Gene expression profiling reveals that the regulation of estrogen-responsive element-independent genes by 17β-estradiol-estrogen receptor β is uncoupled from the induction of phenotypic changes in cell models

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Abstract

Estrogen hormone 17β-estradiol (E2) is involved in the physiology and pathology of many tissues. E2 information is conveyed by the transcription factors estrogen receptors (ER) α and β that mediate a complex array of nuclear and non-nuclear events. The interaction of ER with specific DNA sequences, estrogen-responsive elements (EREs), constitutes a critical nuclear signaling pathway. In addition, E2-ER regulates transcription through interactions with transactivators bound to their cognate regulatory elements on DNA, hence the ERE-independent signaling pathway. Other, the relative importance of the ERE-independent pathway in E2-ERβ signaling is unclear. To address this issue, we engineered an ERE-binding defective ERβ mutant (ERβEBD) by changing critical residues in the DNA-binding domain required for ERE binding. Biochemical and functional studies revealed that ERβEBD signaled exclusively through the ERE-independent pathway. Using the adenovirus infected ER-negative cancer cell models, we found that although E2-ERβEBD regulated the expression of a number of genes identified by microarrays, it was ineffective in altering cellular proliferation, motility, and death in contrast to E2-ERβ. Our results indicate that genomic responses from the ERE-independent pathway to E2-ERβ are not sufficient to alter the cellular phenotype. These findings suggest that the ERE-dependent pathway is a required signaling route for E2-ERβ to induce cellular responses.

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Introduction

Estrogen hormones, particularly the main circulating estrogen, 17β-estradiol (E2), play important roles in the regulation of many tissue functions (Huang et al. 2005a, Deroo & Korach 2006). E2 is also involved in the initiation and development of target tissue malignancies. The E2 information is primarily conveyed by estrogen receptors (ER) α and β (Huang et al. 2005a, Deroo & Korach 2006). ERs are members of the conserved superfamily of hormone receptors and are ligand-activated transcription factors. The effects of E2ER are exerted through a complex array of convergent and divergent signal transduction pathways that mediate genomic events involved in the regulation of mitogenesis, morphogenesis, and apoptosis (Hall et al. 2001, Nilsson et al. 2001, Huang et al. 2005a). The interaction of E2-ER with specific DNA sequences, estrogen-responsive elements (EREs), constitutes a primary genomic signaling pathway (Hall et al. 2001, Nilsson et al. 2001, Huang et al. 2005a). The ERE-bound ER recruits an ensemble of multi-subunit complexes responsible for the alteration of local chromatin structure and the interaction with the basal transcription machinery. The integrated effects of these complexes regulate transcription. This type of E2-ER-mediated signaling is referred to as the ERE-dependent signaling pathway (Hall et al. 2001, Nilsson et al. 2001, Huang et al. 2005a).

In addition, the E2-ER complex regulates the expression of E2-responsive genes through functional interactions with transcription factors bound to their cognate regulatory elements on DNA (Kushner et al. 2000, Safe 2001). In this DNA-dependent and ERE-independent signaling pathway, transcriptional responses are dependent upon the ER-subtype, promoter, and cell-context (Kushner et al. 2000, Safe 2001). However, the relative importance of the ERE-independent pathway in physiology and pathophysiology of E2-ERβ signaling is unknown. We envisioned that a selective regulation of the ERE-independent genes would allow us to begin to address this issue. To accomplish this, we generated an ERE-binding defective ERβ mutant (ERβEBD) which...
renders the receptor nonfunctional at the ERE-dependent pathway, while conserving the regulatory potential at the ERE-independent pathway. We used ER-negative cells as experimental models, with which exogenously introduced ERs were shown to regulate the expression of responsive genes (Licznar et al. 2003, Kian Tee et al. 2004, Stossi et al. 2004, Moggs et al. 2005, Monroe et al. 2005) and to induce phenotypic changes (Garcia et al. 1992, Jiang & Jordan 1992, Zajchowski et al. 1993, Lazennec & Katzenellenbogen 1999, Lazennec et al. 2001, Licznar et al. 2003). In adenovirus-infected cells, we found that genomic responses induced by ERbEBD in response to a physiological level of E2 are insufficient to alter cellular proliferation, death, or motility, in contrast to E2-ERb. These results imply that the ERE-dependent pathway is the required signaling route mediated by the E2-ERb complex.

Materials and methods

Generation of ERb DNA-binding defective mutant (ERbEBD)

The human ERb cDNAs that encode the 530 amino acid ERb have been described previously (Yi et al. 2002a). This ERb cDNA also contains sequences encoding an amino-terminal Flag epitope (Yi et al. 2002a). For the engineering of an ERE-binding defective ERb (ERbEBD), we utilized an overlapping PCR with the ERb cDNA as the template and primers that contain amino acid substitutions to replace glutamic acid and glycine at positions 167 and 168 respectively with alanine residues in the first zinc finger of the DNA-binding domain (DBD) of the receptor.

Restriction and DNA modifying enzymes were obtained from New England Bio-Labs (Beverly, MA, USA) and Invitrogen.

Cell culture

The culturing of MDA-MB-231 and HeLa cells has been described previously (Yi et al. 2002a). U-2 OS cells derived from osteosarcoma were purchased from ATCC (Manassas, VA, USA). U-2 OS cells were grown in McCoy’s 5β medium supplemented with 10% fetal bovine serum (FBS, Invitrogen). In all experiments, the medium was changed every third day.

Transient transfections

Transient transfections for the simulated ERE-dependent and -independent pathways were accomplished as described previously (Yi et al. 2002a, Huang et al. 2004). The transfected cells were treated without or with 10⁻⁹ M of E₂, 10⁻⁷ M of 4-hydroxyl-tamoxifen (4-OHT, Sigma–Aldrich), 10⁻⁷ M ICI 182 780 (Imperial Chemical Industries, Tocris Inc., Ballwin, MO, USA), and 10⁻⁸ M diarylpropionitrile (DPN, Tocris) for 24 or 40 h to assess the effects of ligands on ER-mediated transcriptional responses from the ERE-dependent or -independent signaling pathways respectively.

Generation of a recombinant adenovirus bearing an ER cDNA

Recombinant adenovirus bearing none, the cDNA of Flag-ERb or Flag-ERbEBD were produced using the AdEasy-XL Adenoviral System (Stratagene, La Jolla, CA, USA) as described previously (Huang et al. 2005b). The purified viruses were titered using an Adeno-X Rapid Titer Kit (BD Biosciences, Palo Alto, CA, USA) to determine the multiplicity of infection (MOI).

Western blot (WB), electrophoretic mobility shift assay (EMSA), and immunocytochemistry (ICC)

Transfected or infected cells in a time-dependent manner were processed for WB, EMSA, and ICC as described previously (Muyan et al. 2001, Yi et al. 2002a). For WB, proteins were probed with horseradish peroxidase-conjugated monoclonal Flag antibody (M2-HRP, Sigma–Aldrich) using the ECL-Plus Western Blotting kit (Amersham-Pharmacia). The images were captured using PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). For ICC, we used an ERb-specific antibody (Zymed Laboratories, San Francisco, CA, USA) followed by a fluorescein conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

In situ E2 binding and ERE competition assays

To assess the functionality of ERb species in transfected cells, we used the in situ E2 binding assay and the in situ ERE competition assays as described previously (Huang et al. 2005b).

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed using Flag-M2 antibody-conjugated agarose beads (Sigma–Aldrich) as described previously (Huang et al. 2005b). The generation of a 366 bp PCR fragment indicates the specificity of PCRs.

Endogenous gene expression

MDA-MB-231 cells (100 000 cells/well) plated in six-well tissue culture plates in phenol red-free Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses without or with 10⁻⁹ M E₂ for 48 h to
assess the effects of ER on the expression of the trefoil factor 1 (TFF1), complement factor 3 (C3), matrix metalloproteinase 1 (MMP1), and retinoic acid receptor α (RARA) genes.

The cells were also infected with recombinant adenoviruses in the absence of E2 and maintained for 48 h, the time during which the synthesis of ERs reaches comparable levels (Fig. 1). The infected cells were then treated with 10⁻⁷ M E₂ for 6, 12, or 24 h to confirm the identities of genes determined by microarrays using quantitative PCR (qPCR). At termination, the cells were collected and subjected to total RNA extraction using the RNeasy Mini Kit (Qiagen) for qPCR, which we used custom TaqMan Low-Density Arrays with proprietary primer and probe sequences (Applied Biosystems, Foster City, CA, USA). All qPCRs were carried out at the Functional Genomic Center of the University of Rochester, NY, USA. The expression of the actin β (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was used as the control.

**Figure 1** Generation of an ERE-binding defective ERβ (ERβEBD). (A) ERβEBD was engineered by changing glutamic acid and glycine at positions 167 and 168 of the first zinc finger of the DNA-binding domain of the ERβ respectively to alanine residues. (B) The synthesis of ERβ species. MDA-MB-231 cells were transiently transfected with an expression vector bearing none (V), the ERβ or ERβEBD cDNA. Cell extracts (10 µg) were subjected to western blotting (WB) using a horseradish peroxidase-conjugated monoclonal Flag antibody. MW in KDa is indicated. The cell extracts (20 µg) of transfected cells were also subjected to electrophoretic mobility shift assay (EMSA) without (−) or with (+) a Flag antibody (Ab). ERE denotes unbound and ER–ERE indicates ER-bound radiolabeled ERE. Representative results from three independent experiments of WB and EMSA are shown. (C) The in situ ERE competition assay. MDA-MB-231 cells were transiently transfected with a reporter vector bearing one ERE upstream of the TATA box promoter driving the expression of Firefly luciferase cDNA and the expression plasmid for PPVV, together with various concentrations of expression vector bearing the ERβ or ERβEBD cDNA. The cells were also co-transfected with a reporter plasmid bearing Renilla luciferase cDNA to monitor the transfection efficiency. The cells were then treated without (− E₂) or with (+ E₂) 10⁻⁷ M E₂ for 24 h. The relative luciferase activity was presented as the percentage (%) change compared with control (PPVV alone in the absence of E₂), which was set to 100%. The mean of three independent experiments performed in duplicate has been shown. S.E.M., which was less than 15% of the mean, is not shown for simplicity. (D) Chromatin immunoprecipitation (ChIP) assay. MDA-MB-231 cells were transiently transfected with an expression vector bearing the ERβ or ERβEBD cDNA together with a reporter bearing none (TATA) or one ERE TATA box promoter. The cells were treated without (−) with (+) 10⁻⁷ M E₂ for 1 h prior to ChIP using the Flag antibody-conjugated agarose beads. The size of the DNA fragment is indicated in bp. A representative image from three independent experiments is shown.
real-time RT-PCR amplifications were accomplished using an ABI Prism 7900HT Sequence Detection System with a TaqMan Low Density Array Upgrade (Applied Biosystems). Relative quantification analysis was performed using the comparative C_T method (Livak & Schmittgen 2001).

Cell proliferation

MDA-MB-231 cells (5000 cells/well) plated in 24-well tissue culture plates in phenol red-free DMEM containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for different durations of time. The cells were collected and counted using a hemacytometer (Hausser Scientific, Horsham, PA, USA).

Additionally, we used a colorimetric proliferation assay, the 3-(4,5 dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). The cells, plated and infected with adenoviruses as described for hemacytometer cell counting, were incubated with 200 μl phenol red-free DMEM with 10% CD-FBS containing 60 μM MTT (Invitrogen) for 2 h. The spent medium was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well to dissolve the MTT formazan. The absorbance was measured using a microplate spectrophotometer (SpectraMax Plus; Molecular Devices, Sunnyvale, CA, USA) to estimate the cell number.

For U-2 OS cell proliferation, cells (5000 cells/well) were plated in 24-well tissue culture plates, pre-coated with poly-t-lysine (Sigma–Aldrich), in McCoy’s 5x medium containing 10% FBS, for 24 h. The cells were subsequently incubated with McCoy’s 5x medium containing 10% CD-FBS for an additional 24 h. They were then infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for different durations of time. We used Ad5-ERβ at 40 MOI. At this concentration, the recombinant adenovirus synthesized a concentration of ERβ that is dependent upon E2 for functioning. Ad5-ERβ was used at 50 MOI, which produced levels of receptor comparable to that of ERβ. At the termination of an experiment, cells were subjected to cell counting and MTT assays.

Cell cycle analysis

MDA-MB-231 cells (50 000 cells/well) in six-well tissue culture plates were infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for different durations. The cells were collected and pelleted. For fixing and permeabilization, the pelleted cells were resuspended in ethanol (70%) at 4°C overnight. The cells were subsequently incubated with 1 mg/ml RNase A (Sigma–Aldrich) for 30 min followed by 20 μg/ml propidium iodide (PI) (Sigma–Aldrich) for 10 min. They were then subjected to fluorescence-activated cell sorting (FACS) using EPICS Elite (Coulter Corp., Miami, FL, USA).

Caspase 3/7 assay

MDA-MB-231 cells (12 500 cells/well) plated onto poly-t-lysine coated 96-well tissue culture black plates with clear bottom (BD Biosciences, Franklin Lakes, NJ, USA) in phenol red-free DMEM containing 10% CD-FBS for 24 h were infected with recombinant adenoviruses, in the absence or presence of 10^{-9} \text{ M} \text{ E}_2, for different lengths of time. The cells were then subjected to Apo-ONE Homogeneous Caspase-3/7 Assay (Promega) according to the manufacturer’s protocol. Fluorescence was then measured using a spectrophotometer.

Annexin V assay

To study apoptosis by examining the loss of cell membrane asymmetry as an indicator of middle stages of apoptosis, we used the Vybrant Apoptosis Assay Kit (Invitrogen). This assay is based on the specific recognition of phosphatidyl-serine (PS) by FITC-conjugated annexin V. MDA-MB-231 cells (100 000 cells/well) were infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for different lengths of time. The cells were collected and subjected to annexin V assay, according to the instruction of the manufacturer, prior to FACS analysis.

TUNEL assay

To study the late stages of apoptosis by examining the fragmentation of genomic DNA (Korsmeyer 1999), MDA-MB-231 cells (25 000 cells/well) plated in poly-t-lysine-coated 48-well tissue culture plates in phenol red-free DMEM containing 10% CD-FBS for 24 h and infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for different lengths of time. The cells were then subjected to a TUNEL assay using the DeadEnd Fluorometric TUNEL System (Promega), according to the manufacturer’s protocol. 4',6-diamino-2-phenylindole (DAPI) (Vector Labs, Burlingame, CA, USA) was used to stain cell nuclei. The stained cells were imaged under a microscope with corresponding filters.

Wound-healing assay

MDA-MB-231 cells (200 000 cells/well in 12-well tissue culture plates) were infected with recombinant adenoviruses in the absence or presence of 10^{-9} \text{ M} \text{ E}_2 for 48 h for confluence. A wound was then created using a 1 ml pipette tip. The gap closure was photographed every 24 h. Due to the irregular shape of the edges of a
wound, five randomly selected cross edges were used to obtain a mean gap measure for wound healing.

**Invasion assay**

We used BD Matrigel Invasion Chambers (BD Biosciences, San Diego, CA, USA) for the invasion assay. MDA-MB-231 cells (100,000 cells/well) in six-well tissue culture plates were infected with recombinant adenoviruses in the absence or presence of $10^{-9}$ M E$_2$ for 48 h. The cells were then trypsinized and counted. The same number (25,000 cells/chamber) of cells was seeded on the upper section of the chamber, which contained phenol red-free DMEM without or with $10^{-9}$ M E$_2$. The lower section of the chamber contained phenol red-free DMEM supplemented with 10% CD-FBS and 30 μg/ml fibronectin in the absence or presence of $10^{-9}$ M E$_2$. After 24 h incubation, the cells on the upper section of the membrane were removed using a cotton swab. The cells on the bottom of the chamber membrane were stained with the Diff-Quik Stain Set (Dade Behring, Newark, DE, USA), dried, and mounted onto a glass slide. The images were captured and the stained cells were counted from images.

**Microarray analysis**

To examine the effects of E$_2$ on endogenous gene expression mediated by ERs, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of E$_2$ for 48 h. The infected cells were then treated with $10^{-9}$ M E$_2$ for 6 h. This duration of E$_2$ treatment was expected to induce significant changes in the level of immediate/early gene expression, as observed with the responses to E$_2$ in ER$_{a}$ synthesizing breast cancer cell lines (Frasor et al. 2003). At termination, the cells were subjected to RNeasy Mini kit (Qiagen) for total RNA extraction. The processing of RNA for microarray analysis was carried out at the Functional Genomic Center of the University of Rochester, NY. cDNA synthesis and subsequent fragmentation and biotinylation of cDNA fragments were carried out using the Ovation kit (NuGEN, San Carlos, CA, USA) according to the manufacturer’s procedure. The biotinylated cDNA fragments were then used for hybridization with microarrays. We used Affymetrix HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). The arrays were scanned using the GeneChip Scanner 3000 7G. GeneChip Operating Software (GCOS, Affymetrix) was used for initial processing of the scanner data, including the generation of cel files. Array normalization for the Affymetrix signal method (GCOS) involves multiplying raw signals by a scaling factor such that the trimmed mean (excluding highest and lowest 2%) of all expression scores is 500 arbitrary units for every array.

**Statistical analysis**

Results were presented as the mean ± S.E.M. of, at least, three independent experiments. Student’s t-test was employed for the comparison of means between two groups wherein $P<0.05$ was considered significant.

**Results**

**The functional characterization of the ERE-binding defective ERβ (ERβEBD)**

ERs recognize an ERE as a dimer mediated by a dimerization interface located in the ligand-binding domain and a weak interaction surface in the DBD (Parker 1998). The DBD of ER$_{a}$ contains two zinc finger-like modules that fold to form a single functional domain. Each DBD of the ER$_{a}$ dimer makes analogous contacts with one of the inverted motifs of ERE, which results in a rotationally symmetrical structure (Luísi et al. 1994). Distinct residues in a region of the first zinc finger module of DBD, the P-box, particularly glutamic acid and glycine at positions 203 and 204 respectively, determine the DNA-binding specificity critical for sequence discrimination (Green et al. 1988). The residues in the second zinc finger-like module, the D-box, are involved in the discrimination of half-site spacing through a protein–protein interaction between two ER monomers (Green et al. 1988). The
human ER\(\alpha\) and ER\(\beta\) share a 97% amino acid identity in their DBDs with identical residues at the P- and D-boxes (Mosselman et al. 1996). This structural homology is reflected in the abilities of ERs to bind various ERE sequences with a similar specificity and affinity by interacting with the same nucleotides (Yi et al. 2002b).

Studies showed that the regulation of E\(2\) responsive genes through ERE-independent signaling involves a functional interaction between ERs and a transcription factor bound to its cognate response element on DNA through regions which also encompass the DBD of ERs (Kushner et al. 2000, Safe 2001). Changing the glutamic acid and glycine at positions 207 and 208 of the mouse ER\(\alpha\), and at positions 167 and 168 of human ER\(\beta\) respectively, at the P-box of the first zinc finger of the DBD hinders the ER–ERE interactions, while conserving the capacity to regulate transcription from the ERE-independent signaling pathway (Jakacka et al. 2001, Bjornstrom & Sjoberg 2002). Based on these observations, we engineered an ERE-binding defective ER\(\beta\) variant (ER\(\beta\)\(_{\text{EBD}}\)) bearing alanine residues at positions 167 and 168 to exclusively regulate the DNA-dependent and ERE-independent pathway (Fig. 1A).

The initial biochemical characterization of ER\(\beta\)\(_{\text{EBD}}\) was carried out in transiently transfected ER-negative cell models. Cell extracts, shown for MDA-MB-231 cells, were subjected to WB and EMSA. The detection of receptor proteins by WB using a Flag antibody (M2) directed to the Flag epitope at the amino terminal of the receptors indicated that the receptors were synthesized at similar levels (Fig. 1B, WB). EMSA, using a \(^{35}\)P-end labeled DNA fragment bearing the consensus ERE, revealed that despite the comparable level of synthesis, ER\(\beta\), but not ER\(\beta\)\(_{\text{EBD}}\), interacted in vitro with ERE (Fig. 1B, EMSA).

To ensure that ER\(\beta\)\(_{\text{EBD}}\) is indeed defective in binding to ERE in situ, we used in situ ERE competition and ChIP assays. The in situ ERE competition assay is based on the ability of ER to compete for ERE binding with a designer activator, designated as PPVV, which potently and constitutively induces transcription from an ERE-driven reporter construct (Huang et al. 2005b). The interference of the activator-mediated transcription by unliganded or liganded ERs is then taken as an indication of ER–ERE interaction. The reporter TATA box plasmid bearing none (TATA) or one ERE was co-transfected with an expression vector encoding the PPVV cDNA into cell models, shown for MDA-MB-231 cells, in the absence or presence of varying amounts of an expression vector bearing an ER cDNA without or with a physiological concentration (\(10^{-8}\) M) of E\(2\) for 24 h. The normalized luciferase activity mediated by PPVV alone in the absence of E\(2\) was set to 100%. Alterations in the reporter enzyme activity as a result of a co-transfected ER cDNA without or with E\(2\) are depicted as percentage (%) change compared with the activity induced by PPVV alone. Previously, we (Huang et al. 2005b) and others (Hall & McDonnell 1999) showed that ER\(\beta\), in contrast to ER\(\alpha\), interacts with ERE in situ independent of E\(2\). Consistent with this finding, our results here also reveal that transfection with increasing concentrations of the expression vector bearing ER\(\beta\), but not ER\(\beta\)\(_{\text{EBD}}\), cDNA gradually decreased the luciferase activity induced by PPVV independent of E\(2\) (Fig. 1C), without altering responses from the reporter plasmid bearing the TATA box promoter (data not shown).

A ChIP assay was also employed to corroborate our finding (Fig. 1D). The expression vectors were co-transfected with the reporter TATA box promoter vector bearing none (TATA) or one ERE into MDA-MB-231 cells. The cells were treated without or with a saturating concentration (\(10^{-7}\) M) of E\(2\) for 1 h, and processed for ChIP. We found that ER\(\beta\), but not ER\(\beta\)\(_{\text{EBD}}\) or the parent vector (data not shown), produced a PCR product from cells co-transfected with the reporter vector bearing ERE irrespective of whether the cells were treated with E\(2\) as observed with the in situ ERE competition assay (Fig. 1C). Thus, these results collectively suggest that ER\(\beta\)\(_{\text{EBD}}\) does not interact with ERE in situ as well.

To ensure that ER\(\beta\)\(_{\text{EBD}}\) is functional only in the ERE-independent pathway, the expression vectors were transiently transfected into ER-negative MDA-MB-231, HeLa, or U-2 OS cells. The cells were also co-transfected with a reporter vector containing a promoter driving the expression of the firefly luciferase cDNA as the reporter enzyme. Promoters were derived from the estrogen-responsive TFF1, pS2 and C3 genes that contain ERE sequences, thereby modeling the ERE-dependent signaling pathway (Yi et al. 2002a, Huang et al. 2004). The normalized activity from each reporter construct was compared with the parent expression vector (V) in the absence of E\(2\), with the latter value set to 1. Results showed that the ER\(\beta\)\(_{\text{EBD}}\) had no effect on luciferase activity in the absence or presence of \(10^{-8}\) M E\(2\) from either the TFF1 or the C3 gene promoter in all cell lines tested, shown MDA-MB-231 and HeLa cells (Fig. 2A). In contrast, ER\(\beta\) increased the reporter enzyme activity in response to E\(2\) from both the promoters in all cell lines.

To confirm that the transregulatory function of ER\(\beta\)\(_{\text{EBD}}\) is restricted to the ERE-independent signaling pathway, we used a reporter construct that bears the proximal MMP1 or RARA gene promoter (Huang et al. 2004). The functional interaction of ERs with the Jun/Fos family of proteins bound to an activator protein-1 (AP1) element in the proximal promoter of the MMP1 gene provides the basis for the responsiveness to ERs in a ligand- and cell-type dependent manner in experimental systems (Webb et al. 1995, Paech et al. 1997, Webb et al. 1999, Kushner et al. 2000). Similarly, the interaction of ER with specificity protein-1 (SP1) bound to GC boxes of the proximal promoter is critical for the
ligand-mediated regulation of the RARA gene (Rishi et al. 1995, Sun et al. 1998, Safe 2001). Transiently transfected MDA-MB-231, HeLa or U-2 OS cells were incubated without or with 10^{-9} M E_2, 10^{-8} M of 2,3-bis(4-hydroxyphenyl)propionitrile (DPN), 10^{-7} M of 4-hydroxytamoxifen (4-OHT), and 10^{-7} M ICI 182 780 (Imperial Chemical Industries). DPN is an ER\(_{\beta}\)-potency selective estrogenic compound (Harrington et al. 2003).

Results revealed that while the parent vector had no effect on transcription in the absence or presence of ligands in all cell lines tested, ER\(_{\beta}\) and ER\(_{\beta}\)-EBD mediated the effects of ligands on the reporter enzyme activity similarly independent of promoter and cell type (Fig. 2B). In MDA-MB-231 cells, only DNP and 4-OHT enhanced both ER\(_{\beta}\) and ER\(_{\beta}\)-EBD-induced responses from the RARA promoter construct, while E_2 or ICI had no effect on the reporter enzyme activity mediated by either ER. In contrast to MDA-MB-231 cells, ERs expressed in HeLa cells enhanced the reporter enzyme activity from the MMP1 promoter in response to only 4-OHT. In these cell lines, ER\(_{\beta}\) or ER\(_{\beta}\)-EBD had no effect on the transcriptional responses from the RARA gene promoter in the absence or presence of ligands. In U2-OS cells, on the other hand, E_2 and DNP repressed the reporter enzyme activity mediated by ER\(_{\beta}\) and ER\(_{\beta}\)-EBD from the both the MMP1 and the RARA promoter constructs, whereas 4-OHT and ICI augmented responses to both the receptors.

While the transcriptional responses mediated by ER species varied depending upon the nature of ligand, promoter, and cell context, our findings clearly indicate that ER\(_{\beta}\)-EBD mirrored the effect of ER\(_{\beta}\) on the reporter enzyme activity from reporter constructs emulating the ERE-independent signaling pathway. Our results suggest that ER\(_{\beta}\)-EBD retains its transregulatory function in the ERE-independent pathway, despite the fact that it does not bind to ERE and, consequently, is nonfunctional in the ERE-dependent signaling pathway.

### Regulation of endogenous gene expression by E\(_2\)-ER\(_{\beta}\)s

The experimental reporter systems we used here contain minimal promoter sequences responsive to E\(_2\)-ER signaling. While transient transfection into mammalian cells has been a valuable tool for the understanding of action mechanisms of transfectors, nucleosome deposition onto the non-replicating reporter vector displays an incompletely organized nucleosome array in contrast to the replicative cellular chromatin (Archer et al. 1992, Lee & Archer 1994, Pennie et al. 1995). This results in a more accessible chromatin of the transfected DNA that affects basal and transfactor-regulated transcription compared with the responses observed with cellular chromatin (Archer et al. 1992, Lee & Archer 1994, Pennie et al. 1995).

Moreover, the temporal regulation of endogenous E\(_2\) target gene expression is the result of integrated effects of transcription factors that bind to cognate responsive elements within the regulatory regions. The combinatorial effects of transactors and ER ultimately determine the magnitude and/or direction of the endogenous gene expression (Nunez et al. 1989, Vyhlidal et al. 2000). Consistent with heterologous expression systems, as we have demonstrated here (Fig. 2), the presence of an ERE in the TFF1 gene promoter provides the endogenous responsiveness to E\(_2\)-ER signaling (Nunez et al. 1989). The regulation of the RARA gene expression is, on the other hand, attributed to ER–SP1 interactions in experimental systems (Rishi et al. 1995, Sun et al. 1998, Safe 2001), as we also showed here (Fig. 2) and previously (Li et al. 2004). However, a recent genomics approach identified a long-distance regulatory module that contains a functional ERE responsible for the regulation of the RARA gene expression in breast cancer cell models expressing endogenous ER (Laganiere et al. 2005).

To ensure that ER\(_{\beta}\)-EBD can indeed discriminitely regulate endogenous gene expression, we used MDA-MB-231 cells as a model within which exogenously expressed ERs were shown to induce gene expressions that affect phenotypic characteristics (Garcia et al. 1992, Jiang & Jordan 1992, Zajchowski et al. 1993, Lazennec & Katzenellenbogen 1999, Lazennec et al. 2001, Licznar et al. 2003, Moggs et al. 2005). We also used recombinant adenoviruses for an efficient gene delivery (Huang et al. 2005b).

Recent studies showed that augmented levels of unliganded ERs lead to aberrant gene expression and an altered phenotype through mechanisms that are distinct from those mediated by E_2 (Fowler et al. 2004, 2006). To circumvent this potential problem, we used various concentrations of the recombinant adenovirus bearing ER\(_{\beta}\) cDNA (Ad5-ER\(_{\beta}\)) to obtain an optimal MOI that leads to a level of receptor synthesis which requires E_2 to regulate gene expression and cellular growth. We found that MDA-MB-231 cells infected with Ad5-ER\(_{\beta}\) at 600 MOI synthesize a concentration of ER\(_{\beta}\) that is strictly dependent upon E_2 for function (data not shown). The recombinant adenovirus expressing ER\(_{\beta}\)-EBD cDNA (ER\(_{\beta}\)-EBD) was used at 900 MOI producing comparable levels of receptor to that of ER\(_{\beta}\) (see also Fig. 3B). We therefore used 900 MOI of the parent recombinant adenovirus (Ad5), 600 MOI of Ad5-ER\(_{\beta}\), which was brought to 900 MOI by supplementing with 300 MOI of Ad5, and 900 MOI of ER\(_{\beta}\)-EBD in subsequent experiments.

The synthesis of functional receptor protein was examined by ICC, WB, E_2 binding, and EMSA assays in a time-dependent manner in infected MDA-MB-231 cells. ICC revealed that receptor proteins became detectable in the nuclei of infected cells as a function of time such that at 48 h post-infection nearly all cells showed staining for
Dissection of nuclear E2-ERα signaling

A

Relative luciferase activity fold change

V  ERβ  ERβEBD

E2

TFF1

C3

MDA-MB-231

HelA

B

MDA-MB-231, MMP1

MDA-MB-231, RARA

HeLa, MMP1

HeLa, RARA

U-2 OS, MMP1

U-2 OS, RARA

E2  DPN  4-OHT  ICI
ER\(\beta\) and ER\(\beta_{\text{EBD}}\) (Fig. 3A). This was a reflection of the level of ER synthesis as assessed by WB, which showed that ER\(\beta\) and ER\(\beta_{\text{EBD}}\) were synthesized comparably in a time-dependent manner (Fig. 3B). The in situ \(E_2\) binding assay further showed that \({}^3\text{H}-E_2\) was similarly retained in cells that synthesize ER\(\beta\) and ER\(\beta_{\text{EBD}}\) but not the parent recombinant adenovirus (Fig. 3C). The co-incubation of cells with 4-OHT prevented the retention of radiolabeled \(E_2\). This indicates that \({}^3\text{H}-E_2\) was specifically retained by \(E_2\). E2 binding with E2. Moreover, EMSA further revealed that ER\(\beta_{\text{EBD}}\) did not interact with ERE, while ER\(\beta\) effectively bound to ERE (Fig. 3D).

Based on these observations, we anticipated that ER\(\beta_{\text{EBD}}\) would not regulate endogenous gene expression mediated by the ERE-dependent pathway. Our studies using qPCR revealed that ER\(\beta\) only in the presence of \(E_2\) effectively induced the expression of estrogen-responsive genes mediated by ER–ERE interactions, exemplified by the TFF1 and C3 genes, while ER\(\beta_{\text{EBD}}\) had no effect on the expression of these genes (Fig. 4A). We also observed that \(E_2\)-ER\(\beta\), but not \(E_2\)-ER\(\beta_{\text{EBD}}\), activated the endogenous RARA gene expression. This is consistent with the finding that the regulation of the RARA gene expression is dependent on ER–ERE interactions (Laganiere et al. 2005), which is clearly in contrast to the response from the reporter system we observed here and reported previously (Rishi et al. 1995, Sun et al. 1998, Safe 2001). On the other hand, both ERs in the presence of \(E_2\) repressed the expression of the MMP1 gene that models an ERE-independent signaling-regulated gene.

To further verify the functionality of ER\(\beta_{\text{EBD}}\) and to preliminarily identify the genes mediated by the ERE-independent pathway, we used a global gene expression profiling approach. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of \(E_2\) for 48 h. At this time of post-infection, nearly all cells synthesized receptor proteins at levels that were maximal and comparable (Fig. 3). The cells were then treated with \(10^{-8}\) M \(E_2\) for 6 h, which is expected to induce significant changes in the level of transcription of immediate/early estrogen responsive genes (Frasor et al. 2003).

Expression profiling revealed that the genes regulated by ER\(\beta\) and ER\(\beta_{\text{EBD}}\) are involved in a broad range of function including metabolism, signal transduction, proliferation, apoptosis, and motility (Tables 1 and 2). As summarized in Fig. 4B, \(E_2\)-ER\(\beta\) significantly altered the expression of 41 genes, whereas \(E_2\)-ER\(\beta_{\text{EBD}}\) regulated the expression of 12 genes, 10 of which were also modified by \(E_2\)-ER\(\beta\). The majority of the identified genes were suppressed by both \(E_2\)-ER\(\beta\) and \(E_2\)-ER\(\beta_{\text{EBD}}\). \(E_2\)-ER\(\beta\) enhanced the expression of 12 responsive genes, whereas \(E_2\)-ER\(\beta_{\text{EBD}}\) augmented the expression of three genes.

We further verified the expression of a subset of the identified genes with qPCR. Infected MDA-MB-231 cells were maintained for 48 h in the absence of \(E_2\) (Fig. 4C). The cells were then treated without or with \(10^{-9}\) M \(E_2\) for 6, 12, and 24 h. Total RNA was subjected to qPCR. As observed with microarrays, only ER\(\beta\) in the presence of \(E_2\) induced the expression of the cyclin-dependent kinase inhibitor (CDKN1A, WAF1 p21, CIP1) and hysyl oxidase-like 4 (LOX4) genes. The expression of the follistatin (FST) gene was indeed repressed by only \(E_2\)-ER\(\beta_{\text{EBD}}\).

\(E_2\)-ER\(\beta\) and \(E_2\)-ER\(\beta_{\text{EBD}}\) activated the cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2) and thromboxane A2 receptor (TBXA2R) gene expressions. On the other hand, both receptors repressed the expression of the MMP1, (SERPINB2) serpin peptidase inhibitor, clade member 2, hyaluronan synthase 2 (HAS2), and heparin-binding EGF-like growth factor (HBEGF) genes in the presence of \(E_2\).

Thus, ER\(\beta_{\text{EBD}}\) is capable of modulating the expression of endogenous genes in response to \(E_2\).

**Differential effects of \(E_2\)-ER\(\beta\) and \(E_2\)-ER\(\beta_{\text{EBD}}\) on cell growth**

To examine whether the genomic responses mediated by \(E_2\)-ER\(\beta_{\text{EBD}}\) participate in the regulation of cellular growth, we used proliferation assays. Infected MDA-MB-231 cells were grown for various durations of time in the absence or presence of \(10^{-9}\) M \(E_2\). The cells were then subjected to cell counting (Fig. 5A) and MTT assay (Fig. 5B). Results revealed that ER\(\beta\) or ER\(\beta_{\text{EBD}}\) had no effect on cellular growth in the absence of \(E_2\) (data not shown). On the other hand, \(E_2\) treatment of the
infected cells synthesizing ERβ, but not ERβEBD, decreased the cell number as a function of time. These findings indicate that the E2-ERβEBD signaling has no discernable effect on cell proliferation.

Moreover, despite the comparable synthesis as assessed by WB (Fig. 5C) and the in situ E2 binding assay (data not shown), the ERE-binding defective ERβEBD (Fig. 5D) had no effect on cellular growth in the absence or presence of E2 in infected U-2 OS cells in contrast to E2-ERβ, which effectively suppressed the proliferation (Fig. 5E), as observed with MDA-MB-231 cells.

These results suggest that the ERE-independent pathway does not play a critical role in E2-ERβ signaling to mediate cellular growth.

**Effects of E2-ERβ and E2-ERβEBD on cell cycle**

The absence of an effect of E2-ERβEBD on cellular growth predicts that the ERE-independent pathway mediated by E2-ERβ is also insufficient to alter cell cycle distribution.

To address this prediction, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of 10⁻⁹ M E2 for various durations of time. The cells were then subjected to FACS. The kinetic analysis of histograms (Supplemental data Fig. 1A see Supplementary data in the online of version of the Journal of Molecular Endocrinology at http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/), summarized as the percentile of cells in G1 phase (Fig. 6A), revealed that ERβ, only in the presence of E2, increased the number of cells in the G1 phase and decreased the cell number accumulated in the S and G2 phases (data not shown). On the other hand, ERβEBD had little effect on cell cycle distribution whether or not the cells were treated with E2. Thus, it appears that E2 signaling conveyed by ERβ through the ERE-independent signaling pathway is insufficient to alter cell cycle distribution.

Since the effects of ERβ on the ERE-independent signaling are also dependent on the nature of ligand, we wanted to examine whether ER agonists or antagonists differentially affect cellular growth mediated by ERβEBD. The infected MDA-MB-231 cells were treated without or with 10⁻⁹ M E2, 10⁻⁸ M DPN, 10⁻⁷ M of 4-OHT or ICI (data not shown) for 48 h. The cells were also co-treated with 10⁻⁹ M E2 and 10⁻⁷ M of 4-OHT (or ICI, data not shown), or 10⁻⁸ M DPN together with 10⁻⁷ M of 4-OHT (or ICI, data not shown) for 48 h. These concentrations of ligands are optimally effective to alter the transcription in response to ERβ (Fig. 2 and data not shown).

**Figure 3** Functional ER synthesis in infected model cells. (A) MDA-MB-231 cells were infected with the parent recombinant adenovirus (Ad5) at MOI 900, a recombinant adenovirus bearing ERβ cDNA (ERβ) at 600 MOI together with 300 MOI Ad5 to equalize the total adenovirus titer, or 900 MOI for virus bearing ERβEBD cDNA. The intracellular localization of receptor proteins was examined by immunocytochemistry. Infected cells as a function of time (shown at 18 and 48 h post-infection) were probed with an ERβ-specific antibody followed by a fluorescein-conjugated secondary antibody (FITC). DAPI was used to stain nuclei. (B) Cell extracts (10 μg) of infected cells at indicated times were subjected to WB using the horseradish peroxidase conjugated monoclonal Flag antibody. Molecular weight in KDa is indicated. *Denotes extracts of the anti-flag antibody. **Denotes extracts of the anti-Flag antibody. (C) MDA-MB-231 cells were treated with E2 for various durations of time. The graph represents the mean ± S.E.M. of three independent experiments performed in duplicate. (D) Cell extracts (20 μg) of infected cells for the indicated times were subjected EMSA in the absence (−) or presence (+) of the anti-Flag antibody. **Denotes extracts of the parent adenovirus infected cells at 72 h. ER–ERE indicates the radiolabeled ERE-bound ERs. Unbound radiolabeled ERE is not shown for simplicity. A representative image from three independent experiments is shown.
Figure 4 Effects of ERs on endogenous gene expression. (A) MDA-MB-231 cells were infected with recombinant adenoviruses in the absence (−) or presence (+) of $10^{-9}$ M E$_2$ for 48 h. Total RNA from infected cells was subjected to qPCR for the expression of the TFF1, C3, MMP1 and RARA genes. (B) Summary of genes identified with microarrays. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence of E2 for 48 h. The cells were then treated with $10^{-9}$ M E$_2$ for 6 h. Total RNA was subjected toarrays. Results are the mean of six independent determinations. (C) The verification of subset of genes identified with arrays. The infected MDA-MB-231 cells were maintained in the absence of E2 for 48 h. The cells were then treated without or with $10^{-9}$ M E$_2$ for 6, 12, or 24 h. Total RNA was subjected to qPCR. Results, which are the mean ± S.E.M. of four independent determinations in quadruplicate, are depicted as fold change compared with the level of gene expression in cells infected with the parent recombinant adenovirus (Ad5) in the absence of E2 at 6 h of E2 treatment. The expression of the LOXL4, CDKN1A, FST, CRISPLD2, TBXA2R, SERPINB2, MMP1, HAS2, and HBEGF genes are shown.
were then subjected to FACS (Supplemental data, Fig. 1B http://jme.molecular.endocrinology-journals.org/content/vol40/issue5). The treatment of the infected cells synthesizing ER\(\beta\) with DPN repressed the cell number accumulated in the G1 phase comparable to that observed with the E2 treatment, while the compound had no effect on the growth of cells infected with Ad5 or Ad5-ER\(\beta\)EBD. Furthermore, the ER antagonist 4-OHT (or ICI, data not shown) alone did not affect cell cycle distribution in cells synthesizing either ER\(\beta\) species, but it effectively blocked the effect of E2 or DPN on G1 phase mediated solely by ER\(\beta\). The effects of ligand-ER\(\beta\) on cell cycle distribution were mirrored in cellular growth, which was suppressed only by agonists (Supplemental data, Fig. 1C http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/).

**Table 1** Genes mediated by 17\(\beta\)- estradiol (E\(_2\))- estrogen receptor \(\beta\) (ER\(\beta\))

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Fold change</th>
<th>Process</th>
</tr>
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<tbody>
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<td>SERPINB2</td>
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<td>Anti-apoptosis</td>
<td></td>
</tr>
<tr>
<td>MMP1</td>
<td>-3.57</td>
<td>Collagen catabolism, proteolysis</td>
<td></td>
</tr>
<tr>
<td>EDG1</td>
<td>-2.33</td>
<td>Signal transduction</td>
<td></td>
</tr>
<tr>
<td>HAS2</td>
<td>-2.33</td>
<td>Matrix formation</td>
<td></td>
</tr>
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<td>Cell–cell signaling</td>
<td></td>
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</tr>
<tr>
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<tr>
<td>TGFB2</td>
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<tr>
<td>HBEGF</td>
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<td></td>
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<td></td>
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<td>DKK1</td>
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<td>Signal transduction</td>
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<td>Cell adhesion</td>
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</tr>
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<td>IL18</td>
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<td>Signal transduction</td>
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<tr>
<td>CTGF</td>
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<td>Signal transduction</td>
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<td>Transport vehicle biogenesis</td>
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<td>UGGC</td>
<td>-1.22</td>
<td>Glycosphingolipid biosynthesis</td>
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<td>PIM1</td>
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<td>Cell proliferation, apoptosis</td>
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</tr>
<tr>
<td>CABLES2</td>
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<td>Cell division</td>
<td></td>
</tr>
<tr>
<td>CRISPLD2</td>
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<td></td>
</tr>
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<td>TBX2A2R</td>
<td>-3.16</td>
<td>Signal transduction</td>
<td></td>
</tr>
<tr>
<td>SLITRK4</td>
<td>-5.65</td>
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<td></td>
</tr>
<tr>
<td>LOXL4</td>
<td>24.99</td>
<td>Ion binding</td>
<td></td>
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</table>

**Regulation of apoptosis by E\(_2\)-ER\(\beta\)s**

Since cellular growth encompasses cell proliferation and death, the inability of E\(_2\)-ER\(\beta\)\(_{ERB}\) to affect cell growth suggests that the ERE-independent pathway participates minimally in inducing apoptosis, which is a complex, multiple-step event that culminates in cell death (Korsmeyer 1999). To address this issue, MDA-MB-231 cells were infected in the absence or presence of 10\(^{-6}\) M E\(_2\) for different lengths of time. The cells were then subjected to caspase 3/7, annexin V, and...
Table 2 Genes mediated by 17β-estradiol (E2)-estrogen receptor βE2D (ERβE2D)

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Fold change</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serpin peptidase inhibitor, clade B (ovalbumin), member 2</td>
<td>SERPINB2</td>
<td>−4.76</td>
<td>Anti-apoptosis</td>
</tr>
<tr>
<td>Matrix metalloproteinase 1 (interstitial collagenase)</td>
<td>MMP1</td>
<td>−3.70</td>
<td>Collagen catabolism, proteolysis</td>
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<tr>
<td>Matrix metalloproteinase 3 (stromelysin 1, progelatinase)</td>
<td>MMP3</td>
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<td>Collagen catabolism, proteolysis</td>
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<td>Hyaluronan synthase 2</td>
<td>HAS2</td>
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<td>Matrix formation</td>
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<td>Follistatin</td>
<td>FST</td>
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<td>Heparin-binding EGF-like growth factor</td>
<td>HBEGF</td>
<td>−1.72</td>
<td>Signal transduction</td>
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<td>Interleukin 18 (interferon-γ-inducing factor)</td>
<td>IL18</td>
<td>−1.59</td>
<td>Signal transduction</td>
</tr>
<tr>
<td>Dickkopf homolog 1 (Xenopus laevis)</td>
<td>DKK1</td>
<td>−1.49</td>
<td>Signal transduction</td>
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<td>G-protein-coupled receptor</td>
<td>GPRC5A</td>
<td>−1.35</td>
<td>Signal transduction</td>
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<td>Thromboxane A2 receptor</td>
<td>TBXA2R</td>
<td>1.73</td>
<td>Signal transduction</td>
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<td>PDZ and LIM domain 1 (elfin)</td>
<td>PDLM1</td>
<td>1.98</td>
<td>Signal transduction, cytoskeletal reorganization</td>
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<td>Cysteine-rich secretory protein LCCL domain containing 2</td>
<td>CRISPLD2</td>
<td>4.04</td>
<td>Signal transduction</td>
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</table>

TUNEL assays, which assess early-, mid-, and late-stages of apoptosis respectively.

We found that ERβE2D had no effect on the activity of caspase 3/7 whether or not the cells were treated with E2, while ERβ in the presence of E2 increased the enzyme activities compared with cells infected with Ad5 (Fig. 6B).

Similar results were obtained with annexin V staining of infected cells (Fig. 6C and Supplemental data, Fig. 2A, see Supplementary data in the online version of the Journal of Molecular Endocrinology at http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/). The number of apoptotic cells in a population of cells infected with a recombinant adenovirus bearing a receptor cDNA was compared with the number of apoptotic cells infected with the parent adenovirus in the absence of E2 as a function of time. We observed that the unliganded or E2-bound ERβE2D had no effect on the cell population stained with annexin V, while ERβ in response to E2 increased the number of stained cells.

We corroborated our results with a TUNEL assay by incorporating FITC-conjugated nucleotides into the fragmented genomic DNA (Gorczyca et al. 1998). Infected MDA-MB-231 cells were incubated without or with 10⁻⁹ M E2 for various durations of time. The cells were then subjected to the TUNEL assay. Results, depicted as the number of cells stained with TUNEL (Supplemental data, Fig. 2B http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/) and summarized as a function of time (Fig. 6D), showed that E2-ERβ induced the fragmentation of genomic DNA of the infected cells. In contrast, ERβE2D had no discernable effect.

Collectively, these results indicate that ERβ, but not ERβE2D, induced apoptosis in infected cells treated with E2. These results suggest that the ERE-dependent pathway is the critical E2-ERβ signaling pathway for the induction of apoptosis.

Differential effects of ERβ and ERβE2D on cell migration and invasion

Studies showed that ER-positive breast cancer cell models and ER-negative cell lines expressing ectopically introduced ER cDNA are less motile and invasive than the parent cells (Garcia et al. 1997, Lazennec et al. 2001).

To examine whether the ERE-independent pathway participates in the anti-motogenic effect of E2-ERβ signaling, we used wound-healing and invasion assays. MDA-MB-231 cells infected with recombinant adenoviruses in the absence or presence of 10⁻⁹ M E2 were grown for 48 h to allow the cells to reach near confluence. A wound was then created and the rate of wound closure was assessed. The results showed that while ERβE2D had little effect on wound closure in the absence or presence of E2, ERβ without E2 delayed the wound healing, which was further delayed by the presence of E2 (Fig. 6E and Supplemental data, Fig. 2C http://jme.molecular.endocrinology-journals.org/content/vol40/issue5/).

We also utilized an invasion assay that assesses the capacity of cells to migrate through a reconstituted basement membrane to independently verify our findings. MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of 10⁻⁹ M E2 for 48 h. The cells were then collected, and an equal number of cells were seeded on the reconstituted basement membrane of invasion chambers in the absence or presence of E2. After 24 h incubation, cells on the bottom of the chamber membrane were stained and imaged.
A quantitative analysis of images revealed that ERβ only in response to E2, but not E2-ERβEBD, reduced invasive cell number (Fig. 6F).

These results collectively suggest that the ERE-independent pathway mediated by E2-ERβ signaling plays a minimal role in cellular motility.

Discussion

The integrated effects of the ERE-dependent and ERE-independent nuclear E2-ER signaling are critical for the homeodynamic regulation of estrogen-responsive tissue functions. However, the relative importance of each pathway in physiology and pathophysiology of E2 signaling is unclear. Using an ERE-binding mutant of ERβ (ERβEBD), which acts primarily through the ERE-independent pathway, we show here that genomic responses to E2-ERβEBD are not sufficient to alter phenotypic characteristics of model cells.

The elements of the nuclear action of ERs involve a multi-step regulation in which ERs are converted from an inactive form to a transcriptionally active state mediated by a conformational change upon E2 binding (Parker 1998). The interaction of E2-ER with an ERE constitutes the initial step in the ERE-dependent signaling in which the gene expression is cyclically regulated (Metivier et al. 2003). In the ERE-independent pathway, E2-ER functionally interacts with transcription factors bound to their cognate regulatory elements.
Figure 6 Effects of ERs on cell cycle, death, and motility. (A) To examine the effects of ERs on cell cycle distribution, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence (data not shown) or presence of $10^{-9}$ M E2 for 24, 48, or 96 h. The cells were then subjected to FACS. Results showing the percent of cells in G1 phase as a function of time are the mean ± S.E.M. of four independent experiments. (B) Infected MDA-MB-231 cells in the absence or presence of $10^{-9}$ M E2 as a function of time indicated were subjected to a caspase 3/7 assay. The fluorescence was measured using a fluorometer. Results are the mean ± S.E.M. of three independent experiments. (C and D) To examine the effects of ERs on cell death, infected MDA-MB-231 cells at indicated times were subjected to (C) an annexin V assay using FACS or (D) a TUNEL assay. Results, which are the mean ± S.E.M. of three independent experiments, are summarized as the number of cells bound to annexin V or as the number of cells that incorporated FITC-conjugated dUTP into the fragmented DNA (TUNEL). (E) To assess the role of the ERE-independent signaling pathway in cellular motility, infected MDA-MB-231 cells were incubated in the absence or presence of $10^{-9}$ M E2 for 48 h to allow cells to reach near 100% confluence. A wound was then created and images were captured at 0 h and at every 24 h thereafter. Results, which are the mean ± S.E.M. of three independent experiments performed in duplicate, are summarized as the wound closure at 96 h relative to 0 h. (F) To examine the effects of ERs on cellular invasiveness, MDA-MB-231 cells were infected with recombinant adenoviruses in the absence or presence of $10^{-9}$ M E2 for 48 h. The cells were collected and counted. The same number of cells from each experimental group was then seeded on the upper section of the invasion chamber. After 24 h incubation, those cells with invasive capabilities on the bottom of chamber membrane were counted. Results, which represent the mean ± S.E.M. of three independent experiments performed in duplicate, are percent change compared with cells infected with the parent adenovirus in the absence of E2, which is set to 100%. *Indicates a significant effect compared with the parent adenovirus (Ad5).
elements on DNA to modulate gene expression (Kushner et al. 2000, Safe 2001). This interaction involves the stabilization of transfactor binding to DNA and/or recruitment of co-regulators to the complex (Kushner et al. 2000, Safe 2001). Estrogen regulation of the MMP1 and the insulin-like growth factor-1 (IGF1) genes is, for example, characterized by a functional interaction between the members of the AP1 family proteins and ER (Umayahara et al. 1994, Kushner et al. 2000). The modulation of the FOS (cellular oncogene fos) and the progesterone receptor (PGR) gene expressions, on the other hand, are dependent on the interaction between ER and SP1 (Duan et al. 1998, Schultz et al. 2003). Similarly, ER interactions with the signal transducers and activators of transcription (STATs) family proteins provides a mechanism for the estrogen responsiveness of the β-casein (CSN2) gene (Bjornstrom et al. 2001).

In the ERE-independent signaling, ER establishes interactions with transfactors through regions that also encompass the DBD, while the integrated effects of the amino and carboxyl termini are responsible for the gene expression (Kushner et al. 2000, Safe 2001, Bjornstrom & Sjoberg 2002, Cheung et al. 2005). Studies also demonstrated that the prevention of ER–ERE interactions by changing critical residues in the DBD of the mouse ERα and the human ERβ prevent the transcription from the ERE-dependent pathway, while conserving the regulatory capacity of the receptors in simulated ERE-independent pathways (Jakacka et al. 2001, Bjornstrom & Sjoberg 2004). Consistent with these findings, we also show here that ERβERB, which had no effect on gene expression from the ERE-dependent pathway, mimics the effect of ERβ on transcriptional responses from simulated ERE-independent signaling pathways in a ligand, promoter, and cell context-dependent manner. Although the underlying mechanisms are unclear, distinct conformational changes induced by a specific ligand (Nettles et al. 2007) could alter the extent of interaction with a transfactor specific to each gene, thereby altering the transcription output. Additionally, cellular differences in the concentration of transfactors and/or co-regulatory proteins (McKenna et al. 1999) could contribute to differential effects of ligand-ERs on responses from promoter constructs.

Extending these findings, our microarray and qPCR studies further indicate that ERβERB in the presence of E2 regulates the expression of a number of genes, the majority of which (10 out of 12) are similarly modulated by E2-ERβ in infected MDA-MB-231 cells (Tables 1 and 2). Although the nature of the regulatory elements critical for the E2 responsiveness of the identified genes are unknown and presently being investigated, these genes are also targets for various cytokines and growth factors that utilize transactors involved in cross-talk with ERs. Growth factor-mediated expression of the MMP1 and MMP3 genes, for example, involves the AP1 proteins (Buttice et al. 1996, Aho et al. 1997). Similarly, while the basal expression of the HAS2 gene is primarily controlled by the SP family of proteins (Monslow et al. 2006), the responsiveness of the gene to endocrine and paracrine signaling is mediated by STAT and the NFκB transcription factors (Pasonen-Seppanen et al. 2003, Saavalainen et al. 2005). The protein products of these E2-ERβERB-responsive genes play critical roles in cell growth, death, and migration. Matrix metallopeptidases (MMPs), for example, are a large family of endopeptidases responsible for tissue remodeling and degradation of the extracellular matrix (ECM) that includes collagens, elastins, gelatin, laminin, and matrix glycoproteins (Duffy et al. 2000). MMP1 is involved in the degradation of collagen, while MMP3 participates in the degradation of many ECM substrates including glycoproteins, laminin, fibronectin, and collagen (Duffy et al. 2000). Increased MMP1 and MMP3 gene expressions are associated with advanced stages of breast cancer and are involved in the development of metastasis (Duffy et al. 2000). Similarly, aberrant synthesis of hyaluronan (HA), the major glycosaminoglycan found in the ECM, is implicated in a diverse range of ECM-mediated processes including cellular proliferation, adhesion, and migration (Gotte & Yip 2006). HA is synthesized by the hyaluronan synthase family of transmembrane glycosyltransferases that are encoded by the corresponding HAS1, -2, -3a, and -3b genes (Gotte & Yip 2006). Preferential and high expression of the HAS2 gene appears to be critical for invasiveness of breast cancer cells, including MDA-MB-231 cells (Gotte & Yip 2006). Studies also indicate that an aberrant expression of the SERPINB2 (Andreassen et al. 1997), HBEGF (Ito et al. 2001), DKK1 (Forget et al. 2007), GPRC5A (Nagahata et al. 2005), and TBX2A2R (Watkins et al. 2005) genes alters the proliferation and motility of breast cancer cells.

Considering the ability of ERβERB to alter gene expression, the absence of an effect of ERβERB on the phenotypic characteristics of model cells is surprising. Studies indicate that the suppression of HAS2 (Udabage et al. 2005, Li et al. 2007) or MMP1 (Wyatt et al. 2005) synthesis by a silencing RNA approach decreases the growth and invasiveness of MDA-MB-231 cells. One explanation is that the extent of transcriptional alterations mediated by E2-ERβERB and subsequent changes in RNA levels are not sufficient to modify the corresponding protein concentrations in cells, in contrast to silencing RNA approaches. This is unlikely since the magnitude of E2-ERβ-mediated transcriptional responses was comparable to those modulated by E2-ERβERB (Fig. 3 and Tables 1 and 2), while only E2-ERβ induced alterations in cellular phenotypes. An alternative explanation is that the
integrated regulation of genes involved in a signaling cascade through both ERE-dependent and ERE-independent pathways is crucial for the manifestation of phenotypic alterations in response to E₂-ER signaling. The interaction of CD44 with HA, for example, induces signaling events that promote anchorage-independent cell growth and migration, thereby increasing metastatic spread (Gotte & Yip 2006). Importantly, post-transcriptionally mediated repression of HAS2 leads to a concomitant decrease in its cell surface receptor CD44 transcript (Udabage et al. 2005). A similar mechanism is also suggested for the members of MMP proteins (Wyatt et al. 2005). Thus, the concomitant regulation of the HAS2 and the CD44 gene expression, or genes involved in the MMP signaling cascade, by E₂-ERβ, could be the underlying reason for the efficacy of the receptor to induce phenotypic alterations, in contrast to E₂-ERβ which decreased only the HAS2 gene expression without an effect on cellular phenotype.

Transcript profiling of infected MDA-MB-231 cells synthesizing an ERE-binding defective mouse ERz indicated that E₂ alters the expression of a number of genes involved in various cellular functions (Glidewell-Kenney et al. 2005). However, out of the 29 identified genes, the expression of only the HAS2 and SERPINB2 genes coincides with the altered gene expression reported here. Since transcriptional responses from ERE-independent signaling pathways in experimental systems are also dependent upon ER-subtype, a discordant gene regulation could be critical for differences in subtype-mediated cellular responses.

Augmented synthesis of ERz, as observed in atypical hyperplasia and in situ carcinoma of breast, causes an E₂-independent, tamoxifen-insensitive proliferation through aberrant gene expressions (Fowler et al. 2004, 2006). Our studies presented here were designed to assess the role of the ERE-independent signaling pathway mediated by ERβ to induce cellular responses at concentrations that strictly depend upon E₂ for function. The circumvention of E₂-dependency by increased ERβ levels could contribute to the characteristics of target tissue malignancies through a selective expansion, or altered expression, of target genes mediated by the ERE-independent signaling pathway.

In summary, our results show that genomic responses mediated by the ERE-binding defective ERβ mutant in response to E₂ are insufficient to alter the phenotypic characteristics of model cell lines. These results imply that the ERE-dependent pathway is necessary for E₂-ERβ to regulate growth of the responsive cells. Studies that aim to target specifically ERE-bearing genes through the use of designer transcription factors are underway to address this issue.

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