0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(6):1606–1617

doi: 10.1210/me.2004-0468

Distinctive Actions of Membrane-Targeted *Versus* Nuclear Localized Estrogen Receptors in Breast Cancer Cells

Deshanie Rai, Antonina Frolova, Jonna Frasor, Anne E. Carpenter, and Benita S. Katzenellenbogen

Departments of Molecular and Integrative Physiology (D.R., A.F., J.F., B.S.K.) and Cell and Structural Biology (A.E.C., B.S.K.), University of Illinois and College of Medicine, Urbana, Illinois 61801

Estrogens regulate multiple activities in breast cancer cells, including proliferation. Whereas these hormones are most commonly known to regulate gene transcription through direct interaction with estrogen receptors (ERs) and with specific DNA sequences of target genes, recent studies show that ER also activates a number of rapid signaling events that are initiated at the cell membrane. To study the membrane-initiated effects of estrogen and separate them from the activities initiated by the nuclear localized ER in human breast cancer cells, we generated MDA-MB-231 breast cancer cell lines that have stably integrated either the wild-type nuclear form of ER (WT-ER) or a modified, membrane-targeted ER (MT-ER) that lacks a nuclear localization sequence and is dually acylated with a myristoylation sequence at the N terminus and a palmitoylation sequence at the C terminus. We demonstrate that MT-ER is membrane localized in the absence of estradiol (E2), showing punctate membrane and cytoplasmic speckles after E2 exposure. In contrast to WT-ER, MT-ER was not down-regulated by E2 or by antiestrogen ICI 182,780 exposure, and MT-ER failed to regulate endogenous E2-responsive genes highly up-regulated by WT-ER. Cells expressing MT-ER showed a greater serum response element-mediated transcriptional response that was partially inhibited by antiestrogen ICI 182,780. The MT-ER and WT-ER differentially altered ERK1/2 and Akt activities and the proliferation of breast cancer cells in response to E2. Hence, this study reveals distinct actions of the MT-ER *vs.* the WT-ER in effecting estrogen actions in breast cancer cells. (*Molecular Endocrinology* 19: 1606–1617, 2005)

ESTROGENIC HORMONES regulate multiple activities in breast cancer cells, including cell proliferation and invasiveness. The estrogen receptor (ER) is present in nearly two thirds of breast tumors, and the ER status of breast tumors serves as an important indicator of likelihood of benefit from endocrine therapy (1, 2). It is well accepted that the hormone-occupied ER functions as a versatile transcription factor to either activate or repress gene expression (3, 4). These genomic effects of estrogen involve both 1) direct interaction of the estrogen receptor (ER) with specific DNA sequences termed estrogen response elements (EREs) (5) and 2) indirect tethering of ER to DNA through protein-protein interactions (6, 7).

In addition to these nuclear events, estrogen, like many other steroid hormones, has been demonstrated to be capable of enacting rapid, membrane-initiated signaling events in a variety of cell types (8). These include release of calcium, secretion of prolactin, generation of inositol triphosphate or nitric oxide, and activation of MAPK (9–15), with the response dependent on the nature of the target cell. These findings support the hypothesis that estrogen can exert extranuclear actions either by interacting directly with other growth factor receptors, *e.g*. the epidermal growth factor (EGF) receptor, or through a membrane-associated form of the ER (16). Indeed, evidence for the existence of a cell membrane ER was provided two decades ago (12, 17, 18). More recently, a small pool of endogenous ER has been shown to localize to the plasma membrane in Chinese hamster ovary cells (11) and pituitary tumor cells (10), and to be concentrated in caveolae raft domains of estrogen target cells (19–23). However, the structure and function of this extranuclear form of ER are still unclear.

Therefore, our goal was to use a more direct approach for studying ER membrane-initiated events in breast cancer cells, to elucidate the biological roles and functions of the ER at both the cell surface and nuclear level. To do this, we targeted the ER outside the nucleus and to the cell membrane using fatty acid acylation, *viz.* myristoylation alone or in combination with palmitoylation, and with deletion of the receptor's nuclear localization sequence. Fatty acid acylation is a fundamental biological process whereby proteins are modified with lipophilic moieties, *e.g.* myristate and

First Published Online April 14, 2005

Abbreviations: CAT, Chloramphenicol acetyltransferase; E2, estradiol; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; MT-ER, membrane-targeted ER; SEAP, secreted alkaline phosphatase assay; SRE, serum response element; WT-ER, wild-type ER.

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

palmitate, to direct their binding to the cell membrane lipid bilayer (24). Myristoylation is the permanent cotranslational linkage of the 14-carbon fatty acid myristate to the N-terminal glycine of a protein via an amide bond. Palmitoylation is the reversible posttranslational linkage of the 16-carbon fatty acid palmitate to variably located cysteine residues via a thioester bond (25). Examples of fatty acylated proteins include several Src-related tyrosine kinases, α-subunits of the heterotrimeric G proteins, and the A-kinase-anchoring protein AKAP18 (26).

In this study, we show that membrane-targeted ER does localize outside the nucleus in breast cancer cells and exhibits several unique properties in genomic and nongenomic assays different from the WT-ER in these cells.

RESULTS

Design of Membrane-Targeted-ER (MT-ER) Constructs and Generation of Breast Cancer Cell Lines Stably Expressing MT-ER

The WT-ER and the MT-ER constructs are shown in Fig. 1. The MT-ER contains a myristoylation sequence at the N terminus and a palmitoylation sequence at the C terminus of the receptor. In addition, we deleted amino acids 256–303 from the WT-ER, because we found that constructs containing the myristoylation and palmitoylation sequences on the full-length ER protein that included this nuclear localization sequence-containing region showed some localization in the nucleus despite the presence of these fatty acid modifications introduced to direct localization to the membrane. Hence, our cell characterizations only used MT-ER shown in Fig. 1 that lacked amino acids 256–303 of the WT-ER, the sequence in ER that contains the strong nuclear localization signal.

MDA-MB-231 breast cancer cells, which are ER negative, were stably transfected with either WT-ER or MT-ER using a pcDNA3.1+ plasmid containing a neomycin resistance gene as a vector for stable integration. Positive clones were selected with G418 as detailed in *Materials and Methods*, and after two rounds of selection, various clones were tested for the presence of ER mRNA by real-time quantitative PCR and for ER protein by Western blot with an ERα-specific antibody (Fig. 2). The levels of MT-ER and WT-ER mRNA in the cell clones, as determined by real-time quantitative PCR using standard curves with multiple, differing known amounts of ERα cDNA (Fig. 2A), were compared with the parental MDA-MB-231 cells and with an ERα-containing MDA-MB-231 human breast cancer cell line (denoted 231/ER) previously characterized in this laboratory (27). Of the 20 clones of each receptor type evaluated, 11 clones were positive for MT-ER and 9 were positive for WT-ER (Fig. 2A). The ER mRNA copy number in the MT-ER clones varied from 3500–27,000 per 10 ng RNA, with most clones having copy numbers of approximately 6000–8000. Levels of WT-ER were generally lower than those of MT-ER in most positive clones assayed, but two clones (WT-13 and WT-19) had copy numbers of 6470 and 4160 per 10 ng RNA, similar to those in most MT-ER clones. For our comparative studies, we therefore selected MT-ER clones 6, 11, and 18 and WT-ER clones 13 and 19 because these had similar receptor levels, levels also similar to those in our previously generated MDA-MB-231/ER+ cells (27) and about 20% of that present in the high ER-expressing MCF-7 human breast cancer cells.

These clones, with similar levels of MT-ER or WT-ER RNA, also had similar ER protein levels as determined by immunoblot analysis (Fig. 2B and data not shown), and the mobility of the two ERs was quite similar, the absence of amino acids 256–303 being compensated for by the N- and C-terminal protein modifications in the MT-ER. Intriguingly, incubation of cells with E2 or with the antiestrogen ICI 182,780 revealed that E2 reduced slightly the cellular level of WT-ER protein by 60 min, and ICI greatly reduced the level of WT-ER, as

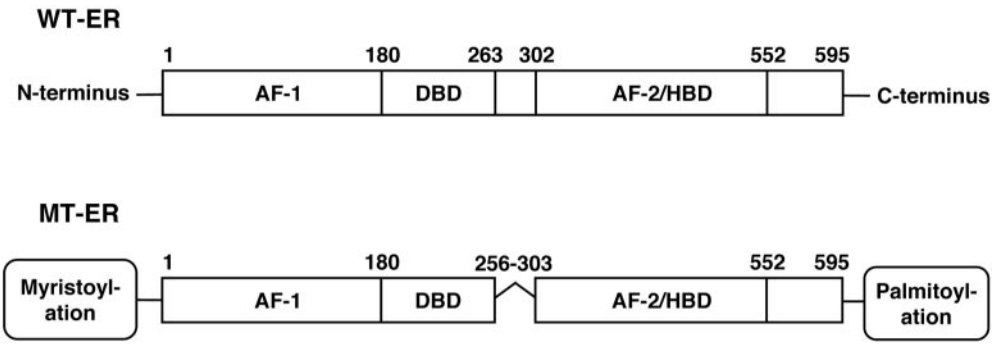

Fig. 1. Schematic Representation of WT- and MT-ERs

The activation function 1 (AF-1), DNA binding domain (DBD), and activation function 2/hormone binding domain (AF-2/HBD) regions of ER are shown. Dual-acylated ΔNLS-ER (MT-ER) was constructed by attaching a myristoylation sequence and a palmitoylation sequence to the N and C terminus, respectively, of ER missing the nuclear localization sequence (NLS, amino acids 256–303).

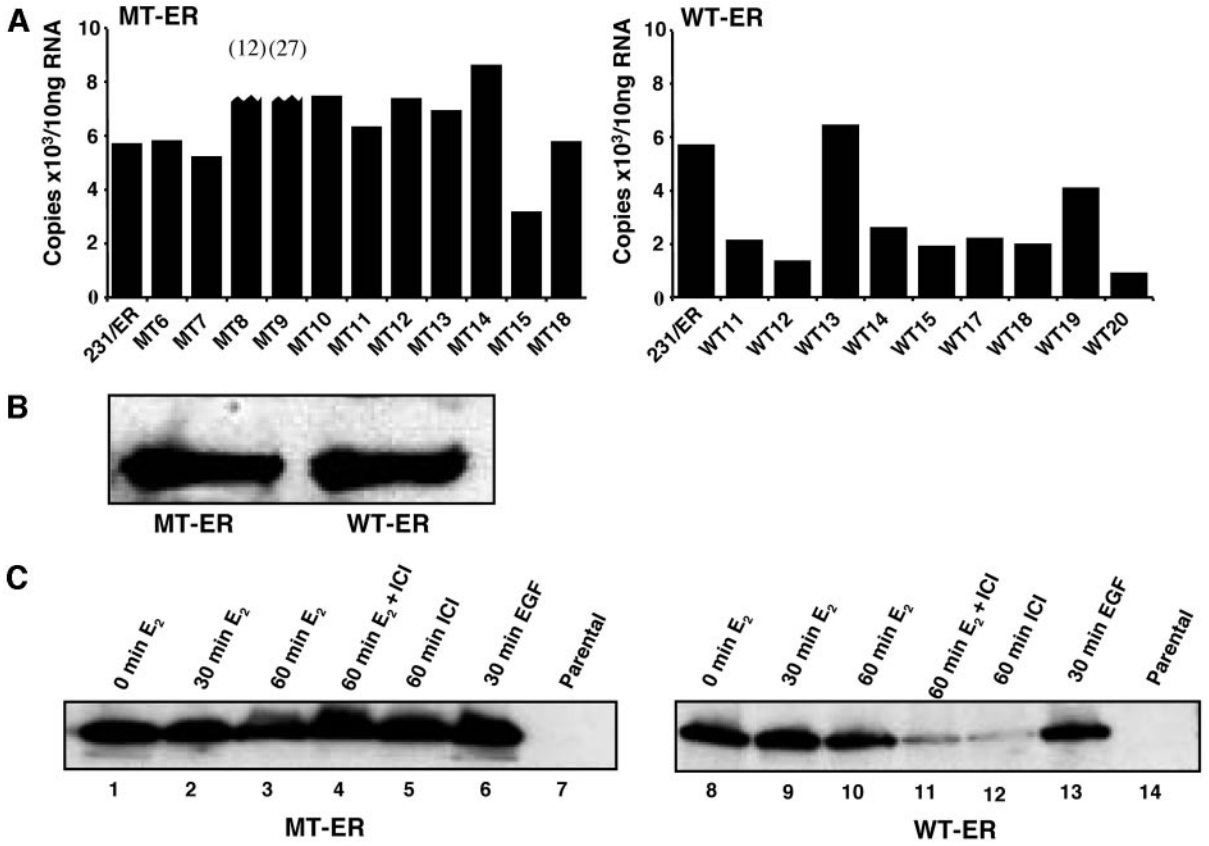

Fig. 2. Characterization of MDA-MB-231 Cell Clones Stably Expressing MT-ER or WT-ER

A, ER mRNA copy number/10 ng total RNA in different cell clones as assessed by real-time quantitative PCR. Values are the mean of closely corresponding values from two separate experiments. B, Western blot of MT-ER and WT-ER from MT-ER cell clone 11 and WT-ER cell clone 19. Cell extracts (200 μg protein per lane) were separated by SDS-PAGE. The ER proteins were detected with the anti-ER H222 antibody. C, Effect of E2 (10^{-8} M), antiestrogen ICI 182,780 (10^{-6} M), or EGF (10 ng/ml) on the level of MT-ER or WT-ER protein in MDA-MB-231 cells treated with compounds for the times indicated. Lanes 7 and 14, containing extracts from parental MDA-MB-231 cells, show no detectable ER as expected. Immunoblots were probed with anti-ER H222 antibody.

expected. In stark contrast, ICI failed to increase turnover of the MT-ER, and E2 likewise had little if any effect on the level of MT-ER protein in the cells (Fig. 2C). As shown in lanes 7 and 14 of Fig. 2C, the parental MDA-MB-231 cells, as expected, lacked any detectable ER protein.

Hormone binding (Scatchard) assays were performed on whole cell protein extracts from MT-ER clone 6 and 11 cells and WT-ER clone 13 and 19 cells to ensure the presence of receptors able to bind hormone. These assays demonstrated similar levels of specific ^{3}H-E2 binding in the WT-ER and MT-ER cells (~0.1–0.15 pmol/mg protein) and affinities for E2 (dissociation constant values) of 0.55 $\pm$ 0.06 nM for WT-ER and 0.36 $\pm$ 0.12 nM for MT-ER (n = 4 experiments). The ^{3}H-E2-specific binding curves obtained in one of the experiments is shown in Fig. 3 and is representative of four separate experiments performed in duplicate with extracts from two different WT-ER and two different MT-ER cell clones. In competitive binding studies not shown, we found that WT-ER and MT-ER have essentially equivalent affinities for the ERα selective ligand propylpyrazoletriol (28, 29) and for the phytoestrogen genistein (30).

Cellular Localization of MT-ER Outside the Nucleus and Primarily to the Cell Membrane

We next investigated the intracellular localization of our WT-ER and MT-ER constructs in MDA-MB-231 cells by fluorescence microscopy immunocytochemistry with an ER-specific antibody (Fig. 4). In the absence of added estrogen, the WT-ER was predominantly nuclear with faint diffuse cytoplasmic staining; upon estradiol addition, WT-ER localized exclusively to the nucleus, as expected (26). In contrast, MT-ER was localized primarily to the cell membrane with some cytoplasmic localization in the absence of estradiol. After estradiol treatment, the MT-ER showed a punctate pattern with localization in the membrane and cytoplasm, these membrane and cytoplasmic speckles probably representing endosomal or microsomal association.

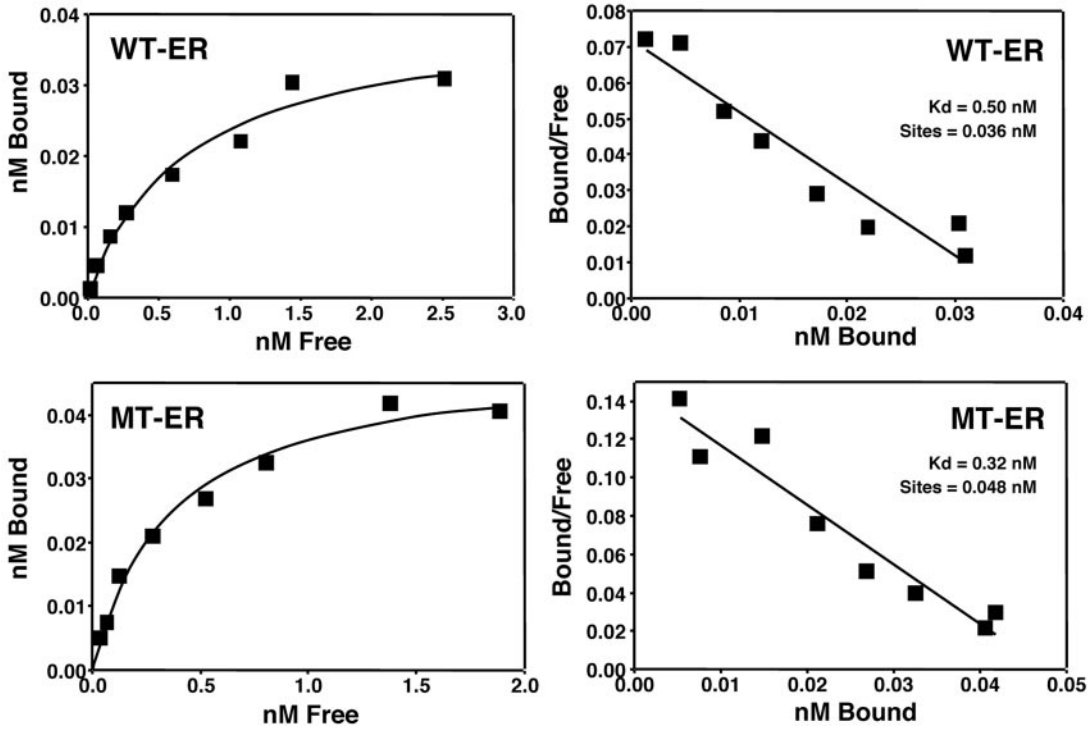

Fig. 3. Hormone Binding of WT-ER and MT-ER in Cell Extracts Monitored by ^{3}H-E2 (Scatchard) Saturation Binding Assay

Extracts were incubated with increasing concentrations of ^{3}H-E2 alone, and ^{3}H-E2 in the presence of a 100-fold excess of radioinert E2. The mean of closely corresponding duplicate determinations at each ligand concentration was determined. Specific ^{3}H-E2 binding (hot minus hot + cold binding) was calculated, and results for a representative experiment are shown. *Left panels* show direct binding curves, and *right panels* show the same data in Scatchard plot format. Similar hormone binding data were obtained in three additional experiments with different WT-ER and MT-ER cell clones.

MT-ER Fails to Regulate Endogenous Estrogen-Responsive Genes or Transfected Estrogen-Responsive Gene Constructs

To examine whether the MT-ER constructs were capable of stimulating the expression of estrogen-responsive genes, we examined their regulation of two endogenous genes, namely pS2 and WISP2, genes that we knew to be robustly regulated by the WT-ER in these cells and in MCF-7 breast cancer cells (31–33). As shown in Fig. 5, WT-ER robustly stimulated pS2 and WISP2 gene expression in a time-dependent manner in response to E2. In contrast, we observed essentially no stimulation of these genes by the MT-ER.

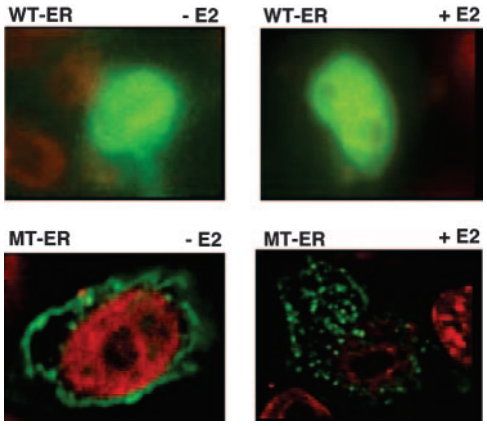

Fig. 4. Cellular Localization of WT-ER and MT-ER Constructs

MDA-MB-231 cells expressing WT-ER or MT-ER were subjected to immunocytochemistry, before and after treatment with 10^{-8} M E2 for 1 h. Cell nuclei are shown in *red*, and ER staining is shown in *green*.

We also evaluated the ability of MT-ER to transactivate ERE or non-ERE-dependent gene constructs shown previously to be stimulated by E2 via the wild-type-ER (WT-ER) in a variety of cell lines (Fig. 6). WT-ER gave marked stimulation of ERE-dependent gene expression and also stimulation of the TGF-β3 promoter reporter, which contains an estrogen-responsive region known to be very different from the estrogen response element (34, 35). In contrast, MT-ER did not activate this ERE or non-ERE-dependent gene transcription. Transient transfection of these two reporter gene constructs along with either WT-ER or MT-ER into parental (ER negative) MDA-MB-231 cells gave the same results as observed in Fig. 6 with the stably transfected cells—stimulation of reporter gene expression by WT-ER but no stimulation of reporter gene expression by MT-ER with E2 treatment (data not shown).

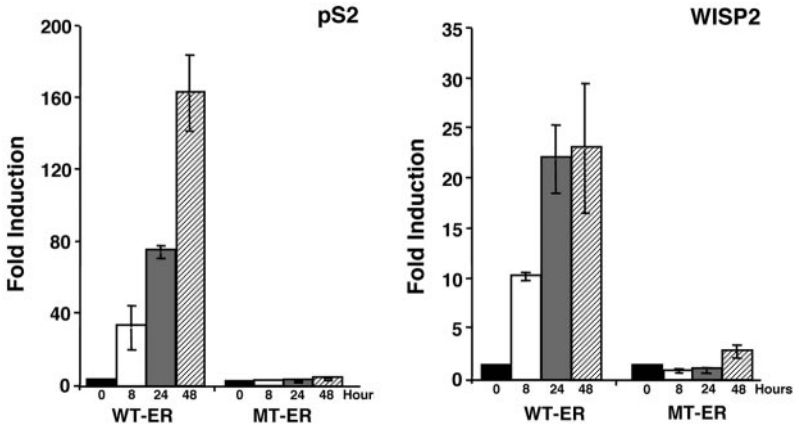

Fig. 5. Effect of WT-ER and MT-ER on Endogenous Estrogen-Responsive Gene Regulation

MDA-MB-231 cells expressing WT-ER or MT-ER were incubated with control ethanol vehicle or with E2 (10^{-8} M) for 0, 8, 24, and 48 h and changes in pS2 and WISP2 mRNA levels were determined by quantitative RT-PCR. Values represent mean ± SD from three separate experiments performed in duplicate with different WT-ER and MT-ER cell clones.

MT-ER More Robustly Activates Serum Response Element (SRE) Activity

The SRE-secreted alkaline phosphatase assay (SEAP) was used to compare SRE activity in WT-ER and MT-ER containing cells. The SRE is recognized by a dimer of the serum response factor, whose binding recruits the ternary complex factor, comprising 3 proteins, Elk-1, SAP1 (serum response factor accessory protein) and SAP2 (36). As seen in Fig. 7, the MT-ER cells had markedly higher SRE-dependent activity compared with the WT-ER, and although these activities were not further increased by estradiol, the SRE-dependent activities of both the MT-ER and WT-ER were significantly reduced by treatment with the antiestrogen ICI 182,780, consistent with ER mediation of at least a portion of the SRE activity that was observed.

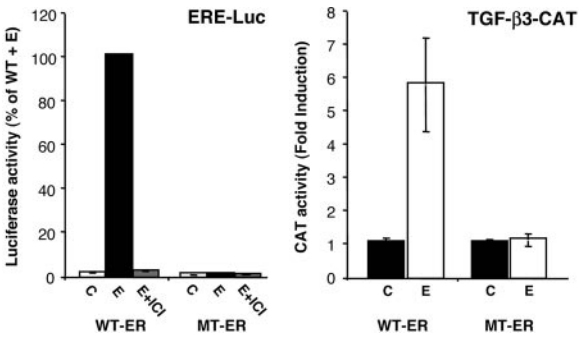

Fig. 6. Effect of WT-ER and MT-ER Constructs on Transcription through ERE-Containing and Non-ERE-Containing Promoters

A, MDA-MB-231 cells expressing WT-ER or MT-ER were transfected with 2ERE-pS2-Luciferase plasmid and internal control β-galactosidase plasmid. At 8 h after transfection, cells were treated with control ethanol vehicle, or E2 (10^{-8} M), or E2 (10^{-8} M) + ICI 182,780 (10^{-6} M) for 24 h. Luciferase activity was measured and normalized by β-galactosidase activity. Values represent mean ± SD from three separate experiments performed in duplicate. B, Cells were transfected with the TGF-β3-CAT reporter and β-galactosidase plasmids, and at 8 h after transfection, cells were treated with control ethanol vehicle or E2 (10^{-8} M) for 24 h. CAT activity was measured and normalized by β-galactosidase activity. Values represent mean ± SD from two separate experiments performed in duplicate.

Differential ERK1/2 Activity and Akt Activity by WT-ER and MT-ER in MDA-MB-231 Cells

To further investigate the effects of the WT-ER and MT-ER in signal transduction, modulation of the activity of the important and commonly studied MAPK pathway, ERK1/2 phosphorylation, was determined (Fig. 8A). In cells containing WT-ER or MT-ER, MAPK activity was high at zero time, consistent with the observations that these MDA-MB-231 cells produce high levels of growth factors. The response of these receptors to estrogen, however, was different. In WT-ER cells, E2 exposure resulted in a reduction in MAPK activity, such that two thirds of the activity was lost by 60 min, an observation made in several repeat experiments (Fig. 8A), and ICI 182,780 cotreatment prevented this down-regulation by E2 (data not presented). In contrast, with the MT-ER cells, E2 treatment resulted in no decrease in MAPK activity (Fig. 8A). Similar findings were made with MDA-MB-231 cells transiently, rather than stably, transfected with WT-ER or MT-ER, *i.e.* a marked decline in phospho-p42/44 MAPK by 60 min of E2 exposure with WT-ER, and no change in phospho-p42/44 MAPK with MT-ER (data not presented).

Because E2 is known to modulate Akt activity in some breast cancer cells (37), we also examined Akt activity. Whereas WT-ER and MT-ER cells had approximately similar levels of basal Akt activity, measured as phospho-Akt and total Akt, E2 treatment

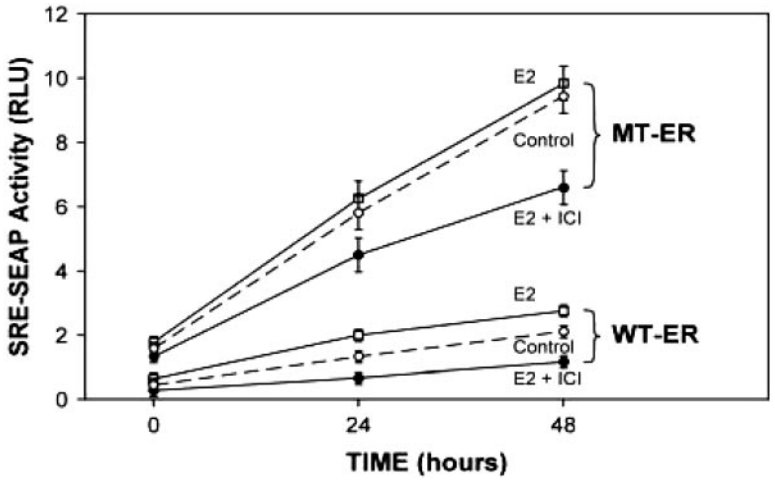

Fig. 7. Assessment of SRE-SEAP Activity Mediated by MT-ER *vs.* WT-ER

MDA-MB-231 cells were transfected with the SRE-SEAP plasmid (1 μg/well) and at 10 h after transfection, serum-deprived cells were treated with control vehicle, E2 (10^{-8} M) or E2 (10^{-8} M) + ICI 182,780 (10^{-6} M) and SEAP activity was measured at 0, 24, and 48 h. Values represent mean ± SD from two separate experiments performed in duplicate. RLU, Relative light units.

resulted in a different temporal profile of Akt activity (Fig. 8B). In both cell types, E2 brought about a rapid (by 5 min) reduction in phospho-Akt, and WT-ER cells showed a more prolonged reduction in phospho-Akt (to ~50% of 0 time level) through at least 4 h *vs.* 1 h for MT-ER cells. Levels of total Akt remained unchanged over time in both cell types.

Differential Effects of Estradiol on the Proliferation of MDA-MB-231 Cells Containing MT-ER *vs.* WT-ER

It is well documented that E2 has no effect on the proliferation of ER-negative MDA-MB-231 cells, but that E2 reduces the proliferation of these breast can-

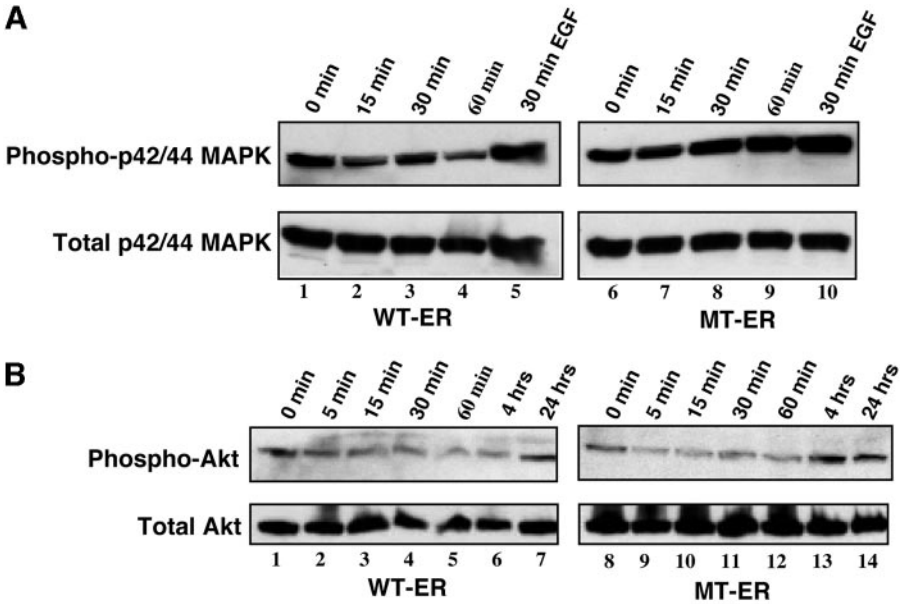

Fig. 8. Effect of E2 Treatment on Phospho-p42/44 MAPK and Total MAPK (A) and Phospho-Akt and Total Akt (B) in MDA-MB-231 Cells Stably Expressing WT-ER or MT-ER

Cells were incubated with 10 nM E2, (or 10 ng/ml EGF for 30 min where noted), for the indicted times before P-p42/p44 MAPK and total MAPK, or P-Akt and total Akt measurement. Similar amounts of protein (50 μg) from each cell extract were analyzed after separation by SDS-PAGE and immunoblotting. Similar profiles were observed in two additional experiments with other WT-ER and MT-ER clones.

cer cells when WT-ER is introduced (31, 38). This decrease in cell proliferation evoked by E2 in ER-containing MDA-MB-231 cells differs from the situation in ER-positive MCF-7 breast cancer cells where E2 enhances cell proliferation (32). We were therefore interested to examine the effect of E2 on the proliferation of these MDA-MB-231 cells containing either WT-ER or MT-ER. As seen in Fig. 9, although MT-ER and WT-ER-containing cells showed similar rates of proliferation, exposure to E2 reduced the proliferation of WT-ER cells, as expected, but we observed that E2 had no effect on the proliferation of the MT-ER-containing cells.

DISCUSSION

Increasing evidence supports the importance of extranuclear estrogen receptors in contributing to the range of estrogen actions in diverse target cells. Our studies, using ERs modified so as to increase their association with the membrane, showed that these MT-ERs localized outside the nucleus and have activities that are distinct from those of the nuclear localized ERs.

Of note, MT-ER failed to activate nuclear ER genomic responses but stimulated some rapid nongenomic effects of estrogen. Hence, in cells containing MT-ERs, serum response element-dependent gene transcription was high and partially inhibited by ICI, implicating ER in at least part of this activity, whereas MAPK activity and cell proliferation rates were high and unaffected by E2 in these same cells. The findings suggest that there is a correlation between suppression of MAPK activation, prolonged reduction of Akt-phosphorylation, and the suppression of cell proliferation by E2 via WT-ERs, and a lack of modulation of both MAPK and proliferation by E2 via MT-ERs. Whereas these observations appear to be consistent with evidence for a role of nuclear ERs and kinase activities in hormonal regulation of cell proliferation (12, 13), this relationship needs to be interpreted cautiously because of limitations in the conditions under which these experiments can be performed. To observe effects of E2 on SRE, MAPK, and Akt activity in these MDA-MB-231 cells, we found that these assays needed to be carried out in the absence of serum. These are conditions under which these nongenomic responses are typically conducted (11, 13, 23, 39). Using 5% charcoal-stripped serum conditions, we found that kinase activity was high and essentially unaffected by E2 (not shown). In contrast, the breast cancer cell proliferation assays cannot be conducted under serum-free conditions because proliferation is negligible. Thus, because these proliferation and kinase assays cannot be performed under comparable conditions, we cannot definitively conclude whether there is a relationship between kinase regulation and proliferation by estrogen in these cells.

The absence of regulation of typical estradiol-ER regulated genes by our MT-ERs implies that activation of these genes requires nuclear-localized ER and that modulation of some kinase cascades alone is insufficient for this gene regulatory activity. It is of note that others have reported that the E domain of the ER alone, if modified with a targeting sequence that keeps it outside of the nucleus, is sufficient to activate some kinase pathways and prevent apoptosis of certain bone cells upon E2 treatment (40–42).

Serine 522 in the ligand binding (E) domain of human ERα has been shown to be necessary for the

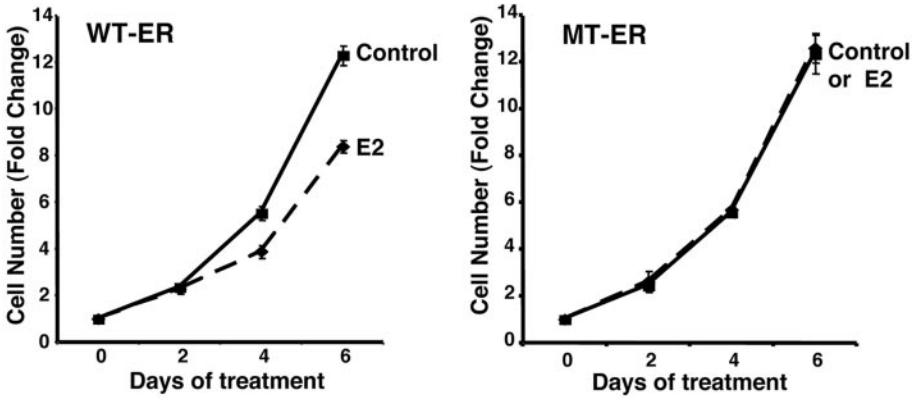

Fig. 9. Differential Effect of Estradiol on the Proliferation of Breast Cancer Cells containing WT-ER or MT-ER

WT-ER and MT-ER MDA-MB-231 cells were treated with control 0.1% ethanol vehicle or E2 (10^{-8} M) for the days indicated before measurement of cell proliferation as described in *Materials and Methods*. Data represent mean ± SD of three separate determinations. Similar findings were obtained in two repeat experiments, with the same and different WT-ER and MT-ER cell clones.

optimal localization and function of ER at the plasma membrane (42). This amino acid in the E domain is also present in our MT-ER, and therefore should allow for interaction with the protein caveolin, an important component of membrane caveoli. It is likely that our MT-ERs are localized in caveolae and are associated with a large complex of proteins, often referred to as a signalosome complex in caveolae and lipid raft regions of the cell membrane (20–22, 39, 43–45). At the plasma membrane, estrogen-liganded ERs have been shown to physically associate with caveolin-1 and a variety of signaling molecules including G proteins, Src, Shc (39) and other components of signal transduction cascades (23). The punctate pattern of the MT-ERs after hormone exposure of cells is consistent with the probable localization of these receptor-signalosome complexes in endosomes and/or microsomes after hormonal treatment. This perinuclear punctate fluorescence pattern has previously been reported for myristoylated and palmitoylated proteins and may reflect localization of the MT-ERs to endosomes (26, 46). Zhang and co-workers (47) recently showed a similar localization pattern in COS cells for ER modified with the membrane localization sequence of GAP-43, a neuromodulin protein (46).

The antiestrogen ICI 182,780 is well known to increase degradation of the nuclear ER, resulting in a marked decrease in intracellular ER levels (48, 49), as observed in our MDA-MD-231 breast cancer cells containing WT-ER. Hence, of interest was our observation that ICI failed to change the intracellular level of the MT-ER. Because the accelerated turnover of the WT-ER in the presence of ICI 182,780 has been shown to result from enhanced proteasomal degradation of the WT-ER, it appears that the MT-ER is not susceptible to this process because its level is unaffected by ICI exposure. Nonetheless, the antiestrogen ICI did inhibit the stimulation of serum response element-dependent activity by the MT-ER, as well as by the WT-ER, and reversed the E2 stimulated down-regulation of MAPK activity by the WT-ER, and the down-regulation of Akt activity by both the WT-ER and the MT-ER, suggesting that this antiestrogen is able to alter the conformation and hence activity of the ER when localized in the membrane/cytoplasm of cells as well as when the receptor is localized in the nucleus. These findings support previous observations made with some point mutants of the WT-ER in which ICI worked effectively as an estrogen antagonist yet failed to enhance the turnover of these altered estrogen receptors (49).

Studies of Razandi and co-workers (11) indicate that cell membrane and nuclear ERs can originate from the same transcript in Chinese hamster ovary cells and that receptors in both compartments bound estradiol with nearly identical affinities, as we observed for our MT-ERs and WT-ERs. ER has interactions with extranuclear localized proteins, such as a truncated MTA1 protein that is highly expressed in aggressive breast cancers (50) and MNAR that links ER to Src (51) and might help to maintain ER outside of the nucleus, thereby fostering increased MAPK signaling and aggressive behavior of tumors. Furthermore, the fact that multiple ER antibodies detecting different epitopes across the ER interact with membrane ER as well as nuclear localized ERs (10) implies that ER in the membrane is likely very similar in amino acid sequence to that of nuclear WT-ER, although an N-terminally truncated form of ER (ER46, 46 kDa) is reported to be more strongly membrane localized than WT-ER in human endothelial cells (52). This form of ER, as well as the full-length ER, has been shown to be palmitoylated in the ligand binding domain (52, 53). We have not directly demonstrated that our MT-ER has been lipidated, as would be expected from the modifications that we have made; however, the membrane localization of the MT-ER suggests that they have occurred. This type of detailed biochemical demonstration of lipidation would be of interest for future work.

In conclusion, we have developed and characterized a model system in which ER can be strongly directed outside of the nucleus and to the cell membrane. This MT-ER failed to activate nuclear ER genomic responses but elicited some rapid nongenomic effects of estrogen. These findings indicate distinctive actions of extranuclear ER and suggest that both nuclear actions and membrane signaling by ERs via intracellular cascades are likely to contribute to the ability of estrogens to influence the functioning of breast cancer and other target cells.

MATERIALS AND METHODS

Chemicals and Materials

Cell culture media were purchased from Life Technologies (Gaithersburg, MD). Calf serum was obtained from HyClone Laboratories (Logan, UT). Estradiol was purchased from Sigma (St. Louis, MO). The antiestrogen ICI 182,780 was kindly provided by Alan Wakeling and Zeneca Pharmaceuticals (Macclesfield, UK). Custom oligonucleotides were purchased from Invitrogen (Carlsbad, CA).

Plasmid Constructs

Several membrane targeted (MT)-ER constructs were generated: Myr-ER (myristoylated ER) and a dual acylated ΔNLS-ER, denoted MT-ER, that lacks the nuclear localization sequence. In the case of Myr-ER, a myristoylation sequence, GAATTCGCCGCCATGGGATGTATAAAATCAAAACGGAAAGAGCTCTTGAATGACGATGAAGGTACC, containing the N-terminal amino acids of the Src tyrosine kinase, Lyn, was ligated to pCMV5 (using the *Eco*RI and *Kpn*I sites). Subsequently, full-length ER was subcloned into the myristoylated pCMV5.

The MT-ER was created by using the HE241G form of ER (a gift from Dr. Pierre Chambon, Strasbourg, France). HE241G is identical with the full-length ER, except that it

lacks the nuclear localization signals contained within amino acids 256–303. First, a palmitoylation sequence ATCGATAGTGGCCCCGCATGCATGAGCTGCAAGTGTGTG-CTCTCCTGAGGATCC from the C terminus of the H-ras protein was attached to pCMV5 using the *Cla*I and *Bam*HI sites. Subsequently, Myr-ER in pCMV5 was subjected to PCR to replace *Bam*HI with a *Cla*I site and to eliminate the stop codon. The sequences of the forward and reverse primers were: 5′-GGTTTCCCTGCCACAGTCTAAATCGATGGC-3′ and 5′GGCATCGATTTAGACTGTGGCAGGGAAACC-3′, respectively. This fragment was subsequently subcloned into the palmitoylated pCMV5 to create dual acylated full-length ER. To create MT-ER, the full-length ER was replaced with HE241G. In this case, the *Eco*RI sites of HE241G were changed by PCR to *Kpn*I and *Cla*I, using HE241G in pSG5 as the template (forward primer, 5′-GAATCCCGGCCAGGTAC-CATGACCATGACC-3′; reverse primer, 5′-GCCAGGGAGCT-CATCGATTGTGGCAGGG-3′). The sequences of all constructs were verified by DNA sequencing.

Cell Culture and Stable Transfections

MDA-MB-231 ER-negative breast cancer cells were routinely maintained as previously described (27, 54). To stably integrate the WT-ER or MT-ER into these cells, cells were grown to 40% confluency in 10-cm plates and then transfected with 10 μg of either WT-ER or MT-ER cDNA in the pcDNA 3.1+ expression vector, which contains a neomycin resistance gene (Invitrogen), using lipofectin in the absence of serum. Five hours later, media were changed to standard culture medium. After 2 d of culture, the medium was supplemented with 800 μg/ml of G418 (a neomycin analog) (Sigma) for a total of 2 wk. Within 4 d, greater than 90% of the cells had died and within 12 d individual colonies containing 10–20 cells were trypsinized and transferred to individual wells of 96-well plates and subsequently to 24-well plates. Positive clones (11 with MT-ER and 9 with WT-ER) were identified by measuring ER mRNA copy number by quantitative RT-PCR, and ER protein expression by Western immunoblot, as described below.

All experiments (transcription assays, Western immunoblotting, hormone binding, gene expression-real time PCR, SRE-SEAP, ERK 1/2 and Akt phosphorylation, and cell proliferation assays) were performed a minimum of three times using at least two different WT-ER cell clones (clones 13 and 19) and at least two different MT-ER cell clones (clones 6, 11, or 18). In some comparative studies not shown but mentioned in the text, experiments were also done with parental MDA-MB-231 cells that were transiently transfected with WT-ER or MT-ER plasmids to confirm that observations similar to those with the stable cell clones were seen in the cells transiently expressing the WT-ER and MT-ERs.

Transcription through ERE-Containing Promoters and Non-ERE-Containing Promoters

Cells were plated in 24-well plates and transfected with 500 ng of an ERE-containing reporter (2ERE-pS2-Luciferase) or with 2 μg TGF-β3-chloramphenicol acetyltransferase (CAT) reporter plasmid, and 500 ng pCMVβ (β-galactosidase) internal control plasmid. At 8 h after transfection, cells were treated with the indicated ligand or 0.1% ethanol control vehicle. Cells were harvested 24 h after hormone treatment. Luciferase or CAT activity was assayed and normalized by β-galactosidase activity as previously described (55, 56).

Western Blotting and Hormone Binding Assays

Whole cell protein extracts were prepared as described (57). Nitrocellulose blots were probed with the human ER-specific H222 monoclonal antibody (kindly provided by Geoffrey Greene, University of Chicago) at 1 μg/ml and the immunoblots were developed using chemiluminescence (Pierce, Rockford, IL).

For hormone binding assays, whole cell extracts were incubated in duplicate with a range of ^{3}H-E2 concentrations alone or with 100-fold excess unlabeled E2 for 1 h on ice. Hydroxylapatite slurry was added and incubated for an additional 15 min on ice. The slurry was washed twice and its radioactivity then determined by scintillation counting (57).

Fluorescence Microscopy

Localization of WT-ER and MT-ER constructs was assessed by immunocytochemistry and additionally using ER constructs subcloned into EGFP vectors (CLONTECH, Palo Alto, CA). Briefly, for cells containing EGFP-ERs, cells on glass coverslips were fixed in 1.6% paraformaldehyde for 30 min, washed in PBS, stained with 4,6-diamidino-2-phenylindole (Sigma) to identify the nuclei and mounted in antifade solution (Molecular Probes, Eugene, OR). For immunocytochemistry, the fixed cells were first incubated with blocking buffer (PBS containing 5% normal goat serum) (Sigma) for 30 min and then stained with a monoclonal rat antihuman ER (H222) (Geoffrey Greene, University of Chicago, IL) antibody overnight at 4 C. FITC goat antirat IgG (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody. After staining with 4,6-diamidino-2-phenylindole, cells were mounted in antifade solution. Fluorescence images were collected on an inverted light microscope (IMT2, Olympus, Success, NY) with a cooled, slow-scan charge-coupled device camera (Photometrics, Tucson, AZ) and optical sections at different planes throughout the cells were collected and deconvoluted as described (58).

Quantitative Real-Time PCR

For quantitative RT-PCR, cells seeded in 10-cm plates were treated with estradiol for 0, 8, 24, and 48 h. Total RNA was isolated using Trizol Reagent (Invitrogen). RNA was reverse-transcribed and the expression of the estrogen-regulated genes pS2 and WISP2 and the expression of WT and MT-ER were measured by real-time PCR using the SYBR Green PCR System (Applied Biosystems, Foster City, CA) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems) as described previously (33, 59). The primer sequences used to detect WT-ER were forward primer: 5′-GGTGGGATACGAAAAAGACCGA-3- and reverse primer: 5′-TCATCTCTCTGGCGCTTGTG-3′. To detect the MT-ER, the primers used were forward: 5′-GC-CGCCATGGGATGTATAAA-3′ and reverse: 5′-AGATGCTTT-GGTGTGGAGGG-3′. Sequences for the pS2 and WISP2 have previously been described (33, 59). The fold change in gene expression was calculated using the ΔΔCt (threshold cycle) method with the ribosomal protein 36B4mRNA (60) as an internal control.

ERK1/2 and Akt Phosphorylation Assays

Stably transfected MDA-MB-231 cells in 10-cm plates were serum deprived for 24 h and then incubated with 0.1% ethanol vehicle control, estradiol (10 nM), or a combination of estradiol (10 nM) and ICI 182,780 (1 μM). Cells were washed with PBS, lysed, and scraped using a cell lysis buffer (Tris-HCl 20 mM, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%, sodium pyrophosphate 2.5 mM, β-glycerophosphate 1 mM, sodium orthovanadate 1 mM, leupeptin 1 μg/ml, and phenylmethylsulfonyl fluoride 10 mM), and sonicated. After heat denaturation, 50 μg of each protein sample were electrophoretically separated on a 10% sodium dodecyl sulfate gel, transferred to nitrocellulose and immunoblotted using the

PhosphoPlus p44/p42 MAPK Kit (Cell Signaling Technology, Boston, MA) or the Akt phosphorylation kit. Phosphorylated forms of p44/p42 MAPK or Akt, as well as total MAPK or total Akt were monitored and quantified.

SRE-SEAP Assay

MDA-MB-231 cells were seeded in six-well plates at 4.5×10^5 cells/well and transfected with 1 μg/well of the SRE-SEAP plasmid (CLONTECH, Palo Alto, CA). After 10 h of transfection, cells were washed with PBS and maintained in serum-free medium for 24 h, at which time cells were treated with control vehicle, estradiol or a combination of estradiol and ICI 182,780 for 0, 8, 24, and 48 h. The amount of SEAP present in the culture medium was quantitated using the Great Escape SEAP Chemiluminescence Detection Kit (CLONTECH). Cells were also transfected with the pTAL-SEAP vector (CLONTECH) as a negative control to determine background signals associated with the culture medium. The pSEAP2-Control vector (CLONTECH) was used as a positive control to confirm transfection and verify the presence of active SEAP in the culture medium.

Cell Proliferation Assays

WT-ER and MT-ER cells were seeded in improved MEM phenol red-free medium supplemented with 5% charcoal dextran-stripped calf serum at 500 cells/well, in 96-well plates. The next day, cells were treated with 0.1% ethanol control vehicle or estradiol for 48 h, after which time fresh media and hormone were added. Cells received fresh media and vehicle or E2 every 48 h. Cell proliferation was assessed over the 6-d time course using the MTS tetrazolium colorimetric Cell Titer 96 Aqueous One Solution Proliferation Assay according to the manufacturer's protocol (Promega, Madison, WI).

Acknowledgments

We thank Kathy Carlson for assistance with the hormone binding assays.

Received November 18, 2004. Accepted April 5, 2005.

Address all correspondence and requests for reprints to: Dr. Benita S. Katzenellenbogen, University of Illinois, Department of Molecular and Integrative Physiology, 524 Burrill Hall, 407 South Goodwin Avenue, Urbana, Illinois 61801-3704. E-mail: katzenel@uiuc.edu.

This work was supported by National Institutes of Health Grant CA 18119 (to B.S.K.).

Current address for D.R.: Bristol-Meyers Squibb, 2400 West Lloyd Expressway, Evansville, Indiana 47712.

Current address for A.E.C.: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, Massachusetts 02142.

REFERENCES

1. Osborne CK 1998 Tamoxifen in the treatment of breast cancer. N Engl J Med 339:1609–1618
2. Katzenellenbogen BS, Frasor J 2004 Therapeutic targeting in the estrogen receptor hormonal pathway. Semin Oncol 31:28–38
3. Hall JM, Couse JF, Korach KS 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276:36869–36872
4. Katzenellenbogen BS, Katzenellenbogen JA 2000 Estrogen receptor transcription and transactivation: estrogen receptor α and estrogen receptor β: regulation by selective estrogen receptor modulators and importance in breast cancer. Breast Cancer Res 2:335–344
5. Weisz A, Rosales R 1990 Identification of an estrogen response element upstream of the human c-fos gene that binds the estrogen receptor and the AP-1 transcription factor. Nucleic Acids Res 18:5097–5106
6. Pfahl M 1993 Nuclear receptor/AP-1 interaction. Endocr Rev 14:651–658
7. Duan R, Porter W, Safe S 1998 Estrogen-induced c-fos protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. Endocrinology 139:1981–1990
8. Edwards DP 2005 Regulation of signal transduction pathways by estrogen and progesterone. Annu Rev Physiol 67:23.1–23.42
9. Pietras RJ, Szego CM 1975 Endometrial cell calcium and oestrogen action. Nature 253:357–359
10. Pappas TC, Gametchu B, Watson CS 1995 Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. FASEB J 9:404–410
11. Razandi M, Pedram A, Greene GL, Levin ER 1999 Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: Studies of ERα and ERβ expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307–319
12. Pietras RJ, Szego CM 1979 Metabolic and proliferative responses to estrogen by hepatocytes selected for plasma membrane binding-sites specific for estradiol-17β. J Cell Physiol 98:145–159
13. Migliaccio A, Di Domenico M, Castoria G, de Flaco A, Bontempo P, Nola E, Auricchio F 1996 Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 15:1292–1300
14. Vasudevan N, Kow LM, Pfaff DW 2001 Early membrane estrogenic effects required for full expression of slower genomic actions in a nerve cell line. Proc Natl Acad Sci USA 98:12267–12271
15. Le Mellay V, Grosse B, Lieberherr M 1997 Phospholipase Cβ and membrane action of calcitriol and estradiol. J Biol Chem 272:11902–11907
16. Levin ER 1999 Cellular functions of the plasma membrane estrogen receptor. Trends Endocrinol Metab 10: 374–377
17. Pietras RJ, Szego CM 1977 Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature 265:69–72
18. Pietras RJ, Szego CM 1980 Partial purification and characterization of oestrogen receptors in subfractions of hepatocyte plasma membranes. Biochem J 191:743–760
19. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER 2002 ERs associate with and regulate the production of caveolin: implications for signaling and cellular actions. Mol Endocrinol 16:100–115
20. Haynes MP, Sinha D, Russell KS, Collinge M, Fulton D, Morales-Ruiz M, Sessa WC, Bender JR 2000 Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via the PI3-kinase-Akt pathway in human endothelial cells. Circ Res 87:677–682
21. Chambliss KL, Yuhanna IS, Mineo C, Liu P, German Z, Sherman TS, Mendelsohn ME, Anderson RG, Shaul PW 2000 Estrogen receptor α and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. Circ Res 87:E44–E52
22. Russell KS, Haynes MP, Sinha D, Clerisme E, Bender JR 2000 Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. Proc Natl Acad Sci USA 97: 5930–5935

23. Razandi M, Pedram A, Park ST, Levin ER 2003 Proximal events in signaling by plasma membrane estrogen receptors. J Biol Chem 278:2701–2712
24. van't Hof W, Resh MD 1997 Rapid plasma membrane anchoring of newly synthesized p59fyn: selective requirement for NH2-terminal myristoylation and palmitoylation at cysteine-3. J Cell Biol 136:1023–1035
25. Berthiaume L, Resh MD 1995 Biochemical characterization of a palmitoyl acyltransferase activity that palmitoylates myristoylated proteins. J Biol Chem 270: 22399–22405
26. McCabe JB, Berthiaume LG 1999 Functional roles for fatty acylated amino-terminal domains in subcellular localization. Mol Biol Cell 10:3771–3786
27. Ediger TR, Kraus WL, Weinman EJ, Katzenellenbogen BS 1999 Estrogen receptor regulation of the Na+/H+ exchanger regulatory factor. Endocrinology 140:2976–2982
28. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA 2000 Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-α-selective agonists. J Med Chem 43:4934–4947
29. Harris HA, Katzenellenbogen JA, Katzenellenbogen BS 2002 Characterization of the biological roles of the estrogen receptors, ERα and ERβ, in estrogen target tissues *in vivo* through the use of an ERα-selective ligand. Endocrinology 143:4172–4177
30. Muthyala RS, Ju YH, Sheng S, Williams LD, Doerge DR, Katzenellenbogen BS, Helferich WG, Katzenellenbogen JA 2004 Equol, a natural estrogenic metabolite from soy isoflavones: convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors α and β. Bioorg Med Chem 12:1559–1567
31. Lazennec G, Katzenellenbogen BS 1999 Expression of human estrogen receptor using an efficient adenoviral gene delivery system is able to restore hormone-dependent features to estrogen receptor-negative breast carcinoma cells. Mol Cell Endocrinol 149:93–105
32. Lazennec G, Alcorn JL, Katzenellenbogen BS 1999 Adenovirus-mediated delivery of a dominant negative estrogen receptor gene abrogates estrogen-stimulated gene expression and breast cancer cell proliferation. Mol Endocrinol 13:969–980
33. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS 2003 Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562–4574
34. Yang NN, Venugopalan M, Hardikar S, Glasebrook A 1996 Identification of an estrogen response element activated by metabolites of 17-β-estradiol and raloxifene. Science 273:1222–1225
35. Yang NN, Venugopalan M, Hardikar S, Glasebrook A 1997 Correction: raloxifene response needs more than an element. Science 275:1249
36. Duan R, Xie W, Burghardt RC, Safe S 2001 Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem 276:11590–11598
37. Martin MB, Franke TF, Stoica GE, Chambon P, Katzenellenbogen BS, Stoica BA, McLemore MS, Olivo SE, Stoica A 2000 A role for Akt in mediating the estrogenic functions of epidermal growth factor and insulin-like growth factor I. Endocrinology 141:4503–4511
38. Jiang SY, Jordan VC 1992 Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. J Natl Cancer Inst 84:580–591
39. Song RX, McPherson RA, Adam L, Bao Y, Shupnik M, Kumar R, Santen RJ 2002 Linkage of rapid estrogen action to MAPK activation by ERα-Shc association and Shc pathway activation. Mol Endocrinol 16:116–127
40. Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104: 719–730
41. Kousteni S, Chen JR, Bellido T, Han L, Ali AA, O'Brien CA, Plotkin L, Fu Q, Mancino AT, Wen Y, Vertino AM, Powers CC, Stewart SA, Ebert R, Parfitt AM, Weinstein RS, Jilka RL, Manolagas SC 2002 Reversal of bone loss in mice by nongenotropic signaling of sex steroids. Science 298:843–846
42. Razandi M, Alton G, Pedram A, Ghonshani S, Webb P, Levin ER 2003 Identification of a structural determinant necessary for the localization and function of estrogen receptor α at the plasma membrane. Mol Cell Biol 23: 1633–1646
43. Wyckoff MH, Chambliss KL, Mineo C, Yuhanna IS, Mendelsohn ME, Mumby SM, Shaul PW 2001 Plasma membrane estrogen receptors are coupled to endothelial nitric-oxide synthase through Gα(i). J Biol Chem 276: 27071–27076
44. Kelly MJ, Wagner EJ 1999 Estrogen modulation of G-protein-coupled receptors. Trends Endocrinol Metab 10: 369–374
45. Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, Liao JK 2000 Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 407:538–541
46. Liu Y, Fisher DA, Storm DR 1993 Analysis of the palmitoylation and membrane targeting domain of neuromodulin (GAP-43) by site-specific mutagenesis. Biochemistry 32:10714–10719
47. Zhang Z, Maier B, Santen RJ, Song RX 2002 Membrane association of estrogen receptor α mediates estrogen effect on MAPK activation. Biochem Biophys Res Commun 294:926–933
48. Dauvois S, Danielian PS, White R, Parker MG 1992 Antiestrogen ICI164,384 reduces cellular estrogen receptor content by increasing its turnover. Proc Natl Acad Sci USA 89:4037–4041
49. Montano MM, Ekena K, Krueger KD, Keller AL, Katzenellenbogen BS 1996 Human estrogen receptor ligand activity inversion mutants: receptors that interpret antiestrogens as estrogens and estrogens as antiestrogens and discriminate among different antiestrogens. Mol Endocrinol 10:230–242
50. Kumar R, Wang RA, Mazumdar A, Talukder AH, Mandal M, Yang Z, Bagheri-Yarmand R, Sahin A, Hortobagyi G, Adam L, Barnes CJ, Vadlamudi RK 2002 A naturally occurring MTA1 variant sequesters oestrogen receptor-α in the cytoplasm. Nature 418:654–657
51. Barletta F, Wong CW, McNally C, Komm BS, Katzenellenbogen B, Cheskis BJ 2004 Characterization of the interactions of estrogen receptor and MNAR in the activation of cSrc. Mol Endocrinol 18:1096–1108
52. Li L, Haynes MP, Bender JR 2003 Plasma membrane localization and function of the estrogen receptor α variant (ER46) in human endothelial cells. Proc Natl Acad Sci USA 100:4807–4812
53. Acconcia F, Ascenzi P, Fabozzi G, Visca P, Marino M 2004 S-palmitoylation modulates human estrogen receptor-α functions. Biochem Biophys Res Commun 316: 878–883
54. Pakdel F, Katzenellenbogen BS 1992 Human estrogen receptor mutants with altered estrogen and antiestrogen ligand discrimination. J Biol Chem 267:3429–3437
55. Sun J, Meyers MJ, Fink BE, Rajendran R, Katzenellenbogen JA, Katzenellenbogen BS 1999 Novel ligands that function as selective estrogens or antiestrogens for es-

trogen receptor-α or estrogen receptor-β. Endocrinology 140:800–804
56. Schodin DJ, Zhuang Y, Shapiro DJ, Katzenellenbogen BS 1995 Analysis of mechanisms that determine dominant negative estrogen receptor effectiveness. J Biol Chem 270:31163–31171
57. Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS 2004 Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) α or ER β in human osteosarcoma cells: Distinct and common target genes for these receptors. Endocrinology 145:3473–3486
58. Nye AC, Rajendran R, Stenoien D, Mancini M, Katzenellenbogen BS, Belmont AS 2002 Alteration of large-scale chromatin structure by estrogen receptor. Mol Cell Biol 22:3437–3449
59. Rajendran RR, Nye AC, Frasor J, Balsara RD, Martini PG, Katzenellenbogen BS 2003 Regulation of nuclear receptor transcriptional activity by a novel DEAD box RNA helicase (DP97). J Biol Chem 278:4628–4638
60. Laborda J 1991 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. Nucleic Acids Res 19:3998

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