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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

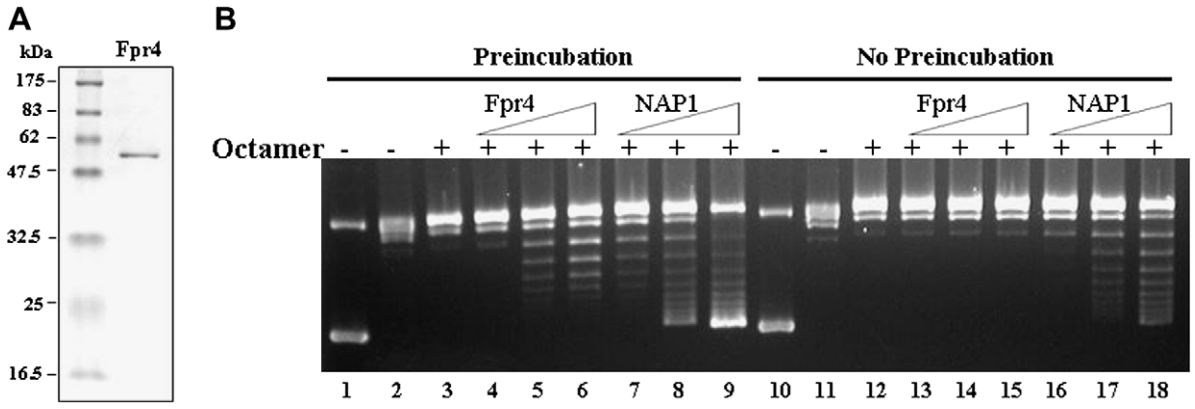

Fig. 1. Nucleosome assembly facilitated by Fpr4. (A) Coomassie blue staining of purified Fpr4 resolved by SDS–PAGE. Left lane: molecular weight markers. Right lane: purified Fpr4 (1 μg). (B) Histone deposition assays resolved in ethidium bromide-stained agarose gels. Lanes 1 and 10: supercoiled plasmid DNA lanes 2 and 11, topoisomerase I-treated, relaxed DNA; lanes 3 and 12, deposition assays without histone chaperones; lanes 4–9: deposition assays with core histones and histone chaperones pre-incubated; lanes 13–18: deposition assays in which core histones and histone chaperones were not pre-incubated. The molar ratios of histone chaperone *vs.* histone octamer are 1:1 (lanes 4, 7, 13, and 16), 2:1 (lanes 5, 8, 14 and 17) and 4:1 (lanes 6, 9, 15, and 18), respectively.

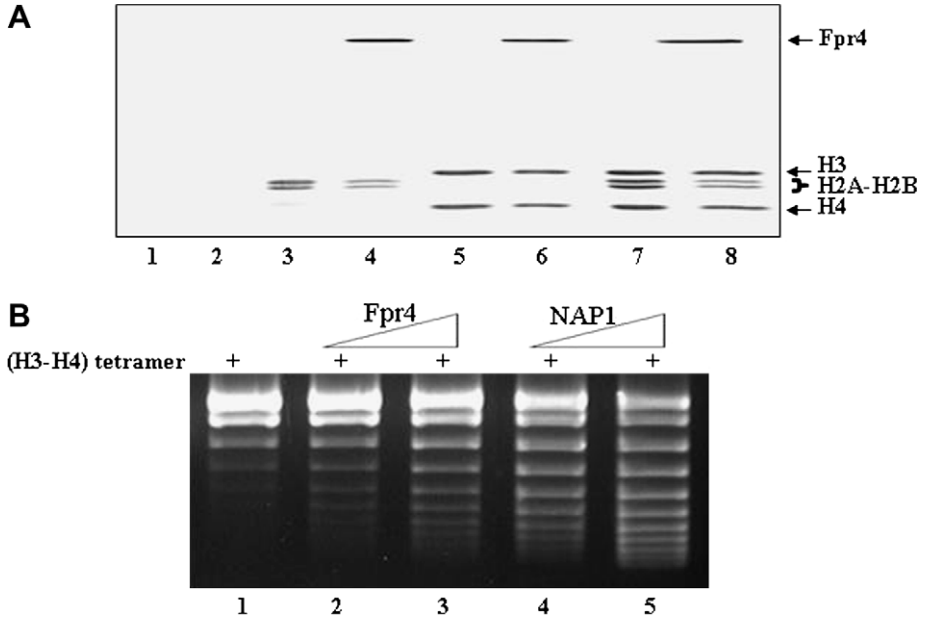

Fig. 2. Fpr4 forms predeposition complexes with histones. (A) Fpr4 forms complexes with both histone H3–H4 and H2A–H2B. Lanes 1 and 2, agarose beads, without Fpr4 attached, do not retain histone H2A–H2B or H3–H4 after incubation; lanes 3, 5, and 7: Histone H2A–H2B, H3–H4, and core histones with all four subunits, respectively, that were incubated with 6His-tagged Fpr4. 40% of the inputs were loaded. Lanes 4, 6, and 8: histones H2A–H2B, H3–H4, and all four core histones retained by the Fpr4 attached onto the agarose beads. (B) Deposition of the histone H3–H4 by Fpr4 and NAP1.

H2A–H2B, has been observed under similar conditions [14]. These results show that Fpr4 forms stable complexes with histones similar to NAP1, which likely provide the biochemical basis for the similarities in their nucleosome assembly activities.

During the nucleosome assembly process, histone chaperones such as NAP1 deposit H3–H4 onto the DNA to form assembly intermediates consisting of $(H3–H4)_2$ tetramers each wrapped around by approximately 60 bp of DNA [10,13]. These intermediates also generate negative coils in the plasmid, which increases its mobility during agarose gel electrophoresis. To determine whether Fpr4 promotes the deposition of H3–H4 onto DNA similar to NAP1, assembly assays were performed with the H3–H4 complex. Fig. 2B shows that both Fpr4 and NAP1 were able to deposit histone H3–H4 to form assembly intermediates. Thus, the results shown in Figs. 1 and 2 indicate that Fpr4 forms pre-deposition complexes with the histones and stimulates their deposition onto DNA for nucleosome assembly like other acidic histone chaperones.

3.2. The histone chaperone activity is in the acidic domain, and inhibited by the PPIase domain

Fpr4 is a FKBP with three well-defined structural domains (Fig. 3A) [6,9]. While the acidic domain bears resemblance to

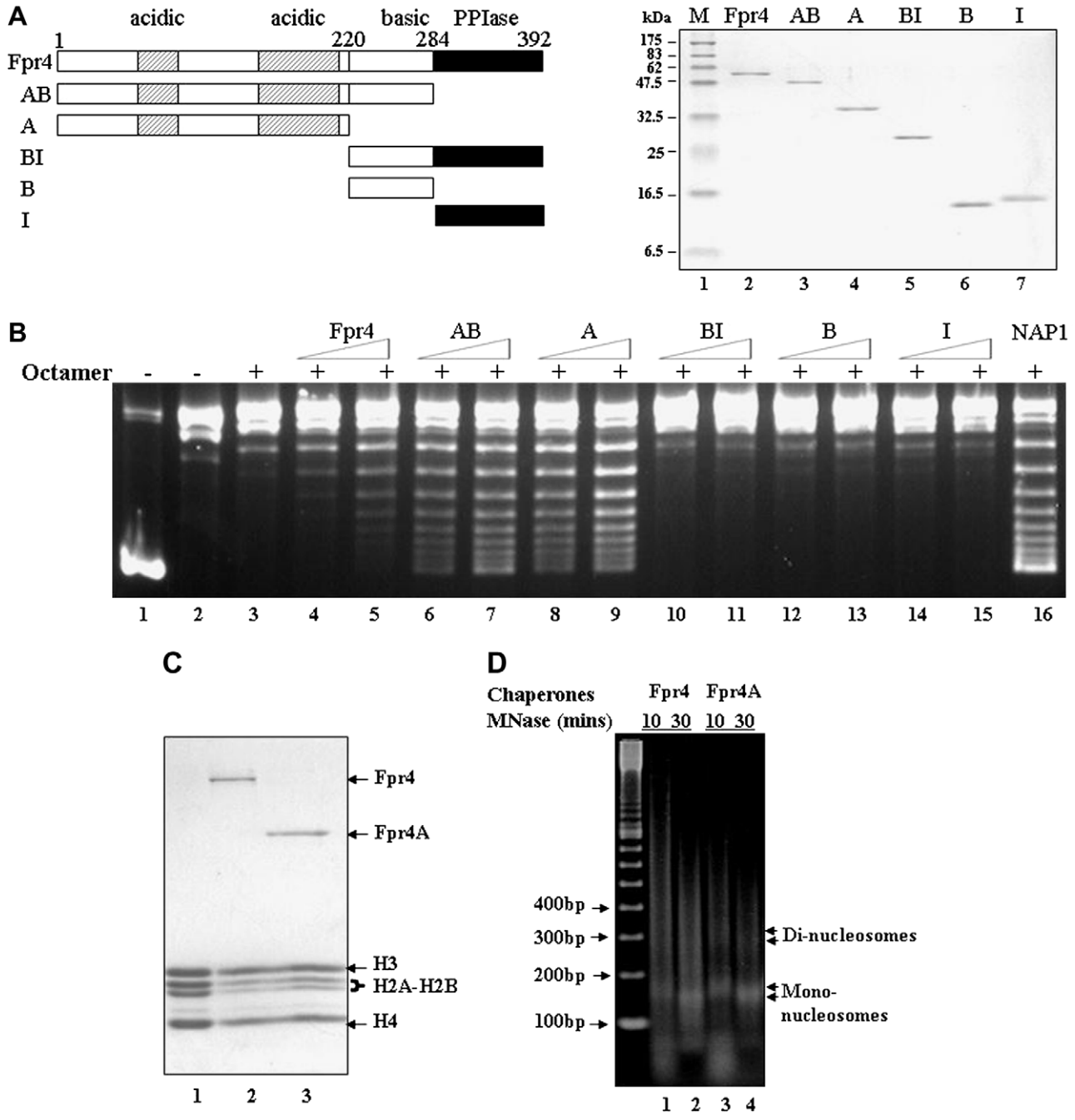

Fig. 3. Histone deposition assays with Fpr4 mutant alleles. (A) The left panel is a schematic representation of Fpr4 and Fpr4 mutant alleles encoded by the expression vectors. The two acidic motifs are shown as shaded boxes. The basic domain and the PPIase domain are shown as open and black boxes, respectively. Numbers on top of the boxes indicate the amino acid residues from which each allele starts and ends, respectively. The right panel shows Coomassie blue staining of purified proteins resolved by SDS–PAGE. Lane 1: size markers with their respective molecular weights labeled on the left. Lanes 2–7: purified Fpr4 and Fpr4 mutant proteins (1 μg each) corresponding to those shown in the schematic representation. (B) Histone deposition assays with Fpr4 alleles. Lane 1: supercoiled plasmid DNA; lane 2, topoisomerase I-treated, relaxed DNA; lane 3, deposition assay carried out without histone chaperone; lanes 4–16: deposition assays carried out with Fpr4 alleles or NAP1 as marked on top of the gel. The molar ratios of histone chaperone *vs.* histone octamer are 1:1 (lanes 4, 6, 8, 10, 12, and 14) and 2:1 (lanes 5, 7, 9, 11, 13, 15 and 16). (C) The acidic domain of Fpr4 forms complexes with histones. Lane 1: core histones used for incubation with 6His-tagged Fpr4. 40% of the inputs were loaded. Lanes 2 and 3: core histones retained by Fpr4 and Fpr4A attached onto the agarose beads, respectively. (D) MNase digestion of histone deposition products. Lanes 1 and 2: histone deposition reactions with Fpr4 (identical to that shown in lane 5 of Fig. 3B) were treated with MNase for 10 and 30 min, respectively. Lanes 3 and 4: histone deposition reactions with Fpr4A (identical to that shown in lane 9 of Fig. 3B) were treated with MNase for 10 and 30 min, respectively. Relevant markers of a 100 bp DNA ladder are marked on the left to serve as a standard for molecular weights. DNA fragments corresponding to mono-and di-nucleosomes are marked on the right.

those found in other histone domains, the PPIase and basic domains are unique to this newly identified chaperone. Although it was previously shown that a mutant allele including both the acidic and basic domains possessed the histone chaperone activity [9], roles of each of the three domains remain to be further defined. We constructed a set of bacterial expression plas-

mids that encode for the acidic domain–basic domain (AB), acidic domain (A), basic domain-PPIase domain (BI), basic domain (B), and the PPIase domain (I) of the protein (Fig. 3A, left panel). These recombinant proteins were purified to >95% homogeneity (Fig. 3A, right panel). Nucleosome assembly assays were performed with these mutant alleles in comparison with full-length Fpr4 and NAP1. Fig. 3B shows that deleting the PPIase domain led to a significant increase in the histone chaperone activity (lanes 4–7), suggesting an inhibitory role for the PPIase domain. Deleting the basic domain, however, did not affect the activity (lanes 8 and 9), indicating that the acidic domain alone possesses the histone chaperone activity. Consistent with this observation, all mutant alleles in which the acidic domain had been removed failed to facilitate nucleosome assembly (lanes 10–15). Like the full-length Fpr4, the acidic domain of the protein also forms stable complex with histones, with higher affinity to H3–H4 than to H2A–H2B (Fig. 3C). The fact that the histone chaperone activity resides solely in the acidic domain underscores our contention that Fpr4 is indeed an acidic histone chaperone. To exam whether the acidic domain-facilitated histone deposition leads to nucleosome formation like the full-length protein [9], products of the Fpr4- and Fpr4A-facilitated histone deposition reactions were digested with Micrococcal nuclease (MNase) at 30 °C. Fig. 3D shows that, after 10 min, DNA fragments of ~160 bp (mono-nucleosomes) were detected in the Fpr4 facilitated reaction (lane 1), while DNA fragments of ~160 bp (mono-nucleosomes) and ~320 bp (di-nucleosomes) were detected in the Fpr4A facilitated reaction (lane 3); Prolonged digestion (30 min) shortened the DNA fragments to ~145 bp and 290 bp, respectively (lanes 2 and 4), consistent with the removing of linker DNA not protected by the nucleosome. These results indicate that Fpr4 and Fpr4A indeed facilitate nucleosomes assembly. The Fpr4 facilitated nucleosome assembly yielded higher levels of non-specific protection (larger DNA fragments with variable sizes) compared to Fpr4A (comparing lanes 1 with 3, and 2 with 4), probably due to the DNA being bound by more histones non-specifically. This is consistent with the observation that Fpr4 is a weaker histone chaperone compared to Fpr4A (Fig. 3B).

The PPIase domain of FKBPs may catalyze isoformic changes in protein substrates as a peptidylprolyl isomerase

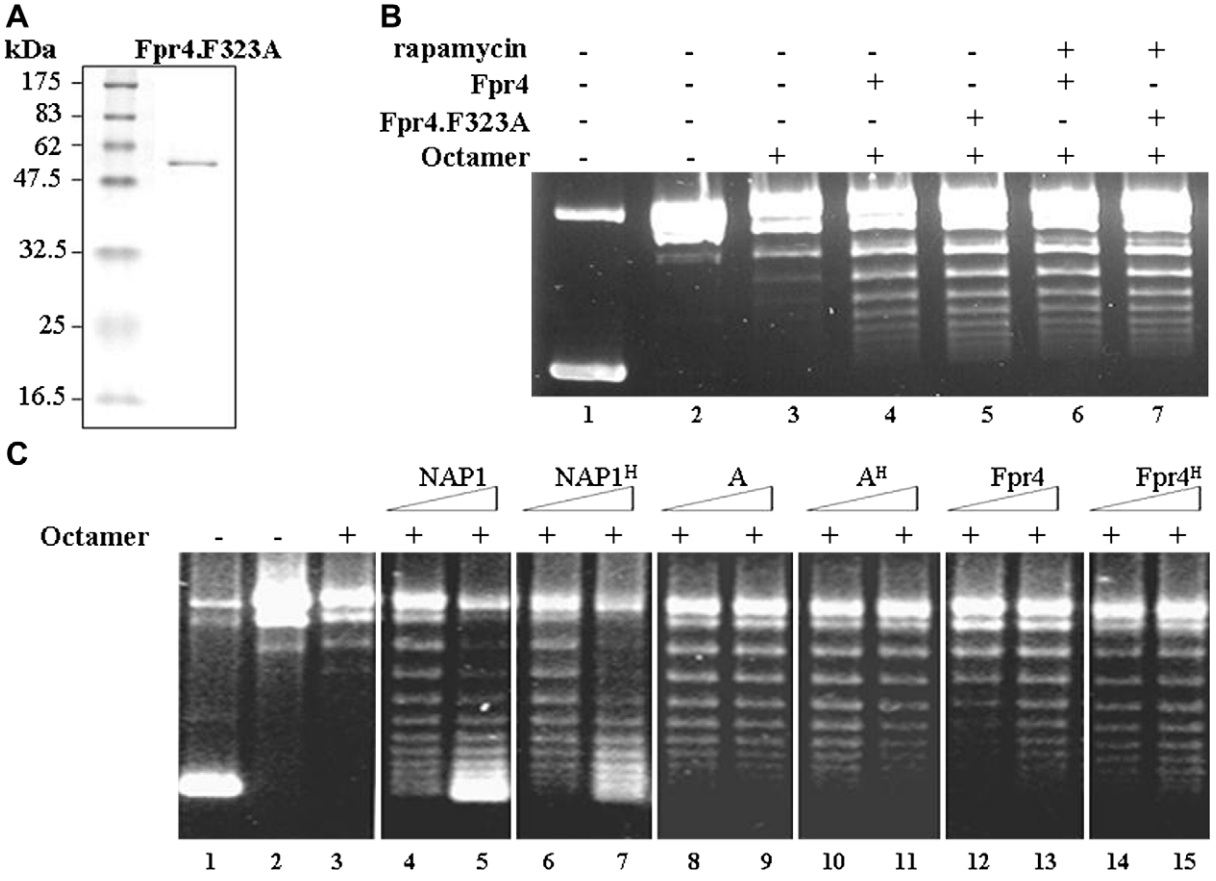

Fig. 4. The inhibitory role of the PPIase domain dependents on its structure, but not enzymatic activity. (A) Coomassie blue staining of purified Fpr4.F323A resolved by SDS–PAGE. Left lane: molecular weight markers. Right lane: purified Fpr4.F323A (1 μg). (B) Nucleosome assembly assays with Fpr4.F323Y and rapamycin. Lane 1: supercoiled plasmid DNA; lane 2, topoisomerase I-treated, relaxed DNA; lane 3: deposition assay carried out without histone chaperone; lanes 4 and 5, deposition assay carried out with Fpr4 and Fpr4.F323A, respectively; lanes 6 and 7, deposition assay carried out with Fpr4 and Fpr4.F323A, respectively, in the presence of rapamycin 1 μg/ml. The molar ratio of histone chaperone *vs.* histone octamer in each reaction is 2:1. (C) Nucleosome assembly assays with heat-treated chaperones Lane 1: supercoiled plasmid DNA; lane 2, topoisomerase I-treated, relaxed DNA; lane 3: deposition assay carried out without histone chaperone. The chaperones NAP1 (lanes 4–7), the acidic domain of FPR4 (lanes 8–10) and full-length FPR4 (lanes 11–15) were either not heated (lanes 4, 5, 8, 9, 12, 13), or pre-heated at 70 °C for 5 min (lanes 6, 7, 10, 11, 14, 15). The molar ratios of histone chaperone *vs.* histone octamer are 1:1 (lanes 4, 6, 8, 10, 12, and 14) and 2:1 (lanes 5, 7, 9, 11, 13, and 15).

and serve as an intracellular receptor for FK506 and rapamycin. Interactions with the immunosuppressants require proper folding of the domain, but not the PPIase activity [18]. Upon binding to the domain, however, the immunosuppressants do inhibit the PPIase activity [19]. Having observed that the PPIase domain inhibits the histone chaperone activity, we decided to examine whether the inhibitory effect is depend on the PPIase activity, perhaps by isomerizing histones, or the tertiary structure of the domain. We purified an Fpr4 mutant allele, Fpr4.F323A (Fig. 4A), which lacks the PPIase activity because of the Phe323Ala substitution at the catalytic center [9]. Nucleosome assembly assays showed that this allele and the wild type Fpr4 had similar levels of activity (Fig. 4B, lanes 4 and 5). Consistent with this observation, rapamycin, which inhibits the PPIase activity, exerted little effect on the histone chaperone activities of Fpr4 and Fpr4.F323A (Fig. 4B, lanes 6 and 7). These results suggest that the inhibition of the histone chaperone activity by the PPIase domain is independent of the PPIase activity, consistent with previous observations that FK506 and mutations reducing the PPIase activity had no obvious effect [9]. The role of the tertiary structure of the PPIase domain was examined by performing nucleosome assembly assays using differentially denatured Fpr4 alleles with or without the domain. We observed that, after heat denaturation at 70 °C for 5 min, NAP1 retained much of its activity in nucleosome assembly assays (Fig. 4C, lanes 4–7), probably because this acidic protein can re-nature very efficiently. This property is shared by the acidic domain of Fpr4, as the same heat treatment resulted in only a slight decrease of its activity like NAP1 (lanes 8–11). In contrast, however, heat denaturation of full-length Fpr4 led to an increase of the histone chaperone activity (lanes 12–15), likely due to less efficient renaturation of the PPIase domain. These observations suggest that a properly folded PPIase domain is required to inhibit the histone chaperone activity of Fpr4.

The histone chaperone NAP1 is known to form a homodimer [23]. If Fpr4 also functions as a homocomplex, removing the PPIase domain may alter the stoichiometry of the complex thus impacting the chaperone activity of the protein. Thus, we examined the oligomeric states of Fpr4 and Fpr4A by sucrose gradient sedimentation. Fig. 5 shows that while NAP1 sedimented with a "*S*" value that equates with a dimeric form of the 48 kDa protein, Fpr4 and Fpr4A, with molecular weights at 44 kDa and 25 kDa, respectively, sedimented at rates consistent with being in monomeric states. These results suggest that both these proteins are likely to function as monomers and the enhanced histone chaperone activity is not due to changes in the oligomeric state.

4. Discussion

Fpr4 belongs to a subfamily of nucleolar FKBPs that may isomerize protein substrates and interact with immunosuppressants. Its *in vivo* function was unknown until Kuzuhara and Horikoshi reported recently that it was required for transcriptional silencing at the rDNA locus [9]. Interestingly, its role in rDNA silencing correlated with a novel histone chaperone activity that facilitated nucleosome assembly through a mechanism different from that shared by other histone chaperones [9]. These observations raised the important question of whether the establishment and/or maintenance of the hetero-

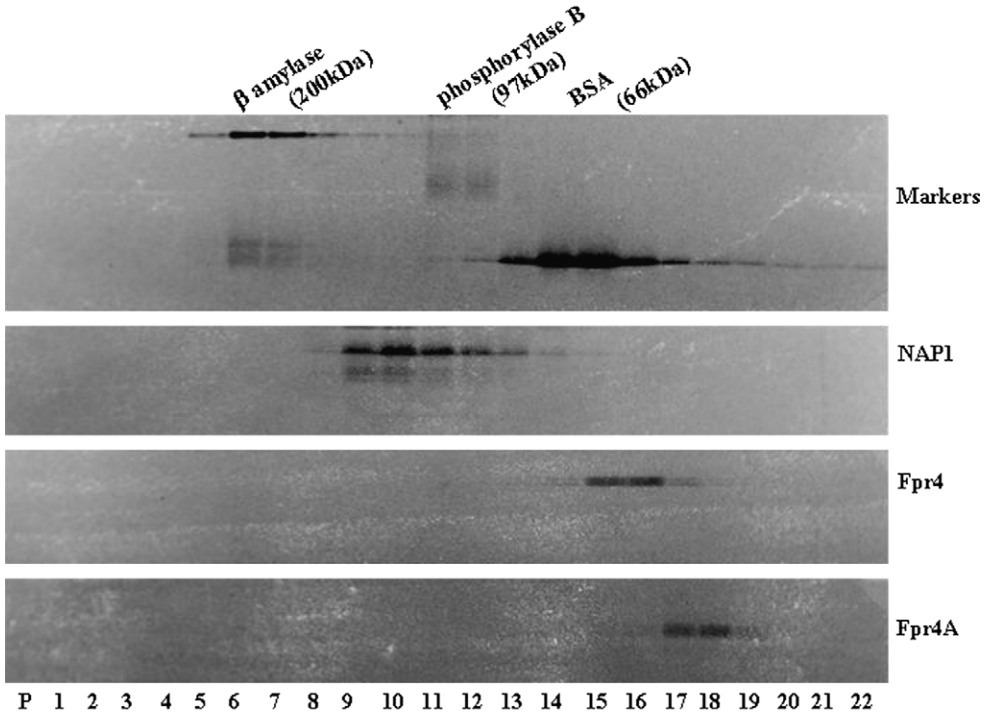

Fig. 5. Coomassie blue staining of SDS–PAGE that shows the distribution of Fpr4 and Fpr4A in sucrose gradients. Marker proteins (β-amylase, phosphorylase B and BSA), NAP1, Fpr4 and Fpr4A were sedimented in four 5–20% sucrose gradients in parallel. Twenty-two fractions, collected from the bottom to the top, were separated by SDS–PAGE.

chromatin structure at rDNA require a unique mode of interaction between histones and the novel histone chaperone. Characterization of the interactions between Fpr4 and histones, therefore, is a critical step towards understanding Fpr4's *in vivo* functions in modulating chromatin structure.

Adopting well-established *in vitro* nucleosome assembly procedures that have been used in many laboratories including ours [11,14,16,20], we observed that Fpr4 and the acidic histone chaperone NAP1 facilitated nucleosome assembly under identical conditions. This deposition process appears to be independent of ATP hydrolysis, as adding ATP to the reactions yielded no detectable effect (Supplemental Data 1). Furthermore, our analyses showed that Fpr4 formed complexes with both histone H2A–H2B and H3–H4 similar to NAP1, and that the acidic domain of Fpr4 was solely responsible for the histone chaperone activity. Together, these results argue that Fpr4 facilitates nucleosome assembly through the same mechanism shared by other acidic chaperones, in contrast to what was reported previously [9]. This information is important for future investigations of Fpr4's role in rDNA silencing and chromatin structure over all.

While the acidic domain of Fpr4 possesses the histone chaperone activity, the PPIase domain appears to play an inhibitory role. This inhibition is independent of the PPIase activity, since neither mutations nor rapamycin treatment, both of which inactivate the activity, had obvious effect on the chaperone activity. The tertiary structure of the PPIase domain, however, is required for the inhibition, as heat denaturation of Fpr4 led to an increase in its histone chaperone activity. Therefore, if Fpr4 indeed functions as a histone chaperone *in vivo*, cellular factors that interact with the PPIase domain directly or through FK506 or rapamycin may regulate its activity. The PPIase domain probably does not compromise the protein's interactions with histones, since Fpr4A and Fpr4 formed stable complexes in a similar manner (Fig. 3C). It remains to be determined whether the domain impedes the transfer of histones from the chaperone to DNA. The basic domain has little effect on the histone chaperone activity, although previously reported as part of the histone chaperone domain [9]. This domain contains a couple of putative nuclear localization sequences (NLSs) and binds to DNA *in vitro* in a non-sequence dependent manner (data not shown). Thus, it may be involved in the nuclear localization and/or chromatin association of Fpr4 at the rDNA locus [9].

A close relative of Fpr4 (~60% identical), Fpr3, is also localized in the nucleolus of the budding yeast cells [6–8]. It has been demonstrated that Fpr3 is required for the meiotic recombination checkpoint, which ensures that DNA breaks created by meiotic recombination are repaired before chromosome segregation [21]. Since the rDNA locus is hyperactive for homologous recombination, the integrity of its chromatin structure is likely a prime target for this checkpoint surveillance [22] that is regulated by Fpr3. Our preliminary studies suggest that Fpr3 is also a histone chaperone *in vitro* (data not shown). It will be interesting to determine in future studies whether the histone chaperone activity of Fpr3 is essential for its role in the checkpoint of meiotic recombination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.093.

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