Characterization of the DNA-binding Domain of the Mouse Uterine Estrogen Receptor Using Site-specific Polyclonal Antibodies*

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The DNA-binding domain of the mouse uterine estrogen receptor (ER) was characterized using site-specific polyclonal antibodies. The peptides used as antigens have sequences corresponding to amino acids 185–190 and 227–245, the two zinc finger regions of the DNA-binding domain of the human ER, and produced antisera designated A-1542 and A-1554, respectively. Mouse uterine nuclear ER and salt-activated 4 S cytosol receptor, as well as 8 S untransformed cytosol receptor, were observed to react with the antisera by Western blot and sucrose density gradient centrifugation analyses indicating that the DNA-binding domain of the 8 S cytosol receptor is not completely masked by heat shock protein 90 or other proteins. Only A-1554 detected a nuclear-specific doublet form of the ER on Western blot analysis. In a gel shift assay, neither antisera altered the pattern of the nuclear ER interaction with the vitellogenin A2 estrogen response element (VRE). In contrast, antiserum A-1554 partially shifted the 8 S cytosol receptor-VRE complex. This concurs with mutational analysis and x-ray crystallography studies with the human ER that have shown that the second finger is not in contact with the DNA. The results of the gel shift assay were confirmed by sucrose density gradient analysis using the same buffer conditions. The nuclear receptor-VRE complex did not react with either antisera, suggesting that when the dimeric nuclear receptor form binds the VRE, the specific receptor epitopes involved with the DNA binding may be blocked and unable to bind the antisera. The cytosol receptor-VRE complex reacted only partially with the second finger antisera A-1554, suggesting that on receptor monomers the second finger epitope is not completely blocked by DNA binding or dimer formation.

Steroid hormone receptors are a class of ligand-inducible, trans-acting regulatory factors that activate gene transcription by binding as dimers to specific DNA sequences termed hormone-responsive elements (1). The receptor proteins for steroids, thyroid hormone, vitamin D, and retinoids comprise a group of proteins which are members of a superfamily of molecular transcription factors (2, 3). Structural analysis indicates steroid hormone receptors are composed of six major functional domains (4, 5). Domains A/B in the NH2-terminal region of the protein comprise a hypervariable region containing transactivation domains. Domain C, the DNA-binding domain contains a 66–68 amino acid region arranged in two zinc-stabilized DNA-binding finger motifs, CI and CII. Mutational analysis has shown that the CI finger determines the specificity of DNA binding by making contacts with bases in the hormone-responsive elements (6). Three amino acid changes at the COOH-terminal base of the CI finger allow ER recognition of glucocorticoid responsive element sequences (7). DNA-bound receptors are believed to be stabilized by contacts with the DNA phosphate backbone contributed by CII (8). Region D contains sequences that function as a nuclear localization signal. Ligand-binding and ligand-dependent transcriptional activation domains are located in domain E.

The non-transformed state of steroid receptors observed in the absence of hormone binding is thought to be composed of a multicomponent protein complex. Most notably, the 90-kDa heat shock protein (hsp90) which constitutes 0.1–2% of cell protein is reported to associate with steroid receptors (9, 10). Proposals were made suggesting that the binding of hsp90 masks the DNA-binding domain (11), thereby preventing DNA binding. Upon hormone binding, hsp90 is released allowing receptor interaction with DNA (12). However, recent studies with native ER suggest that in the absence of hormone and under low salt conditions where hsp90 would be expected to be bound, the receptor can bind specific DNA containing hormone-responsive elements in vitro (13).

Recent reports of NMR (14) and x-ray crystallographic analyses (15) of the glucocorticoid receptor and NMR analysis of the ER DNA-binding domain (8) have shown that the two DNA-binding domain fingers are positioned at a right angular orientation. It is indicated that other regions (TAF-1 and TAF-2) of the protein influence transcriptional activity of the receptor (16, 17) and probably its conformation for interacting with DNA (18). Since NMR and crystallographic studies utilized primarily the DNA-binding domain (8) of the receptor, it remains to be established what influence other domains of the full-length receptor have on the DNA interaction. To further investigate the specific structure and interaction of native mouse uterine ER with DNA, polyclonal antibodies were raised against two specific peptide sequences from the CI and CII regions in the DNA binding region. The antisera were used to characterize

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‡The abbreviations used are: ER, estrogen receptor; ERE, estrogen-responsive element; VRE, vitellogenin A2 estrogen response element; PBS, phosphate-buffered saline; hsp90, heat shock protein 90.
The DNA-binding domain of the estrogen receptor. The amino acid sequence of the DNA-binding domain is indicated above and its zinc finger structure is shown schematically. Underlined portions of the sequence indicate peptides (264 and 267) that were synthesized and used to generate antisera A-1542 and A-1554 respectively. (+) marks indicate charged amino acids. (*) indicates conserved cysteine residues.

**EXPERIMENTAL PROCEDURES**

**Materials—**17β-estradiol (94.7 Ci/mmol) was purchased from Du Pont-New England Nuclear. Electrophoretic reagents were obtained from Bio-Rad. Ultra pure sucrose was obtained from Bethesda Research Laboratories. Estrogen receptor antibody (H222) was a generous gift from Dr. Chris Nolan, Abbott Laboratories.

**Buffers—**The following buffers were used: TE buffer (10 mM Tris, 1.5 mM EDTA, pH 7.4), TEK buffer (TE buffer plus 0.4 M KCl), and TEGM buffer (10 mM Tris, 1.5 mM EDTA, 10% (v/v) glycerol, 3 mM MgCl2). Additional buffers contained 3 mM EGTA, 50 µg/ml each of leupeptin, antipain, chymostatin, and soybean trypsin inhibitor and were designated as TEPI, TEKPI and TEGMPI buffers, respectively. Buffers which contained 10 mM sodium molybdate were designated as TEPIMo and TEGMIPo buffers. TAE buffer contained 6.7 mM Tris, 1.5 mM EDTA, pH 7.4). TEK buffer (TE buffer plus 0.4 M KCl), and TEGM buffer (TE buffer plus 3 mM EDTA) were designated as TEPI, TEKPI and TEGMPI buffers, respectively.

**Animal and Tissue Preparation—**Animal studies were conducted under an approved National Institutes of Health protocol in accord with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals. Female CD-1 mice were obtained from Charles River (Raleigh, NC). Bilateral ovariectomy was performed on animals at 10 weeks of age. Animals were sacrificed by cervical dislocation 2 weeks after ovariectomy. The uteri were removed and immediately frozen on dry ice and stored at −70°C until use.

**Preparation of the Cytosol Receptor and the Ammonium Sulfate-fractionated Cytosol—**The cytosol receptor fraction was prepared from ox/ mouse uteri by homogenization with TEPI or TEKPI buffers (18). The homogenate was centrifuged at 105,000 × g for 50 min to obtain the cytosol. For some studies, ammonium sulfate was added to the cytosol at 40% saturation as previously described (20). The resulting precipitate was collected by centrifugation at 8,800 × g for 30 min, resuspended, and dialyzed against TGM.

**Preparation of Nuclear Receptor—**The nuclear receptor fraction was prepared following treatment of ox/ mouse uteri with either unlabeled estradiol or tritiated estradiol as described previously (13). The uteri were homogenized with TEGMPI buffer and filtered through Nytex (100–125 µm). The filtrate was centrifuged at 2,500 × g for 10 min. The pellet was washed twice with homogenization buffer and resuspended in buffer containing 0.4 M KCl and allowed to extract for 30 min. The suspension was centrifuged at 105,000 × g for 50 min. For Western blots, ER was extracted from the nuclear pellet in 5% SDS and prepared as previously described (21).

**Partially Purified Estrogen Receptor—**Partially purified ER was prepared from ox/ mouse uteri using estrogen affinity column chromatography (20).

**DNA Binding Assays—**Receptor binding to DNA was assayed using sucrose density gradient centrifugation or gel shift assays. The gel shift assay was performed as described previously (13). A [32P]-labeled 56-base pair synthetic DNA fragment containing the vitellogenin A2 ERE (20–40 fmol) was incubated with ammonium sulfate fractionated ER (3.2–4 µg of protein; 3.1 fmol of receptor) with or without 10 nM estradiol or nuclear extract (12.5 µg of protein; 30 fmol of receptor). In some experiments receptor preparations were incubated for 20 h at 0°C with 6 µl of PBS and an aliquot of protein A-Sepharose-purified A-1542, A-1554 antisera or monoclonal antibody H222 as listed in the figure legends. For sucrose density gradient centrifugation, the incubation volume was increased with buffer used for the gel shift assay to provide sufficient sample. Nuclear receptor reaction mixtures were diluted with an equal volume of TAE alone or cytosol receptor was preincubated with a DCC (5% charcoal, 0.5% dextran) TAE solution to absorb free steroid before application to the gradient.

**RESULTS**

**Western Blot Analysis of ER—**Fig. 1 shows a schematic diagram of the highly conserved region of the DNA-binding domain of the ER with the sequence highlighted for peptides 264 and 267 used in antibody production. Reactivity of the nitrocellulose sheet by diffusion blotting (20) and reacted overnight with the rabbit anti-ER peptide antisera at room temperature. Nitrocellulose strips were washed and incubated with [32P]-labeled secondary antibody (rabbit) as described previously (20).

**Sucrose Density Gradient Analysis—**Sucrose density gradients (5–20% or 5–30% w/v) were formed and collected with a Haake Buchler Auto Densi-Flow. Gradients were made in TE, TEK, or TAE buffers as indicated. Gradients were centrifuged for 17 h at 180,000 × g in a Beckman SW60 Ti rotor, and three drop fractions were collected for analysis of radioactivity profile.

**Protein Concentration Determination—**Protein concentrations were determined by the Bradford method using rabbit γ-globulin as the protein standard (24).

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FIG. 3. Western blot analysis of the ER using affinity purified antiserum. Sample preparation and electrophoretic protein separation were performed as outlined in the legend to Fig. 2 and under "Experimental Procedures." Partially purified cytosol ER (20 ng) was probed with preimmune serum (lane 1), antiserum A1542 (lane 2), antiserum A1542 preincubated with peptide 264 (lane 3), affinity purified antiserum A1554 (lane 4), affinity purified antiserum A1554 preincubated with peptide 267 (lane 5), or H222 (lane 6). Arrows indicate full-length ER (65,000) and a major proteolytic fragment (54,000).

two rabbit antisera, A-1542 and A-1554, with partially purified mouse uterine ER was shown by Western blot analysis. Because A-1542 and A-1554 produced a high background on Western blots, they were purified with a peptide-linked Sepharose affinity column. The reactivity of preimmune sera, antisera, and purified antisera toward partially purified cytosol ER is shown in Fig. 2. In agreement with our previous results, multiple bands were observed which resulted from proteolysis of the native 65,000 ER to a truncated 54,000 form (19). The 65,000 full-length receptor and 54,000 fragment form reacted with antisera A-1542 and A-1554 (lanes 3 and 6), and affinity purified A-1554 (lane 7), but not affinity purified A-1542 (lane 4). Antibody A-1554 showed similar reactivity after purification, but exhibited a lower background. A-1542 showed stronger reactivity than A-1554 at the same 200-fold dilution. Samples in Fig. 2, lane 1, were probed with H-222, a monoclonal anti-ER antibody (20), as a positive control. Monoclonal H-222 showed the 65,000 and 54,000 bands, but not the nonspecific lower molecular weight band seen with the serum-probed samples (lanes 2–7). Specificity of the crude A-1542 and purified A-1554 was tested by preincubating each antiserum with the corresponding peptide antigen. The 65,000 and 54,000 immunoreactive bands were not

FIG. 4. Western blot analysis of the nuclear ER. A 500-µg sample of nuclear protein containing ER was applied to each lane, electrophoresed, and transferred to nitrocellulose strips as described in the Fig. 2 legend. The strips were probed with H222 (lane A), preimmune serum (lanes C and E), antiserum A-1554 (lane B), or antiserum A-1542 (lane D).

FIG. 5. Sucrose density gradient analysis of the reactivity of the cytosol ER with A-1542 and A-1554 in high salt (0.4 M KCl) buffer. Mouse uterine cytosol was prepared in TEK/PiMo, labeled with 10 nM [3H]estradiol, and incubated at 0 °C for 20 h with preimmune serum, A-1542 or A-1554 preincubated with PBS, or corresponding peptides in PBS. After DCC treatment, samples were centrifuged on 5–20% sucrose gradients in TEK for 17 h at 180,000 × g. [3H]Ovalbumin (3.6 S) and [125I]γ-globulin (7 S) were used as internal markers. A, ER incubated with preimmune serum (O); A-1542 (Δ); A-1542 and peptide 264 (●). B, preimmune serum (O); A-1554 (Δ); A-1554 and peptide 267 (●).
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FIG. 6. Sucrose density gradient analysis of the reactivity of molybdate stabilized cytosol ER with A-1542 or A-1554 in low salt buffer. Mouse uterine cytosol was prepared in TEPIMo, labeled with 10 nM [3H]estradiol and incubated at 0 °C for 20 h with PBS (○), protein A-Sepharose purified A-1542 (●) or A-1554 (△). After DCC treatment, samples were centrifuged on 5–20% sucrose density gradients in TE buffer for 17 h at 180,000 × g. [14C]Ovalbumin (OV) and [14C]γ-globulin (γG) were used as internal markers.

detected (Fig. 3, lanes 3 and 5), although some nonspecific bands were detected on the nitrocellulose strips.

Our earlier findings demonstrated that monoclonal antibody H-222 or H-226 detected a closely spaced nuclear ER doublet of 66.5 and 65 kDa when analyzed by SDS-polyacrylamide gel electrophoresis (21). Analyses of uterine nuclear ER showed that antisera A-1542 detected two specific bands of the receptor (Fig. 4, lane D) the same as H-222 (lane A). In contrast, only one receptor band was readily detected with A-1554 (Fig. 4, lane B). These findings were confirmed by densitometric analyses (data not presented). The sensitivity of antibody A-1554 was tested by using a 2-fold higher concentration; however, A-1554 was still unable to detect the upper band of the nuclear ER doublet.

Reactivity of Different Molecular Forms of ER—The structure of the different forms of the cytosol ER were analyzed with the DNA-domain-specific antibodies by sucrose gradient centrifugation under high salt conditions (0.4 M KCl, 66.5 kDa), and low salt conditions (no KCl). Under high salt conditions (0.4 M KCl), the receptor exists as a 4 S monomer which shifts to 7–8 S after incubation with the antisera (Fig. 5). A-1542 shifts the 4 S peak less efficiently (Fig. 5A) showing it is less reactive than A-1554 (Fig. 5B). No shifted peaks were observed using antisera precipitated with the corresponding cognate peptides, indicating that the antibodies were site-specific for their respective peptide sequences. Increased concentrations of the antisera did not change the reactivity (data not shown).

In low salt buffers (no KCl) containing sodium molybdate, the cytosol receptor sediments at 8 S on sucrose gradients (Fig. 6). Association of hsp90 and ER is stabilized in the presence of molybdate ions. Incubation of molybdate stabilized ER, and purified antisera results in a 11 S receptor complex (Fig. 6). In low salt buffer, the 8 S receptor was shifted more completely by A-1542 than by A-1554 suggesting in this form the CI region may be more exposed than the CII finger region.

As shown in Fig. 7, we observed specific reactivity and shifting of the 5 S nuclear receptor peak with both antisera in a high salt (0.4 M KCl) gradient. Similar analysis of the nuclear receptor in low salt gradients showed the peaks were shifted more efficiently than in high salt buffer (data not shown). This may indicate the receptor-antibody complex is more easily dissociated by the higher salt concentration in the buffers.

Mobility Shift Analysis of ER—The structural form and accessibility of the DNA-binding domain of the receptor was further investigated by analyzing antibody reactivity in the presence of a specific DNA sequence. 32P-Labeled VRE, which contains the estrogen-responsive element from the vitellogenin A2 gene, was added to the cytosol or nuclear ER preparations before or after antibody addition in the presence of large excess of poly(dI-dC). In the mobility shift assay, neither A-1542 or A-1554 reacted with the 5 S nuclear receptor after addition of the VRE (data not shown). A ternary complex composed of the nuclear receptor, the antibody, and VRE was observed only with monoclonal antibody H-222. Neither DNA-binding domain antisera appeared to have an effect on binding of the nuclear receptor to VRE using this procedure.
Gel shift assay profiles of the cytosol ER, which we have shown is able to bind VRE (13), are shown in Fig. 8. The cytosol receptor produced similar $^3$P-labeled VRE bands as the nuclear receptors. The band in Fig. 8, lane 5, is specific, but is not totally eliminated due to incomplete competition which occurs from time to time in some sample preparations. In addition to the specific bands of the receptor-VRE complex, a small amount of double-shifted complex was observed with A-1554 (arrow, Fig. 8, lane 3). Densitometric scanning of the gel bands indicated approximately 10-15% of the ER-VRE is supershifted with antiserum A-1554. No double shift was detected in the A-1542 sample. The double shift band was also observed in preparations reacted with H-222 (lane 4).

Sucrose Gradient Analysis in Presence of Specific DNA—Antibody-receptor-DNA interactions, binding of the antisera to the cytosol, and nuclear receptors were studied by sucrose gradient analysis in the presence or absence of VRE in the same TAE buffer used in the gel shift assay (Figs. 9 and 10).

The 5 S nuclear receptor-VRE peak was shifted to ~8 S upon addition of VRE (Fig. 9C). The peak at 8-9 S was further shifted by H-222 monoclonal antibody indicating the presence of the ER (Fig. 9A). In the absence of VRE, the 5 S nuclear receptor reacts with both antisera even in the presence of large excess of poly(dI·dC) to form a complex of 11 S (Fig. 9B). In contrast, the 8-9 S ER-VRE form persisted when the nuclear receptor was reacted with either antisera before or after addition of VRE shown with A-1542 (Fig. 9C) and A-1554 (data not presented) indicating these antisera do not bind the nuclear ER when VRE is present. The 8 S cytosol receptor VRE complex can be shifted by H-222 monoclonal antibody (Fig. 10A) but not by the DNA-binding domain antisera (Fig. 10B). The cytosol receptor in the absence of VRE can be shifted by both antisera suggesting that the antibody binding is displaced by VRE binding. The gel shift showed a small amount of ER-VRE-antibody complex (Fig. 8), which is not detectable in the gradient assay, and probably

![Figure 8](image_url)

**FIG. 8. Binding of antiserum 1554 to cytosol ER-VRE complex.** The effect of protein A-Sepharose-purified A-1542 or A-1554 on VRE binding of the cytosol receptor (ERc) was examined using the gel shift assay as described under “Experimental Procedures.” Lanes 1-5, ERc-$^3$P-labeled VRE complexes were formed prior to addition of preimmune serum (lane 1), A-1542 (lane 2), A-1554 (lane 3), or H222 (lane 4). Lane 5 also contains a 100-fold molar excess of unlabeled VRE. Star indicates specific ER-VRE bands. Arrow indicates antibody-shifted ER-VRE complexes.

![Figure 9](image_url)

**Fig. 9. Sucrose density gradient analysis of binding of A-1542 or A-1554 to the nuclear ER in the presence or absence of VRE.** The nuclear ER, prepared as described under “Experimental Procedures,” was incubated with poly(dI·dC) at 0°C for 20 min. Some samples were then incubated with VRE at room temperature for 20 min before or after incubation at 0°C for 20 h with PBS, protein A-Sepharose-purified A-1542 or A-1554, or H222. Samples were centrifuged on 5-30% sucrose density gradients in TAE buffer for 15 h at 130,000 × g. $[^{14}C]$Ovalbumin (OV) and $[^{14}C]$γ-globulin (γG) were used as internal markers. 5 S nuclear ER was incubated with the following reagents in the order listed. A: VRE + PBS (O); VRE + H222 (●). B: A-1542 (O); A-1554 (●). C: VRE + A-1542 (O); A-1542 + VRE (●).
DISCUSSION

Antisera raised against peptides corresponding in sequence to regions of the zinc finger motifs of the ER DNA-binding domain were found useful to probe the structure of the native mouse ER bound to an ERE. A similar approach to analyzing the steroid hormone receptor structure was reported by Traish et al. (25-27) for human ER, the chicken progesterone receptor by Smith et al. (22), and the human glucocorticoid receptor by Wilson et al. (23) and Urda et al. (28), although none of those studies evaluated DNA-bound receptor structures.

In cytosol preparations from unstimulated mouse uterus, the receptor is present as an 8–9 S form under low salt conditions and 4 S in high salt (0.4 M KCl) (29). The exact composition of the 8 S untransformed ER complex is still not clear. It is reported that the non-transformed hetero-oligomer (8–9 S) ER complex includes two molecules of the ER, two hsp90 proteins, and possibly a 59-kDa protein (59) (30). It was also proposed that when bound, hsp90 masked the DNA-binding domain, thereby blocking the ability of the receptor to bind DNA (12). We show here that epitope-specific antibodies A-1542 or A-1554 recognize the 8 S receptor on Western blot and sucrose gradients in the presence of sodium molybdate, which stabilizes the 8 S ER-hsp90 complex. The reaction is inhibited by the specific VRE DNA sequence, but not by nonspecific DNA poly(dC). The data suggest that the peptide antibodies raised against the DNA-binding domain react specifically with the ERE-binding site on the receptor either in its heteromeric state presumably when hsp90 is associated with the ER or in the presence of nonspecific DNA. However, receptor binding to a specific ERE sequence precludes antibody interaction. Reactivity of the DNA domain antibodies with low salt molybdate-stabilized 8 S ER suggests that the zinc finger regions of the DNA-binding domain are not covered, but are accessible to the antisera. This is in agreement with additional studies showing the 8 S cytosol receptor can bind VRE (13, 31, 32). Dalman et al. (33) have used ER-retinoic acid receptor chimeras and an anti-hsp90 antibody to demonstrate that the zinc finger region of the ER is neither necessary nor sufficient for the ER to bind hsp90. Other work has also suggested that hsp90 may be bound to a region of the ligand-binding domain rather than the DNA-binding domain (34). Such a positioning would be consistent with these analyses indicating an available epitope distant from the hsp90-binding site.

Using the gel shift assay, we detected no nuclear receptor-VRE-antibody ternary complex and only a small amount of cytosol receptor-VRE-antibody ternary complex. The nuclear dimeric ER appears to complex with the VRE in such a way that both zinc finger motifs (CI and CII) are inaccessible to the antibodies. Analyses with the ER 8 S cytosol receptor complex was detected only with the CII finger antiserum A-1554, which indicates that the CI finger antibody can not bind the ER in the presence of VRE; however, the CII finger is partially available to bind the antibody. It is conceivable that some of the cytosol receptor binds the VRE as a monomer, for 20 min. Some samples were then incubated with VRE at room temperature for 20 min before incubation at 0 °C for 20 h with PBS, A-1542 or A-1554, or H222. Samples were centrifuged on 5–30% sucrose density gradients in TAE buffer for 15 h at 130,000 x g. [3H]Ovalbumin (OV) and [3H]γ-globulin (yG) were used as internal markers; ER was incubated with the following reactants in the order listed. A: VRE + PBS (O); VRE + H222 (●); B, A-1542 (△); A-1554 (▲); VRE + A-1542 (○); VRE + A-1554 (●).
exposing the CII finger epitope. In contrast, as the ER dimer, both CI and CII of the ER monomers interact and make the epitope unavailable to bind the antibodies. NMR analyses of ER and GR have shown that the CI finger is inserted in the major groove of the DNA where it contacts bases important for sequence recognition (8, 14), and thus would not be available for antibody binding. The CII finger region extends away from the DNA, and thus may be more accessible for antibody binding when ER is bound to VRE. Our results are consistent with the NMR analyses (8) using native ER.

Steric hindrance by VRE interaction appears to prevent binding of the DNA domain antibodies. It would appear that ER has a higher affinity for VRE than for the antiserum, allowing the VRE to displace the antibody from these epitopes on the ER. Even when antibody binding precedes VRE binding, the receptor’s high affinity for VRE, $K_d \approx 0.3 \text{ nM}$ (20), does not allow antibody binding. These are among the first studies to evaluate the structural binding interactions of different forms of the native mouse uterine ER to specific estrogen-responsive DNA sequences. Our findings using a short response element sequence indicate the DNA-binding domain is accessible in different ER forms, and hsp90 or other factors do not appear to be blocking this domain of the ER. Cytosol ER does not appear to require any structural change such as activation in order for the two zinc finger motifs in the DNA-binding domain to be accessible for DNA interactions. These structural properties are somewhat unique to the ER, since cytosol progesterone receptor has been shown to require dissociation of hsp proteins in order to bind DNA (35).

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