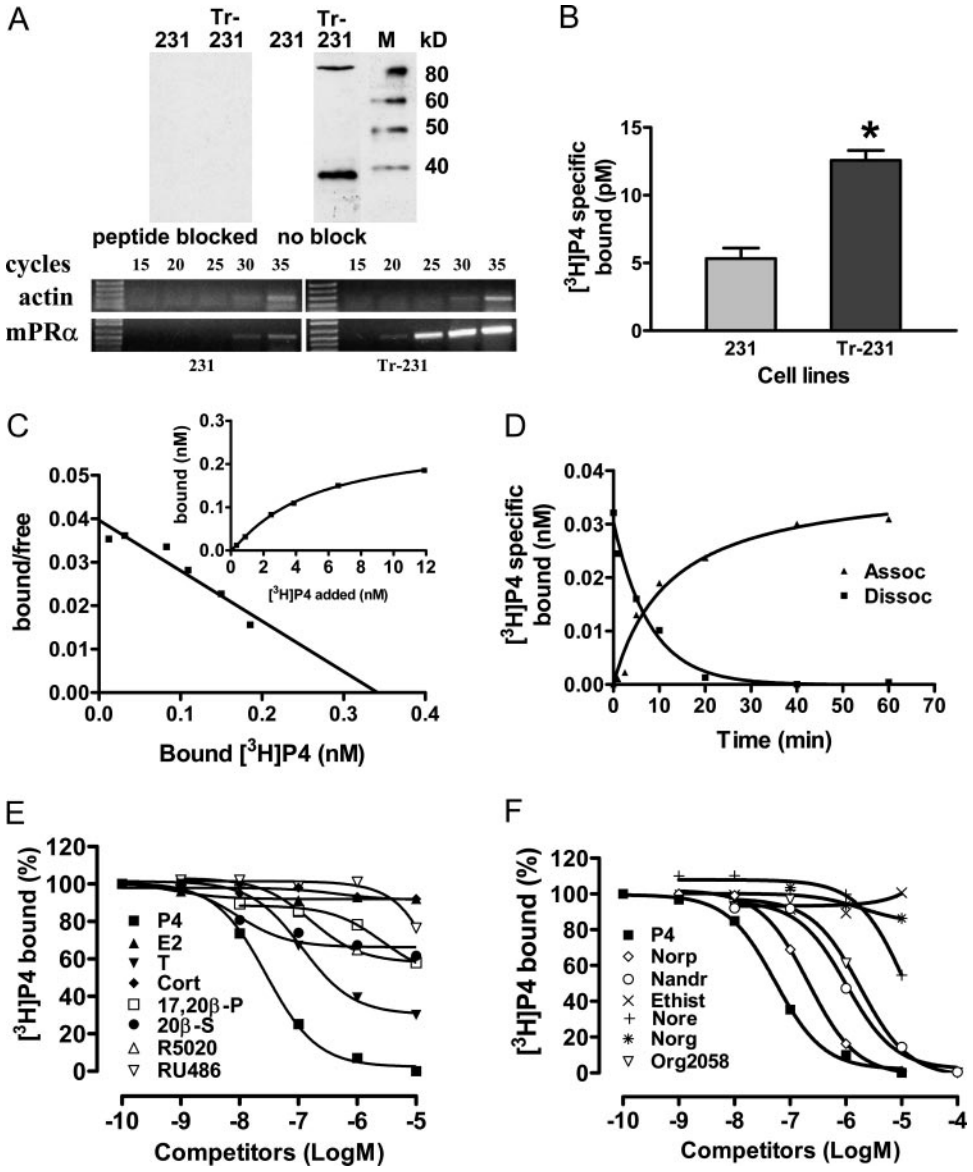

FIG. 2. Progestin binding to plasma membranes of MDA-MB-231 cells stably transfected with hu-mPRα (see Fig. 1 for experimental details and key). A, Detection of hu-mPRα protein and mRNA. B, Specific [^{3}H]-progesterone (P4) binding in a single point assay. C, Representative saturation curve and Scatchard plot of specific [^{3}H]-P4 binding (n = 5). D, Time course of association and dissociation of [^{3}H]-P4 binding. E and F, Competition curves for steroid (E) and progestin (F) binding expressed as a percentage of maximum specific P4 binding. Norp, Norprogesterone; Nandr, nandrolone; Ethist, ethisterone; Nore, norethisterone; Norg, norgestrel.

radioactivity was precipitated with a specific α G_s antibody and control rabbit serum (Fig. 3, E and F).

G protein coupling to seatrout and human mPRαs

Coimmunoprecipitation studies, in which the receptor/G protein complex was first precipitated with antibodies to the G protein α G_i- and G_o-subunits, and subsequently probed with the mPRα antibodies by Western blot analysis, showed direct coupling of st-mPRα (Fig. 4A) and hu-mPRα (Fig. 4B) to an inhibitory (G_i) G protein. No coupling of the mPRαs to the α G_o-subunit could be detected. As predicted, prior treatment with progestins resulted in decreased amounts of the mPRα proteins detected on the Western blots (Fig. 4, A and B). Uncoupling of the G proteins caused decreases in the binding affinities of the mPRαs for their progestin ligands (Fig. 4, C–F). Pretreatment of the plasma membranes with active pertussis toxin (0.5 μg/ml), but not with the inactive form, caused a marked decrease in specific progestin binding to both st-mPRα-transfected (Fig. 4C) and hu-mPRα-transfected cells (Fig. 4D). Similarly, prior incubation of transfected plasma membranes with 25 μM and 50 μM GTPγ-S caused a decrease in specific progestin binding, whereas this treatment did not affect the minor amounts of steroid binding to untransfected cell membranes (Fig. 4, E and F).

Localization, orientation, and mutational analysis

Confocal microscopy of nonpermeabilized mPRα-transfected MDA-MB-231 cells after immunocytochemical stain-

TABLE 1. Rank order of binding affinities of natural and synthetic steroids to plasma membranes prepared from MDA-231 cells transfected with hu-mPRα

Compounds	IC_{50} (Mean)	RBA
Progesterone	87.4	100.0
Norprogesterone	168.6	51.8
Pregna-4,9(11)-diene-3,20-dione	171.7	50.9
5α-Dihydroprogesterone	218.4	40.0
Org7329–0[a]	348.4	25.1
Testosterone	390.5	22.4
11-Desoxycorticosterone	444.1	19.7
11-Methyleneprogesterone	488.8	17.9
5α-Dihydrotestosterone (DHT)	548.3	15.9
Methyl ether testosterone	565.5	15.4
11β-Hydroxyprogesterone	1108.4	7.9
4-Pregnen-21-ol-3,20-dione	1161.2	7.5
Nandrolone	1176.4	7.4
20β-Hydroxyprogesterone	1279.0	6.8
20α-Hydroxyprogesterone	1316.0	6.6
Org2058 (progestin)	1665.0	5.2
11β-Methoxyprogesterone	1756.1	5.0
4-pregnen-16α-methyl-3,20-dione	1882.0	4.6
Promegestone (R5020)	2120.0	4.1
Pregnenolone	2363.2	3.7
4-pregnen-2α-hydroxy-3,20-dione	2366.8	3.7
Demegestone (RU2453)	2809.2	3.1
17α-Hydroxyprogesterone		<1%
Androstenediol		<1%
17α-Methyltestosterone		<1%
Norethisterone		<1%
Drospirenone		<1%
17,20β,21-trihydroxy-4-pregnen-3-one		<1%
17,20β-dihydroxy-4-pregnen-3-one		<1%
Mifepristone (RU486)		NB
Ethisterone		NB
Lynestrenol		NB
MAS[b]		NB
Norgestrel		NB

Each value is the mean of three separate competitive binding assays. IC_{50} is the competitor concentration that causes 50% displacement of [^{3}H]progesterone. RBA, RBA (%) compared with that of progesterone; NB, no binding at 10^{-5} M.

[a] 4-Androstene-3-one 17β-carboxylic acid methyl ester.

[b] 4,4-Dimethyl-5α-cholest-8,14,24,-triene-3α-ol.

ing with antibodies directed against the mPRα N-terminal domains showed both st-mPRα and hu-mPRα are expressed in the cell membrane and suggest the N terminal is extracellular (Fig. 5, Aa and Ba). The specificity was verified by demonstrating that the immunocytochemical reactions were blocked after preincubating the antibodies with their peptide antigens (Fig. 5, Ab and Bb). Neither of the antibodies showed significant immunoreactivity with untransfected cells (Fig. 5, Ac and Bc). No immunoreactivity was also observed on nonpermeabilized hu-mPRα-transfected cells with the antibody directed against the C-terminal domain of hu-mPRα (Fig. 5Bd). The efficacy of the permeabilization and nonpermeabilization procedures was confirmed using an antibody to clathrin, which is present intracellularly (data not shown). The orientation of hu-mPRα in the cell membrane, with the N terminal extracellularly, was independently verified by flow cytometry of transfected cells, which had not been treated with fixatives using the N-terminal and C-terminal hu-mPRα antibodies. Incubation with clathrin antibody confirmed that the harvesting and washing procedure did not damage the cell membranes of the transfected cells making them permeable to the antibody (see supplemental Fig. 1, G and H). Incubation of nonpermeabilized transfected cells, but not untransfected ones, with the N-terminal antibody resulted in a marked increase in immunoflorescence (Fig. 5C), whereas no increase in fluorescence was observed after incubation of nonpermeabilized transfected cells with the C-terminal antibody (Fig. 5D). Specific [^{3}H]-P4 binding was localized by radioreceptor assay in the plasma membrane fractions of cells transfected with hu-mPRα (Fig. 5E). Negligible [^{3}H]-P4 binding was detected in the microsomal and nuclear fractions in the filtration assay or cytoplasmic fractions in the soluble radioreceptor assay. Western blotting of the subcellular fractions with an integrin β3 antibody confirmed lack of cell membrane contamination in these other subcellular fractions (see supplemental Fig. 1D). Similarly, lack of significant contamination of plasma membrane, nuclear, and cytoplasmic fractions with microsomal proteins was confirmed with a spectrophometric reduced NAD phosphate enzyme assay (results not shown). Several potential functional domains of st-mPRα involved in G protein coupling were investigated by mutational analysis (see supplemental Fig. 1, A and B). Truncation of the C-terminal (amino acids RQRVRASLHEKGE deletion, amino acid no. 340–352) resulted in a significant decrease in G protein activation in response to progestin treatment, whereas C-terminal truncation combined with substitution of three amino acids in the third intracellular loop (IL3, KCD changed to VAV, amino acid no. 273–275) completely blocked activation of G proteins after progestin treatment, suggesting that both these intracellular domains near the C-terminal end of st-mPRα are important for G protein coupling (Fig. 5F). Uncoupling of G proteins to the C-terminal st-mPRα mutant was also accompanied with a predicted decrease in ligand binding affinity for the receptor (Fig. 5G).

Phylogenetic analysis

The construction of an unrooted phylogenetic tree (see supplemental Fig. 2) clearly shows the clustering of the sequences into two major groups. The largest group can be further subdivided into two subgroups, one of which represents the large group of bacterial HLY3 proteins. The monocyte to macrophage differentiation protein (MMD) 2 and MMD members of the different species tightly cluster together with the HLY3 proteins. A simpler representation of this unrooted tree excluding the prokaryotes is shown in Fig. 6. Here the major eukaryotic species that were used in the phylogenetic analysis are schematically depicted in a phylogram. The table to the *right* of the phylogram shows the presence of the individual members of the PAQR family identified in humans, adiponectin receptors 1 and 2 (ADR1 and 2), MMD and MMD2, mPRα, β and γ and PAQR3, 4, 6, and 9). Each *dot* represents a gene encoding a protein. *Double dots* indicate gene duplications, resulting in two genes with high similarity. The table summarizes the findings from the phylogenetic tree in the supplement and shows the unique representation of mPRα, mPRβ, mPRγ (PAQR 7, 8, and 5) and PAQR6 in the Chordata. The dense clustering of PAQR6, mPRα, mPRβ, and mPRγ in the dendrogram in supplemental

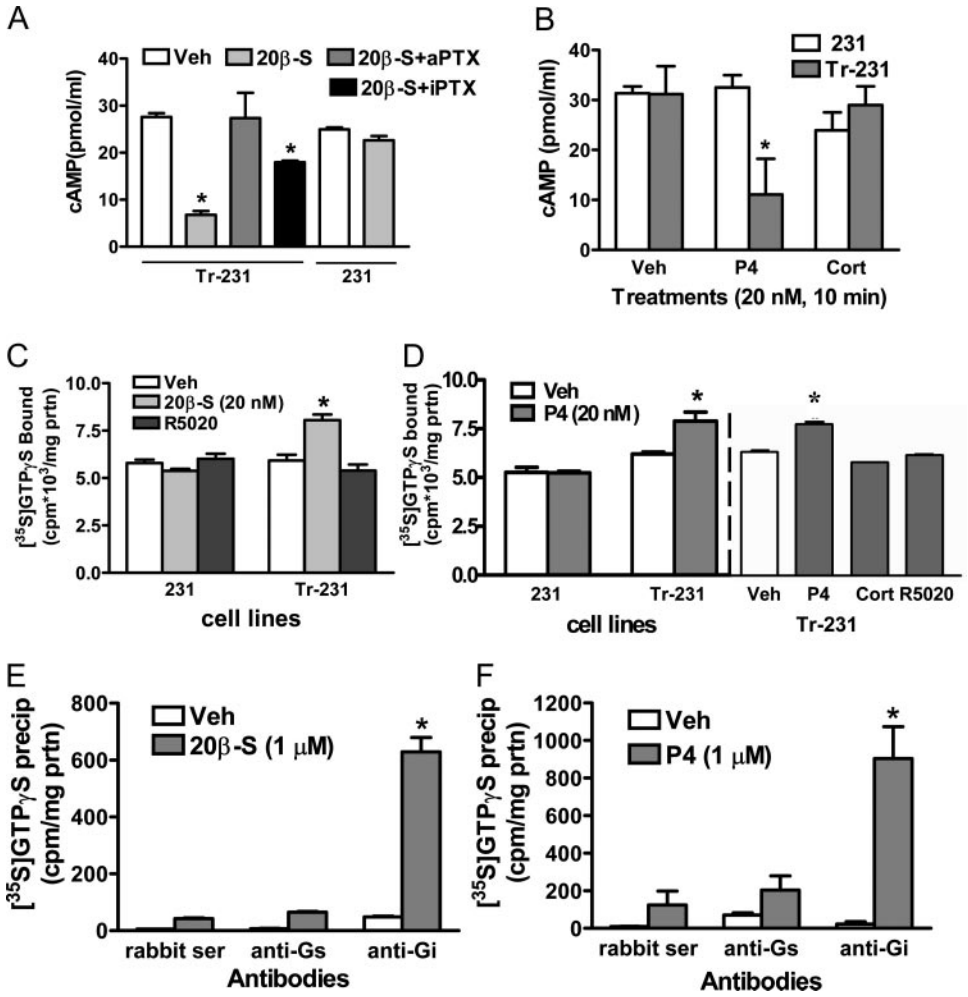

FIG. 3. Activation of G proteins and second messengers in membranes of cells transfected with st-mPRα and hu-mPRα. A, Effects of 15-min treatment with 100 nM 20β-S on cAMP production by membranes of st-mPRα-transfected cells (Tr-231) with or without 30-min pretreatment with activated (aPTX) or heat-inactivated pertussis toxin (iPTX, 0.5 μg/ml). 231-untransfected cells. *, $P < 0.05$, n = 4. B, Effects of 10-min treatment with 20 nM progesterone (P4), 20 nM cortisol (Cort), or vehicle (Veh) on cAMP production by membranes from cells transfected with hu-mPRα. C, Effects of treatment with 20 nM 20β-S or 20 nM R5020 on specific [^{35}S]GTPγ-S binding to membranes of cells transfected with st-mPRα. *, $P < 0.05$ compared with vehicle treatment (Veh), n = 4. D, Effects of treatment with 20 nM progesterone, 20 nM cortisol (Cort), or 20 nM R5020 on specific [^{35}S]GTPγ-S binding to membranes of cells transfected with hu-mPRα. *, $P < 0.05$ compared with vehicle treatment (Veh). E, Immunoprecipitation of [^{35}S]GTPγ-S bound to G protein α-subunits activated on 1 μM 20β-S treatment of st-mPRα-transfected cell membranes with specific Gα_s (anti-G$_s$) and Gα_i (anti-G$_i$) G protein antibodies or control rabbit serum (rabbit ser). *, $P < 0.05$ (n = 4) compared with vehicle control. F, Immunoprecipitation of [^{35}S]GTPγ-S bound to G protein α-subunits activated upon 1 μM progesterone treatment of hu-mPRα-transfected cell membranes with specific antibodies described in E. *, $P < 0.05$ (n = 4) compared with vehicle control.

Fig. 3 further indicates the close structural relationship among members of this subfamily. In contrast, the adiponectin receptors (ADR1 and ADR2, PAQR 1 and 2) and MMD and MMD2 (PAQR11) together with PAQR 3 are found throughout the eukaryotes (Fig. 6).

Figure 7 schematically depicts the proposed parallel convergent evolution of GPCRs and PAQR/HLY3 related proteins. Originating from *Archaebacteria*, bacteriorhodopsin evolved into the rhodopsin like GPCRs in eukaryotes, which in turn gave rise to the other GPCR classes, the Glutamate, Adhesion, Frizzled, and Secretin families as classified by Fredriksson *et al.* (30). In contrast, HLY3 family members are found exclusively in the *Eubacteria*, none have been identified in the *Archaebacteria*. Proteins of various species in the PAQR 10 and 11 members of the PAQR family tightly cluster together with the HLY3 proteins. The sequence comparisons show the PAQR family in eukaryotes shares many structural features with the prokaryotic HLY3 family, suggesting a common bacterial origin. Thus, the PAQR family appears to have arisen from the *Eubacteria* in contrast to members of the GPCR superfamily, which arose from the *Archaebacteria*.

Discussion

The progestin binding results demonstrate that mPRα cDNAs from two distantly related vertebrate species, spotted seatrout and humans, encode membrane-bound progestin binding moieties with all the characteristics of functional steroid membrane receptors. Transfection of MDA-MB-231 cells with these cDNAs resulted in expression of the st-mPRα and hu-mPRα recombinant proteins in the cell membranes and severalfold increases in specific binding of the fish and

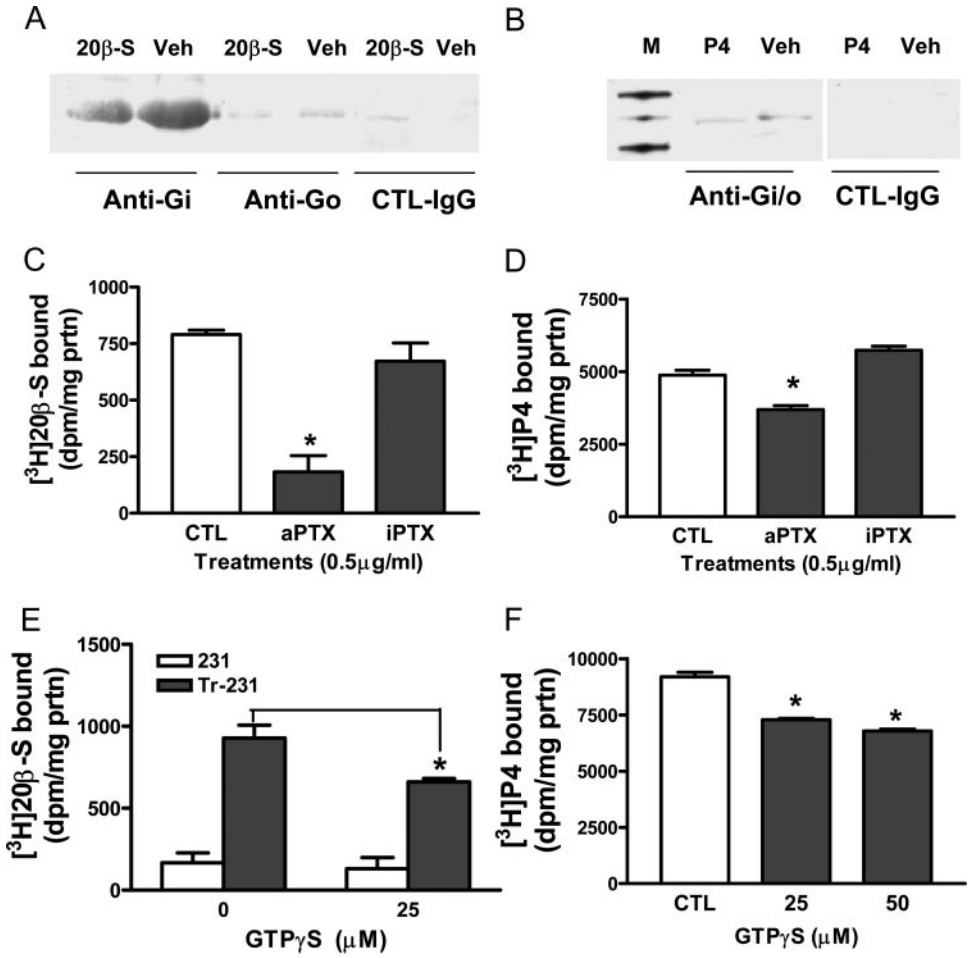

FIG. 4. Coupling of seatrout and human mPRα proteins to G proteins. A and B, Coimmunoprecipitation of st-mPRα (A) and hu-mPRα (B) coupled to G protein α-subunits with specific G protein antibodies followed by immunodetection of the mPRαs by Western blot analysis. Pretreated for 10 min with 100 nM 20β-S or progesterone. C and D, Effects of 30-min pretreatment with 0.5 μg/ml activated pertussis toxin (aPTX) or inactivated pertussis toxin (iPTX) on specific [^{3}H]-20β-S binding to membranes of st-mPRα-transfected cells (C) or on specific [^{3}H]-progesterone binding to membranes of hu-mPRα-transfected cells (D). *, $P < 0.05$, n = 4. E and F, Effects of 30-min pretreatment with 25 μM GTPγS on specific [^{3}H]-20β-S binding to membranes of st-mPRα-transfected cells (E) or pretreatment with 25 or 50 μM GTPγS on specific [^{3}H]-P4 binding to membranes of hu-mPRα transfected cells (F). *, $P < 0.05$, n = 4.

mammalian progestin hormones, 20β-S and progesterone. Saturation analysis and Scatchard plotting indicated that both mPRα proteins have high-affinity, limited-capacity, single binding sites specific for progestin hormones that are characteristic of steroid hormone receptors and distinguish them from other steroid-binding proteins. The binding affinity of 20β-S to the recombinant st-mPRα (Kd 7.58 nM) is similar to its binding affinity to seatrout ovarian and testicular membranes, which express wild-type st-mPRα (Refs. 22 and 31; Kd: ovary 5.0 nM, testis 18.0 nM), whereas the wild-type nuclear progestin receptor in seatrout displays a 4-fold higher progestin binding affinity (Ref. 32; mean cytosolic binding: Kd 1.9 nM). Similarly, the binding affinity of progesterone to the human nuclear progesterone receptor-B (Ref. 33; Kd 0.8 nM) is 5-fold higher than that to recombinant hu-mPRα (Kd 4.2 nM). Information on whether these apparent differences in binding affinities result in differential activation of the two progestin receptor systems, the mPRs only being continuously activated where progestin levels are high near their sites of synthesis and intermittently activated at other target tissues when plasma progestin levels are elevated, may provide clues of the physiological roles of the mPRs during the reproductive cycle.

The high binding specificity of the recombinant st-mPRα produced in the mammalian expression system for 20β-S is consistent with its identity as the major progestin hormone regulating maturation of oocytes and motility of sperm in this species and with previous receptor binding results with seatrout ovarian and sperm membranes (22, 31). The lack of 20β-S binding to the soluble recombinant st-mPRα protein produced in *Escherichia coli* observed in our earlier study is probably due to protein modification deficiencies in this prokaryotic expression system (7). In general, however, the steroid binding characteristics of recombinant st-mPRα and hu-mPRα produced in MDA-MB-231 cell membranes are remarkably similar to those of the recombinant soluble receptors produced in the prokaryotic system (8). The more than 100-fold lower binding affinity of 20β-S and the other teleost progestin, 17,20β-P, for hu-mPRα (RBAs <1%) is noteworthy and warrants

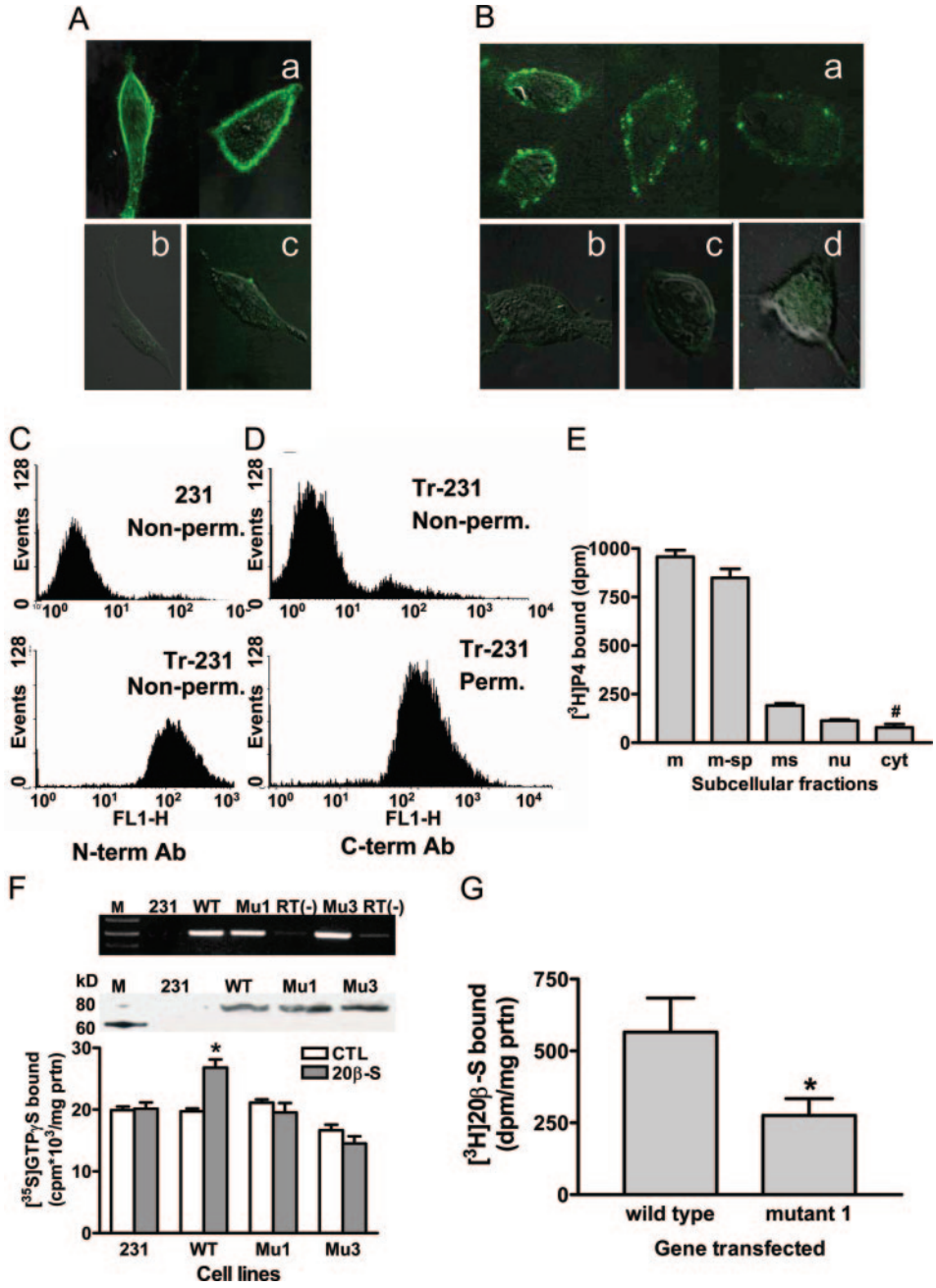

FIG. 5. Localization, orientation, and functional domains of the mPRαs. A and B, Immunocytochemical localization of st-mPRα (Aa) and hu-mPRα (Ba) on external surface of plasma membranes of transfected cells with specific N-terminal directed antibodies. Ab and Bb, Blocked with peptide antigens; Ac and Bc, lack of immunoreaction with nontransfected cells; Bd, lack of immunoreaction with nonpermeabilized cell using C-terminal antibody. C and D, Flow cytometry of cells transfected with hu-mPRα using N-terminal (C) and C-terminal (D) antibodies. C, 231, Background fluorescence on nontransfected cells in the presence mPRα N-terminal antibody; Tr-231 Non-perm., immunodetection of the hu-mPRα N-terminal on the surface of nonpermeabilized transfected cells by flow cytometry using the N-terminal antibody. D, Tr-231 Non-perm., fluorescence of nonpermeabilized transfected cells using the C-terminal antibody; Tr-231 Perm., immunodetection of the hu-mPRα C-terminal in permeabilized transfected cells by flow cytometry using a C-terminal directed antibody. E, Specific binding of [^{3}H]-P4 to subcellular fractions of cells transfected with hu-mPRα. m, Plasma membranes; m-sp, plasma membranes further purified with a sucrose pad; ms, microsomes; nu, nuclear fraction; cyt, cytosolic fraction; #, dextran-coated charcoal used to separate bound from free. F and G, Mutational analysis of st-mPRα functional domains. F, Effects of C-terminal truncation (Mu1) and amino acid substitution in the third intracellular loop (Mu3) on specific [^{35}S]GTPγS binding to cell membranes in response to 20β-S treatment. G, Effects of C-terminal truncation (mutant 1) on specific [^{3}H]-20β-S binding to cell membranes.

investigation, because all the other steroids tested have similar affinities for the two mPRαs. For example, the nuclear receptor agonist R5020 and antagonist RU486 have very low affinities for both mPRαs, whereas testosterone, which shows low affinities for nuclear PRs, has RBAs of 10% and 22% for st-mPRα and hu-mPRα, respectively. The

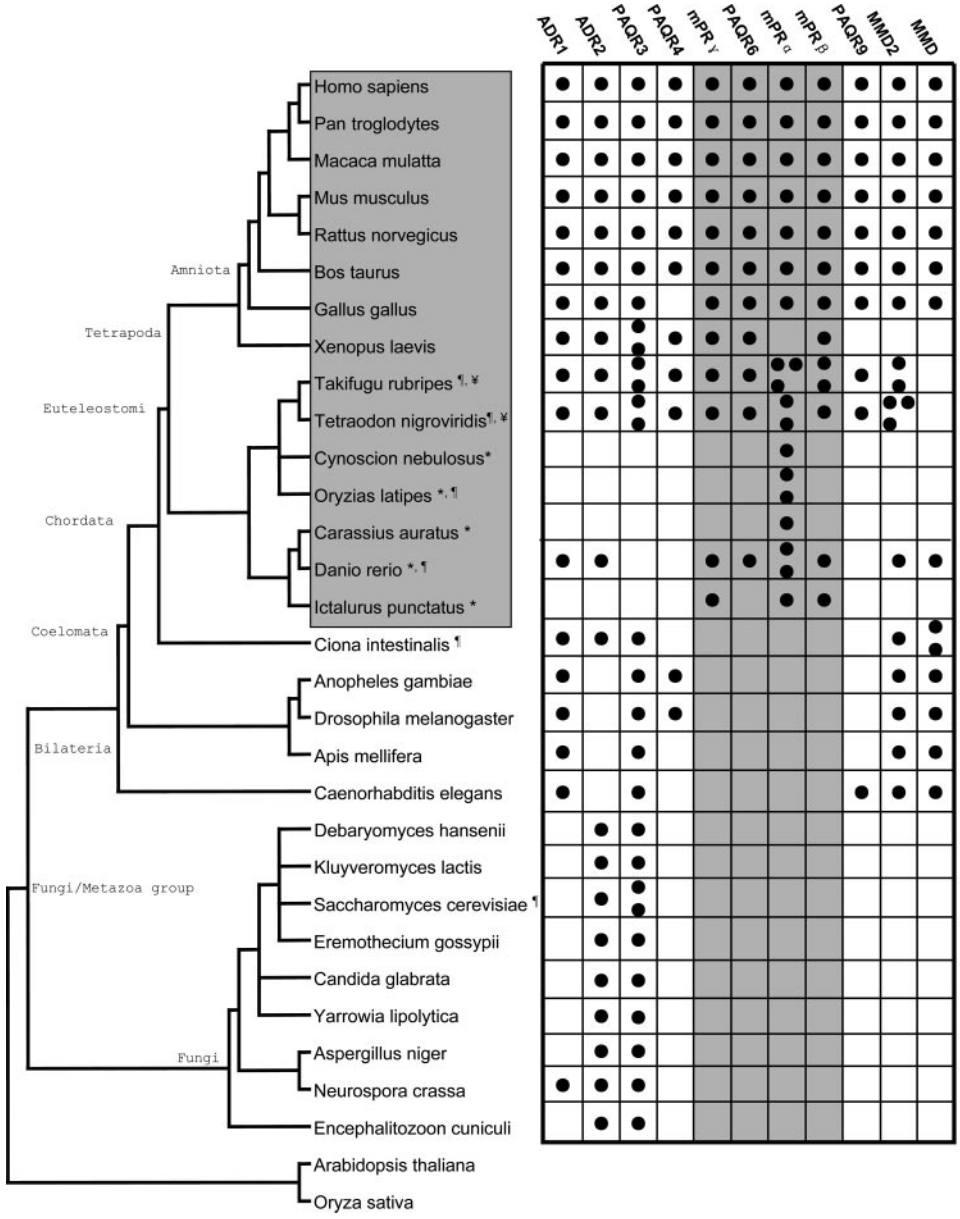

FIG. 6. Schematic representation of the presence of PAQR family members 1–11 among the eukaryotes. The phylogram on the *left* depicts the evolutionary relationships between the different species used in this study. The table on the *right* shows the presence of a particular PAQR family member by a *dot*. A *double dot* indicates a duplication event. *, Missing data, incomplete genome sequence; ¶, gene duplication; ¥, possible redundancy; low-quality sequence shows divergence in phylogenetic tree. The results show that the mPRα, mPRβ, and mPRγ genes first appeared in the *Euteleostomi*.

finding that the steroid specificities of the recombinant seatrout and human mPRαs differ markedly from those of their nuclear progestin receptor counterparts was anticipated because there is no homology in any regions of the mPRs to the ligand binding domain of the nuclear progesterone receptor. Competitive binding assays with more than 30 steroidal compounds revealed different effects of substituting various functional groups on the progesterone nucleus on their binding affinities to hu-mPRα compared with previously published results with human nPR (Refs. 33 and 34 and Table 1). For example, removal of carbon 19 (substitution of a methyl group for hydrogen: norprogesterone, also see R5020 and Organon 2058) decreases binding affinity to mPRα (Table 1) but modestly increases it for the nPR (33, 34). However, removal of the side chain and addition of a hydroxyl at C17 (*i.e.* testosterone, dihydrotestosterone, nandrolone), which practically eliminated binding to the human nPR, only reduced binding with mPRα to 22–7% that of P4 (33, 34). Finally, the hu-mPRα also does not recognize C19 steroids (androgens) with a substitution of a hydrogen on C17 with an ethinyl group (*i.e.* ethisterone, norethisterone, and norgestrel), whereas these steroids have relatively high affinities for the nPR (33, 35). These marked differences in binding affinities suggest selective mPRα modulators, in addition to nPR ones such as R5020, can be developed to

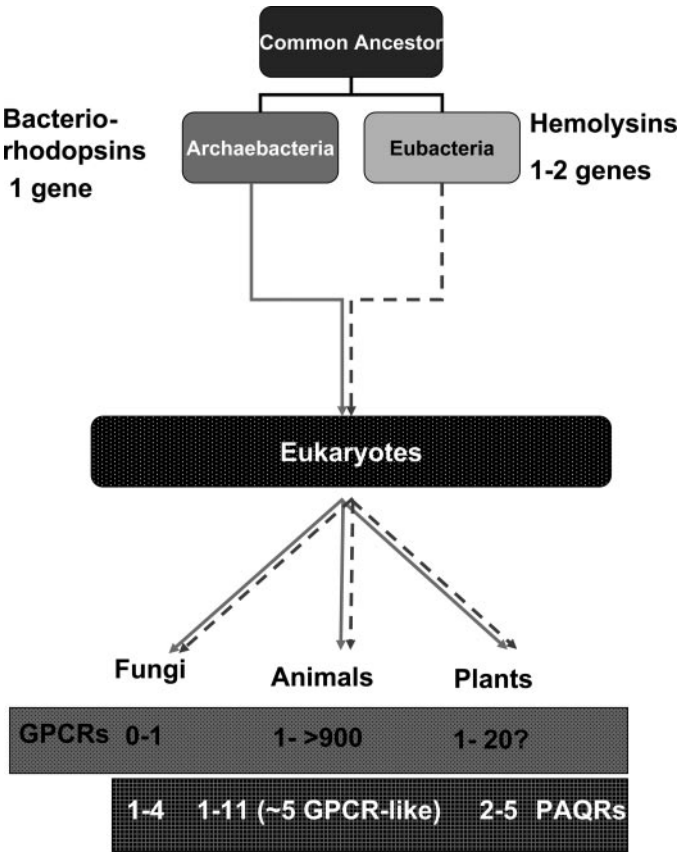

FIG. 7. Schematic depiction of the parallel convergent evolution of the PAQR family and GPCR superfamily. The bacteriorhodopsins are strictly found in *Archaebacteria* and constitute the origin of the GPCR superfamily, whereas the HLY3 genes are strictly found in *Eubacteria*.

independently explore progestin actions in target tissues mediated by each of these two receptor systems.

Identification of the ligand binding pocket by site-directed mutagenesis will be required for definitive proof that the mPRs directly bind progestins and are not merely components of a multiple-protein receptor complex. However, all the results obtained to date with the mPRs are consistent with our previous suggestion that they are true steroid membrane receptors (7). The finding that prokaryotic as well as eukaryotic expression systems produce recombinant st-mPRαs and hu-mPRαs with the binding characteristics of steroid membrane receptors suggests that the ability to bind progestins is an intrinsic property of these proteins and is not dependent on their association with other proteins present only in eukaryotic cells (7, 8). The demonstration that transfection of MDA cells with the various mPRs confers progestin binding specificity to the cell membrane preparations also suggests a direct role for these proteins in ligand binding. Membranes from cells transfected with st-mPRα display a high binding affinity for 20β-S, the principal progestin in this species, whereas those from hu-mPRα-transfected cells have low affinity for this steroid but show highest affinity for progesterone, the major progestin hormone in mammals. In addition, MDA cells transfected with zebrafish mPRα show the highest binding affinity for 17,20β-P, the likely progestin hormone in this species (36). The characteristics of G protein activation and ligand binding to the mPRs on G protein uncoupling are also typical of seven-transmembrane hormone receptors. For example, dissociation of G proteins from GPCRs results in a decrease in ligand binding affinity of the receptors directly coupled to them. The finding that dissociation of the mPRα-G protein complex by pretreatment with GTPγ-S and pertussis toxin causes a decline in progestin binding is further evidence that the progestin binding site is located on the mPRα protein.

There is now evidence that representatives of all three mPR subtypes described in the original papers, α, β, and γ (7, 8), corresponding to PAQR types 7, 8, and 5 (17), display high-affinity, specific and limited-capacity binding to progestins characteristic of steroid membrane receptors. Sequence and phylogenetic analyses reveal that another PAQR type, PAQR 6, is closely related to the mPRs (supplemental Fig. 2) and therefore is a candidate as an additional member of this novel group of membrane progestin receptors. A notable feature of this progestin receptor PAQR subfamily (PAQR 5–9) is that representatives have only been identified in vertebrates, including teleosts and tetrapods, suggesting that they arose at the base of the vertebrate lineage. PAQR 9 could also possibly belong to this subgroup of progestin receptors because it is primarily found in the Chordata (Fig. 6) and is clustered with the mPRs (supplemental Fig. 3). In contrast, all other members of PAQR family, including the adiponectin (PAQR 1, 2) and MMD receptors (PAQR 10, 11), have both invertebrate and vertebrate representatives, indicating they have a more ancient evolutionary origin (Fig. 6). The high similarity of PAQR 10 and 11 with the HLY3 proteins in bacteria suggests that this is the archetypical PAQR in eukaryotes, although the phylogenetic analysis suggests that the ADR2 and PAQR 3 genes appeared first in prokaryotes. The present results with seatrout and human mPRαs indicate that, at least for the PAQR 7 subtype, their ability to bind progestins arose early in vertebrate evolution, before the divergence of the teleost and tetrapod lineages, at least 200 million years ago (37). Members of this progestin PAQR subfamily have not been identified in the urochordate *Ciona* genome, which appear to lack many of the key steroidogenic enzymes (steroid dehydrogenases and cytochrome P450s) and enzymes that metabolize steroids (38, 39). The parallels between the evolution of the progestin membrane receptor subfamily, PAQR 5–8, and that of nuclear steroid receptors are striking. Duplication of nuclear steroid receptors from an ancestral estrogen receptor-like gene in vertebrates also occurred early in vertebrate evolution, coincident with the advent of the jawed vertebrates, to give rise to nuclear progesterone receptors and multiple estrogen receptors and later to give rise to androgen, glucocorticoid, and mineralocorticoid receptors (39–41). Thus, phylogenetic analyses indicate that functional mPRs probably arose around the same time as nuclear progestin receptors (nPRs), coincident with the appearance of critical steroidogenic enzymes and the production of significant quantities of progestins and other steroids in early vertebrates. The appearance of both mPRs and nPRs in early vertebrates likely reflects an intimate and complimentary relationship between the two receptor systems, both of which may be required for a coordinated or varied response of target cells in the reproductive system to progestin hormones. The first evidence of such

an intimate relationship between these two progestin receptor signaling pathways has recently been obtained in human myometrial cells, where progesterone was shown to regulate transactivation of nPR and coactivator function through mPRα- and mPRβ-mediated pathways (26). Complex interactions between these two types of progesterone receptors are expected in some breast cancer cell lines (e.g., MCF-7), which show progesterone activation of nonclassical pathways through both mPRs and nPRs in addition to classical genomic responses through the nPR (4, 42). Information on the differing roles of mPRs and nPRs in nonclassical signaling in these cells may provide insights into why alternative signaling pathways mediated by two different classes of progesterone receptors evolved in vertebrates. Structural analyses of the nuclear steroid receptor ligand binding domains in a wide variety of chordates indicate that the ancestral receptor initially recognized the estrogens produced in vertebrate gonads associated with reproductive activity. It has been suggested that nPRs evolved subsequently to respond to progesterone, a steroid intermediate in estrogen production, by a process called ligand exploitation (40). A similar analytical approach may provide valuable insights into the evolution of mPRs from the ancestral PAQR once information on the structures of the ligand binding domains of mPRs and other members of the PAQR family becomes available.

The results of the present study also demonstrate that both the seatrout and human recombinant mPRα proteins activate inhibitory G proteins (G_i), are coupled to them and share many other critical features of GPCRs. Clear evidence was obtained that mPRαs, like GPCRs, are localized and function as seven-transmembrane receptors in the plasma membranes of cells. However, the mPRαs have also been identified in other cellular compartments in fish oocytes and in certain eukaryotic expression systems (7, 15, 16). Moreover, the mPRαs are similar to GPCRs in that they likely can occur as dimers as well a monomers (43), because an 80-kDa band is also frequently observed on Western blots, the intensity of which varies with the membrane solubilization conditions used (unpublished observation). G protein activation, detected with GPCRs by an increase in specific [^{35}S]GTPγ-S binding to the cell membranes (44), was also demonstrated after progestin treatment of mPRα-transfected cells. The demonstration that progestins activate the inhibitory G protein, G_i, and down-regulate pertussis toxin-sensitive adenylyl cyclase activity in the plasma membranes of MDA-MB-231 cells transfected with receptors indicates the mPRαs function as GPCRs. The activation of an inhibitory G protein by the mPRαs is also consistent with our recent findings on the signaling pathways activated by 20β-S during meiotic maturation of seatrout oocytes (25) as well as those activated by progesterone through the wild-type hu-mPRα in human myometrial cells (26). A unique characteristic of GPCRs is that treatment with nonhydrolyzable forms of guanine nucleotides such as GTPγ-S, and for $G_{i/o}$-protein coupled receptors with pertussis toxin, cause uncoupling of G proteins from the receptors (45, 46), resulting in a decrease in their ligand binding affinities (47, 48). These treatments caused similar decreases in ligand binding to st-mPRα and hu-mPRα. Thus, these results further support the conclusions of the coimmunoprecipitation studies that the mPRαs are directly coupled to G proteins and are not acting through intermediaries. The results of the mutational analyses indicate that two functional domains of GPCRs critical for G protein activation/coupling, the C-terminal domain and the third intracellular loop (49), are also important for activation of G proteins through st-mPRα. The demonstration that the C-terminal of st-mPRα is involved in activation of G proteins suggests it is intracellular in agreement with the model we proposed earlier for the orientation of the receptor in the plasma membrane (8). This orientation is also consistent with the results of the flow cytometry studies with cells transfected with hu-mPRα and probed with N-terminal and C-terminal antibodies. Therefore, several lines of evidence suggest the mPRαs have the same orientation in the plasma membrane as GPCRs.

Although the mPRαs function by activating G proteins and have many characteristics of GPCRs, extensive phylogenetic analysis reveals that they are unrelated to members of the GPCR superfamily, in agreement with our previous conclusions (50), which were subsequently confirmed by Tang *et al.* (18). The mPRs belong to the large eukaryotic family of PAQRs, which in turn is related to the prokaryotic HLY3 family, suggesting they have a common bacterial ancestor. Members of the HLY3 family have only been identified in *Eubacteria*, consisting of Gram-positive and -negative bacteria, and are not present in *Archaebacteria* (18, 50). The results clearly indicate that the eukaryotic PAQR family descended from ancestral HLY3-like proteins in *Eubacteria* and therefore their bacterial origin differs from that of the GPCR superfamily, which descended from *Bacteriorhodopsin*, which is only found in the *Archaebacteria* (51, 52). This would explain the structural similarity but the low degree of sequence similarity between GPCRs and PAQRs. Activation of G proteins by PAQRs has only been demonstrated to date for mPRα and mPRβ (PAQR 7, 8) and predicted to be absent in adiponectin receptors (20), suggesting that this receptor function was acquired early in vertebrate evolution. However, additional studies on G protein activation with other PAQRs will be required to confirm this hypothesis. In conclusion, the mPRαs are coupled to G proteins, activate them on ligand binding, and have many characteristics of GPCRs. However, the mPRαs and members of the GPCR superfamily are unrelated, indicating that hormone signaling through seven-transmembrane receptors coupled to G proteins arose independently more than once during vertebrate evolution, for long thought to be a unique characteristic of the GPCR superfamily.

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Supplementary Figure 1. **(A,B)** Schematic representation of the regions of st-mPRα (**A**) and hu-mPRα (**B**) used for antibody production and mutational analyses. (**C, D**) Adenylyl cyclase activity in plasma membranes prepared from st-mPRα-transfected cells after treatment with 0, 5, 20 and 100 nM of 20β-S for 15 min (**C**); and in hu-mPRα-transfected cells after treatment with the same concentrations of progesterone for 15 min. (**D**) *: $P<0.05$ compared to 0 nM treatment control. N=3. (**E**) G-protein activation on the hu-mPRα-transfected cell membrane by progesterone. [35S]GTPγS binding were significantly increased after the membrane samples were treated with 5 and 20 nM of progesterone. *: $P<0.05$, N=3. (**F**) Western blot of subcellular fractions of MDA cells with human integrinβ3 antibody (Cell Signaling). M, marker; mem, plasma membrane; cyt, cytosol; ms, microsomes. (**G, H)** Immunocytochemical staining of MDA cells with clathrin antibody and Alexa 488 fluorescence antibody. Cells were scraped from the culture dish and washed twice, then either permeabilized with 90% methanol (**G**) or with PBS for control (**H**). Then the cells were blocked with 1% BSA and then incubated with primary and secondary antibodies. The cells were examined under a fluorescence microscope.

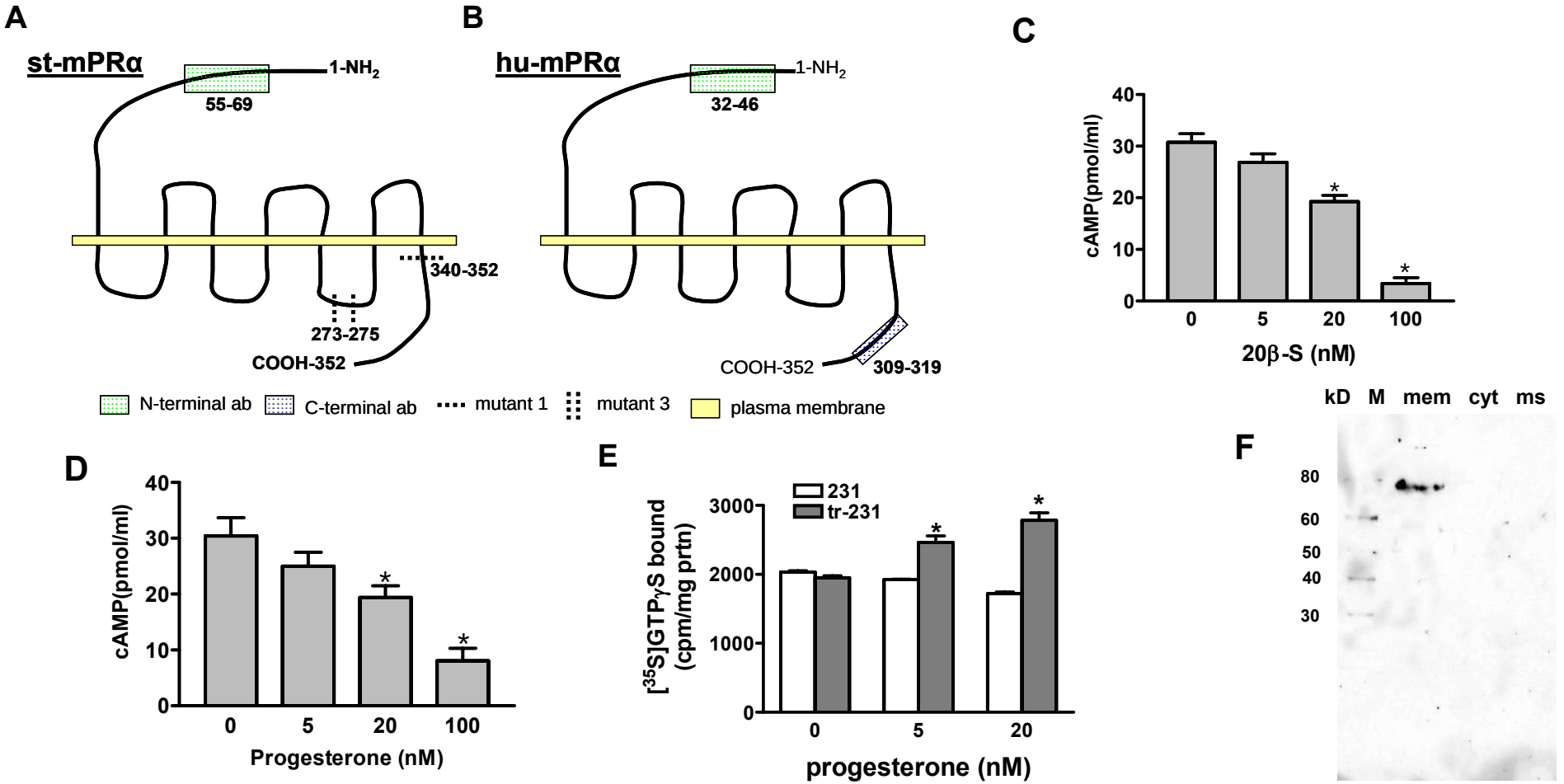
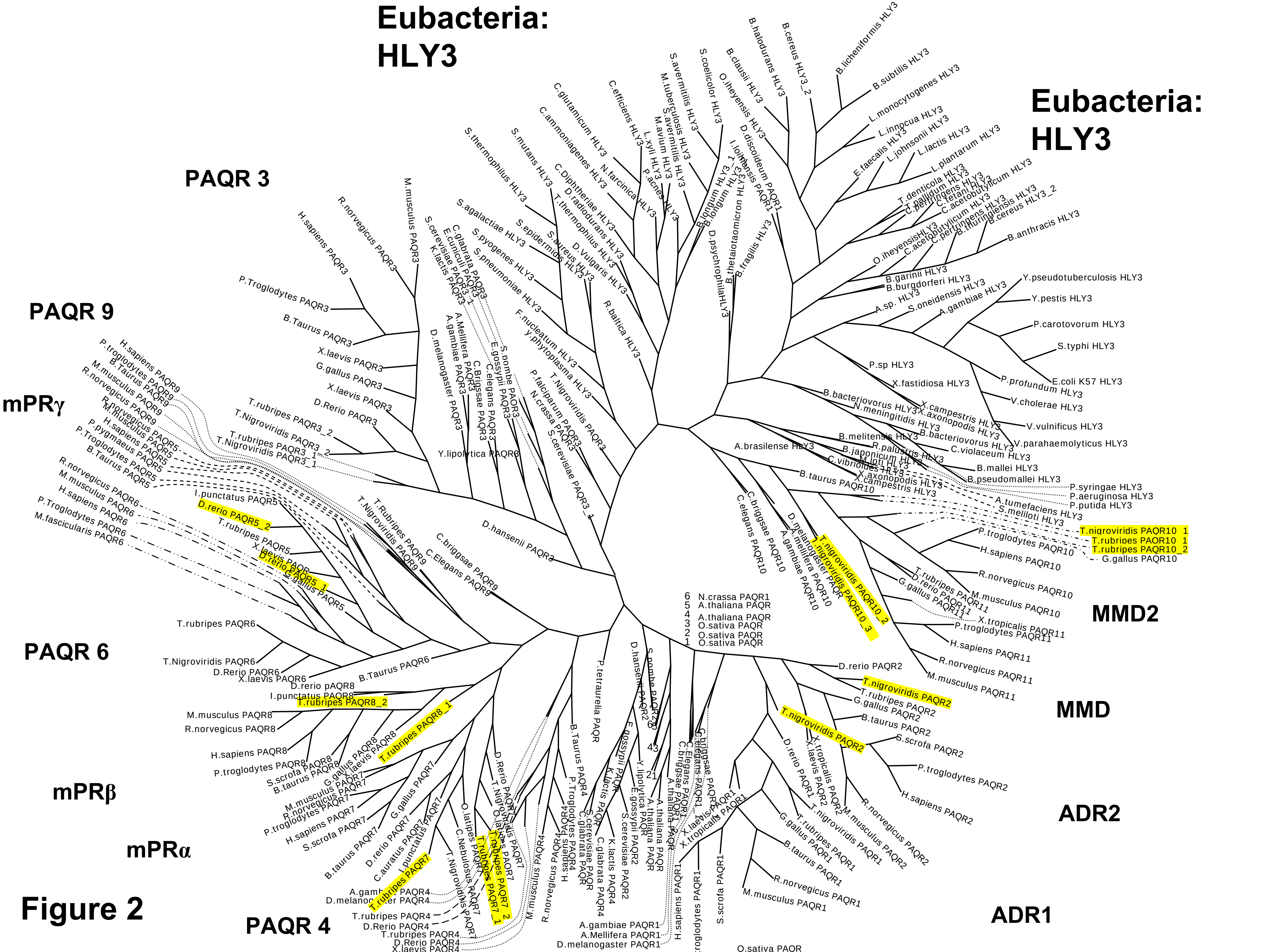

A
st-mPRα
1-NH2
55-69
340-352
273-275
COOH-352
B
hu-mPRα
1-NH2
32-46
COOH-352
309-319
N-terminal ab
C-terminal ab
mutant 1
mutant 3
plasma membrane
C
cAMP(pmol/ml)
40
30
20
10
0
0
5
20
100
20β-S (nM)
D
cAMP(pmol/ml)
40
30
20
10
0
0
5
20
100
Progesterone (nM)
E
231
tr-231
[35S]GTPγS bound (cpm/mg prtn)
3000
2000
1000
0
0
5
20
progesterone (nM)
F
kD
M
mem
cyt
ms
80
60
50
40
30
G
H

Figure 1

Supplementary Figure 2. The total unrooted phylogenetics tree from all sequences, from a diverse set of species that revealed similarity to the PAQR/HLY3 signature. The results from this tree have been used to design figures 6 and 7 in the text. All sub-clusters have been annotated with the current PAQR family gene name nomenclature. The yellow shaded boxes indicate the genes that have undergone duplication in teleost fish and amphibians.



Eubacteria: HLY3
Eubacteria: HLY3
PAQR 3
PAQR 9
mPRγ
PAQR 6
mPRβ
mPRα
PAQR 4
ADR1
ADR2
MMD
MMD2

Figure 2

Supplementary Figure 3. A unrooted phylogenetic tree classifying 4 mammals, showing the high similarity and clustering of mPRα, mPRβ, mPRγ, PAQR6 and PAQR9. These data suggest a possible functional similarity (steroid binding) of PAQR6 and 9.

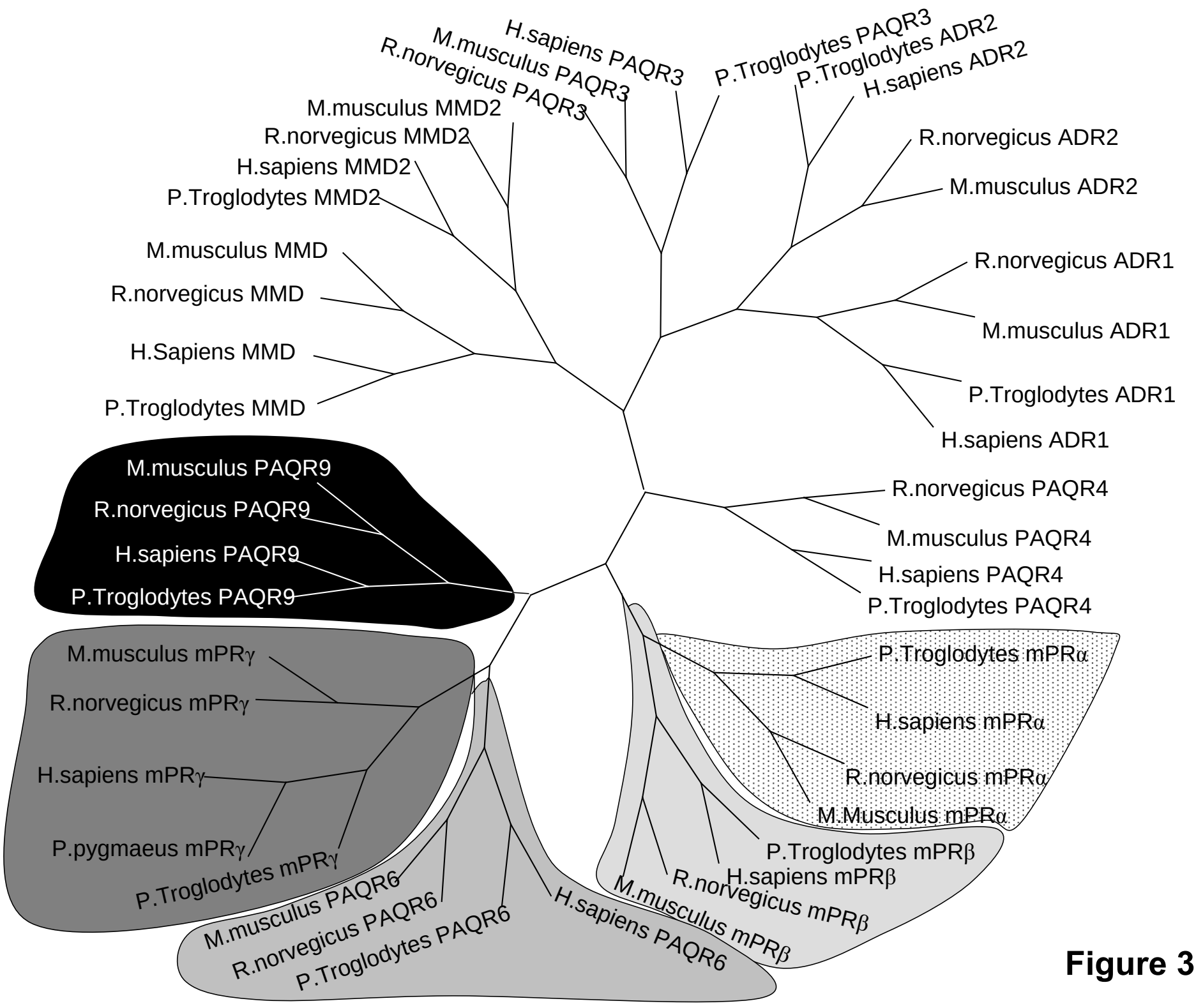

H.sapiens PAQR3
M.musculus PAQR3
R.norvegicus PAQR3
P.Troglodytes PAQR3
P.Troglodytes ADR2
H.sapiens ADR2
M.musculus MMD2
R.norvegicus MMD2
H.sapiens MMD2
P.Troglodytes MMD2
R.norvegicus ADR2
M.musculus ADR2
M.musculus MMD
R.norvegicus MMD
H.Sapiens MMD
P.Troglodytes MMD
R.norvegicus ADR1
M.musculus ADR1
P.Troglodytes ADR1
H.sapiens ADR1
M.musculus PAQR9
R.norvegicus PAQR9
H.sapiens PAQR9
P.Troglodytes PAQR9
R.norvegicus PAQR4
M.musculus PAQR4
H.sapiens PAQR4
P.Troglodytes PAQR4
M.musculus mPRγ
R.norvegicus mPRγ
H.sapiens mPRγ
P.pygmaeus mPRγ
P.Troglodytes mPRγ
P.Troglodytes mPRα
H.sapiens mPRα
R.norvegicus mPRα
M.Musculus mPRα
P.Troglodytes mPRβ
H.sapiens mPRβ
R.norvegicus mPRβ
M.musculus mPRβ
M.musculus PAQR6
R.norvegicus PAQR6
P.Troglodytes PAQR6
H.sapiens PAQR6

Figure 3