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The maize HMGA protein is localized to the nucleolus and can be acetylated in vitro at its globular domain, and phosphorylation by CDK reduces its binding activity to AT-rich DNA

Jing Zhao [a,1], Laju K. Paul [b], Gideon Grafi [b,*]

[a] Department of Plant Sciences, The Weizmann Institute of Science, Rehovot 76100, Israel
[b] French Associates Institute for Dryland Agriculture and Biotechnology, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben-Gurion 84990, Israel

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ABSTRACT

The high mobility group (HMG) proteins are nonhistone chromosomal proteins that participate in diverse nuclear activities including chromatin structure and gene regulation. We previously studied the biochemistry of the maize HMGA protein and its role in transcriptional regulation during maize endosperm development. Here, we extended our study and showed that a strong binding of ZmHMGA to AT-rich DNA requires at least three AT-hook motifs; two motifs showed a significant reduction whereas a single motif was not sufficient for binding. CDK phosphorylation sites situated between AT-hook3 and AT-hook4 were strongly phosphorylated by a SUC1-associated kinase; no in vitro phosphorylation is evident for the AtHMGA protein. Phosphorylation of ZmHMGA reduced its binding to AT-rich DNA in vitro. The maize HMGA protein fused to GFP was localized in the nucleus of transgenic *Arabidopsis* plants tending to concentrate within the nucleolus. Localization to the nucleolus was conferred by the C-terminal portion of the protein containing the AT-hooks. ZmHMGA was acetylated in vitro on its N-terminal globular domain by the human PCAF acetyltransferase. Our results suggest that ZmHMGA participates in nucleolar function and that its role may be regulated posttranslationally by phosphorylation and acetylation.

1. Introduction

The high mobility group (HMG) proteins are among the largest and best characterized nonhistone chromosomal proteins. These proteins are grouped into three families: HMGB (HMG 1/2), HMGN (HMG 14/17) and HMGA (HMG I/Y) [1,2]. HMGA proteins bind specifically to AT-rich DNA through a small DNA binding motif, termed the AT-hook [3], and are involved in diverse nuclear and cellular processes including chromatin organization, DNA repair and gene transcription [1,4–7]. Homologues of mammalian HMGA genes were isolated from a variety of plant species where they appear to be a single-copy gene consisting of two exons and one intron [8,9]. Plant HMGAs display certain unique features: whereas animal HMGA proteins contain three copies of the AT-hook motif and possess a C-terminal acidic region, plant proteins often have four AT-hooks, they lack a C-terminal acidic region, and in addition they contain a unique amino-terminal region that shares homology with the globular domain of histone H1 [2]. Recently, two HMGA-type proteins were isolated from the moss *Physcomitrella* displaying structural differences compared to their higher plant counterparts, namely, they contain six AT hook and their N-terminal domain lacks similarity to linker histone H1 [10]. Beside their binding to a linear AT-rich DNA, HMGA proteins in plants and animals were shown to bind four-way junction DNA thought to be a major intermediate in homologous recombination [11,12]. The spatiotemporal expression of HMGA genes suggest a role for HMGA proteins in growth and differentiation. In adult mammalian tissues, HMGA genes are expressed at very low levels, yet expression is dramatically increased in embryonic cells as well as in neoplastically transformed cells [1,13,14]. Furthermore, a null mutation of the HMG I-C/HMGA2 [15] gene in mice resulted in decreased rate of cell proliferation and in a pygmy phenotype [16]. Plant HMGAs display a wide range of expression pattern in various vegetative and reproductive organs [17,18].

HMGA proteins are subjected to posttranslational modifications, such as phosphorylation and acetylation, that markedly influence their ability to interact with DNA, chromatin, proteins, or protein complexes [1,5]. HMGA proteins can be phosphorylated by various protein kinases including Cdc2 kinase, mitogen-activated protein kinase, protein kinase C, ATM kinase as well as by HIPK2 nuclear kinase [19–24]. Phosphorylation by Cdc2 kinase reduces their DNA-

Abbreviations: HMG, high mobility group; CDK, cyclin-dependent kinase

* Corresponding author. Tel.: +972 8 656 3479; fax: +972 8 659 6742.
E-mail address: ggrafi@bgu.ac.il (G. Grafi).
[1] Present address: Department of Environmental Toxicology, University of California, Davis, CA 95616, USA.

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binding affinity and alters their mode of binding to DNA. Such alterations in the DNA-binding affinity may serve as an important regulatory mechanism for modulating the function of HMGA proteins during the cell cycle [5]. Also, the activity of plant HMGAs may be modulated by phosphorylation during development. Accordingly, ZmHMGA displays differential phosphorylation status during endosperm development becoming hypophosphorylated as cells embark on endoreduplication and synthesis of storage proteins [25]. However, besides phosphorylation not much is known about posttranslational modifications of plant HMGA proteins.

We previously reported biochemical properties of the ZmHMGA protein in vitro and during endosperm development [25]. Here we aimed (1) to characterize the domains in ZmHMGA that mediate binding to AT-rich DNA and undergo phosphorylation and acetylation by CDK and PCAF, respectively, and (2) to determine subcellular localization of the maize HMGA protein. We showed that at least three AT-hooks are required for a strong binding activity to AT-rich DNA. CDK phosphorylation sites between AT-hooks 3 and 4 were strongly phosphorylated by a SUC1-associated kinase, while AtHMGA was not; CDK phosphorylation reduces binding of AT-rich DNA to HMGA. ZmHMGA can be acetylated in vitro at its globular domain by the human PCAF acetyltransferase. We also demonstrated that the maize HMGA is localized in the nucleus tending to concentrate in the nucleolus.

2. Materials and methods

2.1. Construction, expression and purification of GST-HMGA fusion proteins

Construction of the various ZmHMGA clones was performed by PCR in the presence of 10% dimethyl sulfoxide (DMSO, Sigma) using pGEX-ZmHMG-I/Y as template [25] and various combinations of the following primers:

ZmHMGA1-S, 5′-GATATCGGATCCGAGATGGCCACCGACGAAGC-CACC-3′
ZmHMGA16-S, 5′-GAGAGGATCCCCCGAGATGATCCTGGCGGC-GATCGAG-3′
ZmHMGA76-S, 5′-CACAGGATCCAACTACTTCCGCGCGGACGCG-3′
ZmHMGA130-S, 5′-CACAGGATCCGAAGCAGGCCACCGCGGGATG-3′
ZmHMGA191-AS, 5′-GTCGACGAATTCGAGCTCTCAAGCCGCGGCCG-TCTCGCTG-3′
ZmHMGA166-AS, 5′-GAGAGAATTCTCAGGACCCGTCGCCAGCAGG-3′
ZmHMGA136-AS, 5′-CACAGAATTCTCACATCCCGCGGTGGCCTG-CTTC-3′
ZmHMGA80-AS, 5′-GAGAGAATTCTCAGTCCGCGCGGAAGTAGTTG-3′

For preparation of GST-AtHMGA fusion protein, the Arabidopsis HMGA gene (accession number Y10836) was isolated from Arabidopsis cDNA library by PCR using AtHMGA-S, 5′-GATATCGGATCCGAGATGG-CCTTCGATCTCCACCATGGC-3′ and AtHMGA-AS, 5′-GATATCGAATTCTC-AGCACCCAACCGGAGCAACC-3′. All PCR fragments were cut with *Bam*HI and *Eco*RI restriction enzymes and subcloned into the same sites of pGEX-2T. All clones were sequenced to confirm their identity. Expression and purification of GST-HMGA fusion proteins were performed essentially as described [25]. Protein concentration was determined with either the Bradford reagent or the Lowry method (Sigma 690-A). Protein aliquots were stored at −80 °C until use.

2.2. Generation of transgenic plants expressing GFP-ZmHMGA and its derivatives

For preparation of GFP-ZmHMGA and its derivatives, the full-length ZmHMGA, the C-terminal and the N-terminal domains were amplified by PCR. For amplification of the full-length ZmHMGA we used the following primers: ZmHMGA-1-S, 5′-GAA CTA GTA GAT CTA TGG CCA CCG ACG AAG CCA CC, and ZmHMGA-191-AS, 5′-GAG ACC CGG GAG CCG CGG CCG TCT CGC TGG GC, for the N-terminal globular domain (1–80) we used ZmHMGA-1-S and ZmHMGA-80-AS, 5′-GAG ACC CGG GGT TCC GCG CGG AAG TAG TTG TTC, and for amplification of the C-terminal domain (76–190) we used ZmHMGA-76-S, 5′-GAA CTA GTA GAT CTA TGC TCA AGA ACA ACT ACT TCC GCG CGG AC and ZmHMGA-191-AS primers. The PCR products of the full-length ZmHMGA and its derivatives were digested by *Spe*I and *Sma*I restriction endonucleases and subcloned into the same sites of pBluescript SK to generate pBs-ZmHMGA-FL, pBs-ZmHMGA(1–80) and pBsZmHMGA(76–190). To introduce GFP upstream from ZmHMGA we amplified the GFP DNA fragment by PCR using pEGFP-C2 (CLONTECH) as a template and the following primers: EGFP-S, 5′-GAG AAG TAC TAG ATC TAT GGT GAG CAA GGG CGA GGA GCT G, and EGFP-AS, 5′-CTCTGATATCGGATCCCTTGTACAGCTCGTCCATGCCGAG. The PCR product of GFP was digested with *Bgl*II and *BamH*I and subcloned into the *Bgl*II site of the dephosphorylated pbsZmHMGA and its derivatives to generate pBs-GFP-ZmHMGA-FL, pBs-GFP-ZmHMGA(1–80) and pBsGFP-ZmHMGA(76–190). All clones were sequenced to ensure integrity and identity of DNA sequences. Subcloning was then performed into a binary vector pMON530. GFP-fusion ZmHMGA sequences were excised out from pBluescript SK by *Bgl*II and *Eco*RI and subcloned into the same sites of pMON530 downstream from the 35S promoters. The plasmid pMON-GFP-ZmHMGA and its derivatives were introduced into *Arabidopsis* (ecotype WS) using the *Agrobacterium* methodology.

2.3. Electrophoretic mobility shift assay (EMSA), in vitro acetylation, in vitro phosphorylation and analysis of binding activity

EMSAs were performed using the ^{32}P-labeled γZ-AT, a 137-base pair AT-rich fragment derived from the γ-zein promoter region, essentially as previously described [25].

Protein acetyltransferase assays were performed essentially as described [26]. In the standard assay, 30 μl reactions containing 50 mM Hepes (pH 8.0), 10% glycerol, 1 mM DTT, 10 mM sodium butyrate, 1 μl of [^{14}C]-acetyl-CoA (55 mci/mmol), 3 μg of GST fusion proteins, and 100 ng of the human PCAF (kindly provided by M. Oren) were incubated at 30 °C for 1 h. The reaction mixture was fractionated by 15% SDS/PAGE. The gel was dried and read by PhosphorImager.

CDK was purified from protein extracts (1 mg) derived from developing endosperm on glutathione-agarose beads containing GST-SUC1 as described [27]. After washing (five times) with NETN (100 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.0, and 0.5% Nonidet P-40) GST-Suc1-associated kinase was tested for kinase activity in Kinase buffer [80 mM β-glycerophosphate, 20 mM EGTA, 15 mM MgCl2, 1 mM DTT, supplemented with protease inhibitor cocktail (Sigma)] in the presence of nonradioactive ATP or [γ-^{32}P]ATP using the various GST-ZmHMGA derivatives as well as GST-AtHMGA as substrates. Phosphorylated proteins (GST-ZmHMGA-P) were resolved by 15% SDS-PAGE. Gel was dried and autoradiographed.

DNA binding activity of the phosphorylated form of ZmHMGA was analyzed by incubating unphosphorylated GST-ZmHMGA or GST-ZmHMGA-P with γZ-AT2 DNA fragment [0.05 ng/μl; concentration was determined by NanoDrop spectrophotometer, ND-1000 (NanoDrop Technologies)] amplified by the following primers: Z-AT2-F 5′-AAG GTG AAA TTA TGT ATA AGT GTT CTG GAT and Z-AT2-R 5′-CAC ATG TAT TAA GTT GCA CTA GTT ATA TG] at room temperature for 30 min in 100 μl binding buffer (20 mM Tris pH 7.5, 100 mM NaCl, 2 mM EDTA, 1 ng/μl salmon sperm DNA and 10% Glycerol). Following incubation samples were centrifuged at low speed for 5 min, the supernatant, unbound fraction was collected and stored at −20 °C until used. To determine DNA binding activity, we analyzed for the presence of γZ-AT2 DNA fragment in the unbound fraction by