Interactions of the Basic N-Terminal and the Acidic C-Terminal Domains of the Maize Chromosomal HMGB1 Protein†

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Received January 13, 2004; Revised Manuscript Received April 1, 2004

ABSTRACT: Maize HMGB1 is a typical member of the family of plant chromosomal HMGB proteins, which have a central high-mobility group (HMG)-box DNA-binding domain that is flanked by a basic N-terminal region and a highly acidic C-terminal domain. The basic N-terminal domain positively influences various DNA interactions of the protein, while the acidic C-terminal domain has the opposite effect. Using DNA−cellulose binding and electrophoretic mobility shift assays, we demonstrate that the N-terminal basic domain binds DNA by itself, consistent with its positive effects on the DNA interactions of HMGB1. To examine whether the negative effect of the acidic C-terminal domain is brought about by interactions with the basic part of HMGB1 (N-terminal region, HMG-box domain), intramolecular cross-linking in combination with formic acid cleavage of the protein was used. These experiments revealed that the acidic C-terminal domain interacts with the basic N-terminal domain. The intramolecular interaction between the two oppositely charged termini of the protein is enhanced when serine residues in the acidic tail of HMGB1 are phosphorylated by protein kinase CK2, which can explain the negative effect of the phosphorylation on certain DNA interactions. In line with that, covalent cross-linking of the two terminal domains resulted in a reduced affinity of HMGB1 for linear DNA. Comparable to the finding with maize HMGB1, the basic N-terminal and the acidic C-terminal domains of the *Arabidopsis* HMGB1 and HMGB4 proteins interact, indicating that these intramolecular interactions, which can modulate HMGB protein function, generally occur in plant HMGB proteins.

High-mobility group (HMG)[1] proteins represent a heterogeneous family of relatively abundant nonhistone proteins associated with eukaryotic chromatin (*1*, *2*). Proteins belonging to the subgroup of the HMGB proteins (*3*) contain one or two copies of a distinctive DNA-binding motif termed the HMG-box domain, whose global fold is well-conserved, consisting essentially of three α helices, which are arranged in an L shape (*1*, *4*, *5*). The HMG-box domain mediates nonsequence-specific binding of these proteins to linear DNA and high-affinity interactions with distorted DNA structures such as four-way junctions, minicircles, and *cis*-platinated DNA (*2*, *4*−*7*). In complexes with B DNA, the concave surface of the HMG-box domain binds predominantly to the minor groove of the DNA, bending the DNA over 90° (*4*, *5*). HMGB proteins act as architectural components in chromatin facilitating the assembly of nucleoprotein complexes, which are involved, for instance, in the regulation of transcription and recombination (*2*, *4*, *6*, *8*).

Chromosomal HMGB proteins have been identified and characterized from a number of plant species, revealing similarities as well as dissimilarities to their vertebrate, insect, and yeast counterparts (*9*−*15*). On the basis of Northern and Western blot analyses, the *hmgb* genes are considered to be expressed ubiquitously in the plant (*14*, *16*−*18*). The DNA interactions of several plant HMGB proteins have been studied in quite some detail, revealing that they can bind linear DNA nonsequence-specifically with moderate affinity but bind certain DNA structures with high affinity (*10*, *12*, *13*, *19*−*22*). Higher plants have at least five different HMGB proteins (*8*). The various maize HMGB proteins differ in their chromatin association (*23*), in their expression levels (*17*), in some of their DNA interactions (*10*, *24*), in their posttranslational modification by protein phosphorylation (*25*), and in their interaction with the transcription factor Dof2 (*26*). Typically, the plant HMGB proteins have a single HMG-box domain, which is flanked by a basic N-terminal domain and a highly acidic C-terminal domain. While the HMG-box domain of the various plant HMGB proteins is relatively conserved, the basic and acidic flanking regions are variable in length and sequence (*27*). The regions flanking the HMG-box domain modulate the DNA interactions of the maize HMGB1 protein (formerly termed HMGa). Thus, the basic N-terminal domain positively influences the binding to linear DNA, the circularization of short DNA fragments, and the stimulation of the *β*-recombination reaction, whereas the acidic C-terminal domain has the opposite effect (*24*, *28*). Full-length HMGB1 and the individual HMG-box domain displayed similar DNA interactions, suggesting that in the intact protein the basic N-terminal domain and the acidic C-terminal domain functionally "neutralise" each other (*27*).

† This research was supported by a grant from the Danish Research Council to K.D.G.

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[1] Abbreviations: HMG, high-mobility group; EMSA, electrophoretic mobility shift assay; GST, glutathione-*S*-transferase; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; CD, circular dichroism; BNPS-skatole, 2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine.

10.1021/bi0499009 CCC: $27.50
Published on Web 06/02/2004

Here, we have examined possible intramolecular interactions within the maize HMGB1 protein, which may contribute to the regulation of the DNA interactions of the protein. We have analyzed full-length and various truncated versions of HMGB1 by circular dichroism (CD) and fluorescence spectroscopy. Intramolecular cross-linking of the acidic tail demonstrated a preferential interaction with the basic N-terminal domain. Moreover, it is shown that the individual N-terminal domain can bind to DNA, which can support the HMG-box-domain-mediated DNA interactions of HMGB1.

EXPERIMENTAL PROCEDURES

Protein Production. Full-length (M1−E157) and truncated (M1−K123, G35−Y109, and G35−E157) recombinant maize HMGB1 proteins were expressed as 6× His-tagged proteins in *Escherichia coli* and purified by three-step column chromatography as described previously (*28*). The region encoding the N-terminal domain of HMGB1 (amino acid residues M1−G35) was amplified by PCR using primers (5′-AAGGATCCATGAAGGGGGCCAAATCCAA-3′ and 5′-AAGAATTCTTAGCCGGCCTTCCCTTTCCT-3′), Deep-Vent DNA polymerase (New England Biolabs), and the HMGB1 cDNA sequence (*10*) as the template. The resulting PCR product was digested with *Eco*RI and *Bam*HI and inserted into the *Eco*RI/*Bam*HI-digested expression vector pGEX-4T-1 (Amersham Bioscience), which provides an N-terminal glutathione-*S*-transferase (GST) fusion. The final plasmid was checked by DNA sequencing and transformed into the *E. coli* strain BL21. For protein production, the cells were grown to an OD_{600} of 0.8 in 2× YT medium (containing ampicilin), and protein expression was induced by the addition of 2 mM IPTG. After 2 h, the cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-HCl at pH 7.5, 500 mM NaCl, 10 mM 2-mercaptoethanol, 0.5 mM PMSF, and 100 μg/mL benzamidine), and sonicated. The supernatant of the centrifugation (12000*g* for 10 min) was incubated for 1 h with glutathione sepharose 4B (Amersham Bioscience). The sepharose beads were washed with PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 at pH 7.3, 140 mM NaCl, and 2.7 mM KCl), and HMGB1 (M1−G35) was released from the beads by thrombin cleavage of a thrombin site situated between the GST fusion part and the HMGB1 sequence. Thrombin was removed from the HMGB1 (M1−G35) sample by a benzamidine sepharose 4 FF (Amersham Bioscience) chromatography. The *Arabidopsis* HMGB1 and HMGB4 proteins were expressed and purified as described previously (*20*). For certain experiments, maize HMGB1 was phosphorylated using recombinant purified protein kinase CK2α, and the phosphorylation reaction was checked by mass spectrometry as described previously (*25*), revealing that the phosphorylated HMGB1 used in this paper was double-phosphorylated at residue S^{149} and at residue S^{133} or S^{136}.

CD and Fluorescence Spectroscopy. CD measurements were performed using a Jasco J-715 instrument as described previously (*25*). Steady-state fluorescence experiments were performed using a Photon technologies fluorescence spectrophotometer (PTI) at protein concentrations of 1 μM, and the fluorescence emission intensity was recorded at 350 nm. To detect the tryptophane signal, the slit width was set to 4 nm and an excitation wavelength of 296 nm was used. The cuvette was temperature-controlled by a Peltier element, and the temperature of the solution was monitored with a PT100 element submerged into the solution (recording the temperature in the sample cell). Data collected in the range of 20−80 °C were processed with the software Felix (PTI).

DNA Cellulose Binding Assay. The interaction of HMGB1 (M1−G35) and HMGB1 (G35−Y109) with duplex DNA immobilized on cellulose was analyzed as described previously (*28*).

Electrophoretic Mobility Shift Assay (EMSA). A 106-bp fragment of the maize zein gene promoter was amplified by PCR using primers P1 and P4, Taq DNA polymerase (MBI Fermentas), and the plasmid pMS1 as the template, as described previously (*19*). Binding reactions contained binding buffer (10 mM Tris-HCl at pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% (w/v) glycerol, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylenecyanol), various amounts of protein, and 15 ng of the 106-bp fragment. Binding reactions were carried out for 10 min at room temperature, before loading the samples onto 8% polyacrylamide gels in 0.5× TBE buffer. The DNA was stained with SYBR Gold (Molecular Probes) and detected with the Typhoon 8600 Phosphorimager (Amersham Biosciences). Data were analyzed using the Image Quant software.

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) Cross-Linking and Protein Cleavage. To the protein samples (in 10 mM Na-phosphate buffer at pH 7.5, 1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF), a final concentration of 15−20 mM EDC (Pierce) was added from a freshly prepared stock solution, and aliquots of the reaction (∼2 μg of protein) were taken after various times. The cross-linking reaction was quenched by addition of 20 mM 2-mercaptoethanol. The cross-linked samples were either analyzed by SDS−PAGE in 20% polyacrylamide gels or further processed by formic acid cleavage of full-length HMGB1. In the cleavage reactions (*29*), a final concentration of 80% formic acid was added to the protein samples, and they were incubated for 24 h at 37 °C. After lyophilization of the samples, the proteins were resuspended for neutralization in 0.1 M NH_4HCO_3 and lyophilised. HMGB1 (G35−E157) was cleaved using BNPS-skatole (2-(2-nitrophenylsulfenyl)-3-methyl-3-bromoindolenine) (*30*). To 200 μL of the EDC-treated protein sample, 600 μL of acetic acid and 800 μL of a freshly prepared BNPS-skatole solution (1 mg/mL in 80% acetic acid) were added. After 36 h at room temperature, the samples were lyophilised. Finally, the samples of the cleavage reactions were resuspended in SDS-loading buffer and analyzed by SDS−PAGE in 20% polyacrylamide gels containing 8 M urea. Proteins were detected by staining with Coomassie brilliant blue.

RESULTS

DNA-Binding by the Individual Basic N-Terminal Domain of Maize HMGB1. To examine possible intramolecular interactions of the basic and acidic domains flanking the central HMG-box DNA-binding domain of maize HMGB1, full-length and truncated versions of the protein were expressed in *E. coli* and purified (Figure 1), the full-length protein (M1−E157), the protein lacking the acidic C-terminal domain (M1−K123), the protein lacking the basic N-terminal domain (G35−E157), and the individual HMG-box DNA-binding domain (G35−Y109). Previous experiments have

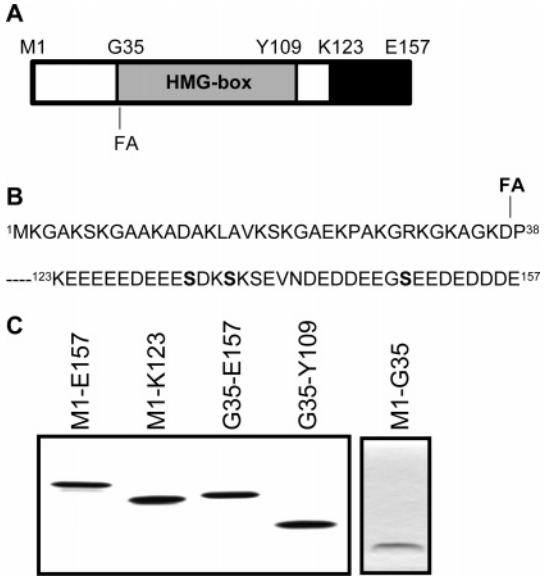

FIGURE 1: Maize HMGB1 protein and the recombinant proteins used in this paper. (A) Schematic representation of the maize HMGB1 protein. The central HMG-box DNA-binding domain is indicated by a gray box, while the acidic C-terminal domain is indicated by a black box. The amino acid positions delineating the full-length and the truncated recombinant proteins used in this paper are indicated, as well as the position of the formic acid cleavage site (FA). (B) Amino acid sequences of the basic N-terminal and the acidic C-terminal domains of HMGB1. The formic acid cleavage site (FA) D^{37}/P^{38} is indicated as well as the serine residues (in bold) in the acidic C-terminal domain, which are phosphorylated by protein kinase CK2 (*25*). (C) Full-length and truncated recombinant maize HMGB1 proteins analyzed by SDS−PAGE in an 18% polyacrylamide gel (left panel) and in a 20% polyacrylamide gel containing 8 M urea (right panel).

demonstrated that the presence of the basic N-terminal domain could enhance the affinity of maize HMGB1 for DNA. Because we hypothesised that this effect is due to direct interactions of the N-terminal domain with DNA (*28*), we have produced the individual N-terminal domain of HMGB1 (M1−G35) (Figure 1) and tested its DNA-binding properties. The purified N-terminal domain (M1−G35) was bound to DNA−cellulose and eluted with increasing salt concentrations. The eluted protein was analyzed by SDS−PAGE and quantified (Figure 2A). As a reference, the experiment was performed in parallel with the individual HMG-box domain (G35−Y109). Both proteins bound to the DNA−cellulose, and the HMG-box domain could be eluted only at higher NaCl concentrations (50% elution for G35−Y109 at ∼220 mM NaCl and for M1−G35 at ∼140 mM NaCl). Using EMSAs, the interaction of M1−G35 and G35−Y109 with a 106-bp DNA fragment of a maize 19 kDa zein storage protein gene promoter was examined. Increasing concentrations of the proteins were incubated with the DNA fragment, and the formation of protein/DNA complexes was analyzed by native gel electrophoresis (Figure 2B). Protein binding to the DNA was evident from the disappearance of the DNA band corresponding to the unbound fragment and from the appearance of rather diffuse complex bands, characteristic of nonsequence-specific protein/DNA interactions. As seen in the DNA−cellulose binding experiment, the affinity of the HMG-box domain for the DNA was higher than that of the N-terminal domain, because G35−Y109 complexed the DNA at lower protein concentrations than M1−G35.

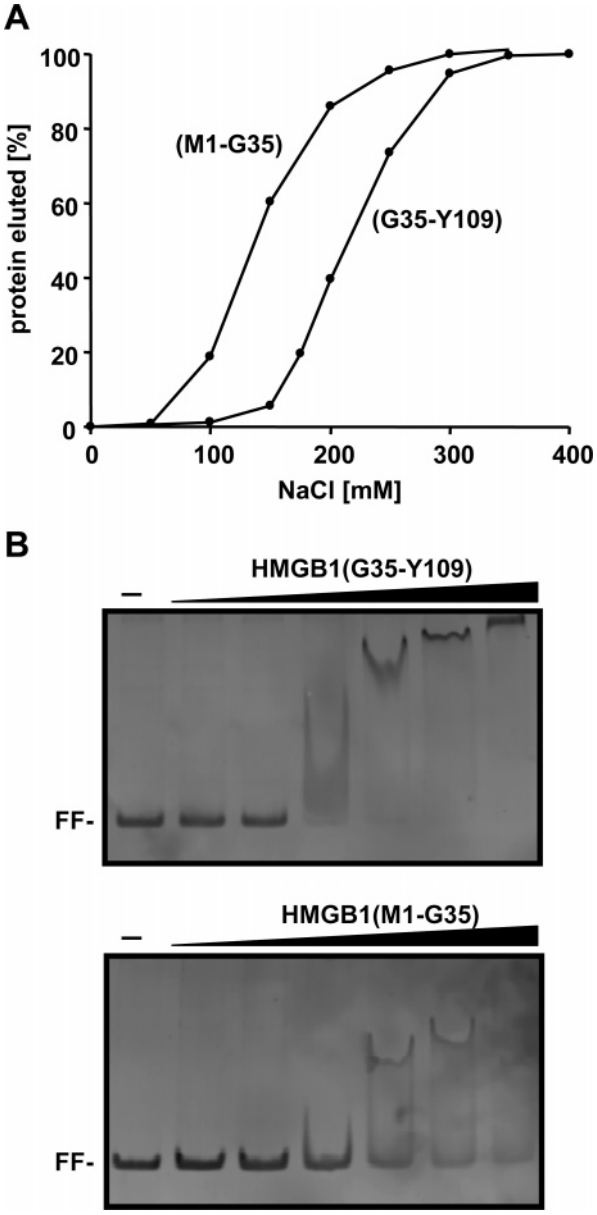

FIGURE 2: DNA binding of the individual N-terminal domain of maize HMGB1. (A) DNA interaction of the N-terminal basic domain analyzed by DNA−cellulose binding. The N-terminal basic domain (M1−G35) and, as a reference, the HMG-box domain (G35−Y109) were bound to DNA−cellulose and eluted with increasing concentrations of NaCl. The eluted proteins were analyzed by SDS−PAGE, stained with Coomassie, and quantified. The graphs represent the mean values of two independent experiments. (B) Binding of the N-terminal domain to a zein gene promoter fragment examined by EMSA. Increasing concentrations (0, 125 nM, 250 nM, 500 nM, 1 μM, and 2 μM) of the N-terminal domain (M1−G35) and, as a reference, the HMG-box domain (G35−Y109) were incubated with a 106-bp DNA fragment of a maize zein gene promoter. The reactants were separated by native PAGE, stained with SYBR Gold, and scanned using a phosphorimager. The migration position of the unbound DNA fragment is indicated (FF).

Contribution of the Domains Flanking the HMG-Box Domain to the Stability of Maize HMGB1. CD wavelength spectra of full-length HMGB1 and the truncated proteins (M1−K123, G35−Y109, and G35−E157) were recorded at 20 °C, resulting in spectra with a shape expected for the α-helical structure (data not shown), because of the largely α-helical conformation found in the HMG-box domain (*2*,

Table 1: Thermal Stability of Full-Length and Truncated HMGB1 Versions Measured by CD and Fluorescence Spectroscopy

HMGB1[a]	T_m fluorescence (°C)[b]	T_m CD (°C)[c]
M1−E157	53	50
G35−E157	58	61
M1−K123	43	47
G35−Y109	45	47

[a] Amino acid positions delineating the full-length and truncated HMGB1 versions. [b] Melting temperature determined by temperature scans monitored by fluorescence spectroscopy. [c] Melting temperature determined by temperature scans monitored by CD spectroscopy at 222 nm.

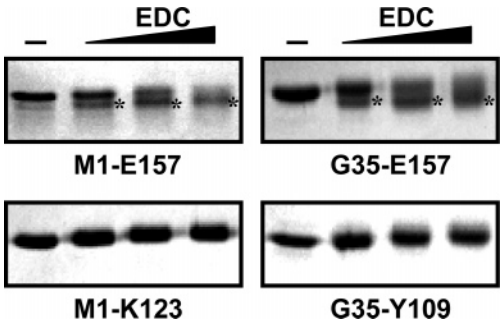

FIGURE 3: Intramolecular cross-linking of full-length and truncated maize HMGB1 proteins. The HMGB1 proteins (M1−E157, G35−E157, M1−K123, and G35−Y109) were reacted with EDC for various times (0, 10, 20, and 40 min) and separated by SDS−PAGE in 18% polyacrylamide gels. The appearance of an intramolecularly cross-linked protein band seen with the M1−E157 and G35−E157 proteins is indicated by an asterisk.

4, *5*). Moreover, these CD spectra recorded for the different versions of HMGB1 (including the individual N-terminal domain) indicated that the N- and C-terminal domains are essentially unstructured in solution. This is in-line with structure predictions using the AGADIR software (*31*) (www.embl-heidelberg.de), which predicted a helix percentage of 1.64 and 0.62 for the N- and C-terminal domains of HMGB1, respectively. The melting temperatures of the different proteins were measured by temperature scans using CD and fluorescence spectroscopy, revealing that the various versions of HMGB1 have different thermal stabilities: G35−Y109 ≈ M1−K123 < M1−E157 < G35−E157 (Table 1). These results indicate that the presence of the acidic tail can stabilize HMGB1 (especially in the absence of the basic N-terminal region) presumably by interacting with the basic parts of the protein (basic regions of the HMG-box domain and/or the basic N-terminal domain). The relative consistency of the CD and fluorescence results suggests that upon thermal denaturation secondary and tertiary structure are lost in parallel.

Intramolecular Interactions within the Maize HMGB1 Protein. Chemical cross-linking using the zero-length agent EDC (which cross-links carboxyl and amino groups) was employed to address the intramolecular interactions of the acidic tail in HMGB1. Full-length and truncated versions of HMGB1 were reacted with EDC for various times and analyzed by SDS−PAGE (Figure 3). Intramolecular cross-linking was evident from the reaction time-dependent appearance of a distinct second protein band of slightly higher electrophoretic mobility than the corresponding noncross-linked protein band. Cross-linking with EDC could be observed only with the proteins that contain the acidic tail

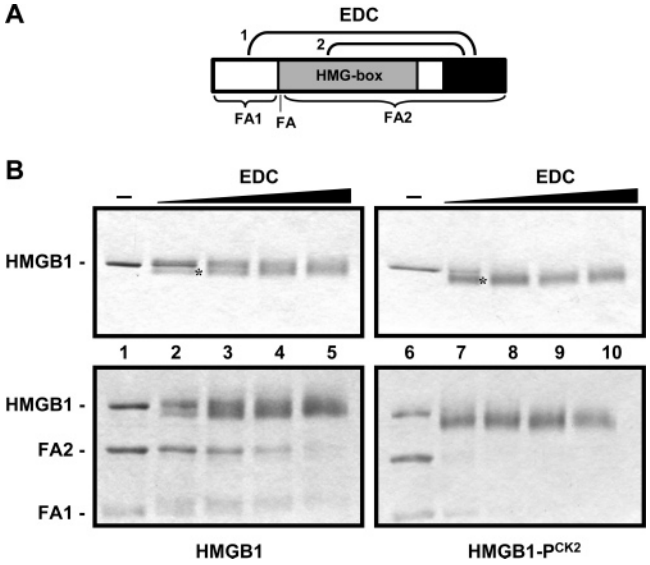

FIGURE 4: Intramolecular cross-linking of nonphosphorylated and CK2-phosphorylated full-length maize HMGB1 followed by formic acid cleavage. (A) Schematic representation of the HMGB1 protein. The formic acid cleavage site (FA) D^{37}/P^{38} is indicated as well as the formic acid cleavage products (FA1 and FA2). The possible EDC cross-links of the acidic tail are indicated on top: EDC may cross-link the acidic tail either (1) with the basic N-terminal domain resulting in a single cross-linked formic acid cleavage product (FA1 and FA2 are covalently linked by EDC) or (2) with the HMG-box domain resulting in two separable formic acid cleavage products (FA1 and FA2). (B) EDC cross-linking of full-length HMGB1. Nonphosphorylated (HMGB1, left panels, lanes 1−5) and CK2-phosphorylated (HMGB1−P^{CK2}, right panels, lanes 6−10) HMGB1 were reacted with EDC for various times (0, 20, 40, 60, and 90 min) and separated by SDS−PAGE (top panels). Another aliquot of the same reaction samples was subsequently cleaved by formic acid treatment, and the reaction products were separated by SDS−PAGE (bottom panels). The protein bands corresponding to the intramolecularly cross-linked product are indicated by an asterisk when they first appear in the course of the cross-linking reaction. The electrophoretic migration position of full-length HMGB1, which is also present after formic acid cleavage because of the incomplete cleavage reaction, is indicated. The migration positions of the formic acid cleavage products (FA1 and FA2) are also indicated.

(M1−E157 and G35−E157), whereas the other proteins could not be cross-linked. In G35−E157, the interaction of the acidic tail is most likely with basic regions in the HMG-box domain, because the N-terminal basic region is lacking in this protein.

To reveal the interaction partner of the acidic tail in the full-length protein, M1−E157 was reacted with EDC for various times (Figure 4B) and formic acid was added to aliquots of the cross-linking reactions. Formic acid can cleave polypeptides between aspartate and proline residues (*29*), and in maize HMGB1, there is a single formic acid cleavage site D^{37}/P^{38}, which is situated between the basic N-terminal domain and the HMG-box domain (indicated in parts A and B of Figure 1). Under the conditions used, formic acid cleaves HMGB1 incompletely, as evident from a portion of noncleaved protein present in the formic-acid-treated samples. SDS−PAGE of the formic acid cleavage products results in three protein bands, the noncleaved protein and the two formic acid cleavage products (FA1 and FA2, indicated in Figure 4A), when the noncross-linked HMGB1 was treated with formic acid (lane 1, bottom panel of Figure 4B). Analysis of the polypeptides of the cleavage reaction by

MALDI/TOF mass spectrometry confirmed the identity of the expected cleavage products (data not shown). Depending on the extent of the EDC cross-linking, the relative amount of product bands of the formic acid cleavage (FA1 and FA2) was reduced, while the diffuse band corresponding to the cross-linked full-length HMGB1 protein became more intense. Formic acid cleavage of the completely cross-linked HMGB1 no longer resulted in the formation of the two cleavage products (lane 5 of Figure 4B), demonstrating that EDC covalently cross-linked the N-terminal basic domain (corresponding to FA1) and the acidic C-terminal domain, because formic acid could no longer cleave HMGB1 into the two separable cleavage products (FA1 and FA2; see Figure 4A). Therefore, in full-length HMGB1, the acidic C-terminal tail interacts preferentially with the basic N-terminal domain rather than with the HMG-box domain. The intramolecular EDC cross-linking of the acidic tail occurred to the same extent, when the cross-linking experiments were performed in the presence of 100 mM NaCl (data not shown).

Because the phosphorylation of serine residues (S^{133}, S^{136}, and S^{149}) in the acidic tail of HMGB1 (detected in vivo and by in vitro phosphorylation) by protein kinase CK2 (indicated in Figure 1B) modulated various properties of the HMGB1 protein such as its thermal stability and certain DNA interactions (*25*), we have examined whether the intramolecular interactions of the acidic tail in the phosphorylated protein differed from those of the nonphosphorylated protein. In vitro phosphorylated HMGB1 (like the nonphosphorylated protein) was reacted with EDC and cleaved by formic acid. Relative to the nonphosphorylated protein, CK2-phosphorylated HMGB1 was remarkably more readily cross-linked by EDC, and accordingly the formic acid cleavage products (FA1 and FA2) indicative of the noncross-linked protein disappear more rapidly in the course of the cross-linking reaction. Cross-linking of nonphosphorylated HMGB1 required an approximately 4-fold more intense cross-linker treatment to obtain a cross-linking efficiency comparable to that of the phosphorylated protein. This finding indicates that phosphorylation of the acidic C-terminal domain of HMGB1 results in a stronger contact of the basic N-terminal and the acidic C-terminal domains in the phosphorylated protein when compared to the nonphosphorylated HMGB1.

The truncated HMGB1 protein (G35−E157) lacking the basic N-terminal domain can also be cross-linked with EDC (Figure 3). To examine the intramolecular cross-link, the protein, cross-linked with EDC for various times, was treated with BNPS-skatole, which cleaves proteins primarily at tryptophan residues. HMGB1 contains a single tryptophan residue that is located in the HMG-box domain (Figure 5A). Cleavage of noncross-linked HMGB1 (G35−E157) results primarily in two cleavage products of the expected size (6657 and 8574 Da, B1 and B2, respectively), while BNPS-skatole cleavage of the EDC cross-linked protein no longer resulted in the two separable cleavage products but resulted in a single cross-linked product comigrating with the noncleaved protein (Figure 5B). Therefore, in the absence of the N-terminal domain, EDC cross-links the acidic tail with the N-terminal part rather than with the C-terminal part of the HMG-box domain (cf. Figure 5A). With the full-length protein, no interaction of the acidic tail and the HMG-box domain was observed (Figure 4). The interaction of the acidic C-terminal domain with the HMG-box domain may explain the severely

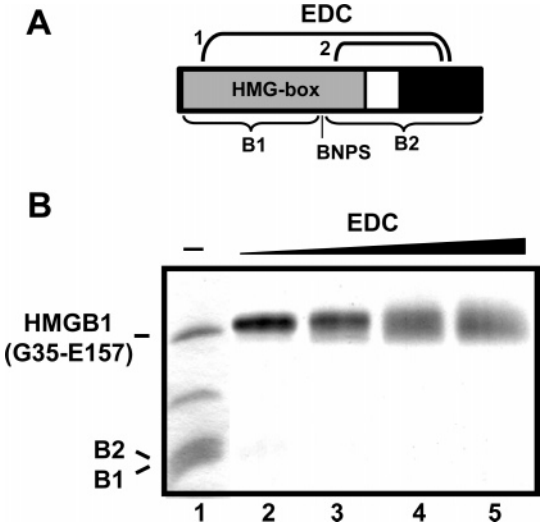

FIGURE 5: Intramolecular cross-linking of HMGB1 lacking the basic N-terminal domain. (A) Schematic representation of the HMGB1 (G35−E157) protein lacking the basic N-terminal domain. (Top) Possible intramolecular EDC cross-links of the acidic tail with the N-terminal part (1) or the C-terminal part (2) of the HMG-box domain are schematically indicated. The two possibilities can be discriminated by cleaving the cross-linked protein at the indicated single tryptophan residue in the HMG-box domain with BNPS-skatole (BNPS). The two expected cleavage products of the noncross-linked protein are indicated below (B1 and B2). (B) HMGB1 (G35−E157) was either untreated (lane 1) or treated with EDC for various times (5, 10, 20, and 40 min, lanes 2−5, respectively), before the protein was cleaved with BNPS-skatole. The protein samples were separated by SDS−PAGE. The electrophoretic migration position of the noncleaved or cleaved and cross-linked proteins are indicated [HMGB1 (G35−E157)], as well as the predominant BNPS-skatole cleavage products (B1 and B2). There is a weak band of an additional cleavage product, which results most likely from nonspecific cleavage.

reduced DNA binding of HMGB1 lacking the N-terminal basic domain (*13*, *28*).

Interaction between the Terminal Domains Affects the DNA Dinding of HMGB1. To test whether the interaction of the basic N-terminal and the acidic C-terminal domains influences the binding of HMGB1 to DNA, the DNA-binding properties of untreated and EDC-cross-linked HMGB1 were compared in EMSAs. Increasing concentrations of the proteins were incubated with a 106-bp DNA fragment of a maize 19 kDa zein storage protein gene promoter, and the formation of protein/DNA complexes was analyzed by native gel electrophoresis (Figure 6). Protein binding to the DNA was evident from the disappearance of the DNA band corresponding to the unbound fragment and from the appearance of rather diffuse complex bands. Binding of untreated HMGB1 to the promoter fragment could be detected at protein concentrations $\geq$500 nM, while DNA binding of the EDC-treated HMGB1 was observed only at protein concentrations $\geq 2\,\mu$M. Therefore, the EDC-mediated covalent link of the basic N-terminal and the acidic C-terminal domains reduced the affinity of HMGB1 for the DNA fragment, presumably because the cross-link affects the positive effect of the basic N-terminal domain on DNA binding.

Intramolecular Interactions of Other Plant HMGB Proteins. Because the basic N-terminal and acidic C-terminal domains of different plant HMGB proteins are relatively variable in length and amino acid sequence (*27*), we have

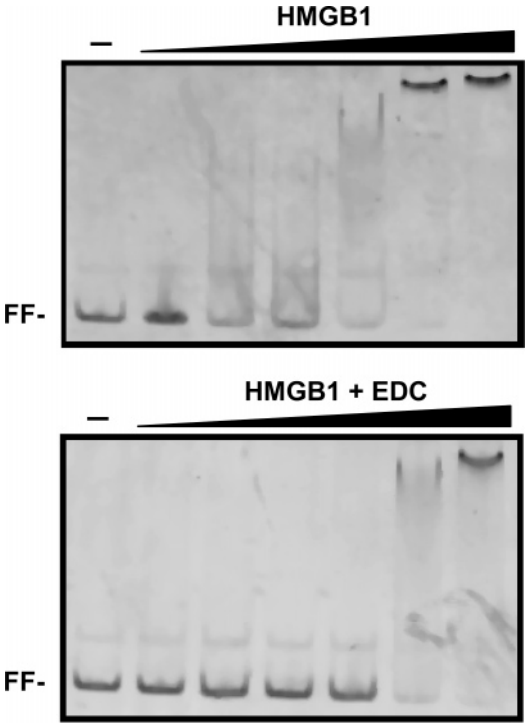

FIGURE 6: Cross-linking by EDC reduces the affinity of HMGB1 for DNA. Binding of untreated HMGB1 and of HMGB1 cross-linked with EDC to a zein gene promoter fragment was examined by EMSAs. Increasing concentrations (0, 250 nM, 500 nM, 750 nM, 1 μM, 2 μM, and 4 μM) of HMGB1 and of HMGB1 treated for 45 min with EDC were incubated with a 106-bp DNA fragment of a maize zein gene promoter. The reactants were separated by native PAGE, stained with SYBR Gold, and scanned using a phosphorimager. The migration position of the unbound DNA fragment is indicated (FF).

tested for intramolecular interactions the *Arabidopsis thaliana* HMGB1 and HMGB4 proteins, which have longer and shorter terminal domains than maize HMGB1, respectively. *Arabidopsis* HMGB1 and HMGB4 were cross-linked with EDC for various times and subsequently cleaved with formic acid. Because both proteins contain a single D/P formic acid cleavage site situated between the N-terminal and the HMG-box domains (D^{49}/P^{50} and D^{31}/P^{32}, in HMGB1 and HMGB4, respectively), SDS−PAGE of the formic acid cleavage products (like with maize HMGB1) resulted in three bands. Depending on the extent of cross-linking, the intensity of the bands corresponding to the separable, noncross-linked cleavage products was reduced, while the diffuse band of the cross-linked full-length proteins became more intense (Figure 7). Therefore, similar to maize HMGB1, the N- and C-terminal domains of *Arabidopsis* HMGB1 and HMGB4 interact, indicating that the interaction of the basic N-terminal and the acidic C-terminal domains is a common feature of plant HMGB proteins.

DISCUSSION

Basic extensions of the HMG-box domain have a positive effect on various DNA interactions of HMGB proteins from different sources (*4*, *5*). In insect and vertebrate HMGB proteins, the functionally most significant basic regions flanking the HMG-box domain are situated C terminal of the HMG-box domain (of domain B in vertebrate HMGB). The basic regions stimulate binding to linear DNA and four-

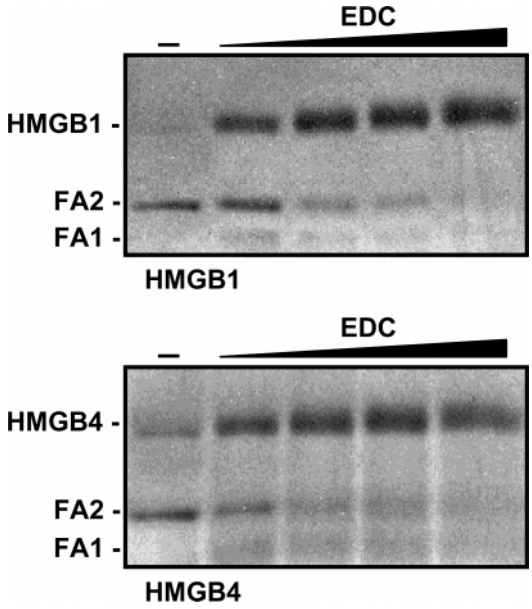

FIGURE 7: Intramolecular cross-linking of *Arabidopsis* HMGB1 and HMGB4 followed by formic acid cleavage. *Arabidopsis* HMGB1 and HMGB4 were reacted with EDC for various times (0, 20, 40, 60, and 90 min) and subsequently cleaved by formic acid treatment, and the reaction products were separated by SDS−PAGE. The electrophoretic migration positions of full-length HMGB1/HMGB4 as well as the migration positions of the formic acid cleavage products (FA1 and FA2) are indicated.

way junctions, supercoiling of plasmid DNA, and circularization of short DNA fragments, while there is only a minor effect on DNA minicircle binding (*32*−*37*). In the yeast NHP6 and plant HMGB proteins, a basic region is found N-terminal of the HMG-box domain (*27*, *38*). Deletion of the 35 amino acid residue basic N-terminal domain (having a net charge of +10) of maize HMGB1 has a severe negative influence (especially in the presence of the acidic tail) on the affinity of HMGB1 for linear DNA and the circularization of short DNA fragments (*28*). Similar results were obtained in studies examining rice HMGB1, which shares ~95% amino acid sequence identity with maize HMGB1 (*13*). Here, we have demonstrated (by DNA−cellulose binding and EMSAs) that the individual basic N-terminal domain of maize HMGB1 binds DNA (indicated in Figure 8), which can explain its marked positive effect on the DNA interactions of HMGB1. In line with this result, the protease-sensitive basic N-terminal domain of HMGB1 was protected from limited tryptic digestion, when the protein was bound to DNA (*28*). The basic N-terminal region (15 amino acid residues, with net charge of +5) of the yeast HMGB protein, NHP6A (which lacks an acidic tail), positively influences DNA interactions (*38*). The solution of the structure of the NHP6A/DNA complex revealed that the HMG-box domain is primarily bound to the minor groove, whereas the basic N-terminal domain wraps over the DNA backbone from the minor groove into the major groove of the DNA (*39*). This situation is likely to be similar for many plant HMGB proteins, because they also contain the proline residue between the N-terminal basic region and the HMG-box domain (P^{38} in maize HMGB1, cf. Figure 1B), which seems to be critical for directing the N-terminal domain into the major groove (*38*, *39*). Therefore, the basic N-terminal domain of the plant and yeast HMGB proteins supports DNA

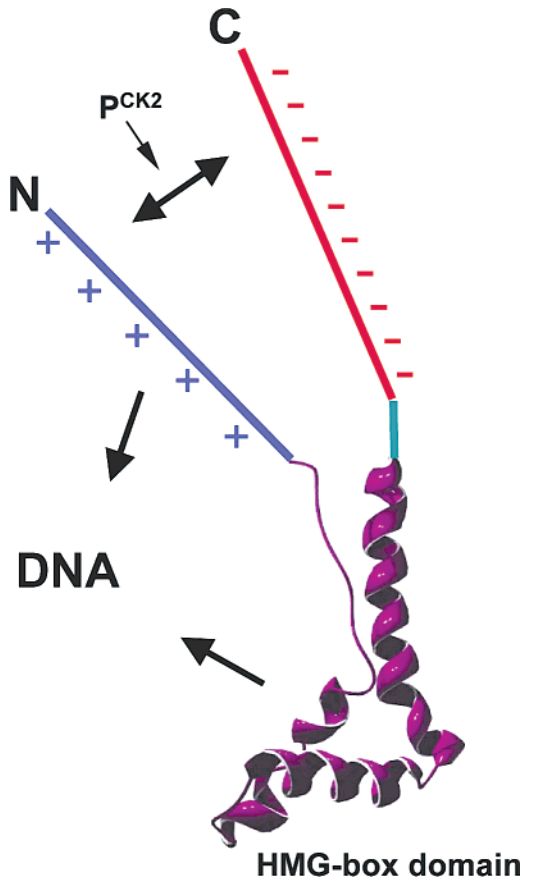

FIGURE 8: Schematic representation of the interactions of maize HMGB1. Because no plant HMGB1 structure has been solved to date, the structure of the rat HMGB1 B domain (amino acid residues P4−A72) (*53*), essentially consisting of three α helices, has been adapted here, because the global fold of this DNA-binding domain is well-conserved (*5*). In maize HMGB1 (and other plant HMGB proteins), the basic N-terminal and the acidic C-terminal domains protrude from the same side of the central HMG-box domain, bringing them into proximity. The basic N-terminal domain and the linker region between the HMG-box domain and the acidic tail are schematically indicated as a straight line in gray, while the C-terminal domain is indicated in black (not drawn to scale). As demonstrated by intramolecular EDC cross-linking, the basic N-terminal domain and the acidic C-terminal domain interact (indicated by a double-headed arrow), which is facilitated by protein-kinase-CK2-mediated phosphorylation (P^{CK2}) of serine residues in the acidic tail. The enhanced intramolecular interaction may explain the observed reduced binding to linear DNA of phosphorylated HMGB1, as well as its increased thermal stability (*25*). In the absence of the basic N-terminal region (in G35−E157), the acidic tail interacts with the N-terminal part of the HMG-box domain forming the concave face of the domain, presumably interfering with the DNA binding of the HMG-box domain, which can explain the severely reduced ability of HMGB1 (G35−E157) to bind DNA (*28*). When bound to DNA, both the concave face of the HMG-box domain and the basic N-terminal domain of full-length HMGB1 interact with the DNA (indicated by arrows). While the HMG-box domain predominantly contacts the minor groove, the basic N-terminal domain may bind the major groove of the DNA (*39*).

binding by direct DNA contacts, and it may facilitate the HMG-box-induced bending of the DNA by neutralizing phosphate charge repulsion in the narrowed major groove of the DNA bent away from the HMG-box domain in the minor groove (*28*, *39*).

The acidic C-terminal domain present in the vertebrate, insect, and plant HMGB proteins negatively influences various DNA interactions (*4*). The acidic tail reduces the affinity of the HMGB proteins for linear DNA and four-way junctions, and it affects the ability to circularize short DNA fragments (*32*, *36*, *37*, *40*−*42*). This negative effect may be brought about by charge repulsion between the acidic tail and the DNA or by interaction of the acidic tail with basic parts of the protein (*4*). Examinations of full-length and truncated mammalian and insect HMGB proteins using various spectrometric methods (CD, NMR, fluorescence) and calorimetric measurements have indicated that the acidic tail interacts with the HMG-box domain(s) in these proteins (*36*, *43*−*46*). From these studies, it was not clear which of the two HMG-box domains in mammalian HMGB1 (HMG-box domain A and/or B) interacts with the acidic C-terminal tail. More recently, intramolecular cross-linking experiments demonstrated that the acidic tail of rat HMGB1 interacts with the central HMG-box (domain B) rather than with the N-terminal HMG-box (domain A) (*47*). Therefore, intramolecular interactions of the acidic tail and HMG-box domain(s) may modulate DNA interactions of the vertebrate and insect HMGB proteins (*32*, *36*, *40*−*42*, *45*).

Deletion of the acidic tail of maize and rice HMGB1 enhanced the binding to linear DNA and the circularization of short DNA fragments, while it had only a minor effect on the recognition of DNA minicircles (*13*, *28*). Using EDC cross-linking, we have examined here whether the acidic C-terminal domain interacts with the basic N-terminal domain and/or the HMG-box domain of maize HMGB1 to modulate the DNA interactions of the protein. EDC cross-linking is observed only with the HMGB1 proteins having the acidic tail, while deletion of the acidic tail abolished intramolecular EDC-mediated cross-linking. Formic acid cleavage of cross-linked HMGB1 demonstrated that the acidic C-terminal domain interacted with the basic N-terminal domain rather than with the HMG-box domain in full-length HMGB1. Because both termini of maize HMGB1 protrude from the same side of the HMG-box domain, the two terminal domains are in proximity to each other (Figure 8), which facilitates interactions between the basic N-terminal and the acidic C-terminal domains. This interaction can also explain the finding that in maize and rice full-length HMGB1 the positive effect of the basic N-terminal domain and the negative effect of the acidic C-terminal domain functionally neutralize each other, resulting in similar DNA-binding properties for full-length HMGB1 and the individual HMG-box domain (*13*, *28*). The conserved eukaryotic protein kinase CK2 (*48*−*51*) can phosphorylate serine residues within the acidic tail of plant and insect HMGB proteins (*25*, *52*). The intramolecular interaction between the basic N-terminal domain and the acidic C-terminal domain of maize HMGB1 is enhanced when the protein has been phosphorylated by CK2, which is consistent with the increased thermal stability of phosphorylated HMGB1 (*25*). Phosphorylation by CK2 increases the negative net charge of the acidic tail, which may strengthen electrostatic interactions with the basic N-terminal domain. Accordingly, the phosphorylation reduced the affinity of maize HMGB1 for linear DNA (*25*), because the positive effect of the basic N-terminal domain on binding linear DNA was limited by the tighter interaction with the phosphorylated C-terminal tail. In line with this assumption, the covalent cross-linking of the two terminal domains resulted in a reduced affinity

of HMGB1 for linear DNA (Figure 6). On the other hand, CK2-mediated phosphorylation had only a minor effect on the affinity of HMGB1 for DNA minicircles, because the basic N-terminal domain has only a marginal effect on the structure-specific binding of HMGB1 to DNA minicircles, which appears to be essentially an endogenous property of the HMG-box domain (*13*, *25*, *28*). Therefore, CK2-mediated phosphorylation of plant HMGB proteins, which reduces the affinity for linear DNA, could favor the interaction with certain DNA structures. This phosphorylation-dependent effect on the DNA interactions is similar to that observed with recombinant plant, insect, and vertebrate HMGB proteins, whose acidic tails have been truncated or extended, presumably affecting intramolecular interactions of the acidic tails because of the alteration in the number of negative charges (*28*, *36*, *40*). In the recombinant maize HMGB1 protein lacking the basic N-terminal region (G35−E157), the acidic tail interacts with the HMG-box domain when the normal interaction partner (M1−G35) is absent. Intramolecular interactions within G35−E157 are indicated by our results obtained by CD and fluorescence spectroscopy and by EDC cross-linking. According to BNPS-skatole cleavage (cleaving at the single tryptophane residue within the HMG-box domain) of EDC cross-linked (G35−E157), the acidic tail interacts with the N-terminal part of the HMG-box domain that forms the DNA-binding surface of the HMG-box domain. This is in-line with previous findings demonstrating that the N-terminally truncated protein is severely affected in its DNA-binding and circularization properties, presumably because the acidic C-terminal domain interferes with the DNA interactions of the HMG-box domain (*13*, *28*).

Because the intramolecular cross-linking experiments with *Arabidopsis* HMGB1 and HMGB4 (like with maize HMGB1) showed an interaction of the terminal domains, the interaction of the basic N-terminal and acidic C-terminal domains might be a general feature of plant HMGB proteins. Various members of the plant HMGB protein family (and their posttranslationally modified variants) are differentially adapted to perform certain architectural functions in different nucleoprotein structures (*24*, *26*). The DNA interactions of the HMGB proteins that are dominated by the central HMG-box domain are markedly modulated by the basic and acidic domains flanking the HMG-box domain (*13*, *28*). In this context, the interaction of the variable terminal domains of the different plant HMGB proteins (eventually modified by phosphorylation) could serve as a molecular mechanism involved in the fine tuning of the functional properties of these architectural factors depending on the structural requirements of specific nucleoprotein complexes (*8*).

ACKNOWLEDGMENT

We thank Hanne Krone Nielsen for excellent technical assistance, Guy Bauw for advice concerning protein cleavage experiments, Daniel Otzen for stimulating discussions and comments on the paper, and Günter Feix for plasmid pMS1.

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BI0499009