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The Role of the C-terminal Extension (CTE) of the Estrogen Receptor \( \alpha \) and \( \beta \) DNA Binding Domain in DNA Binding and Interaction with HMGB*

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HMGB-1/-2 are coregulatory proteins that facilitate the DNA binding and transcriptional activity of steroid receptor members of the nuclear receptor family of transcription factors. We investigated the influence and mechanism of action of HMGB-1/-2 (formerly known as HMGB-1/-2) on estrogen receptor \( \alpha \) (ER\( \alpha \)) and ER\( \beta \). Both ER subtypes were responsive to HMGB-1/-2 with respect to enhancement of receptor DNA binding affinity and transcriptional activity in cells. Responsiveness to HMGB-1/-2 was dependent on the C-terminal extension (CTE) region of the ER DNA binding domain (DBD) and correlated with a direct protein interaction between HMGB-1/-2 and the CTE. Thus the previously reported higher DNA binding affinity and transcription activity of ER\( \alpha \) as compared with ER\( \beta \) is not due to a lack of ER\( \beta \) interaction with HMGB-1/-2. Using chimeric receptor DBDs, the higher intrinsic DNA binding affinity of ER\( \alpha \) than ER\( \beta \) was shown to be due to a unique property of the ER\( \alpha \) CTE, independent of HMGB-1/-2. The CTE of both ER subtypes was also shown to be required for interaction withERE half-sites. These studies reveal the importance of the CTE and HMGB-1/-2 for ER\( \alpha \) and ER\( \beta \) interaction with their cognate target DNAs.

Nuclear hormone receptors comprise a superfamily of ligand-dependent transcription factors that regulate gene expression through interaction with specific hormone response elements (HREs)\(^3\) in target genes. The superfamily can be subdivided into: 1) classical steroid hormone receptors that typically interact with palindromic hexameric HREs as homodimers, 2) non-steroidal or class II nuclear receptors for ligands such as thyroid hormone, retinoic acid, vitamin D, and fatty acids, that function primarily as heterodimers with RXR (retinoid X receptor) bound to direct repeat HREs, and 3) orphan receptors without known ligands that interact with HREs in various dimer and monomer configurations. The nuclear receptors are related through a common domain structure including conserved C-terminal ligand binding (LBD) and centrally located DNA binding domains (DBD), and a variable N-terminal domain that is required in many nuclear receptors for maximal transcription activity (Refs. 1 and 2, reviews). The DBD consists of a highly conserved core with two asymmetric zinc fingers and an \( \sim 30 \) amino acid segment, termed the C-terminal extension (CTE) (Fig. 1A). Within the core DBD, \( \alpha \)-helix 1 extends between the two zinc fingers and makes base specific contacts in the major groove of the HRE DNA. The second \( \alpha \)-helix (helix 2) does not contact DNA but is important for the overall folding of the core DBD (3–5). The CTE is not conserved and adopts different structural motifs dependent on the class of nuclear receptor (6, 7). Nonetheless, the CTE of different receptors does appear to share a functional role to stabilize the receptor-DNA complex by extending the protein-DNA interface beyond that of base-specific contacts made by the core DBD. The CTE of class II receptors (TR and VDR) forms an \( \alpha \)-helix (helix 3) that projects across the minor groove between HRE half-sites, making extensive contacts along the phosphate backbone required for high affinity DNA binding and correct spacing with the RXR heterodimer (8, 9). The CTE of orphan receptors forms an extended loop conformation that makes base-specific contacts in the minor groove immediately \( 5' \) of the HRE. A short peptide motif termed a “GRIP-box” (RXGrZP where \( \lambda \) is any amino acid and \( Z \) is a hydrophobic residue) mediates interaction of orphan receptor CTEs with the minor groove (10, 11). The CTE is also required for monomeric orphan receptor recognition of extended HRE half-sites through interaction with specific tri-nucleotide sequences in the minor groove just \( 5' \) of the HRE (12, 13).

Although no structural information is available as yet, biochemical evidence indicates the CTE of steroid receptors also has a role in mediating high affinity DNA binding, by interacting with high mobility group proteins, HMGB-1 and HMGB-2, that function to facilitate receptor binding to cognate target DNA sites (14). HMGB-1 and closely related HMGB-2 (formerly known as HMG-1 and -2) are members of a family of proteins that bind to duplex B-DNA with moderate affinity and little sequence specificity, but recognize and bind with high affinity to various distorted DNA structures. The nomenclature of HMGB-1 and -2 was adopted in 2001 to designate the canonical HMG “Box” DNA binding domains of these proteins (15). In addition to recognizing distorted DNA targets, the HMG Box also binds in the minor groove and induces sharp bends and distortions in linear duplex DNA. Thus by increasing the flex-

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••The abbreviations used are: HRE, hormone response element; GST, glutathione S-transferase; DTT, dithiothreitol; DBD, DNA binding domain; ER, estrogen receptor; CTE, C-terminal extension; EMSA, electrophoretic mobility shift assay.

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Fig. 1. The estrogen receptor DBD. Panel A, amino acid sequence and structural organization of the core and CTE of the ERα DBD. The DNA recognition α-helix (helix 1) and helix 2 are indicated by gray circles. The conserved glycine-methionine motif is underlined at the boundary of the zinc finger core and CTE. Panel B, sequence alignment of the CTEs of steroid receptors and the orphan receptors SF-1 and ERR. The GRIP-box sequences (RGGR) of SF-1 and ERR and the GRIP-box-like sequence in ERs are underlined.

...ibility of DNA, HMGB-1/-2 are thought to have a general architectural role in the assembly of nucleoprotein complexes involved in regulation of transcription (see reviews in Refs. 16–18). Although HMGB-1/2 enhance DNA binding and transcription activity of all steroid hormone receptors analyzed, including receptors for progesterone (PR), androgen (AR), glucocorticoids (GR), mineralocorticoids (MR), and estrogen (ERα), they have no influence on class II nuclear receptors (19–21). This selective effect on the steroid class of nuclear receptors is dependent on the CTE and correlates with a direct protein interaction between HMGB-1/-2 and the CTE that does not occur with class II receptors (14, 20). HMGB-1/-2 also interacts with select groups of apparently unrelated sequence-specific transcription factors including p53 (22), p73 (23), Hox proteins (24), Oct proteins (25), Rel family members (26), and EBV transcription factors Rta and Zebra (27, 28), to enhance their binding to cognate DNA sequences and transcription activity.

The biological actions of estrogen are mediated by two estrogen receptor subtypes, ERα and ERβ, expressed from separate genes (29). The precise physiological role of the two ER subtypes is not yet well defined. Genetic ablation experiments in mice suggest the effects of estrogen on development and differentiation of female reproductive target tissues are mediated predominantly by ERα, whereas ERβ is more important in development of the ovary and perhaps other non-reproductive tissues (Ref. 30 for review). ERα and ERβ have high and low DNA-binding affinities for estrogen response elements (EREs) than ERβ (35–37), and only ERα is capable of inducing a directed bend in ERE target DNA (38). The differential ability to induce a directed bend in DNA has been attributed to the CTEs of ERα and ERβ (38). The mechanism for the higher DNA affinity of ERα is not known, but is likely due to differences in the CTE or other regions outside the highly conserved core DBDs.

The only member of the steroid class of nuclear receptors that has not been analyzed for interaction with HMGB-1/2 is ERβ. Therefore, in the present study, we sought to further examine the role of the steroid receptor CTE in DNA binding and response to HMGB-1/2 by performing a comparative analysis of ERα and ERβ. HMGB-1/-2 stimulated DNA binding and transcription activity of both ERα and ERβ, and the CTE was required for physical interaction with and functional responsiveness to HMGB-1/-2. The CTE was also found to be responsible for the differential intrinsic DNA binding affinities of ERα and ERβ independent of HMGB-1/-2. Unexpectedly, the CTE was also required for ERα and ERβ interaction with half-site EREs.

**EXPERIMENTAL PROCEDURES**

Expression and Purification of GST Fusion Proteins—The expression vectors for GST-HMGB-1 and the ERα DBD (amino acids 198–286) have been described previously (14). All other DNA-binding domain (DBD) vectors were constructed similarly by subcloning into pGEX2T (Amersham Biosciences), with an in-frame glutathione S-transferase (GST) for expression as a GST fusion protein. Chimeric DBDs were subcloned using “splicing by PCR overlap extension” as previously described (14), and also inserted into pGEX2T (Amersham Biosciences).

DBD-GST fusion proteins were expressed in BL21 bacterial cells and purified by a three-step procedure including affinity chromatography on glutathione-Sepharose resins and thrombin cleavage to remove the GST moiety, DNA cellulose, and FPLC gel filtration by Superdex-30 chromatography. The final product was concentrated by an Amicon stirred cell concentrator (39). Purified DBD concentrations were deter-
mined by comparison to known concentrations of lysozyme on silver stain SDS gel electrophoresis. Relative DNA binding affinity differences were verified using independently purified DBDs, and protein concentrations were determined independently by Lowry assay and UV spectrophotometric quantitation methods using extinction coefficients of 13,260 for both ERα and ERβ DBDs.

Production and Purification of Baculovirus-expressed Recombinant Proteins—Full-length human PR-A and HMGB-2 with N-terminal 6× histidine tags, and human ERα and ERβ with N-terminal FLAG sequences (DYCDDDDK) were expressed from baculovirus vectors in Sf9 insect cells as described (19, 40). For ERα and ERβ, estradiol 17β (200 nm) was added to Sf9 cell cultures to activate receptors during expression in vivo. His-tagged proteins were purified by nickel affinity resins as previously described for PR except that HMGB-2 was dialyzed against 20 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, and 1 mM DTT to exchange the β-mercaptoethanol for DTT and prevent oxidation (14, 16–19). FLAG-tagged ERα and ERβ were purified as described (40) except that the receptors were eluted from anti-FLAG-affinity resins in a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 20% glycerol, 50 μM ZnCl2, 0.2 mM EDTA, 2 mM DTT, 0.1% Nonidet P-40, 0.1 mg/ml FLAG peptide, and 0.5 mg/ml insulin. The eluates were then dialyzed against elution buffer with higher Nonidet P-40 (0.2%) and lacking the FLAG peptide. Purified proteins were analyzed by silver stain or Coomassie Blue-stained SDS gel electrophoresis and judged to be ≥90% pure.

Electrophoretic Mobility Shift Assays (EMSAs)—EMSAs for full-length ERα and ERβ were performed as described previously (14, 19) in a DNA binding reaction containing 10 mM HEPES, pH 7.8, 100 mM KCl, 20% glycerol, 4 mM MgCl2, 1 mM EDTA 0.1 μg poly(dI-dC), 5 μg of ovalbumin, and 0.6 μM 32P-labeled duplex DNA fragments formed from the appropriate oligonucleotides. Binding reactions were carried out for 30 min at 4 °C and samples were then electrophoresed at 4 °C on non-denaturing 5% polyacrylamide gels (40:1 acrylamide/bisacrylamide ratio) containing 2.5% glycerol impregnated in the gels, and the 0.25× TBE (0.02 mM Tris, pH 8.0, 0.02 mM boric acid, 0.5 mM EDTA) as running buffer. Isolated DBDs used a binding reaction containing 10 mM Tris, pH 8.0, 50 mM KCl, 6% glycerol, 1 mM EDTA, 100 ng of poly(dI-dC), and 0.1% Nonidet P-40. Beads were added and incubated in suspension for 1 h (250 μl) at 4 °C. Beads were then pelleted, washed in binding buffer, and protein was eluted in SDS sample buffer and analyzed by Western immunoblot with a rabbit polyclonal antiserum raised against peptide sequences in the ER DBDs (42).

RESULTS AND DISCUSSION

HMGB Enhances the DNA Binding Affinity and Transcriptional Activity of ERβ—Although ERα and ERβ can activate many of the same target genes in response to estrogen, ERβ has been reported to exhibit a 3–4-fold lower affinity for EREs and as much as a 10-fold weaker transcriptional activity than ERα (31–37). Therefore, we sought to determine whether a difference in interaction with HMGB-1/2 contributes to these distinct activities of ERα and ERβ. We previously found that HMGB-1 and -2 are functionally interchangeable with respect to stimulating the DNA binding affinity of steroid receptors in vitro and enhancing transcriptional activity in mammalian cells (19). Therefore, HMGB-1 and -2 were used interchangeably and are collectively referred to as HMGB in the remainder of this article. Receptor DNA binding was analyzed by electrophoretic gel mobility shift assay (EMSA) by varying the concentration of receptors in the presence of a constant amount of 32P-labeled DNA probe. The DNA duplex fragment contains a consensus palindromic ERE based on the well-characterized ERE in the vitellogenin gene (36) that is recognized by human and other species of ER. As shown in Fig. 2 A and B, purified full-length ERα and ERβ bound to ER-Epal in a dose-dependent, saturable manner. Based on apparent dissociation constants (Kd(app)) estimated from the ER concentration at half-maximal DNA binding, ERα bound to ER-Epal with a 4-fold higher affinity than ERβ (Fig. 2B). This affinity difference is consistent with previous reports that ERα has a higher affinity for ER-Epal than ERβ (35–37). For both ERs, addition of HMGB significantly left-shifted the DNA binding curves (Fig. 2B), indicating an increase in binding affinity for ER-Epal. In the absence of HMGB, ERα and ERβ produced two protein-DNA complexes and both contained ERα or ERβ, respectively, as demonstrated by supershifts with ERα- or ERβ-specific antibodies (Fig. 2A). The exact nature of the two mobility ER complexes is not known. Both complexes were obtained in the absence of HMGB, indicating that they do not represent ER complexes containing and lacking HMGB. Because it is well accepted that ER preferentially binds to ER-Epal as a dimer, suggests the faster mobility complex contains an ER dimer, whereas the slower mobility complex contains a higher order ER oligomer (Fig. 2A). At low concentrations of ER (α and β), addition of HMGB stimulated the formation of both mobility complexes, but at high receptor concentrations, HMGB predominantly stimulated the slower mobility complex concomitant with a decrease of the faster mobility complex (Fig. 2A). Multiple mobility ER complexes have been reported previously, but the relevance of the different complexes to ER function is not known (36). HMGB was not retained as a stable component of the stimulated complexes, as they have the same mobility as the unstimulated complexes (Fig. 2A) and are not supershifted by HMGB antibodies (data not shown). Ternary HMGB complexes by EMSAs have not typically been detected with steroid receptors or other transcription factors, suggesting HMGB acts as a DNA chaperone in a transient manner to facilitate binding of transcription factors to their cognate target DNA (18–27).

To determine the influence of HMGB on the transcription activity of ERβ, cell-based transcription assays were performed by transfection of ER negative CV-1 or COS-1 cells with ERα or ERβ DBDs.

Interaction with the CTE of ERα/β DBD

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To determine the influence of HMGB on the transcription activity of ERβ, cell-based transcription assays were performed by transfection of ER negative CV-1 or COS-1 cells with ERα or ERβ DBDs.
expression plasmids, a luciferase reporter gene consisting of five copies of the vitellogenin ERE inserted in place of GRE/PRE sequences of MMTV (MMTV-ERE) as previously described (43), and varying amounts of expression plasmid for HMGB-1. As shown in Fig. 2C, estrogen induction of MMTV-ERE-luc was mediated by ERα/H9251 and ERα/H9252 in a receptor plasmid dose-dependent manner, except that ERα/H9251 mediated a higher level of luciferase expression than ERα/H9252 at all doses. Thus, under these conditions, we confirm the higher transcriptional potency of ERα/H9251 (31, 32). Ectopic expression of HMGB-1 stimulated estrogen-induced ERα-mediated activation of the ERE-luc reporter gene expression in a dose-dependent manner by ~3-fold at the highest amount of HMGB-1. Under the same conditions, HMGB-1 enhanced ERβ mediated transcription by approximately the same amount (Fig. 2D). These are the first data to show HMGB enhancement of ERβ DNA binding and transcription activity and thus extend and complete previous results indicating HMGB is a common co-regulatory protein for all steroid receptors (7, 14, 19, 20, 21). These results also suggest that the higher DNA binding and transcription activity of ERα over ERβ is not due to a lack of ERβ responsiveness to the HMGB. Differences in DNA binding affinity and transcriptional activity of ERα and ERβ were largely maintained under HMGB stimulated conditions.

**Fig. 2.** Full-length ERα has a higher affinity for EREpal than ERβ. Varying amounts of purified ERα (panel A, 0–100 nM) or ERβ (panel B, 0–250 nM) were analyzed by EMSA for binding to a palindromic 32P-labeled ERE duplex DNA (EREpal) in the presence or absence of purified, recombinant HMGB-2 (300 ng). The ER-DNA complexes supershifted with monoclonal antibodies specific to ERα or ERβ. Panel B, free DNA and specific protein-DNA complexes were quantitated by phosphorimaging analysis and the data were plotted as fraction of DNA bound versus ER concentration. Values are the average of at least three independent experiments (n = 3, ± S.E.). Curve fits were performed as described under “Experimental Procedures.” Panel C, CV-1 cells were co-transfected with MMTV-ERE-luc (200 ng/well) and with varying amounts of either ERα or ERβ plasmids as indicated in the figure. At 24 h after transfection, cells were treated for 24 h with or without estradiol 17β (10 nM) and luciferase activity was measured and normalized to an internal β-galactosidase control as described. Values are from a single representative experiment. Panel D, COS-1 cells were co-transfected with MMTV-ERE-luc (200 ng), and a single amount of either ERα (0.1 ng), or ERβ (0.25 ng), with varying amounts of HMGB-1 plasmids (10 to 125 ng), and treated for 24 h with vehicle or 10 nM estradiol 17β. Luciferase activity was normalized to an internal β-galactosidase control (1.5 ng) and expressed as relative units. Values are averages of four independent experiments (n = 4, ± S.E.)

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**A)**

**B)**

**C)**

**D)**
The CTE of ERα and ERβ Is Required for Physical and Functional Interaction with HMGB-1/2—In our previous studies of PR, the CTE was required not only for protein interaction with HMGB and for HMGB enhancement of DNA binding in vitro, but also for HMGB stimulation of transcriptional activity in mammalian cells. This was shown by use of receptor chimeras consisting of swaps between the CTEs of PR and TR. In cell-based transcription assays, enhancement of PR transcription activity by ectopic expression of HMGB was largely dependent on the PR CTE and substantially reduced by the presence of the TR CTE (14).

To determine whether the CTE of ERα and ERβ has a similar role as it does in PR, we analyzed the effect of HMGB on DNA binding by ER DBDs containing or lacking the CTE (Fig. 3A). Full-length ER DBDs exhibited dose-dependent, saturable binding to EREpal. Addition of purified HMGB-2 (300 ng) to ER DBDs (0–8 nM) increased DNA binding in a dose-dependent manner (Fig. 3B). The CTE of ER DBDs was required for HMGB interaction, as shown by the decrease in DNA binding when the CTE was deleted (Fig. 3C).

Panel D shows the effect of HMGB on DNA binding by ER DBDs containing or lacking the CTE. Full-length ER DBDs lacked the CTE, whereas ERαt DBD (0–19 nM) and ERβt DBD (0–10 nM) were incubated with EREpal DNA (0.6 nM) in the absence or presence of purified HMGB-2 (300 ng). The CTE of ER DBDs was required for HMGB interaction, as shown by the decrease in DNA binding when the CTE was deleted (Fig. 3D).

Panel E shows the effect of HMGB on DNA binding by GST-pull-down assay with full and truncated ER DBDs. Bacterial lysates expressing GST or GST-HMGB-1 were immobilized to glutathione-Sepharose beads and incubated with ERα, ERαt, ERβ, and ERβt DBDs. Interacting DBDs were detected by immunoblot with a polyclonal antibody for the ER core DBD (42).
HMG increased the apparent binding affinity of both ER DBDs, enabling the formation of DNA complexes at lower concentrations than observed in the absence of HMG (Fig. 3, B and C). The $K_{d_{app}}$ for ERα DBD binding to EREpal was $0.54 \times 10^{-9}$ M, while ERβ DBD exhibited an ~3-fold lower affinity with a $K_{d_{app}}$ of $1.53 \times 10^{-9}$ M (Table I). Addition of HMG increased the apparent DNA binding affinity of ERα DBD by 4.2-fold ($K_{d_{app}}$ of ERα DBD to 0.13 $\times 10^{-9}$ M) and ERβ DBD by 2.5-fold (0.61 $\times 10^{-9}$ M) (Table I). As with full-length ER, multiple mobility complexes were detected with ER DBDs, including predominantly ER DBD dimers and small amounts of monomer and higher order complexes. HMG enhanced ERα DBD-DNA complexes primarily as dimers and high order oligomers (Fig. 3B). Similar complexes were also observed with the ERβ DBD; however, the higher-order complexes were less abundant than those observed with ERα (Fig. 3B). These results indicate that the DBD of both ER subtypes contain elements sufficient for HMG stimulation of receptor DNA binding, and that the affinity difference of ERα and ERβ for EREpal is largely due to intrinsic differences in their respective DBDs. Truncated DBD constructs, termed ERαt and ERβt, contained the core DBD through the conserved Gly-Met, but lacked most of the CTE (Fig. 3A). The truncated ER DBDs bound to EREpal primarily as dimers with no evidence of higher order complexes (Fig. 3D). In contrast to DBDs with the CTE, addition of HMG had no effect on binding of either truncated ERα or ERβ DBDs to an EREpal (Fig. 3D). Thus, the CTEs of both ERα and ERβ are required for functional response to HMG-2 in terms of enhancement of receptor binding to specific DNA sequences.

To determine whether the ER CTE is also required for direct protein interaction with HMG, we performed GST pull-down assays with full-length (core zinc finger plus CTE) and truncated (monomer and higher order complexes. HMGB enhanced ERα DBD-DNA complexes primarily as dimers and high order oligomers (Fig. 3B). Similar complexes were also observed with the ERβ DBD; however, the higher-order complexes were less abundant than those observed with ERα (Fig. 3B). These results indicate that the DBD of both ER subtypes contain elements sufficient for HMG stimulation of receptor DNA binding, and that the affinity difference of ERα and ERβ for EREpal is largely due to intrinsic differences in their respective DBDs. Truncated DBD constructs, termed ERαt and ERβt, contained the core DBD through the conserved Gly-Met, but lacked most of the CTE (Fig. 3A). The truncated ER DBDs bound to EREpal primarily as dimers with no evidence of higher order complexes (Fig. 3D). In contrast to DBDs with the CTE, addition of HMG had no effect on binding of either truncated ERα or ERβ DBDs to an EREpal (Fig. 3D). Thus, the CTEs of both ERα and ERβ are required for functional response to HMG-2 in terms of enhancement of receptor binding to specific DNA sequences.

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The mechanism by which HMG interacts specifically with the CTE of steroid receptors, and how this interaction enhances DNA binding and transcription, is not known. Facilitated binding of transcription factors to their cognate DNA sites by HMG is thought to be due to the DNA binding and DNA bending properties of HMG, whereby HMG helps to induce or stabilize a DNA conformation that forms the most favorable binding site for the transcription factor. However, because HMG has minimal specificity for binding DNA sequences, recruitment to specific promoters likely requires targeting through protein-protein interaction with a sequence-specific DNA-binding protein. In addition to simply recruiting HMG to specific promoters for the purpose of manipulating DNA structure, our data suggest that protein interaction with HMG may also have a role in directly influencing the DNA binding activity of steroid receptors. The CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding. In contrast, the CTEs of class II and orphan nuclear receptors are structured and directly participate in DNA binding.

### Table I

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**Fig. 4.** The CTE is responsible for the higher DNA binding affinity of ERα than ERβ. Panel A, schematic of ERα/ERβ DBD chimeras. Panel B, varying concentrations of purified ERα/β DBD (0–250 nM) or ERβ/α DBD (0–8 nM) were incubated with EREpal DNA (0.6 nm) in the absence or presence of purified HMG-2 (300 ng). The arrowhead refers to higher order protein-DNA complexes. Free DNA (F), dimer (D), Panel C, quantitative analysis of DNA binding by the ERα (open circles), ERβ (open squares), ERβ/α (closed circles), and ERα/β (closed squares) DBDs to EREpal DNA. Gels in Panel B were quantitated by phosphorimager analysis, and data were plotted as fraction of bound DNA versus DBD concentration. Data are averages of three independent assays ($n = 3$, ± S.E.).
The CTE Is Responsible for the Higher DNA Binding Affinity of ERα—Because the ERα and ERβ core DBDs share greater than 95% amino acid identity, we sought to determine whether the non-conserved CTE region was responsible for the DNA binding affinity difference between the ERα and ERβ. We created chimeric DBDs in which the CTEs were swapped between the ER subtypes (Fig. 4A). The ERβ/α chimera contained the ERβ core DBD fused to the ERα CTE; conversely, the ERα/β chimera consisted of the core DBD of ERα fused to the CTE of ERβ (Fig. 4A). We compared DNA binding of these chimeric DBDs with that of the ERα and ERβ DBDs in quantitative EMSA (Fig. 4, B and C). The binding curve for the ERβ/α DBD chimera was nearly superimposable with that of ERα DBD (Fig. 4C), exhibiting a 6-fold higher apparent DNA binding affinity than that of ERβ DBD (Table I, $K_{dapp} = 0.26 \times 10^{-9}$ M for ERβ/α DBD and $1.53 \times 10^{-9}$ M for the ERβ DBD). Conversely, the ERα/β chimera exhibited ~3 fold lower affinity than that of ERα DBD (Table I, $K_{dapp} = 1.9 \times 10^{-9}$ M for ERα/β and $K_{dapp} = 0.54 \times 10^{-9}$ M for ERα DBD). Thus domain-swapping resulted in a substantial switch in the DNA binding affinities of the two ER subtypes, indicating the CTE is largely responsible for the affinity differences between ERα and ERβ. How the CTE of ERα contributes to a higher DNA binding affinity than ERβ is not known. Schultz et al. (38) showed by similar domain-swapping experiments that the CTE accounted for the differential ability of ERα and ERβ to bend DNA. Deformation of DNA structure could lead to a more stable protein-DNA complex through altering protein-DNA contacts or lowering the energetics of binding.

A previous study by one of the authors (32) showed that ERα and ERβ bind to tandemly arrayed EREs assembled into chromatin in vitro with similar apparent affinities and can initiate chromatin remodeling on those templates with similar efficiencies. Under such conditions (i.e. with multiple EREs), the intrinsic differences in the potencies of the ERα and ERβ activation domains are maximized. Likewise, the intrinsic differences in their DNA binding activities may be minimized. In vivo, where the estrogen-responsive genes studied to date typically
have a single imperfect ERE with or without flanking ERE half-sites, the 3–4-fold differences in the DNA binding affinities of ERα and ERβ that we have observed in our assays with single EREs may play a significant role in ER subtype-specific gene regulation.

ERα and ERβ Exhibit a Similar Ability to Interact with ERE Half-sites Dependent on the CTE—A previous study from Vanacker et al. (44) reported that ERα, but not ERβ, was capable of binding ERE half-site elements in vitro and mediating functional responses from ERE half-sites in cells. Furthermore, ERE half-site binding by ERα required a specific TCA trinucleotide sequence immediately flanking the 5′-end of the ERE half-site, reminiscent of how orphan receptor monomers interact stably with extended half-site HREs through the GRIP box of the CTE. The CTE of ERs (Fig. 1A), but not ERβ, contains a GRIP box-like sequence similar to the orphan receptors SF-1 (steroidogenic factor 1) and ERR (estrogen-related receptor) (11, 45) that require CA dinucleotides at the 5′-end of HRE half-sites. This suggested to us that the ERα CTE might be responsible for the reported ability of ERα, but not ERβ, to bind to ERE half-site elements (44). However, when we compared binding of purified full-length ERα and ERβ to ERE half-site probes containing TCA or the mutated TAG 5′-flanking trinucleotide sequence (Fig. 5A), both ER subtypes bound equally well to either probe (Fig. 5, B and C). Binding of ERα and ERβ to the ERE half-sites was also stimulated in a similar manner by the addition of HMGB-2 (Fig. 5, B and C). As with binding of full-length ERα and ERβ to ERE half-sites, two mobility complexes were obtained with ERE half-site probes, although the slower mobility complex with ERβ is much weaker than that formed by ERα (Fig. 5, B and C). The mobilities of the two complexes on ERE half-sites are similar to those obtained with EREpal (Fig. 2), suggesting they also contain an ER dimer (faster complex) and higher order ER oligomer (slower complex). Because full-length ER dimerizes in solution through the ligand binding domain, ER dimers can interact with ERE half-sites through one of the DBD subunits and would be expected to have the same mobility by EMSA as an ER dimer bound to EREpal.

Because the dimerization domain in the DBD is dependent on DNA binding, the ER DBDs, as expected, bind to the ERE half-sites as monomers instead of dimers (Fig. 6, A and B). The ERα and ERβ DBDs also bound equally to both TCA- and TAG-ERE half-site probes (Fig. 6, A and B), and addition of HMGB increased the affinity of both ER subtype DBDs for the ERE half-sites by a similar 5-fold (Table I). Similar to results with EREpal, the ERα DBD has 3-fold higher affinity than the ERβ DBD for ERE half-sites (Table I). Thus our results do not show a differential ability of ERα and ERβ to recognize ERE half-sites and further suggests that the GRIP box-like sequence of ERα CTE does not confer TCA specificity for sequences flanking the ERE-half as it does with orphan receptors. Why our results differ from that of Vanacker et al. (44) is not known, but could be due to different experimental methods and conditions. They used citro synthesized ERα and ERβ from a rabbit reticulocyte lysate coupled transcription/translation assay, whereas our results were obtained with highly purified, baculovirus-expressed ERs. Receptors may be modified differently in the two systems or may be influenced by other interacting factors in the rabbit reticulocyte lysate system. Additionally, Vanacker et al. (44) analyzed the requirements of flanking sequences indirectly by competition with unlabeled DNA duplexes for binding of ER to the radiolabeled TCA-ERE half-site probe. We analyzed direct binding of ERs to both [32P]TCA and TAG ERE half-sites, which does not have the inherent limitation of estimating relative binding affinities by competition.

Despite the fact that the CTE was not required for ER binding to a full EREpal probe (Fig. 3D), we observed that it was required for binding to ERE half-sites independent of 5′-flanking sequences. Neither of the ER DBDs lacking the CTE interacted with either extended ERE half-site probe, even at the highest concentrations of receptors $-2 \times 10^{-6}$ M (Fig. 6C). As anticipated, addition of HMGB-2 did not promote interaction of these truncated ER DBDs with ERE half-sites, as the CTE is required for protein interaction with HMGB (not shown). Therefore, the CTE is essential for ERα and ERβ interaction with an ERE half-site element.

Most characterized estrogen responsive target genes contain EREs that diverge from the consensus palindrome ERE, or contain multiple half-site HREs, suggesting that the requirement of the CTE for recognition of ERE half-sites may be physiologically relevant. In further support of a role for recognition of divergent target DNA sites, the CTE of the AR DBD was also shown to be required for AR recognition of a novel direct repeat androgen response element (46). How the ER CTE
mediates recognition of ERE half-sites is not known, but presumably occurs in a manner similar to that of class II and orphan nuclear receptors where the CTE makes additional nonspecific DNA contacts outside the HRE to stabilize the specific interactions of the core DBD.

Based on biochemical and structural studies, the CTE of class II and orphan nuclear receptors has been well documented to participate in stabilizing receptor binding to cognate target DNA sequences. In contrast, much less is known about the CTE of the steroid class of nuclear receptor. This paper extends our previous work on the PR CTE, now showing that the CTE of the steroid class of nuclear receptor. This paper explores the role of the CTE and how it interacts with HMGB and/or DNA.

REFERENCES

The Role of the C-terminal Extension (CTE) of the Estrogen Receptor α and β DNA Binding Domain in DNA Binding and Interaction with HMGB
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