TWO HMG DOMAINS OF YEAST MITOCHONDRIAL PROTEIN ABF2P HAVE DIFFERENT AFFINITY TO DNA

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Maintaining mitochondrial genome integrity is essential for the viability of the whole organism. Mitochondrial genome mutations lead to muscular dystrophies, neurodegenerative diseases, and are associated with aging. In this work a baker's yeast (*Saccharomyces cerevisiae*) mitochondria model was used to investigate DNA-binding abilities of different domains of a mitochondrial Abf2p protein which participates in homologous recombination and reparation. A weak non-specific HMG1 binding to linear DNA and a specific HMG1 binding to a branched DNA with a dissociation constant of 510 nM have been discovered. The HMG2 domain itself does not bind to any DNA and either has other functions or demonstrates its DNA-binding activity in a full-length protein only.

Keywords: mitochondria, mitochondrial genome, Abf2p, recombination

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ДВА HMG-ДОМЕНА МИТОХОНДРИАЛЬНОГО БЕЛКА ДРОЖЖЕЙ ABF2P ОБЛАДАЮТ РАЗЛИЧНЫМ СРОДСТВОМ К ДНК

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Поддержание постоянства митохондриального генома имеет большое значение в обеспечении функционирования организма в целом. Мутации в геноме митохондрий могут быть причиной развития мышечных дистрофий и нейродегенеративных заболеваний, установлена также их связь с процессом старения организма. В данной работе исследована ДНК-связывающая способность отдельных доменов митохондриального белка пекарских дрожжей *Saccharomyces cerevisiae* Abf2p, участвующего в процессах гомологичной рекомбинации и репарации. Выявлено, что домен HMG1 неспецифично и слабо связывает линейную ДНК и при этом специфично взаимодействует с разветвленной структурой ДНК с константой диссоциации комплекса 510 нМ. Домен HMG2 сам по себе не обладает способностью связываться с ДНК и, вероятно, предназначен для осуществления других функций либо же проявляет ДНК-связывающую активность в составе полноразмерного белка.

Ключевые слова: митохондрия, митохондриальный геном, Abf2p, рекомбинация

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Mitochondria are intracellular organelles that play a key role in providing most eukaryotic cells with energy through adenosine triphosphate (ATP) synthesis. Mitochondria obtains from the cytoplasm most of the compounds needed to function. However, they have their own genetic information storage and transfer unit — namely their DNA and protein biosynthesis system. Mitochondrial DNA (mtDNA) is typically represented as a closed circular DNA molecule encoding mitochondrial rRNA, mitochondrial tRNA and protein involved in oxidative phosphorylation.

Mitochondrial DNA repair is key to maintaining the normal functioning of the organelle. This is due to the high incidence of mtDNA damage compared with nuclear DNA [1], caused by high concentration of reactive oxygen species in the mitochondria. Homologous recombination is one of the most important ways of repairing double-stranded breaks both in nuclear DNA and mtDNA. Indeed, mitochondrial homologous recombination processes are found in almost all major groups of eukaryotic organisms (plants [2], fungi [3] and invertebrates [4]). It is assumed that DNA recombination mechanisms in the

nucleus and mitochondria are generally similar [5]. The study of homologous recombination in mitochondrial genome became particularly essential after obtaining evidence that this process exists in mammals [5] and that there is link between accumulation of mediated recombination of deletions and several human diseases (muscular dystrophy, neurodegenerative diseases), aging and tumorigenesis process [6].

Brewer's/baker's yeasts (*Saccharomyces cerevisiae*) are extremely convenient object for the study of mitochondrial processes. In this organism, mitochondria have considerable functional similarity with human mitochondria. Moreover, yeast can perform vital functions in the absence of functionally full-fledged mitochondria, allowing for deletion of the genes of certain mitochondrial proteins and study of the phenotypic manifestations of such mutations. The mitochondrial genome of *S. cerevisiae* is a structure (nucleoid) organized in space. The nucleoid contains up to 10 mtDNA copies that interact with different proteins. Mitochondrial DNA located in the nucleoid is composed of three-dimensional organization, closely connected with the mechanisms of replication, transcription, and inheritance. Laying in the nucleoid also protects the DNA from the attack of reactive oxygen species, appearing during oxidative phosphorylation [7].

Abf2p is one of the most widely represented proteins interacting with mtDNA in the mitochondrial nucleoids in yeast. It was extracted for the first time in its pure form in 1979 [8], and the name 'Abf2p' was given to it in 1991 [9]. This protein contains two domains — HMG1 (High-Mobility Group 1) and HMG2 (HighMobility Group 2). Abf2p is presently the most studied among other proteins of yeast mitochondrial nucleoid.

Abf2p is a unique protein with the highest basicity among nucleoid proteins [10]. It generates negative DNA supercoiling when in contact with a circular plasmid in the presence of topoisomerase 1 [10]. Abf2 mutant yeast can support mtDNA growth in YPG medium containing glycerol as a carbon source. However, when culturing in media with fermentable carbon sources such as glucose, there is gradual loss of mtDNA [10]. It is also proved that in Abf2p gene mutation, the number of mitochondrial recombination events when paired with wild-type strain significantly reduces [11]. Besides, Abf2p stabilizes Holliday recombination junction intermediates, which also points to the importance of this protein in recombination [12].

Despite the large number of studies on Abf2p, the molecular mechanisms of its involvement in mtDNA recombination processes remain unexplored. The presence of two HMG domains in mitochondrial DNA-binding protein Abf2p is typical of most HMGB proteins: they are known to bind DNA minor groove with limited specificity or completely non-specific [13]. Besides, it is assumed that HMG domains that make up these proteins bind DNA independently of each other with similar efficiency [13]. However, mitochondrial HMG proteins apparently have much broader functionality than their nuclear homologues. For example, human mitochondrial DNA-binding protein, TFAm, apart from compaction of nucleoids, is a transcription factor and is probably involved in the processes of mtDNA recombination and repair [14]. TFAm is similar to bacterial HU proteins when it comes to its multi-functionality and DNA binding characteristics [14]. It is assumed that Abf2p also possesses multifunctional properties, as it is a structural analog of TFAm in yeast mitochondria.

In this paper, recombinant proteins corresponding to two Abf2p domains — HMG1 and HMG2 — were obtained. The DNA-binding ability of these domains with respect to linear DNA duplex and structure imitating late homologous recombination intermediates (Holliday junction) was studied.

METHODS

Gene cloning and expression

ABF2 gene sites corresponding to domains HMG1 (amino acid residues 27-115) and HMG2 (amino acid residues 112-183) were amplified using primer pairs abfhmg1F (GATACATATGGGTCCTAAAAGGCCC ACATC) / abfhmg1R (CGTCCTCGAGAGGAAGTTTTTCGTCA AACTCC) and abfhmg2F (GGCGCATATGGAGTTTGACGAAAAA CTTCC) / abfhmg2R (GAGGCTCGAGAGCATTATATTCTTGG ATAGC) respectively. Yeast genomic DNA strain BW303 was used as a template. The obtained amplification products were treated with restriction endonucleases *NdeI* and *XhoI* (Thermo Fisher Scientific, USA) and cloned into expression vector pET32a (Novagen, USA). In this way, we obtained the pET32a_HMG1 and pET32a_HMG2 vectors. Conformity of cloned sequences with the reference sequence was verified through Sanger sequencing in a post-genomic lab belonging to the Institute of Physico-Chemical Medicine of Russia, a Federal Medical & Biological Agency. The plasmids obtained were transformed into expression strain Escherichia coli B834 (DE3), single colonies of transformants were transferred to a 2xYT liquid medium with 100 μg/ml of carbenicillin, cultured at 37 °C under vigorous agitation to OD_{600} ~ 0.6–0.8. After that, expression of cloned genes was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.25 mM. Culturing was continued at 30 °C for 3 hours. The cells were then pooled by centrifugation (3000 g, 10 min) and re-suspended in the starting buffer (25 mM sodium phosphate buffer with pH 7.4; NaCl 500 mM; imidazole 20 mM). The re-suspended cells were sonicated in 4 pulses by 15 seconds each at 20 % amplitude. The cell lysates obtained were centrifuged at 17,000 g for 20 min. Supernatants containing recombinant proteins were pooled and further purification of the target product was carried out by metal-chelate affinity chromatography in 1 ml HisTrap column filled with Ni-NTA sepharose (GE Healthcare, USA), using high-performance protein chromatography system AKTA Purifier (GE Healthcare System, US) according to manufacturer's recommendations. Elution of target proteins was monitored by absorbance at 280 nm wavelength. A fraction with the target protein was immediately transferred into the storage buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA) using a 5 ml HiTrap Desalting column that contained Sephadex G-25. Efficient protein transfer to the buffer for storing was monitored through the absorption peak ratio at 280 nm wavelength to the conductivity of the solution.

Protein concentrations were determined by absorbance at 280 nm wavelength on NanoPhotometr spectrophotometer (Implen, Germany) according to known molar extinction coefficient (12090 $M^{-1} \times cm^{-1}$ for both proteins) and taking into account exact molecular weights: 12632.39 12 Da for HMG1 and 10177.58 Da for HMG2.

Assembling DNA structures

The structures used (DNA duplex and Holliday structure) were collected from the following oligonucleotides - x-FAM (FAM-AGTCTAGACTGCAGTTGAGTCCTTGCTAGGAC GGATCCCT), x-com (AGGGATCCGTCCTAGCAAGGACTCAA CTGCAGTCTAGACT), b (AGGGATCCGTCCTAGCAAGGGGC TGCTACCGGAAGCTTCT), r (AGGAATTCAACCACCGCTCAA CTCAACTGCAGTCTAGACT), h (AGAAGCTTCCGGTAGCAGC CTGAGCGGTGGTTGAATTCCT) – which are similar to those in the study by Duckett & Lilley [15]. Linear double-stranded

DNA was pooled from x-FAM and x-com oligonucleotide, while Holliday structure was collected from x-FAM, b, r and h oligonucleotides. The pooling was performed as follows. 5l of 1 μm of solution of labeled oligonucleotide (x-FAM) and 5l 2 mM of solutions of other oligonucleotides were added to 20 mcl of double buffer (Tris-HCl 25 mM, pH 7.5; NaCl 150 mM), and if necessary, adjusting the volume of the reaction mixture to 40 μm of deionized water. The reaction mixture was heated in a water bath to a temperature of 95 °C, and then passively cooled over 3 hours to 25 °C.

Electrophoretic mobility shift assay (EMSA)

Each of the reaction mixtures consisted of 2 mcL of 5-fold EMSA buffer (100 mM Tris-HCl, pH 8.0; 1 M NaCl; 1 mg/ml BSA; 35 % glycerol), 1 mcL of 100 nM of solution of DNA structures and various concentrations of the recombinant protein. The total volume of each reaction mixture was 10 mcL and if necessary, brought to the final volume using deionized water.

The reaction mixtures were incubated for 15 minutes in the dark at room temperature. Thereafter, they were applied to 6% polyacrylamide gel (20 × 20 cm) prepared on TBE buffer (90 mM Tris-borate, 2 mM EDTA). Before applying the samples, the gel was subjected to preliminary electrophoresis at 400 V for 40 minutes with active cooling to a temperature of 10°C. Electrophoretic separation of samples was performed for 120 minutes under the same conditions. After electrophoresis, the gel was scanned using Storm 860 scanner (GE Healthcare, USA) with blue fluorescence excitation. The resulting images were analyzed using the ImageJ program, determining the area for each track and the fluorescence band intensities of the bound and free DNA. By ratio of integral values of the fluorescence band intensities to the sum of the band areas in the track, free and bound DNA concentrations were calculated. Dissociation constants were calculated using the formula:

$$K_D = [Df] \times [P_0 - Db] / [Db],$$

where [Df] is the free DNA concentration, [Db] is bound DNA concentration and [P0] is protein concentration. All concentrations were expressed in nM. At least three independent experiments were carried out for each DNA/recombinant protein pair.

RESULTS

Obtaining recombinant proteins

In order to clarify the role played by each of the HMG domains of mitochondrial protein Abf2p in performing its functions, recombinant proteins corresponding to both domains were obtained. After isolation and purification of these proteins, their degree of purity was assessed using denaturing electrophoresis (Fig. 1). The resulting protein preparations had sufficient purity for further research. The recombinant proteins yielded 8 mg/l culture for HMG1 and 4.2 mg/l culture for HMG2.

Analysis of interaction of individual HMG domains with linear and cruciform DNA

As already noted, the HMG domains of HMGB proteins are presently believed to bind DNA independently of each other with similar efficiency. To verify whether this is true of Abf2p, we analyzed the binding of linear DNA duplex of 40 base pairs (bp) and synthetic cruciform structure that mimics the Holliday structure, with the obtained recombinant proteins through EMSA.

HMG1 domain in the conditions used by us almost did not interact with the linear DNA (Fig. 2, A). It was only at high protein concentration (500 nM) that lower band intensity corresponding to free DNA was observed. However, there was no formation of complex (which would have been visible as a clear band at the top of the gel). From this, it can be concluded that HMG1 weakly and nonspecifically interacts with linear DNA. However, it effectively bound with cruciform structure. DNA/protein complexes were detected even at a protein concentration of 100 nM (Fig. 2, A, C1). Through three independent experiments, we calculated the apparent dissociation constant of the HMG1 complex with cruciform DNA. The constant was equal to 510 ±11.78 nM. At the same time, the HMG2 domain did not form complexes with linear DNA duplex nor with cruciform structure. Even at concentrations of recombinant protein equal to 1 μm, there was no reduction in the intensity of the band corresponding to free DNA (Fig. 2, B).

According to various literature sources, full-length protein Abf2p forms a complex with linear DNA with a constant from 40 to 150 nM, that is, considerably smaller than HMG1 should [11, 15]. It was assumed that increased affinity of the full-length protein to DNA with respect to HMG1 is due to interaction of the two domains. To verify this assumption, we conducted an EMSA experiment after incubation with a mixture of two recombinant proteins. The results showed that addition of HMG2 does not

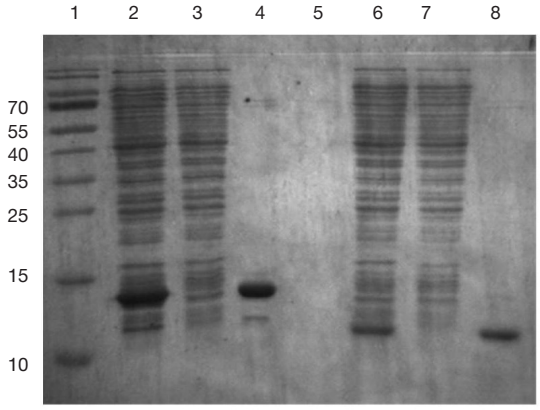

Fig. 1. Isolation and purification of recombinant proteins corresponding to HMG1 and HMG2 domains of the Abf2p protein

Recombinant proteins were purified by metal affinity chromatography on Ni sepharose. 1 — molecular weight markers (the molecular weights of marker proteins are shown on the left); 2, 6 — damaged cell lysates; 3, 7 — factions that didn't interact with affinity column; 4, 8 — purified preparations of recombinant proteins HMG1 and HMG2 respectively.

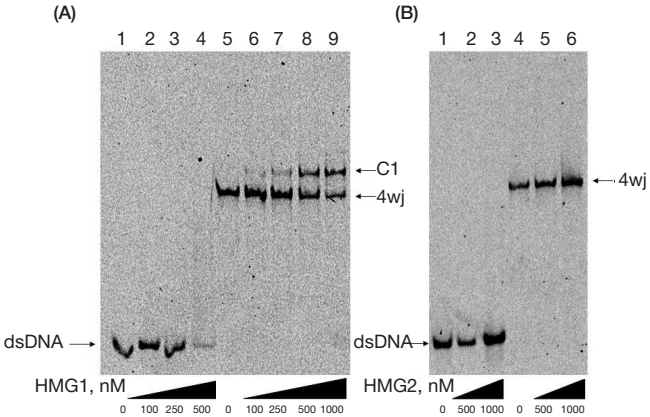

Fig. 2. Analysis of the binding of recombinant proteins corresponding to HMG1 and HMG2 domains with DNA using EMSA

Linear DNA duplex (double-stranded DNA - dsDNA) and cruciform DNA (4-way junction — 4wj) at a concentration of 10 nM were incubated with increasing concentrations of recombinant proteins, after which the reaction mixtures were separated in 6% polyacrylamide gel. **(A)** Binding with DNA of HMG1 domain; 1–4 — interaction with DNA duplex, 5–9 — binding with cruciform DNA; C1 — resulting complex. **(B)** Binding with DNA of HMG2 domain; 1–3 — interaction with DNA duplex, 4–6 — binding with cruciform DNA. The lower part of the figure shows the used concentration of recombinant proteins (in nM).

affect the characteristics of DNA binding with HMG1 (data not shown).

DISCUSSION

Based on data obtained, the following can be concluded. HMG1 domain makes a major contribution to the DNA binding activity of Abf2p. Here, the HMG2 domain does not possess direct DNA-binding activity in vitro. However, it is important for performance of this function by the full-length protein since the dissociation constant of the HMG1/linear DNA complex, according to our results, is more than 1 μm, while the dissociation constant of the same complex with Abf2p is lower by 1–2 orders of magnitude. It should be highlighted that HMG1 can, similarly to full-length protein, specifically bind cruciform DNA structure. This feature is characteristic of many HMGB proteins involved in maintaining DNA integrity of the process, namely repairing by recombination [14]. There are reports that Abf2p is important for homologous recombination of yeast mitochondrial DNA, and apparently, the specificity of binding of full-length protein is achieved directly by HMG1 [11, 12]. The role of HMG2 in the various functions of Abf2p is less obvious. As our data show, HMG2 by itself does not bind to a linear or branched DNA in the selected experimental conditions. This is quite unusual for HMG domain. Nevertheless, relying on the results obtained, it is not clear whether HMG2 can exhibit DNA-binding activity inside a full-length protein. Apparently, apart from strengthening the DNA-binding properties of HMG1, the second domain may be involved in Abf2p interaction with other proteins, for example, in attracting enzymes needed for repair and recombination.

CONCLUSIONS

The study conducted allowed to establish that the HMG1 domain of yeast mitochondrial protein Abf2p is not specific and weakly binds linear DNA, at the same time forming a specific complex with cruciform DNA with a dissociation constant of 510 nM. HMG2 cannot bind with DNA by itself *in vitro.*

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