The ligand-mediated nuclear mobility and interaction with estrogen-responsive elements of estrogen receptors are subtype specific

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Abstract

17β-Estradiol (E2) plays important roles in functions of many tissues. E2 effects are mediated by estrogen receptor (ER) α and β. ERs regulate transcriptions through estrogen-responsive element (ERE)-dependent and ERE-independent modes of action. ER binding to ERE constitutes the basis of the ERE-dependent pathway. Direct/indirect ER interactions with transcription complexes define ERE-independent signaling. ERs share functional features. Ligand-bound ERs nevertheless induce distinct transcription profiles. Live cell imaging indicates a dynamic nature of gene expressions by highly mobile ERs. However, the relative contribution of ER mobility at the ERE-independent pathway to the overall kinetics of ER mobility remains undefined. We used fluorescent recovery after a photo-bleaching approach to assess the ligand-mediated mobilities of ERE binding-defective ERs, ERE-EBD. The decrease in ERα mobility with E2 or the selective ER modulator 4-hydroxyl-tamoxifen (4HT) was largely due to the interaction of the receptor with ERE. Thus, ERα bound to E2 or 4HT mediates transcriptions from the ERE-independent pathway with remarkably fast kinetics that contributes fractionally to the overall motility of the receptor. The antagonist Imperial Chemical Industries 182 780 immobilized ERαs. The mobilities of ERβ and ERE-EBD in the presence of ligands were indistinguishable kinetically. Thus, ERβ mobility is independent of the nature of ligands and the mode of interaction with target sites. Chimeric ERs indicated that the carboxyl-termini are critical regions for subtype-specific mobility. Therefore, while ERs are highly mobile molecules interacting with target sites with fast kinetics, an indication of the hit-and-run model of transcription, they differ mechanistically to modulate transcriptions.

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Introduction

17β-Estradiol (E2) plays critical roles in many physiological and pathophysiological processes of a wide range of tissues (Huang et al. 2005a, Zhao et al. 2010). E2 effects are primarily mediated by transcription factors, estrogen receptor (ER) α and β, that convey E2 signaling through estrogen-responsive element (ERE)-dependent and -independent pathways.

Kinetic biochemical assays indicate that the unliganded ERα interacts, albeit inefficiently, with EREs cyclically with a time scale of 20 min (Shang et al. 2000, Metivier et al. 2003, Reid et al. 2003). The binding of E2 to ERα leads to a structural reorganization that increases the stability of the ERα dimer (Tamrazi et al. 2002) and the affinity of ERα to co-regulatory proteins (Yi et al. 2002b, Tamrazi et al. 2005). The interaction of E2–ERα with ERE extends the duration of promoter engagement to 40–60 min (Shang et al. 2000, Metivier et al. 2003, Reid et al. 2003). This is due to the sequential recruitment of preformed co-activator complexes for initiation and the subsequent dissociation of complexes from promoter for termination of transcription (Shang et al. 2000, Metivier et al. 2003, Reid et al. 2003). This episodic ERα–ERE engagement led to the transcriptional ratchet model that suggests ordered and directional events for ERE-driven gene expressions.

Fluorescent protein technologies together with quantitative live cell imaging also indicate a dynamic transcriptional regulation (Stenoien et al. 2000, 2001a,b, Sharp et al. 2006, Zwart et al. 2010). These approaches demonstrated that unliganded ERα exhibits rapid rates of exchange with chromatin, residence time measured in milliseconds. Although the binding of E2 to ERα decreases the mobility of the receptor, the exchange still occurs in seconds, in a clear contrast with longer cycling times determined by kinetic biochemical assays. These fast interactions of ERα with promoters support the alternative hit-and-run model for transcription.

Along with E2, the activity of ERα is modulated by the selective ER modulator (SERM) tamoxifen and antagonist Imperial Chemical Industries 182 780 (ICI) (McDonnell 1999). The binding of SERMs or antagonists to ERα alters the nuclear mobility and the ability of ERα to interact with co-regulators and chromatin (McDonnell 1999, Stenoien et al. 2001a, Yi et al. 2002b).
E2–ERs also regulate transcription by interacting with transcription factors (Kushner et al. 2000, Safe 2001). This nuclear signaling route is called the ERE-independent signaling pathway, which participates in the fine-tuning of cellular responses by regulating the expression of a subset of estrogen-responsive genes (Li et al. 2008, Nott et al. 2009). However, the underlying mechanisms or the kinetics of events at the ERE-independent signaling pathway remains poorly defined.

Although encoded by a distinct gene, ERβ shares structural features with ERα reflected in similar mode of action through signaling pathways (Huang et al. 2011). ERβ, nevertheless, regulates transcription with distinct potency and profile in response to ligands at signaling pathways (Huang et al. 2005a, Zhao et al. 2010). As the nuclear mobility is the sum of ER actions at target sites on chromatin, we addressed how ligands affect the nuclear mobility of an ERE binding-defective ER variant (ERαERD or ERβERD) that functions exclusively at the ERE-independent pathway to obtain initial insights into mechanisms of ER-mediated gene expressions. To address this issue, we used green fluorescence protein (GFP) fusion-ERs and fluorescent recovery after a photo-bleaching (FRAP) approach.

We found that the ligand-mediated nuclear mobility of ERα largely reflects the ability of the receptor to interact with ERE, whereas the mobility of ERβ is independent of the nature of ligands and the ability of ERβ to bind to ERE. Thus, ERs are highly mobile molecules interacting with target sites with fast kinetics, an indication of the hit-and-run model of transcription, and they differ mechanistically to modulate transcriptions.

Materials and methods

Plasmids

The expression vectors bearing human ERα and ERβ cDNAs encoding 595 and 530 amino acid long receptors respectively and the cDNA encoding the designer transcription factor PPVV were described previously (Yi et al. 2002a, Huang et al. 2004). The AF2 mutant of ERα contains a three amino acid replacement (D538A, E542A, and D545A) that blocks the ligand-dependent activation function (AF2) of ERα (Tzukerman et al. 1994, Sathya et al. 2002, Yi et al. 2002a). We initially used an AF2 mutant of ERβ that contains analogous mutations to that of ERα as we described previously (Yi et al. 2002a). However, the presence of the GFP at the amino-terminus of this mutant renders the receptor toxic to cells as they died before experimentation. To circumvent this problem, we used a point mutation that changes only the Glu residue at position 493 to a Lys in ERβ that prevents AF2 (An et al. 1999). The ERαERD were described previously (Li et al. 2008, Nott et al. 2009). The ERαERD contains Ala, Ala, and Glu residues at positions 203, 204, and 211 respectively that replace Glu, Gly, and Arg at the corresponding positions in the DNA recognition helix of the first zinc finger critical for ERα–ERE interactions (Nott et al. 2009). The replacement of Glu and Gly at positions 167 and 168 respectively in the DBD of ERβ with Ala residues generates the ERβERD (Li et al. 2008). The chimeric ERαN or ERβN generated by genetically exchanging sequences that encode the entire amino-terminal region of ERβ or ERα with that of ERα or ERβ, were also described previously (Yi et al. 2002a). cDNAs also contain sequences that encode a Flag epitope at the amino-terminus.

For the engineering of GFP fusion proteins, a restriction enzyme site was engineered at the 5’ of the start codon of ER cDNAs using an overlapping PCR. The engineered cDNAs were inserted into the 3’ end of the reading frame of the GFP-cDNA in the pAcGFP-C1 expression vector (Clontech) with appropriate restriction enzyme sites. For comparative analysis of GFP-ERs in some biochemical assays, we also generated a GFP cDNA with sequences that encode a Flag epitope at the amino-terminus of the protein. All constructs were sequenced to ensure the fidelity of encoding sequences.

We assessed the effect of ligands on ERE-driven gene expression using reporter vectors that emulate the ERE-dependent signaling pathway. For the simple TATA box promoter, we used the reporter pGL3 (Promega Corp.) plasmids bearing a TATA box promoter with single (ERE) or two EREs (2XERE) (Sathya et al. 2002, Yi et al. 2002a). We also used the pGL3 reporter vector bearing the promoter of the trefoil factor 1, TFF1, or pS2 gene (TFF1-Luc) (Yi et al. 2002a). To simulate ERE-independent signaling, we used an MMP1-Luc reporter plasmid that bears a fragment of the proximal promoter of the matrix metalloproteinase 1, MMP1, gene with single AP1 response element (Webb et al. 1995, Huang et al. 2004, Li et al. 2004) or an RARA-Luc reporter vector derived from the proximal promoter of the retinoic acid receptor α, RARA, gene that contains two GC-boxes (Sun et al. 1998, Huang et al. 2004, Li et al. 2004). In all reporter vectors, promoters drive the expression of the firefly luciferase cDNA as the reporter enzyme. A reporter vector driving the expression of the Renilla luciferase cDNA (Promega) was used to assess transfection efficiency (Yi et al. 2002a, Huang et al. 2004). The ratio of the firefly/Renilla luciferase activities of the cell lysate was determined using a dual luciferase assay kit (Promega Corp.) to obtain the relative luciferase activity.

The Flag M2 antibody, ERα-specific H-222, and ERβ-specific antibody D7N were purchased from...
Tissue culture plates were incubated with 10 K 300 ng expression vector containing the cDNA for an 
protein, carrying the PPVV cDNA together with 0, 75, 150, or 250 ng expression vector modified with one ERE and 300 ng expression vector were added into a given reaction to equalize the total amount of plasmid DNA. A vector bearing the Renilla luciferase cDNA was used as an internal control in the amount of 0.5 ng to normalize the transfection efficiency. Four hours after transfection, cells were maintained in fresh medium supplemented with 10% CD-FBS in the absence or presence of 10^{-9} M E_2, 10^{-7} M 4HT, or ICI for 24 h.

**Transient transfections**

Transient transfections for simulated ERE-dependent and ERE-independent pathways were accomplished as described previously (Yi et al. 2002a, Huang et al. 2004, Li et al. 2004). Transfected cells were treated without or with 10^{-7} M E_2 in the absence or presence of 10^{-7} M 4HT and/or 10^{-7} M ICI for 24 h to examine the effects of ligands on ER-mediated transcriptional responses from the ERE-dependent and ERE-independent signaling pathways.

**In situ E_2 binding assay**

To assess the synthesis and function of GFP fusion ERs in transfected cells, we used an in situ E_2 binding assay described previously (Huang et al. 2005b, Li et al. 2008). Briefly, transiently transfected cells in 48-well tissue culture plates were incubated with 10^{-7} M of (2,4,6,7,16,17^-H) E_2 (118 Ci/mmol, NEN Life Sciences, Boston, MA, USA) in the absence or presence of 10^{-6} M 4HT or ICI for 1 h. Cells were then washed extensively with PBS, collected, and radioactivity remaining in cells was measured in a scintillation counter. By the use of in situ ligand binding assay, we estimate that transiently transfected HeLa and MDA-MB-231 cells synthesize about 5-5- and 4-fold respectively more ERz compared with MCF-7 cells, a breast adenocarcinoma cell line that endogenously synthesizes ERz (Eckert et al. 1984).

**In situ competition for ERE binding assays**

In situ competition for ERE binding assay (Huang et al. 2005b) was used to assess the ability of GFP-ERs to interact with ERE in situ. This assay is based on the interference of a constitutively active potent activator (PPVV)-mediated transactivation from a single ERE-driven promoter construct by unliganded or ligand-bound ERs. The extent of interference is then taken as an indication of ER–ERE interactions (Huang et al. 2005b). In brief, cultured cells in 48-well tissue wells were transfected with 125 ng simple TATA box promoter with one ERE and 300 ng expression vector carrying the PPVV cDNA together with 0, 75, 150, or 300 ng expression vector containing the cDNA for an ER. Appropriate amounts of the parent expression vector were added into a given reaction to equalize the total amount of plasmid DNA. A vector bearing the Renilla luciferase cDNA was used as an internal control in the amount of 0.5 ng to normalize the transfection efficiency. Four hours after transfection, cells were maintained in fresh medium supplemented with 10% CD-FBS in the absence or presence of 10^{-9} M E_2, 10^{-7} M 4HT, or ICI for 24 h.

**Western blot and electrophoretic mobility shift assay**

Transiently transfected cells with expression vectors in six-well tissue culture plates were maintained for 24 h. Cell extracts (10 μg) were subjected to western blot (WB) and electrophoretic mobility shift assay (EMSA) as detailed previously (Li et al. 2008, Nott et al. 2009). For WB, proteins were probed with the HRP-conjugated monoclonal Flag antibody (M2-HRP, Sigma–Aldrich). We also used HC-20 and D7N antibodies (Santa Cruz Biotechnology, Inc.) specific to ERα and ERβ respectively to detect receptor proteins, which were visualized with a second antibody conjugated with HRP. The ECL-Plus Western blotting kit (GE Life Sciences, Piscataway, NJ, USA) was used for the detection of receptor proteins. For EMSA, we used the Flag or a receptor-specific antibody to assess the specificity of ER–ERE interactions. Images from WB and EMSA were analyzed by PhosphorImager (Storm 860, GE Life Sciences) and were quantified with ImageQuant (GE Life Sciences).

We also examined the effects of ER ligands on the detergent extractability and intracellular level of receptor proteins with WB. Cells maintained in six-well tissue culture plates in 10% CD-FBS containing media for 24 h were then transiently transfected for 24 h. Cells were subsequently incubated with fresh medium supplemented with or without 10^{-9} M E_2, 10^{-7} M 4HT, or ICI for 1 h. At the termination of an experiment, cells were collected, pelleted, and subjected to protein extraction using 50 μl of a high salt extraction buffer, HSB (400 mM KCl, 20% glycerol, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), and 1/14 (v/v) protease inhibitor cocktail). After the HSB or RIPA extraction, the remaining pellet was also subjected to 50 μl 1 × Laemmli buffer (LB) (60 mM Tris–Cl, pH 6–8, 2% SDS, 2 mM DTT, 1 mM PMSF, and 1/14 (v/v) protease inhibitor cocktail). After the HSB or RIPA extraction, the remaining pellet was also subjected to 50 μl 1 × Laemmli buffer (LB) (60 mM Tris–Cl, pH 6–8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue), to extract insoluble protein aggregates. In addition, we used 100 μl LB to obtain total cell lysate (TCL) by extracting both soluble and insoluble proteins. Ten micrograms of total protein estimated with Nanodrop...
Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP) assay was described previously (Huang et al. 2005b). In brief, cells grown in six-well tissue culture plates were co-transfected with expression vector and the reporter vector bearing the TATA box promoter with single ERE. Twenty-four hours after transfection, cells were incubated in fresh medium with or without 10⁻⁷ M E₂, 4HT, or ICI for 1 h. Cells were then subjected to ChIP using the Flag-M2 antibody-conjugated agarose beads (Sigma–Aldrich). The generation of a 366 bp PCR fragment by ChIP indicates the specificity of PCRs. Due to the difficulty of assessing the interaction of ER with EREs of endogenous genes by ChIP in transiently transfected cells, we used recombinant adenovirus-infected MDA-MB-231 cells with which we previously carried out experiments to assess the interactions of E₂–ERs with (Huang et al. 2005b), and transcriptional responses from Li et al. (2008), Nott et al. (2009) and Huang et al. (2011), the ERE of TFF1. MDA-MB-231 cells, 100 000 cells/well in six-well tissue culture plates, were infected with recombinant adenoviruses in media with 10% CD-FBS for 48 h. We used recombinant adenovirus bearing ERα, ERαEBD, ERβ, or ERβEBD cDNA with sequences encoding a Flag epitope at the amino-terminus at 100, 150, 600, or 900 multiplicity of infection (MOI) respectively together with the parent recombinant adenovirus bearing no cDNA at varying MOI to equalize the total amount of adenovirus, 900 MOI, used for infections. At these MOIs, the synthesis of ERs was comparable (Li et al. 2008, Nott et al. 2009, Huang et al. 2011). Forty-eight hours after infections, cells were incubated in fresh medium supplemented without or with 10⁻⁹ M E₂, 10⁻⁷ M 4HT, or ICI for 1 h. We also used 10⁻⁸ M ERα-selective propyl pyrazole triol agonist or 10⁻⁸ M ERβ-selective diarylpropionitrile agonist for 1 h. At these concentrations, ligands maximally affected transcriptional responses from reporter constructs induced by ERs or E₂–ERs (data not shown). Cells were collected and subjected to ChIP using M2-Flag antibody-conjugated agarose beads (Sigma–Aldrich) as described (Huang et al. 2005b). The production of a 315 bp PCR product indicates specific ER–ERE interactions (Supplementary Figure 3A, see section on supplementary data given at the end of this article).

Live cell microscopy and FRAP

HeLa or MDA-MB-231 cells were grown in 35 mm glass bottom coverslip dishes (MatTek Corp., Ashland, MA, USA) in medium containing 10% CD-FBS without phenol-red for 24 h. Cells were then transiently transfected with 1.5 µg of an expression vector bearing the GFP-fusion receptor cDNA. Twenty-four hours after transfections, cells were treated with or without various concentrations (10⁻¹⁰ to 10⁻⁷ M) of E₂ for 1 h before FRAP analysis. We observed in preliminary studies that 10⁻⁹ M E₂ maximally affected the intracellular mobility of both ERα and ERβ. Based on these findings, we used 10⁻⁹ M E₂ in subsequent FRAP assays. We also treated cells with 10⁻⁷ M 4HT or ICI, a concentration that maximally affected the nuclear mobility of ERs in preliminary experiments. FRAP was performed using an Olympus FV1000 laser scanning confocal microscope containing a full-stage incubator equilibrated to 37 °C housed at the URMC Confocal and Conventional Microscopy Core. Cells were imaged live using a 60× 1.4 NA oil immersion objective.

Cells were initially examined with differential interference contrast (DIC) to assess the cellular health. To prevent experimental artifacts due to over-expression of GFP-fusion ERs (Stenoien et al. 2001a,b), cells with low fluorescence intensities (600–1500 arbitrary fluorescence unit) were selected for FRAP analysis. Photo-bleaching was accomplished using a tunnel region of interest (ROI) of the FRAP module. A tunnel ROI as a 9×9 pixel area (4468 µm²) was used for all photo-bleaching experiments. It should be noted that the FRAP results were independent of the shape of ROI, as we obtained similar results from stripe bleaching in preliminary experiments (data not shown). A single z plane was bleached with the SIM scanner capabilities using the 405 nM laser set to 30% power for 0.2 s and simultaneously imaged in freerun (~0.25 ms intervals) mode.

FRAP analysis was carried out with FV1000 Olympus post-processing software. Briefly, frames of the time-lapse data were moved to the point of photo-bleaching and graphs were obtained for the bleached ROI. Another ROI at a size and fluorescence intensity that corresponded to those of the experimental ROI before photo-bleaching within the same cell was used as the control to assess the background and alterations in total cellular fluorescence due to photo-bleaching (Supplementary Figure 1, see section on supplementary data given at the end of this article). All data were exported to Excel for further analysis. The fluorescence intensity of the control ROI throughout the post-bleach period was used to normalize the recovery of bleached ROI by dividing the fluorescence value of the bleached ROI with that of the control ROI at each time point of imaging. Fluorescence is expressed as relative fluorescence units where zero (0) is the fluorescence after photo-bleaching (time 0) and one (1) is the fluorescence of the bleached area reached to pre-bleach levels. Images were exported as Tagged Image File Format (tif) and movie (mov) files. Adobe Photoshop
Results

The synthesis and function of GFP-ERs in HeLa cells

The interaction of ERα with permutations of a core palindromic DNA sequence 5′-GGTCAnnnTGACC-3′, or ERE as well as ERE half-sites (Kato et al. 1995, Ansari et al. 2012), constitutes the ERE-dependent signaling pathway (Huang et al. 2005a, Zhao et al. 2010). The recognition of an ERE by the ERα dimer is mediated by two zinc-binding motifs in each DBD monomer that fold to form a single functional unit (Schwabe et al. 1993). Distinct residues particularly Glu203 and Gly204 in the DNA recognition helix of the first zinc finger of DBD of the human ERα are critical for DNA sequence discrimination (Schwabe et al. 1993) and also for binding to EREs (DeNardo et al. 2007). We showed that changing Arg211, a conserved amino acid among nuclear hormone receptors critical for receptor–DNA interactions, to Glu211 together with Ala203 and Ala204, which replace Glu203 and Gly204 respectively, generates ERαDBD that functions only at the ERE-independent signaling pathway (Nott et al. 2009). Similarly, changing Glu176 and Gly176 in the first zinc finger motif of the DBD of the human ERβ to Ala also generated ERβDBD that regulates gene transcriptions exclusively through the ERE-independent signaling pathway (Bjornstrom & Sjoberg 2002, Li et al. 2008).

To examine the effects of ligands on the kinetics of nuclear movement of EREDBD, we initially assessed the synthesis and biochemical features of GFP fusion receptors in transiently transfected HeLa cells derived from an ER-negative cervical carcinoma. Cellular extracts were subjected to WB using an antibody specific to the Flag epitope present at the amino-terminus of the resulting protein. Cell extracts (10 μg) were subjected to WB using a HRP-conjugated monoclonal Flag antibody. Molecular mass in kDa is indicated. (A) The synthesis of functional GFP-ERs in HeLa cells. HeLa cells were transiently transfected with an expression vector bearing none (V) or an ER cDNA with or without sequences encoding GFP (G) genetically conjugated to the 5′ of ER coding sequences. Constructs also contain sequences that encode for a Flag epitope present at the amino-terminus of the resulting protein. Cell extracts (10 μg) were subjected to WB using a HRP-conjugated monoclonal Flag antibody. Molecular mass in kDa is indicated. (B) In situ binding assay. Twenty-four hours after transient transfections with an expression vector bearing none (V) or an ER cDNA with (G) or without GFP, HeLa cells were incubated in medium containing 10⁻⁷ M of ³H-E₂ in the absence or presence of 10⁻⁶ M 4HT (+ 4HT) for 1 h. The medium containing the radioactive ³H-E₂ was removed and cells were extensively washed with PBS before dislodging. Radioactivity retained in cells was then quantified by scintillation counting. The graph represents the mean ± S.E.M. of three independent experiments performed in duplicate. (C) The interaction of ERs with ERE in vitro. Cell extracts (10 μg) of transfected cells were also subjected to electrophoretic mobility shift assay (EMSA) without (−) or with (+) a receptor-specific antibody (Ab). ERE specifies unbound and ER-ERE denotes ER-bound radiolabeled ERE. Asterisk denotes the free ERE lane. A representative result from a minimum of two independent experiments of WB or EMSA is shown.
mobilities to those of corresponding parent ERs, the GFPERa and GFP-ERβ retarded the migration of the radiolabeled ERE, whereas GFP-ERZEBD, GFP-ERβEBD, or the ERZEBD showed no binding.

To further ensure that GFP-ERs in response to ligands mimic the effects of the parent ERs on the transcription and GFP-ERZEBD are functional only at ERE-independent signaling pathways, we used reporter vectors with promoters emulating ERE-dependent and ERE-independent signaling routes (Supplementary Figures 2 and 3, see section on supplementary data given at the end of this article). For the simulated ERE-dependent signaling pathway, we used reporter plasmid bearing two ERs in tandem located upstream of the simple TATA box promoter (2XERE-Luc) or the proximal promoter region derived from the gene MMP1 (TFF1-Luc) bearing an ERE. ER concentration (10^(-10) M) of E2 from both promoters in transfected cells (Supplementary Figure 2A). Although the extent of activations was lower than those induced by ERa, GFP-ERa also increased the enzyme levels in response to E2. The treatment of cells with 10^(-7) M 4HT or 10^(-7) M ICI alone had little effect on transcriptional responses mediated by ERs or GFP-ERs. However, 4HT or ICI effectively countered the effect of E2 on the reporter enzyme when cells were co-treated, whereas ERZEBD or GFP-ERZEBD did not affect the enzyme activity whether or not cells were exposed to ligands alone or in combination. Similarly, ERβ or GFP-ERβ, but not the ERE binding-defective counterparts, augmented the activity of the reporter enzyme only in the presence of E2, which was blocked by the co-treatment of cells with 4HT or ICI (Supplementary Figure 3A). Thus, GFP-ERs, but not the ERZEBD with or without GFP, in response to ligands mimic the effects of the parent ERs on the transcription of the reporter enzyme mediated through the ERE-dependent signaling pathway.

We previously showed that MMP1 is a target gene for E2-ER signaling, as ERs in response to 10^-9 M repress the expression of MMP1 through the ERE-independent signaling pathway (Li et al. 2008, Nott et al. 2009). Simulated systems suggest that the functional interaction of ERs with AP1 bound to an AP1 element provides the basis for the regulation of MMP1 gene promoter in an ER subtype, nature and concentration of ligand, promoter and cell type-dependent manner (Webb et al. 1995, Kushner et al. 2000). Similarly, the interaction of ER with SP1 bound to GC boxes is critical for the ligand-mediated regulation of the RARA gene promoter in reporter assays (Sun et al. 1998, Safe 2001). To ensure that GFP-ERs also mimic the effects of the parent ERs on transcription, an expression vector bearing none or an ER cDNA was transfected into HeLa cells together with MMP1-Luc or the RARA-Luc reporter vector (Supplementary Figure 2B and 3B). We did not observe a significant effect of 10^-9 M E2 mediated by ERs with or without GFP on the activity of the reporter enzyme from promoters, which could be due to the promoter composition in reporter constructs. However, 10^-7 M 4HT mediated transcriptional responses to ERs similarly. ICI at 10^-7 M also affected the luciferase activity mediated by ERa proteins, but not by ERβ with or without GFP. Importantly, ERZEBD mimicked the effects of the parent ERs on transcriptions in response to ligands from reporter vectors emulating ERE-independent signaling pathways. Thus, the presence of GFP at the amino-termini of ERs does not affect the transregulatory functions of the receptors at simulated ERE-independent signaling pathways as well.

**Ligand-mediated nuclear mobility of GFP-ERs**

To examine the effects of ligands on nuclear mobility of ERs with or without ERE binding function, transfected HeLa cells were treated with a vehicle (EtOH, 0.01%) for 1 h and then subjected to FRAP analysis (Fig. 2). GFP-ERa showed a diffuse distribution throughout the nucleus but it is excluded from nucleoli. After a 0.2 s photo-bleaching, the bleached area equilibrated to pre-bleach levels within 1 s with a half-maximum recovery rate (1/2mRR) of <0.2 s. On the other hand, the treatment of cells with 10^-9 M E2 or 10^-7 M 4HT for 1 h reduced the mobility of the receptor. Fluorescence after photo-bleaching was fully recovered within 40 s of post-bleaching with a 1/2 mRR of about 5 s. By contrast, the treatment of cells with 10^-7 M ICI immobilized GFP-ERa as no fluorescence recovery was observed (up to 15 min, data not shown) in post-bleaching. Consistent with previous studies (Stenoien et al. 2000, 2001a,b, Sharp et al. 2006, Zwart et al. 2010), our results also demonstrate that GFP-ERa in the unliganded state is a highly mobile molecule and shows different kinetics of mobility in response to ligands.

Similar to the unliganded GFP-ERa, GFP-ERZEBD in the absence of a ligand showed a rapid mobility with 1/2 mRR of <0.2 s (Fig. 3). Although the treatment of cells with 10^-9 M E2 or 10^-7 M 4HT for 1 h slowed the nuclear mobility of GFP-ERZEBD with a 1/2 mRR of about 1 s, the full fluorescence recovery occurred within 10 s of post-bleaching, much faster kinetics than that observed with E2 or 4HT-ligated ERa. These results indicate that the E2 or 4HT-mediated decrease in the nuclear mobility of ERa is primarily due to the interaction of the receptor with ERE. ICI, on the other hand, prevented the mobility of GFP-ERZEBD in the majority of cells (more than 80%). However, in the remaining cell population, GFP-ERZEBD in response to ICI showed mobility with varying 1/2 mRRs (Supplementary Figure 4, see section on supplementary data given at the end of this article). These results
Figure 2 The assessment of nuclear mobility of GFP-ERα by FRAP. HeLa cells transiently transfected with GFP-ERα for 24 h were treated without (NL) or with $10^{-8}$ M E$_2$, $10^{-7}$ M 4HT, or ICI for 1 h. Cells were then subjected to FRAP analysis. Images were obtained before bleaching (pre-bleach, PB), at bleaching for 0.2 seconds (bleach, B), and at the indicated times in seconds after bleaching. The overlay image (Overlay) was generated with the superimposition of images from DIC and GFP. The time-dependent equilibration of the bleached area (within the white circle) was used to estimate the recovery rate of ER in response to ligands. The recovery rate was based on a control ROI with the size and fluorescence intensity that corresponded to those of the ROI (bleached area) before photo-bleaching within the same cell to normalize the background and alterations in total cellular fluorescence after bleaching (Supplementary Figure 1). The control ROI values obtained throughout the post-bleach period were then used for data normalization. Fluorescence intensity is expressed as the relative fluorescence (RF) where zero (0) is the RF at the photo-bleaching (time 0) and one (1) is the fluorescence of the bleached area equilibrated to pre-bleach levels. Graph represents the normalized mean fluorescence recovery of GFP-ERα with or without a ligand in three independent experiments with a minimum of five individual cells per experiment. S.E.M., which was <15% of the mean, is not shown for simplicity.
suggest that the DBD of ERα contributes to but it is not sufficient for the ICI-mediated immobilization of ERα.

GFP-ERβ (Fig. 4A) and GFP-ERβ_{EBD} (Fig. 4B) displayed similar patterns of intra-nuclear distribution and kinetics of mobility. In cells synthesizing ERβ or ERβ_{EBD} in response to the vehicle control, the fluorescence recovery of the region after a 0.2-s photobleaching occurred with a $1/2$ mRR of about 1 s that reached pre-bleach fluorescence intensities within 40 s of post-bleaching. These results, as shown for ERβ (Damdimopoulos et al. 2008), indicate that the mobilities of the unliganded ERβ variants are kinetically slower than the corresponding ERα species. This was also the case for the E_2- or 4HT-ligated ERβ proteins. Treatment of cells with $10^{-9}$ M E_2 or $10^{-7}$ M 4HT decreased the mobility of the receptors that was reflected in $1/2$ mRRs of about 15 s with full recoveries occurring within 90 s after bleaching. Remarkably, both GFP-ERβ and GFP-ERβ_{EBD} in the presence of $10^{-7}$ M ICI displayed mobilities that were kinetically indistinguishable from those of the E_2- or 4HT-bound receptors, in contrast to ERα species that were stationary in the presence of ICI. Thus, the ability of ERβ to bind to ERE is uncoupled from the nuclear mobility of the receptor independent of the nature of ligand.

In addition to alterations in the stability, turnover, and intracellular location of ERα (Dauvois et al. 1992), ICI rapidly sequesters the receptor to a sub-compartment that also involves the nuclear matrix resistant to detergent and salt extractions (Stenoien et al. 2000, 2001a,b, Long & Nephew 2006, Lupien et al. 2007). This sequestration appears to be responsible for the immobilization (Stenoien et al. 2000, 2001a,b, Reid et al. 2003) and the absence of interaction with ERE (Reid et al. 2003) of the receptor. By contrast, ICI does not affect the turnover of ERβ (Peekhaus et al. 2004, Long et al. 2010).

Figure 3 The kinetics of nuclear mobility of GFP-ERα_{EBD}. Transiently transfected HeLa cells were treated without or with ligands for 1 h and subjected to FRAP analysis as described in the legend of Fig. 2. Graph represents the normalized mean fluorescence recovery of GFP-ERα_{EBD} in three independent experiments with a minimum of five individual cells per experiment. S.E.M., which was <15% of the mean, is not shown for simplicity.
Indeed, ERα (Fig. 5A) and ERβ (Fig. 5D) with or without GFP showed different intracellular levels in the presence of ICI. Transiently transfected HeLa cells synthesizing ERα or GFP-ERα for 24 h were treated with or without 10⁻⁹ M E₂, 10⁻⁷ M 4HT, or ICI for 1 h. Cells were then subjected to protein extractions using a buffer containing high salt (HSB) or detergent (RIPA, data not shown). Extracts (10 µg) were then subjected to WB. While E₂ or 4HT had minimal effect on ERα levels at 1 h posttreatment, ICI substantially reduced the receptor level in HSB extracts. This was inversely correlated with the detection of a higher receptor amount in ICI, but not E₂ or 4HT, treated cell extracts obtained with 1× LB to solubilize the insoluble aggregates following HSB extractions. By contrast, WB of TCLs generated with LB to extract both soluble and insoluble protein aggregates revealed that ligands had minimal effects on total receptor levels. This suggests that ICI-mediated rapid immobilization of ERα variants is primarily independent of the receptor degradation.

A rapid sequestration of ERα with or without GFP by ICI to a nuclear sub-compartment resistant to HSB extraction also predicts that ICI prevents the interaction of GFP-ERα with ERE, as shown previously for ERα (Reid et al. 2003). To address this point, we employed ChIP assay (Fig. 5B). The expression vector bearing none or an ER cDNA was co-transfected with the reporter TATA box promoter vector bearing one ERE into HeLa cells. Cells were treated with or without 10⁻⁹ E₂, 10⁻⁷ 4HT, or ICI for 1 h and processed for ChIP using a Flag antibody. Results revealed that the binding of apoERα to ERE is augmented when cells were treated with E₂ or 4HT. ICI effectively prevented ERα–ERE interaction, as there was no PCR product. In clear contrast, E₂ or ICI had minimal effects on the binding of ERβ to ERE (Fig. 5E). On the other hand, 4HT enhanced the binding of ERβ to ERE. As expected, ERαEBD or ERβEBD did not interact with ERE whether or not cells were treated with a ligand.

To correlate the intracellular mobility of GFP-ERs to ERE binding using ChIP with various antibodies directed to different structural domains of fusion receptor with or without Flag epitope proved to be difficult. To circumvent this problem, we used an in situ ERE binding competition assay (Huang et al. 2005b). This assay is based on the ability of ER to compete for
Figure 5 Effects of ligands on protein levels and ERE interactions of ER and EREBD with or without GFP. (A) Transiently transfected HeLa cells for 24 h were incubated with fresh media supplemented with or without 10⁻⁹ M E₂, 10⁻⁷ M 4HT, or ICI for 1 h. Cells were then collected, washed, re-suspended in 1 ml PBS, and divided into two equal portions. One portion of collected cells was pelleted and subjected to protein extraction using a HSB. The remaining pellet was subjected to 1 × LB to extract insoluble receptor aggregates. The other portion of the suspended cells was pelleted and the pellet was suspended with LB to extract both soluble and insoluble proteins for TCL. 10 μg total protein was subjected to 10–18% SDS-PAGE. Proteins with (G) or without GFP were probed with a receptor specific antibody. All experiments were replicated at least two independent times. (B) ChIP of transiently transfected HeLa cells. Cells co-transfected with expression vector expressing an ERα cDNA and the reporter vector bearing the TATA box promoter with single ERE for 24 h were treated without (NL) or with 10⁻⁹ M E₂, 10⁻⁷ 4HT, or ICI for 1 h. Cells were then subjected to ChIP using Flag-M2 antibody-conjugated agarose beads. A 366 bp PCR fragment indicates the ER–ERE interactions. Experiments were replicated at least three independent times. (C) The in situ ERE binding competition assay. HeLa cells were co-transfected with 125 ng the TATA box promoter with one ERE that drives the expression of the firefly luciferase cDNA as the reporter enzyme and 300 ng expression plasmid bearing the designer transcription factor, PPVV, without (0 ng ER) or with 75, 150, or 300 ng expression vector bearing an ER cDNA with or without GFP. Cells were then grown in the medium supplemented without (NL) or with 10⁻⁹ M E₂, 10⁻⁷ M 4HT, or ICI for 24 h. Normalized luciferase activity is presented as percent change compared with the control (PPVV, 0 ng ER) without ligand, which was set to 100. Graph represents the mean of three independent experiments performed in duplicate; S.E.M., which was < 15% of the mean, is not shown for simplicity. (D) Transfected HeLa cells with an expression vector bearing none (V) or an ERβ cDNA were treated without (NL) or with 10⁻⁹ M E₂, 10⁻⁷ M 4HT, or ICI for 1 h. Cells were collected, pelleted, and subjected to protein extraction using HSB. 10 μg total protein was subjected to 10–18% SDS-PAGE. Proteins were probed with a receptor-specific antibody. The image is from an experiment that was repeated at least three independent times. (E) ChIP assays for in situ interactions of ERβ and ERβEBD with ERE in HeLa cells were carried out using the M2-Flag antibody-conjugated agarose beads as described for ERα proteins. A representative image from an experiment repeated three independent times is shown. (F) Transient transfections of HeLa cells for the assessment of the binding of ERβ proteins with (G) or without ERE using the in situ ERE binding competition assay are accomplished as described for ERα. The mean of three independent experiments performed in duplicate without the S.E.M., which was < 15% of the mean, is shown.
ERE binding with a designer activator, PPVV, that constitutively and potently induces transcription from the TATA box promoter construct bearing single ERE (IEREGATA) at which ERs have minimal effect on transcription (Huang et al. 2005b). Thereby, interference of activator-mediated transcription by unliganded or liganded ERs is taken as an indication of ER–ERE interaction.

The reporter IEREGATA plasmid was co-transfected with an expression vector encoding the PPVV cDNA into HeLa cells in the absence (0) or presence of varying amounts (75, 150, and 300 ng/well) of an expression vector bearing an ER cDNA (Fig. 5c). Cells were then treated with or without 10–9 M E2, 10–7 M 4HT, or ICI for 24 h. As PPVV does not bind to a ligand and consequently ligands do not affect the transregulatory potential of PPVV (Huang et al. 2005b), the normalized luciferase activity mediated by PPVV alone in the absence of a ligand was set to 100%. Alterations in the reporter enzyme activity as a result of a co-transfected ER in the absence or presence of a ligand are depicted as percentage change compared with the activity induced by PPVV alone (0 ng ER). Similar to results obtained with ChIP assay, E2 or 4HT increased the ability of ERα and GFP-ERα to interact with ERE reflected in a further repression of the PPVV-induced luciferase activity by the unliganded ERα with or without GFP. The treatment of cells with ICI, on the other hand, had no effect on enzyme levels induced by PPVV. This suggests that ICI prevents the binding of ERα or GFP-ERα to ERE. The effect of ERα in the absence or presence of ligand on PPVV-mediated enzyme activity requires ERE interactions as ERαEBD or GFP-ERαEBD did not alter enzyme levels whether or not cells were exposed to a ligand. Thus, the decrease in the nuclear mobility of ERα mediated by E2 or 4HT is dependent upon the ability of the receptor to interact with ERE, whereas ICI sequesters the majority of the receptor to and immobilizes at a nuclear sub-compartment, thereby preventing ERα–ERE interactions.

In clear contrast to ERα, the short-term treatment (1 h) of cells with ICI as E2 or 4HT did not affect intracellular levels of ERβ or ERβEBD with or without GFP (Fig. 5d). The absence of an effect of ICI, as E2 or 4HT, on levels and mobilities of ERβ proteins also predicts that ICI does not alter ERβ–ERE interactions in situ. Indeed, ChIP (Fig. 5e) or the in situ ERE binding competition assay (Fig. 5f) revealed that E2 or ICI did not affect the binding of ERβ or GFP-ERβ to ERE, whereas 4HT increased ERβ–ERE interactions. As expected, the ERβEBD with or without GFP did not bind to ERE. These findings imply that the ligand-mediated nuclear mobility of ERβ is independent of nature of ligands and the ability of ERβ to bind to ERE.

Effects of ligands on transregulatory function and nuclear mobility of GFP-ERα in MDA-MB-231 cells

To examine whether or not the effects of ligands on the nuclear mobilities of ERs are cell type specific, we also used ER-negative MDA-MB-231 cells derived from a breast adenocarcinoma. Exogenously introduced ERz or ERβ in MDA-MB-231 cells modulates genomic and cellular responses in the presence of E2 (Garcia et al. 1992, Zajchowski et al. 1993, Lazennec et al. 2001, Li et al. 2008, Nott et al. 2009, Huang et al. 2011). In this cell line, 4HT acts as an ERs subtype-specific agonist by mimicking the effects of E2 on cellular responses when mediated by the ERE-dependent signaling pathway, whereas ICI is an antagonist for both ER subtypes (Bentrem et al. 2001, Tonetti et al. 2003). The EREBD do not interact with the ERE sequence of the estrogen-responsive TFF1 gene with or without ligands, while ligands differentially alter the parent ER–ERE interactions (Supplementary Figure 5A, see section on supplementary data given at the end of this article). Moreover, providing evidence for a functional ERE-independent signaling pathway, we recently showed that the DNA binding-defective ERs participate in the fine-tuning of phenotypic features of MDA-MB-231 cells by regulating the expression of a subset of estrogen-responsive genes (Li et al. 2008, Nott et al. 2009).

Transient transfections of MDA-MB-231 cells with heterologous reporter vectors emulating ERE-dependent and ERE-independent signaling pathways revealed that the GFP fusion-ERs mimic the abilities of the parent receptors to regulate transcription in response to ligands (Supplementary Figures 5 and 6, see section on supplementary data given at the end of this article).

The nuclear motilities of GFP-ERα with or without ligands in MDA-MB-231 cells (Fig. 6) showed patterns indistinguishable from those observed in HeLa cells. However, the rate and the time of the total recovery of ERα variants in response to E2 or 4HT were about twofold slower than those of the receptor synthesized in HeLa cells. The 1/3mRR of the unliganded GFP-ERα or GFP-ERαEBD was <0.2 s with a total recovery within 5 s after photo-bleaching. The treatment of cells with 10–9 M E2 or 10–7 M 4HT in cells synthesizing GFP-ERα increased the 1/3mRR of the bleached region to about 11 and 9 s respectively with a full fluorescence recovery occurring within 60 s of post-bleaching. ICI at 10–7 M effectively halted the fluorescence recovery of GFP-ERα. As observed in HeLa cells, ICI also prevented the recovery of the bleached region in the majority of cells (more than 80%) synthesizing GFP-ERαEBD, while the rate of fluorescence recovery vastly varied in individual cells in the remaining population (data not shown). These findings support our conclusion that the DBD contributes to ICI-mediated immobilization of ERα. On the other hand, the unliganded GFP-ERαEBD...
showed a very rapid recovery with a $\frac{1}{2}$ mRR of $0.2$ s with a full recovery occurring within 1 s. This was similar to the rate of recovery of GFP-ER$_{EBD}$ in cells exposed to E$_2$ or 4HT with the fluorescence equilibration occurring within 10 s of post-bleaching. Thus, the E$_2$- or 4HT-mediated decrease in the nuclear mobility of ERx is dependent upon the ability of the receptor to interact with ERE and is independent of cell type.

The fluorescence recovery of GFP-ER$_{β}$ in the absence or presence of a ligand was kinetically similar to that of GFP-ER$_{EBD}$ in MDA-MB-231 cells and mirrored those observed in HeLa cells wherein the overall rate of recovery was faster for both receptor species. In the absence of ligand, the bleached region synthesizing ER$_β$ or ER$_{βEBD}$ recovered within 40 s of post-bleaching with a $\frac{1}{2}$ mRR of about 1 s. Treatment of cells with E$_2$, 4HT, or ICI slowed the rate of fluorescence recovery to about 25 s with a full recovery within 120 s post-bleaching. Thus, the ability of ER$_β$ to bind to ERE is not reflected in the nuclear mobility of the receptor, which is also independent of the nature of ligand and cell context.

**Structural domains responsible for ER subtype-specific nuclear mobility**

The amino- and carboxyl-termini of ERs functionally differ (Cowley & Parker 1999, Hall & McDonnell 1999, Yi et al. 2002a, Huang et al. 2005b). To examine the roles of structural termini on the nuclear mobility of ERs, we used GFP fusion ER chimera proteins. In ERx$_β_C$ and ERx$_{N\beta_C}$, the entire amino-termini of the receptors are genetically interchanged (Yi et al. 2002a). We found in transiently transfected HeLa cells that the fluorescence recoveries of ERx$_β_C$ with or without a functional ERE binding (data not shown) were kinetically similar to those observed with ERx$_β$s in the absence or presence of ligands (Fig. 7). Conversely, ERx$_{N\beta_C}$ or the ERE binding-defective ERx$_{N\beta_C}$ mimicked the nuclear mobilities of ERx$_α$s with or without a ligand (data not shown). These results indicate that the carboxyl-termini are the structural basis for the difference in the nuclear mobility of ER subtypes in the absence or presence of a ligand.

Distinct conformational features of ER carboxyl-termini induced by a ligand determine the formation of a functional co-regulator interacting surface responsible for AF2 of the receptors. We also addressed whether the changing of the critical residues that prevent AF2 affects the subtype-specific nuclear mobility of ERs in response to ligands. To examine this issue, we used the GFP-fusion ERs with abrogated AF2 (ERx$_{AF2}$). In transiently transfected HeLa cells, the nuclear mobility of the unliganded, E$_2$- or 4HT-bound GFP-ER$_{αAF2}$ showed kinetics of mobility (Fig. 8A) similar to that of ERx$_{β}$ (Fig. 2). GFP-ERx$_{AF2}$ was also mobile when ICI was present. This suggests that the absence of AF2 renders ERx$_β$ mobile when ICI is present. ICI, as E$_2$ or 4HT, also had minimal effects on the FRAP of the ERE binding-defective receptor with
abrogated AF2, GFP-ERβ_{EBD+AF2} (Fig. 8B). ERβ_{AF2} or ERβ_{EBD+AF2} without or with GFP was HSB extractable (Fig. 8C). Moreover, ICI-ERβ_{AF2} gained the ability to interact with ERE in situ (Fig. 8D) in stark contrast to ICI-ERz, which was immobile due to the sequestration to a nuclear sub-compartment resistant to HSB extraction. However, ERz_{AF2} with or without GFP was transcriptionally inactive when cells were treated with ICI, E2, or 4HT (Fig. 8E). Thus, it appears that the ligand-mediated nuclear mobility and the ability to interact with and to induce transcription from target sites of ERz are discernible. A much faster kinetics of mobility of ERz_{EBD} compared with that of ERz in response to E2 or 4HT also indicate that the mobility of ERz at the ERE-independent signaling pathway contributes fractionally to the overall nuclear mobility of the receptor.

The prevention of AF2 did not alter the pattern or the kinetic of mobility of the GFP-ERβ_{AF2} mutant compared with that of the GFP-ERβ in the absence or presence of ligands (Fig. 9A). The pattern of fluorescence recovery of GFP-ERβ_{EBD+AF2} was also similar to that of GFP-ERβ_{AF2} (Fig. 9B). Interestingly, however, the nuclear movement of GFP-ERβ_{EBD+AF2} occurred at slower kinetics than GFP-ERβ_{EBD} or GFP-ERβ_{AF2} in the absence or presence of ligands. The fluorescence level of the bleached region in cells synthesizing ERβ_{EBD+AF2} in the absence of ligand reached pre-bleach levels with a ½mRR of about 6 s, whereas the fluorescence recovery in cells synthesizing GFP-ERβ_{EBD} or GFP-ERβ_{AF2} was about 1 s. The treatment of cells with E2, 4HT, or ICI decreased the mobility of ERβ_{EBD+AF2} similarly, reflected in a ½mRR of about 20 s in comparison with liganded GFP-ERβ_{EBD} that showed recovery rates of about 15 s. This suggests that integrated effects of the DBD and the LBD of ERβ are important for the mobility characteristics of ERβ.

Ligands did not affect the intracellular levels of the receptor species (Fig. 9C). The treatment of cells with or without a ligand did not alter the ability of ERβ_{AF2} or GFP-ERβ_{AF2} to interact with ERE in situ (Fig. 9D), despite the fact that the receptors were transcriptionally silent at simulated ERE-dependent and ERE-independent signaling pathways (Fig. 9E). Showing similar intracellular levels in the absence or presence of a ligand (Fig. 9C), ERβ_{EBD+AF2} with or without GFP did not bind to ERE (Fig. 9D) nor did it modulate the reporter enzyme levels whether or not cells were treated with a ligand (Fig. 9E).

Thus, the nuclear mobility of ERβ is independent from the nature of ligand and from the ability of the receptor to interact with target sites. These results imply that ERβ mediates gene transcription through the ERE-dependent and ERE-independent signaling pathways with similar kinetics.

**Discussion**

ERs are highly mobile proteins partitioned dynamically between the nucleoplasm and target sites on the chromatin that constitute the ERE-dependent and ERE-independent signaling pathways. We here assessed the relative contribution of ER mobility at the ERE-independent signaling pathway to the overall mobility of receptors to gain insights into mechanisms of action.

Our observations revealed several distinct features of ERβ mobility compared with ERz. These are as follows: i) ERβ mobility with or without ligands is slower than ERz mobility. ii) The interaction of ERβ with ERE is augmented with 4HT but not with E2 or ICI, whereas E2 and 4HT enhance and ICI prevents ERz-ERE interactions. iii) ICI does not sequester ERβ with or without ERE binding and/or ICI functions to a nuclear sub-compartment, whereas the sequestration of ERz is dependent on AF2. iv) The ability of ERβ to interact with and to induce transcription from target sites is largely uncoupled from the receptor mobility. v) Cooperation between DBD and LBD contributes to ERβ motility. Based on these observations, we conclude that while ICI immobilizes ERz to a sub-nuclear
compartment, E₂ or 4HT decreases ERβ mobility by increasing ERβ–ERE interactions. We therefore suggest that ERβ in response to E₂ and 4HT mediates transcriptions from the ERE-independent pathway with remarkably fast kinetics that contributes fractionally to the overall motility of the receptor. On the other hand, the ligand-mediated mobility of ERβ is independent of the nature of ligands or the mode of interaction with target sites. It therefore appears that although ERs interact with target sites with fast kinetics, they use distinct mechanisms to regulate transcriptions at signaling pathways.

We show here, as previous studies (Sharp et al. 2006, Zwart et al. 2010), that 4HT, as E₂, decreases the mobility of ERα by enhancing ERα–ERE interactions (Shang et al. 2000, Huang et al. 2005b). However, the underlying mechanism(s) remains unclear. The binding of tamoxifen to ERα alters conformation (Paige et al. 1999) that affects co-activator recruitment (Yi et al. 2002b). Tamoxifen-ERα can also recruit co-repressors for transcription repression (Lavinsky et al. 1998, Delage-Mourroux et al. 2000, Shang et al. 2000). While 4HT is an antagonist for ERα in HeLa cells, it acts as an agonist in MDA-MB-231 cells (Bentrem et al. 2001, Tonetti et al. 2003). 4HT was also augmented ERα–ERE interactions. 4HT-ERα showed mobility similar to E₂–ERα. On the other hand, ERαEBD with or without AF2 was kinetically much faster when bound to 4HT or...
E\textsubscript{2} compared with ER\textsubscript{a}. Our findings therefore imply that E\textsubscript{2} and 4HT-mediated decreases in ER\textsubscript{a} mobility are due to the residency time of the receptor on ERE independent of transcription. By contrast, ICI immobilized ER\textsubscript{a}. ICI binding prevents ER-co-regulator interactions (Yi et al. 2002\textsubscript{b}) but drives ER\textsubscript{a} to interact with cytokeratins through LBD (Long & Nephew 2006). Leading to the association of ICI-ER\textsubscript{a} with nuclear matrix (Long & Nephew 2006, Lupien et al. 2007), this could result in the immobilization and complete prevention of ERE interactions, as shown here and previously (Reid et al. 2003). However, it was also shown that a fraction of ICI-ER\textsubscript{a} remains associated with the prolactin promoter array, which is composed of 52 prolactin gene promoters containing multiple EREs (Sharp et al. 2006). It is possible that while the majority of ER\textsubscript{a} bound to ICI is immobilized to a sub-nuclear region, a fraction of ER\textsubscript{a} bound to EREs of the promoter array cooperatively and hence stably (Yi et al. 2002\textsubscript{b}) cannot be readily sequestered away from the array in contrast to the single ERE of the reporter system and the endogenous gene we used here. Another puzzling observation is that while ICI immobilized ER\textsubscript{a}, ICI-ER\textsubscript{a} still modulated the reporter gene transcription from ERE-independent pathways. Immobilization of ER\textsubscript{a} by ICI could prevent the interaction of ER with co-regulators/transfactors thereby countering the ER\textsubscript{a}-mediated repressed or activated state of transcriptions.

The changing (Fig. 8) or deletion (Sharp et al. 2006) of critical residues to block AF2 rendered ICI-ER\textsubscript{a} mobile in cells. This was reflected in the increased extractability of ER\textsubscript{a} with HSB or detergent likely due to the inability of the receptor to interact with cytokeratins (Long & Nephew 2006). Nevertheless, ICI-ER\textsubscript{a,AF2} was transcriptionally silent despite the fact that the receptor interacted with ERE. Moreover, the increased mobility of ER\textsubscript{a,EBD+AF2} regardless of the nature of ligand strengthens the conclusion that the duration of ERE occupancy reflects the

Figure 9 The mobilities of GFP-ER\textsubscript{a,AF2} and GFP-ER\textsubscript{a,EBD+AF2} in HeLa cells. The transfection and processing of cells synthesizing (A) GFP-ER\textsubscript{a,AF2} or (B) GFP-ER\textsubscript{a,EBD+AF2} for FRAP were carried out as described in the legend of Fig. 2. (C) Intracellular level of receptor proteins were analyzed with high salt extracts of transfected HeLa cells, which were treated and processed as described in the legend of Fig. 8C. The effects of ligands on in situ ERE binding abilities of ER\textsubscript{a,AF2} and ER\textsubscript{a,EBD+AF2} with (G) or without GFP were assessed as described in the legend of Fig. 8D. The s.e.m., which was <15% of the mean, is not shown for simplicity. (E) The effects of ligands on transcription by ER\textsubscript{a,AF2} and ER\textsubscript{a,EBD+AF2} with (G) or without GFP were assessed as described in the legend of Fig. 8E. Graphs show the mean of three independent experiments performed in duplicate. The s.e.m., which was <15% of the mean, is not shown.
ligand-mediated mobility of ERα independent of transcription status. This lends further credence to the hit-and-run model of transcription for ERα regardless of signaling pathway.

In contrast to ERα, ERβ and ERβ<sup>EBD</sup> showed indistinguishable mobility rates independent of the nature of ligands and the ability of the receptor to interact with target sites and cellular context. Consequently, it appears that ERβ mediates gene transcriptions through the ERE-dependent and ERE-independent signaling pathways with similar kinetics. ERs share structural features reflected in similar functional properties. ERs nevertheless exhibit distinct trans-regulatory potentials at signaling pathways. The amino-termini are critical regions that contribute to subtype-specific transcriptional responses. In contrast to ERα, the ERβ amino-terminus impairs ER–ERE interactions (Huang et al. 2005b), lacks an AF (Cowley & Parker 1999, Yi et al. 2002a), and does not interact with the carboxyl-terminus (Yi et al. 2002a). However, the amino-termini do not appear to contribute to distinct receptor mobility. We observed that the mobilities of the ERZβ<sub>SC</sub> chimeras were kinetically similar to those observed with ERβ variants. ERβ<sub>SC</sub>, on the other hand, mimicked ERα mobility in response to ligands. These imply that the carboxyl-termini are critical regions in defining mobility differences of ERs. Studies also showed that the carboxyl-termini contribute to transcriptional potencies of ERs (Yi et al. 1998, Kressler et al. 2002). Moreover, some co-regulator interactions with ERs are specific to the nature of the ligand. The unliganded ERα interacts with co-repressors SMRT/NCoR (Lavinsky et al. 1998, Webb et al. 2003). The binding of E<sub>2</sub> releases co-repressors from ERα (Lavinsky et al. 1998, Webb et al. 2003). The unliganded ERβ also interacts with SMRT/NCoR through the carboxyl-terminus (Webb et al. 2003). However, the binding of E<sub>2</sub> does not promote co-repressor dissociations (Webb et al. 2003). By contrast, the binding of 4HT or ICI releases SMRT/NCoR from ERβ but not from ERα (Lavinsky et al. 1998, Webb et al. 2003). As ERβ requires E<sub>2</sub> to regulate transcription from the ERE-dependent signaling pathway, E<sub>2</sub> binding could act as a switch to convert ERβ to an active state by concurrently recruiting co-activators likely through a distinct surface.

We also found that 4HT enhanced the ERβ–ERE interaction in contrast to E<sub>2</sub> or ICI. Although is unclear, distinct trans-conformational changes in ERβ-DBD mediated by the binding of 4HT to LBD could underlie the effect of 4HT on ERβ–ERE interactions. We observed that 4HT- or ICI-bound ERβ, as ERβ<sub>AF2</sub>, showed mobility similar to E<sub>2</sub>–ERβ despite the fact that the receptor was transcriptionally inactive at the ERE-dependent pathway. Furthermore, ERβ<sub>EBD</sub> mobility was indistinguishable from that of ERβ independently of ligands. However, ERβ<sub>EBD</sub> showed slower mobility than ERβ<sub>EBD</sub>. This suggests that the cooperation of AF2 with the ability of the receptor to interact with target sites is a critical feature for the nuclear mobility of ERβ.

In summary, our results indicate that while ERs use a hit-and-run mode of action, they differ mechanistically to modulate transcriptions. The use of integrated promoter arrays mimicking various signaling pathways would yield further insights into mechanisms of ER actions. This in turn could aid in the development of better strategies to combat estrogen target tissue malignancies.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1550/JME-12-0097.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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**References**


Dauvois S, Danielian PS, White R & Parker MG 1992 Antiestrogen ICI 164,384 reduces cellular estrogen receptor content by increasing its turnover. PNAS 89 4037–4041. (doi:10.1073/pnas.89.9.4037)


Garcia M, Deroo C, Freiss G & Rochefort H 1992 Activation of estrogen receptor transfected into a receptor-negative breast cancer cell line decreases the metastatic and invasive potential of the cells. PNAS 89 11538–11542. (doi:10.1073/pnas.89.23.11538)


Huang J, Li X & Muyan M 2011 Estrogen receptors similarly mediate the effects of 17β-estradiol on cellular responses but differ in their conformational changes in ERα and ERβ. Journal of Biological Chemistry 286 3999–4004. (doi:10.1074/jbc.M001327290)


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