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The FK506-binding protein, Fpr4, is an acidic histone chaperone

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Abstract **Fpr4, a FK506-binding protein (FKBP), is a recently identified novel histone chaperone. How it interacts with histones and facilitates their deposition onto DNA, however, are not understood. Here, we report a functional analysis that shows Fpr4 forms complexes with histones and facilitates nucleosome assembly like previously characterized acidic histone chaperones. We also show that the chaperone activity of Fpr4 resides solely in an acidic domain, while the peptidylprolyl isomerase domain conserved among all FKBPs inhibits the chaperone activity. These observations argue that Fpr4, while unique structurally, deposits histones onto DNA for nucleosome assembly through the well-established mechanism shared by other chaperones.**

Keywords: Histone chaperone; Nucleosome assembly; FKBP

1. Introduction

Fpr4 is a member of the FK506-binding protein (FKBP) family defined by a structural domain that interacts with immunosuppressants and catalyzes the *cis–trans* conversion of prolyl bonds in protein substrates [1–3]. The best studied FKBPs, which contain essentially only the peptidylprolyl isomerase (PPIase) domain, function as intracellular receptors of the immunosuppressants FK506 and rapamycin that block antigenic responses and/or cell cycle progression [2–5]. The overwhelming majority of FKBPs contain other structural elements in addition to the PPIase domain [1,6]. Their cellular functions, however, remain largely unknown. A subfamily of these FKBPs consists of nucleolar proteins such as Fpr4 of the budding yeast *Saccharomyces cerevisiae* that share distinctive structural features: an acidic domain in the NH2-terminus, a basic domain in the middle, and the conserved PPIase domain at the COOH-terminus [6–8]. Interestingly, a recent study demonstrates that Fpr4 possesses a novel histone chaperone activity *in vitro*, and acts as a chromatin component required for transcriptional silencing at the rDNA locus *in vivo* [9].

Histone chaperones are acidic proteins or protein complexes that promote the assembly of histones and DNA into nucleosomes, the basic units of eukaryotic chromatin each consisting of an octameric histone core wrapped around by 146 bp DNA [10–13]. A consensus model, emerged from extensive studies of histone chaperones including nucleosome assembly protein 1 (NAP1), histone regulator A (HIRA), anti-silencing factor 1 (ASF1), and chromatin assembly factor 1 (CAF1), suggests that the assembly of nucleosomes involves: (1) the formation of pre-deposition complexes of histone chaperones with histone H3–H4 and H2A–H2B, respectively, and (2) sequential deposition of the H3–H4 tetramer and H2A–H2B dimers onto the DNA [10,13]. Because the pre-deposition complexes are critical intermediates of nucleosome assembly, pre-incubating chaperones and histones is needed for efficient deposition of histones onto DNA *in vitro* [13,14]. The newly identified histone chaperone Fpr4, however, was reported to function in an unexpected manner: it facilitated effective nucleosome assembly when exposed to core histones and DNA simultaneously, but lost the activity completely after pre-incubation with core histones [9]. How this unique histone chaperone manages to deposit histones onto DNA and how each of its well-defined structural domains contributes to this novel activity, therefore, are intriguing questions critical for further investigation of Fpr4's *in vivo* roles in gene silencing and perhaps nucleosome assembly.

We report here that Fpr4 forms complexes with both H2A–H2B and H3–H4, like the acidic histone chaperone NAP1. Fpr4 pre-incubated with core histones facilitates the deposition of the histones onto DNA. When exposed to core histones and DNA at the same time, however, it exerts much diminished chaperone activity. Furthermore, we demonstrate that the histone chaperone activity of Fpr4 resides in the acidic domain of the protein, while the PPIase domain inhibits the histone chaperone activity. These results suggest that Fpr4, a novel histone chaperone that belongs to the FKBP family, facilitates nucleosome assembly in a manner similar to other acidic histone chaperones. The signature PPIase domain of FKBPs, however, inhibits the activity.

2. Materials and methods

2.1. Plasmid construction

We have previously isolated a cDNA clone that contains the entire *FPR4* open reading frame of *Saccharomyces cerevisiae* (data not shown). To construct the expression vectors, DNA fragments that encode for the various alleles of Fpr4 as shown in Fig. 3 were PCR amplified from the cDNA clone. Each forward primer carries a *Bam*HI cutting site near its 5′ end, while each backward primer carries an *Xho*I cutting site near its 5′ end. For alleles with C-terminal deletions (AB, A, and B), a stop codon was inserted following the coding sequence

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Abbreviations: FKBP, FK506-binding protein; PPIase, peptidylprolyl isomerase

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in the backward primer. These DNA fragments were digested with *Bam*HI and *Xho*I, and cloned into the pET28 plasmid (Novagen, Madison, WI), which encodes a six-histidine tag at the N-terminus of each fusion protein. pFPR4.F323A (Fig. 4A) was constructed by substituting codon 323 (TTT) with GCT in pFPR4, using the quick change mutagenesis kit (Stratagene, San Diego, CA) following the manufacturer's instructions.

2.2. Purification of proteins

To purify Fpr4 and Fpr4 mutant alleles, the expression vectors were introduced into the *E. coli* strain BL21. Cells were grown in LB medium supplemented with kanamycin (30 μg/ml) at 30 °C to $OD_{600} \sim 0.8$ and induced with 0.4 mM isopropyl-D-thiogalactopyranoside (IPTG) at 25 °C for 4 h. Cells from one liter cultures were then harvested and resuspended in 20 ml lysis buffer containing 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0, 5% glycerol, 0.1% Triton X-100, and 10 mM imidazole. The cell suspensions were sonicated and the lysates were subjected to centrifugation at 16000 rpm for 20 min. The supernatant was applied onto an Ni^{2+}-NTA column (2 ml, Novagen) pre-equilibrated with wash buffer containing 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0, 5% glycerol, 0.1% Triton X-100, and 20 mM imidazole. The column was washed with 20 ml wash buffer and then eluted with an elution buffer containing 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0, 5% glycerol, 0.1% Triton X-100, and 250 mM imidazole. For Fpr4, Fpr4-F323A, Fpr4AB, Fpr4BI and Fpr4B, proteins eluted from the Ni^{2+}-NTA columns were dialyzed against a buffer containing 0.2 M NaCl, 20 mM Tris–HCl, pH 8.0, 5% glycerol, and 0.1% Triton X-100, and then applied onto a heparin column (1 ml). After washing the column with 10 ml of the dialysis buffer, proteins retained on the column were eluted with buffer containing 0.5 M NaCl, 20 mM Tris–HCl, pH 8.0, 5% glycerol, 0.1% Triton X-100. The Fpr4A and Fpr4I eluted from the Ni^{2+}-NTA column were dialyzed and further purified similarly using an ion-exchange (Fast Q) column. All purified proteins were dialyzed against the histone deposition reaction buffer (0.15 M NaCl, 0.5 mM DTT, and 20 mM Tris–HCl, pH 8.0) before protein concentrations were determined using BCA protein assay kit (Pierce, Milwaukee, WI). Each of these proteins (at 0.5, 1 and 2 μg) was resolved by SDS–PAGE, along with pure BSA (Sigma, St. Louis, MO) also at 0.5, 1 and 2 μg, respectively. After Coomassie blue staining, the relative intensities of the Fpr4 bands compared to those of BSA at the same quantity were quantified to determine the purities of the samples (all >95%). The purification of chicken erythrocyte histones, recombinant yeast NAP1, and topoisomerase I has been described previously [15–17].

2.3. Histone deposition and MNase digestion

Core histones or histone H3–H4 (4 pmol) were incubated with various forms of histone chaperones, including Fpr4, Fpr4 mutants, and NAP1, at 0, 4, 8 or 16 pmol, in 15 μl reaction buffer containing 0.15 M NaCl, 0.5 mM DTT, and 20 mM Tris–HCl, pH 8.0, at 30 °C for 30 min. The plasmid, T7/T3–19 (2234 bp) [15–17], was pre-treated with topoisomerase I (20 u/μg DNA) for 5 min and then added (0.4 μg in 1 μl) into each of the chaperone–histone mix and incubated for an additional 60 min. The reactions were stopped by adding equal volume of stop buffer (glycerol 25%, 60 mM Tris–HCl, pH 8.0, 30 mM EDTA, 0.5% SDS) and were resolved in 1.2% agarose gel by electrophoresis followed by ethidium bromide staining. Alternatively, for histone deposition assays without pre-incubation, the chaperones, histone octamers and topoisomerase I-treated circular plasmid were added to test tubes sequentially with ~1 s for each interval. The reactions were then incubated at 30 °C for 60 min and analyzed as described above. To examine the formation of nucleosomes, products of the histone deposition reactions were digested by Micrococcal nuclease (MNase) (0.3 U Mnase/μg DNA, 0.5 mM $CaCl_2$, and 3 mM $MgCl_2$) at 30 °C for 10 and 30 min, respectively. The reactions were terminated with 'stop' buffer as described above and electrophoresed on an 1.5% agarose gel.

For histone deposition assays carried out in the presence of rapamycin, rapamycin was added to the chaperone–histone pre-incubation reactions at 0.1 μg/ml. When heat denaturation was applied for analysis of the thermal stabilities of the chaperones, NAP1, the acidic domain and full-length Fpr4 were heated at 70 °C for 5 min and then renatured at 23 °C for 10 min before being incubated with histones for deposition assays.

2.4. Protein–protein interaction

The 6His-tagged Fpr4 or Fpr4A (100 pmol) was mixed with 100 pmol of histone H2A–H2B, histone H3–H4, or core histones in the reaction buffer (20 mM Tris–HCl, pH 8.0, 20% glycerol, 0.2 mM EDTA, 0.1% Nonidet P-40, 2 mM phenyl methylsulfonyl fluoride, 300 mM KCl, and 30 mM imidazole) and incubated at 30 °C for 30 min. Ni^{2+}-NTA (Novagen) agarose beads pre-equilibrated with reaction buffer were then added to the reactions and the samples incubated at 4 °C for 2 h on a rotary shaker. After the beads were extensively washed with reaction buffer, the proteins bound to the beads were eluted with reaction buffer containing 250 mM imidazole, and resolved by SDS–PAGE in an 18% gel. The retention ratios were calculated based on the relative intensity of the protein bands as determined by densitometer.

The oligomeric states of Fpr4 and Fpr4A were examined by sucrose gradient analysis. Sucrose gradients (4.2 ml) composed of 5–20% sucrose, 100 mM NaCl, 40 mM Tris, 0.5 mM EDTA, pH 8.0 were prepared and overlayed with 100 μl of the protein sample (50 μg). Sedimentation was in a SW60 Ti rotor at 55000 rpm for 10 h at 4 °C. Fractions were collected and analyzed on SDS–PAGE.

3. Results

3.1. Fpr4 facilitates nucleosome assembly like a typical acidic histone chaperone

Fpr4 was reported to facilitate nucleosome assembly in a way different from the consensus model shared by other histone chaperones [9]. To gain insight into how this novel histone chaperone deposits histones onto DNA, we purified a recombinant Fpr4 to >95% homogeneity (Fig. 1A) and compared its nucleosome assembly activity with that of NAP1. Fig. 1B shows the assembly of nucleosomes, which generates negative coils in a circular plasmid and increases mobility of the DNA during agarose gel electrophoresis. When Fpr4 or NAP1 was pre-incubated with core histones for 30 minutes, both proteins stimulated nucleosome assembly in a dosage dependent manner (lanes 4–9), although higher levels of activities for NAP1 were observed. On the other hand, when the chaperones, histones, and DNA were added into the reaction mixtures sequentially with ~1 s in interval, undetectable or very low levels of nucleosome assembly was observed with Fpr4 and NAP1 (lanes 12–18), respectively. Thus, pre-incubation with core histones enables Fpr4, very much like NAP1, to deposit histones to DNA more effectively, in contrast to what was reported previously [9].

Having observed that Fpr4 and NAP1 facilitated nucleosome assembly under the same conditions, we decided to examine whether Fpr4 forms stable complexes with histones H3–H4 and H2A–H2B similar to NAP1. The recombinant Fpr4, which contains a 6Histine tag at the NH2-terminus, was incubated with three different histone samples of H2A–H2B, H3–H4, or all four subunits, respectively. The same histone *vs.* Fpr4 ratio was maintained in all three reactions. Histones that formed complexes with Fpr4 were then retrieved by Ni^{2+}-NTA agarose beads and resolved by SDS–PAGE electrophoresis. Coomassie blue staining of the proteins (Fig. 2A) suggests that ~30% of H2A–H2B (lanes 3 and 4) and ~40% of H3–H4 (lanes 5 and 6) from the inputs were retained by Fpr4, while neither H2A–H2B nor H3–H4 binds to the agarose beads non-specifically (lanes 1 and 2). The H3–H4 complex was also retrieved at higher levels than H2A–H2B from the histone sample with all four subunits (lanes 7 and 8), suggesting a stronger affiliation between Fpr4 and H3–H4. Higher binding affinity between NAP1 and H3–H4, compared to