

The Two *Plasmodium falciparum* Nucleosome Assembly Proteins Play Distinct Roles in Histone Transport and Chromatin Assembly^{*[5]}

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The malarial parasite *Plasmodium falciparum* has two nucleosome assembly proteins, PfNapS and PfNapL (Chandra, B. R., Olivieri, A., Silvestrini, F., Alano, P., and Sharma, A. (2005) *Mol. Biochem. Parasitol.* 142, 237–247). We show that both PfNapS and PfNapL interact with histone oligomers but only PfNapS is able to deposit histones onto DNA. This property of PfNapS is divalent cation-dependent and ATP-independent. Deletion of the terminal subdomains of PfNapS abolishes its nucleosome assembly capabilities, but the truncated protein retains its ability to bind histones. Both PfNapS and PfNapL show binding to the linker histone H1 suggesting their probable role in extraction of H1 from chromatin fibers. Our data suggests distinct sites of interaction for H1 versus H3/H4 on PfNapS. We show that PfNapS and PfNapL are phosphorylated both *in vivo* and *in vitro* by casein kinase-II, and this modification is specifically inhibited by heparin. Circular dichroism, fluorescence spectroscopy, and chymotrypsin fingerprinting data together suggest that PfNapL may undergo very small and subtle structural changes upon phosphorylation. Specifically, phosphorylation of PfNapL increases its affinity 3-fold for core histones H3, H4, and for the linker histone H1. Finally, we demonstrate that PfNapS is able to extract histones from both phosphorylated and unphosphorylated PfNapL, potentially for histone deposition onto DNA. Based on these results, we suggest that the *P. falciparum* NapL is involved in the nucleocytoplasmic relay of histones, whereas PfNapS is likely to be an integral part of the chromatin assembly motors in the parasite nucleus.

Malaria is a protozoan disease that claims 1–3 million victims every year (2–4). Efforts to eradicate malaria in the most severely affected regions have been largely ineffective, and the disease burden on social and economic development is increasing (2–4). It is important to understand the fundamental biological processes of *Plasmodium falciparum*, as it is a major human scourge. A molecular understanding of essential biological processes may provide avenues for the development of a new generation antimalarials. Chromatin assembly and remodeling are vital cellular processes that are pivotal during various stages like replication, transcription, recombination, and repair in eukaryotic cells.

The major players in this process include multisubunit chromatin remodeling enzymes, histone-modifying enzymes, and histone chaperones (5–9). The latter are acidic molecules that interact with histones and prevent nonspecific aggregation of DNA with histones. Nucleosome assembly protein (Nap), chromatin assembly factor-1 (CAF-1), antisilencing function-1 (Asf1), and histone regulatory protein A (HirA) are histone chaperones that are involved in the deposition of core histones onto DNA (10–13). Nucleosome assembly proteins function along with chromatin-remodeling enzymes such as ACF (ATP utilizing chromatin assembly and remodeling factor) and CHD1 (chromodomain containing protein) to generate uniformly spaced nucleosomes (10, 14, 15). There is increasing evidence that the Nap family proteins function beyond histone deposition onto DNA. Yeast Nap1 exchanges histone H2A-H2B dimers from nucleosomes resulting in chromatin fluidity, which assists processes like transcription (16, 17). Nap1 interacts with proteins like Clb2p, B-type cyclin, and Gin4p kinase, suggesting its role in mitotic progression (18–21). Tobacco Nap1 interacts with tubulin and colocalizes with mitotic spindle suggestive of its role in microtubule dynamics (22, 23). Nap proteins in different organisms have complex patterns of intracellular localization. Human Nap1 is cytoplasmic, whereas human Nap2 and *Drosophila* Nap1 localize to the nucleus specifically in the S phase (10, 24, 25). Yeast Nap1 and a few plant Nap proteins shuttle between the nucleus and cytoplasm (21, 23). Several Nap family proteins get phosphorylated with casein-kinase II, and the phosphorylation status of human Nap-2 changes during the cell cycle (23, 26, 27). Deletion of the Nap gene in *Drosophila* and mouse leads to embryonic lethality (25, 28, 29) suggesting the importance of this protein family in eukaryotic cells.

We have recently described two nucleosome assembly proteins from *P. falciparum*, PfNapS and PfNapL (1). Both of these proteins form complexes with histone tetramer [(H3-H4)₂] as well as histone octamer (formed by two heterodimers of H2A-H2B and a tetramer of histone H3-histone H4) (1). Here, we show that PfNapS and PfNapL, apart from interacting with core histones, also interact with the linker histone H1. Native gels and ELISA⁴-based assays indicate that DNA is able to compete with the interaction of PfNapS and PfNapL with core histones. Plasmid supercoiling assays suggest that PfNapS is able to deposit histones onto DNA, whereas PfNapL is not. This is in agreement with the nuclear localization of PfNapS and the predominantly cytoplasmic positioning of PfNapL (1). Histone deposition by PfNapS requires divalent cations, and the process is ATP-independent. Our current studies indicate that PfNapS is phosphorylated both *in vivo* and *in vitro* by casein kinase-II, and this modification is inhibited by heparin, a specific inhibitor for CK-II. Circular dichroism spectroscopy, tryptophan fluores-

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⁴ The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; CK-II, casein kinase II; DTT, dithiothreitol.

cence studies, and mild proteolysis data suggest that phosphorylation may induce small and subtle structural changes in PfNapL. We show that the histone binding activity PfNapL is enhanced significantly upon phosphorylation. Finally, we provide data to show that PfNapS is able to displace both unphosphorylated and phosphorylated PfNapL from PfNapL-histone complexes. Together, our analysis provides a model of histone relay from cytoplasm to the nucleus in *P. falciparum* based on these two nucleosome assembly proteins.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Proteins—The proteins PfNapS and PfNapL were expressed and purified as described earlier (1). PfNapS-HT (30–220 amino acid residues, HT refers to a construct missing terminal subdomains from 1 to 29 and 221 to 269) was cloned into pET28a, expressed, and purified like full-length PfNapS (1). Calf thymus histones were obtained commercially from Roche and used as described earlier (1, 30). Briefly, a total of 2 mg each of H2A, H2B, H3, and H4 lyophilized calf thymus histones (Roche Diagnostics) were dissolved in unfolding buffer (50 mM Tris-HCl, pH 8.0, 6 M urea, 2 mM EDTA, 10 mM DTT, 2 M NaCl) by rocking for 4 h at 20 °C. The concentration of histones was kept at 1 mg/ml. The unfolded histones were transferred to a 3.5-kDa cut off dialysis bag and dialyzed against refolding buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM DTT, 2 M NaCl, 1 mM phenylmethylsulfonyl fluoride, 5% glycerol) with three changes at 8-h intervals. The contents were concentrated in a centrprep (Millipore) and loaded onto a S200 (Amersham Biosciences) GPC column in buffer containing 50 mM Tris-HCl, pH 8.0, 500 mM NaCl. The histone octamers and tetramers were collected in separate fractions (a chromatogram is provided in the supplemental information). The purified fractions were buffer-exchanged into histone storage buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 500 mM NaCl), stored in 25- μ l aliquots at 1 mg/ml concentration, and transferred to –70 °C.

Plate-based Histone Binding Assays—A total of 100 ng of each of the individual core histones H2A, H2B, H3, H4, and linker histone H1 were coated on an ELISA plate and allowed to interact with 25 ng of PfNapS or PfNapL proteins. The ELISAs were developed as described earlier (1). In assays where phosphorylated PfNapS or PfNapL were used, commercially available casein kinase-II (New England Biolabs) was used to phosphorylate recombinant proteins (see below).

Phosphorylation Assays—A total of 1 μ g of PfNapS was phosphorylated with either commercial casein kinase II (New England Biolabs) or with *P. falciparum* lysate in a buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂ and 5 μ Ci of [γ -³²P]ATP at 30 °C for 1 h. In cases where parasite lysate was used, PfNap proteins were immunoprecipitated from the reactions. The phosphorylated samples were resolved by 12% SDS-polyacrylamide gel electrophoresis and autoradiographed. Heparin (Sigma) was added at a final concentration of 20 ng/ μ l of the reaction mixture for inhibition studies.

Histone Binding Assays Using Native Gel Electrophoresis—Equimolar quantities of PfNapS, PfNapS-HT, or PfNapL were incubated with 1 μ g of either histone tetramer or histone octamer in a buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, and 1 mM DTT at 37 °C for 1 h. A total of 1 μ g of plasmid DNA (pBluescript, 2.9 kb) was included in competition reactions. The complexes so formed were resolved on 7.5% native polyacrylamide gels in 1 \times TAE buffer at room temperature and later stained with Coomassie Brilliant Blue R250.

DNA Supercoiling Assays—The assays were performed according to Ref. 14 with following changes: the plasmid DNA (pBluescript, 2.9 kb) was relaxed by topoisomerase I (Promega) for 1 h. A total of 1 μ g of relaxed plasmid from this reaction (along with topo I) was used for

each supercoiling assay by incubating 1 μ g of PfNapS or PfNapL or PfNapS-HT with 1 μ g of either histone tetramer or octamer in buffer containing 20 mM Tris-HCl, pH 8.0, 50 mM KCl, 10 mM MgCl₂, 1 mM DTT, and 1 mg/ml bovine serum albumin at 37 °C for 1 h. The samples were deproteinized and analyzed on a 5% native polyacrylamide gel in 1 \times TAE buffer. The gel was stained with ethidium bromide and visualized under UV light.

Circular Dichroism and Fluorescence Spectroscopy—Phosphorylated and unphosphorylated PfNapS and PfNapL were buffer exchanged into 10 mM sodium phosphate buffer, pH 8.0. Far UV CD spectra were recorded on Jasco J810 at 25 °C in a quartz cuvette of 0.2-cm path length between wavelengths 190 and 250 nm, at a scan speed of 200 nm/min over three accumulations. CK-II phosphorylated PfNapS and PfNapL were buffer-exchanged into 10 mM sodium phosphate buffer, pH 8.0. The fluorescence measurements were recorded at room temperature in a PerkinElmer LS 50B spectrometer with the excitation slit of 5 nm and emission slit of 10 nm at a scan speed of 900 nm/min. The intrinsic tryptophan fluorescence spectra of phosphorylated and unphosphorylated proteins were recorded from 310 to 500 nm with excitation at 280 nm. Fluorescence intensities were averaged across four scans.

Chymotrypsin Fingerprinting—A total of 100 μ g of PfNapL was phosphorylated using casein kinase II (New England Biolabs) according to published protocols (100- μ l volume). An identical reaction without ATP was also done, and this was used as unphosphorylated PfNapL control in the chymotrypsin digestion experiment. A total of 100 ng of chymotrypsin was added to the 100 μ g of PfNapL and incubated at 37 °C. After the indicated time points, 10 μ l of reaction mixture was taken out from both phosphorylated and unphosphorylated PfNapL tubes, and digestion was stopped by adding protease inhibitor mixture and loading buffer. The samples were boiled and resolved by 15% SDS-PAGE and then stained with Coomassie Brilliant Blue R250.

RESULTS

PfNapS and PfNapL May Occupy the Same Binding Site on Histones—Prior to the study of histone deposition on DNA by PfNapS and PfNapL, we addressed the histone binding properties of PfNapS and PfNapL in the presence of plasmid DNA by native PAGE techniques (Fig. 1). The addition of DNA to PfNapS did not change its mobility on native gels suggesting that PfNapS may not interact with DNA on its own (Fig. 1A, lane 2). The histone tetramers and octamers alone did not enter native gels because of their basic charge character (Fig. 1A, lanes 3 and 5, respectively). Even in the presence of DNA, the histone tetramers and octamers did not enter native gels (Fig. 1A, lanes 4 and 6) because they are known to form higher order aggregates with DNA in the absence of histone chaperones as reported in previous studies (6). However, the complexes of PfNapS interacted with both histone tetramer and octamer and migrated on native gels like a specific macromolecular assembly (Fig. 1A, lanes 7 and 9). Further, DNA could compete this interaction suggesting that histones have higher affinity for DNA than PfNapS (Fig. 1A, lanes 8 and 9). PfNapS-HT behaved similarly to full-length PfNapS in its interaction with the histone octamer (Fig. 1B). The protein PfNapL also formed complexes with the histone tetramer and histone octamer, and again DNA could compete this interaction (Fig. 1C). We then incubated PfNapS and PfNapL together and compared their interaction with histones (Fig. 1D). In the absence of histones, PfNapS and PfNapL retained their individual mobility and did not form a complex with each other (Fig. 1D, compare lane 7 with lanes 1 and 4). In the presence of histone tetramer or octamer, most of the PfNapS was bound to histones, whereas there was negligible binding seen with PfNapL. These data indicate that PfNapS has a higher affinity for histones as compared with

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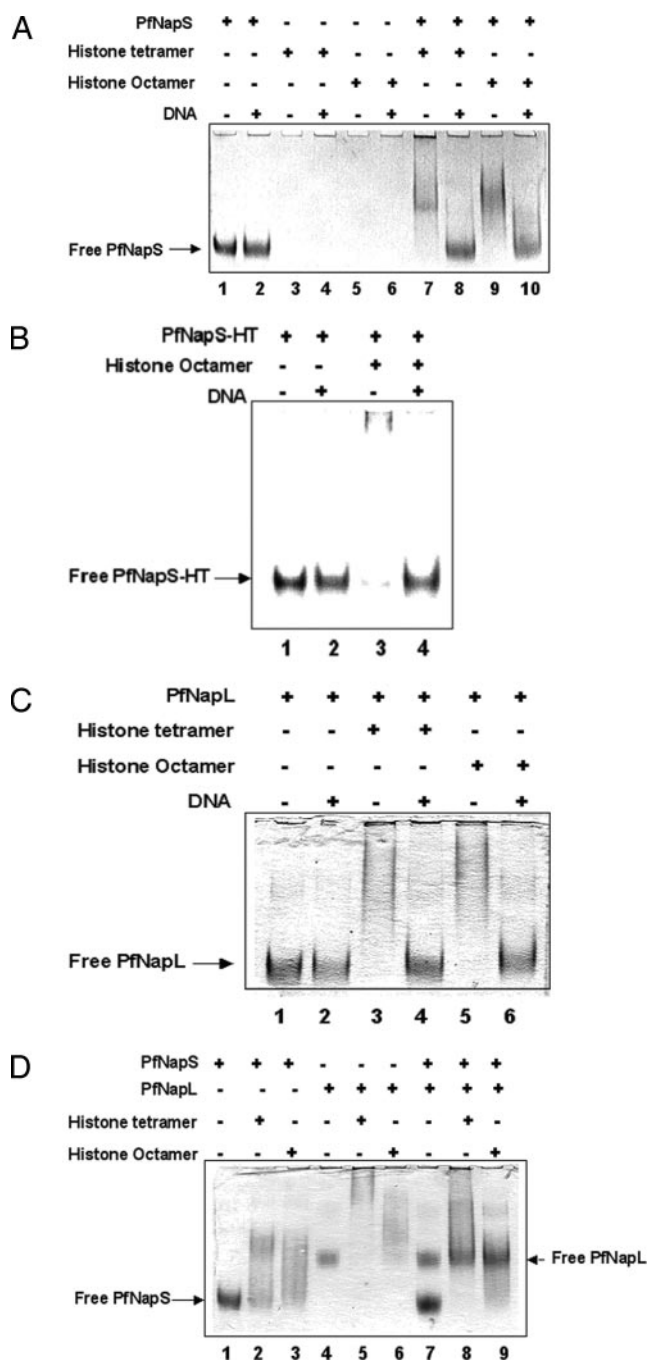


FIGURE 1. Effect of DNA on the interaction of PfNapS and PfNapL with histones. Equimolar quantities of PfNapS (A), PfNapS-HT (B), PfNapL (C), and PfNapS with PfNapL (D) were incubated with 1 μ g of either histone tetramer or histone octamer, either in the presence or absence of 1 μ g of plasmid DNA (as indicated). The complexes were resolved on a 7.5% native PAGE and stained with Coomassie Brilliant Blue R250.

PfNapL and suggest that both PfNapS/PfNapL are likely to occupy the same binding site on histones.

The Terminal Subdomains of PfNapS Are Required for Both Proper Release of Histone Cargo on DNA and for Nucleosome Assembly—DNA supercoiling is a standard assay to study histone deposition onto plasmid DNA (14). For further dissection of histone-Nap-DNA interactions, we incubated DNA with histones and PfNap proteins, deproteinized the samples, and analyzed the migration of the complexes on ethidium bromide-stained native polyacrylamide gels (Fig. 2A). Neither PfNapS nor the histone octamer alone induced supercoiling in DNA,

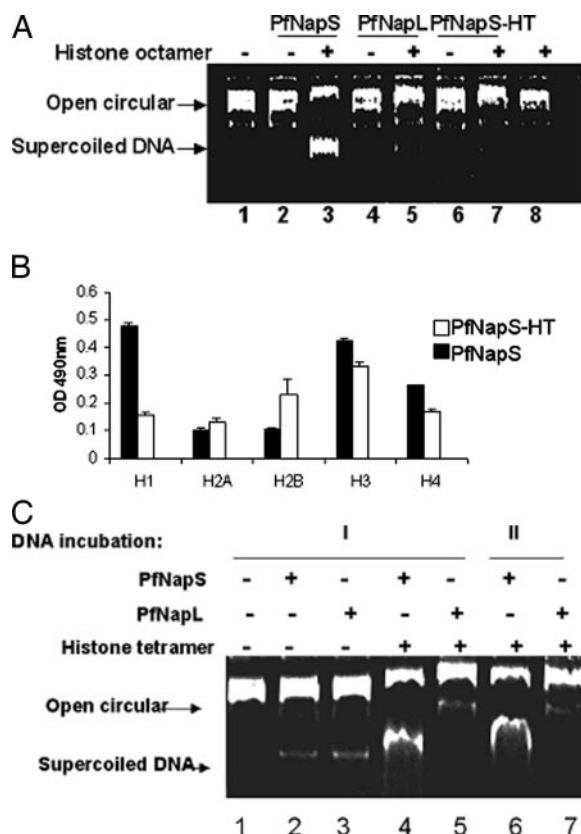


FIGURE 2. DNA supercoiling assay to study histone deposition on DNA by *P. falciparum* Nap proteins. A, PfNapS, PfNapL or PfNapS-HT (1 μ g) was incubated at 37 $^{\circ}$ C with 1 μ g of plasmid DNA (pBSK, 2.9 kb, previously relaxed by topoisomerase I) and 1 μ g of histone octamer (as indicated in the figure). The samples were then deproteinized and analyzed on a 5% native PAGE, and the DNA was visualized by ethidium bromide staining. B, 100 ng of individual core histones, H2A, H2B, H3, H4, and linker histone H1 were coated on an ELISA plate and allowed to interact with 25 ng of PfNapS or PfNapS-HT. The binding of PfNapS (black bars) and PfNapS-HT (white bars) with histones is shown. C, PfNapS or PfNapL (1 μ g) was incubated with 1 μ g of histone tetramer and 1 μ g of plasmid DNA (pBSK previously relaxed by topoisomerase I) at 37 $^{\circ}$ C for 1 h (DNA incubation-I, lanes 1–5) or DNA was added after 30 min of PfNapS/PfNapL incubation with histone tetramer (DNA incubation-II, lanes 6 and 7).

and DNA in presence of either of these migrated mostly in open circular form. However, in the presence of PfNapS and histone octamer, relaxed DNA was converted to supercoiled forms suggesting deposition of histone octamers onto DNA (Fig. 2A, lane 3). Deletion of the head-(1–29) and acidic-(221–269) subdomains from PfNapS (PfNapS-HT) abolished its histone deposition property (Fig. 2A, lane 7). We then tested whether the histone deposition activity was lost by PfNapS-HT because of its inability to interact with histones. We did not find any significant difference between PfNapS and PfNapS-HT in their interaction with core histones, but PfNapS-HT binding to linker histone H1 was reduced by 3-fold (Fig. 2B). These data suggest that the terminal subdomains of PfNapS are required for the proper release of core histones onto DNA. In contrast to PfNapS, PfNapL was unable to deposit histones in identical assays (Fig. 2A, lane 5).

Because PfNapS has higher affinity for histones H3 and H4 than for H2A and H2B, we tested whether PfNapS could deposit histone tetramers onto DNA and facilitate subnucleosome assembly as well, using the same plasmid supercoiling assays (Fig. 2C). Once again, PfNapS was able to deposit histone tetramer onto DNA (Fig. 2C, lane 4), whereas PfNapL was not (lane 5). We also tested whether PfNapL could stimulate or cooperate with PfNapS in histone deposition. The addition of PfNapL did not change the extent of DNA supercoiling induced by PfNapS alone (data not shown). In earlier studies on histone deposition by other

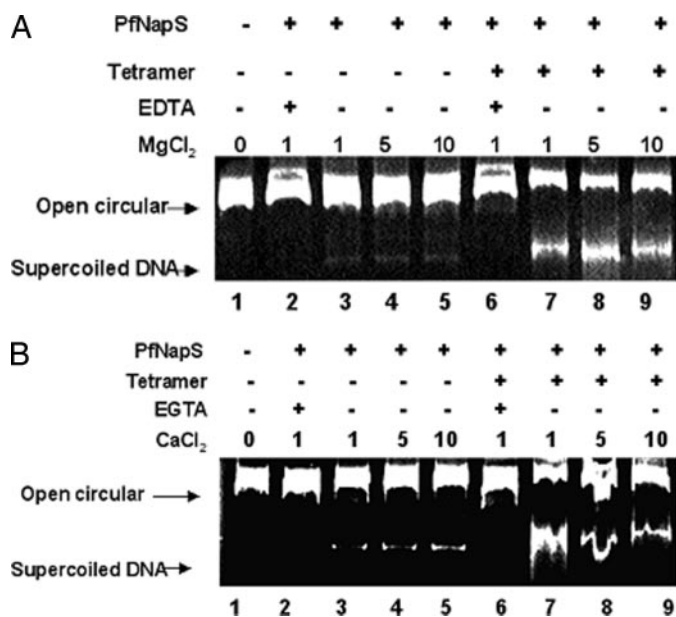


FIGURE 3. Effect of divalent cations on histone deposition activity of PfNapS. PfNapS (1 μ g) was incubated with 1 μ g of plasmid DNA (previously relaxed with topoisomerase I) and 1 μ g of histone tetramer in buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, and varying amounts of MgCl₂, EDTA (A) and CaCl₂, EGTA (B) (as indicated) at 37 °C for 1 h. The samples were deproteinized and subjected to 5% native PAGE and visualized by ethidium bromide staining.

eukaryotic Nap proteins (14), Nap proteins were first preincubated with histones for 30 min, and later DNA was added. We tested the above method, as well as simultaneous addition of all three components (Nap proteins, histones, and DNA) in the supercoiling assays (Fig. 2C). We did not observe any significant difference in the results from these alternative methods.

PfNapS Requires Divalent Cations for Histone Deposition onto DNA—The concentrations of Mg²⁺ and Ca²⁺ in the nucleoplasm are in the range of 2–4 mM and 4–6 mM, respectively (32, 33). We tested whether PfNapS-mediated histone deposition was dependent on divalent cations (Fig. 3). The addition of 1 mM EDTA completely abolished the histone deposition by PfNapS (Fig. 3A, lane 6), whereas optimal activity was observed in the presence of 1–10 mM MgCl₂. Similarly, EGTA abrogated histone deposition by PfNapS (Fig. 3B), and the activity was optimal in 1–10 mM CaCl₂. It is likely that the acidic nature histone chaperones provides multiple divalent coordination sites where Mg²⁺ and Ca²⁺ may be accommodated.

Phosphorylation of PfNapS by Casein Kinase II and with *P. falciparum* Extracts—Many of the eukaryotic Nap proteins as well as PfNapL get phosphorylated with casein kinase-II (1, 23, 26, 27). *P. falciparum* extract was obtained from mixed asexual stages of the parasite and used for phosphorylation of PfNapS (Fig. 4, lane 2). To test whether PfNapS is a target of CK-II, we used commercial recombinant enzyme in kinase assays (Fig. 4, lanes 3 and 4). Heparin, a specific inhibitor for CK-II, inhibited PfNapS phosphorylation by both parasite lysate as well as by recombinant CK-II (Fig. 4, lanes 1 and 3, respectively).

Effect of Phosphorylation on PfNapS and PfNapL Conformations—Circular dichroism spectroscopy studies were performed to study the secondary structural changes in PfNapL and PfNapS upon phosphorylation (Fig. 5). Characteristic spectra of a protein containing both α helical and β sheet structures were obtained for both PfNapL and PfNapS. Circular dichroism spectra of unphosphorylated and phosphorylated PfNapL/PfNapS suggested the possibility of very small and subtle conformational changes in the structures of these proteins upon

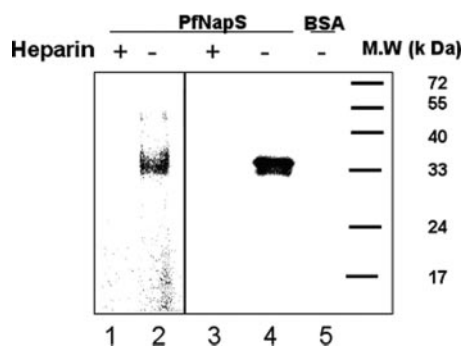


FIGURE 4. Phosphorylation of PfNapS by *P. falciparum* extract and casein kinase II. 1 μ g of PfNapS was phosphorylated with *P. falciparum* extract (lanes 1 and 2) or commercial casein kinase II (lanes 3 and 4) either in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of heparin (20 ng/ μ l). 1 μ g of bovine serum albumin (BSA) was used instead of PfNapS as a control protein in the phosphorylation reaction (lane 5).

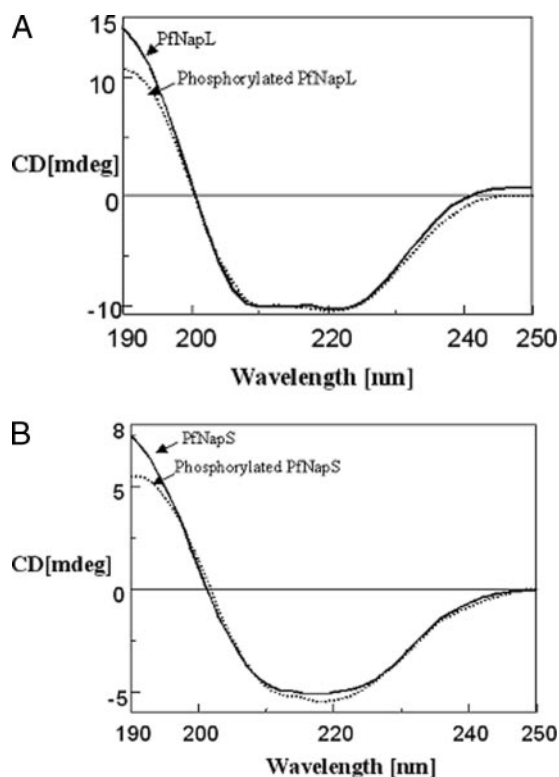


FIGURE 5. Far UV circular dichroism spectra of phosphorylated and unphosphorylated PfNapL (A) and PfNapS (B). PfNapL and PfNapS were phosphorylated with casein kinase II and buffer exchanged with 10 mM sodium phosphate buffer (pH 8.0). Far UV CD spectra of phosphorylated and unphosphorylated proteins were recorded between wavelengths 250 and 190 nm. Spectra taken are the average of three scans and were subtracted from the buffer spectra. The spectra in **bold** are that of unphosphorylated proteins, and the spectra with **dotted lines** are of phosphorylated proteins.

phosphorylation (Fig. 5). We also measured tryptophan fluorescence of these proteins in phosphorylated and unphosphorylated states. Fluorescence was quenched in the case of phosphorylated PfNapL, suggesting the burial of the tryptophans Trp-103 and/or Trp-190 upon phosphorylation. PfNapS also showed fluorescence quenching upon phosphorylation (Fig. 6). PfNapS has five tryptophan residues, Trp-80, Trp-159, Trp-183, Trp-188, and Trp-209. Fluorescence measurements indicated emission maxima at 370 nm for this protein. Residues Trp-80 and Trp-159 are the most conserved tryptophans in PfNapS-like family of proteins, and these residues align with Trp-103 and Trp-190 of PfNapL, respectively. Overall, these data suggested the possibility of small and subtle conformational changes in PfNapL and PfNapS.

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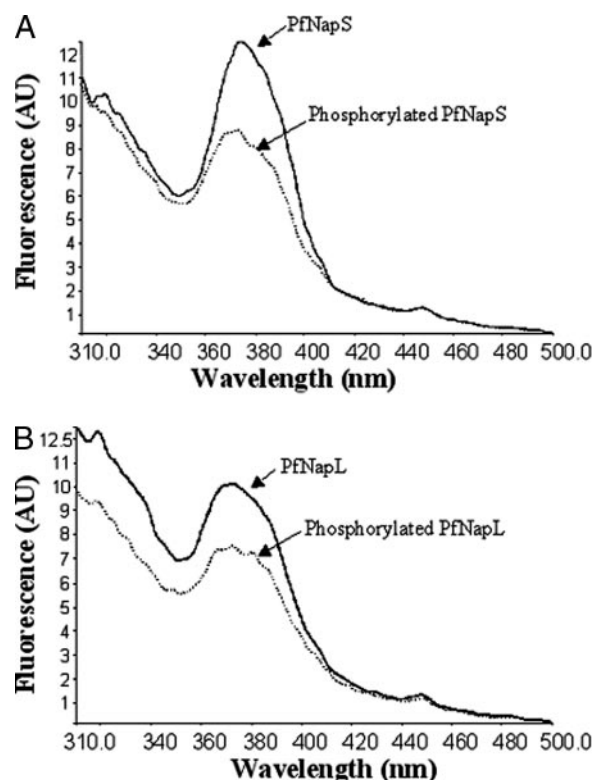


FIGURE 6. Tryptophan fluorescence spectra of phosphorylated and unphosphorylated PfNapS (A) and PfNapL (B). Both PfNapS and PfNapL were phosphorylated with casein kinase II. The intrinsic tryptophan fluorescence spectra of phosphorylated and unphosphorylated proteins were recorded from 310 to 500 nm with excitation wavelength at 280 nm. Fluorescence intensities are the average of four scans. The spectra in *bold* are that of unphosphorylated proteins, and the spectra with the *dotted line* are of phosphorylated proteins.

Effect of Phosphorylation on the Interaction of PfNapL and PfNapS with Histones—Because both PfNapL and PfNapS can be phosphorylated by casein kinase II, we studied whether this modification had any effect on the histone binding and histone deposition properties of these proteins (Fig. 7). Although both PfNapS and PfNapL bound to the linker histone H1 with specificity, PfNapS binding to H1 was 3-fold higher than the PfNapL-H1 interaction. However, upon phosphorylation, PfNapL binding to H1, H3, and H4 increased 3–4-fold, whereas there was no significant difference in its binding to H2A and H2B (Fig. 7A). Because phosphorylated PfNapL showed increased binding with histone H3 and H4, we tested whether it was also capable of depositing histone tetramer and histone octamer onto DNA. Supercoiling assays indicated that phosphorylated PfNapL was still unable to deposit histones on DNA (data not shown). This suggested that the phosphorylation of PfNapL controlled only its histone binding affinity. The phosphorylation on PfNapS, however, did not have any observable effect on its interaction with histones or on its ability to mediate histone deposition (Fig. 7B). Given that phosphorylation enhances the PfNapL-histone interaction, we decided to probe for the conformational changes induced upon phosphorylation by mild proteolysis. The chymotrypsin digestion of phosphorylated and unphosphorylated PfNapL produced several polymorphic bands (Fig. 7C) suggesting the likelihood of small and subtle structural changes. As shown in Fig. 7C, *bands 1 and 2* are protected after phosphorylation. There is also a partial protection of *band 3* at all of the time points considered. *Bands 4 and 5* appear to be protected in the unphosphorylated PfNapL state. Overall, chymotrypsin fingerprinting data hint at very small and subtle conformational changes

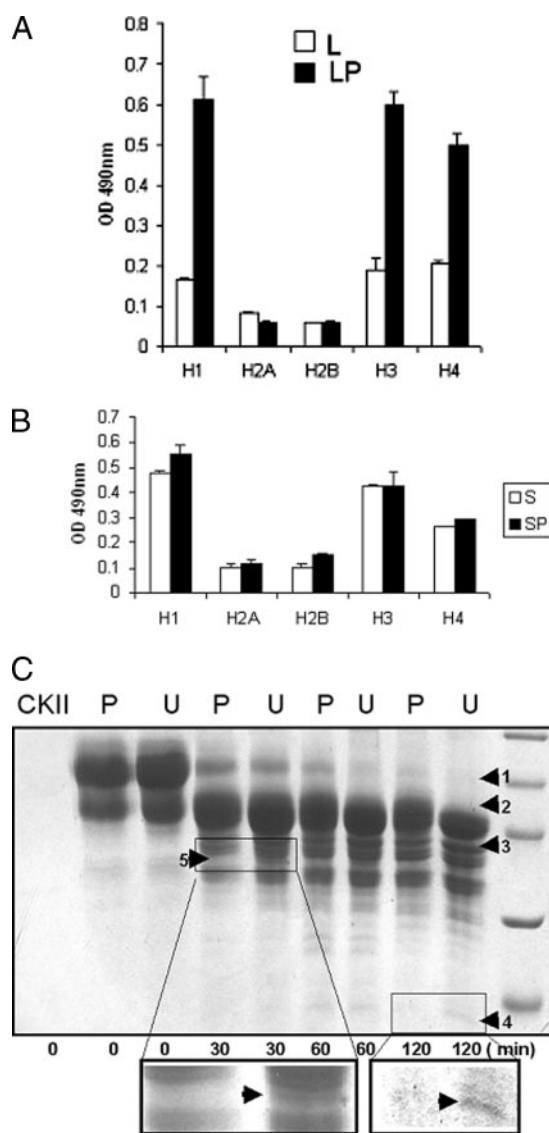


FIGURE 7. Effect of phosphorylation on PfNapS and PfNapL interaction with histones. 100 ng of individual core histones, H2A, H2B, H3, H4, and linker histone H1 were coated on an ELISA plate and allowed to interact with 25 ng of phosphorylated and unphosphorylated PfNapS or PfNapL. The binding of phosphorylated and unphosphorylated PfNapL (A) and PfNapS (B) are shown. *White bars* represent unphosphorylated protein, and *black bars* represent phosphorylated samples. C, the fingerprint of PfNapL digestion with chymotrypsin in both phosphorylated (P) and unphosphorylated (U) states. This digestion was performed for indicated time points (in minutes) in identical sets for phosphorylated and unphosphorylated PfNapL, as indicated under “Materials and Methods.” The *arrows* show polymorphic bands in these samples. The lane indicating CK-II is casein kinase II control along with chymotrypsin only. Proteolytic *bands 4 and 5* are magnified for clarity.

in PfNapL upon phosphorylation. These changes may lead to a 3-fold enhanced PfNapL-histone interaction.

PfNapS Replaces PfNapL on Histones—In our earlier experiments (Fig. 1D), we had demonstrated that when both PfNapS and PfNapL were present together, PfNapS had a higher affinity for histones than PfNapL, as judged by migrations of complexes on native gels. The proteins PfNapS and PfNapL did not interact with each other, as determined both by ELISA-based plate assays and native gel shifts. We addressed whether PfNapS was able to extract histones already bound to PfNapL (Fig. 8). When both PfNapS and PfNapL were incubated with histone octamers, most of the PfNapS was bound to histones, whereas there was negligible binding with PfNapL (Fig. 8, *lane 2*). Even when the histone octamer had been incubated with PfNapL (phosphorylated or

unphosphorylated) 30 min prior to PfNapS addition, most of PfNapS was bound to histones, leaving PfNapL free (Fig. 8, lanes 3 and 4). We then incubated phosphorylated PfNapL with PfNapS and allowed them to interact with core histones. This competition of PfNapS for histones was seen even in the presence of phosphorylated PfNapL (Fig. 8, lane 4). The same effect was observed when phosphorylated PfNapL was incubated earlier with core histones before the addition of PfNapS (Fig. 8, lane 5). Therefore, these data together suggest that PfNapS was able to extract the histone oligomers from preformed histone-PfNapL complexes.

DISCUSSION

We have analyzed in considerable detail the biochemical functions of the two nucleosome assembly proteins from *P. falciparum*. Our studies indicate that these proteins play distinct roles for the parasite. In addition to their previously characterized interactions with individual and oligomeric histones (1), in this study we demonstrate that PfNapS and PfNapL bind to the linker histone H1 as well. In *Xenopus* and HeLa cells, the H1-Nap1 complexes are integral for extraction of H1 from chromatin fibers, which results in an extended and transcriptionally active chromatin conformation (34, 35). Given the nuclear association of PfNapS (1), this protein is likely to have the ability to modulate gene expression like other H1 interacting histone chaperones (29). The terminal subdomains of PfNapS appear to make critical contacts with H1

histone, as PfNap-HT bound to H1 with lesser affinity. The deletion of terminal subdomains of PfNapS, however, does not alter its binding to H3 or H4 histones, suggesting distinct sites of interaction for H1 *versus* H3/H4 on PfNapS.

The protein PfNapS is able to deposit both histone tetramer and histone octamer on DNA, whereas PfNapL does not possess this activity. This property of PfNapS is consistent with its nuclear association in both asexual and sexual stages of the parasite life cycle, whereas PfNapL is predominantly cytoplasmic (1). The deletion of PfNapS terminal subdomains does not effect its interaction with core histones but reduces its interaction with linker histone H1 and abolishes its ability to deposit core histones onto DNA. This suggests that the terminal subdomains of PfNapS are involved in the proper release of the core histones onto DNA. We propose that the acidic PfNapS C-terminal subdomain might assist in the ordered release of histones, which otherwise aggregate on DNA, by altering the histone release dynamics on DNA. Although PfNapS/PfNapL do not interact with each other, they do compete for histones, suggesting that same surfaces on histones maybe involved in binding to these proteins. This competition between PfNapS and PfNapL is likely to be determined by the relative strengths of Nap-histone interactions rather than by a direct cross-talk between PfNapS and PfNapL.

Like other eukaryotic Nap proteins (23, 26, 27), the two *P. falciparum* Nap proteins are phosphorylated with casein kinase II. Similar to the case of human Nap2 (27), the phosphorylation of PfNapS does not change its interaction with histones or its histone deposition activity. Casein kinase II-mediated phosphorylation is known to change the conformation of some proteins (36, 37). Our data suggest the possibility of a very small and subtle structural change in PfNapL upon phosphorylation. The PfNapL residue Trp-103 is conserved in nucleosome assembly proteins from various organisms, whereas Trp-190 is very close to threonine 186, a putative casein kinase II phosphorylation site. Histone binding assays indicate that phosphorylated PfNapL has higher affinity for histones H1, H3, and H4 explaining the role of phosphorylation. Our experiments suggest that CK-II-mediated phosphorylation up-regulates PfNapL affinity for histones but does not impart histone deposition ability. However, the 3-fold increase in histone binding of PfNapL after phosphorylation has little effect on histone transfer dynamics. PfNapS precluded the binding of histones to PfNapL and could compete for

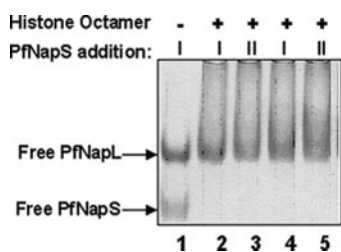


FIGURE 8. Competition of PfNapS with phosphorylated and unphosphorylated PfNapL for histones. Equimolar quantities of PfNapS and PfNapL were incubated with 1 μ g of histone octamer, and the complexes were resolved on a 7.5% native PAGE and stained with Coomassie Brilliant Blue R250. PfNapS addition I (lanes 1, 2, and 4) indicates that PfNapS was incubated along with PfNapL and histones. PfNapS addition II (lanes 3 and 5) indicates that PfNapS was added 30 min after the incubation of PfNapL and histones. PfNapL was unphosphorylated in lanes 1–3 and phosphorylated in lanes 4 and 5.

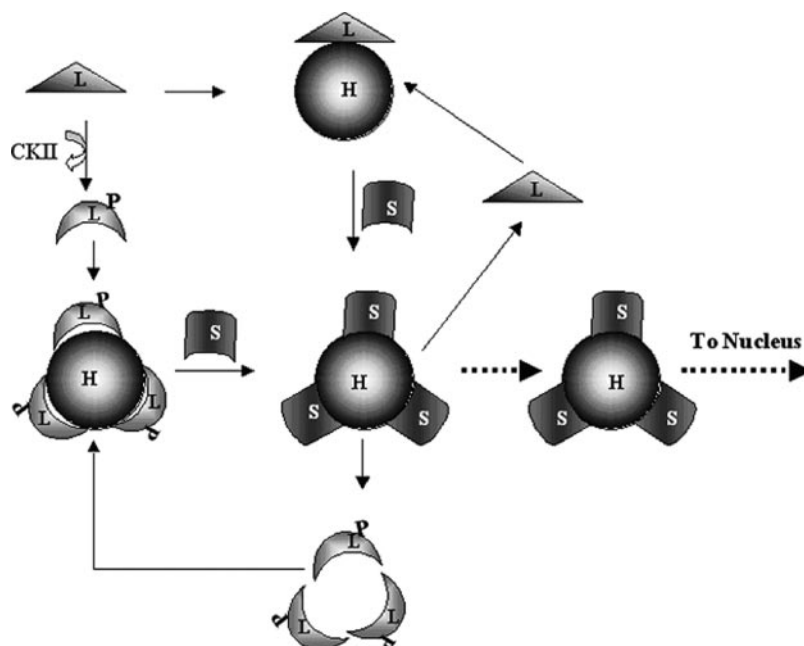


FIGURE 9. Model for *in vitro* relay of histones by *P. falciparum* nucleosome assembly proteins. PfNapL (L) binds to histones poorly but can deliver histones to PfNapS (S, phosphorylated or unphosphorylated) readily. PfNapL upon phosphorylation (L^P) by CK-II may undergo a very small conformational change and thereby bind 3-fold better to the histones, when compared with the unphosphorylated form of PfNapL. Phospho-PfNapL can then present histone cargo more efficiently to PfNapS. PfNapS hijacks histones from Phospho-PfNapL/histone complexes and shuttles the histones into the nucleus. Free PfNapL are recycled for subsequent relay of histones. The presented model does not imply any specific stoichiometry of the components.

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histones already bound to both phosphorylated and unphosphorylated PfNapL, indicating its higher affinity for histones and its ability to extract the same from PfNapL (Fig. 8). PfNapS is able to disrupt the preformed PfNapL-histone complex, highlighting a potential relay of histones between PfNapL, PfNapS, and DNA. This idea is further strengthened by the fact that PfNapL is predominantly cytoplasmic, and PfNapS is essentially associated with the nucleus (1). Taken together, this work provides evidence for a cellular pathway that the histone cargo can take in *P. falciparum*, from cytoplasm to the nucleus. We propose a model for the relay of histones by *P. falciparum* Nap proteins to summarize our results (Fig. 9). The cytoplasmic PfNapL binds to histones poorly in an unphosphorylated state, but CK-II phosphorylates PfNapL and increases the affinity of the PfNapL-histone interaction. This PfNapL-histone complex is readily broken down in the presence of PfNapS. The histone cargo is therefore offloaded from PfNapL to PfNapS by virtue of the higher affinity for histones displayed by PfNapS, which can then shuttle the PfNapS-histone complex to the nucleus. The translocation of this complex to the nucleus will then enable the deposition of histones onto DNA during the nucleosome assembly process. This would be followed by release of free PfNapS for subsequent cycling.

Our observations highlighting the role of PfNapS in nucleosome assembly are further strengthened by the recent protein-protein interaction studies in *P. falciparum*, which show PfNapS as the part of chromatin remodeling complex and in direct interaction with ISWI (38), a protein that regulates transcription and chromatin assembly (39). ISWI is an ATPase and is part of multiple remodeling machines like NURF (nucleosome remodeling factor), ACF (ATP utilizing chromatin assembly and remodeling factor), and CHRAC (chromatin accessibility complex) in higher eukaryotes (40, 41). Nap proteins from other eukaryotes are shown to act in concert with ATP-utilizing chromatin assembly and remodeling factors (10, 14, 15). Together, our analysis suggests that PfNapS-mediated histone deposition appears to be functioning along with the ATPases such as ISWI to produce uniformly spaced nucleosomes. It is to be noted that the yeast two-hybrid studies indicate that PfNapL is not part of the chromatin-remodeling complex, but instead it interacts with nine ribosomal proteins (42). This is also consistent with our findings, which demonstrate that PfNapL cannot deposit histones on DNA, but it can interact with both core histones and linker histone H1. It is likely that PfNapL might be involved in binding to nascent chains of histones that come off the ribosomes and in their subsequent transfer to PfNapS.

The occurrence of PXXP motifs in both PfNapS and PfNapL offers the opportunity for protein-protein interactions involving these two chaperones and components of chromatin/signaling networks (43). Further, the occurrence of acidic residue homorepeats in PfNap proteins seems to be functionally relevant to the histone binding dynamics and in this case may not reflect the amino acid composition peculiarities of *P. falciparum* proteins (31). In summary, our data suggest a distinct and non-overlapping functional roles for *P. falciparum* NapS and NapL proteins in chromatin assembly and histone transport, respectively. The elucidation of additional protein motors involved in chromatin organization is necessary for greater understanding of the cellular networks that maintain chromatin structure within the parasite.

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