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Nature of Functional Estrogen Receptors at the Plasma Membrane

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Although rapid signaling by estrogen at the plasma membrane is established, it is controversial as to the nature of the receptor protein. Estrogen may bind membrane proteins comparable to classical nuclear estrogen receptors (ERs), but some studies identify nonclassical receptors, such as G protein-coupled receptor (GPR)30. We took several approaches to define membrane-localized estrogen-binding proteins. In endothelial cells (ECs) from ERα/ERβ combined-deleted mice, estradiol (E2) failed to specifically bind, and did not activate cAMP, ERK, or phosphatidyinositol 3-kinase or stimulate DNA synthesis. This is in contrast to wild-type ECs, indicating the lack of any functional estrogen-binding proteins in ERα/ERβ combined-deleted ECs. To directly determine the identity of membrane and nuclear-localized ER, we isolated subcellular receptor pools from MCF7 cells. Putative ER proteins were trypsin digested and subjected to tandem array mass spectrometry. The output analysis identified membrane and nuclear E2-binding proteins as classical human ERα. We also determined whether GPR30 plays any role in E2 rapid actions. MCF7 (ER and GPR30 positive) and SKBR-3 (ER negative, GPR30 positive) cells were incubated with E2. Only MCF7 responded with significantly increased signaling. In MCF7, the response to E2 was not different in cells transfected with small interfering RNA to green fluorescent protein or GPR30. In contrast, interfering RNA to ERα or ER inhibition prevented rapid signaling and resulting biology in MCF7. In breast cancer and ECs, nuclear and membrane ERs are the same proteins. Furthermore, classical ERs mediate rapid signals induced by E2 in these cells. (*Molecular Endocrinology* 20: 1996–2009, 2006)

STEROID HORMONES INCLUDING estrogen rapidly activate signal transduction (1). Estrogen acts *in vitro* and *in vivo* to stimulate second messenger generation (cAMP, cGMP), kinase and phosphatase activation, and calcium flux (reviewed in Ref. 2). These and other signals are generated often within seconds to minutes. Many of these signals result from G protein activation and contribute to gene transcription (genomic effect) (2–4). This is mediated, in part, through modulating nuclear estrogen receptor (ER) function or recruiting coactivator proteins. In addition, rapid signaling modulates existing protein structure and function (nongenomic effect) (5, 6). The importance of these generated signals to the totality of cellular actions of estrogen *in vitro* and *in vivo* has increasingly been established (reviewed in Ref. 7). As examples, target cells proliferate (8), survive (9, 10), and migrate (11), and estrogen-induced rapid signaling contributes in these regards.

The vast majority of studies have implicated rapid actions of steroids to originate at the cell surface rather than in the nucleus. Estrogen activates ERK and induces the survival of multiple cells that are engineered to express only a membrane-localized E domain of ERα (12, 13). In contrast, the same cells that express only a nuclear-localized, ligand-binding domain fail to support these actions of estradiol (E2) (13). Similarly, cells transfected to express a nuclear localization sequence-deficient ERα show kinase activation by E2 (14). Finally, membrane-restrained compounds such as E2-BSA or estrogenic compounds that activate only membrane -localized steroid-binding proteins capably activate rapid estrogen signaling (12, 15). These findings make it imperative to understand the structural and functional aspects of endogenous estrogen-binding proteins localized to the cell membrane.

ERs translocate to the plasma membrane where the receptors are probably tethered to the cytoplasmic face of the bilayer and are contained within caveolae rafts (16–18). The exact nature of these localized proteins remains controversial. Much data support the idea that membrane-localized ERs are very similar to the classical nuclear ERs. Using a series of antibodies to multiple epitopes of the nuclear ERα, plasma mem-

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Abbreviations: DERKO, ERα and ERβ deleted; E2, Estradiol; EC, endothelial cell; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GFP, green fluorescent protein; GPR, G protein-coupled receptor; MS, mass spectrometry; PD, PD98059; PI3K, phosphatidylinositol 3-kinase; WTM, wortmannin.

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brane ERs were identified in several cell types (19, 20), indicating broad similarity of the two receptor pools of this isoform. Introduction of antisense oligomers to ERα into pituitary cells diminished detection of ERα at the plasma membrane by immunohistochemistry (20). Expression of the cDNAs for either ERα or ERβ in ER null cells resulted in the codetection of membrane and nuclear ERs, indicating that the two cellular pools could arise from a single transcript (21). More recently, endogenous ERα and ERβ receptors of 67 and 54 kDa, respectively, have been identified in the caveolae and cell membranes from endothelial cells (ECs). These studies used antibodies against the classical nuclear ERα and ERβ (22). Finally, ECs from the DERKO mouse (ERα and ERβ deleted) do not show membrane or nuclear ERα or ERβ by immunoblot, in contrast to wild-type ECs that endogenously express both ER isoforms at both locations (23).

Alternatively, multiple reports involving several cell types describe nonclassical steroid-binding proteins that functionally mediate rapid actions of estrogen. In neurons, alternative ER or ER-X have been characterized to have slightly altered receptor pharmacologies, compared with classical ERs in breast or uterus (24, 25). It has even been reported that estrogen binds putative ERs to signal in cells that have been traditionally thought to be ER null (26). The latter report, however, has not been the experience of dozens of laboratories that report a lack of estrogen binding or E2 action in Chinese hamster ovary and COS cells. Furthermore, the protein sequences, membrane-localization mechanisms, and structure/functional relationships of these putative ERs have largely not been determined (24–26). Finally, it has not been established that these receptors function in cells from DERKO mice.

It has been proposed that an orphan GPR, GPR30 (27), is an alternative ER (28). This protein has been reported to respond to E2 at the plasma membrane, or in the endoplasmic reticulum (28, 29). GPR30 has been implicated to mediate E2 signaling, but often these reports used ER-negative cells in which overall E2 biological action is unproven (28–31). The original reports on this protein indicated an unusual pharmacology, in that 17β-estradiol (17β-E2) or ICI 182780 (ER antagonist) each activated GPR30. This interaction occurred through unknown mechanisms to signal downstream kinase activation and second messenger generation via epidermal growth factor receptor (EGFR) transactivation (28, 30). More recent work showed a low-capacity (very modest) binding of GPR30 by sex steroid (31). E2 binding resulted in modest second messenger generation, as shown primarily in GPR30-transfected, overexpressing cells (31). Another study indicated that GPR30-E2 interactions required micromolar (not nanomolar) E2 (32). More definitively, expression of antisense oligonucleotides to GPR30 did not affect E2 activation of ERK or cell proliferation in breast cancer cells (33). However, a more physiological interaction between E2 and GPR30 has been reported in keratinocytes (34). Thus, a role for GPR30 as an ER remains controversial.

In this report we address many of these controversies. We determined the lack of any functional estrogen-binding protein in ECs from DERKO mice. We also isolated and analyzed the membrane and nuclear estrogen-binding proteins in MCF7 cells by mass spectrometry (MS). Finally, we carried out a series of studies to determine whether GPR30 or classical ERs support E2 action in both ER-positive and -negative breast cancer cells.

RESULTS

DERKO ECs Lack Estrogen Binding and Signaling

We previously determined that DERKO ECs lack classical ERα and ERβ in the plasma membrane, compared with wild-type ECs (23). This led us to conclude that the genes that code for the classical ER isoforms produce the membrane ER proteins. Whether nuclear and membrane ER proteins are identical could not be determined from this approach.

Here we find that both nuclear and membrane ERα and ERβ at the expected sizes are seen in ECs from wild-type littermates but are absent in ECs from the DERKO mouse (Fig. 1A). This justified asking the question, "Are there any functional E2-binding proteins at the membrane of ERα/ERβ-deleted cells?" To address this, we first carried out studies in DERKO ECs, and found virtually no binding of labeled E2 in the whole cell. This contrasts to strong binding of E2 in wild-type ECs (Fig. 1B). In addition, no binding was detected specifically in cell membrane or cytoplasmic fractions of DERKO ECs (data not shown).

Importantly, we then determined whether E2 could activate rapid signaling in the DERKO ECs. As seen in Fig. 1C, E2 stimulated cAMP and ERK activity in wild-type EC, but not in DERKO ECs. Similarly, there was no evidence that E2 stimulated phosphatidylinositol 3-kinase (PI3K) activity (AKT phosphorylation) in the DERKO ECs, in contrast to wild-type ECs that support this function (Fig. 1D). Finally, E2 stimulated EC thymidine incorporation, as an index of DNA synthesis, in wild-type ECs (Fig. 1E). This occurred in an ERK-dependent and PI3K-dependent fashion, because the MAPK kinase inhibitor PD98059 (PD) and PI3K inhibitor wortmannin (WTM) blocked this E2 action. It is well documented that E2 stimulates ERK and PI3K in these cells via membrane ERs (17, 18, 35). By contrast, no thymidine incorporation was measured in DERKO ECs incubated with E2 (Fig. 1E). We also found comparable expression of GPR30 in wild-type or DERKO ECs, yet vastly different signaling by E2 (Fig. 1F). We conclude that the ability of E2 to activate rapid signaling and resulting cell biology in EC requires the classical ERα or ERβ. Furthermore, our results do not support the idea that functional estrogen-binding proteins exist in DERKO cells.

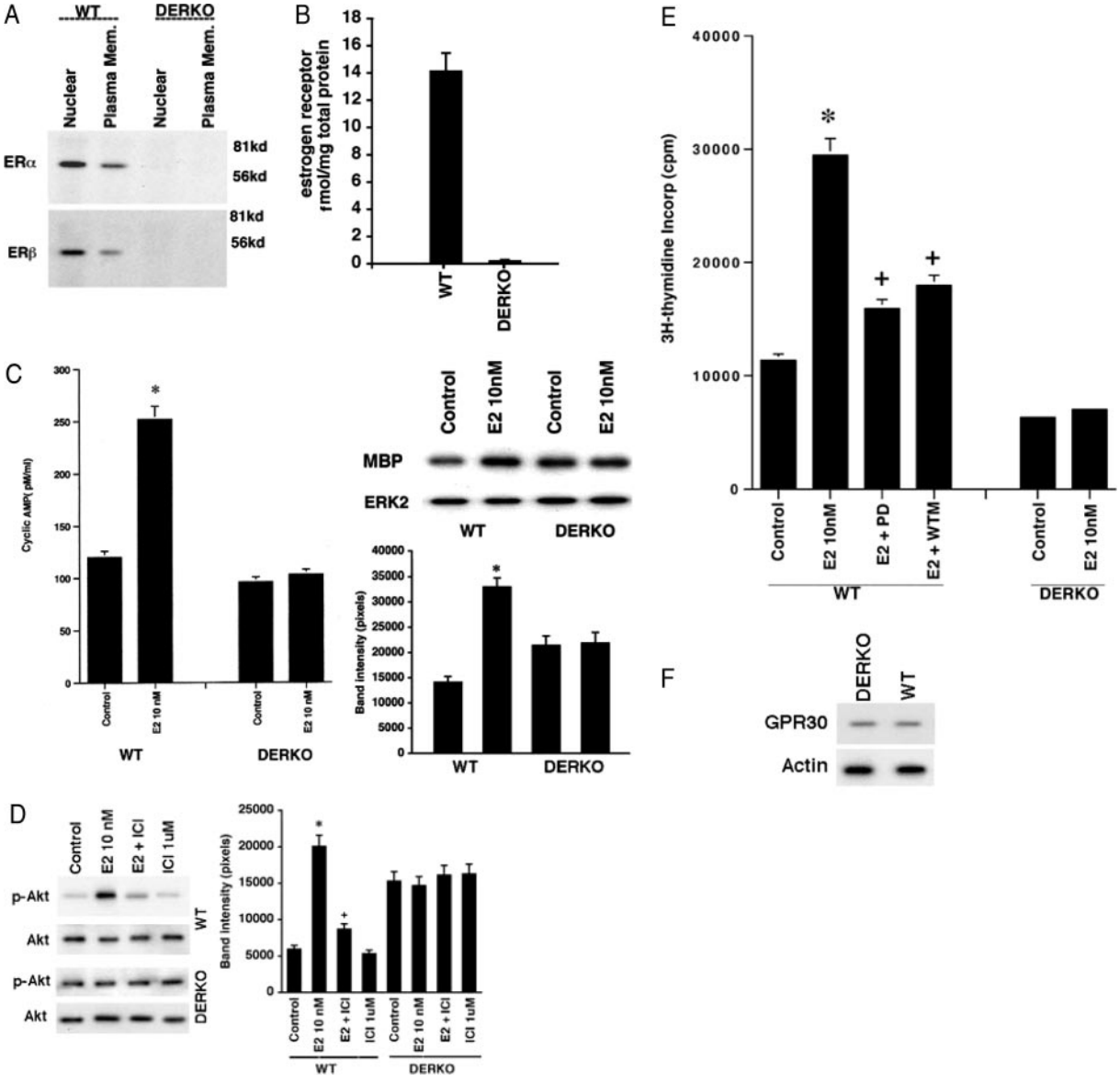


Fig. 1. E2 Binding and Signaling in ECs

A, Lack of nuclear or membrane ERα or ERβ in DERKO ECs. ECs were isolated from the brain capillaries of wild-type (WT) or DERKO mice and briefly cultured in the presence of E2. Cells were processed for membrane or nuclear fractions by sucrose gradiant centrifugation, proteins were separated by SDS-PAGE, and Western immunoblots with antibodies to the C terminus of ERα or N terminus of ERβ were carried out. A representative study is shown and was repeated. B, Specific binding of [^{3}H]17β-E2 to whole WT or DERKO ECs. The cells were incubated with 1 nM labeled E2 (specific activity, 170 Ci/mmol) for 1 h in the presence or absence of various concentrations of unlabeled E2 (0.1–100,000 nM E2) at 37 C. Specific binding was calculated by the LIGAND program (B_{max}, as bound fmol/mg protein) and shown in the *bar graph* (three experiments combined). C, E2 activates cAMP and ERK in WT but not DERKO ECs. WT or DERKO ECs were incubated with 10 nM E2 for 5 min (cAMP) or 8 min (ERK), and second messenger (*left*) or kinase activity (*right*) was determined. Three studies were combined for cAMP and ERK shown in *bar graph* form. Mean and SEM were calculated for the cAMP results and analyzed by ANOVA + Schefe's test. *, $P < 0.05$ for control *vs.* E2 in WT EC. D, E2 activates PI3K in WT but not DERKO ECs. Cells were exposed or not exposed to E2 for 15 min, and Ser473 phosphorylation of AKT (PI3K-induced activation site) was determined. The studies were repeated three times and data were combined for the *bar graph*. *, $P < 0.05$ for control *vs.* E2; +, $P < 0.05$ for E2 *vs.* E2 + ICI 182780. E, E2 stimulates DNA synthesis in WT but not DERKO ECs. Cells were incubated with E2 ± PD or WTM, and thymidine incorporation was carried out over 24 h. Data are combined from three experiments and analyzed as above. *, $P < 0.05$ for control *vs.* E2; +, $P < 0.05$ for E2 *vs.* E2 plus PD or WTM. F, Immunoblot of GPR30 protein in WT or DERKO ECs. A representative study, repeated once, is pictured. Actin is shown for protein loading. MBP, Myelin basic protein.

Isolation and Characterization of Membrane ERs from MCF7 Cells

To determine the identity of endogenous estrogen-binding proteins at the plasma membrane of a target cell (breast cancer), we approached this by MS analysis. MCF7 cell lysate fractions from the nucleus and the plasma membrane were isolated by sucrose gradient centrifugation. The lack of contamination of the cell fractions after isolation was previously published (13, 36) and is shown here (Fig 2A).

The membrane and nuclear fractions were individually passed through separate E2-sephadex beads. The eluates from the beads were then separated by

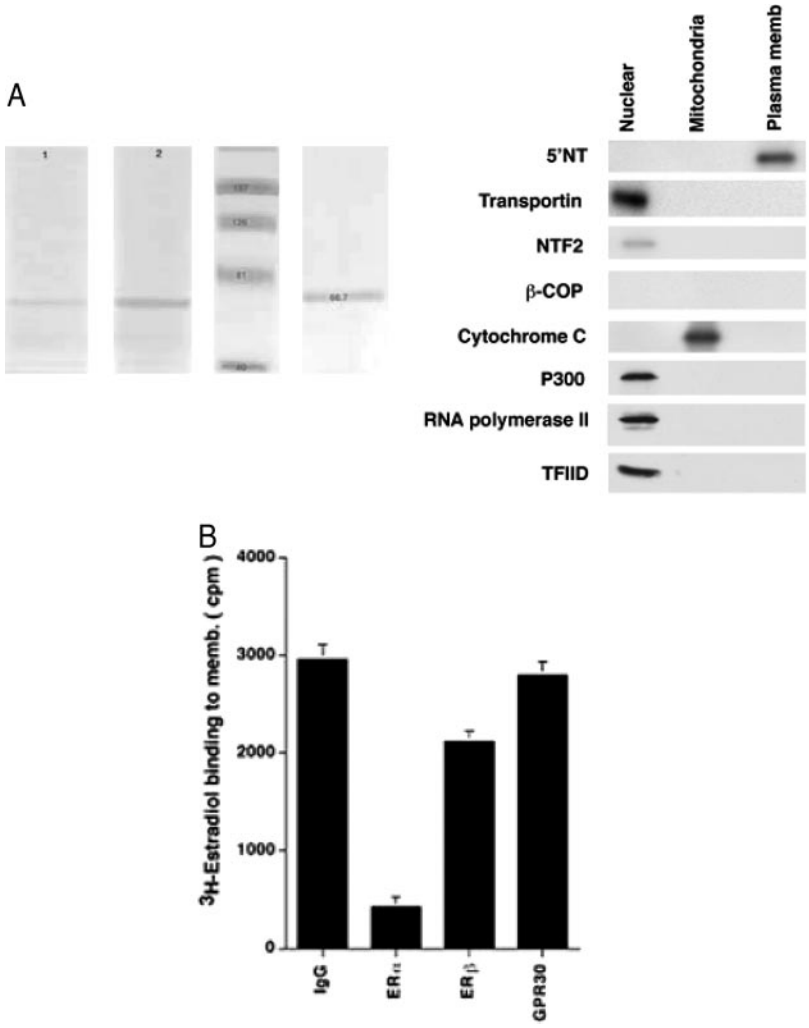


Fig. 2. Nature of the Membrane ER in MCF7 Cells

A (*left*), Protein bands from the plasma membrane and nucleus, respectively, separated by SDS-PAGE. MCF7 cells were subjected to sucrose gradient centrifugation to isolate the membrane and nuclear fractions. The two fractions were individually added to separate affinity columns of E2-sephadex. The eluants from the column were mixed in sodium dodecyl sulfate buffer, boiled, and separated by PAGE. The gel was stained with Gel Code Blue dye (Pierce Chemical Co.) and then dried. Molecular weight markers and recombinant human ERα protein were run in parallel on the gel. The membrane (lane 1) and nuclear (lane 2) bands were prominent at approximately 67 kDa and were cut out for MS analysis. A (*right*), Lack of contamination of membrane, mitochondrial, and nuclear fractions of MCF7 cells. The cell fractions were isolated by sucrose gradient centrifugation, and proteins from each fraction were separated by SDS-PAGE. Western blot was performed with antibodies against nuclear (transportin, P300, NTF2, RNA polymerase II, TFIID), mitochondrial (cytochrome C), cytoplasmic (β-COP), and membrane-localized proteins [5′-nucleotidase (NT)]. B, E2 significantly binds to ERα but not GPR30 in plasma membranes from MCF7 cells. Plasma membranes from MCF7 cells were isolated and solubilized with a small amount of detergent. Protein-normalized membrane aliquots were incubated with high-concentration antibodies to ERα, ERβ, GPR30, or with IgG (control), each linked to sepharose. After centrifuging the beads into a pellet, nonbound membrane protein in the supernatant was incubated with [^{3}H]E2, and bound E2 was isolated on GC/ Whatman nylon filters. Counts of bound [^{3}H]E2 were determined by β-scintillation counting. The single study shown comprised three replicates per each condition, and the entire study was repeated. 5′-NT, 5′-nucleotidase; TFIID, Transcription factor IID; β-COP, coat protein.

SDS-PAGE and stained for proteins (Fig. 2A). Only one discrete band appeared, notably at approximately 67 kDa, in both the membrane and nuclear fractions of MCF7 cells. This was comparable to the position on gel of recombinant human ERα protein, run in a parallel well. Importantly, no antibodies were used in the isolation of the proteins, including antisera for ERα. This prevented bias toward isolating only known ER protein. Approximately eight times more cell lysate was used to isolate membrane receptors, compared with the protein used for nuclear receptor isolation, consistent with the fact that nuclear/membrane ERs exist in an approximately 9:1 ratio (21). Each prominent gel band was cut out.

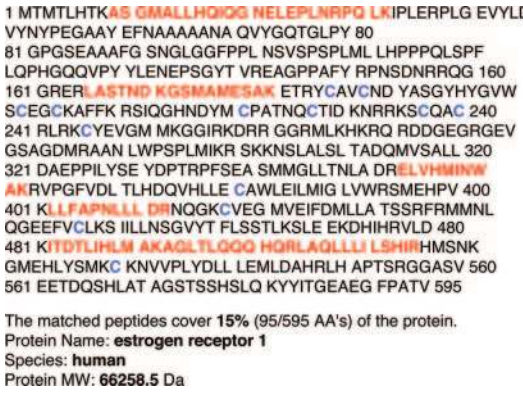
The bands at approximately 67 kDa were analyzed in the Mass Spectrometry Facility at the Ben May Cancer Institute, University of Chicago. The proteins constituting the prominent nuclear and membrane bands were trypsin cleaved and analyzed by electrospray MS. The output was further interrogated using two rigorous protein analysis programs, Mascot and Spectrum Mill (see *Materials and Methods*). Thirteen small to moderate size peptides resulted from the trypsin digests in each fraction and were all scored as 100% identical to classical human ERα (Table 1). Analysis of the membrane peptides showed coverage of 15% of the classical human ERα, and analysis of the nuclear proteins showed 19% coverage, ranging from the A/B domains to the E/F domains of the sex steroid receptor. Overall, the results were interpreted as unlikely to be a protein other than ERα, at a significance level of greater than $P < 0.00000000001$. Furthermore, although some peptides from the nuclear and membrane samples partially overlapped at several portions of ERα, there were also unique peptides in each cell fraction, covering differing portions of the receptor protein (representative peptides described in Table 1).

Only a small amount of human skin keratin proteins 1 and 9 were also found in the samples. These proteins are frequently found in samples analyzed by MS and are felt to be containments of handling the gel band (personal communication from Dr. Alexander Schilling, Director of the Mass Spectrometry Facility). A protein band of the size of ERβ was not detected

Table 1. Mass Spectrometry Sequence of Representative, Large Peptides from the Tryptic Digest of a Protein Band from the Membrane (*Top*) and Nucleus (*Bottom*) of MCF7 Cells

Mr(exp)	Mr(calc)	ΔMr	Peptide Sequence
2657.62	2657.42	0.20	(8–32)ASGMALLHQIQGNELEPLNR PQLK
1639.90	1639.75	0.15	(166–180)LASTNDKGSMAMESAK
2255.57	2255.64	0.07	(343–362)ELVHMINWAKR, ox methionine
1292.4	1291.9	0.5	(402–412)LLFAPNLLLDR
3270.78	3270.7	0.08	(482–515)ITDTLIHLMAKAGLTLQQQHQ RLAQLLLILSHIR, ox methionine
2657.8	2657.4	0.4	(8–32) ASGMALLHQIQGNELEPLNR PQLK
1846.06	1845.89	0.17	(143–158)EAGPPAFYRPNSDNRR
2353.03	2351.99	1.03	(212–231)SIQGHNDMCPATNQCTIDK+2 Carbamide
1284.9	1284.8	0.1	(402–412)LLFAPNLLLDR
1629.85	1629.7	0.15	(436–449)MMNLQGEEFVCLK
3270.81	3270.70	0.11	(482–515)ITDTLIHLMAKAGLTLQQQHQ RLAQLLLILSHIR, ox methionine

Peptide fragments were found to match the NCBI database for human ERα by MASCOT and Spectrum Mill programs. Peptide cleaved fragments from the membrane (*below left*) and nucleus (*below right*) protein bands that cover human ERα are shown in *red*. Cystine is shown in *purple* for reference.



after PAGE, as this protein is present in small amounts in MCF7 cells (see below).

To further rule out other E2-binding proteins in the cell membranes of the MCF7 cells, solubilized membrane fractions were subjected to preclearing of potential E2-binding proteins. Excess antibody to ERα (H222), ERβ, GPR30, or IgG (control) was bound to Protein A sepharose beads and incubated with solubilized membrane protein. This retained the specific proteins from the membrane fractions onto the antibody-conjugated beads. Nonretained membrane protein was then incubated with labeled estradiol, and specific binding was determined.

As seen in Fig. 2B, E2 bound strongly to solubilized membranes that were first incubated with IgG-sepharose (control), indicating one or more E2-binding proteins were not retained on the antibody-conjugated beads. However, ERα antibody-bead complexes removed 88% of subsequent E2 binding to the cell membrane. ERβ antibody-bead complexes removed the rest of the E2 binding, this being consistent with a very small amount of ERβ detected at the membrane by immunoblot (our unpublished data). Importantly, beads complexed with GPR30 antibody failed to remove E2 binding to membranes. Because this latter result was very similar to IgG antibody/bead exposure, little binding could have occurred to the orphan GPR. It is possible that solubilization or immunodepletion of the membranes disrupted ER-GPR30 binding that could have participated in an E2-binding complex. However, we previously reported that binding of small G proteins to ER was readily apparent in membranes solubilized by our procedure (21, 23). These results support the idea that classical ER proteins mediate the binding of E2 at the membrane.

E2 Does Not Function in ER-Negative Breast Cancer Cells

We then investigated the potential role of GPR30 for E2 signaling in breast cancer cells. GPR30 has been described at the plasma membrane or in the endoplasmic reticulum, and some investigations reported that this protein mediates second messenger generation, kinase activation, or calcium signaling by E2 (28–30). We used ER-positive breast cancer cells that are reported to produce GPR30 (MCF7), and ER-negative breast cancer cells that express this orphan GPR (SKBR-3) (28–31). Also, we briefly compared HCC-1569 breast cancer cells that lack both ER and GPR30 (data not shown) for binding E2. In membranes prepared from HCC-1569 or SKBR-3 cells, comparably insignificant binding of isotope-labeled E2 was seen (Fig. 3A, *left*). This supports the lack of any meaningful estrogen-binding protein at the membranes in these cells. In contrast, similar numbers of MCF7 cells showed 2.6 fmol of estrogen-binding protein at the membrane.

For further analysis, we carried out saturation binding using various concentrations of labeled E2 in MCF7 cell membranes. E2 bound membrane ER with an affinity [dissociation constant (K_d)] of 0.19 nM, and a binding capacity (B_{max}) of 5.3 pM (Fig. 3A, *right*). We also confirmed that GPR30 is expressed in both MCF7 and SKBR-3 cells, with some protein localized to the plasma membrane, but much of the orphan GPR residing in other, undetermined cell locations (Fig. 3B).

To determine the mediation of the rapid effects of E2, we examined multiple signals. In MCF7 cells, E2 activated ERK and PI3K, cAMP, and calcium. The ER inhibitor, ICI 182780, substantially blocked all these effects (Fig. 3C). These results suggest that ER mediates E2-induced rapid signaling in ER-positive breast cancer cells. In contrast, E2 did not significantly activate ERK, PI3K, or cAMP in SKBR-3 cells. Interestingly, E2 modestly stimulated an increase in calcium flux in these cells, but this was unaffected by ICI 182780, small interfering RNA (siRNA) to GPR30, or control siRNA to green fluorescent protein (GFP) (also see Fig. 4). Furthermore, 10 nM E2 caused a 4-fold greater increase in calcium in MCF7 than in SKBR-3 cells. Thus, potential interactions of the sex steroid with any putative binding protein do not have any recognizable and significant outcome in these ER null breast cancer cells.

We also compared cell cycle progression and cell survival responses in MCF7 and SKBR-3 cells. E2 stimulated a 260% increase in DNA synthesis (indicative of G_1/S progression) in the MCF7 cells, significantly inhibited by ICI 182780 (Fig. 3D, *left*). In SKBR-3 cells, a very modest stimulation of thymidine incorporation in response to E2 was seen, unaffected by ICI 182780. Furthermore, E2-induced a 75% survival of MCF7 breast cancer cells after UV-radiation that was 85% reversed by ICI 182780 (Fig. 3D, *right*). In SKBR-3 cells, there was a statistically insignificant 17% decrease in cell death in E2-incubated cells. We and others previously showed that the ability of membrane ER/E2 to stimulate ERK and PI3K contributes to survival in ER-positive breast cancer cells (8, 23). This coincides with previous demonstrations of rapid signaling from cell membrane ER that are required for similar E2 actions in a variety of cells (7–10).

Because only MCF7 cells demonstrated meaningful responses to E2, we asked whether GPR30 might contribute to these functions of the sex steroid in this cell that produces both putative binding proteins. To accomplish this, we silenced GPR30, using interfering RNA. As seen in Fig. 4A, *left*, approximately 80% knockdown of the GPR30 protein occurred with a specific siRNA (siRNA4) to this orphan GPR, not seen with the GFP control siRNA. Actin expression was unaffected by either siRNA, and transfection efficiency was about 82%, determined by expressing a second control, fluorescent siRNA (latter data not shown). The ability of E2 to activate rapid signaling to cAMP (Fig. 4A, *right*), ERK (Fig. 4B), PI3K (Fig. 4C), and calcium (Fig. 3C) was not affected by GPR30 knockdown. Fur-

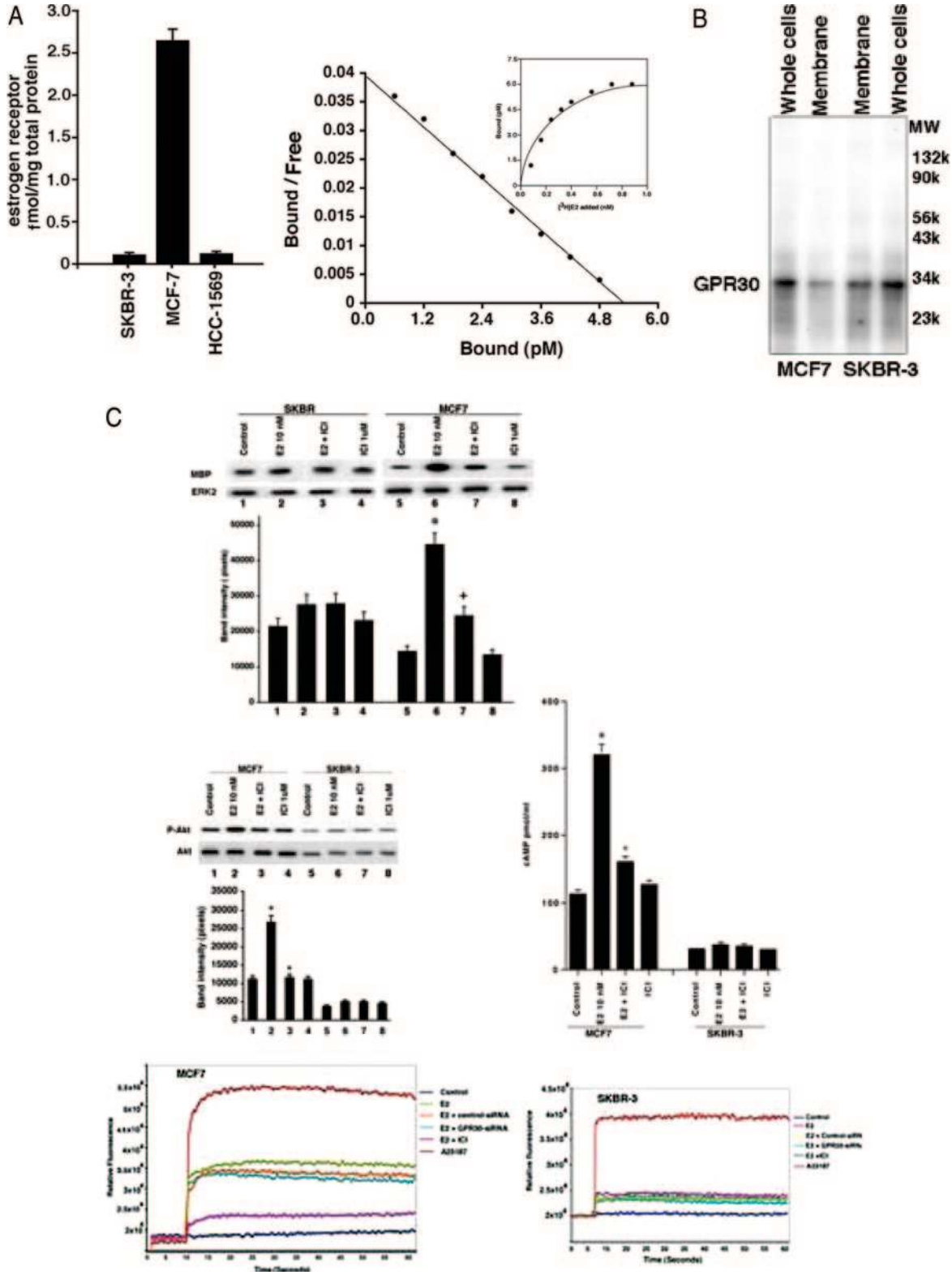
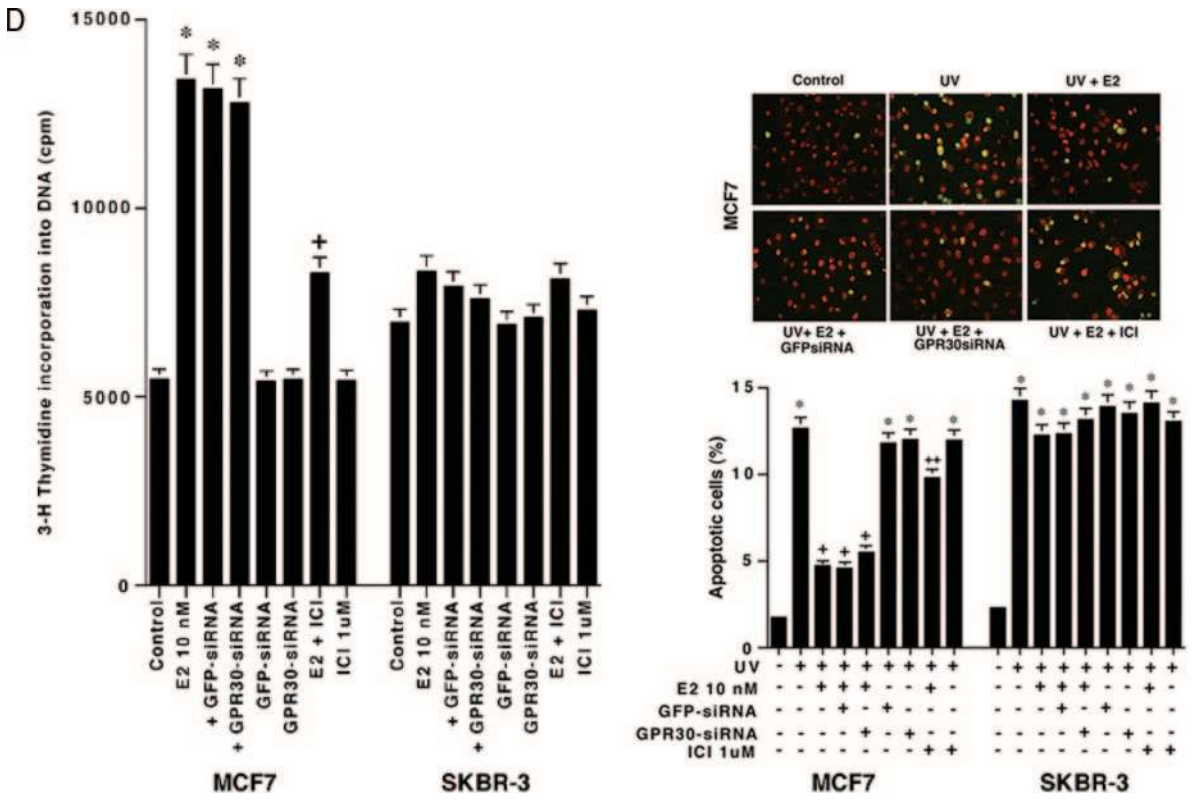


Fig. 3. Binding and Signaling by E2 in Breast Cancer Cells

A (*left*), Membranes from HCC-1569 and SKBR-3 cells (ER negative) and MCF7 cells (ER positive) were incubated with 1 nM [^{3}H]E2 for 1 h, with or without cold E2 (0.1 nM to 1 μM). Specific competition binding was determined, and analyzed for B_{max}, normalized for total protein. The data are from three studies. A (*right*), Saturation binding on the membranes of MCF7 cells. [^{3}H]E2 (0.1–1.5 nM) was added to membranes fractionated from 10^6 MCF7 cells, and binding to equilibrium occurred for 1 h at 37 C. Each *point* was done in triplicate. The *graph* represents the output by Scatchard analysis of binding from one experiment, using the



LIGAND computer program. The *inset* is the saturation binding curve, and the study was repeated a second time. B, GPR30 protein in the whole-cell lysate and in membrane fractions of SKBR-3 and MCF7 cells. The representative Western blot was repeated. C, E2 activates multiple rapid signals in MCF7 but not in SKBR-3 cells. Cells were incubated with 10 nM E2 ± 1 μM ICI 182780 for 1–15 min depending on the assay. ERK (8 min) and PI3K (15 min) activity was determined, with total kinase protein shown for each condition. cAMP generation (5 min) and calcium flux (multiple readings over 0–60 sec) were also determined as described in *Materials and Methods*. Calcium results shown are the mean of triplicate determinations for each condition in one experiment, and the study was repeated a second time. AG23187 is a calcium ionophore, serving as positive control. Three experiments were combined for ERK, PI3K, and cAMP generation. *, $P < 0.05$ for control *vs.* E2; +, $P < 0.05$ for E2 *vs.* E2 + ICI. D, E2 stimulates DNA synthesis (*left*) and cell survival (*right*) in MCF7 but not SKBR-3 cells. Cells were labeled with [^{3}H]thymidine, and uptake was measured at 24 h by β-scintillation counting, as an indicator of G_1/S cell cycle progression. *, $P < 0.05$ for control *vs.* condition; +, $P < 0.05$ for E2 + ICI *vs.* E2 for data combined from three experiments, three replicates per condition in each study. UV-induced cell death is shown for selected conditions by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining in MCF7 cells (*left side* of figure) and SKBR-3 cells (*right side* of figure). Five hundred cells per condition were counted in each experiment, and the *bar graph* represents three experiments combined for each cell type. *, $P < 0.05$ for control *vs.* condition; +, $P < 0.05$ for UV *vs.* UV + E2; ++, $P < 0.05$ for UV + E2 *vs.* same plus ICI 182780. ICI, ICI 182780; MW, molecular weight.

thermore, E2-induced thymidine incorporation in MCF7 was significantly inhibited by ICI 182780, but was unaffected by GPR30 silencing (Fig. 3D, *left*). A similar lack of GPR30 importance was seen for cell survival induced by E2 in these cells (Fig. 3D, *right*, lanes 2, 3, and 5 on the *left side* of the figure).

In contrast, siRNA to ERα significantly prevented ERK, PI3K, cAMP, and calcium signaling (Fig. 5). Knockdown of ERβ also had a modest effect on ERK activation, an insignificant effect on cAMP generation and calcium signaling, and no effect on PI3K activity, as stimulated by E2: these findings indicate the importance of the ERα isoform. We conclude that E2 does not utilize GPR30 for rapid signaling in MCF7 cells but does require mainly classical ERα.

DISCUSSION

The nature of ER-binding proteins that localize to and support rapid E2 signaling from the plasma membrane is still debated. Here we sought to clarify several controversies surrounding this important issue.

Membrane and nuclear-localized estrogen-binding proteins from MCF7 cells were isolated in an unbiased fashion and analyzed by MS. We report that membrane and nuclear ER in these human breast cancer cells are the same proteins, classical ERα. In supporting studies, no appreciable specific binding of E2 was found after immunodepleting ERα (primarily) and ERβ (secondarily) from MCF7-solubilized membrane fractions. This indicates that no residual E2-binding pro-

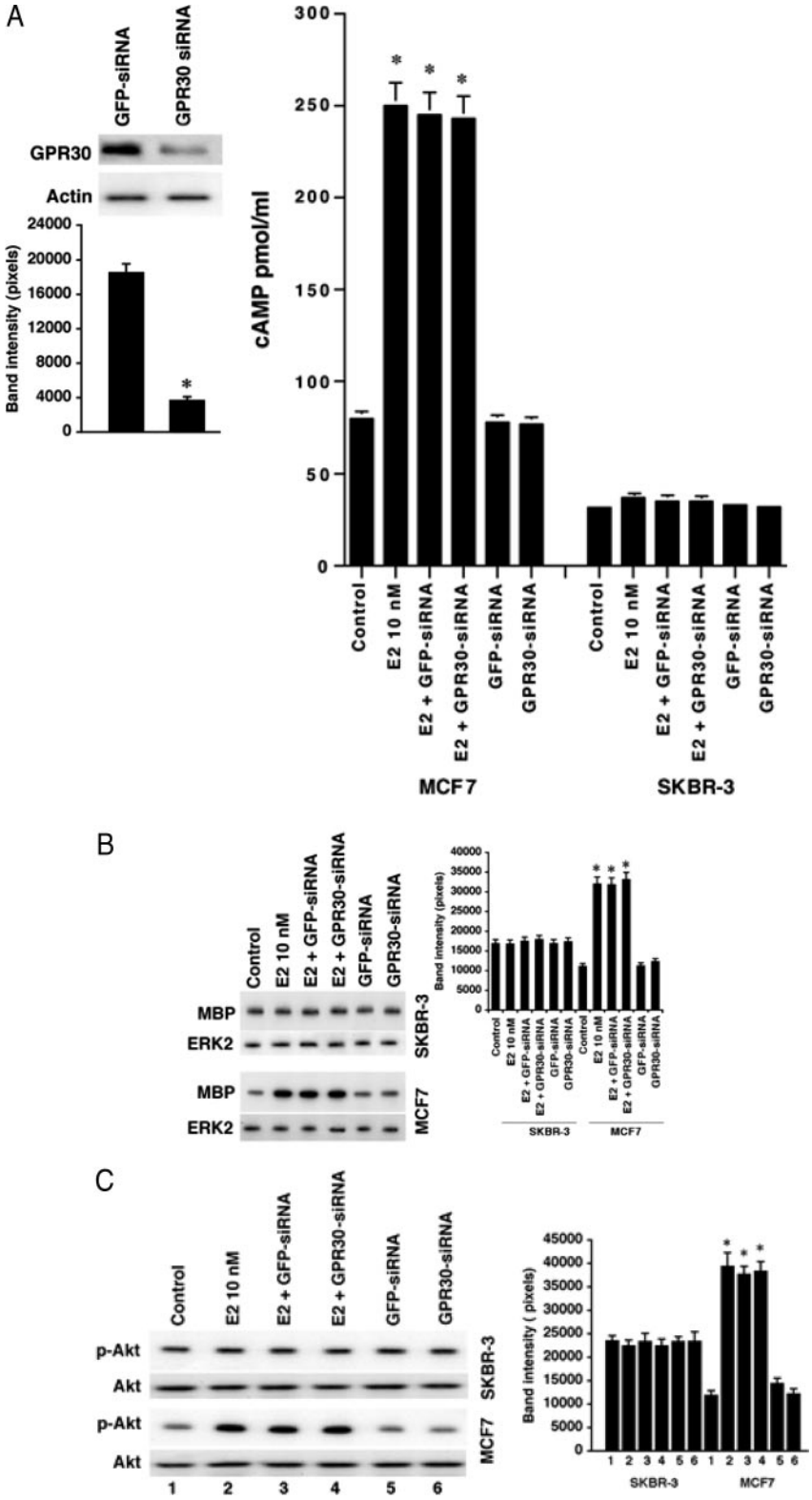


Fig. 4. Knockdown of GPR 30 Does Not Affect E2-Induced Functions in MCF7 Cells

A (*left*), MCF7 were transfected with several different siRNAs to GPR30 or GFP (control), and the cells then were used 48 h later for GPR30 immunoblot. Actin is shown as a loading and specificity control. Transfection efficiency was 82 ± 4 (SD)% over three experiments, determined by expression of a fluorescent, control siRNA (not shown). A (*right*), After 48 h, the cells were incubated with 10 nM E2, and cAMP was determined at 5 min. The *bar graph* represents three studies combined. *, $P < 0.05$ for control *vs.* condition. B, ERK activation in response to E2. A representative study after 8 min exposure to E2 is shown, repeated once. ERK2

teins remain in the absence of classical ER. We cannot rule out the presence of a small amount of another E2-binding protein that shares epitope homology to ERα. However, we used several different antibodies against N- and C-terminal motifs (data not shown), making it less likely that another protein was inadvertently precipitated. Also, immunodepleting GPR30 did not significantly influence E2 binding at the membrane.

Supporting this theme, we report that in ECs or breast cancer cells, E2 requires a classical ER to rapidly activate multiple signals to downstream cell biology. In ECs, wild-type, and not the combined ERα/ERβ-deleted cell, shows estrogen binding and activation of multiple rapid signals that are known to originate at the membrane. This is despite the fact that wild-type and DERKO EC contain comparable amounts of GPR30. In ECs, E2/ER have been reported to stimulate G_i-dependent PI3K activity and resulting nitric oxide generation (37).

In ER null breast cancer cells, E2 failed to significantly activate multiple signals or enact cell physiology. A modest cell survival response to E2 was seen in radiated SKBR-3 cells. However, it has been reported previously that E2 directly acts as an antioxidant to prevent cell death, unrelated to ER binding (38). Perhaps such a mechanism underlies this modest steroid action in the SKBR-3 cells, effects not comparable to ER-mediated signaling in MCF7 cells. Consideration of whether E2 functions in ER-negative cells is also relevant to clinical medicine and breast cancer biology. In women, aromatase inhibitors are ineffective in modulating the behavior of ER-negative breast cancer (39). Aromatase inhibitor compounds might be expected to have some therapeutic effects if E2 bound and functioned through alternative ER (such as GPR30) in classical ER-negative tumors.

In contrast, E2 up-regulated cAMP, ERK, PI3K, and calcium flux in MCF7 cells, all inhibited by ICI 182780. Additionally, the ability of E2 to stimulate DNA synthesis or cell survival in these breast cancer cells required an ER that was inhibited by ICI 182780. Finally, silencing ERα in MCF7 cells significantly inhibited rapid signaling by E2. We also asked whether there is functional cooperation between ER and GPR30 in MCF7 cells. In cells expressing a control siRNA, E2 rapidly and strongly activated multiple signals that led to cell cycle progression and cell survival. Comparable signaling and outcome response was found in MCF7 that showed approximately 80% GPR30 protein knockdown. We conclude that ER, and not GPR30, is required for rapid E2 actions in these cells.

When estrogen-binding proteins other than ERα or ERβ are further considered, previous reports of alternative ERs failed to show 1) the presence of functional receptors in cells from DERKO mice, and 2) the isolation and characterization of such receptors from the membrane. The receptor pharmacology of classical ER at the membrane may be somewhat different in various cell types (40, 41), depending on differences in receptor orientation, membrane domain localization, or unique binding/scaffolding partners. Importantly, it is largely unproven that E2 carries out any cell physiological actions mediated through alternative ERs. By contrast, rapid signaling and cell actions *in vivo* result from classical ER isoforms (42, 43).

More recent studies of nonclassical estrogen-binding proteins implicate GPR30. As recently reported, E2 stimulates calcium mobilization and PI3K activation in the endoplasmic reticulum though binding this orphan GPR (29). The location of GPR30 is in dispute: many earlier studies indicated a plasma membrane localization of this typical heptahelical protein (27, 28, 30), but this was notably absent in the more recent report (29). Furthermore, initial studies indicated that 17-β-E2 or ICI 182780 each activated GPR30 by unknown mechanisms and transmitted a downstream signal to the activation of the EGFR (28, 30). EGFR transactivation then resulted in rapid kinase activation.

Subsequent studies of E2 engagement of GPR30 at the membrane revealed an extremely low-capacity binding interaction and quite modest generation of cAMP, the latter determined mainly in cells transfected to overexpress GPR30 (31). In GPR30 overexpressing-cells, 100 nM E2 generated approximately 30% increase over baseline cAMP (31). This is compared with a 200% increase above basal levels, generated by endogenous ER responding to a 1 logarithm lower concentration of E2 as shown here in MCF7 cells.

Many of the GPR30 studies did not show a cell physiological function resulting from the interaction of E2 and GPR30 in cells expressing endogenous GPR30 (28–31). We confirmed that native SKBR-3 cells (ER negative) produce abundant GPR30, found at the plasma membrane and other locations in the cell. Despite this, E2 could not signal to cell biology. Similarly, we and others have reported in many ER null cells that E2 does not stimulate rapid signaling: However, this does occur after transfection of classical ER (13, 14, 22, 33). Thus, endogenous GPR30 supports no discernible functions of E2 in these cells.

Our results contrast to Revankar *et al.* (29), who showed that COS7 cells transfected to overexpress either ERα or GPR30 responded to E2 with a strong activation of intracellular calcium mobilization. These investigators did not determine calcium signaling in SKBR-3 cells that produce abundant endogenous GPR30 protein. We did find an insignificant activation

total protein blots are shown for normalization of results. *Bar graph* is three combined studies; $P < 0.05$ for control *vs.* E2 or E2 +siRNA(s). C, PI3K activation. AKT phosphorylation at serine 473 (PI3K-induced activation site) and total AKT protein (for normalization) are shown. A representative study is shown, and the *bar graph* is three experiments combined. *, $P < 0.05$ for control *vs.* condition. MBP, Myelin basic protein.

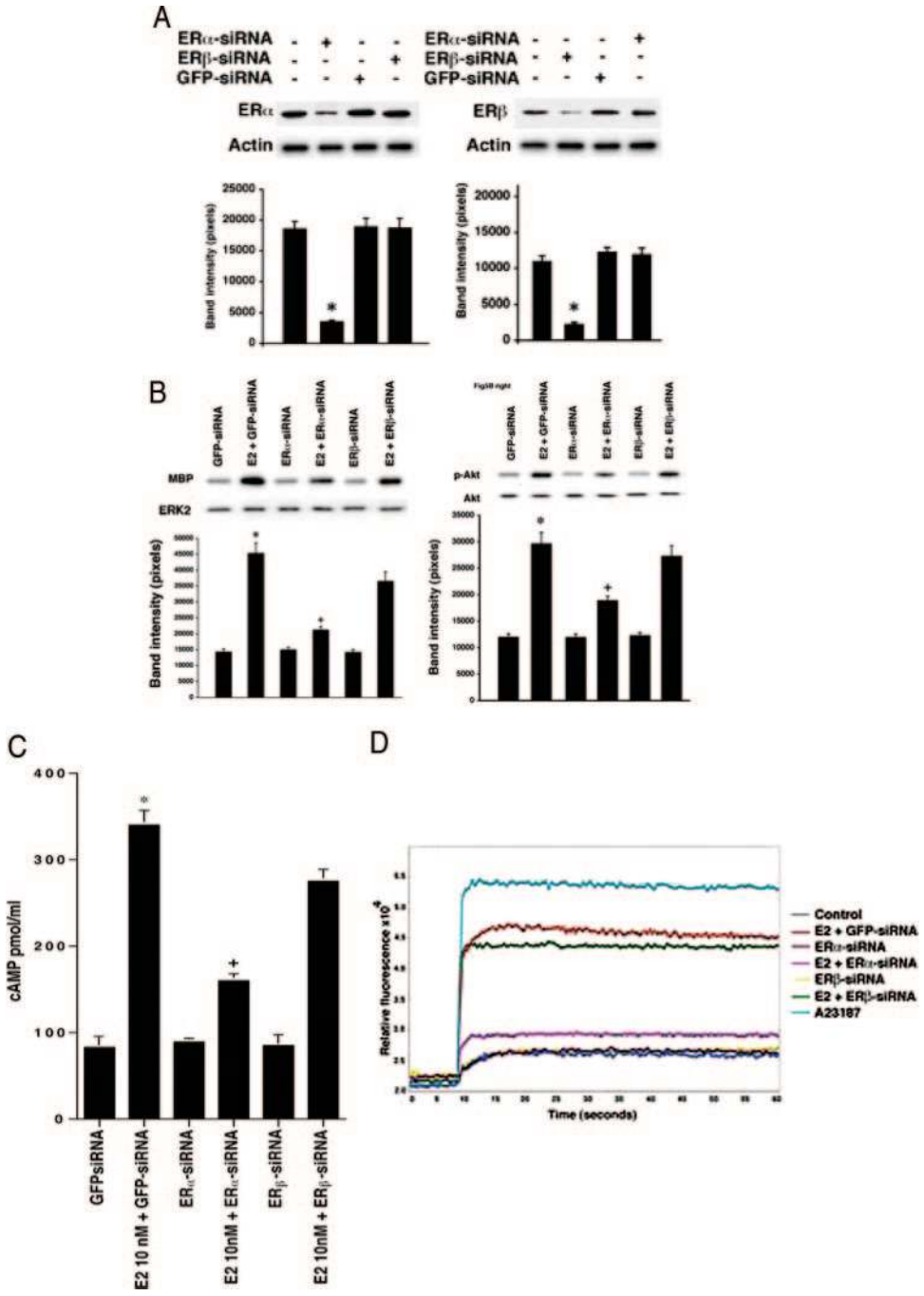


Fig. 5. siRNA to ERα Prevents E2-Induced Signaling

MCF7 cells were transfected to express siRNA to GFP, ERα, or ERβ, recovered over 48 h, and specific protein expression was then determined by Western blot (panel A). At 48 h post-transfection, ERK and PI3K activity (panel B, *left* and *right*) and cAMP generation (C) were determined. The representative kinase studies and the cAMP *bar graph* data reflect three combined experiments. *, $P < 0.05$ for control (GFP-siRNA) *vs.* E2 + siRNA to GFP; +, $P < 0.05$ for E2 + siRNA to GFP *vs.* E2 + siRNA to ERα. D, E2-induced calcium in MCF-7 cells. After transfection of GFP, ERα or ERβ siRNA, MCF-7 cells were recovered and exposed to 10 nM E2. Calcium was measured repetitively over 60 sec. Data from a representative study are shown reflecting triplicate determinations per condition, and the study was repeated once. MBP, Myelin basic protein.

of calcium by E2, unrelated to ER or GPR30 in SKBR-3 cells. Previously, direct activation of the Maxi-K calcium channel was triggered by pharmacological concentrations of E2 (44). Perhaps such a mechanism accounts for the modest stimulation of calcium reported here in SKBR-3 cells.

However, recent studies in endometrial cancer cells have implicated both ER and GPR30 in E2-induced

c-Fos transcription, and cell proliferation (45) and previous studies have implicated this orphan GPR in rapid signaling by E2 to transcription in keratinocytes (34). Thus, in some cell contexts, GPR30 may play a contributory role. It remains to be demonstrated whether GPR30 acts independently of ER in transmitting rapid E2 signaling (45).

In summary, E2 fails to activate multiple pathways in cells that lack classical ER even when GPR30 is present. Consistent with these findings, we report that the membrane and nuclear receptors in MCF7 are the classical ERα. We suggest that future reports of alternative estrogen-binding proteins should 1) isolate and analyze the proteins, 2) establish physiological functions of estrogen mediated through the endogenous putative receptors, and 3) define whether endogenous classical ER are also required for E2 action.

MATERIALS AND METHODS

Isolation of Membrane and Nuclear ER

One hundred dishes ($\sim 10^9$) of MCF7 cells were grown to near confluence in DMEM/F12 medium. The cells were lysed, and nuclear and membrane fractions were isolated by sucrose gradient centrifugation. Cell fractions were dialyzed and the membrane receptors were solubilized with 0.2% Nonidet P-40. The purity and lack of contamination of the two cell fractions have been extensively validated previously (13, 36) and were further validated by the presence of integral proteins in cell fractions, determined by Western immunoblotting. Antibody to ERα (MC-20, directed against the C terminus of this protein) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) for this purpose.

Membrane and nuclear fractions were then passed through an affinity column composed of E2-sephadex beads (kindly provided by Dr. Geoffrey Greene, University of Chicago) (46). The proteins were released from the E2-sephadex by sodium dodecyl sulfate (2%)-containing buffer and subjected to denaturing by boiling. Proteins were separated on 8% gels by SDS-PAGE, and the resulting bands were visualized using Gel-Code Blue staining reagents (Pierce Chemical Co., Madison, WI). The excised membrane and nuclear protein bands were further analyzed.

Electrospray MS of Proteins

Each protein band was destained, and the gel slice was washed with water and then with 0.5% formic acid in acetonitrile to dehydrate the gel. The gel slice was digested (50 mM ammonium carbonate, pH 9.0; 250 mM $CaCl_2$; and 12.5 ng/ml of modified Trypsin enzyme (final enzyme-protein ratio, 1:5). The gel in tube was rehydrated and placed in a clean tube with 100 ml of an extracting solution containing 5% formic acid. Equal volume of 5% formic acid-acetonitrile (50:50) was added to solubilize any hydrophobic material remaining in the gel.

The sample was injected onto a trapping column (Zorbax Stablebond C18, 300 A pore) connected to a reversed-phase column (75 mm id Zorbax Stablebond C18 (300 A pore), connected to a 1100 nano/capillary LC/MSD XCT system (Agilent Technologies, Palo Alto, CA), equipped with an orthogonal nanospray interface and a nanopump. The samples were chromatographed using a solvent system of A) 0.1% formic acid and 10% acetonitrile and B) 0.1% formic acid and 100% acetonitrile at a flow rate of 300 nl/min, using a 15–55% gradient over 60 min. The instrument completed a mass scan from 400-2200 daltons in 1 sec and optimized the acquisition of quantitative data in MS mode as well as qualitative data in MS/MS mode.

Data analysis was carried out using both Mascot (Matrix Science, Boston, MA) (47) and SpectrumMill software platforms (Agilent Technologies, Palo Alto, CA). Raw data from the MS data files were first subjected to protein library search and spectral interpretation by the Sherenga (48) module. The results for all proteins detected from both platforms received a search score. A general requirement for a positive identification (at $P < 0.01$ or greater) is that the library matches agree between both search platforms and that at least four distinct peptides for the tentatively identified protein are present.

Cells

ECs from the brain capillaries of wild-type and DERKO mice were prepared as previously described (23). MCF7, SKBR-3, and HCC-1569 cells were from American Type Culture Collection (Manassas, VA). DNA synthesis (thymidine incorporation) and apoptosis assays (TUNEL) were conducted as previously described (21, 23). Cells were transfected to express siRNAs to GFP, or to GPR30 (duplex 4, antisense strand, 5′-PCUUCAGCGAAUCUCACUCCUU-3′) (Dharmacon, Lafayette, CO). ERα or ERβ siRNAs (QIAGEN, Chatsworth, CA) were reported to be directed against the following DNA sequences, respectively; human esr1, 5′-AAGCCCAAATGTGTTGTGGCC-3′, and esr2, 5′-AAGGTGGGATACGAAAAGACC-3′. siRNA (2–3 μg) was used in each well of six-well plates or 100-mm dishes of cells. Fluorescent control siRNA (QIAGEN) was used to determine transfection efficiency over three experiments.

Signaling Assays

The activities of ERK, PI3K, and cAMP were determined as previously described (23, 35), all within 5–15 min exposure of cells to E2. In some experiments, the cells were pretreated with 1 μM ICI 182780 for 20 min before addition of E2. For some signaling studies, data from three experiments were combined and analyzed for statistical significance by calculating a mean $\pm$ SEM and comparing conditions by ANOVA plus Schefe's test, at a significance level of $P < 0.05$. In additional experiments, siRNAs to GPR30, ERα or ERβ, or GFP (control) were introduced into cells with oligofectamine and recovered overnight, and GPR30 or ER protein knockdown was determined by Western blot after 48 h, as we described previously (36). Transfection efficiency was determined using an Alexa-fluor 488-tagged, scrambled double-stranded RNA oligonucleotide (QIAGEN), with Hoechst nuclear stain. Antibody to GPR30 was kindly provided by Dr. Eric Prossnitz (29) and was used at a dilution of 1:2500. For cell signaling assays, the assays proceeded 48 h after siRNA transfection.

Calcium activity reflected both extracellular calcium influx through membrane channels and intracellular calcium mobilization from stores and was determined as follows. Calcium was measured in MCF7 and SKBR-3 cells by loading with Fluo-4 NW (Molecular Probes, Inc., Eugene, OR). The cells were cultured in 96-well microplates (poly-D-lysine-coated) to subconfluence (40,000–50,000 cells per well) and grown overnight. The next day, some cells were transfected with siRNA as described. After 24 h, the cells were synchronized overnight in medium lacking fetal bovine serum and phenol red. The medium was removed to eliminate sources of baseline fluorescence, particularly esterase activity. Fluo-4 NW (100 μl) in loading solution was carefully added to each well, and the cells were incubated at 37 C for 30 min. The microplate was then transferred into a 37 C prewarmed, Nowastar Spectrofluorometer (BMG Labtech, Offenburg, Germany).

The instrument was preset and loaded with test solutions at 2× concentration, subsequently injecting 50 μl of control or test substances to each well, followed immediately by 250 cycles of reading over 60 sec. Each condition was done in triplicate, and the study was repeated a second time. Fluorescence was measured at excitation, 494 nm, and emission at 516 nm.

E2 Binding Studies

Competition binding studies on whole ECs or on the membranes of MCF7 cells were conducted as follows: 1 nM [^{3}H]E2 was added to tubes containing 10^4 ECs or membranes fractionated from 10^6 MCF7 cells, each tube also containing 0.01 nM-1 μM unlabeled E2. Binding to equilibrium (21) was carried out for 1 h at 37 C. Saturation binding on MCF-7 cell membranes was done using a set of tubes containing [^{3}H]E2 at 0.1–1.5 nM (total binding). Another set of identical tubes also contained 100-fold excess unlabeled E2 (nonspecific binding). Binding was carried out at 1 h and 37 C, and specific binding was determined after passing the mixture through GF/C Whatman filters under mild vacuum. Labeled E2 bound to membrane protein was quantified by β-scintillation counting of the washed filters, subtracting nonspecific binding from total binding. Binding data were subjected to Scatchard analysis using the LIGAND computer program. Each point was done in triplicate and the study was repeated a second time.

For the preclearing studies, antibodies to ERα, ERβ (L-20, directed against the C terminus of this protein, Santa Cruz Biotechnology) GPR30, or IgG (control) were linked to sepharose beads for 2 h, and then added to protein-normalized, separate aliquots of solubilized membrane fractions from MCF7 cells. After 2 h, the membrane proteins bound to the antibody-bead complexes were removed by pelleting through centrifugation. The supernatants (unbound membrane proteins) were then incubated with 1 nM [^{3}H]E2 for 1 h, and binding was determined after passing the mixture through filters as described above.

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