Phosphorylation by protein kinase C weakens DNA-binding affinity and folding stability of the HMGB1 protein

*Xi Wang, Luis Marcelo F. Holthauzen, Jonathan M Paz-Villatoro, Karina G. Bien, Binhan Yu, and Junji Iwahara**

Department of Biochemistry and Molecular Biology, Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-1068, USA

Abstract: The HMGB1 protein typically serves as a DNA chaperone that assists DNA-repair enzymes and transcription factors but can translocate from the nucleus to the cytoplasm and even to extracellular space upon some cellular stimuli. One of the factors that triggers the translocation of HMGB1 is its phosphorylation near a nuclear localization sequence by protein kinase C (PKC), though the exact modification sites on HMGB1 remain ambiguous. In this study, using spectroscopic methods we investigated the HMGB1 phosphorylation and its impact on the molecular properties of the HMGB1 protein. Our nuclear magnetic resonance (NMR) data on the full-length HMGB1 protein showed that PKC specifically phosphorylates the A-box domain, one of the DNA binding domains of HMGB1. Phosphorylation of S46 and S53 was particularly efficient. Over a longer reaction time, PKC phosphorylated some additional residues within the HMGB1 A-box domain. Our fluorescence-based binding assays showed that the phosphorylation significantly reduces the binding affinity of HMGB1 for DNA. Based on the crystal structures of HMGB1-DNA complexes, this effect can be ascribed to electrostatic repulsion between the negatively charged phosphate groups at the S46 side chain and DNA backbone. Our data also showed that the phosphorylation destabilizes the folding of the A-box domain. Thus, phosphorylation by PKC weakens the DNA-binding affinity and folding stability of HMGB1.

The HMGB1 protein is a multi-functional DNA-binding protein with two HMG-box domains referred to as A-box and B-box.[1] HMGB1 typically functions as a DNA chaperone that assists transcription factors, DNA repair/recombination enzymes, chromatin remodeling factors through binding to distorted non-B-form DNA structures in the nuclei.[1,2] Upon particular cellular stimuli (e.g., by interleukin-1β or lipopolysaccharide), HMGB1 molecules translocate to the cytoplasm and are even released via secretory lysosomes to extracellular space for signaling to induce innate immune response of other cells.[3,4] Lys acetylation and Ser phosphorylation trigger the translocation of HMGB1.[5] Protein kinase C (PKC) enzymes, including PKCα, can phosphorylate HMGB1 and were proposed to play a role in translocation of HMGB1 from the cell nuclei.[6,7]

Other aspects of the HMGB1 phosphorylation by PKC have not been well characterized. Although Oh et al. showed that PKC phosphorylates Ser residues of HMGB1 within or near the nuclear localization sequences (NLS), they used Ser-to-Ala mutations for multiple residues (S35, S39, S42, S46, S53, and S181) simultaneously and did not provide residue-specific information.[6] A later study proposed the significance of the HMGB1 residues S39, S53, and S181 as the PKC phosphorylation sites,[7] whereas another study suggested the importance of S46.[8] Although these data are qualitatively consistent with the predicted PKC sites (**Figure 1A**),[9] the phosphorylation sites and their modification efficiency remain to be determined more definitively. Regarding the impact of phosphorylation by PKC, an increase in DNA-binding affinity was reported for the rat HMGB1 protein,[10] whereas an opposite result was reported for an insect HMGB1 homologue.[11] Under the current situation with seemingly inconsistent information, more quantitative investigations are desired to understand how PKC affects HMGB1's molecular properties.

In this study, using nuclear magnetic resonance (NMR) spectroscopy, we investigated HMGB1 phosphorylation by PKCα and its impact on the molecular properties of human HMGB1. NMR is

useful to study protein phosphorylation at an atomic level.[12-18] Combining NMR and other biophysical techniques, we characterized the impact of phosphorylation on its DNA-binding affinity and stability of HMGB1.

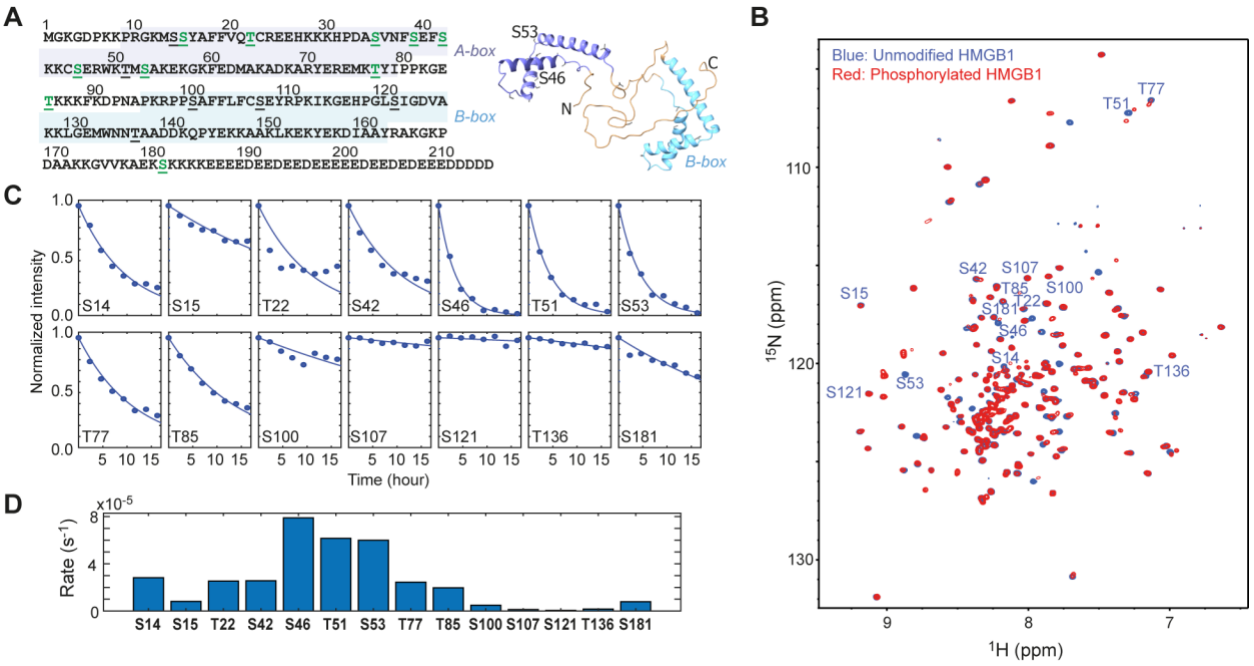


Figure 1. PKCα-induced phosphorylation of the full-length HMGB1 protein occurs in the A-box domain. (**A**) Human HMGB1 amino-acid sequence (GenBank CAG33144.1). All Ser/Thr residues are underlined, among which the PKC phosphorylation sites predicted by the NetPhos 3.1 server (https://services.healthtech.dtu.dk/services/NetPhos-3.1/)[19] are indicated in green (a score > 0.5). (**B**) Overlaid ^{1}H-^{15}N TROSY spectra recorded for the unmodified and phosphorylated HMGB1 protein. The latter was recorded after 12 hours of the phosphorylation reaction using 1.6 μg/ml PKCα and 2 mM ATP together with PKC lipid activator. Signals from the Ser/Thr residues of the unmodified HMGB1 protein, which were assigned in our previous study,[20] are annotated. NMR signals from the S35 and S39 NH groups were unanalyzable due to line broadening. More spectra recorded at different time points are shown in Figure S1 in the SI. (**C**) Time courses of signal intensities for the NMR signals from the Ser and Thr residues of the unmodified HMGB1 protein. The solid lines represent the best curves obtained with a mono-exponential decay function. Values are normalized with I_r/I_o, where I_o and I_r represent the original signal intensity and the intensity for the reaction mixture, respectively. (**D**) Apparent decay rates for the signals from unmodified HMGB1.

By NMR, we monitored the reaction of HMGB1 phosphorylation by PKC. Series of ^{1}H-^{15}N transverse relaxation optimized spectroscopy (TROSY)[21] spectra were recorded for ^{15}N-labeled full-length HMGB1 immediately after mixing with PKCα, ATP, and activator lipids in a pH 7.4 buffer containing 100 mM NaCl. **Figure 1B** shows an overlay of the initial spectrum and the spectrum after 12 hours of the phosphorylation reaction. While some signals of the unmodified

protein became weaker, new signals from the phosphorylated HMGB1 protein gradually appeared. Some of the new signals were in the spectral region characteristic for phosphorylated Ser/Thr residues (^{1}H, 8.7 – 9.1 ppm; ^{15}N, 116-121 ppm)[22]. However, the new signals were difficult to assign because there were many species due to multiple sites becoming phosphorylated at different rates. Therefore, we focused on analyzing decays of the signals from the unmodified HMGB1 protein in the reaction time course.

The time courses of the signal intensities for the unmodified Ser/Thr residues in HMGB1 are shown in **Figure 1C**. The signals from unmodified S46 and S53 (and some residues near these residues) rapidly became weaker and disappeared while the signals from other Ser/Thr residues remained strong. Rapid decays in signal intensities were found only for residues in the A-box domain, but not for those outside the A-box domain. **Figure 1D** shows the signal decay rates for the Ser/Thr NH groups of the unmodified HMGB1 protein. These rates do not necessarily represent the phosphorylation rates because phosphorylation of nearby residues can also cause the decay indirectly. For instance, while the signal from T51 rapidly decayed, a new peak appeared nearby the original signal (**Figure 1B**). Phosphorylation usually causes a larger change in ^{1}H chemical shifts.[22] Considering it along with the virtually identical decay rates for T51 and S53, it is likely that the new peak appearing nearby the original T51 signal is due to phosphorylation of S53, though we cannot completely rule out the possibility that only small perturbation occurs for the phosphorylated T51 amide group due to the lack of a hydrogen bond with phosphate. Nonetheless, the rapid decay rates for S46 and S53 represent efficient phosphorylation of these residues.

Judging from prior studies on the intra-molecular interactions involving the C-terminal acidic tail,[20,23-26] the low efficiency in phosphorylation for residues outside A-box may be partly due to the tail. For example, the low phosphorylation efficiency of S181, which is predicted to be a PKC

site, may be due to strong interactions between the surrounding basic residues (K180, K182, K183, K184, and K185) and the acidic tail. In this regard, interactions with other molecules (e.g., histone H1) that can displace the C-terminal tail of HMGB1 may affect the phosphorylation efficiency.

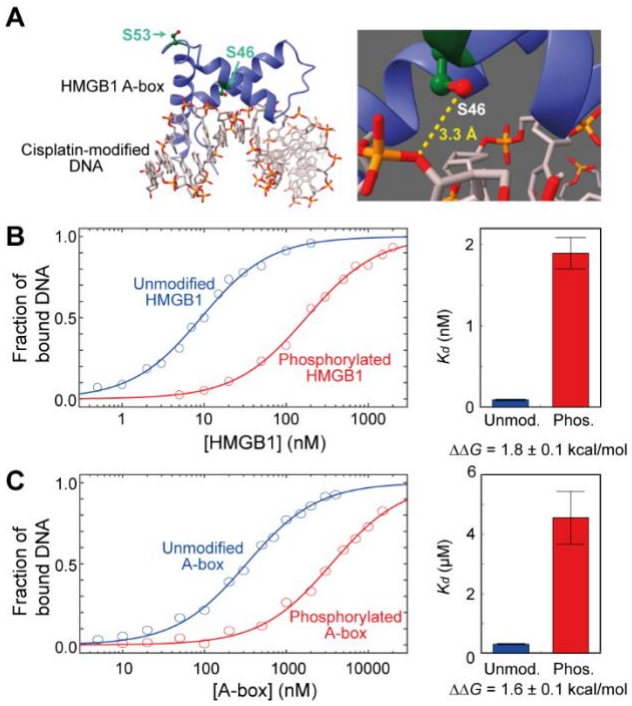


Figure 2. Impact of phosphorylation on the DNA-binding affinity of HMGB1. (**A**) Locations of S46 and S53 in the crystal structure of a complex of the HMGB1 A-box domain with cisplatin-modified DNA (PDB 1CKT)[27]. (**B, C**) Binding isotherm data for FAM-labeled 20-bp DNA in which a GG step is modified with cisplatin[20] (Panel B, the full-length HMGB1 protein; Panel C, the A-box domain). The experiments were conducted using a buffer of 10 mM potassium phosphate (pH 7.5), 100 mM KCl, and 1 mM DTT.

In **Figure 2A**, the efficient phosphorylation sites, S46 and S53, are indicated in the crystal structure of the HMGB1 A-box domain bound to cisplatin-modified DNA (PDB 1CKT)[27]. Because the distance from the S46 O_γ atom to the closest phosphate group is only 3.3 Å, it appears that electrostatic repulsion between the phosphoserine and DNA phosphate would make the DNA-binding affinity of HMGB1 weaker. However, Ugrinova et al. reported that HMGB1 phosphorylated by PKC increased the binding affinity by an order of magnitude.[10] Perplexed by their data, we decided to assess the impact of PKC-induced phosphorylation on the binding affinity of HMGB1 for cisplatin-modified DNA in our laboratory.

HMGB1 is known to bind strongly to non-B-form DNA such as G-quadruplex,[25,28,29] four-way junction,[30,31] and cisplatin-modified DNA[27,32]. Through fluorescence-based titration assays used in our previous study on HMGB1,[20] we examined the impact of PKC-induced phosphorylation on the DNA-binding affinities (**Figure 2B**). Unlike the data of Ugrinova et al.,[10] our data clearly show that PKC-induced phosphorylation weakens the binding affinity of HMGB1 for cisplatin-modified DNA. The dissociation constants K_d for the complexes with cisplatin-modified DNA were 9 (± 1) nM for the unmodified full-length HMGB1 protein and 182 (± 12) nM for the phosphorylated protein. The change in binding free energy $\Delta\Delta G$ upon S46/S54 phosphorylation is calculated to be +1.8 (± 0.1) kcal/mol. For a further test, we also conducted the same experiments for a partial construct (HMGB1 residue 1-84) that contains only the A-box domain (**Figure 2C**). The dissociation constants K_d were 0.33 (± 0.03) μM for the unmodified A-box domain and 3.6 (± 0.5) μM for the phosphorylated A-box domain. The change in binding free energy upon phosphorylation ($\Delta\Delta G$) was +1.6 (± 0.1) kcal/mol. Although the K_d values are different between the isolated A-box domain and the full-length HMGB1 protein, the $\Delta\Delta G$ values for them were close, which is reasonable because the phosphorylation of the full-length HMGB1 protein occurs in the A-box domain. The positive $\Delta\Delta G$ represents an energetic penalty, which can stem from the vicinity of the negatively charged phosphoserine and DNA phosphate at the molecular interface.

When we monitored the PKCα-catalyzed phosphorylation reactions for the isolated A-box domain by NMR, many broad signals due to additional phosphorylation were observed after completion of S46/S56 phosphorylation (**Figure 3A**; see also **Figure S2**). The large number of additional broad signals in a narrow chemical shift range made us speculate that hyperphosphorylation may enhance unfolding of the A-box domain. To investigate this possibility, we conducted circular dichroism (CD) experiments for the unmodified,

phosphorylated, and hyperphosphorylated states of the A-box domain (**Figure 3B**). The CD spectra suggested that the α-helical content of the hyperphosphorylated state is significantly lower. The thermal denaturation data recorded by monitoring CD at 222 nm showed that hyperphosphorylation causes a significant decrease in the melting temperature of the A-box domain. These data show that phosphorylation destabilizes the A-box domain and enhances its unfolding. This effect of hyperphosphorylation may be relevant to HMGB1 *in vivo* because the UniProt database indicates 7 phosphorylation sites that were experimentally identified within A-box. We should also note that enhancement of the unfolded state through phosphorylation has also been observed for some other proteins.[13,14]

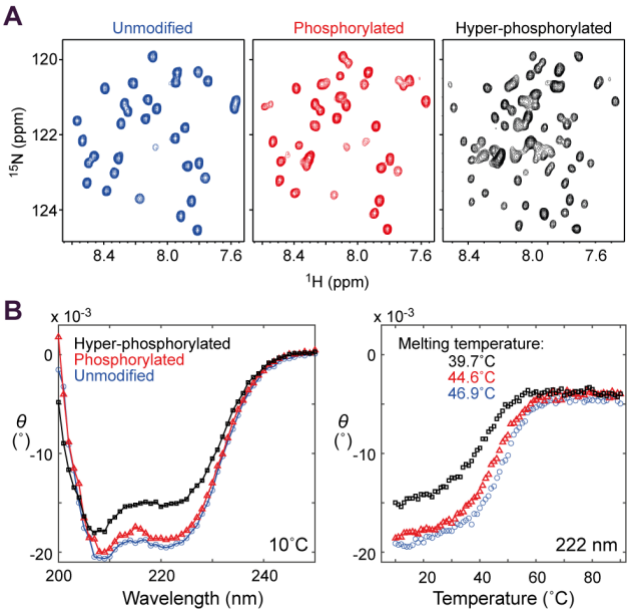


Figure 3. Hyperphosphorylation destabilizes the A-box domain. (**A**) Zoomed ^{1}H-^{15}N HSQC spectra recorded for the A-box domain. The full HSQC and ^{31}P NMR spectra are shown in Figure S2 in the SI. (**B**) Circular dichroism spectra and thermal denaturation data for the unmodified, phosphorylated, and the hyperphosphorylated A-box domain samples. The protein concentration in the CD experiments was 10 μM for each.

In conclusion, our study has provided spectroscopic information about HMGB1 phosphorylation by PKCα. This kinase phosphorylates the HMGB1 residues S46 and S53 efficiently and other residues in the A-box domain more slowly, while the B-box domain remains unphosphorylated.

Compared to the unmodified state, the phosphorylated HMGB1 protein exhibits weaker DNA-binding affinity, presumably due to electrostatic repulsion between DNA phosphate and phosphorylated S46 at the protein-DNA interface. Hyperphosphorylation of the A-box domain significantly destabilizes its folding.

ASSOCIATED CONTENT

Supporting Information. The following information is available free of charge.

Materials and Methods; additional NMR data on phosphorylation [Figures S1-S2] (PDF)

Acknowledgements

This work was supported by Grant R35-GM130326 from National Institutes of Health (to. J.I.), Grant MCB-2026805 from National Science Foundation (to J.I.), and Grant H-2104-20220331 from the Welch Foundation (to J.I.).

AUTHOR INFORMATION

Corresponding Author

* Junji Iwahara. Email: j.iwahara@utmb.edu

References

1. Tang, D., Kang, R., Zeh, H. J., and Lotze, M. T. (2023) The multifunctional protein HMGB1: 50 years of discovery, *Nat Rev Immunol 23*, 824-841.

2. Stros, M. (2010) HMGB proteins: interactions with DNA and chromatin, *Biochim Biophys Acta 1799*, 101-113.

3. Venereau, E., Schiraldi, M., Uguccioni, M., and Bianchi, M. E. (2013) HMGB1 and leukocyte migration during trauma and sterile inflammation, *Mol Immunol 55*, 76-82.

4. Bonaldi, T., Talamo, F., Scaffidi, P., Ferrera, D., Porto, A., Bachi, A., Rubartelli, A., Agresti, A., and Bianchi, M. E. (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion, *Embo J 22*, 5551-5560.

5. Chen, R., Kang, R., and Tang, D. (2022) The mechanism of HMGB1 secretion and release, *Exp Mol Med 54*, 91-102.

6. Oh, Y. J., Youn, J. H., Ji, Y., Lee, S. E., Lim, K. J., Choi, J. E., and Shin, J. S. (2009) HMGB1 is phosphorylated by classical protein kinase C and is secreted by a calcium-dependent mechanism, *J Immunol 182*, 5800-5809.

7. Lee, H., Park, M., Shin, N., Kim, G., Kim, Y. G., Shin, J.-S., and Kim, H. (2012) High mobility group box-1 is phosphorylated by protein kinase C zeta and secreted in colon cancer cells, *Biochem Biophys Res Commun 424*, 321-326.

8. Taira, J., and Higashimoto, Y. (2014) Evaluation of in vitro properties of predicted kinases that phosphorylate serine residues within nuclear localization signal 1 of high mobility group box 1, *J Pept Sci 20*, 613-617.

9. Nishikawa, K., Toker, A., Johannes, F.-J., Songyang, Z., and Cantley, L. C. (1997) Determination of the Specific Substrate Sequence Motifs of Protein Kinase C Isozymes *, *J Biol Chem 272*, 952-960.

10. Ugrinova, I., Zlateva, S., and Pasheva, E. (2012) The effect of PKC phosphorylation on the "architectural" properties of HMGB1 protein, *Mol Biol Rep 39*, 9947-9953.

11. Wiśniewski, J. R., Schulze, E., and Sapetto, B. (1994) DNA binding and nuclear translocation of insect high-mobility-group- protein-1 (HMG1) proteins are inhibited by phosphorylation, *Eur J Biochem 225*, 687-693.

12. Pufall, M. A., Lee, G. M., Nelson, M. L., Kang, H. S., Velyvis, A., Kay, L. E., McIntosh, L. P., and Graves, B. J. (2005) Variable control of Ets-1 DNA binding by multiple phosphates in an unstructured region, *Science 309*, 142-145.

13. Díaz-Moreno, I., Hollingworth, D., Frenkiel, T. A., Kelly, G., Martin, S., Howell, S., García-Mayoral, M., Gherzi, R., Briata, P., and Ramos, A. (2009) Phosphorylation-mediated unfolding of a KH domain regulates KSRP localization via 14-3-3 binding, *Nat Struct Mol Biol 16*, 238-246.

14. Mitrea, D. M., Grace, C. R., Buljan, M., Yun, M. K., Pytel, N. J., Satumba, J., Nourse, A., Park, C. G., Madan Babu, M., White, S. W., and Kriwacki, R. W. (2014) Structural polymorphism in the N-terminal oligomerization domain of NPM1, *Proc Natl Acad Sci U S A 111*, 4466-4471.

15. Bah, A., Vernon, R. M., Siddiqui, Z., Krzeminski, M., Muhandiram, R., Zhao, C., Sonenberg, N., Kay, L. E., and Forman-Kay, J. D. (2015) Folding of an intrinsically disordered protein by phosphorylation as a regulatory switch, *Nature 519*, 106-109.

16. Gibbs, E. B., Lu, F., Portz, B., Fisher, M. J., Medellin, B. P., Laremore, T. N., Zhang, Y. J., Gilmour, D. S., and Showalter, S. A. (2017) Phosphorylation induces sequence-specific conformational switches in the RNA polymerase II C-terminal domain, *Nat Commun 8*, 15233.

17. Monahan, Z., Ryan, V. H., Janke, A. M., Burke, K. A., Rhoads, S. N., Zerze, G. H., O'Meally, R., Dignon, G. L., Conicella, A. E., Zheng, W., Best, R. B., Cole, R. N., Mittal, J., Shewmaker, F.,

and Fawzi, N. L. (2017) Phosphorylation of the FUS low-complexity domain disrupts phase separation, aggregation, and toxicity, *Embo J 36*, 2951-2967.

18. Sun, X., Dyson, H. J., and Wright, P. E. (2021) A phosphorylation-dependent switch in the disordered p53 transactivation domain regulates DNA binding, *Proc Natl Acad Sci U S A 118*, e2021456118.

19. Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., and Brunak, S. (2004) Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence, *Proteomics 4*, 1633-1649.

20. Wang, X., Greenblatt, H. M., Bigman, L. S., Yu, B., Pletka, C. C., Levy, Y., and Iwahara, J. (2021) Dynamic Autoinhibition of the HMGB1 Protein via Electrostatic Fuzzy Interactions of Intrinsically Disordered Regions, *J Mol Biol 433*, 167122.

21. Pervushin, K., Riek, R., Wider, G., and Wüthrich, K. (1997) Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution, *Proc Natl Acad Sci U S A 94*, 12366-12371.

22. Hendus-Altenburger, R., Fernandes, C. B., Bugge, K., Kunze, M. B. A., Boomsma, W., and Kragelund, B. B. (2019) Random coil chemical shifts for serine, threonine and tyrosine phosphorylation over a broad pH range, *J Biomol NMR 73*, 713-725.

23. Knapp, S., Muller, S., Digilio, G., Bonaldi, T., Bianchi, M. E., and Musco, G. (2004) The long acidic tail of high mobility group box 1 (HMGB1) protein forms an extended and flexible

structure that interacts with specific residues within and between the HMG boxes, *Biochemistry 43*, 11992-11997.

24. Stott, K., Watson, M., Howe, F. S., Grossmann, J. G., and Thomas, J. O. (2010) Tail-mediated collapse of HMGB1 is dynamic and occurs via differential binding of the acidic tail to the A and B domains, *J Mol Biol 403*, 706-722.

25. Wang, X., Mayorga-Flores, M., Bien, K. G., Bailey, A. O., and Iwahara, J. (2022) DNA-mediated proteolysis by neutrophil elastase enhances binding activities of the HMGB1 protein, *J Biol Chem 298*, 102577.

26. Yu, B., Wang, X., Tan, K. N., and Iwahara, J. (2024) Influence of an Intrinsically Disordered Region on Protein Domains Revealed by NMR-Based Electrostatic Potential Measurements, *J Am Chem Soc 146*, 14922-14926.

27. Ohndorf, U. M., Rould, M. A., He, Q., Pabo, C. O., and Lippard, S. J. (1999) Basis for recognition of cisplatin-modified DNA by high-mobility-group proteins, *Nature 399*, 708-712.

28. Amato, J., Cerofolini, L., Brancaccio, D., Giuntini, S., Iaccarino, N., Zizza, P., Iachettini, S., Biroccio, A., Novellino, E., Rosato, A., Fragai, M., Luchinat, C., Randazzo, A., and Pagano, B. (2019) Insights into telomeric G-quadruplex DNA recognition by HMGB1 protein, *Nucleic Acids Res 47*, 9950-9966.

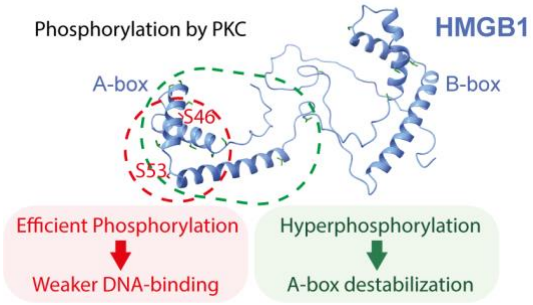
29. Amato, J., Madanayake, T. W., Iaccarino, N., Novellino, E., Randazzo, A., Hurley, L. H., and Pagano, B. (2018) HMGB1 binds to the KRAS promoter G-quadruplex: a new player in oncogene transcriptional regulation?, *Chem Commun (Camb) 54*, 9442-9445.

30. Bianchi, M. E., Beltrame, M., and Paonessa, G. (1989) Specific recognition of cruciform DNA by nuclear protein HMG1, *Science 243*, 1056-1059.

31. Webb, M., and Thomas, J. O. (1999) Structure-specific binding of the two tandem HMG boxes of HMG1 to four-way junction DNA is mediated by the A domain, *J Mol Biol 294*, 373-387.

32. Jung, Y., and Lippard, S. J. (2003) Nature of Full-Length HMGB1 Binding to Cisplatin-Modified DNA, *Biochemistry 42*, 2664-2671.

TOC Graphic (For Table of Contents use only)



SUPPORTING INFORMATION

Phosphorylation by the protein kinase C weakens DNA-binding affinity and folding stability of the HMGB1 protein

Xi Wang, Luis Marcelo F. Holthauzen, Jonathan M Paz-Villatoro, Karina G. Bien, Binhan Yu, and Junji Iwahara

Department of Biochemistry and Molecular Biology, Sealy Center for Structural Biology and Molecular Biophysics, University of Texas Medical Branch, Galveston, Texas 77555-1068, USA

1. Materials and Methods

Proteins

The ^{15}N-labeled human HMGB1 protein expressed in *Escherichia coli* strain BL21 (DE3) was purified as described by Wang et al.[1] The ^{15}N-labeled A-box domain of the HMGB1 protein was prepared as described by Sahu et al.[2] Recombinant Protein kinase Cα was purchased from Sigma-Aldrich (cat no. 14-484). 5.0 mM DTT was used to maintain the reduced (i.e., thiol) state of the HMGB1 cysteine residues.

Phosphorylation reactions

Full-length HMGB1 or A-box protein was dissolved in 400 μL buffer containing 10 mM potassium phosphate (pH 7.4), 100 mM NaCl, 5 mM DTT and 10 mM $MgCl_2$. The protein solution was mixed with a 100 μL solution of PKC lipid activator (Sigma-Aldrich; cat no. 20-133A) containing 0.5mg/ml phosphatidylserine and 0.05mg/ml diacylglycerol in 20mM MOPS (pH 7.2), 25mM β-glycerol phosphate, 1mM sodium orthovanadate,1mM dithiothreitol and 1mM $CaCl_2$. The phosphorylation reaction was started by adding a 4 μL solution of PKCα and 2 mM ATP.

NMR-based monitoring of phosphorylation

NMR experiments were conducted using a Bruker Avance III spectrometer operated at a ^{1}H frequency of 750 MHz. To monitor phosphorylation by PKC, series of ^{1}H-^{15}N TROSY spectra were recorded at 25˚C immediately after adding the PKC enzyme and ATP to a solution of 100 μM ^{15}N-labeled full-length HMGB1 or 250 μM ^{15}N-labeled A-box. A cryogenic TCI probe was used to record these spectra. The NMR spectra were processed with NMR-Pipe[3] and analyzed with NMRFAM-SPARKY[4] and NMR-View[5], based on our previous NMR chemical shift assignment data.[1,2]

Chromatographic isolation of phosphorylated A-box

A phosphorylation reaction mixture of A-box was diluted at least 5-fold with a buffer of 50 mM Tris-HCl (pH 7.5), and 1 mM EDTA. Using a Cytiva AKTA Pure system, the solution was loaded onto a Source-S cation-exchange column (10 ml) equilibrated with a buffer of 50 mM Tris-HCl (pH 7.5), and 1 mM EDTA. After the column was washed at 0 mM NaCl, the proteins were eluted with a gradient of 0 – 600 mM NaCl in the same buffer. A reaction mixture at the reaction time of 6 hours was used to isolate the A-box proteins phosphorylated at the major phosphorylation sites. Another reaction mixture at 3 days was used to isolate the hyperphosphorylated A-box protein. The phosphorylation was confirmed through 1D ^{31}P NMR experiments using the Bruker Avance III 750-MHz spectrometer with a broadband DiffBB probe.

Fluorescence-based measurements of DNA-binding affinity

FAM-labeled cisplatin-modified 20bp DNA was prepared as described by Wang et al.[1] Binding affinity of phosphorylated full-length HMGB1 or A-box was measured using FAM fluorescence anisotropy. Protein titration was conducted in a buffer of 10mM potassium phosphate

(pH 7.5), 100 mM KCl and 1mM DTT with 1 nM FAM-labeled cisplatin-modified DNA. The dissociation constant K_d was determined from the anisotropy data using MATLAB (MathWorks, Inc), as previously described.[1]

Circular dichroism experiments

Circular dichroism (CD) experiments were conducted using a Jasco J-815 CD spectrometer. CD wavelength scans were performed at 10 °C from 200 to 250 nm. Thermal denaturation experiments were then carried out at 222 nm at temperatures from 10 to 90 °C, at a scan rate of 1 °C/min. All measurements were made using a 1 mm quartz cuvette filled with 500 μL of each sample. Measured samples were 0.1 mg/ml proteins in 40 mM potassium phosphate (pH 7.4), 100 mM KCl, and 2 mM DTT. The melting temperatures (T_m) were determined through fitting with Sigma Plot.

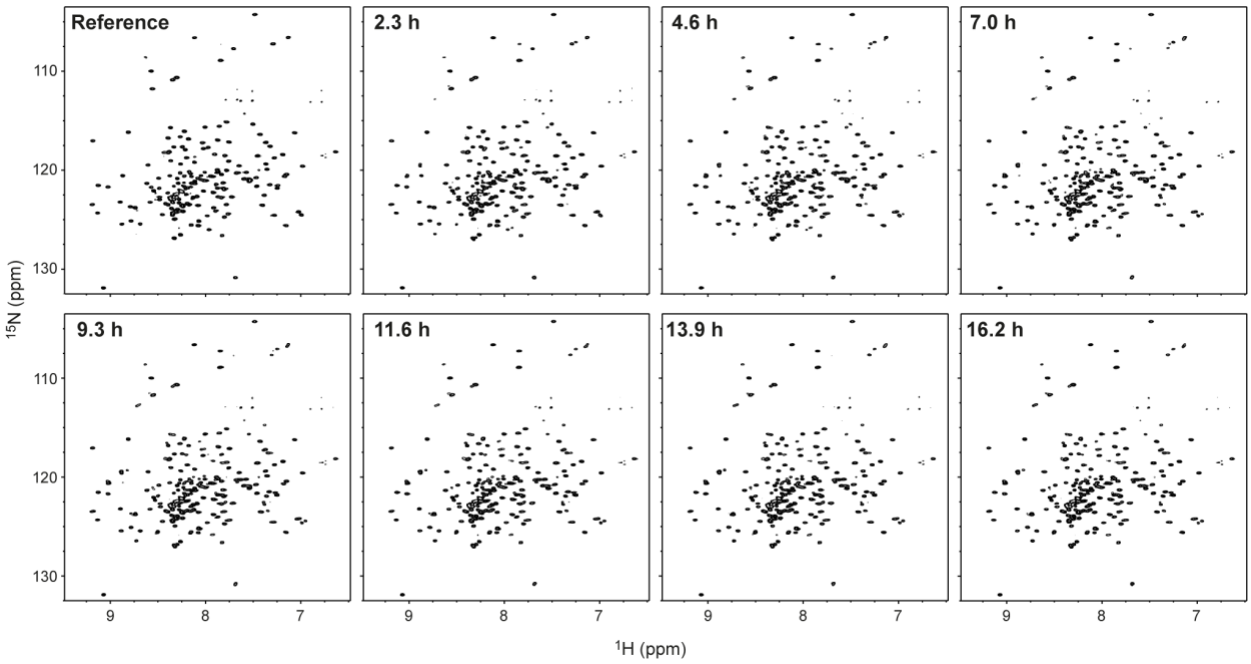


Figure S1. Series of ^{1}H-^{15}N TROSY spectra recorded for the reaction mixture of the full-length ^{15}N HMGB1 protein being phosphorylated by PKCα. These spectra were used for the data shown in Figure 1.

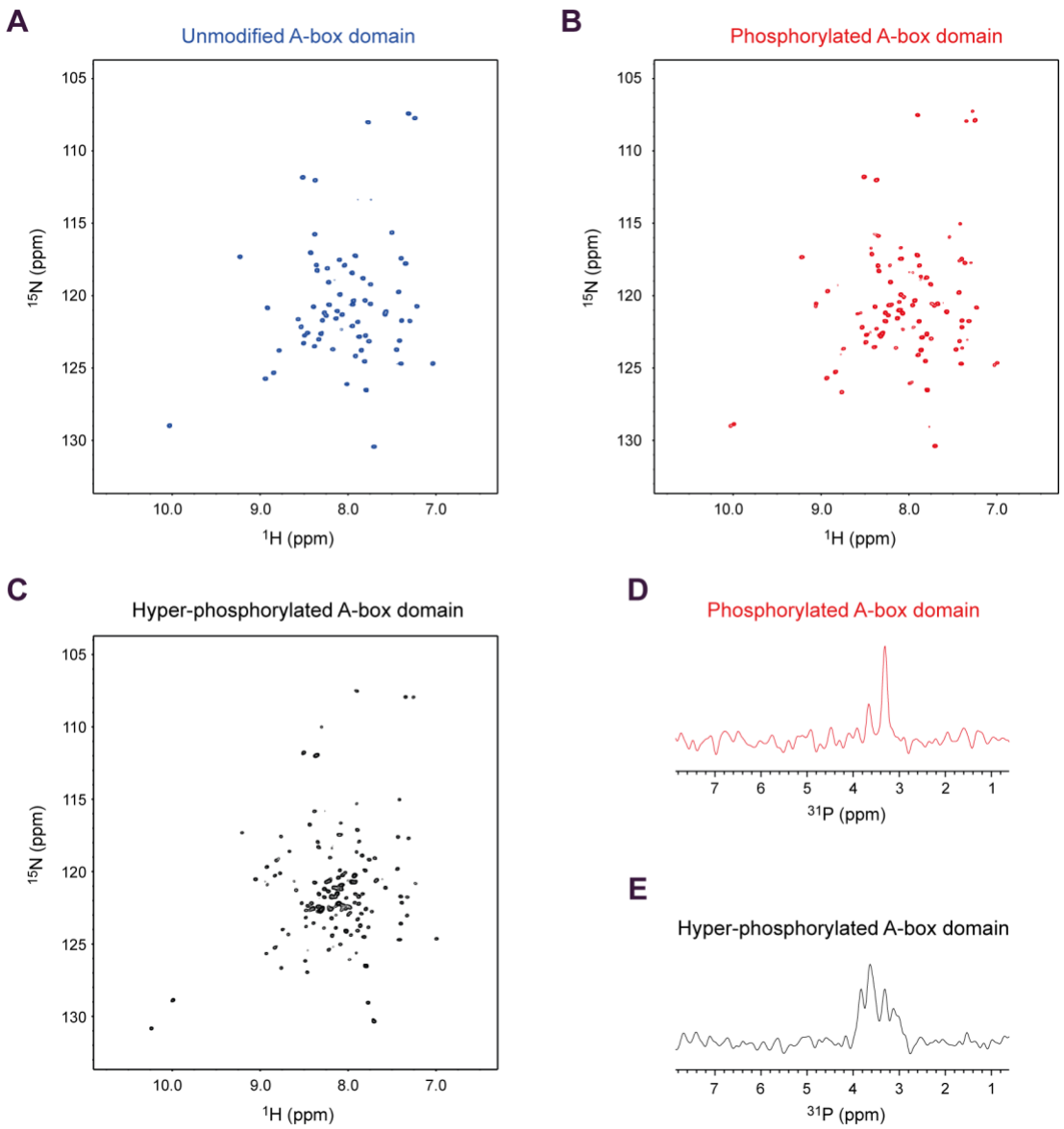


Figure S2. Additional NMR data on phosphorylation of the ^{15}N-labeled HMGB1 A-box domain. (**A, B, C**) Full-range of ^{1}H-^{15}N HSQC spectra recorded for the unmodified (A), phosphorylated (B), and hyperphosphorylated (C) A-box domains. The spectra in Figure 3A are the same as these but are zoomed to show the broad line-shapes of signals from the hyperphosphorylated A-box domain more clearly. (**D, E**) ^{31}P NMR spectra recorded for the phosphorylated (D) and hyperphosphorylated (E) states of the A-box domain.

References for SI

(1) Wang, X.; Greenblatt, H. M.; Bigman, L. S.; Yu, B.; Pletka, C. C.; Levy, Y.; Iwahara, J. Dynamic Autoinhibition of the HMGB1 Protein via Electrostatic Fuzzy Interactions of Intrinsically Disordered Regions. *J Mol Biol* **2021**, *433*, 167122.

(2) Sahu, D.; Debnath, P.; Takayama, Y.; Iwahara, J. Redox properties of the A-domain of the HMGB1 protein. *FEBS Letters* **2008**, *582*, 3973.

(3) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **1995**, *6*, 277.

(4) Lee, W.; Tonelli, M.; Markley, J. L. NMRFAM-SPARKY: enhanced software for biomolecular NMR spectroscopy. *Bioinformatics* **2015**, *31*, 1325.

(5) Johnson, B. A.; Blevins, R. A. Nmr View - a Computer-Program for the Visualization and Analysis of Nmr Data. *Journal of Biomolecular Nmr* **1994**, *4*, 603.