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Activation of the Novel Estrogen Receptor G Protein-Coupled Receptor 30 (GPR30) at the Plasma Membrane

E. Filardo, J. Quinn, Y. Pang, C. Graeber, S. Shaw, J. Dong, and P. Thomas

Departments of Medicine (E.F., J.Q., C.G.) and Pediatrics (S.S.), Brown University, Providence, Rhode Island 02903; and Marine Science Institute (Y.P., J.D., P.T.), University of Texas at Austin, Port Aransas, Texas 78373

G protein-coupled receptor 30 (GPR30), a seven-transmembrane receptor (7TMR), is associated with rapid estrogen-dependent, G protein signaling and specific estrogen binding. At present, the subcellular site of GPR30 action is unclear. Previous studies using antibodies and fluorochrome-labeled estradiol (E2) have failed to detect GPR30 on the cell surface, suggesting that GPR30 may function uniquely among 7TMRs as an intracellular receptor. Here, we show that detectable expression of GPR30 on the surface of transfected HEK-293 cells can be selected by fluorescence-activated cell sorting. Expression of GPR30 on the cell surface was confirmed by confocal microscopy using the lectin concanavalin A as a plasma membrane marker. Stimulation of GPR30-expressing HEK-293 cells with 17β-E2 caused sequestration of GPR30 from the cell surface and resulted in its codistribution with clathrin and mobilization of intracellular calcium stores. Evidence that GPR30 signals from the cell surface was obtained from experiments demonstrating that the cell-impermeable E2-protein conjugates E2-BSA and E2-horseradish peroxidase promote GPR30-dependent elevation of intracellular cAMP concentrations. Subcellular fractionation studies further support the plasma membrane as a site of GPR30 action with specific [^{3}H]17β-E2 binding and G protein activation associated with plasma membrane but not microsomal, or other fractions, prepared from HEK-293 or SKBR3 breast cancer cells. These results suggest that GPR30, like other 7TMRs, functions as a plasma membrane receptor. (*Endocrinology* 148: 3236–3245, 2007)

SEVEN-TRANSMEMBRANE RECEPTORS (7TMRs) comprise the largest family of cell surface receptors, with more than 800 members in the human genome (1). 7TMRs are activated by a wide spectrum of extracellular signals, including light, odorants, vasoactive substances, and peptide hormones. Simple mechanistic models depict 7TMRs as receptors that exist as active or inactive conformers (2, 3). In general, ligand-unoccupied receptors favor an inactive conformation that is bound to a heterotrimeric G protein complex consisting of a GDP-bound Gα-GTPase subunit protein and associated Gβγ dimer (4). During engagement of their cognate ligands, 7TMRs are transformed into an active conformation, in which the 7TMR acts as a GDP/GTP exchange factor and promotes the release of GDP and the binding of GTP to the Gα subunit. Subsequent catalysis of the bound GTP by the Gα-GTPase results in the dissociation of the Gα subunit protein from the Gβγ components, with activated Gα-GTPase stimulating plasma membrane-associated enzymes or ion channels, which, in turn, promote second-messenger signaling (4). Free Gβγ subunit protein complexes released from activated 7TMRs also serve as a functional signaling unit that may stimulate matrix metalloproteinase (MMP)-dependent cleavage and release of plasma membrane-tethered epidermal growth factor (EGF)-like polypeptides from the cell surface (5). This ultimately results in the activation of EGF receptors (EGFRs) and subsequent downstream activation of EGFR-regulated lipid and protein kinases and is referred to as 7TMR-mediated EGFR transactivation (5, 6). 7TMR signaling is attenuated after ligand activation as a consequence of receptor sequestration and internalization into clathrin-coated vesicles, events consistent with the plasma membrane as a site of action for 7TMRs.

Recent evidence supports a role for the 7TMR G protein-coupled receptor 30 (GPR30) in rapid estrogen action (7) and in breast cancer metastasis (8). Both ectopic expression and RNA interference studies have shown specific estrogen binding (9, 10), activation of G proteins (9, 11) and adenylyl cyclase (9, 12, 13), and release of pro-heparin-bound (HB)-EGF (11). These results suggest that GPR30 acts similarly to other 7TMRs, as a cell surface receptor. However, analysis of GPR30-dependent ligand binding with Alexa dye 594-conjugated 17α-[4-aminomethyl-phenyl-ethnyl]-estra-1,3,5(10)-triene 3,17β-diol required detergent permeabilization, implying that GPR30 may function uniquely among 7TMRs as an intracellular receptor (10). This study further showed that expression of a fusion protein constructed of GPR30 and green fluorescent protein resulted in a diffuse intracellular staining pattern that exhibited colocalization with the KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum retention marker. In general, 7TMRs are well expressed within the endoplasmic reticulum and within submembranous vesicles, observations that have been associated with their trafficking to the plasma membrane during receptor biogenesis (14) and their reuptake after agonist stimulation (15). In this regard, intra-

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Abbreviations: β1AR, β Adrenergic receptor 1; con A, concanavalin A; DAPI, 4′,6-diamidino-2-phenylindole; E2, 17β-estradiol; EGF, epidermal growth factor; EGFR, EGF receptor; Endo H, endoglycosidase H; ER, estrogen receptor; GPR30, G protein-coupled receptor 30; HA, hemagglutinin; HB-EGF, heparan-bound EGF; HRP, horseradish peroxidase; mAB, monoclonal antibody; MMP, matrix metalloproteinase; NADPH, reduced NAD phosphate; PNGase F, N-glycosidase F; PRF, phenol red-free; TBS-T, Tris-buffered saline containing 0.05% Tween 20; 7TMR, seven-transmembrane receptor.

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cellular staining of endogenous 7TMRs, as observed in cytochemical and histochemical analyses, is common (16–18) and may reflect slow egress during receptor biosynthesis and receptor downmodulation during reuptake.

GPR30 bears the structural characteristics that define 7TMRs, with an amino-terminal signal peptidase cleavage site and seven hydrophobic domains that serve to anchor GPR30 into the plasma membrane. Cleavage and release of signal peptides from nascent 7TMRs does not appear to be an absolute requirement for the trafficking of 7TMRs to the plasma membrane, because small epitope tags fused to the amino-terminal end of 7TMRs, *e.g.* hemagglutinin (HA)-tagged adrenoceptors (19) and Flag-tagged olfactory receptors (20), are retained at the plasma membrane. Thus, removal of a signal peptide sequence is not necessary for surface expression, and the structural determinants encoded by 7TMRs that define surface expression are unclear. However, an intracellular location appears to be incompatible with the mechanism by which 7TMRs associate with heterotrimeric G proteins and promote intracellular signaling. Previous work has shown that GPR30 is a $G\alpha_s$ protein-coupled receptor (9) that also triggers proHB-EGF release via $G\beta\gamma$ subunit protein and Src-like kinases (for review, see Refs. 7 and 21). Thus, GPR30 regulates the activity of two plasma membrane-associated enzymes, adenylyl cyclase, which is an integral membrane protein (12), and MMPs, which are either integrated into the plasma membrane or associated with its exoplasmic surface (22).

In this study, we investigate more thoroughly the subcellular location of GPR30 and its site of action by using a variety of experimental approaches. As a rule, 7TMRs are poorly expressed in endogenous settings, and most of the information regarding their function and trafficking has been developed from their study in heterologous cell models. Therefore, experiments were conducted with HEK-293 cells transfected with HA-tagged GPR30 as well as with SKBR3 cells expressing endogenous GPR30. The experiments outlined here support the concept that GPR30 is a plasma membrane receptor that promotes rapid estrogen signaling.

Materials and Methods

Animals and cell culture

All animal handling and experimental procedures with live animals were reviewed and approved by the Institutional Animal Care and Use Committee at Rhode Island Hospital. As described below (see below, *Antibodies*), hybridomas were prepared from female BALB/C mice obtained from Charles River Laboratories (Wilmington, MA). Human SKBR3 breast cancer cells and HEK-293 embryonal kidney cells were obtained from the American Tissue Culture Collection (Manassas, VA) and cultured in phenol red-free (PRF) DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum as described previously (9).

Construction of HA-GPR30 protein and generation of stable HEK-293 transfectants expressing surface HA-GPR30

An HA-epitope tag was incorporated at the amino terminus of human GPR30 by PCR stitching using molecular clone GPR-BR (GenBank accession no. U63917) (23) as template. For this purpose, a PCR product encoding full-length GPR30 protein was synthesized using forward (5′ CACCGAATTCAGAGACATG**TACCCATACGACGTCCCAGACTACGC**GGATGTGACTTCCCAAGCC 3′) and reverse (5′ CAAGGCTGTCTAGACGGCACTGCTGAACCT 3′) oligonucleotide primers containing *Eco*RI and *Xba*I restriction sites, respectively (underlined). The nucleotide sequence encoding the HA epitope YPYDVPDYA nonamer is shown in bold. The amplified product was cleaved with *Eco*RI and *Xba*I, purified by agarose gel electrophoresis, and subcloned into pcDNA3.1Zeo (+) for expression (Invitrogen, La Jolla, CA). The resultant molecular clone, named HA-GPR30Zeo, was transfected into HEK-293 cells using Lipofectamine (Invitrogen). Three days after transfection, 500 μg/ml Zeocin (Invitrogen) was added to the growth media. Two weeks later, more than 100 drug-resistant colonies were counted, and all of the cells in these colonies were pooled together and propagated by cultivation in PRF-DMEM/F-12 with 5% fetal bovine serum in the absence of the drug. Immunofluorescence analysis with the HA antibody showed that a relatively small proportion of the cells displayed significant cell surface expression of HA-GPR30. Therefore, transfected cells expressing HA-GPR30 on the cell surface were enriched from the Zeocin-resistant population by fluorescence-activated cell sorting using rabbit HA-specific polyclonal antibody (see *Immunofluorescent analysis*). Transfectants were sorted based on their mean intensity fluorescence at which the highest staining (upper one percentile) cells were gated under sterile conditions, expanded in culture, and then subjected to a second round of sorting. The resultant cell line, HEK-293 (HA-GPR30), was passed for several months in cell culture and remained cell surface positive for GPR30. HEK-293 [HA-tagged β adrenergic receptor 1 (HA-β1AR)] cells were generated in a similar manner by transfection and drug selection using HA-β1AR plasmid DNA kindly provided by Brian Kobilka (Stanford University, Stanford, CA). Flow cytometry-assisted selection was not necessary to establish detectable HA-β1AR on the surface of HEK-293 cells.

Antibodies

Rabbit anti-HA epitope antibody and agarose beads conjugated with HA monoclonal antibody (mAB) H7 were purchased from Abcam (Cambridge, MA) and Sigma (St. Louis, MO), respectively. Goat $G\alpha_s$ subunit-specific antibodies (sc-26766) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified rabbit GPR30 C-terminal peptide antibodies were described previously (11). Monospecific GPR30 antibody, 2F2, was generated in BALB/C mice that were immunized with synthetic peptide CAVIPDSTEQSDVRFSSAV from the C terminus of human GPR30 that was conjugated to keyhole limpet hemocyanin using the bifunctional cross-linker *N*-maleimidobenzoyl-*N*-hydroxysuccinimide ester. Mice were injected intraperitoneally at 3-wk intervals with GPR30 peptide-keyhole limpet hemocyanin conjugate mixed in Titer-max adjuvant (Sigma) and boostered intravenously with peptide 3 d before fusion. Spleens were then harvested, and lymphocytes were isolated and fused with mouse 8653 myeloma cells using polyethylene glycol and seeded into semi-solid containing selective media (hypoxanthine/aminopterin/thymidine) as described in the Clonal Cell-Hy Hybridoma Cloning kit (Stem Cell Technologies, Vancouver, British Columbia, Canada). Supernatants from 952 resultant hybridomas were screened by ELISA for the presence of antibodies reactive to immobilized GPR30 C-terminal peptide. Hybridoma supernatants that were peptide reactive were rescreened for their capacity to immunoprecipitate recombinant GPR30 protein. Hybridomas positive by immunoprecipitation were subcloned in semi-solid media. GPR30 mAB 2F2 was purified from recloned culture supernatants by affinity peptide chromatography.

Immunofluorescent analysis

Adherent cultures of HEK-293 cells stably transfected with GPR30 or vector were detached in PBS supplemented with 0.5 mM EDTA and collected by centrifugation. The cell pellet was resuspended in ice-cold PRF DMEM/F-12 media, recentrifuged, and washed in the same media for two additional cycles. Cells were then resuspended at 10^6/ml and incubated with rabbit HA antibody (Abcam) or control rabbit IgG at 0.5 μg/ml for 1 h at 4 C. Cells were then washed free of primary antibody by three successive washes in ice-cold PRF-DMEM/F-12 media and then exposed to Alexa 488-conjugated goat antirabbit IgG (Invitrogen) at 2.5 μg/ml for 1 h at 4 C. After this incubation period, cells were then washed three times in PRF-DMEM/F-12 media, and surface fluorescence was measured on 10^4 cells using a FACSort analyzer (BD Biosciences, Franklin Lakes, NJ) with an argon-ion 488 nm laser and CellQuest software.

To measure the surface expression of GPR30, HEK-293 cells stably

transfected with HA-GPR30 or HA-β1AR were plated in PRF-DMEM/F-12 media on fibronectin-coated glass coverslips and then fixed in 4% paraformaldehyde-PBS. Cells were then reacted with rabbit anti-HA antibodies (Abcam) or control rabbit IgG (0.5 μg/ml) for 30 min at 22 C. After incubation in primary antibody, cells were washed three times in PBS and stained with Alexa 594-derivatized concanavalin A (con A) (100 μg/ml) and Alexa 488-derivatized antirabbit antibodies diluted at 1:800 for 30 min (Invitrogen). Cells were then washed in PBS and mounted in Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA). To measure trafficking of GPR30 from the cell surface, quiescent, adherent HEK-293 (HA-GPR30) cells were labeled with rabbit anti-HA antibodies, as above, then stimulated with 17β-E2 (1 nM) for various lengths of time, and fixed in 4% paraformaldehyde. Fixed cells were treated for 60 sec in 0.05% Triton X-100 in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl and blocked in 5% BSA for 15 min. Permeabilized cells were exposed to anticlathrin mouse mAB (ab2371; Abcam) (6 μg/ml) for 30 min, washed, and stained with Alexa 488 antimouse and Alexa 594 antirabbit antibodies for 30 min (each at 1:800). Excess antibody was removed by washing in PBS, and coverslips were mounted in Vectashield with DAPI. Cells were visualized using a Nikon (Melville, NY) E600 microscope equipped with epifluorescence.

Confocal images were acquired using a Nikon PCM 2000 microscope using argon (488 nm) and green helium-neon (543 nM) lasers. Serial optic sections were performed with Simple 32, C-imaging computer software (Compix, Cranberry Township, PA). z-Axis serial sections were collected at 0.5 μm with a 60× PlanApo objective and a scan zoom of 1×. Images were processed and reconstructed in NIH Image shareware.

Immunoprecipitation and deglycosylation

HEK-293 cells stably transfected with HA-GPR30 or HA-β1AR were grown to confluence in 10-cm culture dishes, washed three times with ice-cold PBS, and lysed in radioimmunoprecipitation assay-buffered detergent [50 mM Tris (pH 7.6), 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 2 mM phenylmethylsulfonylfluoride plus protease inhibitors (Complete TM)]. Total cell protein was determined by bicinchoninic acid assay. Total protein (1 mg) was cleared with protein G-agarose beads to minimize the interaction of nonspecific proteins with immunoabsorbent beads. Precleared cellular protein was incubated with 3 μg GPR30 mAB 2F2 overnight, and antigen-antibody complexes were collected with protein G-agarose beads for 1 h at 4 C. Immunoabsorbed proteins were eluted with Laemmli sample buffer containing β-mercaptoethanol at ambient temperature, size fractionated by SDS-PAGE, and electrotransferred to nitrocellulose. The filter was blocked with nonfat dried milk (5%) prepared in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) overnight. Eluted proteins were visualized by blotting with rabbit GPR30 peptide antibodies diluted 1:500 in TBS-T for 2 h at room temperature. Rabbit GPR30 antibodies were detected using secondary goat antirabbit horseradish peroxidase (HRP) and ECL.

Deglycosylation of immunopurified HA-GPR30 protein was performed with N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) according to the specifications of the manufacturer (New England Biolabs, Beverly, MA). Sample incubation in the absence of enzyme was performed as a control. Reactions were stopped in Laemmli sample buffer containing β-mercaptoethanol.

Intracellular calcium mobilization and measurement of cAMP

Cells were seeded in fibronectin-coated eight-well cover glass chambers (Lab-Tek II; Nalge Nunc, Naperville, IL), serum starved in PRF-DMEM/F-12, and then loaded with fluo-4 (Invitrogen) (5 μg/ml for 15 min). Cover glass chambers were placed in a microscope stage incubator (20/20 Technologies, Eugene, OR) at 37 C and imaged using a inverted scope (Nikon TE2000E), with a 20× PlanApo objective and a cooled CCD camera (Roper CoolsnapHQ; Photometrics, Tucson, AZ). Images were collected every second for 10 min. Data were quantified and analyzed using Metavue Software (Molecular Devices, Sunnyvale, CA) and Microsoft (Seattle, WA) Excel. Background was subtracted, and fluorescence was expressed relative to starting values. To measure intracellular cAMP, HEK-293 transfectants were subcultured in six-well plates to near confluence. Cells were then incubated in serum-free PRF-DMEM/F-12 medium for 3–5 h and treated with charcoal-stripped E2-BSA or E2-HRP or water-soluble E2 for 10 min. Cells were then digested with 0.1 M HCl, and cAMP concentrations were measured in cytosolic fraction samples using an EIA kit following the instructions of the manufacturer (Cayman Chemical, Ann Arbor, MI).

Preparation of subcellular fractions

Cells were washed with PBS, scraped from the plates, suspended in HEPES buffer (9), and centrifuged at 5000 × *g* for 5 min. The cell fraction was resuspended in HEPES buffer and homogenized, followed by sonication for 10 sec. The nuclear fraction was obtained by centrifugation of the cell homogenate at 900 × *g* for 7 min. A crude plasma membrane fraction was obtained by centrifuging the supernatant at 20,000 × *g* for 30 min. A 3500 × *g* spin for 10 min was used initially before the 20,000 × *g* spin to remove the heavy mitochondrial fraction, but this step was eliminated in subsequent experiments because it had no effect on the binding results. The plasma membrane fraction was further purified using a sucrose pad (1.2 M sucrose in HEPES buffer, centrifuged at 6900 × *g* for 45 min) as described previously (24). Microsomal (pellet) and cytoplasmic (supernatant) fractions were obtained by centrifugation at 100,000 × *g* for 1 h of the remaining supernatant after the 20,000 × *g* spin. Subcellular fractions were stored at −80 C for up to 2 d before analysis. Cytochrome *c* reductase [reduced NAD phosphate (NADPH)] activity, an enzyme marker of the endoplasmic reticulum, was measured in subcellular fractions using a spectrophometric assay (kit CY0100; Sigma).

Western blot analysis of subcellular fractions

Western blot analysis for subcellular fractions was performed as described previously (9). Briefly, subcellular fractions were mixed with the 5× reducing lane marker sample buffers (ImmunoPure; Pierce, Rockford, IL) and incubated for 10 min at 22 C. Samples were electrophoresed and blotted according to standard procedures. Equal protein loading was confirmed by the Lowry technique before loading the samples onto the gel. In addition, the protein content of the samples was verified by gel electrophoresis followed by Coomassie blue staining of the gel. Rabbit GPR30 peptide antibody was used at a dilution of 1:500 in an overnight incubation at 4 C after blocking with 5% nonfat milk in TBS-T buffer for 1 h. The membrane was subsequently washed three times, then incubated for 1 h at room temperature with HRP-conjugated goat antirabbit IgG (Pierce), and then treated with enhanced chemiluminescence substrate (SuperSignal; Pierce) and exposed on film (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Estrogen receptor binding assay

Specific [^{3}H]17β-E2 binding assays for the plasma membrane, microsomal and nuclear subcellular fractions were performed according to general procedures described previously (9). Specific [^{3}H]17β-E2 binding of each subcellular fraction was obtained by subtraction of nonspecific binding [mixture of 4 nM [^{3}H]17β-E2, 1 μM 17β-E2, and subcellular sample (~250 μg protein)] from total binding (same mixture but without 17β-E2) after removing unbound (free) ligand. Bound [^{3}H]17β-E2 from membranous fractions was captured on GF/B glass filters (Whatman, Haverhill, MA). The percentage recovery of protein samples after filtration ranged from 61.0% (microsomes) to 91.7% (plasma membrane) (data not shown). Dextran-coated charcoal was used to separate bound from unbound [^{3}H]17β-E2 in a soluble receptor assay in cytosolic fractions (25).

[^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding to the subcellular fractions were assayed as described previously (9) with a few modifications. Briefly, the subcellular samples (150–200 μg protein) were incubated with 10 μM GDP and 0.5 nM [^{35}S]GTPγS (~12,000 cpm, 1.0 Ci/mol) in 300 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 15 min in the presence of 100 nM 17β-E2 (dissolved in ethanol, final ethanol concentration or 0.1%) or buffer containing 0.1% ethanol as a control. Nonspecific binding was deter-

mined by addition of 500 nM GTPγS to the mixture. At the end of the incubation period, 300 μl of stop solution (10 mM GDP/GTPγS in Tris buffer) was added to the reaction tube, and 200 μl aliquots were filtered through GF/B glass fiber filters, followed by several washes with the same buffer and subsequent scintillation counting.

Coimmunoprecipitation of $G\alpha_s$ subunit protein with GPR30

SKBR3 cells were incubated with 100 nM 17β-E2 or no treatment (controls) for 20 min, followed by two washes with buffer. Plasma membrane and microsomal fractions were prepared as described above and incubated overnight at 4 C with 1:200 of goat anti-$G\alpha_s$ subunit protein antibody (Santa Cruz Biotechnology) following procedures described previously (26). The fractions were subsequently incubated for 2 h at 4 C with protein-A agarose beads (Santa Cruz Biotechnology). The beads were washed several times, and the immunoprecipitates were eluted by boiling for 10 min in SDS sample buffer. The solubilized immunoprecipitates were run on a 10% Tris-glycine SDS-polyacrylamide gel, the proteins were transferred to nitrocellulose membranes, and the membranes were blocked, incubated overnight with GPR30 antibody (1:500), and visualized as described above for the Western blot analyses.

Statistical analysis

Association between categorical groups was evaluated using the Student's *t* test. Two-tailed *P* values of 0.05 or less were considered to be statistically significant.

Results

GPR30 is expressed on the cell surface and promotes intracellular signaling

7TMRs are type-I, N-linked glycoproteins, whose amino terminus faces the extracellular environment (27). To address whether GPR30 protein can be detected on the cell surface by cytofluorescence, recombinant GPR30 protein was engineered containing an HA epitope tag on its amino terminus and expressed in HEK-293 cells. After transfection and drug selection, the expression of GPR30 on the surface of intact HEK-293 (HA-GPR30) cells was evaluated by flow cytometry using HA antibodies after enrichment by fluorescence-activated cell sorting. The HA-enriched HEK-293 (HA-GPR30) cells exhibited an average relative mean intensity fluorescence that was 8- to 10-fold greater than HEK-293 control cells, indicating that HA-GPR30 is expressed on the cell surface (Fig. 1).

Epifluorescent images collected from HA-GPR30 and HA-β1AR cells immunostained with rabbit HA antibodies clearly demonstrate that HA is concentrated at the cell surface (Fig. 2A), albeit the surface expression of HA-β1AR was slightly higher than that of HA-GPR30. Specific HA immunostaining of submembranous vesicles was observed during permeabilization of either cell type with Triton X-100. No immunoreactivity was observed in either HEK-293 transfectant (intact or permeabilized) that was stained with rabbit control IgG. Localization of HA-GPR30 within the plasma membrane was confirmed by confocal microscopy using Alexa 594-labeled con A, a lectin that specifically binds to α-mannosyl saccharides expressed in the core structures of plasma membrane glycoproteins, as a plasma membrane marker (Fig. 2B). A circumferential staining pattern was observed in serial Z-section stacks prepared from paraformaldehyde-fixed HA-GPR30 cells that were stained with Alexa 594-conjugated con A (Fig. 2B, *red*). HA rabbit antibodies, which

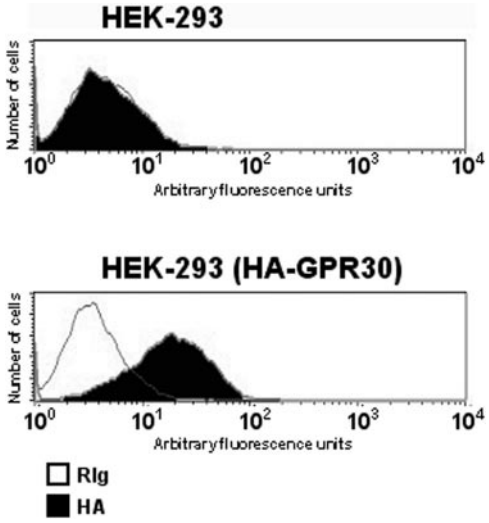

FIG. 1. Expression of GPR30 on the cell surface by flow cytometry. Histograms generated from flow cytometry analysis of HEK-293 cell lines stably expressing HA-tagged GPR30 or vector. Cells were stained with rabbit anti-HA antibody (HA; *black*) or control IgG (Rig; *white*). The x-axis values are displayed on a logarithmic scale as arbitrary fluorescence units, and the y-axis values are plotted on a linear scale as number of cells.

were detected with Alexa 488-conjugated secondary antibodies, showed a similar cell surface staining pattern compiled from these optical sections (*green*). Coexpression of HA-GPR30 and con A is clearly observed when the HA- and con A-stained sections are registered (Fig. 2B, *yellow*). In contrast, control rabbit IgG showed no appreciable reactivity, and reactivity was not detected in cells that were costained with control antibodies and con A. These immunofluorescent data strongly suggest that the HA epitope of the HA-GPR30 protein is expressed on the cell surface.

Cellular expression of HA-GPR30 and its relative content of N-linked glycan side chains were assessed by comparing the relative mobilities on SDS-polyacrylamide gels of immunopurified GPR30 proteins that were untreated or treated with glycosidases (Fig. 3). GPR30 immunoprecipitates were prepared from HEK-293 (HA-GPR30) and control HEK-293 (β1AR) cells using GPR30 peptide mAB 2F2. Immunoprecipitated proteins were eluted and analyzed by immunoblotting with rabbit GPR30 antibodies. The predominant protein species specifically detected by the GPR30 antibody possessed an apparent molecular mass of 44 kDa, although other species with larger apparent molecular masses were also detected. Removal of N-glycan chains by treatment with PNGase F or Endo H resulted in the appearance of major bands with molecular masses of 40 and 39 kDa, respectively, which closely approximates the predicted molecular mass of the unmodified GPR30 core protein fused to the HA-epitope tag.

Addition of the HA tag to GPR30 did not compromise receptor functionality as assessed in two independent analyses. First, the ability of 17β-E2 to stimulate a rise in intracellular calcium was determined (Fig. 4A). Vector-transfected HEK-293 cells did not activate calcium fluorochrome in response to 17β-E2 but remained sensitive to calcium ionophore (*trace A*). In contrast, GPR30-expressing cells gen-

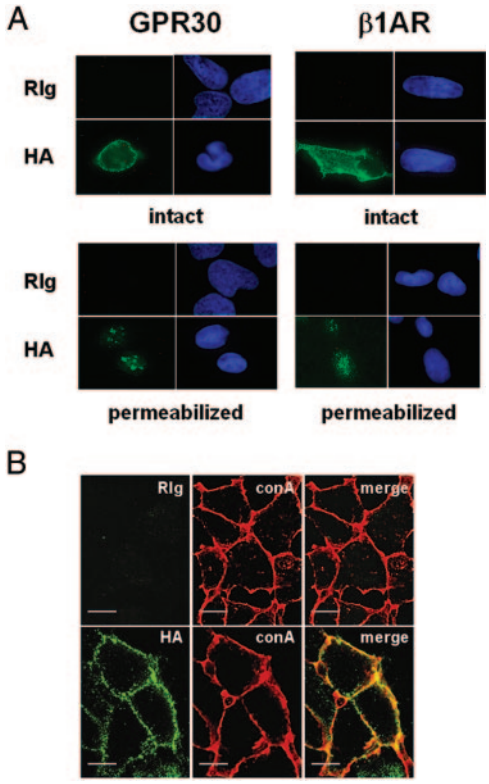

FIG. 2. Subcellular localization of GPR30 in paraformaldehyde-fixed cells. A, Epifluorescent images of paraformaldehyde-fixed, intact (*top*) or Triton X-100-permeabilized (*bottom*) HEK-293 cells expressing HA-GPR30 or HA-β1AR cells were immunostained with rabbit HA antibodies or rabbit control IgG (Rlg). Cell-associated antibodies were detected using Alexa 488-derivatized antirabbit antibodies (*green*). Nuclei are stained using DAPI (*blue*). B, Confocal microscopy of paraformaldehyde-fixed HEK-293 (HA-GPR30) cells costained with Alexa 594-labeled con A (*red*), which specifically binds α-mannosyl saccharides in plasma membrane glycoproteins, and GPR30 peptide or control rabbit antibodies (*green*). Colocalization of GPR30 with con A is observed in the merge (*yellow*). *Scale bars*, 10 μm.

erated a rapid increase in intracellular calcium after treatment with 10 nM 17β-E2 (*trace B*) but not with 17α-E2 (*trace C*). This response was rapid with peak levels (~3-fold increase) measured within 60 sec, indicating that HA-GPR30 functions in HEK-293 cells to trigger E2-mediated intracellular signaling. Second, we have shown previously that ectopic expression of GPR30 in HEK-293 cells results in activation of $G\alpha_s$ protein and accumulation of intracellular cAMP in response to E2 stimulation (9). To determine whether GPR30 acts at the cell surface to promote estrogen-dependent intracellular signaling, intracellular cAMP concentrations were measured in HA-GPR30-expressing HEK-293 cells after stimulation with cell-impermeable E2 conjugates (Fig. 4B). Mean basal intracellular cAMP concentrations of 18.5 pmol were measured in quiescent cells. Exposure to 20 nM 17β-E2 for 10 min resulted in a significant increase in intracellular cAMP (22.0 pmol). Similar increases in intracellular cAMP were observed after short-term exposure to the same concentrations of charcoal-stripped E2-BSA or E2-HRP (22.0 and 24.0 pmol, respectively). Together, these results suggest that functional HA-GPR30 is expressed on the cell surface and promotes rapid E2 signaling as measured by

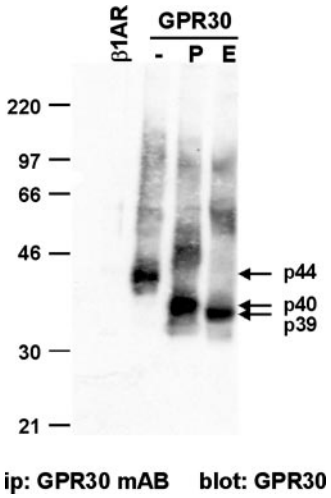

FIG. 3. Cellular expression of HA-GPR30 protein. Detergent lysates (0.5 mg) from HEK-293 (GPR30) cells or HEK-293 (β1AR) control cells were immunoprecipitated (IP) with the mouse GPR30 mAB 2F2. Immunoprecipitated protein was left untreated (−) or deglycosylated by incubation with PNGase F (P) or Endo H (E). Samples were subsequently resolved by 10% SDS-PAGE and blotted with affinity-purified rabbit GPR30 antibodies. Molecular mass standards (kilodaltons) are indicated at *left*. Gels shown are representative of three independent experiments.

stimulation of adenylyl cyclase and release of intracellular calcium.

Sequestration and trafficking of GPR30 from the plasma membrane after E2 stimulation

7TMRs are sequestered from the cell surface and redistributed to clathrin-coated pits after ligand stimulation (28–31). To measure the trafficking of HA-GPR30 from the cell surface and to determine its distribution pattern with clathrin, immunofluorescent analyses were conducted on HA-GPR30 or control HA-β1AR cells that were prelabeled with HA antibody before E2 stimulation (Fig. 5). Antibody-labeled, adherent cells expressing either receptor were left untreated or exposed to 17β-E2 for 5 or 15 min, fixed, permeabilized with Triton X-100, and immunostained with clathrin-specific antibodies (Fig. 5). Before stimulation with 17β-E2, HA-β1AR was localized to the cell perimeter, as expected, and 17β-E2 treatment did not induce HA-β1AR internalization from the cell surface or result in its association with clathrin (Fig. 5A). By comparison, in untreated cells, HA-GPR30 was localized in patches at the cell surface (Fig. 5B), in part attributable to the fact that these cells express significantly less receptor than is present in HA-β1AR cells (data not shown). Surprisingly, to a small extent, HA-GPR30 colocalized with clathrin in untreated cells (Fig. 5B). However, codistribution of GPR30 with clathrin was more readily observed within 5 min of hormone treatment. By 15 min, the majority of the prelabeled GPR30 was coexpressed with clathrin in a punctate staining pattern, with little GPR30 associated with the cell periphery, suggesting that 17β-E2 stimulation resulted in its redistribution to clathrin-coated vesicles. Clathrin codistribution with GPR30 was not observed in cells stimulated with the control hormone 17α-E2 (data not shown), indicating the specificity of this response.

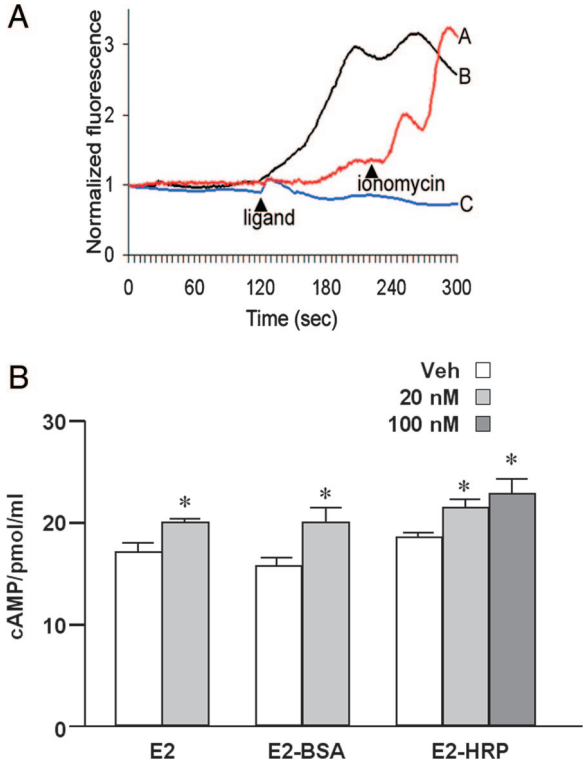

FIG. 4. HA-GPR30 promotes intracellular signaling after stimulation with 17β-E2 or membrane-impermeable 17β-E2 conjugates. A, 17β-E2 promotes GPR30-dependent release of intracellular calcium. Fura-loaded HEK-293 (vector) cells (*trace A*) or HEK-293 (HA-GPR30) cells (*traces B* and *C*) were stimulated with 10 nM 17β-E2 (*traces A* and *B*) or 17α-E2 (*trace C*) at 120 s. Calcium ionophore was added at 220 sec in A. B, HEK-293 (HA-GPR30) cells were exposed to the indicated concentrations of charcoal-stripped E2-HRP or E2-BSA or water-soluble 17β-E2 or control vehicle (Veh) for 10 min. Intracellular cAMP was measured by ELISA. The data shown represent means ± SD of three separate experiments. *, $P < 0.05$ relative to the control treatment group, Student's paired *t* test.

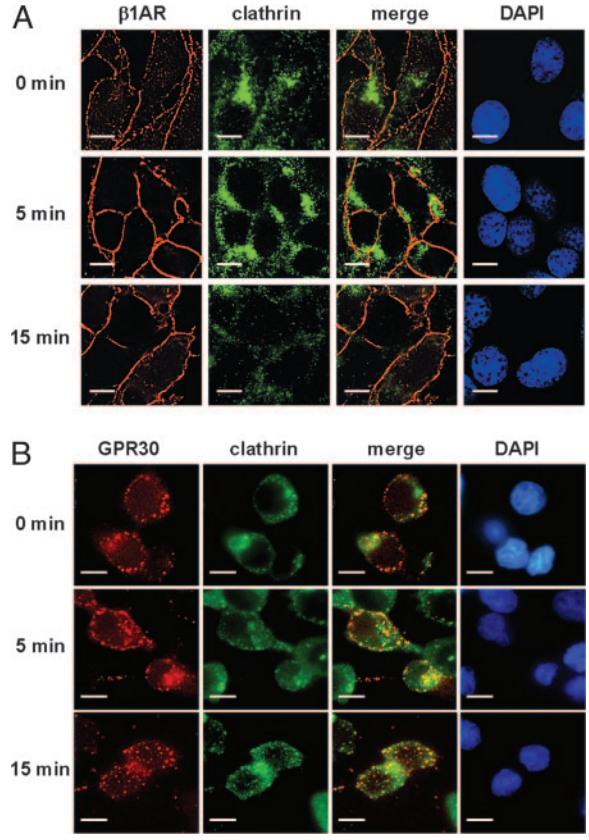

FIG. 5. Codistribution of GPR30 and clathrin after 17β-E2 stimulation. HEK-293 (HA-GPR30) cells (B) or HEK-293 (HA-β1AR) control cells (A) were prelabeled with rabbit HA antibody. After removing excess antibody, cells were left untreated or stimulated with 17β-E2 (1 nM; 0, 5, or 15 min) and then fixed in paraformaldehyde. Cells were then permeabilized and incubated with mouse clathrin antibody. Cell-associated antibodies were detected using Alexa 594-conjugated antirabbit antibodies (*red*) and Alexa 488-conjugated antimouse antibodies (*green*). Codistribution is demonstrated in the merge (*yellow*). Nuclei are detected by DAPI (*blue*).

Collectively, these data imply that GPR30 traffics from the plasma membrane and likely enters clathrin-coated pits after 17β-E2 stimulation.

Estrogen binding and G protein activation are associated with plasma membrane fractions

De novo expression of GPR30 protein in HEK-293 cell membrane fractions is associated with specific estrogen binding and 17β-E2-dependent G protein activation (9). This previous study showed that these activities are GPR30 dependent because HEK-293 cells neither express detectable GPR30 mRNA as assessed by RT-PCR nor do their purified membranes contain GPR30 protein as analyzed by Western blotting. To examine the ability of other subcellular fractions to support GPR30-dependent estrogen binding and G protein activation, enriched plasma membrane, microsomal, nuclear, and cytoplasmic fractions were prepared from HEK-293 (HA-GPR30) cells. As shown in Fig. 6A, NADPH oxidase was greatly enriched in microsomal fractions relative to all other subcellular fractions, which had similar low residual levels of enzyme activity in the spectrophotometric assay, indicating that the endoplasmic reticulum was essentially absent from the nonmicrosomal fractions. Both crude and partially purified plasma membrane fractions displayed high specific [^{3}H]17β-E2 binding activity (Fig. 6B), 6- to 8-fold higher than that in any other subcellular fractions on a milligram per protein basis. Treatment with 100 nM 17β-E2 caused G protein activation in the plasma membrane fractions as shown by an approximately 50% increase in specific [^{35}S]GTPγS binding activity, whereas no activation of this signal transduction pathway was detected in microsomes or other subcellular fractions (Fig. 6C). Significant G protein activation was also observed after treatment with a lower concentration of 17β-E2 (20 nM) (see the supplemental figure published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). These binding activities were positively associated with the expression of GPR30 protein (Fig. 6D), indicating that functional estrogen receptors (ERs) were associated with enriched plasma membrane fractions that had the highest concentrations of GPR30 protein.

Expression of endogenous GPR30 protein in SKBR3 breast cancer cells is associated with estrogen binding (9, 10). Therefore, the distribution of endogenous GPR30 protein and ERs was also assessed in subcellular fractions prepared from

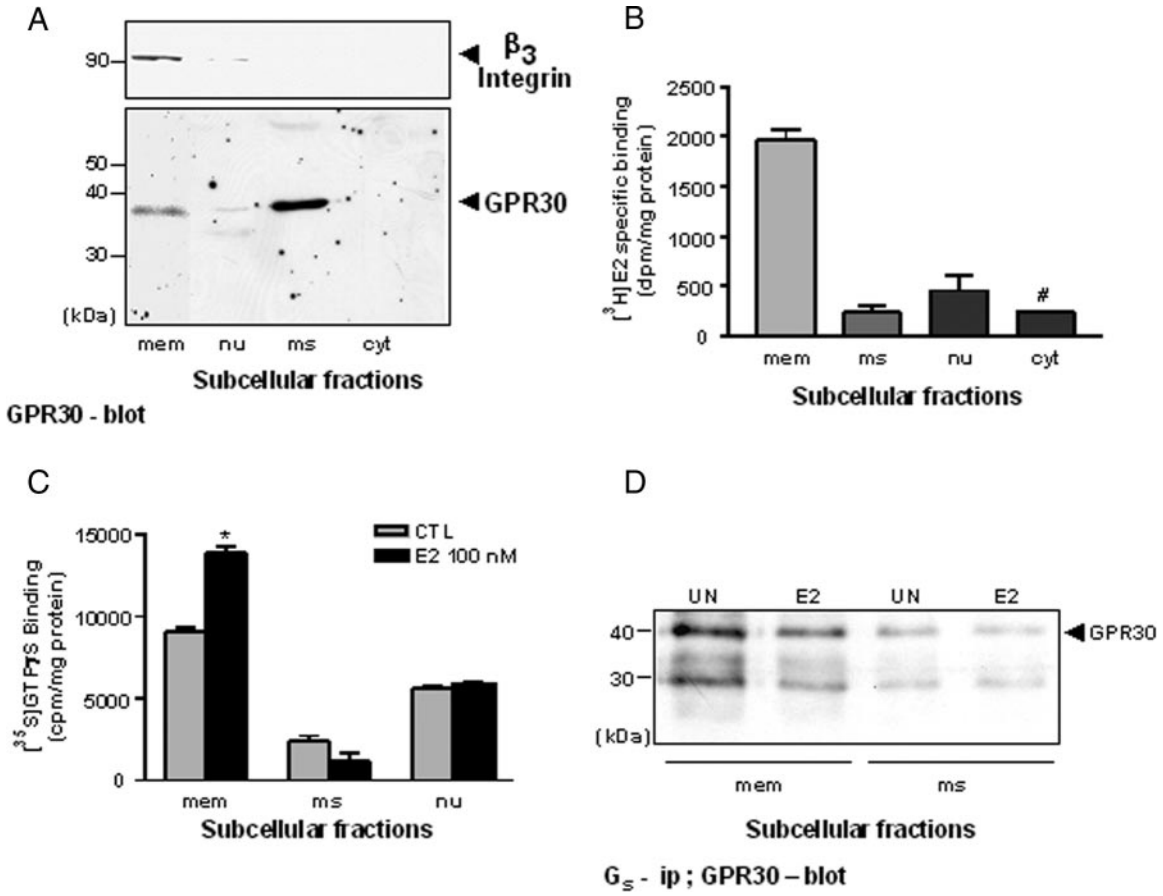

FIG. 6. Binding characteristics of subcellular fractions from SKBR3 cells. A, Western blot analyses of subcellular fractions using human integrin β3 antibody (1:1000) (*top gel*) and human GPR30 peptide antibody (*bottom gel*), 40 μg protein loaded per lane. n = 2. B, [^{3}H]17β-E2 binding to subcellular fractions. For key to symbols, refer to the legend of Fig. 7. Means ± SD are shown, n = 3. C, [^{35}S]GTPγS binding to the subcellular fractions after treatment with 100 nM 17β-E2 or vehicle (Veh). Means ± SD are shown, n = 3, *, $P < 0.05$ compared with corresponding untreated (UN) group (Student's paired *t* test). CTL, Control. D, Coassociation of Gα_s subunit protein with GPR30 in plasma membrane or microsomal fractions that were treated with 100 nM 17β-E2 or vehicle. After treatment, Gα_s protein was immunoprecipitated (IP) from 500 μg protein from each fraction using Gα_s-specific antibody. Associated GPR30 protein was detected by immunoblotting with rabbit GPR30 peptide antibodies. Molecular weight standards are designated at *left*. Location of GPR30 protein is denoted at *right* (n = 2).

SKBR3 cells by Western blotting, radioreceptor assay, and GTP binding (Fig. 7). As noted previously (10), Western blotting analysis demonstrated that, although GPR30 protein is detectable within plasma membrane fractions, a significant portion of GPR30 protein is retained within the microsomal fractions (Fig. 7A). However, 17β-E2 binding activity (Fig. 7B) and estrogen-enhanced GTP binding (Fig. 7C) is not readily measurable within the microsomal fractions or other intracellular fractions but instead is concentrated within the plasma membrane fraction. This finding is consistent with the classical model of 7TMR activation supporting the coupling of GTP with the Gα subunit dissociated from ligand-occupied receptors. To directly test this concept, the association of Gα_s protein with GPR30 in various cellular fractions was measured (Fig. 7D). Significantly more GPR30 was found coupled to G_s in plasma membrane fractions than in microsomal fractions, a finding consistent with the concentration of G_s protein within the plasma membrane. 17β-E2 treatment triggered Gα_s uncoupling from GPR30, indicating G protein activation (Fig. 7D). These results are consistent with general models that have been proposed for 7TMRs describing their relative activity states (3, 4). These models indicate that high-affinity 7TMRs coupled to Gα subunit proteins accumulate in the plasma membrane. Subsequent to ligand binding and Gα protein release, they assume a conformation associated with low affinity for ligand and are transported to intracellular vesicles for 7TMR recycling or receptor degradation in late endosomes.

Discussion

Estrogen has long been known to trigger rapid biochemical signaling events, including activation of second-messenger cascades (13, 32–34) and lipid/protein kinases (35–38). Similarly, other steroid hormones and their antihormones also elicit rapid actions (39–42). Although some debate exists regarding the receptor(s) that promote these effects, it is primarily recognized that the rapidity by which these actions occur is consistent with the plasma membrane as a site of origin for these signaling events, a hypothesis that is supported by the fact that both heterotrimeric G proteins (43–45) and EGFRs (46–48) have been implicated in rapid estrogen

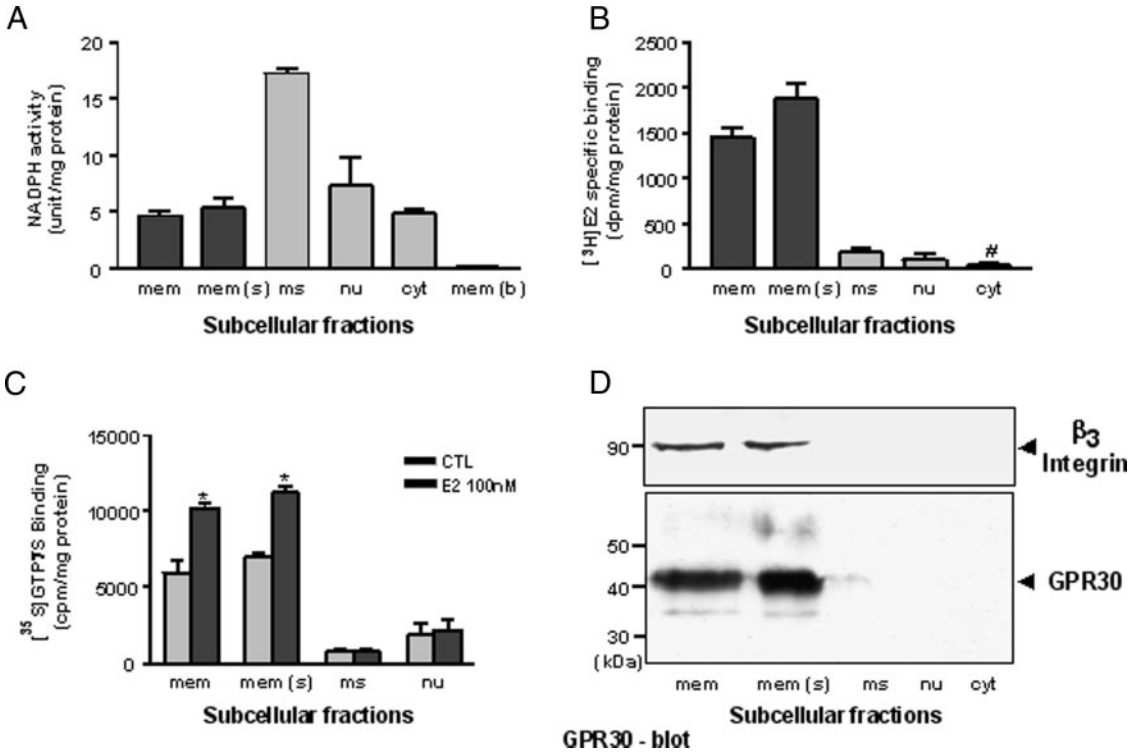

FIG. 7. Binding characteristics of subcellular fractions from HEK-293 (HA-GPR30) cells. A, Cytochrome *c* reductase (NADPH) activities in the subcellular fractions. mem, Plasma membrane; mem (s), plasma membrane fractions purified by the sucrose pad method; ms, microsomes; nu, nuclear; cyt, cytosol; mem (b), boiled plasma membrane. Means ± SD are shown, n = 3. B, Specific [^{3}H]17β-E2 binding to subcellular fractions. Dextran-coated charcoal was used to separate bound from free in the cytosolic fraction (#). Means ± SD are shown, n = 3. Nonspecific E2 binding was 50.3 dpm/mg protein for HEK-293 (HA-GPR30) (<6.6% of specific E2 binding). C, [^{35}S]GTPγS binding to the subcellular fractions after treatment with vehicle (Veh) or 5, 20 (see the supplemental figure), and 100 nM 17β-E2. Means ± SD are shown, n = 3. *, $P < 0.05$ compared with corresponding untreated group (Student's paired *t* test). D, Western blot analyses of subcellular fractions with human integrin β3 (*top gel*) (1:1000; Cell Signaling Technology, Danvers, MA) and GPR30 (*bottom gel*) antibodies, 15 μg protein loaded per lane (n = 2).

action. Previous studies have shown that GPR30 regulates adenylyl cyclase (for review, see Ref. 7) and also triggers extracellular release of proHB-EGF through the activation of membrane-tethered MMPs (22). Thus, GPR30 promotes its actions through two plasma membrane-associated enzymes, yet studies with fluorescently labeled E2 conjugates as well as green fluorescent protein-GPR30 fusion proteins have shown that GPR30 accumulates in the endoplasmic reticulum, and perhaps in other tubuloreticular compartments, and in intracellular vesicles but could not be detected in the plasma membrane (10). This result was interpreted to suggest that GPR30 may function intracellularly (10), although this subcellular site is inconsistent with the known mechanisms by which 7TMRs promote cell signaling. This apparent paradox casts some confusion regarding the receptor mechanism by which estrogen promotes rapid signaling.

Here, evidence is provided that GPR30, like all other members of the 7TMR superfamily, is a plasma membrane receptor. We show that estrogen binding and G protein activation is strongly associated with plasma membrane fractions (Figs. 6 and 7). Moreover, the fact that GPR30 is detectable in the plasma membrane (Figs. 1, 2, and 5), becomes sequestered from the cell surface, and codistributes into clathrin-coated vesicles (Fig. 5) is consistent with the current modeling of 7TMRs as plasma membrane receptors. Evidence has been provided previously suggesting that the known estrogen receptors ERα and ERβ (47–49) or variants of them (50) may associate with the plasma membrane and be linked to nongenomic signaling. These studies have suggested that ERs may function as membrane ERs (48–50). In light of the data presented here that GPR30, a structurally distinct ER belonging to the 7TMR family, may also promote rapid 17β-E2 actions from the plasma membrane, we propose that this receptor be distinguished from the known ERs by applying the name 7TM-ER.

Expression of recombinant GPR30 protein and subsequent selection with antibodies directed against an N terminally located epitope tag allowed for facile detection of GPR30 on the cell surface by a cytofluorescent technique. The abundant expression of GPR30 within the intracellular compartment (Figs. 2A and 7C) may be a product of receptor trafficking patterns observed for other 7TMRs. Retention of 7TMRs within the endoplasmic reticulum is a common feature of 7TMR biogenesis (14) as a consequence of multiple regulatory events, including carbohydrate processing (51), disulfide bond exchange (52), and proteolytic editing (53). Additional complexity is provided by the fact that specific chaperone proteins have been identified, *e.g.* DrIP (D receptor-interacting protein) (14) and RAMPs (receptor activity-modifying proteins) (54, 55), that allow for 7TMR export. Intracellular retention as a consequence of receptor endocytosis is a common fate for 7TMRs (15, 56) and provides yet

another explanation as to why 7TMRs may concentrate intracellularly. Perhaps for these reasons, concentrated plasma membrane expression of 7TMRs is not observed in nature, and perturbations in 7TMR function linked with human disease appear to be the consequence of dysregulated receptor trafficking and activity (57). Localization of endogenous GPR30 in the plasma membrane of SKBR3 cells (Fig. 7) suggests that this location cannot be easily dismissed as anomalous receptor distribution as a result of epitope tagging and overexpression of recombinant GPR30. Surface expression of endogenous GPR30 is further supported by the recently published immunoelectron microscopy data demonstrating that GPR30 concentrates within the plasma membrane of pyramidal neuronal cells of the rat hippocampus (58).

Our study does not exclude the possibility that GPR30 may signal from the endoplasmic reticulum, or other intracellular locations, by a currently undefined molecular mechanism. However, using a classic radioreceptor assay and isotopically labeled [^{3}H]17β-E2, we did not obtain any evidence for the presence of a functional ER in the intracellular compartment. The reasons for the apparent discrepancies between these results and those obtained by Revankar *et al.* (10), who detected binding in the intracellular compartment but not on the plasma membrane using E2-Alexa dyes, are unclear at present. The binding affinity of GPR30 for its natural ligand, 17β-E2, like that of other 7TMRs, is dramatically decreased when the receptor is not coupled with its Gα protein (9), presumably attributable to a conformational change in the ligand binding pocket (3, 4, 59). The present study shows that much more GPR30 is coupled to its G_s protein in the plasma membrane than in the endoplasmic reticulum (Fig. 7D). Likely differences in the conformational state of the ligand binding pockets of GPR30 proteins in the two compartments could partly explain their apparent different binding affinities for [^{3}H]17β-E2 and Alexa dye 594 conjugated estrogen. Additional studies directed at identifying the biological and physiological roles of GPR30 in estrogen action are warranted.

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Address all correspondence and requests for reprints to: E. Filardo, Department of Medicine, Rhode Island Hospital, 593 Eddy Street, Aldrich Building, Room 718, Providence, Rhode Island 02903. E-mail: edward_filardo@brown.edu.

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