Previously, we and others reported that the high mobility group proteins, HMGB-1/-2, enhance DNA binding in vitro and transactivation in situ by the steroid hormone subgroup of nuclear receptors but did not influence these functions of class II receptors. We show here that the DNA binding domain (DBD) is sufficient to account for the selective influence of HMGB-1/-2 on the steroid class of receptors. Furthermore, the use of chimeric DBDs reveals that this selectivity is dependent on the C-terminal extension (CTE), amino acid sequences adjacent to the zinc finger core DBD. HMGB-1/-2 interact directly with the DBDs of steroid but not class II receptors, and this interaction requires the CTE. This in vitro interaction correlates with a requirement of the CTE for maximal HMGB-1/-2 enhancement of DNA binding in vitro and transcriptional activation in cells. Finally, class II receptor DBDs have a much higher intrinsic affinity for DNA than steroid receptor DBDs, and this affinity difference is also dependent on the CTE. These results reveal the importance of the steroid receptor CTE for DNA binding affinity and functional response to HMGB-1/-2.

Nuclear hormone receptors comprise a superfamily of transcription factors that regulate diverse metabolic processes by binding to response elements in the enhancer regions of specific genes. This superfamily consists of three receptor subclasses: 1) the steroid hormone receptors for progesterone (PR), estrogen (ER), glucocorticoids (GR), androgens (AR), and mineralocorticoids (MR); 2) class II receptors for thyroid hormone (TR), retinoids (RAR and RXX), vitamin D3 (VDR), prostaglandins (PPAR), oxyysterols, and bile acids; and 3) orphan receptors for which no endogenous ligand has been identified (1-4). Each of the receptor subclasses is characterized by a unique mechanism of action with respect to dimerization and DNA sequence recognition. Steroid receptors form homodimers that optimally recognize hexameric DNA elements arranged as inverted repeats separated by three unspecified base pairs. PR, GR, AR, and MR bind to the core hexamer AGAACA, whereas ER recognizes AGGTCA (2). Class II receptors preferentially function as heterodimers with RXR and recognize the AGGTCA hexamer arranged as direct repeats. Variable spacing between the direct repeats determines the RXR heterodimer binding specificity. Class II receptors, particularly TR, can also recognize an inverted repeat as homodimers, or half-sites as monomers. Orphan receptors can bind to the AGGTCA hexamer arranged either as a direct repeat, palindrome, or half-site as heterodimers with RXR, homodimers, or monomers (1, 5-7).

DNA-bound nuclear receptors activate transcription through assembly of a coactivator protein complex (8-10). Some of these coactivators possess enzyme activities that are thought to facilitate access of general transcription factors to chromatin templates (11, 12). Additionally, the coactivator complex may serve as a protein bridge to facilitate assembly of the basal transcription apparatus (13, 14). We and others have identified another group of co-regulatory proteins, the high mobility group proteins 1 and 2 (HMGB-1/-2), that facilitate steroid receptor interaction with specific target DNA sequences and appear to be essential for maximal transcriptional activation by this subgroup of nuclear receptors (15-20).

HMGB-1/-2 proteins are ubiquitous, conserved, non-histone chromatin proteins that bind to the minor groove of DNA in a structure-specific, sequence-independent manner (21, 22). A clear physiological role for these proteins has not been defined; however, they have been implicated in processes that require manipulation of DNA structure and assembly of higher order nucleoprotein complexes, such as DNA replication and repair, recombination, and transcription (23, 24). In addition, HMGB-1/-2 proteins enhance DNA binding and transcriptional activity of a number of eukaryotic transcriptional activators, including octamer transcription factors (Oct-1, Oct-2, and Oct-6) (25), homeodomain protein HOXD9 (26), p53 (27), viral transcription factors (28, 29), Rel family members (30), and the steroid hormone receptors (15-20). However, not all sequence-specific transcription factors are influenced by HMGB-1/-2. Paull et al. showed that only a subset of transcriptional activators were influenced by loss of the HMGB proteins, NHP6A and 6B, in another group of co-regulatory proteins, the high mobility group proteins 1 and 2 (HMGB-1/-2), that facilitate steroid receptor interaction with specific target DNA sequences and appear to be essential for maximal transcriptional activation by this subgroup of nuclear receptors (15-20).
yeast (31). Additionally, we and others have shown that both HMGB-1/2 enhance DNA binding and transcriptional activity of the steroid hormone subclass of nuclear receptors while not affecting these functions of class II receptors, including RAR, VDR, or RXR (18, 20, 26).

The nuclear receptor core DBD consists of two zinc fingers and two α-helices. Helix1 makes base-specific contacts in the major groove, while helix2 maintains the overall structural fold of the core DBD (32–38). Both biochemical and structural analyses of the nuclear receptor DBDs have shown that this domain is highly conserved across all nuclear receptors. In addition to the core DBD, sequences located immediately C-terminal to the second zinc finger, termed the C-terminal extension (CTE), directly participate in DNA binding by the class II and orphan receptors. Unlike the core DBD, CTE sequences are not conserved among nuclear receptors, and the CTE adopts different structural motifs (39). However, these divergent structures share a common function to extend the protein–DNA interface beyond that of base-specific contacts in the major groove thus stabilizing DNA binding. Crystallization of a TRβ-RXRα DBD heterodimer bound to a direct repeat element demonstrated that the TRβ CTE forms an additional α-helix (helix3) distinct from the core DBD that projects across the minor groove of the DNA helix making contacts along the phosphate backbone (34).

NMR analysis of the RXR DBD in solution also revealed a third α-helix in the CTE (33). However, the crystal structure of the RXR DBD-DNA complex had a CTE with an extended structure, suggesting that the RXR CTE undergoes a conformational change upon binding to DNA (37, 38). The orphan receptor CTE contains a short, conserved amino acid sequence, termed the “GRIP-box”, with the consensus RXGZR, where “X” is any amino acid and “Z” is a hydrophobic residue. Structural analysis of orphan receptor DBDs bound to DNA as either a homodimer (RevErb, (40)) or monomer (NGFI-B, (36)), revealed the CTE lying along the minor groove of DNA in an extended conformation distinct from that observed in either of the class II receptor DBDs. This GRIP-box creates an additional protein–DNA interface beyond that of the core DBD that interacts with specific base pairs. In the absence of DNA the orphan receptor CTE is unstructured suggesting that it also undergoes a conformational change upon association with DNA (35). Biochemical analysis has also demonstrated the importance of the CTE for high affinity DNA binding because point mutations or truncation of the class II and orphan receptor CTEs reduces or abolishes DNA binding (33, 41–46). These results taken together suggest that the class II and orphan nuclear receptors have a bipartite DBD consisting of the core DBD that makes base-specific contacts with core hormone response elements (HRs) and the CTE that provides additional, largely nonspecific DNA contacts that stabilize the protein–DNA complex.

There is no structural evidence for the existence of an equivalent CTE in the steroid class of nuclear receptors. Structural studies of the ERα and GR DBDs complexed to DNA and in solution either lacked the comparable length CTE sequences in the expressed DBD constructs or the CTE was unstructured (32). Aside from an earlier report that sequences C-terminal to the core ERα DBD are important for DNA binding stability (47), no other biochemical data are available to suggest that the CTE of steroid receptors directly participates in DNA binding as it does with other nuclear receptors.

Here, we determine the mechanistic basis for the selective influence of HMGB-1/2 on the steroid subclass of nuclear receptors. Through biochemical analysis of purified DBDs, we show that HMGB-1/2 selectively influence DNA binding by the DBDs for steroid but not class II receptors. By use of chimeric DBDs, in which the CTE was swapped between steroid and class II receptors, we demonstrate that the CTE is responsible for the differential influence of HMGB-1/2 on the two classes of nuclear receptors. Furthermore, class II receptor DBDs exhibited a much higher intrinsic affinity for their target DNAs than the steroid receptor DBDs, a difference also attributed to the CTE. Finally, we show that the CTE of steroid receptors is required for direct interaction with HMGB-1/2 and for maximal HMGB-1/2-enhanced DNA binding in vitro and transcriptional activation in cells. These results demonstrate that the CTE of steroid receptors plays a role in DNA binding but acts differently than class II DBDs by a mechanism that involves interaction with HMGB-1/2.

**EXPERIMENTAL PROCEDURES**

**Bacterial Expression Vectors—**Expression vectors for RXRα (aa. 130–223) and TRβ (aa. 97–207) DBDs have been previously described (6, 33) and were obtained from Ron Evans, Salk Institute, San Diego, CA, and Thomas Perlmann, Ludwig Institute for Cancer Research, Stockholm, Sweden, respectively. All other DBD vectors were subcloned by PCR amplification of DNA encoding the appropriate DBD and insertion into the BamHI and EcoRI restriction sites of the GST fusion vector, pGEX2T (Amersham Biosciences). PCR-generated fragments were purified by dideoxy sequencing (Sequenase 2.0, USB, Cleveland, OH) and were determined to be in frame with the GST tag. An EcoRI-SalI fragment containing the HMGB-1 cDNA was excised from the pBlueBacHis2B-HMGB-1 plasmid, previously described (18), and inserted into the EcoRI and SalI sites in pGEX4T1 (Amersham Biosciences). Cloning junctions were sequenced by dideoxy sequencing.

Chimeric DNA binding domains were subcloned using “splicing by overlap extension” (48). In brief, this technique involved two consecutive PCR reactions. The first reaction amplified the core DBDs and CTEs separately using primers containing sequences complementary to the “splice junction”. In the second step, the PCR products were mixed with primers to the distal ends. Upon denaturation, reannealing, and amplification, the final PCR product contained the core DBD of one receptor fused at a precise junction to the CTE of another. The chimeric PCR products were inserted into pGEX2T (Amersham Biosciences) using BamHI and EcoRI restriction sites in the polylinker and were sequenced to verify that the DBDs were in frame with the GST tag and that the chimeric junction was correct.

**Expression and Purification of GST Fusion Proteins—**GST fusion proteins were purified from BL21 cells as previously described (49). Purified recombinant proteins were dialyzed against 20 mM Tris, pH 8.0, 50 mM NaCl, 1 mM DTT, and 1 mM ZnCl2. Purified proteins were analyzed by silver stain of SDS-PAGE gels and judged to be at least 90% pure.

**Production and Purification of Baculovirus-expressed Proteins—**Recombinant baculovirus vectors that express FLAG-tagged human ERα and His-tagged human TRβ and RXRα were obtained from W. Lee Kraus, Cornell University, Ithaca, NY, Nancy Weigel, Baylor College of Medicine, Houston, TX, and Dave Clemm, Ligand Pharmaceuticals, San Diego, CA, respectively. Receptors and HMGB-2 were expressed in Sf9 insect cells in Grace’s insect medium supplemented with 10% fetal bovine serum as previously described (18). 200 μM T3 for TRβ and 200 nM estradiol for ERα were added to Sf9 cell cultures for the last 24 h of expression. No ligand was added for HMGB-2 or RXRα.

Baculovirus expression and purification of His-tagged, human PR-A has previously been described (18). His-HMGB-2-expressing Sf9 cell pellets were lysed in a buffer containing 20 mM Tris, pH 8.0, 400 mM NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 5 mM imidazole, and a protease inhibitor mixture (50). His-TRβ or His-RXRα-expressing Sf9 cells were lysed in a buffer containing 20 mM Tris, pH 8.0, 400 mM NaCl, 10% glycerol, 5 mM ZnCl2, 5 mM β-mercaptoethanol, 2 mM imidazole, and a protease inhibitor mixture. All His-tagged proteins were purified by metal affinity resins as previously described for PR (18) 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 to prevent oxidation of HMGB-2 molecules. Receptors were dialyzed against 20 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 50 μM ZnCl2, and 5 mM DTT. Purified proteins were analyzed by silver stain of SDS gels and judged to be at >80% purity.

FLAG-tagged ERα was purified using a method previously described (51) except ERα was eluted from FLAG affinity resin 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 a buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 20% glycerol, 50 μM ZnCl₂, 0.2 mM EDTA, 2 mM DTT, and 0.2% Nonidet P-40.

Electrophoretic Mobility Shift Assays (EMSA)—EMSAs for full-length TRβ and RXRs were performed as described previously (18). Briefly, components of DNA-binding reactions were incubated for 30 min on ice in 10 mM HEPES, pH 7.8, 50 mM KCl, 4 mM MgCl₂, 1 mM DTT, and 12% glycerol in the presence of 0.2 μg of poly(dI-dC), and 1 μg of ovalbumin as carrier protein (25 μL of total volume). Labeled, specific oligonucleotide (0.6 mM) was added, and reactions were incubated for 30 min on ice. DNA binding reactions were then electrophoresed on non-denaturing 5% polyacrylamide gels (40:1 acrylamide/bisacrylamide ration) in 0.25× TBE buffer (0.02 M Tris borate, pH 8.0, 0.02 mM boric acid, 0.5 mM EDTA) at 4 °C. Antibody supershifts of TRβ/RXRs heterodimers were performed with a TRβ mouse IgG monoclonal antibody (clone 2386/G10) prepared against a synthetic peptide corresponding to amino acids 31–50 in the N terminus. EMSA for various receptor DBDs used similar methods, except that the binding reactions were carried out in 10 mM Tris, pH 8.0, 50 mM KCl, 6% glycerol, 1 mM DTT, 100 ng of poly(dI-dC), and 0.1% Nonidet P-40 or regulatory CA630 (Sigma) in 20 μL of total volume and were electrophoresed on 6% polyacrylamide gels. After electrophoresis, gels were dried, autoradiographed, and free [32P]DNA and [32P]DNA-protein complexes were quantitated by direct scanning of gels for radioactivity by a series 400 Amersham Biosciences PhosphorImager. Data were expressed graphically as the normalized fraction of DNA bound versus DBD concentration. The fraction-bound DNA was calculated as 1–(free DNA/(bound DNA + free DNA)). The data were normalized so that the fraction DNA bound at saturation was set to 1.0. All other values were calculated relative to 100%. For varying amounts of purified HMGB-2 (100–500 ng) or ovalbumin (500 ng). The heterodimer-DNA complex was supershifted with a monoclonal antibody specific to TRβ. Quantitative effects of HMGB on DNA binding affinity of TRβ/RXRs heterodimer. Varying concentrations of TRβ–RXRα heterodimers were incubated with a DR4 oligonucleotide (0.6 mM) in the absence (open circles) or presence (closed circles) of HMGB-2 (300 ng). The free DNA and specific protein-DNA complexes were quantitated by phosphorimaging analysis, and the data was plotted as fraction of DNA bound versus TRβ–RXRα heterodimer concentration. C, Cos1 cells were cotransfected with a PR-B expression vector (1 ng/well) and a PREC tk-LUC reporter gene (200 ng/well) or with a TRβ (0.5 ng/well) expression vector and a DR4-LUC reporter gene (200 ng/well) with or without increasing amounts of an HMGB-1 expression plasmid (Δ–50-fold of excess over transfected receptor plasmid). Cells were treated with ethyl phenyl (white bars), 10 mM R5020 (gray bars), or 100 mM T3 (black bars) for the last 24 h of transfection. Relative luciferase activity was calculated as described under "Experimental Procedures." The values are averages of three independent experiments (n = 3, ± S.E.).
FIG. 2. A, schematic of nuclear receptor core DBDs for human PR and TRβ. B, CTE sequences for steroid and class II receptor DBDs used in this study. Expressed DBDs contain the core DBD (A) consisting of the zinc fingers through the highly conserved GM diamin acid motif and enough C-terminal sequence (B) to encompass a CTE based on either the TRβ or RXRα DBD structure. C, purification of PR DBD651. The DBD was expressed in bacteria as a glutathione S-transferase fusion protein and purified in a two-step procedure as described under “Experimental Procedures.” GST-PR DBD651 fusion protein was expressed in bacteria as a glutathione-Sepharose column containing the partially purified GST-PR DBD651 fusion protein (lane 1), eluted from glutathione-Sepharose column (lane 2), the thrombin cleavage product containing free DBD and GST-moiety (lane 3), and the final purified PR DBD651 (lane 4) are shown. Fractions were analyzed by SDS-PAGE on a 12% polyacrylamide gel and subsequent silver staining.

FIG. 3. HMGB-2 stimulates DNA binding by steroid receptor DBDs. A, EMSA of PR DBD651. Varying concentrations of purified, recombinant PR DBD651 (0–1000 nM) were incubated with a palindromic PRE oligonucleotide (0.6 nM) in the absence (top panel) or presence (bottom panel) of purified, recombinant HMGB-2 (1000 nM). D, monomer complex and M, monomer complex. B, quantitative analysis of PR DBD651–DNA binding in the absence (open circles) or presence (closed circles) of HMGB-2. Gels as in A were quantitated by phosphorimaging analysis, and data was plotted as fraction of bound DNA versus DBD concentration. Data are averages of three independent assays (n = 3, ± S.E.). Curve fits were performed as described under “Experimental Procedures.” C, quantitative analysis of GR DBD binding to the PRE/GRE oligonucleotide (as described in B). D, quantitative analysis of ERα DBD binding to a palindromic ERE oligonucleotide (as described in B).

RESULTS

HMGB-1/-2 Selectively Enhance DNA Binding and Transactivation of a Steroid Receptor, PR, but Not a Class II Nuclear Receptor, TRβ—In previous studies we showed that HMGB-1/-2 increased steroid receptor DNA binding affinity in vitro and transactivation in intact cells but had no effect on class II receptors (18). The class II receptors analyzed included RXR, RAR, and VDR but not thyroid hormone receptor (TRβ) (18). Because TRβ has the best characterized CTE of the class II receptors, it was important for the present study to examine the effects of HMGB-1/-2 on the DNA binding and transactivation properties of TRβ. To complete our comparative analysis we examined the effects of HMGB-1/-2 on TRβ DNA binding and transactivation using PR as a steroid receptor control. TRβ binds as a heterodimer with RXRα to a direct repeat element separated by four intervening base pairs (DR4). In EMSA, purified TRβ formed a weak protein–DNA complex with the DR4 oligonucleotide, which was unaffected by the addition of HMGB-2 or an unrelated control protein, ovalbumin (Fig. 1A). Addition of purified RXRα induced formation of a TRβ–RXRα heterodimer complex, which was also unaffected by either HMGB-2 or ovalbumin. The TRβ–RXRα heterodimer complex was specific as shown by a supershift with a TR-specific antibody (Fig. 1A). The influence of HMGB-2 on the affinity of the TRβ–RXRα heterodimer for DR4 was determined by varying the concentration of the TRβ–RXRα heterodimer in the presence and absence of HMGB-2 (Fig. 1B). As shown in Fig. 1B,
the saturation DNA binding curves for the TRβ-RXRα heterodimer are overlapping in the presence or absence of HMGB-2, indicating that HMGB-2 does not affect the affinity of the TRβ-RXRα heterodimer for specific DNA. This contrasts with the effect of HMGB-1/-2 on DNA binding of purified PR; as previously reported, HMGB-1/-2 dramatically increase the apparent DNA binding affinity of PR for a PRE by 20–50-fold (15, 18).

To determine the influence of HMGB-1/-2 on transactivation by TRβ in cells, Cos-1 cells were transfected with expression plasmids for TRβ, HMGB-1, and a TRβ-dependent reporter gene (DR4-LUC); PR-B and a PREα tk-LUC reporter were included as a steroid receptor control. As expected, HMGB-1 enhanced PR-mediated, hormone-dependent transactivation of the PREα tk-LUC reporter gene by as much as 9-fold at the highest concentration of HMGB-1 plasmid (Fig. 1C). Under the same conditions HMGB-1 had no effect on T3-dependent, TR-mediated transactivation of DR4-LUC. These results support our previous observation that HMGB-1/2 stimulate both DNA binding and transactivation by steroid hormone receptors but does not affect these functions of class II receptors (18).

The Selective Influence of HMGB-1/-2 on Steroid Receptors Is Attributed to the DNA Binding Domain—To determine whether the DNA binding domain is sufficient to account for the differential effect of HMGB-1/2 on steroid versus class II receptors, we next analyzed the effect of HMGB-1/2 on the DNA binding properties of expressed DBDs from these two classes of nuclear receptors. The DBDs of PR, GR, and ERα were used as representative of the steroid subclass of receptors, while the TRβ and RXRα DBDs were analyzed as representative of class II receptors (Fig. 2). Expressed DBDs contained sequences corresponding to the core zinc binding modules through the conserved glycine-methionine (GM) motif (Fig. 2A) as well as C-terminal sequence sufficient to contain the CTE of class II receptors and equivalent length sequences from steroid hormone receptors (Fig. 2B). The DBDs were purified to near homogeneity as judged by silver stain SDS gels. By way of example, a purification of PR DBD651 is shown in Fig. 2 (90% purity) (49). The purified DBDs were then analyzed for DNA binding to their respective DNA elements in the presence and absence of recombinant, purified HMGB-2.

Fig. 3A shows results from gel mobility shift assays using the PR DBD670 (aa 552–651) as representative of steroid receptors (Figs. 2B and 3A). Increasing concentrations of PR DBD651 were incubated with a single concentration of [32P]-labeled, PRE/GRE-containing DNA oligonucleotide. The PR DBD651 exhibited dose-dependent binding to the PRE/GRE, primarily as a dimer, with some monomer complex also apparent (Fig. 3A). Binding was specific as determined by antibody supershift of the complex and competition with a specific oligonucleotide (20) (data not shown). Addition of HMGB-2 caused the DBD to bind DNA at lower concentrations indicating an enhancement of DNA binding. Estimation of the DBD concentrations at which half-maximal binding was achieved indicates that the apparent dissociation constants (Kapp) for the PR DBD651-PRE complex increased 9-fold from 140 × 10⁻¹⁰ m to 16 × 10⁻⁹ m in the absence and presence of HMGB-2, respectively (Table I). Because the PR DBD651 has a shorter CTE than that described for the TRβ DBD used in crystallization (see Fig. 2, A and B), we also examined DNA binding and HMGB-1/-2 effects on a PR DBD construct containing additional C-terminal sequence, PR DBD670 (aa 562–670), equal in length to that of the TRβ DBD (Fig. 2B). As summarized in Table I, the longer PR DBD670 had a DNA binding affinity similar to PR DBD651 (Kapp = 150 × 10⁻¹⁰ m and 17 × 10⁻⁹ m, respectively) and HMGB-1/2 stimulated DNA binding by approximately the same extent (8.7-fold).

We next analyzed the effects of HMGB-2 on DNA binding properties of a class II receptor DBD, TRβ. Addition of increasing concentrations of purified TRβ DBD to a constant amount of the DR4 probe yielded dose-dependent formation of both monomer- and dimer-DNA complexes (Fig. 4A). The monomer complexes constituted a greater proportion of bound DNA than dimer. Unlike the steroid receptor DBDs, HMGB-2 did not influence either TRβ DBD monomer- or dimer-DNA complexes. We quantitated the influence of HMGB-2 on the affinity of the TRβ DBD for a DR4 element (Fig. 4B) as well as an inverted repeat element, TREpal (Table I). TRβ bound both elements with a similar Kapp (3.8 × 10⁻⁹ m on DR4 and 2.4 × 10⁻⁹ m on TREpal, Table I), which were unaffected by HMGB-2 (Kapp = 3.8 × 10⁻⁹ m and 2.4 × 10⁻⁹ m, respectively) than the PR DBD constructs (Kapp values = 140 × 10⁻¹⁰ m for PR DBD651 and 150 × 10⁻⁹ m for PR DBD670, Table I). The affinity of PR DBDs for the PRE/GRE only approached that of the TRβ DBD in the presence of HMGB-2 (Kapp = 16–17 × 10⁻⁹ m; Table I). Thus the TRβ DBD has a substantially higher intrinsic affinity for its target DNA than the PR DBD.

To extend these analyses to other steroid and class II receptor DBDs, we also tested the influence of HMGB-1/-2 on the binding affinity of purified GR and ERα DBDs for their respective GRE and ERE palindromic target elements (Fig. 3, C and D), RXRα DBD for a DR1 element (Fig. 4C), and TRβ-RXRα

| Receptor DBD | DNA element | Kapp (× 10⁻⁹ m) | Fold HMGB effect |
|--------------|-------------|----------------|-----------------|
| Steroid receptors | PRE/GRE | 150 | 17 | 8.8 |
| PR<sub>651</sub> | PRE/GRE | 140 | 16 | 8.7 |
| PR<sub>642</sub> | PRE/GRE | 19 | 4.7 | 4.0 |
| GR | PRE/GRE | 16 | 2.1 | 7.6 |
| ERα | ERE | 5.5 | 0.82 | 6.7 |
| Class II receptors | | | | |
| TRβ | DR4 | 3.8 | 2.6 | 1.5 |
| RXRβ | DR1 | 3.1 | 1.8 | 1.7 |
| TRβ/RXRα | DR4 | 5.7 | 4.6 | 1.2 |
| Chimeric receptors | PRE | 24 | 10 | 2.4 |
| TR<sub>β</sub> | DR4 | 51 | 9.9 | 5.1 |
| TREpal | 75 | 13 | 5.8 |

TABLE I
Effects of HMGB-1/-2 on the apparent DNA binding affinities of nuclear receptor DNA binding domains

Kapp values were determined from the average curve of at least three independent experiments.
The Selective Effect of HMGB-1/-2 on the Steroid Receptor DBDs

Interaction of HMGB-1/-2 with the CTE of the Steroid Receptor DBDs

DBD heterodimer for a DR4 element (Fig. 4D). Both class II receptor DBDs had substantially higher intrinsic DNA binding affinities than the GR or PR DBDs and a slightly higher affinity than the ERα DBD. Addition of HMGB-2-stimulated DNA binding by the steroid receptor DBDs but had no effect on either class II receptor DBD (Table I). These results suggest that high affinity DNA binding is intrinsic to class II receptor DBDs, while steroid receptor DBDs require the accessory protein, HMGB-1/-2 for a comparable affinity.

The Selective Effect of HMGB-1/-2 on the Steroid Receptor DBD Is Dependent on the CTE—To determine whether the CTE is responsible for the higher intrinsic affinity of class II receptor DBDs and the selective effect of HMGB-1/-2 on steroid receptors, we constructed chimeric DBDs that swapped the CTE between PR and TRβ DBDs. The chimeras were spliced using overlap extension to create a precise boundary between the core DBD and CTE without introducing alterations in the peptide sequence (48). As illustrated in Fig. 5A, the TR<sub>PRCTE</sub> chimera contains the core zinc binding modules of PR through the conserved glycine-methionine motif (aa 562–632) fused to the CTE sequences from TRβ (aa 170–207) (Fig. 5A). This chimera is expected to retain the DNA binding specificity of PR for an inverted repeat PRE/GRE. Conversely, the TR<sub>PRCTE</sub> chimera DBD contains the TRβ core DBD (aa 97–169) fused to the CTE region of PR (aa 633–670) and should retain the binding specificity of TRβ for DR4. DNA binding by these chimeric DBDs was assessed by quantitative gel mobility shift assays as was done with wild-type PR and TRβ DBD constructs in Figs. 3 and 4. The PR<sub>PRCTE</sub> DBD bound to the PRE/GRE with a 6-fold higher affinity (K<sub>d</sub> = 24 × 10<sup>-9</sup> M; Table I) than the PR DBD<sub>670</sub> (K<sub>d</sub> = 150 × 10<sup>-9</sup> M), while addition of HMGB-1/-2 only increased the affinity of the PR<sub>PRCTE</sub> for a PRE by 2.4-fold as compared with the 8.8-fold effect observed with the PR DBD constructs (Table I and compare Figs. 3B and 5B). Conversely, the TR<sub>PRCTE</sub> chimera bound to both DR4 and TREpal elements with 13–30-fold lower affinities than the TRβ DBD construct (Table I and compare Figs. 4B and 5C). Unlike the TRβ DBD that was unaffected by HMGB-1/-2, the TR<sub>PRCTE</sub> chimera exhibited a 5- to 6-fold increase in apparent affinity for the DR4 and TREpal in response to addition of HMGB-2 (Fig. 5C and Table I). The apparent binding affinities of the TR<sub>PRCTE</sub> for DR4 and TREpal increased from 51 × 10<sup>-9</sup> M to 9.9 × 10<sup>-9</sup> M and 13 × 10<sup>-9</sup> M in the absence and presence of HMGB-2, respectively (Table I). Thus swapping the CTE between the PR and TR DBDs resulted in a nearly quantitative reversal of the DNA binding properties of these two classes of nuclear receptor DBDs with respect to binding affinity and response to HMGB-1/-2. These data suggest that the CTE is responsible for both the differences in intrinsic DNA binding affinity of the PR and TRβ DBDs for their target DNA sequences and for response to HMGB-1/-2.

The CTE Is Required for Protein Interaction with HMGB-1/-2 and for Full Stimulatory Effects of HMGB-1/-2 on Receptor Activities—We previously demonstrated a physical interaction of HMGB-1/-2 with PR in the absence of DNA that was not observed with a class II receptor, VDR (18). To examine protein interaction with other nuclear receptors, we performed in vitro pull-down assays with GST-HMGB-1. As shown in Fig. 6A, both full-length PR (A and B-form) and ERα interacted specifically with GST-HMGB-1, as compared with the minimal nonspecific interaction with GST alone. In contrast, no specific interaction was observed between HMGB-1 and full-length RXRα or TRβ (Fig. 6A). To narrow the region of steroid receptors required for interaction with HMGB-1/-2, similar pull-down assays were performed with various baculovirus-expressed domains of PR. PR domain constructs containing both the DBD and CTE bound to GST-HMGB-1/-2, but those that lacked the DBD and CTE did not (data not shown) indicating that the PR DBD and CTE were required for interaction with HMGB-1. To determine whether the DBD alone was sufficient to mediate HMGB-1/-2 interaction and to test the involvement of the CTE, we performed pull-downs with three different PR DBD constructs (Fig. 6B): the PR DBD<sub>651</sub>, which contains the full-length CTE equivalent to the TRβ CTE; PR DBD<sub>670</sub>, which contains an intermediate length CTE; and PR DBD<sub>642</sub>, in which most of the CTE was truncated. PR DBD<sub>670</sub> and PR DBD<sub>651</sub> bound to GST-HMGB-1 with similar efficiency, while PR DBD<sub>642</sub> exhibited little or no specific interaction (Fig. 6B). As anticipated, the RXRα DBD did not interact with HMGB-1 (Fig. 6B). These results indicate that HMGB-1 interaction is mediated by the DBD and requires the CTE.

We next sought to determine whether the CTE region was also important for mediating the stimulatory effect of HMGB-1/-2 on steroid receptor DNA binding. Using quantitative EMSA, we analyzed the effect of HMGB-1/-2 on the DNA binding affinity of the three PR DBD constructs used in pull-down assays (Fig. 6B). As summarized in Table I, PR DBD<sub>670</sub> and PR DBD<sub>651</sub> exhibited similar intrinsic DNA binding affinities in the absence of HMGB-1/-2 and responded similarly to HMGB-1/-2 addition with an ~9-fold increase in apparent DNA binding affinity. HMGB-1/-2 also stimulated DNA binding by PR DBD<sub>642</sub>; however, the increase (4-fold) was substantially reduced in comparison to the other DBDs (Table I). Unexpect-
edly, PR DBD_642 had a significantly higher intrinsic DNA binding affinity than PR DBD_670 or PR DBD_651 (Table I), suggesting that the CTE represses PR-DNA binding in the absence of HMGB-1/-2.

To examine whether the CTE is also involved in HMGB-1/-2 enhancement of PR-B transactivation in situ, a chimeric receptor, PRtrPR, was created that consisted of full-length PR-B containing the TR\textsuperscript{H9252}/CTE (aa 97–207) in place of the PR CTE (aa 633–670) (Fig. 7A). The ability of HMGB-1/-2 to influence transactivation by the PRtrPR chimera was directly compared with effects on PR-B in transient transfection assays. As shown in Fig. 7B, cotransfection of HMGB-1 increased hormone-dependent transactivation mediated by PRtrPR; however, the magnitude of the effect was significantly reduced as compared with wild-type PR-B. This reduction in response to HMGB-1 was not due to differences in protein expression of the two receptors because the levels of PR-B and PRtrPR were comparable and unaffected by HMGB-1 co-expression as determined by immunoblot (data not shown). We also constructed a chimeric receptor that lacked the N terminus, but retained the DBD-CTE and thus is the minimal PR construct capable of mediating hormone-dependent gene transactivation (DHLBD and DHtrLBD, Fig. 7A). As shown in Fig. 7C, the chimeric DHtrLBD also had a similar reduced response to HMGB-1/-2 in comparison to the wild-type DHLBD. These results together with the reduced activity of HMGB-1/-2 on PR DBD_642-DNA binding suggest that interaction of HMGB-1/-2 with the CTE is important for enhancement of PR-DNA binding and transactivation.

DISCUSSION

The present study reveals that the CTE of steroid receptors plays a role in DNA binding but acts by a distinct mechanism from class II nuclear receptors. By use of chimeric DBDs and truncation mutants, we demonstrate that the CTE is responsible for the differential ability of HMGB-1/-2 to selectively increase the DNA binding affinity of steroid receptor DBDs and is required for a direct protein interaction between steroid receptors and HMGB-1/-2 that does not occur with class II nuclear receptors. We also show that the CTE is responsible for a higher intrinsic DNA binding affinity of class II DBDs. These results taken together demonstrate that the CTE of steroid receptors is a site required for interaction with HMGB-1/-2 and for maximal enhancement of steroid receptor-DNA binding and transactivation by HMGB-1/-2.

No other study to our knowledge has directly compared the apparent binding affinities of different classes of nuclear receptor DBDs under the same conditions. This comparison demon-
The PRtrPR chimera contains the TRDBD resulted in a 13-fold reduction in the affinity of the steroid receptor DBDs, particularly the PR and GR DBDs (Table I), suggesting fundamental differences in the way the DBDs of these two nuclear receptor subclasses interact with DNA. Fusing the TRβ CTE to the PR core DBD increased the affinity of the PR DBD for PREs 6-fold, whereas fusing the PR CTE to the core TR DBD resulted in a 13–30-fold reduction in the affinity of the TRβ DBD for its target DNA (Fig. 5 and Table I). These CTE domain swapping experiments indicate that the observed affinity differences between the steroid and class II receptor DBDs are largely attributable to the CTE.

The ERα DBD appears to be unique among the steroid receptor DBDs, because it bound to DNA with a higher apparent affinity than the other steroid receptor DBDs analyzed (Table I). ERα recognizes the core DNA hexamer AGGTCA, a sequence bound by the majority of the nuclear receptor superfamily with the exception of the GR subgroup of steroid receptors, including PR, GR, MR, and AR, which all bind to AGAAACA. Comparison of the GR and ERα DBD-DNA crystal structures demonstrates that ERα makes more direct and water-mediated DNA contacts in the major groove than does GR, a possible mechanism for the observed affinity differences.

Additionally, the ERα CTE (aa 261–264) contains a sequence motif similar to the GRIP-box sequence in the orphan receptor SF-1. The GRIP-box forms an extended structure that interacts in the minor groove flanking HREs and is important for stability of orphan receptor-DNA interaction. Other steroid receptor CTEs, such as those in PR or ERβ, do not contain a similar GRIP-box-like sequence. If the ERα CTE contains a bona fide GRIP-box this could account for the affinity differences observed between the ERα DBD and other steroid receptor DBDs. Nonetheless, the ERα DBD behaved like other steroid receptors in that HMGB-1/-2 increased its DNA binding affinity 7-fold (Table I). It is of interest to note that the DNA binding affinity of ERα DBD in the presence of HMGB-1/-2 is higher than the intrinsic affinities of the class II DBDs (Table I) further illustrating that ERα has some unique properties that do not fit with the steroid or class II receptor classifications.

Whereas the steroid receptor DBDs have a low relative DNA binding affinity, addition of HMGB-1/-2 enhanced this affinity 7–9-fold, such that the Kdapp approached that of the class II receptor DBDs. In contrast, HMGB-2 had no effect on DNA binding by RXRs or TRβ DBDs (Figs. 3 and 4, and Table I). This selective interaction with the steroid receptor DBDs is not dependent on the DNA target element because HMGB-1/-2 enhanced PR DBD binding to both inverted repeat and half-site elements (Fig. 3) (20) but did not affect binding by the TRβ DBD to either direct or inverted repeat elements (Table I). These results suggest that a feature of steroid receptor DBDs and not the target DNA is the important determinant for the selective influence of HMGB-1/-2. Indeed, we show that the DBD of steroid receptors mediates a protein interaction with HMGB-1/-2 that was not detected with class II receptor DBDs.
(Fig. 6). That HMGB-1/-2 interaction with the steroid receptors is mediated through the DBD is not entirely surprising since HMGB-1/-2 interaction with the OCT and HOX transcription factors, is also mediated by the DBD (25, 26). A correlation was observed between the ability of HMGB-1/2 to stimulate receptor activity and to make a direct contact with the receptor. HMGB-1/2 directly interacted with the steroid receptors (PR and ER) and stimulated both DNA binding and transactivation by these receptors. The class II receptors (TRβ and RXRα) did not interact with HMGB-1/2, nor did they respond functionally to HMGB-1/2 (18) (Fig. 6). This correlation highlights the importance of protein-protein interactions in HMGB-1/-2 stimulation of steroid receptor activity and is consistent with previous observations that HMGB-1/2 selectively increased DNA binding and transactivation by full-length steroid receptors but not class II receptors (18, 20, 26).

HMGB-1/2 interaction with the steroid receptor DBD was dependent on the CTE, since truncation of the CTE resulted in a loss of detectable protein interaction (Fig. 6). Whether the CTE is a binding site for HMGB-1/2 or confers a conformation on the DBD that is required for interaction remains to be determined. Truncation of the CTE also resulted in a significant reduction in HMGB-1/2 enhancement of PR DBD-DNA binding (Table I). Likewise, the chimeric PR construct, which contained the TRβ CTE in place of its own CTE, also exhibited a substantial reduction in HMGB-1/2 stimulation of PR-mediated transactivation in cells (Fig. 7). Thus, we also observed a correlation between the requirement of the CTE for physical association with HMGB-1/2 and the ability of HMGB-1/2 to stimulate PR DNA binding in vitro and PR-mediated transactivation in situ. However, some residual stimulatory activity of HMGB-1/2 in the absence of the PR CTE was still observed in DNA binding and transfection assays suggesting that physical interaction with the CTE does not account for all the HMGB-1/2 effects on PR activity. The mechanism for this residual HMGB-1/2 activity in the absence of the CTE is not known but may be explained by interaction of HMGB-1/2 with DNA or a weak interaction between HMGB-1/2 and the core DBD that is not detectable under our assay conditions.

The CTEs of class II and orphan receptors have clearly been shown to play an important role in DNA binding through extension and stabilization of protein-DNA contacts through interactions with DNA in the minor groove. Only a few reports exist to support a similar role in DNA binding of the steroid receptor CTE. Truncations of the ERE CTE led to an acceleration of the off-rate of the ER DBD from DNA and an increased sensitivity to the ionic strength of EMSA buffers (47) suggesting a role of the CTE in stability of the ER-DNA complex. Additionally, truncation of the GR and AR CTEs to a length shorter than twelve amino acids reduced the DNA binding affinity (59). In the same study, the CTE and the second zinc finger of AR were also required for AR recognition of a direct repeat androgen response element; a function attributed to the different dimerization mode required to bind to the direct versus inverted repeat (59).

The present results support a model whereby HMGB-1/2 selectively interact with steroid receptors through a direct protein interaction with the DBD that is dependent on the CTE and that this interaction somehow increases the DNA-binding affinity of the receptor. The mechanism by which HMGB-1/2 interaction enhances steroid receptor-DNA binding remains to be determined. One possibility is that the HMGB-1/2 recruited by steroid receptors stabilizes the receptor-DNA complex by extending the protein-DNA interface thus substituting for the CTE of other nuclear receptors. However, the existence of a stable ternary DBD/HMGB/DNA complex is questionable since it is very difficult to capture HMGB-1/2 in the final high affinity PR-DNA complex suggesting that HMGB-1/-2 interaction is transient (18). An alternative mechanism favored by our data is that HMGB-1/-2 interaction either induces or stabilizes a conformation that enables the CTE to directly interact with DNA. This model suggests that the steroid receptor CTE serves a function similar to that of other nuclear receptor CTEs but requires the additional step of HMGB-1/2 interaction to participate in DNA binding. The latter mechanism is supported by the observation that truncation of nearly the entire CTE of PR (PR DBD<sub>445</sub>) resulted in an increase in the intrinsic affinity of the DBD for DNA and a reduction of the stimulatory effect of HMGB-1/-2. This suggests that the PR CTE in the absence of HMGB-1/2 has a repressive effect on DNA binding and that HMGB-1/-2 relieve this repression. We believe that the CTE of steroid receptors is structurally unstable and that the role of HMGB-1/2 is to stabilize the CTE.

Allosteric modification of steroid receptors is a common theme in gene regulation by steroid hormones. Steroid binding induces a conformational change in the receptor to promote dissociation of the heat shock proteins and enable co-activator recruitment. Specific DNA can also allosterically modify steroid receptors to allow for promoter-specific gene regulation and potential recruitment of specific coactivator proteins. The present results suggest that interaction between HMGB-1/2 and the DBD-CTE of steroid receptors provides another dimension to allosteric regulation of steroid receptors by inducing a conformation that results in higher affinity DNA binding.

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Note Added in Proof—After submission of the manuscript a similar report showed that the C-terminal extension region of all steroid receptor DNA binding domains was required for the stimulatory influence of HMGB-1 on specific DNA binding (Verrijdt, G., Haelens, A., Schoenmakers, E., Rombauts, W., and Claessen, F. (2002) Biochem. J. 361, 97–103).

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The C-terminal Extension (CTE) of the Nuclear Hormone Receptor DNA Binding Domain Determines Interactions and Functional Response to the HMGB-1/-2 Co-regulatory Proteins
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