0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(2):624–632

doi: 10.1210/en.2004-1064

Identity of an Estrogen Membrane Receptor Coupled to a G Protein in Human Breast Cancer Cells

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Although nonclassical estrogen actions initiated at the cell surface have been described in many tissues, the identities of the membrane estrogen receptors (mERs) mediating these actions remain unclear. Here we show that GPR30, an orphan receptor unrelated to nuclear estrogen receptors, has all the binding and signaling characteristics of a mER. A high-affinity (dissociation constant 2.7 nM), limited capacity, displaceable, single binding site specific for estrogens was detected in plasma membranes of SKBR3 breast cancer cells that express GPR30 but lack nuclear estrogen receptors. Progesterone-induced increases and small interfering RNA-induced decreases in GPR30 expression in SKBR3 cells were accompanied by parallel changes in specific estradiol-17β (E2) binding. Plasma membranes of human embryonic kidney 293 cells transfected with GPR30, but not those of untransfected cells, and human placental tissues that express GPR30 also displayed high-affinity, specific estrogen binding typical of mERs. E2 treatment of transfected cell membranes caused activation of a stimulatory G protein that is directly coupled to the receptor, indicating GPR30 is a G protein-coupled receptor (GPCR), and also increased adenylyl cyclase activity. The finding that the antiestrogens tamoxifen and ICI 182,780, and an environmental estrogen, ortho,*para*-dichlorodiphenyldichloroethylene (o,p′-DDE), have high binding affinities to the receptor and mimic the actions of E2 has important implications for both the development and treatment of estrogen-dependent breast cancer. GPR30 is structurally unrelated to the recently discovered family of GPCR-like membrane progestin receptors. The identification of a second distinct class of GPCR-like steroid membrane receptors suggests a widespread role for GPCRs in nonclassical steroid hormone actions. (*Endocrinology* 146: 624–632, 2005)

IN ADDITION TO the classic genomic mechanism of steroid action mediated by intracellular receptors belonging to the nuclear steroid receptor superfamily, there is now convincing evidence that steroids also exert rapid, nongenomic steroid actions initiated at the cell surface by binding to membrane receptors (1–3). However, despite intensive research the identities of steroid membrane receptors remain unclear and surrounded by controversy (4–6). Investigations of nonclassical estrogen signaling suggest nuclear estrogen receptors (ERs), ERα and ERβ, or ER-like proteins are likely candidates for the membrane ERs (mERs) mediating these estrogen actions in a variety of target cells, including endothelial, neuronal, and pituitary cells (7–11). However, evidence has also been obtained for the involvement of novel mERs unrelated to nuclear ERs (nERs) in nonclassical estrogen actions in several other cell types, many of which are associated with G proteins (12–16). Our recent discovery of a hitherto unknown family of membrane progesterone receptors (mPRs), unrelated to nuclear steroid receptors, but instead with characteristics of G protein-coupled receptor (GPCRs) (17, 18), prompted us to search for other GPCRs with characteristics of steroid membrane receptors. The orphan GPCR-like protein, GPR30, is widely distributed in neural, breast cancer, placental, heart, ovarian, prostate, hepatic, vascular epithelial, and lymphoid tissues and shows structural sequence homology to receptors for angiotensin, interleukin, and a variety of chemokines, suggesting it may be a peptide receptor (19–22). However, a broad range of chemotactic peptides and angiotensins showed no binding affinity for GPR30 (20, 23). Instead, evidence was obtained for an involvement of GPR30 in estrogen-induced transactivation of epidermal growth factor receptor and adenylyl cyclase activity in SKBR3 breast cancer cells that lack nERs (24–26), suggesting GPR30 may be a novel mER. The present results demonstrate that expression of GPR30 in cells lacking ERα and ERβ is associated with the presence of high affinity, limited capacity, and specific 17β-estradiol (E2) binding to their plasma membranes characteristic of mERs. Evidence is presented that GPR30 is directly coupled to a stimulatory G protein to up-regulate adenylyl cyclase activity and is a GPCR.

First Published Online November 11, 2004

Abbreviations: Bmax, Maximal binding capacity; CTX, cholera toxin; DDE, o,p′-DDE; DTT, dithioerythritol; E1, estrone; E2, 17β-estradiol; E2α, 17α-estradiol; E3, estriol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GPR30, orphan GPCR-like protein; G_s, stimulatory G protein; HEK, human embryonic kidney; ICI, ICI182,780; Kd, dissociation constant; mER, membrane estrogen receptor; mPR, membrane progestin receptor; nER, nuclear ER; P4, progesterone; RBA, relative binding affinity; SDS, sodium dodecyl sulfate; si, small interfering; $t_{1/2}$, halftime; Tmx, tamoxifen.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

Materials and Methods

Chemicals

The steroids E2, 17α-estradiol (E2α), estrone (E1), estriol (E3), cortisol, testosterone, and progesterone (P4) and the synthetic estrogen diethylstilbestrol were purchased from Steraloids (Newport, RI). The antiestrogen tamoxifen (Tmx) and the fungal metabolite, zearalenone, were purchased from Sigma-Aldrich Corp. (St. Louis, MO). The ortho,*para* derivative of the pesticide ortho,*para*-dichlorodiphenyldichloroethylene,

o,p′-DDE (DDE), was purchased from Chem Service (West Chester, PA). The synthetic antiestrogen ICI182,780 (ICI) was purchased from Tocris (Ellisville, MO). 17β-[2,4,6,7-^{3}H]estradiol ([^{3}H]E2); approximately 89 Ci/mmol, was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All other chemicals, buffers, and media were purchased from Sigma-Aldrich unless noted otherwise.

Cell culture and transfections

Human SKBR3 cells and human embryonic kidney (HEK)293 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM/Ham's F-12 medium without phenol red supplemented with 10% fetal bovine serum (FBS) and 100 μg/ml of gentamicin, with changes of medium every 1–2 d. SKBR3 cells were transiently transfected with GPR30 small interfering (si)RNA (100 nM), or nonspecific, presynthesized siRNA (control) using Lipofectamine 2000 (Life Technologies Inc., Gaithersburg, MD) at 25 C, following the manufacturer's procedures (Dharmacon, Lafayette, CO) to interfere with GPR30 expression, and experiments were conducted 18 h later. HEK293 cells were transfected with a GPR30 construct, consisting of the full-length cDNA ligated into the pBK-CMV expression vector (25), using Lipofectamine 2000 and grown to confluence. Geneticin (500 μg/ml) was added and the geneticin-resistant cells containing the GPR30 construct were propagated to generate stable cell lines (selectively maintained with 500 μg/ml geneticin). Cells reached 80% confluence after 3 d in culture (~2×10^8 cells, ~0.6 mg cell membrane protein/150 mm dish) and were replaced with fresh media containing 0 or 5% FBS 1 d before experiments.

Treatments

GPR30 expression and receptor binding were up-regulated in SKBR3 cells by incubating them for 16 h in FBS-free media with 200 nM P4 or E2 or media alone, followed by repeated washes with buffer before measurement of E2 binding. The effects of uncoupling G proteins on E2 binding affinity was investigated with membranes of transfected HEK293 cells pretreated with 0 or 25 μM GTPγ-S at 25 C for 30 min. Cells were also incubated with 10 μg/ml activated cholera toxin [CTX; activated with 4 mM dithioerythritol (DTT)], inactive CTX (inactivated by boiling), or media alone for 30 min at 37 C immediately before preparation of the cell membrane for assay of E2 binding. Cells were collected with a cell scraper and washed twice with fresh media before preparation of plasma membranes. All experiments were repeated at least three times with different batches of cultured cells.

Membrane preparation and solubilization

Plasma membrane fractions of healthy human placental tissue (obtained from a live birth at Women and Infant's Hospital, Providence, RI, following National Institutes of Health guidelines), and cells were obtained after homogenization and centrifugation procedures described previously (27, 28). Placental tissue plasma membranes were further purified by centrifuging the membrane pellet with a sucrose pad (1.2 M sucrose) at 6500 × *g* for 45 min (17, 29). Membranes were solubilized with 12 mM Triton X-100 in four volumes HEPES buffer (25 nM HEPES, 10 mM NaCl, 1 mM DTT) for 30 min, followed by removal of the detergent with polystyrene adsorbents (2:1 volume-weight; SM-2, Bio-Rad Laboratories, Hercules, CA) and subsequent removal of the adsorbents by filtration (G-8 filter, Fisher Scientific, Fair Lawn, NJ) before the addition of loading buffer for Western blot analyses (17).

ER binding assays

General procedures used in our laboratory for assaying saturation, association, and dissociation kinetics and steroid specificity of ligand binding to steroid membrane receptors (27–29) were used to measure [^{3}H]E2 binding to plasma membrane preparations. For saturation analysis, one set of tubes contained a range (0.5–8.0 nM) of [^{3}H]E2, ~89 Ci/mmol) alone (total binding) and another set also contained 100-fold excess (50–800 nM) E2 competitor (nonspecific binding). For competitive binding assays, tubes contained 4 nM [^{3}H]E2 and the steroid competitors (concentration range 1 nM to 100 μM; dissolved in 5 μl ethanol, 1% of the total volume, which does not affect [^{3}H]E2 binding in the assay). After a 30-min incubation at 4 C with the membrane fractions, the reaction was stopped by filtration (GF/B filters, Whatman), the filters were washed and bound radioactivity measured by scintillation counting. The displacement of [^{3}H]E2 binding by the steroid competitors was expressed as a percentage of the maximum specific binding of E2. Each assay point was run in triplicate, and the assays were repeated using different batches of cultured cells for each test chemical.

Western blot analysis

Solubilized membrane proteins were resolved by electrophoresis and Western blot analysis performed as described previously (17), using a GPR30 polyclonal antibody generated against a C-terminal 19-amino acid peptide fragment (24) (dilution 1:1000) in an overnight incubation. The membrane was 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 before incubation with the GPR30 antibody. The membrane was subsequently washed several times and then incubated for 1 h at room temperature with horseradish peroxidase conjugated to goat antirabbit antibody (Cell Signaling, Beverly, MA), and visualized by treatment with enhanced chemiluminescence substrate (SuperSignal, Pierce, Rockford, IL).

cAMP measurement

Plasma membranes (1.5 mg/ml) were incubated in buffer [20 mM KCl, 12 mM $MgCl_2$, 3 mM EDTA, 2 mM ATP, 0.2 mM DTT, 10 mM creatine phosphate, 1 U creatine kinase, 1 U pyruvate kinase, and 20 mM HEPES (pH 7.5)] with or without 100 nM of the test compounds for 20 min at 25 C. A standard concentration of 100 nM was chosen for comparison of the effects of compounds with low binding affinities for the receptor, although E2 has previously been shown to be effective in SKBR3 cells at a much lower concentration, 1 nM (25). Activated CTX (10 μg/ml) was coincubated with 100 nM E2 in some experiments. The reaction was terminated by boiling the samples for 10 min. cAMP concentrations were measured in cytosolic fractions using an enzyme immunoassay kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI).

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

Binding of [^{35}S]GTPγ-S to plasma membranes (~10 μg protein) was assayed following the procedure of Liu and Dillon (30) with few modifications. Plasma membranes were incubated with 10 μM GDP and 0.5 nM [^{35}S]GTPγ-S (~12,000 cpm, 1.0 Ci/mol) in 250 μl Tris buffer [100 nM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.6 mM EDTA, 0.1%BSA, and 50 mM Tris-HCl (pH 7.4)] at 25 C for 30 min in the presence of 100 nM of the test compounds. Nonspecific binding was determined by addition of 100 μM GTPγ-S. At the end of the incubation period, 100-μl aliquots were filtered through GF/B glass fiber filters (Whatman), followed by several washes and subsequent scintillation counting.

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

Immunoprecipitation of the G protein α-subunits coupled to [^{35}S]GTPγ-S was performed as described elsewhere (30). Plasma membranes (~20 μg protein) of transfected HEK293 cells were incubated with 1 μM E2 for 30 min at 25 C in 250 μl Tris buffer containing 4 nM [^{35}S]GTPγ-S, 10 μM GDP, and protease inhibitor cocktail (Sigma-Aldrich). The incubation was stopped by addition of 750 μl ice-cold buffer containing 100 μM GDP and 100 μM unlabeled GTPγ-S. Samples were centrifuged at 20,000 × *g* for 15 min and the pellet resuspended in immunoprecipitation buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 5 mM EDTA, 25 mM Tris-HCl (pH 7.4), and protease inhibitors. Specific antisera to the α-subunits of G proteins (G_i, Gs, Sigma-Aldrich, dilution 1:500) were incubated with the samples for 6 h at 4 C. Protein A-Sepharose was added, and after an overnight incubation, the immunoprecipitates were collected by centrifugation (12,000 × *g* for 2 min) and washed in buffer (50 mM HEPES, 100 μM NaF, 50 mM sodium phosphate, 100 mM NaCl, 1% Triton X-100, and 1% SDS). The pellets were boiled in 0.5% SDS and the radioactivity in the immunoprecipitated [^{35}S]GTPγ-S-labeled G protein α-subunits counted.

RT-PCR of GPR30

Total RNA was extracted with Tri-reagent (Sigma-Aldrich). Reverse transcription was performed by adding 1–3 μg RNA to the 10-μl reaction mix containing 1× first-strand buffer (10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate, 50 ng/μl oligo-dT primer, and 100 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and the mixture was incubated for 2 h at 42 C. The PCR was conducted in 30 μl PCR SuperMix (Invitrogen) consisting of 0.5 μl of the reverse transcription reaction and 0.2 μM of each of the primers. The following gene-specific primers for GPR30 were designed according to the GPR30 sequence from GenBank (accession no. BC011634): 1) sense, 5′-GGC TTT GTG GGC AAC ATC-3′; antisense, 5′-CGG AAA GAC TGC TTG CAG G-3′; 2) sense, 5′-TGG TGG TGA ACA TCA GCT TC-3′; antisense, 5′-TGA GCT TGT CCC TGA AGG TC-3′; and 3) sense, 5′-GCA GCG TCT TCT TCC TCA CC-3′; antisense, 5′-ACA GCC TGA GCT TGT CCC TG-3′. After an initial denaturation for 5 min at 94 C, the PCR was performed on the Eppendorf Mastercycler for 35 cycles with the cycling profile of 30 sec at 94 C, 30 sec at 55 C, and 2 min at 72 C followed by a 10-min extension at 72 C. The PCR (5 μl) was electrophoresed on an agarose gel (1%) containing ethidium bromide to visualize the products. For semiquantitative RT-PCR, 25 cycles of PCR were performed (linear portion of cycle/product curve).

Statistics

Linear and nonlinear regression analyses for all receptor binding assays and calculations of dissociation constant (Kd) and binding capacity were performed using GraphPad Prism for Windows (version 3.02; GraphPad Software, San Diego, CA). Student's paired *t* test was used for paired comparisons and one-way ANOVA and Tukey tests for multiple comparisons (Sigma Stat, SPSS, Chicago, IL).

Results

Estrogen binding to plasma membranes of SKBR3 breast cancer cells

Saturation analysis and Scatchard plotting of [^{3}H]E2 binding to SKBR3 cell plasma membranes showed the presence of a single, high-affinity (Kd 2.7 nM), saturable, low-capacity [maximal binding capacity (Bmax) 114 pM]-specific estrogen binding site (Fig. 1A). The binding was displaceable and the kinetics of association and dissociation of binding were rapid, with halftimes ($t_{1/2}$) of 5.5 and 8.1 min, respectively (Fig. 1B). Competitive binding assays showed that steroid binding was specific for E2. P4, cortisol, and testosterone had

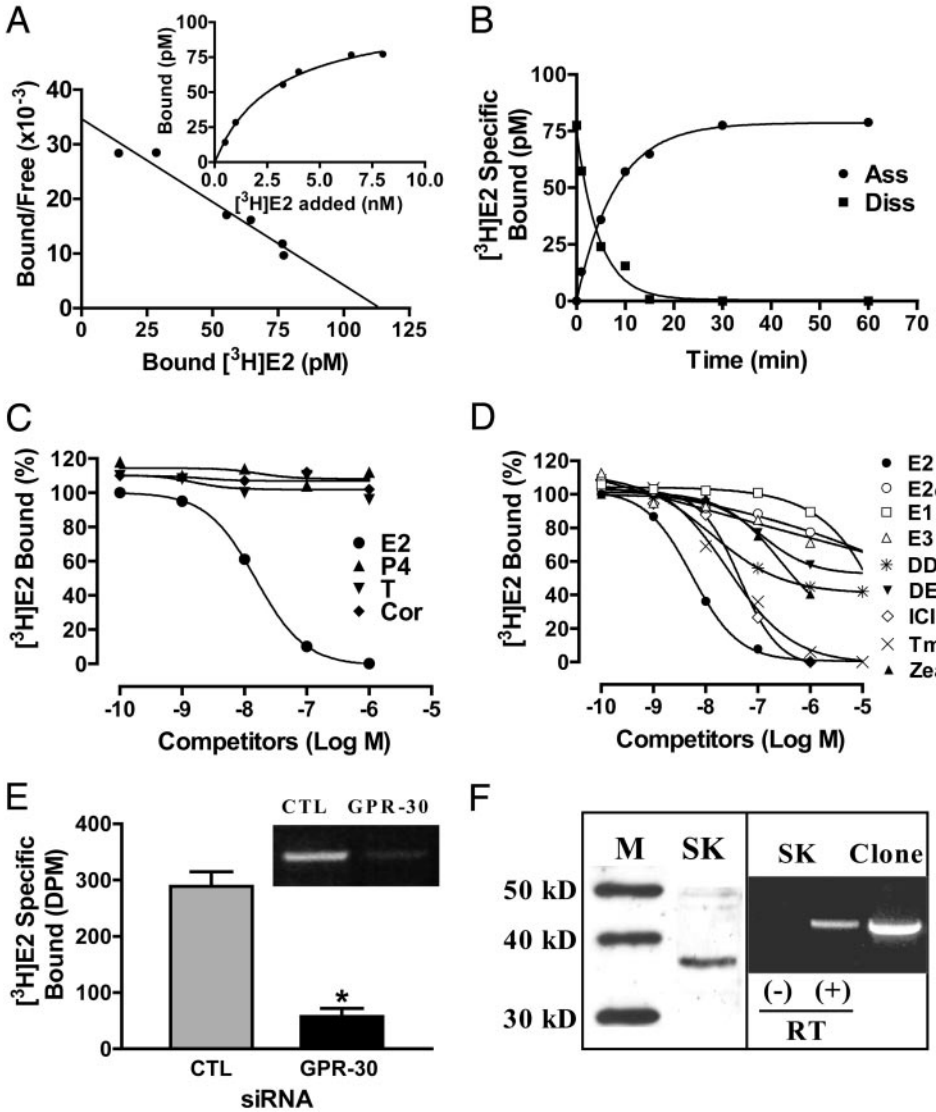

FIG. 1. Estrogen binding characteristics of plasma membranes from SKBR3 cells (ERα-, ERβ-, GPR30+). A, Representative saturation curve and Scatchard plot of specific [^{3}H]E2 binding. B, Time course of association (Ass) and dissociation (Diss) of specific [^{3}H]E2 binding. C, Competition curves of steroid binding expressed as a percentage of maximum specific [^{3}H]E2 binding. D, Competition curves of binding by estrogens. E1, E1; DES, diethylstilbestrol; Zea, zearalenone E, Effects of transfection with 100 nM GPR30 siRNA (GPR siRNA) on specific [^{3}H]E2 binding to cell membranes 18 h later. CTL, Nonspecific control siRNA. *Inset*, RT-PCR results. F, Detection of GPR30 protein in SKBR3 (SK) cell membranes by Western blot analysis and GPR30 mRNA in cells by RT-PCR. M, Protein molecular weight standards; clone, control GPR30 plasmid; (−)RT, lacking reverse transcriptase. (n = 6; * $P < 0.05$, Student's *t* test).

no affinity for the receptor at concentrations up to 1 μM (Fig. 1C). As observed previously for some nERs and other estrogen membrane receptors, the inactive estradiol isomer, E2α, failed to significantly displace E2 binding (Fig. 1D). An unexpected finding was that the other natural estrogens, E3 and E1, had very low affinities for the receptor, less than 0.1% that of E2. In contrast, ICI and Tmx were effective competitors, with relative binding affinities (RBAs) approximately 10% that of E2. Interestingly, comparatively low concentrations (0.1–1 μM) of the xenoestrogen o,p′-DDE also caused significant displacement of [^{3}H]E2 binding. Treatment of the cells with GPR30 siRNA caused decreased expression of GPR30 mRNA that was accompanied by an 80% decrease in specific [^{3}H]E2 membrane binding (Fig. 1E). A major immunoreactive band was detected in plasma membranes of SKBR3 cells by Western blotting, and GPR30 mRNA was detected in SKBR3 cells by RT-PCR (Fig. 1F). Immunocytochemical analysis of whole SKBR3 cells demonstrates that GPR30 protein is concentrated at the cell periphery, consistent with its function as a membrane receptor (see Supplemental Fig. 1B and the supporting text, published on The Endocrine Society's Journals online web site at http://endo.endojournals.org). The absence of ERα and ERβ mRNA in SKBR3 cells was confirmed by PCR using three sets of specific primers for each receptor (see Supplemental Fig. 1A and supporting text).

Estrogen binding to plasma membranes of HEK293 cells transfected with GPR30

HEK293 cells do not express GPR30 mRNA and protein (Fig. 2A) or ERα or ERβ mRNA (see Supplemental Fig. 2 and supporting text) as shown by RT-PCR and Western blot analysis. Therefore, we investigated whether they acquire the ability to specifically bind E2 upon transfection with a cDNA encoding GPR30. Expression levels of GPR30 mRNA and protein in the transfected HEK293 cells and membranes (Fig. 2A) were similar to those in SKBR3 cells (data not shown). Significant amounts of specific estrogen binding were detected in plasma membranes of cells transfected with

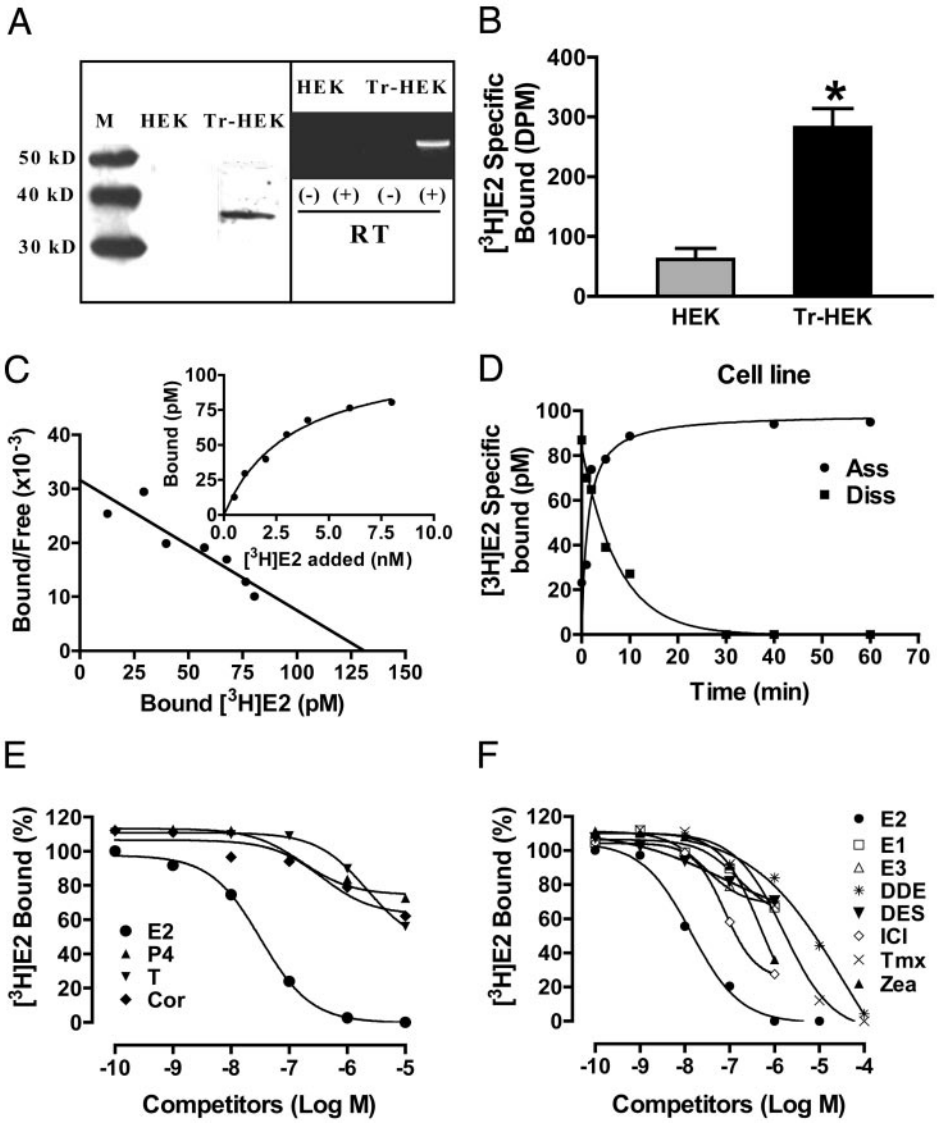

FIG. 2. Estrogen binding to plasma membranes of HEK293 cells (ERα−, ERβ−) stably transfected with GPR30. A, Detection of GPR30 protein in transfected (Tr-HEK) cell membranes by Western blot analysis and GPR30 mRNA in cells by RT-PCR. HEK, Untransfected control HEK293 cells. B, Single-point assay of specific [^{3}H]E2 binding to cell membranes of HEK293 cells and cells transfected with GPR30 (see key in A). C, Representative saturation curve and Scatchard plot of specific [^{3}H]E2 binding to membranes of transfected cells. D, Time course of association (Ass) and dissociation (Diss) of specific [^{3}H]E2 binding. E, Competition curves of steroid binding. F, Competition curves of estrogen binding. (see Fig. 1 for key for steroid abbreviations) (n = 6, *, $P < 0.05$, Student's t test).

GPR30, whereas negligible specific binding was detected in the plasma membranes of untransfected cells (Fig. 2B). Saturation analysis showed high-affinity (Kd 3.3 nM), saturable (Bmax 100 pM) specific [^{3}H]E2 binding, and the Scatchard plot indicated a single binding site (Fig. 2C). The kinetics of association/dissociation of [^{3}H]E2 binding to the recombinant protein produced in HEK393 cells were rapid with halftime ($t_{½}$ sec) of 1.3 and 4.9 min (Fig. 2D). Competitive binding studies showed that binding was specific for E2 and certain estrogens. E1, E3, diethylstilbestrol, and nonestrogenic compounds failed to significantly displace [^{3}H]E2 at concentrations up to 10 μM, whereas Tmx, ICI, o,p′-DDE, and the mycotoxin estrogenic compound, zearalenone, displayed significant binding affinity (Fig. 2, E and F). The binding affinities of most of the compounds for the recombinant GPR30 protein in plasma membranes from transfected HEK293 cells were similar to their affinities for the wild-type GPR30, with the exception of the antiestrogens ICI and Tmx, which displayed somewhat lower binding affinities. Immunocytochemical analysis showed that GPR30 was detected only on the plasma membranes of transfected whole cells (see Supplemental Fig. 2 and supporting text).

Activation of signal transduction pathways

Coincubation with 100 nM E2 caused a significant increase in specific [^{35}S]GTPγ-S binding to membranes from HEK293 cells transfected with GPR30 but not to membranes of untransfected cells (Fig. 3A), whereas E2α treatment was ineffective (Thomas, P., Y. Pang, and J. Dong, unpublished observations). Immunoprecipitation of the membrane-bound [^{35}S]GTPγ-S with specific G protein α-subunit antibodies showed that the majority of the GTPγ-S is bound to the α_s-subunit on E2 treatment (Fig. 3B), which suggests the receptor activates a stimulatory G protein (G_s). Treatment with 100 nM E2 caused a similar increase in specific [^{35}S]GTPγ-S binding to membranes of SKBR3 cells expressing wild-type GPR30, whereas E1 and E3, which display low binding affinities for the mER in this cell line, failed to activate the G proteins (Fig. 3C). Two other compounds with higher RBAs for the mER, Tmx and DDE, also significantly increased [^{35}S]GTPγ-S binding to SKBR3 cell membranes, suggesting they mimic the actions of E2 on this nonclassical signaling pathway. Adenylyl cyclase activity, measured as an increase in cAMP content, was significantly increased in transfected HEK293 cells after 15 min treatment with 100 nM E2 and ICI but not in untransfected cells (Fig. 3D), in agreement with previous findings in SKBR3 cells (26). Moreover, the estrogen-induced increase in cAMP concentrations was blocked by prior treatment with activated CTX (Fig. 3E), which is consistent with coupling of GPR30 to a stimulatory G protein and activation of this pathway. E1 and E3 did not alter cAMP production, but other compounds with higher RBAs for the mER in transfected cells, Tmx, ICI, and o,p′-DDE significantly increased cAMP (Fig. 3F).

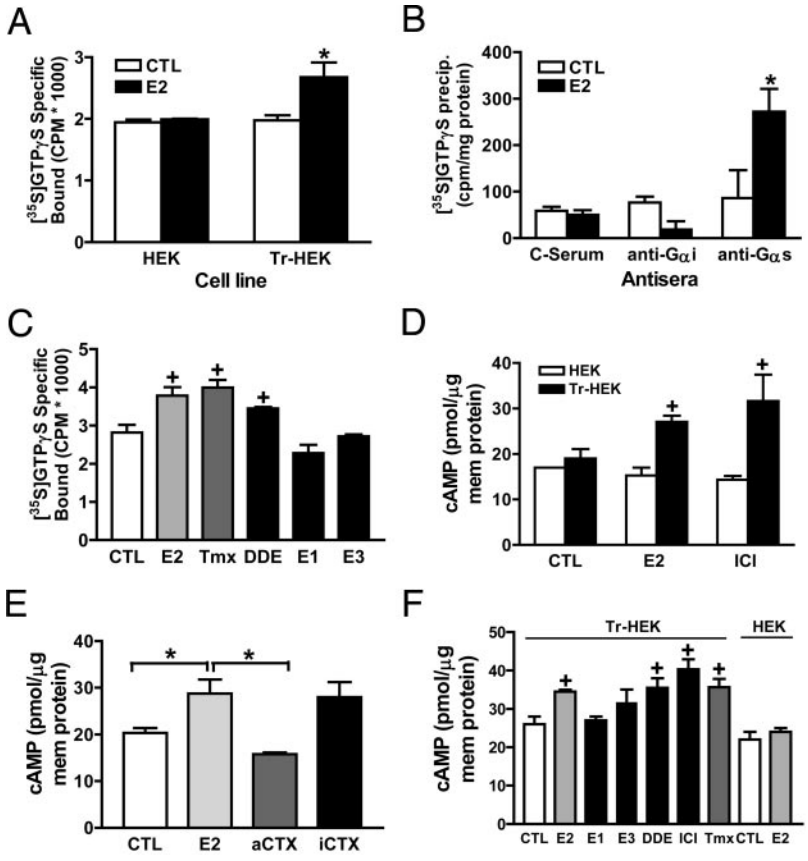

FIG. 3. Coupling of GPR30 to G proteins and activation of adenylyl cyclase in SKBR3 and transfected HEK293 cells. A, Effects of 20 min treatment with E2 (100 nM) on specific [^{35}S]GTPγ-S binding to G proteins in membranes of transfected (Tr-HEK) and untransfected (HEK) HEK293 cells. B, Immunoprecipitation of [^{35}S]GTPγ-S bound to G proteins transfected with specific $G\alpha_s$ (anti-$G\alpha_s$) and $G\alpha_i$ (anti-$G\alpha_i$) G protein antibodies or control rabbit serum (C-serum). CTL, Control untreated membranes. HEK293 cells were treated with E2 or media (CTL) before membrane solubilization. C, Effects of 20 min treatment with various estrogenic compounds (100 nM) on specific [^{35}S]GTPγ-S binding to membranes of SKBR3 cells. D, Effects of 20 min treatment with 100 nM E2 or ICI on cAMP production by transfected (Tr-HEK) and untransfected HEK293 cells. E, Effects of 20 min pretreatment with 10 μg/ml CTX (aCTX, active; iCTX, inactivated) on cAMP production by transfected HEK293 cells in response to 100 nM E2. F, Effects of 20 min treatment with various estrogenic compounds (100 nM) on cAMP production by transfected (TR-HEK) and untransfected HEK293 cells (n = 6; *, $P < 0.05$, Student's t test; †, $P < 0.05$, one-way ANOVA).

Modulation of estrogen binding to membranes

There was more than a 50% decrease in [^{3}H]E2 binding to membranes of transfected cells after pretreatment with either CTX or GTPγ-S (Fig. 4, A and B), which causes uncoupling of G proteins from their receptors. Plasma membranes of SKBR3 cells cultured for 2 d in FBS-free media had low amounts of specific E2 binding. Pretreatment of the cells with 200 nM P4 or E2 for 16 h in FBS-free media caused dramatic 9- and 2.5-fold increases in receptor binding, respectively, which paralleled the increases in GPR30 mRNA and protein expression (Fig. 4, C and D). The hormonal treatments did not alter ERα and ERβ mRNA expression levels, which remained undetectable by RT-PCR analysis. However, a transcript for the nuclear P4 receptor (nPR-A) was detected by RT-PCR (see Supplemental Fig. 3 and supporting text). Saturation analysis and Scatchard plotting also showed the presence of a high-affinity (Kd 6.3 nM) limited capacity (Bmax 1.4 nM) single E2 binding site on human placental plasma membranes (Fig. 4E). Immunocytochemistry and Western blots showed specific GPR30 staining of human placenta synctiotrophoblast and other cell types and a 38-kDa protein band, respectively (Fig. 4F).

Absence of GPR30 in several estrogen-responsive cells

GPR30 mRNA was not detected by RT-PCR in three well-characterized models of nonclassical E2 action: sheep endothelial cells (9) and rat pituitary (GH_3/B6, F10, 8) and hypothalamic (SK-N-SH) (31) cells (see Supplemental Fig. 4 and supporting text).

Discussion

The steroid binding and signal transduction experiments clearly demonstrate that GPR30 has all the characteristics of mERs that distinguish them from other previously described estrogen binding moieties. The finding that the plasma membranes of SKBR3 cells lacking ERα and ERβ mRNAs and proteins (Supplemental Fig. 1) (24, 32–35) but expressing GPR30 (24) show high-affinity, limited capacity, displaceable, specific binding to E2 suggests the presence of a previously unknown ER in these cells. The orphan GPCR-like protein, GPR30, is a candidate for a novel ER because it is expressed and is a requisite signaling intermediary in estrogen-dependent activation of adenylyl cyclase and epidermal growth factor receptor (EGFR) in SKBR3 in breast

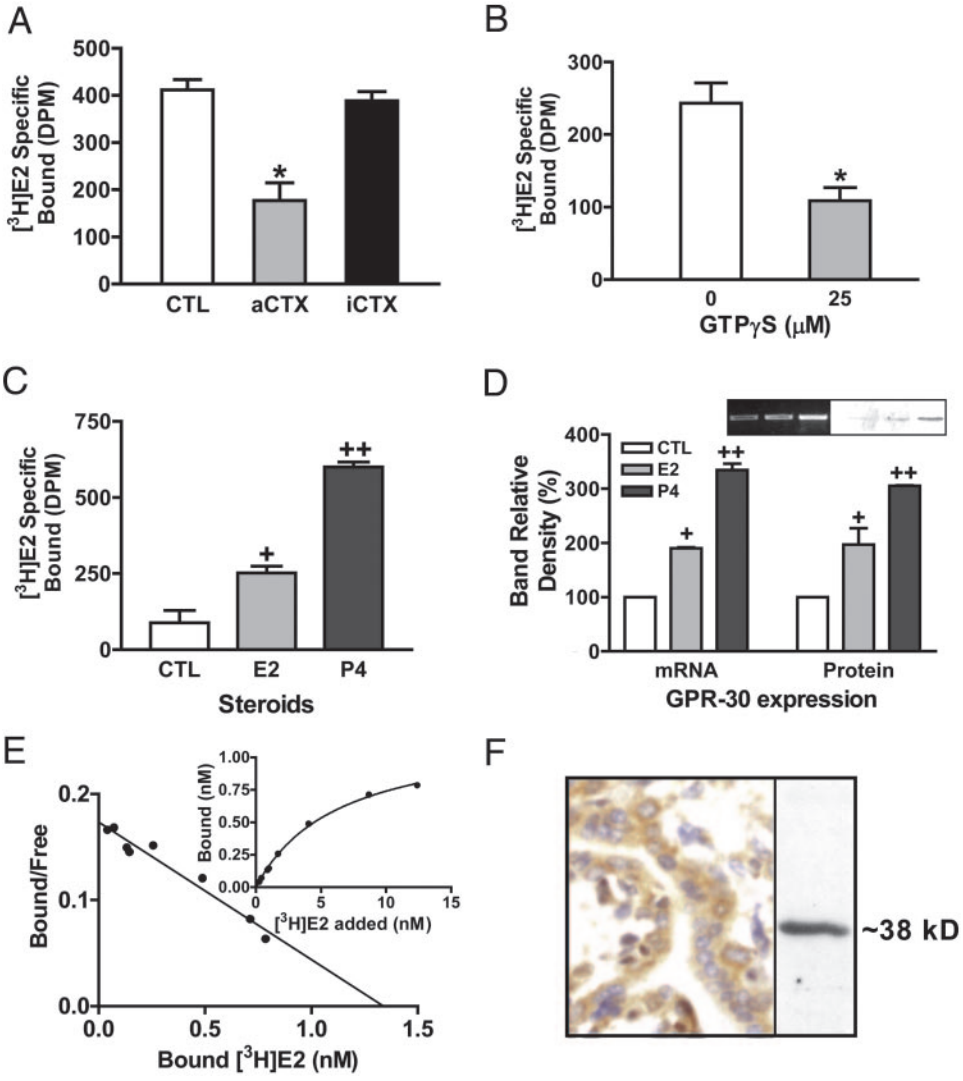

FIG. 4. A, Effects of pretreatment with 10 μg/ml CTX (aCTX, active; iCTX, inactivated) on specific [^{3}H]E2 binding to cell membranes of transfected HEK293 cells. B, Effects of pretreatment with 25 μM GTPγ-S on specific [^{3}H]E2 binding to cell membranes of transfected HEK293 cells. C and D, Effects of 16 h treatment of SKBR3 cells with 100 nM P4, E2, or media alone (CTL) on mER binding activity (C) and GPR30 mRNA and protein expression by semiquantitative RT-PCR (D). E, Saturation curve and Scatchard plot of specific [^{3}H]E2 binding to human placenta cell membranes. F, Immunocytochemistry and Western blot of human placental tissues and cell membranes, respectively, using a monoclonal GPR30 antibody (n = 6; *, $P < 0.05$, Student's t test; †, $P < 0.05$, ††, $P < 0.001$, one-way ANOVA).

cancer cells that lack known ERs (Supplemental Fig. 1) (24–26). Evidence in support of this hypothesis was obtained from experiments showing that both P4-induced increases and siRNA-induced decreases in GPR30 expression in SKBR3 cells were accompanied by parallel changes in specific E2 binding. Moreover, the observation that a tissue that expresses GPR30, human placenta (19), also shows similar high-affinity E2 binding in its plasma membranes suggests GPR30 is a functional mER in human tissues. Direct evidence that GPR30 binds estrogens and has the signal transduction characteristics of a mER was obtained from the studies with transfected HEK293 cells, which lack both nuclear ERα and ERβ (Supplemental Fig. 2) (36). Untransfected HEK293 cells showed negligible E2 binding and E2 activation of G proteins in their membrane fractions. However, plasma membranes of cells transfected with GPR30 displayed specific E2 binding almost identical with that of the SKBR3 cells and characteristic of mERs identified previously (20–25).

The steroid binding characteristics of the recombinant GPR30, like those of the wild-type receptor, fulfill all the criteria for its designation as a mER. Both forms of GPR30 display high affinity and saturable E2 binding with Kds of approximately 3.0 nM, similar to the affinities of other mERs (28). E2 consistently occupies a single binding site in cell and tissue membrane preparations as shown in the Scatchard plots. Moreover, E2 readily dissociates from the binding site, a critical feature of steroid receptors. The kinetics of association and dissociation were rapid, with $t_{1/2}$ less than 10 min, which is characteristic of membrane steroid receptors (27, 29). In addition, it was demonstrated that GPR30 can act as a mER in transfected cells to transduce the signals of estrogenic compounds with high RBAs for the receptor, resulting in activation of a stimulatory G protein and up-regulation of adenylyl cyclase activity, whereas E3 and E1, which have low RBAs for the receptor, were inactive. Finally, the decrease in mER binding in transfected cell membranes observed after treatment with agents causing uncoupling of G proteins from GPCRs, GTPγ-S, and CTX (30, 37), indicates the mER is directly coupled to G protein and is a GPCR, consistent with its identity as GPR30. To our knowledge this is the first report of a protein structurally unrelated to nuclear ERs that has the characteristics of an ER. The discovery of this novel mER provides a plausible mechanism by which estrogens can initiate rapid steroid actions at the cell surface and act in certain nER-negative target cells. The existence of this mER-mediated signaling pathway also explains some of the pleiotropic actions of estrogens in breast and other estrogen target tissues.

The identification of a novel mER that activates a G_s indicates that estrogen and antiestrogen signaling in human breast cancer is more complex than previously recognized. Several characteristics of the receptor have important implications for the development of the disease. Estrogens can activate pathways involved in proliferative responses, such as MAPK via EGFR transactivation and c-*fos* expression, in nER-negative breast cancer cells via GPR30 (24, 38). Recent studies show that G_s-coupled GPCRs, in addition to G_q protein-coupled ones, can stimulate EGFR transactivation (39). GPR30 transactivates the EGFR by release of heparan-bound epidermal growth factor from the cell surface by a G$\beta\gamma$-Src-Shc signaling pathway (26). G_s-coupled receptors can signal to Src and Shc via β-arrestin scaffolds (40), and this could provide an alternative mechanism by which they transactivate EGFR. Estrogen also attenuates the EGFR-to-ERK signaling axis by cAMP-dependent signaling (25) via GPR30, indicating an additional role of this novel receptor in regulating EGF action. Interestingly, GPR30 is abundantly expressed in human primary breast carcinomas and breast cancer cells lines that are nER positive but shows no or minimal expression in ER-negative breast cancer tissues and cells (19).

The observation that GPR30 is up-regulated by P4 confirms the results of a previous study (41) and raises the possibility of coordinate hormonal control by P4 of GPR30 and the nERs in cells expressing both receptors. The nuclear P4 receptor is a potential mediator of the P4 up-regulation of GPR30 in SKBR3 cells, whereas estrogen is presumably acting via a nER-independent mechanism, possibly through GPR30 and activation of adenylyl cyclase (42), although additional experiments will be required to confirm this. Environmental contaminants that are weak nER agonists (xenoestrogens), such as polychlorinated biphenyls (PCBs) and the DDT derivative, o,p′-DDE, have also been implicated in tumorigenesis in breast and other estrogen target tissues, presumably via nER activation (43, 44). The finding that o,p′-DDE is an agonist for GPR30 receptor activity demonstrates that xenoestrogens can also activate this alternative estrogen signaling pathway in breast cancer cells, as has been shown for mERs in other tissues (14, 27). Interestingly, the RBA of o,p′-DDE binding to GPR30 and nERs are similar (45), possibly indicating a similar susceptibility of these two estrogen signaling pathways to interference by this xenoestrogen. Interference with nontraditional steroid actions by xenoestrogen binding to steroid membrane receptors has previously demonstrated for the mPR on fish gametes (46). The present results extend this novel mechanism of endocrine disruption to a second GPCR-like steroid receptor and suggest that interactions of xenoestrogens with ligand binding sites is a shared feature of both nuclear and GPCR-like steroid receptors.

The results also have profound implications for the treatment of breast cancer. Patients treated for ER-positive breast cancers are frequently administered the antiestrogen Tmx to prevent reoccurrence of tumor growth (47). However, our results show that Tmx and the nER antagonist ICI have opposite actions on the alternative mER-mediated pathway, acting as estrogen agonists by binding to GPR30 and activating G proteins. The agonist activity of the pure ER antagonist ICI was expected because we found it has a high RBA for GPR30 and it has previously been shown to mimic estrogen actions initiated at the cell membrane in a broad range of targets, including SKBR3 cells (25, 28). The identification of GPR30 as a mER facilitates investigations on its role in the physiology and pathology of breast, prostate, placenta, ovarian, neural, and vascular tissues and also provides a potential target for therapeutic intervention.

The discovery of a second class of ERs unrelated to nERs provides an entirely new model to explore the structural requirements for estrogen binding and activation of receptor proteins. The marked differences in the RBAs of some es-

trogens to GPR30 and their affinities to ERα and ERβ and a third distinct ER subtype in fishes was expected, considering the lack of structural similarity between GPR30 and the nERs (48, 49). Initial binding studies with a limited number of nER ligands suggests GPR30 has a higher specificity for E2 binding than the nERs; all the other estrogens tested had RBAs of 10% or lower for the membrane receptor. Interestingly, the presence of other functional groups on the D ring of the steroid molecule in the vicinity of the 17 position or alteration of the 17β-OH configuration dramatically decreases binding to GPR30, E1, E3, and E2α having RBAs less than 1% that of E2, whereas these changes result in relatively modest decreases in binding affinity to the nERs (48, 49). In contrast, alteration of the four carbon ring structure to produce Tmx or addition of a large side chain at the 7 position to produce ICI caused only minor decreases in RBA to GPR30, similar to that observed with some nERs (48, 49).

Although the identities of mERs remain uncertain and a topic of intense debate, there is a growing body of evidence indicating a role for nER or ER-like proteins in many tissues showing rapid, cell surface-mediated estrogen actions (7–12). The absence of GPR30 in several well-characterized cell models of rapid, nongenomic estrogen actions, sheep endothelial cells and rat pituitary and hypothalamic cells (Supplemental Fig. 3) (8, 9, 31), suggests that not all these estrogen actions are mediated via GPR30 and that at least two classes of mERs are present in vertebrates. The physiological significance of the presence of both types of mERs in certain cell types, such as MCF-7 cells is unclear (38, 50). However, the Kd of E2 binding to membranes of SKBR3 and HEK293 cells expressing GPR30 in the present study ranged from 2.7 to 3.3 nM, 10-fold higher than that reported for membranes of Chinese hamster ovary cells transfected with ERα (7) and may be indicative of a higher threshold concentration for activation of GPR30-dependent signaling pathways by estrogens.

GPR30 and the mPRs we discovered previously represent two distinct classes of GPCRs with no sequence homology and few apparent structural similarities. Thus, there is no indication that these mERs and mPRs arose from a common ancestor, unlike members of the nuclear steroid receptor superfamily (51). The C-terminal domain of GPR30 is longer than that of the mPRs (47 *vs.* 12 amino acids), the DRY sequence involved in signal transduction in intracellular loop 2 is absent in the mPRs, whereas the length of the second extracellular loop in GPR30 (10–20 amino acids) is shorter than that of the mPRs (~50 amino acids). On the other hand, both receptors have seven-transmembrane domains, N-terminal glycosylation sites, and two conserved cysteines in the first two extracellular loops, which can form disulfide bonds to help stabilize the structure, basic features of GPCRs (19, 20). In addition, both receptors have large N-terminal extracellular domains, 57–75 amino acids long, that could possibly be involved in ligand binding. The discovery of two apparently unrelated families of GPCR-like membrane steroid receptors raises interesting evolutionary questions regarding their origins, such as whether the ancestral proteins were receptors for nonsteroidal ligands that subsequently acquired new functions (neofunctionalization) to bind and transduce specific steroid signals and, if so, whether the receptors have retained their responses to these nonsteroidal ligands. Information on the tissue distribution, regulation, and ligand specificity of these receptors should provide insights into the evolution and functions of this new class of steroid receptors.

Acknowledgments

Received August 16, 2004. Accepted November 4, 2004.

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This work was supported by American Cancer Society Award (to E.J.F. and P.T.) and Environmental Protection Agency STAR Grant R-82902401 (to P.T.).

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