Comparative analysis of the influence of the high-mobility group box 1 protein on DNA binding and transcriptional activation by the androgen, glucocorticoid, progesterone and mineralocorticoid receptors

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We performed a comparative analysis of the effect of high-mobility group box protein 1 (HMGB1) on DNA binding by the DNA-binding domains (DBDs) of the androgen, glucocorticoid, progesterone and mineralocorticoid receptors. The affinity of the DBDs of the different receptors for the tyrosine aminotransferase glucocorticoid response element, a classical high-affinity binding element, was augmented up to 7-fold by HMGB1. We found no major differences in the effects of HMGB1 on DNA binding between the different steroid hormone receptors. In transient transfection assays, however, HMGB1 significantly enhances the activity of the glucocorticoid and progesterone receptors but not the androgen or mineralocorticoid receptor. We also investigated the effect of HMGB1 on the binding of the androgen receptor DBD to a subclass of directly repeated response elements that is recognized exclusively by the androgen receptor and not by the glucocorticoid, progesterone or mineralocorticoid receptor. Surprisingly, a deletion of 26 amino acid residues from the C-terminal extension of the androgen receptor DBD does not influence DNA binding but destroys its sensitivity to HMGB1. Deletion of the corresponding fragment in the DBDs of the glucocorticoid, progesterone and mineralocorticoid receptor destroyed their DNA binding. This 26-residue fragment is therefore essential for the influence of HMGB1 on DNA recognition by all steroid hormone receptors that were tested. However, it is dispensable for DNA binding by the androgen receptor.

Key words: DNA-binding domain, sex-limited protein, steroid receptor, transcription, tyrosine aminotransferase.

INTRODUCTION

The high-mobility group (HMG) box proteins are small, non-histone chromatin proteins that are known to be integral parts of transcriptionally active enhancers (such as interferon β enhanceosome [1,2] and the viral BHLF-1 gene promoter [3]). Their DNA-binding domains (DBDs) are formed by two so-called HMG boxes, each consisting of three α-helices, arranged in an L shape [4,5]. Each HMG box contains two DNA-binding surfaces oriented perpendicular to each other. HMG boxes can interact with particular three-dimensional DNA structures such as four-way junction DNA [6]. In addition, HMG boxes can interact in a non-sequence-specific manner with linear or bent DNA [7]. DNA binding by HMG boxes widens the minor groove and causes a significant bend in the DNA path [7–9]. Owing to their ability to recognize and induce bent or distorted DNA, HMG-box proteins are generally considered to participate in the formation of nucleoprotein complexes involved in transcriptional regulation and recombination events [7,10]. Several DNA binding proteins, such as the RAG-1/-2 recombinase [10], several transcription factors {for example p53 [11], HOXD9 [12], Oct 1, 2 and 6 [13] and the TATA-box-binding protein ('TBP') [14]} can bind and recruit HMG-box proteins to the DNA. This additional binding of the HMG-box proteins is proposed to stabilize the protein–DNA complex and subsequently to enhance the functionality of these proteins.

HMGB1 and HMGB2, two HMG-box proteins, are modular proteins containing a duplicated N-terminal region composed of two HMG boxes (A and B) and a highly acidic C-terminal part [5]. Several reports describe the recruitment of HMGB1/HMGB2 to DNA by steroid hormone receptors and the subsequent increase in transcriptional activity in transient transfection assays [15–17]. This phenomenon could not be demonstrated for the members of the retinoic acid receptor–retinoid X receptor (RXR) family of nuclear receptors [15].

Androgen receptor (AR), glucocorticoid receptor (GR), progesterone receptor (PR) and mineralocorticoid receptor (MR) form the class I subgroup of the nuclear receptor superfamily [18,19]. These steroid receptors are known to interact in a head-to-head conformation with partial palindromic repeats of their core 5′-TGTTCT-3′ binding sequence with a three-nucleotide spacer [20–22]. The only exception to this rule is the AR, which, next to its interaction with 'classical' palindromic repeats, has recently been shown to interact with partial direct repeats of the core steroid-receptor-binding sequence [23–25]. Our group recently proposed a key role for residues within the C-terminal extension (CTE) of the AR-DBD in the interaction of the receptor with directly repeated motifs, indicating that the AR possibly interacts with these AR-specific elements in a way that is similar to the members of the class II nuclear receptors [26]. The different members of the class II subfamily of nuclear receptors interact with direct repeats of their 5′-TGACCT-3′ core recognition sequence with variable spacer lengths [18,27]. Crystal structures show the presence of a helix in the CTE of the RXR [28] and thyroid hormone receptor (TR) [29] that was not present in the crystal structure of the GR-DBD [20]. In the RXR

and TR, these helices in the CTE (called the T-box and the A-box for each protein respectively) make extensive contacts with the minor groove of the DNA surrounding the core binding element and are proposed to stabilize the protein–DNA interaction greatly [28,29]. HMGB1/HMGB2 has recently been proposed to substitute for the lack of a minor-groove-interacting surface in the DBDs of the steroid receptors and hence to increase the stability of the receptor–DNA complex [15,17]. The presence of HMGB2 in PR–DNA complexes, and a low-affinity but specific interaction of HMGB2 with PR, was demonstrated by Boonyaratanakornit et al. [15], whereas HMGB2 did not interact with the vitamin D receptor [15]. The DBD of the PR was found to contain the necessary elements required for the influence of HMGB2 on DNA binding [17].

In view of our findings on the alternative binding of the AR to DNA, we wished to analyse and compare, in DNA binding assays *in vitro*, the effect of the presence of HMGB1 on binding of the AR-DBD to the classical palindromic and the recently described AR-specific directly repeated motifs. We also compared the effects of HMGB1 on recognition of the tyrosine aminotransferase glucocorticoid response element (TAT-GRE), a classical high-affinity binding element, by the DBDs of the AR, GR, PR and MR. In functional studies we compared the effects of HMGB1 co-transfection on the ability of the different steroid receptors to increase the expression of a reporter gene controlled by the E1b promoter and one or two copies of the TAT-GRE. In addition we describe a deletion fragment of the AR-DBD that fails to interact with a directly repeated androgen response element (ARE) motif but is able to interact with a classical palindromic motif, independently of the presence of HMGB1. The same deletion in the GR-DBD, the MR-DBD and the PR-DBD completely destroys their interaction with DNA.

EXPERIMENTAL

General techniques

Restriction and modifying enzymes were purchased from Gibco-BRL Life Technologies (Grand Island, NY, U.S.A.), MBI Fermentas (Heidelberg, Germany), Amersham Pharmacia Biotech (Uppsala, Sweden), Promega Corp. (Madison, WI, U.S.A.) and Roche Molecular Biochemicals (Mannheim, Germany). PCR reactions were performed on a Progene thermocycler (Techne, Cambridge, U.K.) with *Taq* DNA polymerase obtained from Takara (Takara, Shuzo Co. Ltd, Shiga, Japan) or Gibco-BRL Life Technologies. PCR primers and oligonucleotides used in gel-shift assays were purchased from Eurogentec (Seraing, Belgium). [α-^{32}P]dCTP was purchased from Amersham Pharmacia Biotech.

Production of glutathione S-transferase (GST) fusion proteins

The steroid receptor DBDs depicted in Figure 1 were cloned in the pGEX-2TK GST fusion vector (Amersham Pharmacia Biotech) for bacterial expression. The DNA-binding portion of the AR containing the CTE was named AR1. The fragment lacking the CTE was called AR3. The DNA-binding domains of the other steroid receptors were termed accordingly. The construction of AR1, AR3, GR1, GR3, PR1 and MR1 was as described in [23]. For the generation of PR3 and MR3 respectively, the following downstream primers (5′-ggggaattcTCAA-CCTCCAAGGACCATGCCAGC-3 and 5′-ggggaattcTC-AAGCTCCTAAGTTCATGCC-3′) were used to perform a PCR reaction on plasmids containing PR1 and MR1. PCR fragments were cloned in frame in the pGEX-2TK vector after digestion with *Eco*RI and *Bam*HI.
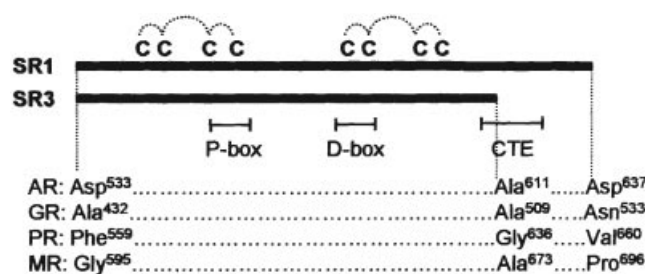

Figure 1   Diagram of the receptor DBD fragments used in the binding assays

The thick lines are a linear representation of the SR1 and SR3 receptor DBD fragments used in band-shift assays. The zinc-co-ordinating cysteine residues creating the two zinc-fingers are depicted above the top line and are connected by dotted lines. The locations of the P-box, the D-box and the CTE are also indicated. The amino acid residues at the N-terminal end and the C-terminal ends of the SR1 and SR3 fragments are depicted.

The cDNA of rat HMGB1 was cloned by performing a PCR reaction on a mammalian expression vector containing the full-length rat HMGB1 (a gift from Dr D. Edwards) with a vector primer as upstream primer and 5′-gggaattccggatccTCACTTCT-TTTTCTTGCTCTTCTCAGC-3′ containing a stop codon and corresponding to nt 531–554 in the HMGB1 cDNA. The PCR product was digested with *Bgl*II and *Eco*RI and inserted into *Bam*HI/*Eco*RI-digested pGEX-2TK plasmid. This resulted in the cloning of a deletion fragment of rat HMGB1 containing the N-terminal and central DBD of the protein but lacking 30 residues of the acidic C-terminal tail. The resulting fragment is known to contain the full capacity of HMGB1 to promote DNA binding by the PR (M. E. Bianchi, personal communication). The inserts of all expression vectors generated were verified by sequence analysis. Proteins were grown and purified as described previously [23]. In brief, 100–200 ml bacterial cultures were grown overnight in Luria Broth medium containing 0.1 μg of ampicillin per ml of culture at 37 °C. Cell cultures were spun and resuspended in PBS containing PMSF (0.5 mM) and aprotinin (100 i.u./ml). After sonication in an AT200-Bioruptor Sonicator (Cosmo Bio, Seraing, Belgium), soluble protein was separated from debris by centrifugation at 7700 *g* for 45 min. The supernatant was applied to a GST–Sepharose 4B column (Amersham Pharmacia Biotech) and incubated overnight at 4 °C. The next day, the Sepharose beads were washed several times with PBS, protein was cleaved from the GST portion with 8–16 units of thrombin and eluted in 2 bed vol. of PBS. The amount, purity and fragment lengths of the polypeptides generated were checked by SDS/PAGE. This typically generated 50–400 μg of protein (more than 90 % pure). Protein concentrations were determined with the Coomassie Protein Assay Reagent (Pierce).

Gel-shift assays

Electrophoretic mobility-shift assays were performed essentially as described previously [23,25]. In brief, radiolabelled probe (final concentration 0.3 nM) was incubated with receptor DBD in 20 μl of binding buffer [10 mM Hepes (pH 7.9)/2.5 mM MgCl$_2$/0.05 mM EDTA/8 % (v/v) glycerol/50 mM NaCl/0.1 % (v/v) Triton X-100/1 mM dithiothreitol containing 50 ng of poly(dI-dC)] for 30 min on ice in the presence or absence of either the recombinant HMGB1 or an equal amount of BSA (Pierce). Protein-bound DNA was separated from the free probe by non-denaturing 5 % PAGE at 100 V for 1.5 h. Gels were dried and radioactivity was determined by scanning in a Phosphor-

Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.). For the calculation of apparent dissociation constants (K_s values) for DNA binding by the different receptor DBD fragments, constant amounts of radiolabelled probes were incubated with increasing amounts of protein in the presence of 200–600 ng of BSA or an identical amount of recombinant HMGB1. For each data point (each amount of protein), the percentage of radioactivity in the retarded band, after subtraction of background, relative to the total amount of radioactivity in that lane was calculated. For each binding curve the data points are means $\pm$ S.E.M. for at least three independent experiments with at least two different preparations of HMGB1. The resulting sets of data points were analysed with the SigmaPlot software package (SPSS, Chicago, IL, U.S.A.). Best fits were calculated to curves with allosteric Hill kinetics. K_s values are computer estimates of the concentrations of protein that cause the retardation of a radiolabelled nucleotide that is 50% of maximal. Oligonucleotides used as radiolabelled probes contained the sequences of known steroid-receptor-binding sites, as indicated in each experiment, composed of the two hexamer half-sites, the original three-nucleotide spacer, the original four nucleotides flanking the hexamer repeat, and *Nhe*I and *Xho*I overlapping ends. Double-stranded oligonucleotides were obtained by the hybridization of two complementary oligonucleotides and radioactively labelled by a fill-in reaction with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of [α-^{32}P]dCTP. The motifs used in the experiments described here are the rat TAT-GRE (5′-TGTACAggaTGTTCT-3′ [28]), the mouse sex-limited protein hormone response element (*slp*-HRE2) (5′-TGGTC-AggcAGTTCT-3′ [25]) and the *slp*-HRE2 mutant -4T-A (5′-TGG**A**CAggcAGTTCT-3′) [25] (the hexamer motifs are in capitals, the three-nucleotide spacer is in lower-case letters).

Transfection assays

HeLa cells were purchased from the American Type Tissue Culture Collection (Manassas, VA, U.S.A.) and maintained in DMEM (Dulbecco's modified Eagle's medium) containing 1000 mg/l glucose, supplemented with 10% (v/v) fetal calf serum (Gibco-BRL Life Technologies), penicillin (100 i.u./ml) and streptomycin (100 μl/ml). For transfection experiments, cells were plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) at 7×10^4 cells per well and grown in DMEM containing dextran-coated charcoal-stripped serum (5%). On day 2, cells were transfected with LIPOFECTAMINE 2000 (Gibco-BRL, Life Technologies) in accordance with the manufacturer's instructions. The DNA mixture consisted of (per well): 700 ng of luciferase expression vector driven by the E1b promoter containing one or two copies of the rat TAT-GRE (a gift from Dr G. Jenster), 100 ng of cytomegalovirus (CMV)-driven β-galactosidase expression vector (Stratagene), 100 ng of a mammalian expression vector containing the cDNA encoding the appropriate receptor and either 100 ng of CMV-driven rat-HMGB1-expressing plasmid or 100 ng of pGEM15. The luciferase reporter vector containing one copy of the rat TAT-GRE was generated by excision of a *Pae*I fragment from the vector containing the two copies, resulting in the deletion of the downstream motif. The receptor-expressing plasmids were pSG5-hAR, pSG5-rGR, pRSV-hPR and pCMV-rMR. On day 3, media were replaced, either with or without the addition of the appropriate hormones: methyltrienolone (R1881, 1 nM), dexamethasone (10 nM), progesterone (100 nM) and aldosterone (10 nM). On day 4, cells were harvested by incubation for 15 min in 100 μl of 1 $\times$ passive lysis buffer (Promega); 20 μl of cellular extract was used for the

quantification of luciferase in a Microlumat LB 96P Luminometer (EG&G Berthold, Bad Wilstadt, Germany) with luciferase assay reagent (Promega) in accordance with the manufacturer's instructions. The β-galactosidase activity in 10 μl extracts of each sample was measured with the β-galactosidase chemiluminescent reporter gene assay system (Tropix, Bedford, MA, U.S.A.). To correct for transfection efficiencies, the luciferase value of each sample was normalized by its β-galactosidase activity. The reported values are averages of at least three independent experiments performed in duplicate.

RESULTS AND DISCUSSION

Comparative analysis of the effect of HMGB1 on DNA binding by AR-DBD, GR-DBD, PR-DBD and MR-DBD

We performed gel-shift assays with the rat TAT-GRE, a known binding element for AR and GR [30] and commonly used in experiments investigating DNA binding of, and transactivation by, the AR, GR and PR [15,31]. The protein fragments used were the 105-residue AR-DBD (here termed AR1 as described by Schoenmakers et al. [23]), the 102-residue GR-DBD (GR1 in [23]), the 102-residue PR-DBD (by analogy called PR1) and the 102-residue MR-DBD (MR1; see also Figure 1). Constant amounts of radiolabelled probe were incubated with increasing amounts of receptor fragment in the presence of BSA or the same amount of recombinant HMGB1. The curves in Figure 2 show the different sets of data points generated for each receptor fragment used, as well as the best fits to curves with allosteric Hill kinetics calculated by the SigmaPlot software. Representations of gel-shift experiments with the indicated amounts of receptor DBD with or without additional proteins (600 ng of BSA or 600 ng of HMGB1) are shown at the right of the graphs. The affinities of the different DBDs for the TAT-GRE are comparable for each steroid receptor. K_s values for TAT-GRE binding by AR1, GR1 and MR1 were 33.5 ± 2.8, 19 ± 0.4 and 25 ± 0.8 nM respectively. The lower affinity (K_s 52.9 ± 2.3 nM) of PR1 is probably explained by a lower concentration of active protein because we could not avoid degradation when growing and purifying the receptor fragment. We therefore conclude that the interaction of the different receptor fragments with the TAT-GRE is comparable between the different receptors. From the right panels in Figure 2 it is clear that the presence of BSA does not alter the affinity of any receptor DBD for the motif when compared with the absence of additional protein. From the binding curves and the panels at the right, it is clear that all receptor fragments have a significantly higher affinity for the rat TAT-GRE in the presence of recombinant HMGB1 than in the presence of BSA. The increases in affinity (decreases in K_s values) were 4.0-fold, 7.1-fold, 2.0-fold and 4.5-fold for binding of the AR-DBD, GR-DBD, PR-DBD and MR-DBD respectively. This confirms previously reported results on the PR-DBD [17] and corroborates findings on the influence of HMGB1/HMGB2 on DNA binding by full-size AR and GR [15]. We find here a significant difference in sensitivity to the presence of HMGB1 of the GR-DBD compared with the other steroid receptors used (a 7-fold increase in GR1 binding compared with a 3–4-fold increase for AR1, PR1 and MR1).

The presence of HMGB1 in the binding mixtures of the receptor DBDs with their binding elements did not result in the supershift of the retarded protein–DNA complex that would be expected if HMGB1 were present in the complex. The same phenomenon was observed by Senkus and Edwards [17]. We believe that this indicates that HMGB1 rapidly dissociates from the complex or that the binding is destroyed during electro-
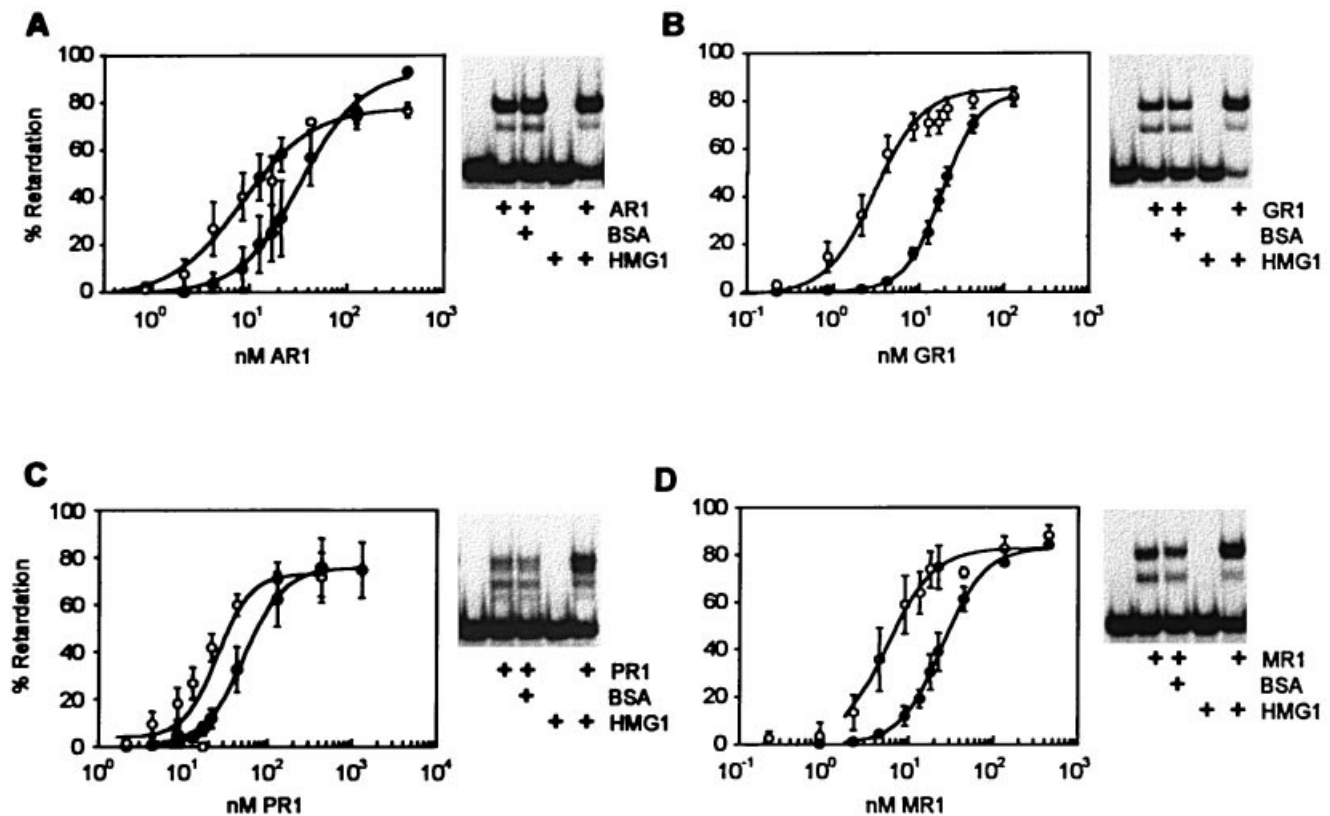
Figure 2   Comparison of the influence of HMGB1 on TAT-GRE binding by the AR-DBD, the GR-DBD, the PR-DBD and the MR-DBD

Left panels: binding curves of the different receptor DBDs for the rat TAT-GRE: AR1 (**A**), GR1 (**B**), PR1 (**C**) and MR1 (**D**). For each amount of protein (*x*-axis) the radioactivity in the upshifted band as a percentage of the total amount of radioactivity in that lane, after the subtraction of background, was calculated and plotted on the *y*-axis. Results are means $\pm$ S.E.M. for at least three independent experiments with at least two different HMGB1 preparations. Best fits to allosteric binding with Hill kinetics were calculated. Symbols: $\bigcirc$, results obtained in the presence of 600 ng HMGB1; $\bullet$, results obtained in the presence of 600 ng BSA in the binding mixture; in each case the best fit is represented by the curve. The K_s values in the absence and the presence of HMGB1 respectively were as follows: (**A**) 33.5 and 8.3; (**B**) 19.1 and 2.7; (**C**) 52.9 and 25.6; (**D**) 25.0 and 5.6. Right panels: overview of one data point from the left panels. The TAT-GRE probe was incubated with the 4 ng of AR1 (**A**), 3 ng of GR1 (**B**), 5 ng of PR1 (**C**) and 5 ng of MR1 (**D**) in the presence of either 600 ng BSA or HMGB1 as indicated.

phoresis. We were also unable to detect a stable interaction of GST-bound GR1 with full-size HMGB1 or GST-bound HMGB1 with full-size GR (translated *in vitro*) in the absence or presence of DNA (results not shown). In bandshift assays with full-size PR, Boonyaratanakornkit et al. [15] found HMGB2 in the protein-bound DNA as demonstrated by a supershift of the retarded complex in the presence of an antibody against HMGB2. However, they also reported the need to use a longer oligonucleotide (43 bp) because it was not possible to demonstrate the presence of HMGB1 in the protein–DNA complex when a 32 bp probe was used [32]. All oligonucleotides used in the present study were 33 bp long. Experiments with a 43 bp motif containing the rat TAT-GRE element were uninterpretable owing to high non-specific interaction of HMGB1, even in the absence of receptor DBD (results not shown).

Functional analysis of the influence of HMGB1 co-transfection on steroid receptor transcriptional activation in transfection experiments

To investigate the functional implications of the presence of HMGB1 on steroid receptor action, we transiently transfected HeLa cells with a luciferase reporter vector driven by the E1b promoter and containing one or two copies of the rat TAT-GRE motif that was used in the DNA binding assays (Figure 3). Cells

were always co-transfected with 100 ng of a eukaryotic expression plasmid expressing either AR, GR, PR or MR. Co-transfection with HMGB1 was performed by adding 100 ng of a CMV-driven expression plasmid containing the HMGB1 cDNA in the transfection mixture.

As shown in Figure 3, HMGB1 co-transfection resulted in a 2–4-fold increase in transcriptional activation by the GR and PR when using either reporter construct. These results are in agreement with those of Boonyaratanakornkit et al. and Senkus and Edwards, who reported a 4-fold stimulation of PR activity by HMGB1 in HeLa cells [15,17]. ER transactivation in transiently transfected HeLa cells was stimulated 2–5-fold by the addition of an HMGB1 expression plasmid in equal amounts as the ER expression plasmid [16]. Surprisingly, we observed no stimulation of AR- or MR-driven reporter gene expression. As with the AR, Boonyaratanakornkit et al. reported an approximate 25-fold increase of transcriptional activity after HMGB1 co-transfection in COS cells. However, in those experiments, AR was inactive in the absence of HMGB1. In our experiments, androgen stimulation resulted in a 95-fold increase in reporter gene expression compared with samples that were not stimulated with hormone (Figure 3B). In addition, in the experiments by Boonyaratanakornkit et al. basal transcription of the reporter construct in the absence of hormone increased significantly after HMGB1 co-transfection. One possible explanation could be
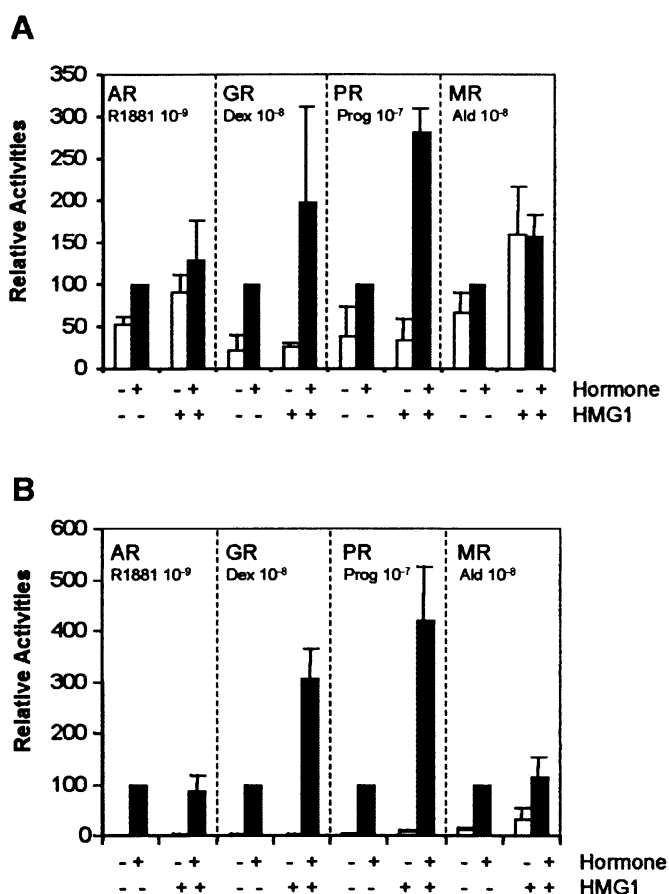
A

B

Figure 3   Influence of HMGB1 co-expression on the functionality of the different steroid hormone receptors in transient transfection assays

HeLa cells were transiently transfected with 700 ng of a luciferase reporter plasmid driven by the E1b promoter and containing one (**A**) or two (**B**) copies of the rat TAT-GRE, 100 ng of a β-galactosidase expression plasmid, 100 ng of the appropriate steroid receptor expression plasmid and 100 ng of either empty pGEM15Zf(−) or a CMV-driven expression plasmid containing the rat HMGB1 cDNA. Luciferase activities are depicted relative to the activity of the samples in the absence of HMGB1 and stimulated with the indicated amount of hormone; the latter activity was set at 100. Open columns, activities in the absence of hormone [in the AR and GR co-transfected samples in (**B**) they are too small to be plotted]; filled columns, activities of the samples stimulated with the indicated amounts of hormone. Results are means $\pm$ S.E.M.

provided by a recent report by Hofman et al. [33] that highlights an important consideration that needs to be taken into account when performing co-transfection assays in COS cells. Co-transfection of a large excess of empty expression vector DNA can severely impair steroid receptor action by decreasing receptor expression. The same effect was observed when expression plasmids containing short cDNA were used. However, the effect was inversely proportionate to the length of the cDNA and independent of the function of the expressed protein. The effect was particularly evident in COS, but much smaller in DU145 and HeLa cells.

The absence of any stimulatory effect of HMGB1 co-transfection on AR and MR action was rather surprising because both receptor DBDs were sensitive to the presence of HMGB1 in DNA binding assays *in vitro* (Figure 2). Many different mechanisms can explain the apparent contradictions between the DNA binding affinities *in vitro* and the transcriptional activity. For example, when analysing the effects of point mutations in selective and non-selective AREs, we found no strict correlation

between the affinity *in vitro* of the receptor DBD for a motif and the fold induction of transcriptional activity through the same motif [25]. One possibility could be that other receptor domains, not present in the fragments used in the binding assays, might contribute to this difference. For example, it has recently been shown that the CTE of the DBD of the AR harbours a function that strongly inhibits the ligand-dependent AF2 function [34]. It is therefore tempting to speculate that an increase in the affinity of the receptor for the response element might affect such inhibitory function.

The absence of influence of HMGB1 on AR function might be a possible explanation for the observations that HMGB1 knockout mice display a clear deficiency in GR function [35], whereas the action of some other steroid receptors seems less impaired (M. E. Bianchi, personal communication). A loss of AR function would result in, for example, a total or partial androgen insensitivity syndrome or testicular feminization, which was not observed in Hmgb1$^{-/-}$ mice.

Influence of HMGB1 on AR–DBD interaction with the androgen-specific, directly repeated *slp*-HRE2 and a non-specific partly palindromic point mutant

Because of our interest in the study of AR–DBD interaction with different classes of DNA motif, we wished to compare the effect of HMGB1 on the binding of the AR-DBD to an AR-specific, partly directly repeated motif (the *slp*-HRE2; 5′-TGGTCAgcc-AGTTCT-3′) and its non-specific point mutant (*slp*-HRE2 mutant −4T-A; 5′-TGG<u>A</u>CAgccAGTTCT-3′), which has a partly palindromic structure. We have described the *slp*-HRE2 as being essential for the androgen specificity of the *slp* enhancer [25] due to a specific interaction of the AR, but not the GR, with this motif. Mutation of the T at position −4 relative to the central spacer nucleotide resulted in a binding of the GR-DBD as well as a gain of glucocorticoid responsiveness of the *slp* enhancer [25]. Binding affinities of the AR1 polypeptide to the wild-type (K_s 418 ± 15 nM) and mutated (K_s 56.6 ± 2 nM) *slp*-HRE2 were comparable to those reported earlier (Figure 4) [25]. The presence of HMGB1 increased the binding affinity of AR1 to the wild-type and the mutated *slp*-HRE2 motif by 3.5-fold and 2.1-fold respectively. AR-DBD binding to both elements seems therefore sensitive to the presence of HMGB1.

Deletion mutant of the AR-DBD interacts with its recognition motif independently of the presence of HMGB1

We investigated the influence of HMGB1 on DNA binding by using deletion mutants of the steroid receptor DBDs, lacking an 26-residue peptide fragment of the C-terminal tail (SR3; see also Figure 1) and resulting in the deletion of part of the hinge region and 8 residues of the CTE. Our group previously reported that this deletion in the AR-DBD results in a complete loss of binding of the AR-DBD to the AR-specific ARE of the rat probasin promoter, whereas interaction with the classical palindromic C3(1)ARE motif remains unaltered [24]. The same deletion of the GR-DBD results in a complete loss of DNA binding to the C3(1)ARE [24]. In our current hypothesis, the AR might have a dual mode of interaction with different types of ARE: it dimerizes in the classical head-to-head configuration on a classical palindromic element, whereas it would dimerize in a head-to-tail conformation on a directly repeated element. The latter binding conformation involves a large portion of the CTE and hinge region [23]. This would greatly resemble the binding of, for example, a vitamin D receptor–RXR heterodimer to a direct repeat of the class II receptor core-binding element with a three-nucleotide spacer (DR3) [36]. The DNA binding of such hetero-
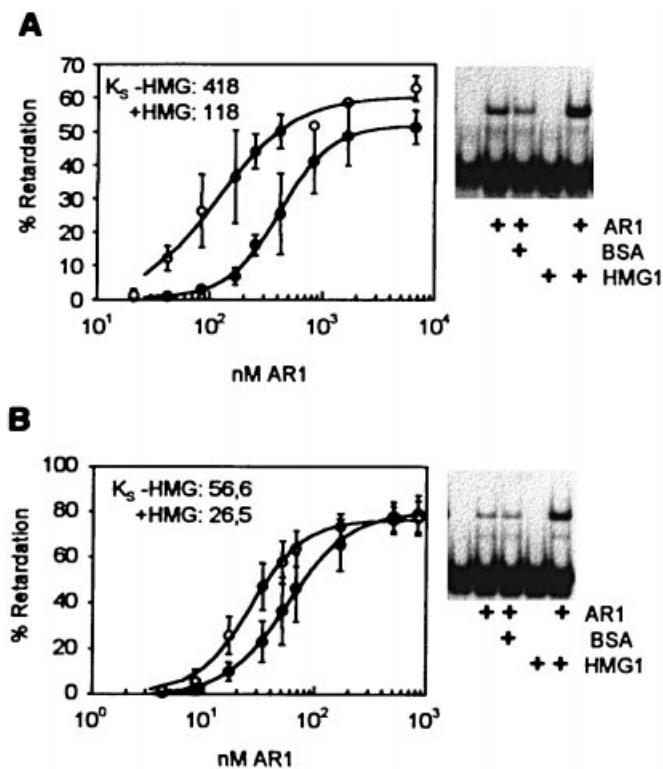
Figure 4   Comparison of the influence of HMGB1 on binding of the wild-type and mutated *slp*-HRE2 by the AR-DBD

Results are represented as in Figure 2. The DNA used in these experiments was the wild-type *slp*-HRE2 (5′-TGGTCAnnnAGTTCT-3′) (**A**) and its −4T-A mutant (5′-TGGACAnnnAGTTCT-3′) (**B**). The amounts of AR1 used in the right panels of (**A**) and (**B**) were 10 and 2 ng respectively.

Figure 5   Analysis of the influence of HMGB1 on DNA binding by the R3 deletion mutants

(**A**) DNA binding curves of the R3 deletion mutants of the AR-DBD (●), the GR-DBD (○), the PR-DBD (▼) and the MR-DBD (▽) are depicted. The rat TAT-GRE was used as a radiolabelled probe. (**B**) Comparison of the DNA binding curves of the AR3 fragment with the rat TAT-GRE in the presence of 600 ng BSA or 600 ng HMGB1. Results are depicted as in Figure 2. The K_s values in the absence and the presence of HMGB1 were 23.1 and 19.8 respectively The amount of AR3 used in the right panel was 5 ng.

dimers is independent of the presence of HMGB1/HMGB2 [15]. We analysed the effect of the presence of HMGB1 on the interaction of the AR3 fragment with the TAT-GRE (Figure 5). The presence of HMGB1 did not significantly alter the affinity of the protein fragment for the DNA, indicating that HMGB1 needs at least part of the AR-CTE and hinge region for the stimulation of receptor DNA binding. It remains to be determined whether or not this indicates that steroid receptors recruit HMGB1 to the DNA by direct interaction between their DBDs and HMGB1. HMGB1/HMGB2 has been proposed to substitute for the lack in the DBDs of the steroid receptors of a structured CTE, such as the A-box in TR and the T-box in RXR, interacting with the DNA minor groove and thereby stabilizing the protein–DNA complex [15,17]. It is therefore conceivable that HMGB1/HMGB2 needs to interact closely with part of the CTE or hinge region of the steroid receptors.

However, our findings do indicate that the AR is capable of interacting with its recognition motif independently of HMGB1, which is not true for the GR, PR or MR, and thus might interact with its binding motif in a way that is qualitatively different from the other steroid hormone receptors.

General conclusion

We have compared in binding assays *in vitro* the sensitivity of the DNA-binding domains of each of the steroid receptors to the presence of HMGB1. We have found that the GR-DBD is significantly more dependent on the presence of HMGB1 than are the AR, PR and MR. In functional assays, surprisingly, co-
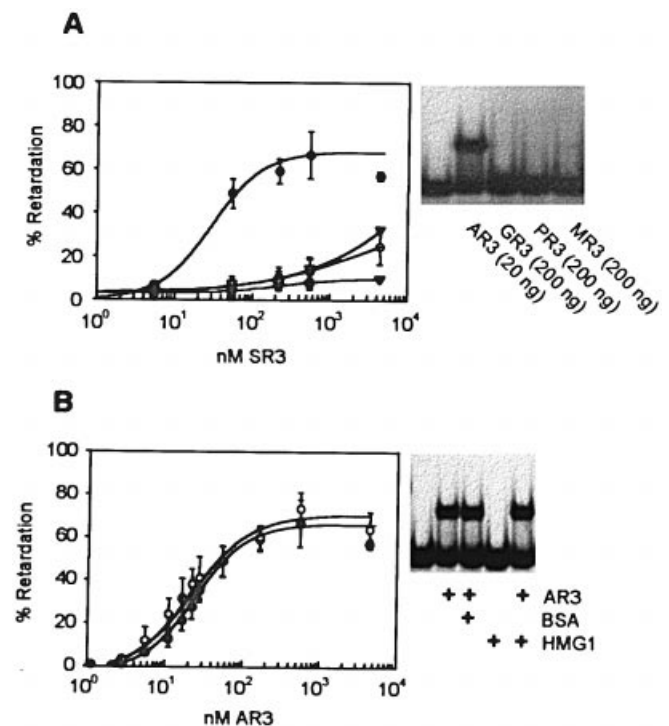
transfection of an HMGB1 expression plasmid has no effect on AR or MR function, in contrast with GR and PR. These findings are in accordance with the results of Calogero et al. [35] that HMGB1 knock-out mice display a predominant loss of GR function. We also demonstrate that no significant difference exists in the influence of HMGB1 on AR-DBD binding to direct or inverted repeats of its core recognition element. Finally, we have shown that a deletion mutant of the AR-DBD but not the GR-DBD, PR-DBD or MR-DBD, is capable of binding to DNA independently of the presence of HMGB1, indicating that the AR might resemble in this respect the members of the class II nuclear receptor family, the DNA binding of which is independent of the presence of HMGB1/HMGB2. Our results provide further insights into the mechanism of DNA binding by steroid receptors, in particular the AR, and the mechanisms involved in the specificity of transcriptional responses regulated by different steroid receptors sharing similar recognition sites.

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