The DNA Binding Domain of Estrogen Receptor α Is Required for High-Affinity Nuclear Interaction Induced by Estradiol†

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ABSTRACT: Estrogen receptor α (ER) is a member of the nuclear hormone receptor family, which upon binding estrogen shows increased apparent affinity for nuclear components (tight nuclear binding). The nuclear components that mediate this tight nuclear binding have been proposed to include both ER−DNA interactions and ER−protein interactions. In this paper, we demonstrate that tight nuclear binding of ER upon estrogen occupation requires ER−DNA interactions. Hormone-bound ER can be extracted from the nucleus in low-salt buffer using various polyanions, which mimic the phosphate backbone of DNA. The importance of specific ER−DNA interactions in mediating tight nuclear binding is also supported by the 380-fold lower concentration of the ERE oligonucleotide necessary to extract estrogen-occupied ER from the nucleus compared to the polyanions. We also demonstrate that estrogen-induced tight nuclear binding requires both the nuclear localization domain and the DNA binding domain of ER. Finally, enzymatic degradation of nuclear DNA allows us to recover 45% of tight nuclear-bound ER. We further demonstrate that ER−AIB1 interaction is not required for estrogen-induced tight nuclear binding. Taken together, we propose a model in which tight nuclear binding of the estrogen-occupied ER is predominantly mediated by ER−DNA interactions. The effects of estrogen binding on altering DNA binding in whole cells are proposed to occur through estrogen-induced changes in ER−chaperone protein interactions, which alter the DNA accessibility of ER but do not directly change the affinity of the ER for DNA, which is similar for both unoccupied and occupied ER.

Estrogen plays a major role in reproduction and disease processes such as breast cancer, and therefore, ER[1] is a major molecular target for therapeutics. Identification of additional molecular targets can be facilitated by an improved understanding of the biochemical mechanism of ER activation and function. Estrogen signaling is a complex and ordered process in which ligand binding activates the ER to affect transcription (*1*, *2*). One of the most rapid events upon ER occupation with estrogen in the whole cell is a change in the association of ER with the nucleus (*3*−*5*). ER is a nuclear protein in the absence or presence of hormone, although some studies have also shown a subset of ER to be localized in or near the plasma membrane (*6*−*9*). However, hormone treatment of cells changes the biochemical properties of ER as measured by changes in receptor solubility from lysed cells. Early biochemical studies of ER demonstrated that unoccupied ER was present in the postribosomal or cytosolic supernatant after hypotonic cellular lysis, whereas hormone treatment shifted ER to the insoluble pellet (*10*, *11*). This shift is termed ER tight nuclear binding. ER can be solubilized from the pellet with high salt (e.g., 0.6 M NaCl) or a combination of salt and detergent. Since ER is predominantly nuclear in the presence or absence of hormone, tight nuclear binding reflects a change in the apparent affinity of ER for all nuclear components as opposed to a change in cellular localization. The nuclear components with which ER interacts to cause tight nuclear binding may include DNA, proteins, or both. Although this large increase in the apparent affinity of ER for the nucleus is a very early event in estrogen signaling that affects the whole population of ER, its mechanistic basis remains unknown.

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[1] Abbreviations: ER, estrogen receptor α; LBD, ligand binding domain; BRG-1, brahma-related protein 1; Brm, brahma; CBP, CREB-binding protein; SRC, steroid receptor coactivator; ERE, estrogen response element; mutERE, mutant estrogen response element; DBD, DNA binding domain; FRAP, fluorescence recovery after photobleaching; E_2, 17β-estradiol; pcv, packed cell volume; LS, low-salt; HS, high-salt; RARα, retinoic acid receptor α; CREB-1, cAMP responsive element binding protein 1; Sp1, specificity factor 1; AIB1, amplified in breast cancer 1; PP, polyphosphate; DV, decavanadate; GR, glucocorticoid receptor; NLS, nuclear localization sequence; WT, wild-type; ED_{50}, 50% effective dose; EtOH, ethanol; hsp, heat shock protein; Hop, hsp organizing protein.

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ER has been shown to interact with a large number of nuclear components, and some of these associations probably play a role in tight nuclear binding induced by hormone (*12*). Binding of estrogen to ER causes a conformational change in the ligand binding domain (LBD) that results in changes in protein partners that are bound. Hormone occupation of ER promotes a decreased level of interaction with some chaperone proteins, while inducing recruitment of coactivators, subunits of chromatin remodeling complexes (BRG-1, Brm), and mediator complexes (TRAP220/DRIP205) (*13*−*15*). The chaperone complex associated with unoccupied nuclear receptors has been characterized well (*16*). Some coactivator proteins have intrinsic histone acetyltransferase activity, and in combination with chromatin remodeling complexes, they are thought to prime the chromatin for activation of transcription (*15*, *17*). The mediator complex is responsible for recruiting the basal transcriptional machinery to the promoter (*17*). Important coactivators for ER transcriptional activation include the CREB-binding protein (CBP) and the family of p160 proteins: SRC-1, SRC-2, and AIB1/SRC-3 (*15*, *17*). AIB1 is overexpressed in both breast cancer and the MCF-7 breast cancer cell line and plays an important role in ER signaling in breast cancer (*18*, *19*). Studies using chromatin immunoprecipitation have allowed for the construction of a model of estrogen activation of transcription, where ER recruits coactivator proteins, chromatin remodeling factors, mediator complexes, and basal transcriptional machinery in a coordinated temporal manner (*20*−*22*). DNA binding of ER to specific sequences termed estrogen response elements (EREs) is a major feature for estrogen activation of transcription at many genes. ER can bind DNA directly via a highly conserved DNA binding domain (DBD) that contains two zinc finger-like motifs each consisting of four cysteines (*23*). The DBD of ER binds with high affinity to a consensus element that consists of an inverted palindrome with the sequence 5′-GGTCAnnnT-GACC-3′. ER can also bind with high affinity to variations of this consensus sequence. Significantly, ER demonstrates strong binding to DNA in a non-sequence-specific manner, albeit with an affinity 100−1000-fold lower than that of an ERE (*24*). In contrast to ER−protein interactions, hormone has little effect on binding of ER to DNA in vitro (*25*−*29*).

Hormone dramatically alters the distribution of ER within the nucleus investigated using live cell imaging (*30*−*32*). The unoccupied receptor initially displays a diffuse nuclear distribution pattern, and estrogen binding causes the receptor to localize to punctate foci within 30 min of treatment. The dynamic, mobile nature of ER in the nucleus is demonstrated by FRAP analysis in which the rate of recovery of unliganded ER is less than 1 s (*33*, *34*). Addition of estradiol slows the recovery time, but only to 5−6 s, suggesting that liganded ER is also extremely mobile. Interaction of ER with chromatin sites is also a very dynamic process as seen in experiments of GFP-ER visualized using live cell imaging. ER rapidly exchanges with chromatin sites in both the unoccupied and occupied states, though the residence time is estrogen-dependent (*34*, *35*). Estradiol treatment results in a longer residence time compared to that with either the absence of ligand or the addition of the antagonist tamoxifen. This rapid mobility of occupied ER in vivo contrasts with its lack of solubility, or tight nuclear binding, observed upon cell lysis.

In this study, we identify the molecular basis for estrogen-induced tight nuclear binding of ER. In previous work, we showed that relatively low concentrations of the polyanion decavanadate could solubilize the occupied ER from nuclei (*36*). Here we combine the use of polyanions and ER mutant proteins to demonstrate the requirement for the DBD of ER in estrogen-induced tight nuclear binding. In addition, we show that binding of AIB1 to ER is not necessary for tight nuclear binding. We propose a model for reconciling the requirement of the DBD for estrogen-induced tight nuclear binding despite the relatively modest effects of hormone on binding of ER to DNA.

EXPERIMENTAL PROCEDURES

Cell Culture. MCF-7 and HeLa cells were obtained from ATCC. The HEK293 cell lines stably expressing WT ER, ΔC-ER, ΔNLS-ER, or C202/205H-ER were generated as previously described (*37*, *38*). Cells were routinely cultured in phenol red-free DMEM and supplemented with 10% fetal bovine serum, 1 mM pyruvate, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10 μg/mL insulin. HEK293 ER cell lines were maintained by periodic hygromycin selection (200 μg/mL, Invitrogen). To prepare cell extracts, cells were grown to 70% confluence followed by culturing in phenol red-free DMEM supplemented with 5% dextran-coated charcoal-stripped fetal bovine serum, 1 mM pyruvate, 2 mM L-glutamine, and 0.1 mM nonessential amino acids for approximately 20−24 h prior to ligand binding. C202/205H ER HEK293 cells were treated with 10 μM MG132 for 30 min prior to all experiments to limit ER degradation.

Cell Extract Preparation. MCF-7 or HEK293 ER cells were treated with 10 nM 17β-estradiol (E_2) for 30 min, or HeLa cells were treated with 1 μM dexamethasone. The following steps were performed at 4 °C unless otherwise noted. Medium was removed, and calcium- and magnesium-free phosphate-buffered saline was added. Cells were lifted either by scraping (MCF-7) or by pipetting (HEK293 ER, HeLa). Cells were pelleted by centrifugation at 150*g* for 5 min and resuspended in 4 packed cell volumes (pcv) of low-salt buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 0.5 mM AEBSF, 1 μM leupeptin, 2 μg/mL aprotinin, and 50 mM NaF. Increasing concentrations of either polyphosphate (chain length of 25, Sigma), decavanadate [chain length of ≅10, Calbiochem, prepared as previously described (*36*)], double-stranded ERE oligonucleotide (chain length of 21), double-stranded mutant ERE oligonucleotide (chain length of 21), or Benzonase (Calbiochem) were added to the low-salt buffer as indicated in figures. The forward oligonucleotide for ERE was 5′ATGAGGTCACAGTGACCTAGC3′, and the forward oligonucleotide for mutERE was 5′ATGAGATCACAGT-GATCTAGC3′. Double-stranded ERE and mutERE were prepared as described previously (*39*). Cells were lysed by either vortexing (HEK293) or dounce homogenization using a B pestle (MCF-7 and HeLa) in low-salt buffer. The low-salt fraction was prepared by collecting the supernatant following centrifugation at 436000*g* (Beckman TLA120 ultracentrifuge) for 10 min at 4 °C. The high-salt extract was prepared by resuspending the remaining pellet in 4 pcv of a high-salt extraction buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 1.5 mM $MgCl_2$, 0.5 mM AEBSF, 1 μM leupeptin, 2 μg/mL aprotinin, 50 mM

NaF, and 0.6 M NaCl for 30 min. The high-salt supernatant was collected by centrifugation at 436000*g* for 10 min at 4 °C. Extracts were flash-frozen on dry ice and stored at −80 °C.

Western Blot Analysis. Equal volumes of low-salt extract (LS) and high-salt extract (HS) were prepared in Laemelli sample buffer and separated by SDS−PAGE. Protein was transferred to nitrocellulose paper, and Western blot analysis was performed. Primary antibodies used for each protein were as follows: 1:1000 ER (HC-20, Santa Cruz), 1:1000 retinoic acid receptor α (RARα) (C-20, Santa Cruz), 1:2000 CREB binding protein (CBP) (C-20, Santa Cruz), 1:500 cAMP responsive binding protein 1 (CREB-1) (240, Santa Cruz), 1:500 brahma related gene 1 (BRG-1) (H-88, Santa Cruz), 1:2000 specificity protein 1 (Sp1) (PEP-2, Santa Cruz), 1:1000 amplified in breast cancer 1 (AIB1; steroid receptor coactivator-3) (BD Biosciences Labs), and 1:2000 glucocorticoid receptor (GR) (generously provided by J. Harmon, Uniformed Services University). Blots were washed in 1× TBS-T [25 mM Tris-HCl (pH 7.4), 135 mM NaCl, 3 mM KCl, and 0.1% Tween 20] followed by incubation with either anti-rabbit IgG or anti-mouse IgG horseradish peroxidase-linked secondary antibodies (Amersham), and proteins were detected by ECL reagents (Amersham). Blot images were scanned, and densitometry analysis using NIH image was used to measure protein levels in the LS and HS samples. The total density units from LS and HS fractions for each polyanion or Benzonase concentration were totaled and set to 100%. The percentage of the assayed protein recovered in the LS and HS fraction for each polyanion concentration was then calculated and graphed. The %ER recovered in the LS fraction as a function of the log-[polyanion] was subjected to a fit to a sigmoidal dose−response curve utilizing the Prism 4 software package from GraphPad Software, Inc. An EC_{50} with the standard error was calculated for data sets that could be fit to a sigmoidal curve, and the values are reported in Results.

Immunofluorescence. Cells were grown on glass coverslips (Fisher) and fixed in 4% paraformaldehyde (Sigma) for 20 min. Cells were rinsed twice with wash buffer (1× PBS and 0.1% Triton X-100), and blocking buffer (1× PBS, 0.1% Triton X-100, and 3% goat serum) was added for 30 min. Cells were rinsed with wash buffer twice for 2 min followed by a single wash in 1× PBS. Primary ER antibody (HC-20) was diluted 1:500 in wash buffer and incubated overnight at 4 °C. Cells were rinsed three times at room temperature in wash buffer for 2 min each. The secondary antibody AlexaFlour 488 chicken anti-rabbit IgG (Molecular Probes) was diluted 1:500 in wash buffer and added to the cells for 2 h at room temperature. Cells were rinsed once with 1× PBS, and a 1:50000 dilution of DAPI (Sigma) counterstain in 1× PBS was added for 2 min at room temperature. Cells were rinsed two additional times for 2 min each with wash buffer. Coverslips were mounted on glass slides using Fluoromount-G (Southern Biotech) and visualized using an Olympus fluorescent microscope at 400× magnification, captured by digital photography, and processed using Adobe Photoshop.

RESULTS

Solubility Properties of ER from Hormone-Treated Cells. Figure 1A shows the increased apparent affinity of wild-type ER for nuclear components induced by estradiol in MCF-7 cells. We used a two-step, serial extraction protocol on the cells to solubilize ER. Cells were lysed in a low-salt buffer without NaCl or detergent, and the supernatant after centrifugation was the low-salt (LS) fraction. The pellets containing the nuclei were resuspended in the same buffer with the addition of 0.6 M NaCl, and the supernatant recovered following centrifugation was the high-salt (HS) fraction. Western blot analysis was performed to compare the ER recovery in LS and HS fractions (Figure 1A). Treatment of the cells with estradiol resulted in a shift of ER to the HS fraction indicative of an increased apparent affinity of ER for nuclei. Immunoblotting for actin was used as a loading control. The change in the apparent affinity of estradiol-occupied ER for the nucleus is termed tight nuclear binding. Approximately half of the ER in the EtOH control

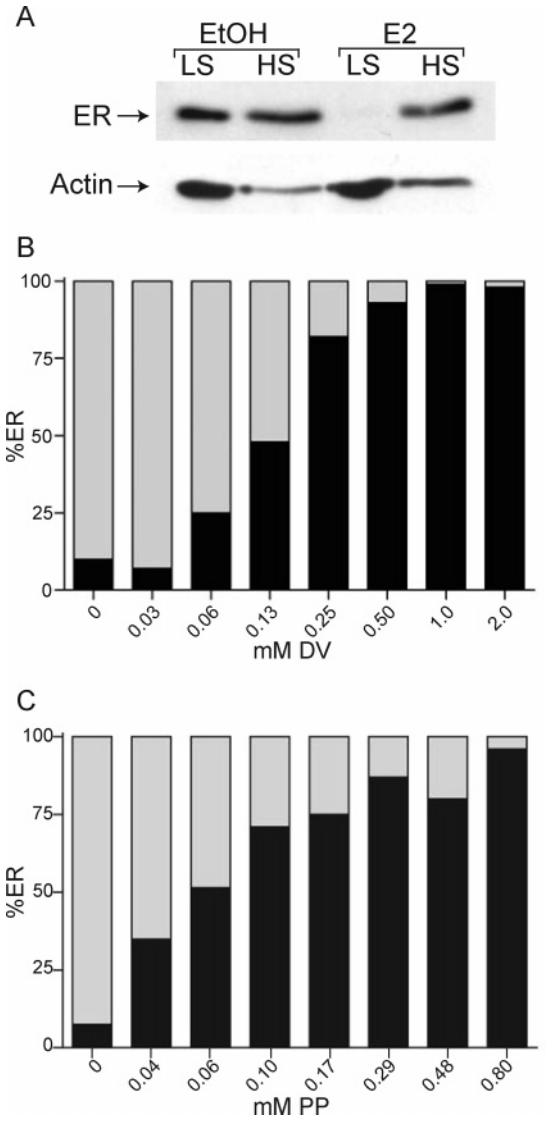

FIGURE 1: Solubilization of ER by polyanions. (A) Western blot for ER and actin in low salt followed by high-salt fractions separated by SDS−PAGE from cells treated for 30 min with 0.1% EtOH or 10 nM E_2. Graphical representation of %ER in LS (black bars) fractions prepared as described for panel A from E_2-treated MCF-7 cells but with increasing concentrations of (B) decavanadate or (C) polyphosphate added to the low-salt buffer. Sequential HS fractions (gray bars) were prepared as described for panel A for each decavanadate or polyphosphate concentration. ER was detected by Western blotting and quantitated as described in Experimental Procedures.

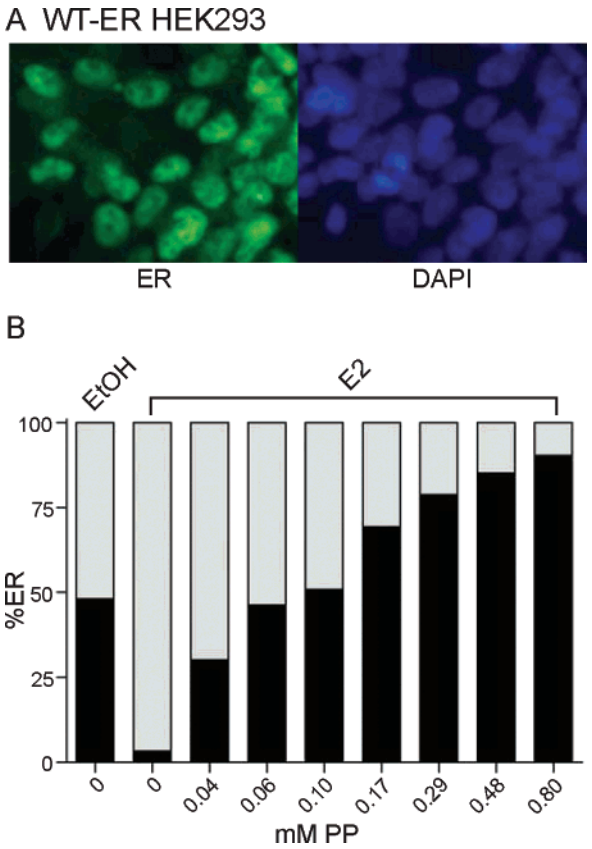

FIGURE 2: Solubilization of WT ER from WT ER HEK293 cells by polyphosphate. (A) Immunofluorescence detection of ER in E_2-treated WT ER HEK293 cells (left). DAPI staining of cells (right). (B) Graphical representation of %ER in LS (black bars) fractions with increasing concentrations of polyphosphate and their sequential HS (gray bars) fractions from EtOH- or E_2-treated WT ER HEK293 cells.

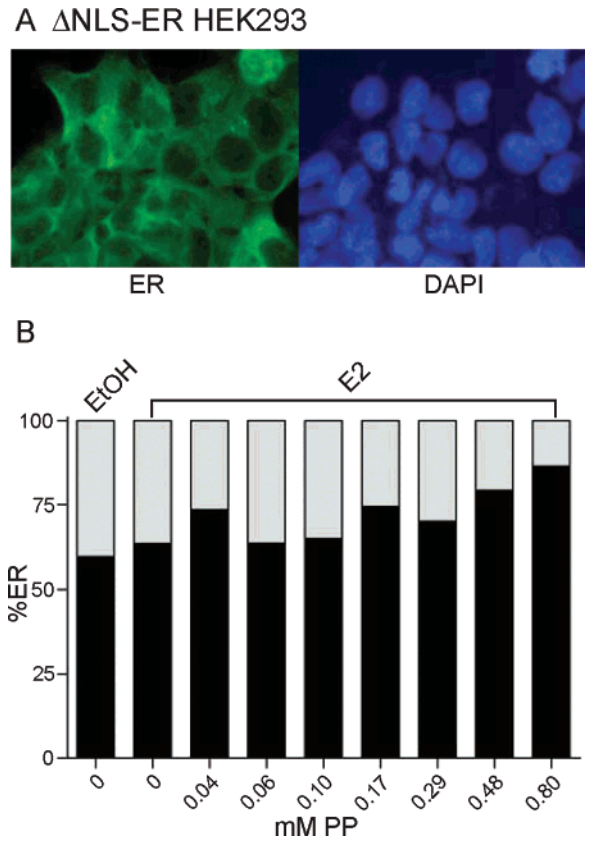

FIGURE 3: Nuclear localization sequence required for tight association of ER with nuclear components. (A) Immunofluorescence detection of ER in E_2-treated ΔNLS-ER HEK293 cells (left). DAPI staining of cells (right). (B) Graphical representation of %ER in LS (black bars) fractions with increasing concentrations of polyphosphate and their sequential HS (gray bars) fractions from EtOH- or E_2-treated ΔNLS-ER HEK293 cells.

was observed in the HS fraction probably due to the limited estrogen withdrawal (20−24 h) of cells in this and subsequent experiments. We routinely extracted some residual ER from the insoluble pellet remaining after the high-salt extraction using SDS. However, we did not pursue this population of ER in this study. We used this assay to assess the ability of polyanions to solubilize the tight nuclear-bound ER into the low-salt extract by adding the polyanions to the low-salt buffer. Figure 1B shows the extraction of ER from estradiol-treated cells by the addition of increasing concentrations of decavanadate to the low-salt buffer (black bars). The proportion of ER present in the serial high-salt buffer extraction is shown by the gray portion of each bar. We set a measure of the effectiveness of decavanadate extraction as the concentration required to solubilize 50% of the tightly bound ER from MCF-7 nuclei and termed this effective dose 50 (ED_{50}). The data were fit to a sigmoidal dose−response curve to determine the ED_{50}. The ED_{50} for decavanadate extraction of ER was 0.13 ± 0.01 mM decavanadate. Figure 1C shows that another polyanion, polyphosphate, behaved like decavanadate to increase the solubility of estrogen-occupied ER. The ED_{50} for polyphosphate extraction was 0.05 ± 0.01 mM. We hypothesized that the polyanion was competing for ER with nuclear components that bind the activated estradiol-occupied ER.

Both the Nuclear Localization Signal and the DNA Binding Domain of ER Are Required for Estradiol-Induced Tight Nuclear Binding. We used a set of ER mutants to test the importance of different functional regions of the ER protein for tight nuclear binding. Each mutant ER, as well as WT ER, was stably expressed in HEK293 cells. As seen in Figure 2A, the estradiol-occupied WT ER had strong nuclear localization when stably expressed in HEK293 cells, as detected by immunofluorescence on whole cells. Figure 2B shows that more than 95% of the stably expressed WT ER also exhibited tight nuclear binding after estradiol treatment. Figure 2B also shows the efficient solubilization of WT ER from the HEK293 nuclei with polyphosphate. The ED_{50} for WT ER in HEK293 cells was 0.08 ± 0.02 mM polyphosphate, which is equivalent to the ED_{50} for MCF-7 cells (Figure 1C). WT ER stably expressed in the HEK293 cells behaved like endogenously expressed ER in MCF-7 cells as in Figure 1B.

Figure 3A shows the loss of nuclear localization in cells expressing an ER mutant in which two nuclear localization signals (NLS) were deleted (ΔNLS-ER cell line). Other studies have shown that the ΔNLS-ER mutant cannot induce ER-regulated gene expression by E_2 (*40*). ΔNLS-ER failed to display an increase in its apparent affinity for nuclear components when the cells were treated with estradiol as shown in Figure 3B. Approximately 60% of ΔNLS-ER was soluble with low-salt buffer in both the EtOH control and the estradiol-treated cells. However, despite a lack of cellular localization to the nucleus or an effect of estradiol, 40% of the ΔNLS-ER was in the HS fraction. This small population of tightly nuclear bound ER was not effectively solubilized

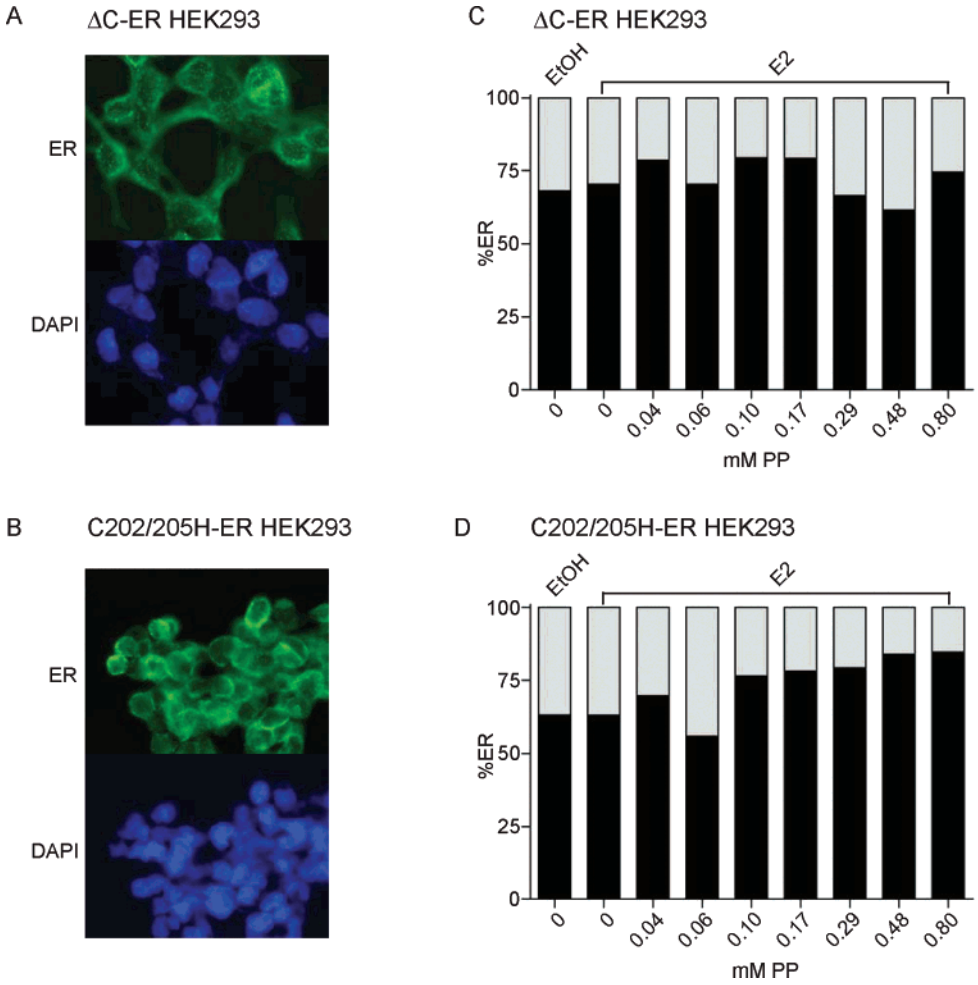

FIGURE 4: Functional DNA binding domain of ER that is necessary for tight association of ER with nuclear components. Immunofluorescence detection of ER in E_2-treated (A) ΔC-ER HEK293 cells and (B) C202/205H-ER HEK293 cells (top panels). DAPI staining of cells (bottom panels). Graphical representation of %ER in LS (black bars) fractions with increasing concentrations of polyphosphate and their sequential HS (gray bars) fractions from EtOH- or E_2-treated (C) ΔC-ER HEK293 and (D) C202/205H-ER HEK293 cells.

with polyphosphate over the concentration range used, and the data did not fit to a sigmoidal dose−response curve. This contrasts with the results for WT-ER, which showed tight nuclear binding in response to estradiol and polyanion extractability of that population of ER. These results indicate that the NLS region is required for estradiol-induced tight nuclear binding of ER.

Figure 4 shows the behavior of two well-characterized DNA binding mutants of ER when stably expressed in HEK293 cells. ΔC-ER lacks the entire DNA binding domain and shows punctate staining in the nucleus as well as some cytoplasmic localization (Figure 4A). The C202/205H-ER mutation disrupts the first zinc finger and abolishes DNA binding (*41*). This mutant receptor shows strong perinuclear and some nuclear staining as seen in Figure 4B. Despite some nuclear localization for both of these DNA binding mutants, neither exhibited estradiol-induced tight nuclear binding as shown in panels C and D of Figure 4. Approximately 70% of ΔC-ER and 60% of C202/205H-ER were recovered in the LS fraction from either ethanol- or estradiol-treated cells. The small portion of ΔC-ER in the HS fraction was not solubilized by addition of up to 0.8 mM polyphosphate to the LS buffer. The portion of the C202/205H-ER in the HS fraction exhibited limited solubilization by polyphosphate, but an ED_{50} was not determined as the data did not fit a sigmoidal dose−response curve. These results were similar to that seen with ΔNLS-ER. The DNA binding mutant ERs in the HS fraction were less sensitive to polyphosphate extraction than the WT ER. Thus, the DNA binding domain of ER is required for the estradiol-induced tight nuclear binding. The efficient solubilization of this population of WT ER from nuclei by polyanions suggests that the mechanism involves competition with chromosomal DNA for ER binding.

Other DNA Binding Proteins Were Also Solubilized by Polyphosphate. If polyanions were competing for proteins bound to chromosomal DNA, we expected other transcription factors to also be solubilized by polyanions. As shown in Figure 5, the transcription factors glucocorticoid receptor (GR), retinoic acid receptor α (RARα), CREB-1, and Sp1 were all solubilized from cells by decavanadate or polyphosphate in low-salt buffer. Similar to ER, GR and RARα are ligand-activated transcription factors and members of the nuclear receptor family (*42*). However, unlike ER, unliganded GR is localized to the cytoplasm, and upon ligand binding, GR is translocated to the nucleus where it associates with nuclear components (*43*). As seen in Figure 5A, all of the unoccupied GR was recovered in the LS fraction, while dexamethasone treatment of the cells resulted in 90% of GR displaying an increased apparent affinity for nuclear components. Increasing concentrations of the polyanion decavanadate showed a dose-dependent increase in the degree

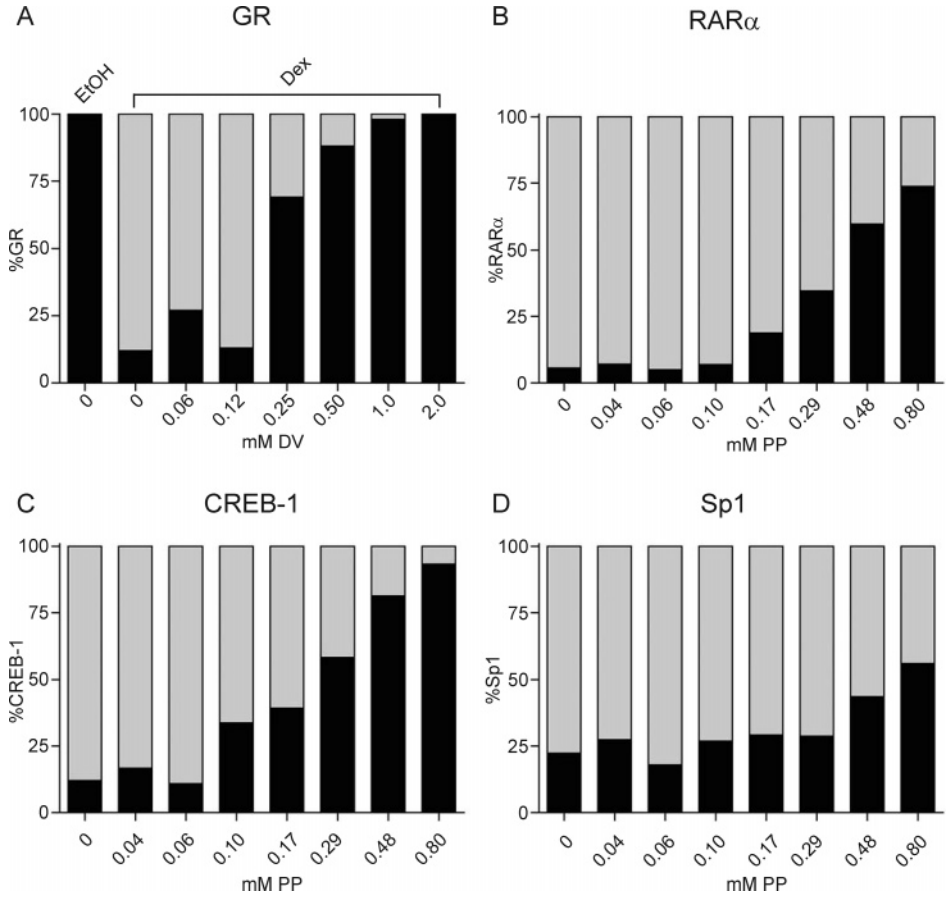

FIGURE 5: Solubilization of other DNA binding proteins by polyanions. Graphical representation of (A) %GR prepared from EtOH- or dexamethasone-treated HeLa cells, (B) %RARα, (C) %CREB-1, and (D) %Sp1 prepared from E_2-treated MCF-7 cells in LS (black bars) fractions with increasing concentrations of either DV or PP and their sequential HS (gray bars) fraction.

of solubilization of occupied GR with an ED_{50} of 0.18 ± 0.01 mM decavanadate. RARα differs from GR in that the unliganded RARα is localized to the nucleus, and it differs from ER in that it has a high apparent affinity for nuclei even in the absence of ligand (*44*). As seen in Figure 5B, greater than 90% of the unoccupied RARα was recovered in the HS fraction. Increasing concentrations of the polyanion polyphosphate showed a dose-dependent increase in the degree of solubilization of unoccupied RARα with an ED_{50} of 0.34 ± 0.02 mM polyphosphate. CREB-1 and Sp1 are transcription factors that do not bind a ligand (*45*, *46*). As seen in Figure 5C, more than 85% of CREB-1 was present in the HS fraction, demonstrating a high apparent affinity for nuclear components. CREB-1 exhibited a dose-dependent solubilization by polyphosphate with an ED_{50} of 0.30 ± 0.12 mM polyphosphate. As seen in Figure 5D, more than 75% of Sp1 was present in the HS fraction, showing a high apparent affinity for nuclear components. Sp1 also showed some solubilization by polyphosphate but was less sensitive than CREB-1 or RARα. The ED_{50} for Sp1 solubilization could not be determined well over this concentration range of polyphosphate. Sp1 and ER also interact in an estrogen-dependent manner to induce gene expression of GC-rich elements by tethering of ER to Sp1 (*47*). However, we did not observe an effect of estrogen on the extractability of Sp1 by polyphosphate in our experiments.

We also tested the ability of polyanions to solubilize two coregulator proteins from the nucleus. CREB binding protein (CBP) and BRG-1 do not have direct high-affinity binding for DNA but associate with transcription factors (*48*, *49*). Both CBP (Figure 6A) and BRG-1 (Figure 6B) exhibited a high apparent affinity for nuclei with ~90 and ~70% recovered in the HS fraction, respectively. CBP exhibited a dose-dependent increase in solubility by polyphosphate with an ED_{50} of 0.10 ± 0.01 mM polyphosphate. However, only a small portion of total CBP (~60%) was solubilized even at the highest polyphosphate concentration. The solubility of BRG-1 was more limited, and an ED_{50} could not be determined over this polyphosphate concentration range.

AIB1 Interaction Was Not Required for Estradiol-Induced Tight Nuclear Binding of ER. Estradiol occupation of ER induces protein−protein interactions, particularly with co-activator proteins such as AIB1 (*1*, *19*). AIB1 is highly overexpressed in MCF-7 cells compared with other ER coactivators (*50*). We considered the possibility that the ER−AIB1 interaction plays a role in the tight nuclear binding of ER induced by estradiol. The L540Q-ER mutant is a dominant negative mutant that does not interfere with DNA binding but disrupts the coactivator interaction surface (*51*). This mutant was stably expressed in HEK293 cells and displayed estradiol-induced tight nuclear binding as seen in Figure 7A. More than 75% of the L540Q-ER was recovered in the HS fraction from cells treated with estradiol. Increasing concentrations of polyphosphate solubilized the L540Q receptor in a dose-dependent manner with an ED_{50} of 0.41 ± 0.45 mM polyphosphate. As seen in Figure 7B, estrogen caused a very modest decrease in the solubility of AIB1 from MCF-7 cells where estradiol-occupied ER and

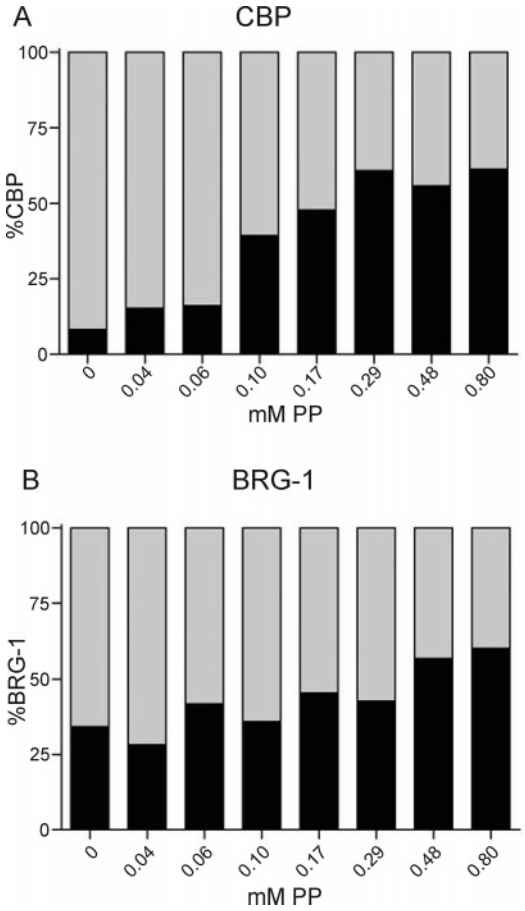

FIGURE 6: Polyanion solubilization of coregulator proteins. Graphical representation of (A) %CBP and (B) %BRG-1 in LS (black bars) fractions with increasing concentrations of polyphosphate and their sequential HS fraction from E_2-treated WT ER HEK293 cells.

AIB1 interact endogenously. However, the %AIB1 in the LS fraction was not sensitive to polyphosphate extraction at various concentrations. AIB1 showed little tight nuclear binding in ethanol- or estradiol-treated cells with 75 or 65% recovered in the LS fraction, respectively. We concluded that estradiol-induced tight nuclear binding of ER did not require its interaction with AIB1.

Binding of ER to Chromosomal DNA Underlies Tight Nuclear Binding Induced by Estradiol. The requirement for both the nuclear localization and DNA binding domains of ER for estradiol-induced tight nuclear binding suggested that binding to chromosomal DNA could account for the decreased solubility of the liganded receptor. We hypothesized that the polyanions, decavanadate and polyphosphate, were functioning as DNA mimics and were competing for binding of ER to chromosomal DNA. This hypothesis predicts that an estrogen response element (ERE)-containing oligonucleotide should also release ER from nuclei and do so with greater efficiency than nonspecific polyanions. As seen in Figure 8A, the estradiol-occupied WT ER was solubilized in a dose-dependent manner by addition of a double-stranded ERE-containing oligonucleotide to the LS buffer. The ED_{50} for ERE solubilization of ER was at 0.34 ± 0.04 μM ERE, which was 380-fold lower than the ED_{50} for polyphosphate for the same stable cell line. We also tested for oligonucleotide sequence specificity with a 2 bp ERE mutant (mutERE) added to the low-salt buffer. As seen in Figure 8B, the mutERE also increased the solubility of liganded ER, but

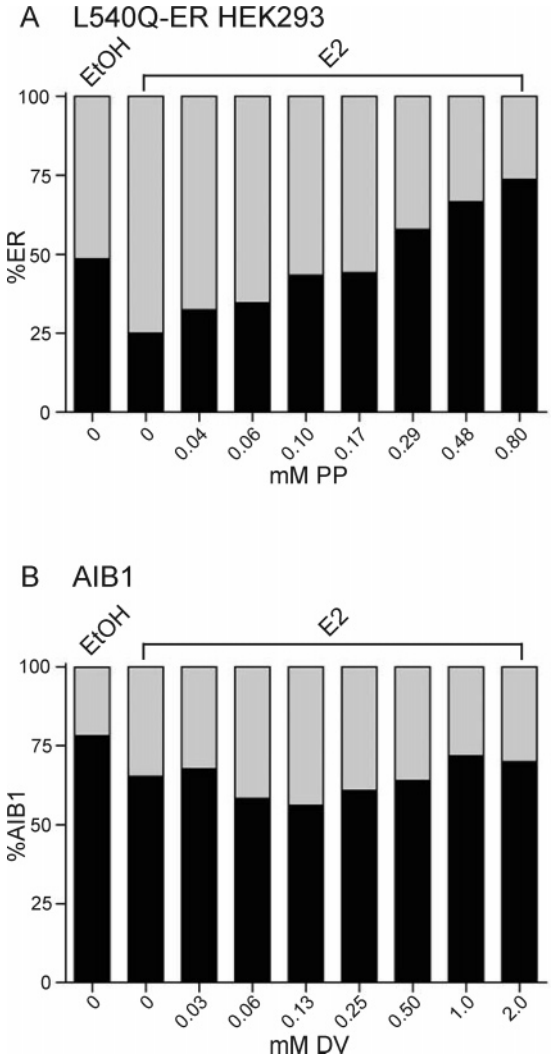

FIGURE 7: Interaction of AIB1 with ER does not affect ER solubility. (A) Graphical representation of %ER present in LS (black bars) fractions with increasing concentrations of polyphosphate and their sequential HS (gray bars) fraction from EtOH- or E_2-treated L540Q-ER HEK293 cells. (B) Graphical representation of %AIB1 extracted in LS (black bars) fractions with increasing concentrations of decavanadate and their sequential HS (gray bars) fraction from E_2-treated MCF-7 cells.

the ED_{50} of 2.3 ± 7.7 μM was 7-fold higher than for the consensus ERE. Similar results were obtained for oligonucleotide extraction of ER from estradiol-treated MCF-7 cells (data not shown).

A reciprocal prediction from the hypothesis given above is that DNA degradation should also solubilize occupied ER. Increasing concentrations of the enzyme Benzonase, which degrades both DNA and RNA, was added to the low-salt buffer. As seen in Figure 8C, Benzonase solubilized approximately 45% of estrogen-occupied ER at the highest concentration of 1000 units/mL.

DISCUSSION

The experiments presented here address the molecular basis for one of the most rapid responses of the ER to hormone treatment, a change in the apparent affinity of ER for nuclear components termed tight nuclear binding. ER is a nuclear protein, but its fractionation behavior upon cell lysis shows a dramatic dependence on hormone treatment

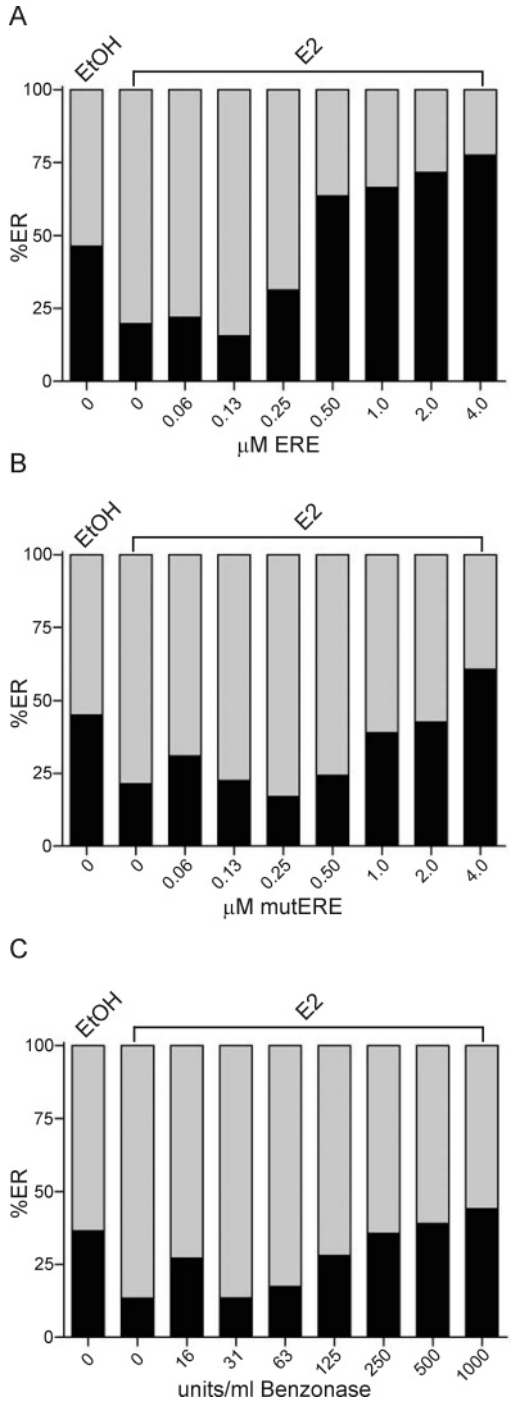

FIGURE 8: ER solubilization by disruption of ER−DNA interactions. Graphical representation of %ER present in LS (black bars) fractions with increasing concentrations of (A) ERE, (B) mutERE, or (C) Benzonase and their sequential HS (gray bars) fraction from E_2-treated WT ER HEK293 cells.

(*12*). Occupied ER can be solubilized by high salt or low concentrations of polyanions such as decavanadate and polyphosphate (*10*, *11*, *36*). Furthermore, a DNA oligonucleotide with a specific binding site for ER efficiently solubilized occupied ER at a 380-fold lower concentration than polyanions. In addition, extraction by the consensus ERE is more efficient than that by the mutERE, showing that the efficacy of ER extraction by an oligonucleotide is sequence-dependent. The polyanions are apparently acting as DNA mimics, consistent with their chemical similarity to the polyphosphate backbone of DNA. The ER appears to interact with the polyanions in the same way that it can bind to the polyphosphate backbone of nonspecific DNA (*24*).

We conclude that the mechanism of polyanion or oligonucleotide solubilization of the occupied ER from nuclei is competition for ER binding with the chromosomal DNA. This conclusion is further supported by the solubilization of ER via degradation of the chromosomal DNA with the Benzonase nuclease. This is in agreement with a previous report, which shows that DNase treatment increases the level of solubilization of ER (*52*). Furthermore, we found that the DBD of the ER was required for estradiol-induced tight nuclear binding. The NLS sequence of ER was also required, but we expect that this is an upstream event with respect to estradiol-induced tight nuclear binding.

It is important to note that there is a population of ER that is found in the pellet after hypotonic cell lysis for each of the ER DBD mutants. This population of ER is not dependent on estradiol or the DNA binding domain for its association with the pellet. Furthermore, it is less sensitive to extraction with polyanions than the portion of ER that exhibits tight nuclear binding in response to estradiol. The nature of this subfraction of ER is unknown, but presumably, it is associated with elements in the pellet other than chromosomal DNA.

Our conclusion, that binding to chromosomal DNA is the mechanism underlying estradiol-induced tight nuclear binding by ER, must be reconciled with other data showing very limited effects of estradiol on the interaction of ER with DNA. A number of studies have demonstrated that ligand is not required for ER to bind DNA in vitro and has little effect on the affinity of the interaction (*25*−*29*). Promoter interference and chromatin immunoprecipitation assays show that unliganded ER can bind to DNA in whole cells, though full gene activation requires hormone (*53*−*55*). Live cell imaging studies also show unliganded ER associating with DNA in vivo, though the kinetics of binding are increased by estradiol (*34*). The simple interpretation of these data is that the intrinsic ability of ER to bind DNA is the same both in vitro and in vivo and is not affected by estradiol. A large mass of nuclear DNA will be accessible to ER despite chromatin packaging, and we expect it can behave as an adsorbent in much the same way as the solid phase in DNA affinity chromatography. The presence of such a mass of potential binding sites means that although ER has a fast rate of dissociation from any single site, it will immediately encounter another site and bind again. The effect is for ER to partition into the solid phase of chromosomal DNA and display the behavior we term tight nuclear binding. This would be dependent on the DNA binding domain consistent with our results. Solubilization of ER is accomplished by adding a small soluble molecule, such as polyphosphate or an oligonucleotide, into the buffer that can compete with binding of ER to the chromosomal DNA. Thus, the observation of ER tight nuclear binding can be understood simply by its affinity for DNA, but its dependence on ligand suggests the regulatory role of other interactions. In addition, ChIP assays show that the ability to recruit ER to an ERE is greatly increased by E_2 (*20*). Furthermore, the rapid mobility of unoccupied ER visualized in the nuclei of whole cells also suggests that partitioning of ER into the chromosomal DNA is inhibited (*31*, *32*).

Our work is consistent with a model in which interaction of ER with DNA in vivo is regulated by chaperone proteins. A large body of work has characterized the unliganded nuclear receptor complexes containing chaperone proteins that are recovered in the cytosol of hormone-withdrawn cells or tissues (*16*). The unoccupied ER in this complex fails to bind DNA in vitro; however, addition of estradiol causes dissociation of ER from most of the chaperones, and ER−DNA binding activity is recovered. This is consistent with our observation that the DBD of ER and estradiol are required for tight nuclear binding. When cells are treated with estradiol, inhibition of DNA binding by chaperone proteins is lost. Our data on solubilization of both GR and RARα by polyanions are also consistent with this model. Unoccupied GR also forms a complex with chaperone proteins, and we observe both hormone-induced tight nuclear binding and polyanion solubilization. Unoccupied RARα is not found in a chaperone complex and is tightly nuclear bound even without hormone, but it is also sensitive to polyanion extraction as would be predicted from the model.

The model described above, in which estrogen-dependent association of ER with chaperone proteins regulates ER access to DNA, does not predict the high mobility of the occupied ER or the ability of unoccupied ER to bind DNA in whole cells that is observed in live cell imaging studies (*34*). A modification to the model is suggested by the work of the DeFranco and Hager labs in which the mobility of the glucocorticoid and progesterone receptors in nuclei from hormone-treated cells was dependent on both ATP and a minimum set of five chaperone proteins: hsp90, hsp70, hsp40, p23, and Hop (*56*). These five chaperone proteins are also part of the unoccupied receptor complex for PR, GR, and probably ER (*57*). Upon hypotonic lysis of cells, nuclear ATP and chaperone protein levels are depleted and nuclear receptor mobility is lost (*56*). We propose that interaction of ER with chaperones prevents the ER from partitioning into the chromosomal phase which would result in very limited movement of ER to candidate sites of action. Upon hypotonic cell lysis and loss of nuclear ATP, the dynamic interaction of ER with chaperones is lost. In the case of occupied ER, chaperone binding is lost, and we observe ER partitioning into the chromosomal DNA. In the case of the unoccupied ER, a relatively stable chaperone complex that cannot bind DNA is recovered.

The work presented here on the importance of the ER DBD for tight nuclear binding pulls together a large body of knowledge of the interaction of ER with the nucleus which supports a model in which chaperone proteins play a regulatory role. We have virtually no molecular understanding of how the estradiol-occupied ER interacts with the same chaperones found in the unoccupied ER complex. An understanding of these complexes and their regulation by hormone will improve our understanding of estrogen signaling.

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