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HMGB1 interacts differentially with members of the Rel family of transcription factors

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Abstract

HMGB1 is an architectural factor that enhances the DNA binding affinity of several proteins. We have investigated the influence of HMGB1 on DNA binding by members of the Rel family. HMGB1 enhances DNA binding by p65/p50 and p50/p50, but reduces binding by p65/p65, c-Rel/c-Rel, p65/c-Rel, and p50/c-Rel. In pull-down assays, HMGB1 interacts directly with the p50 subunit via its HMG boxes and this interaction is weakened by the presence of the acidic tail. Functionally, HMGB1 is required for the NF-κB-dependent expression of the adhesion molecule VCAM-1.

Keywords: HMGB1; Rel proteins; NF-κB; Protein–protein interaction; Transcription

The HMG box family of architectural transcription factors comprises the mammalian high mobility group proteins HMGB1 and HMGB2 (formerly, HMG1 and HMG2 [1]) as well as the *Drosophila* dorsal switch protein, DSP1 [2]. HMGB1 is characterized by a tripartite structure consisting of two DNA binding domains, HMG boxes A and B, and an acidic C-terminus composed of 30 glutamic and aspartic residues. This structure is conserved in the C-terminus of the DSP1 protein; however, the N-terminal half contains additional glutamine-rich regions, which are not present in the mammalian HMGB1/2 proteins. The *dsp1* and *Hmgb1* genes have similar intron–exon boundaries and it has been proposed that they are derived from a common ancestral gene [3]. Though structurally related, DSP1 and HMGB1 do not appear to be functional orthologs: most notably, the DSP1 protein is expressed ubiquitously during embryogenesis, and its absence causes homeotic phenotypes, but is expressed only in ovaries and in brain of adult flies [4].

HMGB1 is a ubiquitously expressed, highly abundant nuclear protein with more than one million copies per single nucleus. It recognizes with high affinity specific DNA structures like kinked or bent DNA and four-way junctions. HMGB1 also binds double-stranded DNA, albeit with reduced affinity and little sequence specificity. Binding occurs in the minor groove and HMGB1 itself introduces sharp bends and distortions in the double helix. HMGB1 interacts with many apparently unrelated proteins by recognizing short amino acid sequences [5]; several of these interactors are transcription factors (including p53, Hox proteins, and steroid receptors), and HMGB1 enhances their binding to cognate DNA sites (for reviews see [6,7]). The phenotype of *Hmgb1*$^{-/-}$ mice is in agreement with the role of HMGB1 as a regulator of transcription. Mice die shortly after birth due to hypoglycemia and show a defect in the activation of genes responsive to the glucocorticoid receptor [8]. However, HMGB1 is also able to repress transcription by interacting with the TATA-binding protein, TBP, like its *Drosophila* relative DSP1 [9–11].

The Rel family of transcription factors (which includes NF-κB) is characterized by a conserved structure in the N-terminus, called the Rel homology domain (RHD), which contains the sequences for DNA binding,

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dimerization, and the nuclear localization signal. The Rel protein family can be divided into two groups based on differences in structure, function, and mode of synthesis. The first group consists of the subunits p50 and p52, which are synthesized as precursors. The second group comprises the mammalian proteins p65, RelB, and c-Rel as well as the *Drosophila* proteins Dorsal and Dif, which are not synthesized as precursors and contain in addition to the RHD one or more transcriptional activation domains. Members of the two groups bind to DNA as homo- or heterodimers, even though not all combinations have been found yet (reviewed in [12,13]).

NF-κB is an inducible transcription factor that plays a key role in the expression of a variety of genes involved in inflammation, cell survival, apoptosis, cell differentiation, and cancer. It is one of the most common transcription factors and is found in virtually all tissues. In unstimulated cells the dimer is bound by the inhibitor protein IκB, which masks the nuclear localization signal and hence retains it in the cytoplasm. Activation of cells with various stimuli initiates a signalling cascade that leads to the phosphorylation of IκB, which in turn targets the IκB protein for ubiquitination and subsequent degradation. As a result, NF-κB translocates to the nucleus where it binds its cognate sites. Frequently, NF-κB synergizes with other transcription factors to activate its target genes; in some cases NF-κB can also repress target genes. In *Drosophila*, the protein responsible for the switch of Dorsal from activator to repressor is DSP1 [2]. DSP1 interacts directly with Dorsal and also interacts with the mammalian Rel protein p50 and weakly with p65 [14,15]. Conversely, the mammalian HMGB1 also has been shown to interact with Dorsal [15], but potential interactions between the mammalian Rel proteins and HMGB1 remained to be elucidated. We show here that HMGB1 interacts directly with the p50 subunit and facilitates the binding to DNA of the p65/p50 and p50/p50 dimers, whereas it reduces the binding of the p65/p65, c-Rel/c-Rel, and p50/c-Rel dimers. Moreover, TNFα induced expression of VCAM mRNA, which is regulated by the NF-κB subunits p65/p50, requires the presence of HMGB1.

Materials and methods

Cloning, expression, and purification of proteins. The plasmids pRNHMG1/M1-V176 to express HMGB1 (AB), pT7-HMG1bA encoding box A, and pT7-HMG1bB encoding box B have been described elsewhere [16]. The plasmid pT7-7-rHMG1cm, encoding the full-length HMGB1 protein, was a gift of Prof. J.O. Thomas (Cambridge). Expression and purification of the single boxes, HMGB1 (AB), and the full-length protein was performed as described [16,17]. HMGB1 purified from calf thymus was a gift of Dr. Jordi Bernues (Barcelona).

The plasmids c-RelpRSET, Rc/CMVhp65, and Rc/CMVhp50 encoding for c-REL, p65 and p50, respectively, were provided by Dr. Luisa Guerrini (Milano). In vitro translated proteins were synthesized using the TNT T7 coupled reticulocyte lysate system kit (Promega) according to instructions provided by the manufacturer. The pBluescript SK vector was purchased from Stratagene.

Electromobility shift assay. The double-stranded oligonucleotide containing the HIVκB binding site was labeled using T4-polynucleotide kinase and [γ-^{32}P]ATP. Binding reactions were performed in a total volume of 20 μl with a final buffer concentration of 12.5 mM Hepes, pH 7.9, 80 mM KCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM DTT, 8% glycerol, and 0.8 μg/μl poly(dI–dC). In case of the reaction containing the p50 homodimer, poly(dI–dC) was omitted. The protein was incubated in the binding buffer for 10 min on ice and 2 μg antibody was added, where required, for an additional 30 min. Following addition of the oligonucleotides a further 30 min incubation at room temperature was performed and the reaction products were separated on a 6% non-denaturing polyacrylamide gel in 0.5× TBE and 4% glycerol.

The following oligonucleotides were used:

HIVκB	(5′-TAGGGACTTTCCGCTGGGGACTTTCCAG-3′, 5′-CTGGAAAGTCCCCAGCGGAAAG TCCCTA-3′)
NS	(5′-CCTCCCTCGAGTCACACCCC-3′, 5′-GGGGTGTGACTCGAGGGAGG-3′)
ε-NFκB	(5′-CCTCCGGGGGTTCCCACCCC-3′, 5′-GGGGTGGGAACCCCCGGAGG-3′).

Antibodies against p65, p50, and c-Rel were purchased from Santa Cruz.

Protein–protein interaction. The HMGB1 protein or its different domains were coupled to tosyl-activated Dynabeads M-280 (Dynal). Interaction assays between the in vitro translated, ^{35}S-labeled proteins and the coupled protein were performed as described [18].

Cell culture and TNF-α stimulation. The mouse embryonic fibroblast cell lines C1, derived from a *Hmgb1*$^{-/-}$ mouse, and VA1, derived from a wild type littermate, have been described earlier [8]. Cells were cultivated in DMEM with Glutamax (Life Technologies), 10% FCS, and 1% penicillin/streptomycin. For TNF-α stimulation cells were plated into 6-well plates and stimulated after overnight culture with 100 U/ml TNF-α for the indicated time.

RT-PCR analysis. Total RNA was extracted with Trizol (Life Technologies) according to the instructions of the manufacturer. Four μg of total RNA was treated with DNAseI and subsequently reverse transcribed with Superscript II Rnase H reverse transcriptase (Life Technologies) as described by the manufacturer, using oligo(dT) as a template primer. The cDNA was subsequently diluted 1:320, amplified by PCR, and quantified with a Light Cycler instrument (Roche) and the LightCycler—Fast Start DNA master SYBR green I kit (Roche) according to instructions of the manufacturer. The following primer pairs were used:

VCAM-1	(5′-CTCCAGACATTTACCCAGTT-3′, 5′-TCACAGCCAATAGCAGCACA-3′)
β-Actin	(5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, 5′-CTAGAAGCATTGCGGTGGACGATGGAGGG-3′)
HPRT	(5′-AGTTCTTTGCTGACCTGCTG-3′, 5′-CGCTCATCTTAGGCTTTGTA-3′).

Results and discussion

Interactions of members of the Rel family of transcription factors with HMGB family members have already been reported: the *Drosophila* Rel protein, Dorsal, interacts with DSP1 and with mammalian HMGB1 [2,14,15]. Here, we analyze the interplay between the mammalian HMGB1 and the mammalian Rel proteins.

Different members of the Rel family of proteins have specific binding preferences to NF-κB DNA binding motifs. We have therefore chosen an oligonucleotide containing the HIV-κB site, which is recognized by all

Rel proteins [19]. We then analyzed the effect of HMGB1 on the binding of the different mammalian Rel family members, translated in vitro. Fig. 1A shows a specific shift of the c-Rel homodimer; the amount of the c-Rel–DNA complex is strongly reduced after addition of the anti-c-Rel antibody, but not of an unspecific antibody. The specificity of the binding was confirmed by competition with specific or non-specific oligonucleotides, respectively (Fig. 1B, lanes 17 and 18). Binding of the c-Rel homodimer to the HIV-κB binding site is reduced when HMGB1 is added (Fig. 1B, lanes 6–9 and 11–14). The origin of HMGB1, whether purified from calf thymus or produced in bacteria, is irrelevant. HMGB1 on its own is not able to produce a gel shift of the HIV-κB fragment (lanes 5 and 10) nor can the in vitro translation mix be programmed to produce c-Rel (V, lane 15).

We next investigated the effect of HMGB1 on the binding of the c-Rel/p50 heterodimer to the HIV-κB site. HMGB1 also reduces the binding of this heterodimer (Fig. 2B). Again the effect was independent of the source of HMGB1 used. The specificity of the binding was shown by addition of specific or non-specific competitor oligonucleotides (Fig. 2B) and confirmed with specific or non-specific antibodies, respectively (Fig. 2A).

Also binding of the p65/c-Rel heterodimer to the κB binding site is reduced by HMGB1 (Fig. 3A). At variance to what has been observed with other dimers containing c-Rel, the p65/c-Rel heterodimer is supershifted both by anti-p65 and anti-c-Rel antibodies.

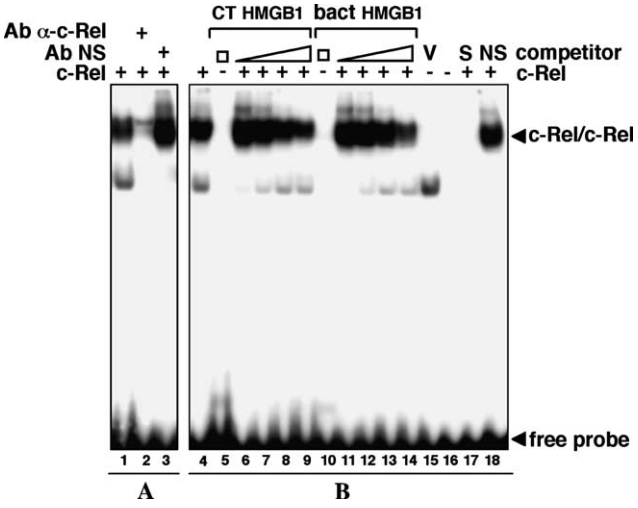

Fig. 1. HMGB1 decreases binding of the c-Rel/c-Rel dimer to DNA. (A) EMSA of in vitro translated c-Rel with or without addition of specific (α-c-Rel) or unspecific antibody (NS). The second arrow shows migration of the unbound HIV-κ B probe (free probe). (B) EMSA of in vitro translated c-Rel (where indicated with +) and effect of addition of increasing amounts (100, 300, 600, and 900 ng) of HMGB1, purified from calf thymus (CT) or produced in bacteria. S indicates the presence of specific, N of unspecific competitor DNA. V shows the shift produced by the in vitro translated empty vector.

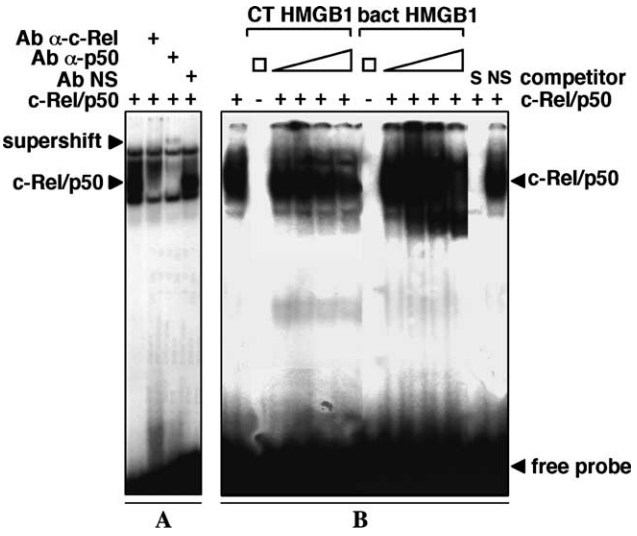

Fig. 2. HMGB1 decreases binding of the c-Rel/p50 dimer to DNA. (A) EMSA of in vitro translated c-Rel/p50. Addition of a specific antibody against p50 (α-p50) creates a supershift as indicated by an arrow, whereas addition of an unspecific antibody (NS) does not. Addition of an antibody against c-Rel inhibits the formation of the complex. The lower arrow shows migration of the unbound probe (free probe). (B) EMSA of in vitro translated c-Rel/p50 (where indicated with +) and effect of addition of increasing amounts (100, 300, 600, and 900 ng) of HMGB1 (either bacterially expressed or isolated from calf thymus). Other symbols are used as described for Fig. 1.

Thus, HMGB1 reduces the binding capabilities of the c-Rel homodimers as well as the two c-Rel containing heterodimers p50/c-Rel and p65/c-Rel.

Previous analysis showed that binding of the p65 subunit of NF-κB to DNA was not or only weakly positively affected by the *Drosophila* HMGB protein, DSP1 [14]. Addition of mammalian HMGB1, however, decreased the binding of the p65 homodimer to DNA, as was the case for the other Rel dimers investigated above (Fig. 3B). To exclude effects due to the identity of the binding site used, we also tested the binding to a different oligonucleotide containing a NF-κB binding site from the Igε promoter [20,21]. However, HMGB1 interfered with the binding of this target DNA as well (data not shown).

The classical NF-κB heterodimer is composed of the p65 and p50 subunits. The binding of this heterodimer to DNA is enhanced by the addition of HMGB1 (Fig. 4A), which is similar to the effect of DSP1 [14]. A positive effect was also seen for the binding of the p50 homodimer (Fig. 4B), which again parallels the effect of DSP1 on p50/p50 [14]. In our case, we observed no ternary complex between NF-κB, DNA, and HMGB1; on the contrary, DSP1 produces a ternary complex with p50 homodimers or p50/p65 heterodimers on a NF-κB site [14]. The absence of a ternary complex is a typical feature of the effect of HMGB1 on DNA binding of transcription factors and has also been observed for the

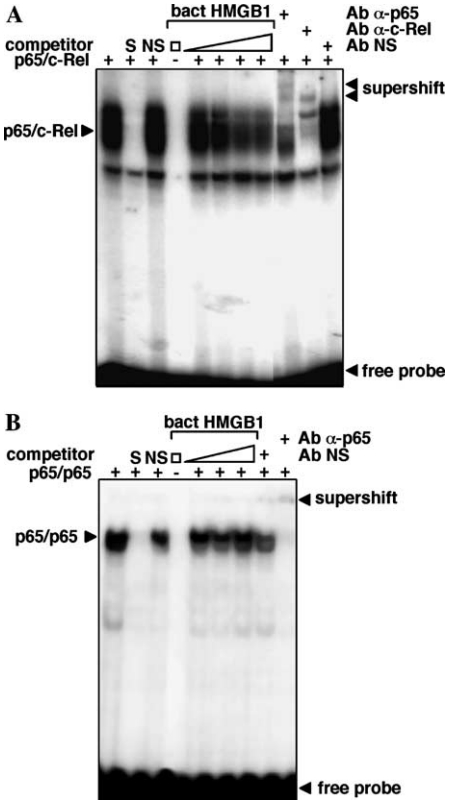

Fig. 3. HMGB1 decreases binding of p65/c-Rel (A) and p65/p65 (B) to DNA. Symbols used as described for Fig. 1.

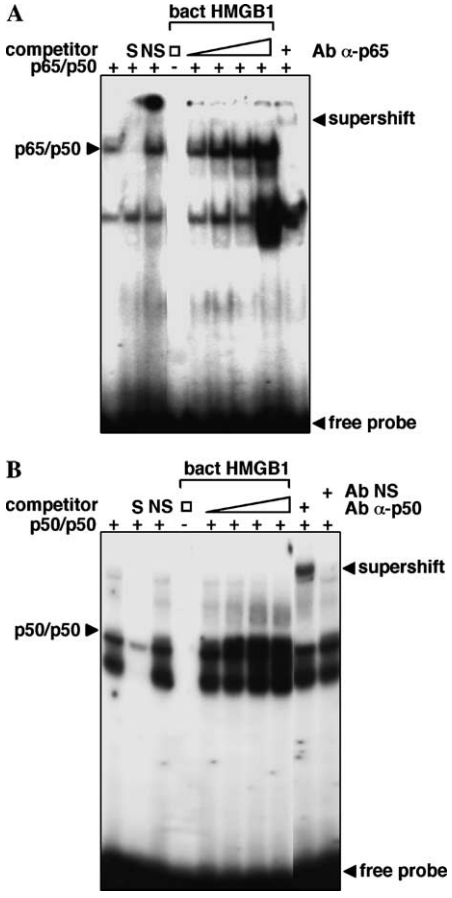

Fig. 4. HMGB1 increases binding of p65/p50 (A) and p50/p50 (B) to DNA. Symbols used as described for Fig. 1.

enhancement of binding of steroid hormone receptors, HoxD9 and p53 [18,22,23].

To investigate direct interactions between the Rel proteins and HMGB1 in the absence of DNA, we used pull-down assays (Fig. 5). Only the p50 subunit bound to HMGB1 coupled to magnetic beads, whereas p65 and c-Rel did not interact directly with HMGB1. None of the Rel proteins bound unspecifically to the BSA control. The interaction between HMGB1 and p50 is mediated by the two HMG boxes, which are both able to bind p50, albeit weakly. The strongest interaction occurred with a truncated HMGB1 protein, which contained both HMG-boxes but lacked the acidic tail, suggesting that the acidic tail interferes with the binding surface for p50. The direct interaction of HMGB1 readily explains the enhanced binding of the p50 homodimer and the p50/p65 heterodimer to the NF-κB binding site. However, other mechanisms must account for the reduced binding capabilities of the other Rel homo- and heterodimers, in particular that of the p50/c-Rel heterodimer.

In order to verify the functional importance of HMGB1 for NF-κB transcriptional activity, we compared the expression of an NF-κB target in mouse embryonic fibroblasts (MEFs) obtained from *Hmgb1*$^{-/-}$ mice or wild type littermates. VCAM-1, an adhesion molecule, is expressed in fibroblasts and other cells under the direct control of NF-κB (both the p50 homodimer and the p50/p65 heterodimer). The p50/p65 heterodimer which is translocated to the nucleus in response to TNF-α [24].

We stimulated wild type and *Hmgb1*$^{-/-}$ MEFs with TNF-α, and measured VCAM-1 mRNA levels several times after stimulation. Fig. 6 (lower panel) shows that in wild type MEFs the expression of VCAM mRNA is induced after 1 h to over fivefold the original level and then declines slowly over time. On the contrary, VCAM mRNA starts from about the same level in *Hmgb1*$^{-/-}$ MEFs, but TNF-α does not increase its level significantly. As control we used two housekeeping genes, β-actin (Fig. 6, upper panel) and *Hprt* (Fig. 6, middle panel). Actin has a high level of expression and *Hprt* a moderate one; the basal levels were the same in the 2 cell lines. Expression of these two genes was indeed unchanged after stimulation with TNF-α, in both cell lines. These data show that HMGB1 is necessary for NF-κB-dependent VCAM mRNA expression. Moreover, it has been shown that in TNF-α-stimulated fibroblasts the predominant form of NF-κB is the p65/p50 heterodimer [24], whose DNA binding affinity is indeed enhanced by HMGB1.

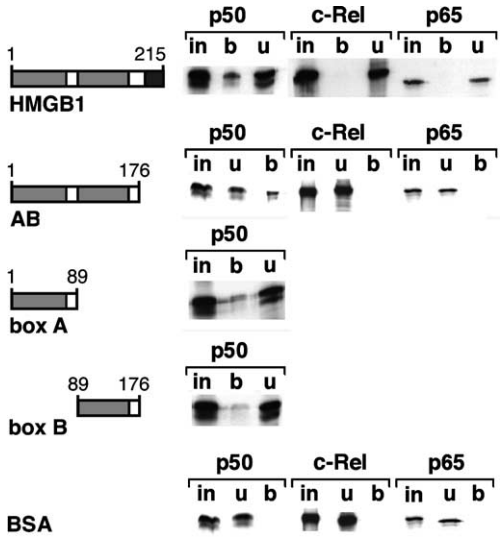

Fig. 5. HMGB1 interacts in vitro with p50 via the HMG boxes. Shown in the left panel is a schematic representation of the domain structure of HMGB1 and its truncated versions (amino acid number is indicated above). HMGB1 identifies the full-length protein, AB the protein lacking the acidic tail, and box A and box B the two single HMG boxes, respectively. These were immobilized onto Dynabeads. The right panel shows the result of the pull-down assay: "in" indicates the input of in vitro translated Rel protein, "b" the fraction bound to HMGB1 or HMGB1 truncations, and "u" the unbound fraction.

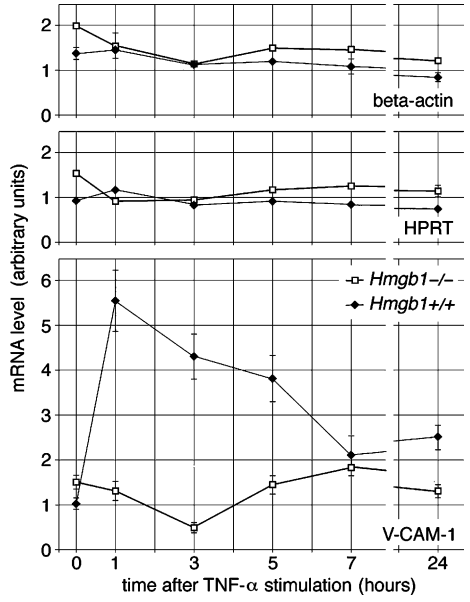

Fig. 6. HMGB1 is required for TNF-α stimulated VCAM-1 expression. Mouse embryonic fibroblasts derived from *Hmgb1*$^{-/-}$ (empty squares) or wild type (black diamonds) littermates were stimulated with 100 U/ml TNF-α for the indicated time. On the vertical axis are expression levels of VCAM-1, HPRT, and β-actin mRNA, as determined by quantitative RT-PCR. Each point represents the mean and standard error of four to six experiments.

HMGB1 is an architectural transcription factor which binds to the minor groove of DNA and is able to bend DNA. The DNA bound by Rel proteins is bent to various degrees as determined by the crystal structures (reviewed in [25]). On the other hand, mutations that produce excessive bending in the NF-κB binding site abrogate protein binding [26]. It seems therefore likely that DNA bending is important for Rel protein binding, and that HMGB1 might modulate the DNA binding activities of the various Rel family members by bending the target DNA. Since the different Rel dimers recognize the target DNA in a slightly different way, bending of the NF-κB site by HMGB1 could result in either enhanced or reduced stability of the Rel–DNA complexes. This is similar to what has been proposed for the interaction of HMGB1 with steroid hormone receptors and with p53 [22,23]. Interestingly, another minor groove binding protein which bends DNA, HMGA1 (formerly HMG-I(Y) [1]), also stimulates binding of certain Rel dimers to the NF-κB site in the β-interferon gene and also interacts directly with specific Rel subunits [27]. NF-κB thereby synergizes with other transcription factors, which ultimately leads to the formation of a multiprotein complex which recruits chromatin remodelling machines and RNA pol II on the β-interferon gene (reviewed in [28]). Whether HMGB1 is able to play a similar complex role in modulating gene expression by NF-κB remains to be elucidated, but our work definitely establishes that HMGB1 can modulate the binding of NF-κB to its target sequences, and that this translates into a readily measurable effect on the expression of the VCAM gene.

Acknowledgments

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