DNA organization by the apicoplast-targeted bacterial histone-like protein of *Plasmodium falciparum*

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Received March 4, 2008; Revised July 10, 2008; Accepted July 11, 2008

**ABSTRACT**

Apicomplexans, including the pathogens *Plasmodium* and *Toxoplasma*, carry a nonphotosynthetic plastid of secondary endosymbiotic origin called the apicoplast. The *P. falciparum* apicoplast contains a 35 kb, circular DNA genome with limited coding capacity that lacks genes encoding proteins for DNA organization and replication. We report identification of a nuclear-encoded bacterial histone-like protein (PfHU) involved in DNA compaction in the apicoplast. PfHU is associated with apicoplast DNA and is expressed throughout the parasite’s intra-erythrocytic cycle. The protein binds DNA in a sequence nonspecific manner with a minimum binding site length of ~27 bp and a $K_d$ of ~63 nM and displays a preference for supercoiled DNA. PfHU is capable of condensing *Escherichia coli* nucleoids in vivo indicating its role in DNA compaction. The unique 42 aa C-terminal extension of PfHU influences its DNA condensation properties. In contrast to bacterial HUs that bend DNA, PfHU promotes concatenation of linear DNA and inhibits DNA circularization. Atomic Force Microscopic study of PfHU–DNA complexes shows protein concentration-dependent DNA stiffening, intermolecular bundling and formation of DNA bridges followed by assembly of condensed DNA networks. Our results provide the first functional characterization of an apicomplexan HU protein and provide additional evidence for red algal ancestry of the apicoplast.

**INTRODUCTION**

The apicoplast is the nonphotosynthetic plastid of apicomplexan parasites that include the genera *Plasmodium* and *Toxoplasma* and is believed to have arisen from a secondary endosymbiotic event involving an ancestral protist and an alga (1). The apicoplast is essential for parasite survival; it is the site for Type II fatty acid biosynthesis, nonmevalonate pathway of isoprenoid biosynthesis, and synthesis of heme intermediates within the parasite (2,3). Biochemical pathways operative within this organelle provide novel sites for drug intervention against malaria. Due to its essentially prokaryotic nature the processes of DNA replication, transcription and translation within the apicoplast are also validated drug targets (4–6).

Each sporozoan cell of *P. falciparum* carries a single apicoplast with apicoplast DNA (plDNA) copy number estimates varying from 1 to 15 (7,8). The ~35 kb, A+T-rich, circular double-stranded plDNA molecules of *P. falciparum* replicate via the D-loop/bi-directional ori mechanism at the late trophozoite-early schizont stage of the intraerythrocytic cycle. PlDNA replication origins (ori) localize within the inverted repeat (IR) region of the plDNA molecule (9–11). Although there is extensive sequence similarity between plDNA of *P. falciparum* and *T. gondii* they have distinct in vivo topology. The former is circular while the latter occurs as an oligomeric series of linear tandem arrays of the 35 kb genome. Light microscopy studies on *T. gondii* apicoplast genome have suggested that it is organized into a nucleoid that segregates into two equal portions during apicoplast division (8,12). The fact that a single plDNA circle is ~12 μm in circumference and several molecules have to be packed into an organelle with a diameter of only ~0.3 μm (8) as well as replicate and divide into daughter molecules without getting tangled is indicative of the involvement of a DNA-compacting protein in plDNA organization.

The apicoplast genome primarily encodes components of the transcription and translation machinery of the organelle (3,6) but lacks genes encoding any DNA organization or replication protein. Thus, all major protein...
components involved in pIDNA organization/replication
must be nuclear-encoded and imported into the organelle.
There is accumulating evidence on the components of
the pIDNA replication machinery. A ~220 kDa multi-domain
polypeptide (PipREX) that contains DNA polymerase as
well as DNA primase, DNA helicase and 3’–5’ exonu-
clease activities (13) has been identified as a key enzyme
for pIDNA replication. Genes encoding putative apico-
plast-targeted glyrase A and B have also been identified
on Chr12 of P. falciparum and glyrase B has been function-
ally characterized (14,15). Ciprofloxacin and novobiocin,
that target bacterial DNA glyase, specifically inhibit repli-
cation of P. falciparum pIDNA and also reduce parasite
growth in culture (4,15) thus validating malarial apico-
plast DNA replication as a drug target.

The dearth of information on proteins involved in orga-
nization of the P. falciparum apicoplast genome prompted
us to investigate putative candidates from the parasite
genome database. The prokaryotic nature and putative
red algal origin of the apicoplast suggested the possible
involvement of a histone-like protein (‘heat unstable’
or HU) (16) that is the primary organizational component
of bacterial nucleoids, dinoflagellate chromosomes as well
as red algal chloroplast genomes (17–19). A gene encoding
a HU-ortholog that carries a conserved BHL domain
(bacterial histone-like domain) together with a predicted
N-terminal apicoplast targeting sequence was identified on
Chr.9 of the P. falciparum nuclear genome. HU proteins
are small basic proteins of prokaryotic origin that are
structurally distinct from eukaryotic histones (20),
belong to the DNABII family of DNA-binding proteins,
and exhibit hetero- or homo-dimeric DNA binding. HU
proteins also have regulatory effects on DNA replication,
recombination and transcription (21–25). In bacteria,
HU proteins together with the structurally related IHF
(integration host factor), organize chromosomal DNA
into periodic nucleosome-like structural units (26).
Assuming an even distribution across the chromosome,
in vivo concentrations of these proteins in Escherichia
coli (27) provide for binding of one dimer only approxi-
ately every 125 bp, indicating that filament formation
by HU could occur only locally (28). The presence of
10 other DNA-organization proteins such as histone-
like nucleoid structuring protein (H-NS) and factor for
inversion stimulation (Fis) that are also involved in
DNA organization in E. coli (27) and their sequence
preferences and co-operativity in binding DNA would
further influence filament formation and condensation
of E. coli DNA.

We report characterization of the P. falciparum
nuclear-encoded bacterial histone-like protein (PfHU)
and its interaction with apicoplast DNA. PfHU is
expressed throughout the intra-erythrocytic phase of para-
site growth, exhibits DNA binding and is capable of
condensing DNA. The unique C-terminal domain of
PfHU influences its interaction with DNA. Atomic
Force Microscopic analysis shows that the protein is
capable of forming both DNA bridges and bundles.

MATERIALS AND METHODS

Parasite culture
P. falciparum (strains 3D7 and NF54) was cultured in
human RBCs maintained in RPMI-1640 supplemented
with 0.5% AlbumaxII (Invitrogen). The parasites were
synchronized with sorbitol (29).

Cloning, expression and purification of recombinant PfHU
The gene encoding the predicted 22.5 kDa P. falciparum
bacterial histone-like protein (PlasmoDB ID PFI0230c)
was amplified by PCR. DNA encoding unprocessed
PfHU (PfHUup) (1–189 aa) was amplified using upstream (HU-f:
5’-CGCGATCATGTAATATTATTTTG-3’
TT-3’) and downstream (HU-r: 5’-CGCGTCGACATA
ATACCTTTTACATTTTTC-3’) primers carrying BamHI and Sall sites, respectively with P. falciparum
cDNA as template. The sequence encoding predicted
processed PfHU (PfHU) (54–189 aa) was amplified using the
forward primer HU-p (5’-CGGGATCATGTCGACATA
GCGATTACAAAAG-3’) and HU-r as the reverse primer.
Processed PfHU lacking 41 amino acids of the
C-terminal end (PfHU) was amplified using HU-p as
the forward primer and ΔHU-r (5’-CGCGTCGACCTT
ATTAACCTTTGAAAACCTTTG-3’) as the reverse primer.
The fragments were cloned into pQE30 (Qiagen,
USA) and sequenced to confirm their identity. Soluble
recombinant PfHUup, PfHU and PfHUΔC proteins
carrying the N-terminal RGS-6XHis tag were obtained after
E. coli JM109 cells co-transformed with the RIG plasmid
(gifted by Prof. W.G.J. Hol) were induced with 0.1 mM
IPTG at 20°C for 18 h. The RIG plasmid carries tRNA
genes whose transcripts recognize rare codons for the
amino acids R, I and G in P. falciparum DNA expressed in
E. coli (30). The recombinant proteins were purified
on Ni-NTA Superflow (Qiagen, USA) and dialysed in
a buffer containing 50 mM Tris–HCl (pH 7.6), 200 mM
NaCl. PfHU and PfHUΔC were further purified on a
SP sepharose column. Final concentration of purified protein
was determined by BCA assay. Far UV CD spectra
analysis of purified PfHU showed the presence of both
α-helices and β-sheets and was comparable to other HU
proteins thus indicating correct folding (data not shown).
Chemical-crosslinking of PfHU was carried out with
0.1% glutaraldehyde in 10 mM NaH2PO4 and 50 mM
NaCl for 30 min at 37°C.

The gene encoding Bacillus subtilis HB (HSu), was
PCR-amplified from B. subtilis genomic DNA (upstream
primer, 5’-CGGGATCATGTAATATTATTTTG-3’
and downstream primer, 5’-CGGCTGACATA
TTTTTCCGCACTGCTTTTAAG-3’) and cloned in
the pQE30 expression vector. The ~12 kDa recom-
binant protein carrying a N-terminal His-tag was purified
by Ni-NTA chromatography and used as positive control
in DNA circularization assays.
Antibodies against PfHU<sub>p</sub> were raised in rabbits and mice using purified PfHU<sub>p</sub>. The titer of the raised antiserum was determined by ELISA.

For preparation of total parasite lysate, parasites were released by 0.05% saponin lysis, washed with PBS, and suspended in 1x SDS loading buffer containing protease inhibitors (Protease Arrest, GBiosciences, USA). After brief sonication, the cell lysate was separated on a 15% SDS–polyacylamide gel. Western blotting was carried out (6) and the blot was developed using a chemiluminescent system (Amersham Biosciences, UK).

For immunoprecipitation, parasite cultures at 6–8% parasitaemia were harvested when cells were predominantly at the late trophozoite stage. Cells were washed with PBS and parasites were released by 0.05% saponin lysis. The parasite pellet was washed with PBS and lysed in chilled immunoprecipitation (IP) buffer (30 mM Tris–HCl pH 8.0, 300 mM NaCl, 1 mM EDTA, 1% v/v Triton X-100, 1% v/v Igepal and protease inhibitor cocktail) on ice for 30 min. After brief sonication, the lysate was centrifuged at 12,500 r.p.m. for 10 min. The supernatant was ice for 30 min. After brief sonication, the lysate was centrifuged at 12,500 r.p.m. for 10 min. The supernatant was precleared by addition of 3 mg Protein A sepharose CL-4B and centrifuged at 10,000 r.p.m. for 2 min at 4°C. After centrifugation, the cell lysate was separated on a 15% gel and transferred onto a nitrocellulose membrane. The membrane was probed with mouse antibody (rabbit anti-PfHUp serum) for 2 h on ice precleared by addition of 3 mg Protein A sepharose CL-4B and centrifuged at 12,500 r.p.m. for 10 min. The supernatant was incubated with 5 mg Protein A sepharose at 4°C overnight with 5 mg Protein A sepharose at 4°C with concomitant mixing. Sepharose beads were pelleted at 12,000 r.p.m. for 2 min at 4°C and washed five times with chilled IP buffer followed by two PBS washes. Immuno-precipitated proteins were obtained by treating the beads with nonreducing SDS lysis buffer. The sample was electrophoresed on a 15% gel and transferred onto a nitrocellulose membrane. The membrane was probed with mouse anti-HU<sub>p</sub> serum as primary antibody and anti-mouse HRP conjugate as secondary antibody followed by development of the blot using a chemiluminescent detection system.

Electrophoretic mobility shift assay (EMSA)

Agarose gel electrophoresis was carried out with complexes of plasmid pBR322 and PfHU<sub>p</sub> or PfHU<sub>ΔC</sub> as a function of protein concentration. Four hundred nanograms of supercoiled pBR322 (New England Biolabs, USA) or linear pBR322 DNA was used in the binding reaction with PfHU<sub>p</sub> or PfHU<sub>ΔC</sub> in a reaction buffer containing 50 mM Tris–HCl pH 7.5, 0.1 mM EDTA. The reaction was incubated at 37°C for 40 min followed by electrophoresis in a 1% agarose gel at room temperature in 1x TAE buffer. The gel was stained with ethidium bromide (0.5 μg/ml) for 1 h, destained with 1x TAE and photographed.

For EMSAs on polyacrylamide gels, the binding reaction (20 μl) was carried out in 20 mM Tris–HCl, 0.1 mM EDTA and incubated for 30 min at room temperature. The gel was electrophoresed in 0.25x TBE.

*K<sub>d</sub>* determination was carried out by incubating 100 femtomoles of 5'-end-labeled 30 bp double-stranded oligo probe with increasing concentrations of protein as described in Ghosh and Grove (31). The region of a lane from the complex up to the free probe was taken as complex. *K<sub>d</sub>* was calculated from the Hill equation, *f* = *f*<sub>max</sub> × ([PfHU<sub>p</sub>]<sup>f</sup>)/(K<sub>d</sub> + [PfHU<sub>p</sub>]<sup>f</sup>), where *f* is the fraction complex ([PfHU<sub>p</sub>/DNA]<sub>f</sub>), *f*<sub>max</sub> is fraction complex at maximal saturation and [PfHU<sub>p</sub>] is protein concentration. The Hill coefficient (*n*) was set to one for single-site binding. Signals for bound and free probe were quantitated using OptiQuant1 software in Cyclone phosphorimager (Packard). *K<sub>d</sub>* was calculated by curve-fitting using nonlinear regression in GraphPad PRISM software.

Supercoiling assay

Negatively supercoiled pBR322 (200 ng/reaction) was reacted with 2 units of *E. coli* topoisomerase I (New England Biolabs, USA) in 10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA and 100 μg/ml BSA for 2 h at 37°C. The relaxed DNA was incubated with increasing concentrations of PfHU<sub>p</sub> or PfHU<sub>ΔC</sub> and the volume was adjusted with 1x dilution buffer (20 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 100 μg/ml BSA) and incubated for 1 h at 37°C. Reactions were terminated with proteinase K (0.5 μg/μl) and 0.5% SDS and incubated at 37°C for 1 h. The DNA was electrophoresed on 0.8% agarose gel in 0.5 x TBE for 14 h.

In vivo condensation assay

*Escherichia coli* JM109 cells co-transformed with pQE30-HU<sub>p</sub> + RIG or the parent vector pQE30 + RIG as control were grown till the cultures reached an O.D. of 0.5. After induction with 0.5 mM IPTG for 3 h at 20°C, 1 ml culture was withdrawn and cells were washed twice with 1x TBS. Washed cells were suspended in 100 μl TBS, fixed with 0.5% glutaraldehyde and stained with 1.0 μg/ml DAPI for 30 min at 37°C. Cells were washed three times with 1x TBS and visualized in a fluorescence microscope (Leica DM5000B).

DNA-circularization assay

The ability of PfHU<sub>p</sub> to induce DNA circularization in the presence of T4 DNA ligase was assayed by incubating 2.5 ng of a 136 bp, 32P 5'-end-labeled DNA probe with or without PfHU<sub>p</sub> (or PfHU<sub>ΔC</sub>) in a 20 μl reaction containing 1x ligase buffer at 25°C for 30 min. The 136 bp DNA was derived from a 369 bp PCR-amplified fragment from a *Mycobacterium* gene. The 369 bp fragment was digested with *TaqI* to give the 136 bp probe containing 5' overhangs at both ends. The incubation of the DNA probe with PfHU<sub>p</sub> was followed by addition of 4.5 U of T4 DNA ligase (Promega, USA) and further incubation at 22°C for 2 h. If samples were to be treated with BAL31 exonuclease (Fermentas, USA) after ligation, the ligation reaction was added to an equal volume of 2x nuclease buffer containing 1U of BAL31. The digestion reaction was incubated at 30°C for 15 min. The DNA was extracted...
with phenol–chloroform, collected by ethanol precipitation and suspended in DNA loading dye. After electrophoresis on 7.5% 0.25× TBE-PA gel, the gel was dried and autoradiographed.

For the transformation assay, linear pBR322 (5ng) was incubated with PfHU_p and T4 DNA ligase as in the ligation assay above followed by protein removal by Proteinase K treatment (10μg/reaction) and transformation of E. coli DH5α cells.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assay was performed as described by Gissot et al. (32) with some modifications. Thirty milliliters of trophozoite stage P. falciparum culture was subjected to protein–DNA crosslinking using 1% (v/v) formaldehyde at 37°C for 15 min. Parasites were released by 0.05% saponin lysis and the parasite pellet was suspended in 1 ml ChIP buffer [30 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Triton X-100 and 1% v/v Igepal]. Cells were incubated on ice for 20 min, sonicated nine times (Branson Digital Sonifier 450) at 30% amplitude for 10 s each with 1 min cooling between sonications. After centrifugation at 12,500 r.p.m. for 10 min at 4°C, the supernatant containing soluble chromatin was pre-cleared with 50μl of 50% Protein A sepharose CL-4B and 20 μg of sheared salmon sperm DNA for 2 h at 4°C. The beads were pelleted at 10,000 r.p.m. for 2 min. Preimmune serum or anti-PfHU_p serum (1:75 dilution) was added to the supernatant and incubated at 4°C for 2 h. Forty microliters of 50% Protein A sepharose and 20 μg salmon sperm DNA was added to the mixture and incubated overnight at 4°C with continuous mixing. The sepharose beads were pelleted at 2000 r.p.m. at 4°C for 1 min and washed three times with ChIP buffer. This was followed by two washes with 1× TE and one wash with 1× TE and 0.01% SDS. Chromatin was eluted using 1× TE supplemented with 1% SDS. Chromatin was reverse cross-linked for 6 h at 65°C and treated with proteinase K (20 μg) for 2 h at 37°C. DNA was extracted with phenol–chloroform followed by ethanol precipitation. DNA from input and ChIP samples were resuspended in 100 and 20 μl TE, respectively. For PCR-amplification, 0.5 μl of input DNA and 2 μl of the ChIP sample were used as template. Primers for the nuclear gene PfHU and a plDNA sequence RIII (11) were used to amplify nuclear and apicoplast DNA, respectively. The PCR products were analysed on 1% agarose gel.

Confocal microscopy

P. falciparum cultures were processed for immunofluorescence labeling and confocal microscopy according to the method of Tonkin et al. (33). For mitochondrial labeling, live cells were incubated in 25 ng/ml MitoTracker Deep Red 633 (Molecular Probes) in PBS for 20 min at 4°C prior to fixation. Cells were washed with PBS and fixed in solution using 4% paraformaldehyde and 0.0075% glutaraldehyde in PBS for 30 min. After one wash with PBS, fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After another PBS wash, cells were treated with ~0.1 mg/ml sodium borohydride in PBS for 10 min. Cells were washed once with PBS, blocked in 3% BSA/PBS for 1 h and incubated overnight with rabbit anti-PfHU_p serum (1:50 dilution in PBS containing 3% BSA) at 4°C. After three washes (10 min for each wash) with PBS, the cells were incubated with AlexaFluor488 tagged anti-rabbit secondary antibody for 2 h at room temperature and allowed to settle onto coverslips coated with poly-l-lysine (100 μg/ml). The coverslips were then washed three times in PBS and mounted in anti-fade mounting medium (Oncogene, USA). For apicoplast co-localization studies, the D10-ACPleader-GFP line in which GFP is an apicoplast marker was used. Mouse anti-PfHU_p Ab (1:25) or rabbit anti-GFP Ab (1:1000) (Molecular Probes) was used as primary antibody with Texas Red tagged anti-mouse Ab (Molecular Probes) or Alexa Fluor488 tagged anti-rabbit Ab (Sigma) as secondary antibodies. The slides were viewed in a confocal laser-scanning microscope (Zeiss LSM 510) under a 63× oil immersion lens.

Atomic Force Microscopy (AFM)

Atomic force microscopy of DNA–PfHU complexes was carried out by using freshly cleaved mica treated with vapors of 3-aminopropyl triethoxysilane (APTES) at room temperature for 2 h for immobilization of samples (18). For sample preparation, PfHU_p and linear pBR322 DNA were mixed at different dimer/bp ratios in 20μl of AFM buffer (20 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 1 mM EDTA). The final concentration of DNA in the mix was ~1 ng/ml. After incubation for 10 min at room temperature, 10 μl of sample was dropped on APTES-coated mica and allowed to bind for 2 min at room temperature. The mica was washed extensively with deionized water, blotted at the edge and air-dried.

AFM imaging was carried out using the PicoSPM equipment (Molecular Imaging, AZ, USA). Images were obtained in the AAC mode with 225 μm long cantilevers that had resonance frequency of 65 kHz and force constant of 2.8 N/m. DNA contour length (in absence of protein) was calculated to be 1.403 μm (SD = 0.156 μm, n = 95), which is close to the expected contour length (1.439 μm) of the 4361 bp pBR322.

RESULTS

Structure modeling of PfHU reveals extensive fold conservation

The nuclear DNA-encoded 22.5 kDa P. falciparum protein (PlasmoDB ID PF10230c) was identified as a candidate protein for DNA organization in the apicoplast due to the presence of a predicted bipartite apicoplast-targeting sequence that is the characteristic of nuclear-encoded proteins targeted to the organelle (34). The sequence has also been recently annotated as ‘bacterial histone-like protein, putative’ in the Plasmodium database (www.plasmodb.org). PfHU has a predicted 53 aa long signal and transit peptide with a high score in the apicoplast-targeting sequence prediction software Plasmo AP. This is followed by a conserved bacterial histone-like (BHL) domain found
in histone-like proteins in bacteria, dinoflagellates, red algal chloroplasts as well as in the related apicomplexan *T. gondii* (35) (Figure 1). PfHU also carries a 42 aa uncon- served C-terminal extension beyond the BHL domain.

Alignment of the PfHU sequence with selected HU homologs from other organisms demonstrates significant sequence conservation within the BHL domain (Figure 1A) suggesting overall similarity of structure and function with other HU proteins. PfHU exhibits maximum homology with the *T. gondii* HU (30% identity) followed by the chloroplast HU of the red alga *Cyanidioschyzon merolae* (28% identity). Hydrophobic

**Figure 1.** Sequence alignment and structure model of PfHU. (A) ClustalW alignment of PfHU with bacterial (*Bacillus stearothermophilus, B. subtilis, Thermotoga maritima*), red algal chloroplast (*Cyanidioschyzon merolae*) and apicomplexan (*Toxoplasma gondii*) HU proteins. Conserved residues described in the text are marked with asterisk. (B) Structure of the PfHU dimer modeled on the crystal structure of *B. stearothermophilus* HU. The position of the leucine residue (L63) that replaces the conserved proline of other HU proteins is indicated. The 42 aa C-terminal domain could not be modeled on any known protein structure and is not depicted in the figure.
residues in the BHL core, notably phenylalanine residues at positions 29, 47, 50 and 79, are conserved in PfHU. These residues are part of the dimerization signal and are involved in the formation of an aromatic hydrophobic core involving inter-subunit stacking (36,37). PfHU has an additional F54 residue that may also contribute to formation of the hydrophobic core. Structural analysis has shown that a number of arginine residues (R55, R58, R61) within the DNA-binding arms participate in hydrogen bonding or electrostatic contacts to the DNA (38). Of these, R55 and R58 are conserved in PfHU while R61 is replaced by a lysine. Surface-exposed lysine residues (K3, K18, K86) that line the body of the protein and have been shown to contribute to DNA binding (39) are also conserved in PfHU suggesting their contribution to wrapping of DNA around the protein.

Structure modeling of PfHU on the *Bacillus Stearothermophilus* structure (PDB ID: 1huu) (40) was carried out by SWISS-MODEL using the alignment mode. Extensive fold conservation in the PfHU BHL domain was revealed. The 42 aa C-terminal extension could not be modeled as it lacked significant homology with any known protein and contained regions of high disorder predicted using DisEMBL (41). The structure model of the PfHU homodimer is shown in Figure 1B. The predicted homodimer structure is comprised of a largely α-helical body with the α1- and α2-helices being formed by residue K3–T14 and K18–E37 of the predicted processed protein, respectively. β1 (residue 142–144), β2 (residue G48–R55) and β3 (residue S73–F81) strands form the saddle-like β sheet structure. β2 and β3 (R58–K61 and D68–E72, respectively) are part of the DNA-binding arm of PfHU. The DNA-binding arm of the PfHU monomer (residues 53–75) contains eight positively charged residues but lacks the highly conserved proline (P63) that is implicated in induction and/or stabilization of DNA binding by intercalating between base pairs (42). The DNA-binding arms of Hu proteins have been described as disordered in crystal structures (36,43) and NMR studies show that the homodimer DNA-binding arms are folded at the tips and are flexible in solution (44).

**PfHU specifically binds apicoplast DNA**

The processed form of PfHU (PfHU)p was expressed as a soluble protein in *E. coli*. Although the expected size of the 6XHis-tagged processed protein is ~18 kDa, purified PfHU_p ran at ~23 kDa on SDS–PA gels (Figure 2A) with the intact protein comprising ~93% of the purified fraction. The observed difference in size may be attributed to the presence of nonglobular domains in the protein or an excess of positively charged residues; such differences between expected and observed sizes on SDS–PA gels have also been observed for other *P. falciparum* proteins (15). The unprocessed form of PfHU (PfHU_up) carrying the apicoplast-targeting sequence was also expressed in *E. coli* and resolved at ~25 kDa on SDS–PA gels (Figure 2A). Purified recombinant processed PfHU lacking the 42 aa C-terminal domain (PfHU_A) ran at ~15 kDa, above its expected size of ~13 kDa, together with some truncated products. The truncated products were recognized by anti-His Ab in western blots (data not shown) indicating that they contain deletions from the C-terminal end. The major 13.5 kDa band would lack the α2-helix in addition to the C-terminal domain but is likely to retain DNA binding. The two minor truncation products would additionally lack both the β1 and β3 strands and are thus unlikely to bind DNA. The 15 kDa band and its major truncated product of ~13.5 kDa that together comprised ~85% of the total purified protein are referred to as the ‘C-terminal deletion’ (PfHU_A) and were used for all calculations for quantitation of PfHU_A for DNA-binding experiments.

When purified recombinant PfHU_p was subjected to SDS–PAGE analysis, an additional band of ~44 kDa was observed together with the ~23 kDa monomeric form at high protein concentration (Figure 2B, i). The 44 kDa band corresponds to the size expected for the dimeric form of PfHU_p. Anti-His-antibody recognized the band in lane 3 using mouse anti-PfHU_p antibody in a Western blot. Lane 1 represents western blot confirming that it was the PfHU_p dimer that either remained associated even after boiling in the presence of 0.1% SDS (Figure 2B, ii) or the PfHU_p monomers reassociated in 0.1% SDS after removal from heat.
Treatment of purified PfHU with 8 M urea caused dissociation of the dimer (data not shown) suggesting that hydrophobic interactions play a major role in dimerization of the protein in vitro. Glutaraldehyde-mediated crosslinking of PfHU demonstrated that the protein existed predominantly as a dimer in solution (Figure 2C). A minor tetrameric form was also seen upon crosslinking.

Antibodies raised against recombinant PfHU specifically recognized a ~22 kDa protein in western blots together with a fainter band at ~24 kDa (Figure 2E). This size difference corresponds to the difference observed for recombinant PfHU and PfHU indicating that the lower and upper bands represent processed and unprocessed forms of PfHU, respectively. Rabbit anti-PfHU serum immunoprecipitated the processed form of PfHU that was specifically recognized by mouse anti-PfHU serum (Figure 2D). A specific RT-PCR product of the length expected after cleavage of the single intron in the PfHU gene was also amplified from total parasite RNA (data not shown). These results confirm that PfHU is translated in the parasite. PfHU was also detected in parasite lysates from synchronized cells at different intra-erythrocytic stages (Figure 2E). Expression of the protein was observed at all stages and comparison with parasite tubulin levels indicated constitutive PfHU expression during the P. falciparum erythrocytic cycle.

P. falciparum carries three DNA genomes- nuclear, apicoplast and mitochondrial (45). To address the possibility of PfHU also serving as a mitochondrial/nuclear DNA organization protein, we carried out immunofluorescence detection of PfHU using anti-PfHU antibody in confocal microscopy. PfHU localized to an organellar structure close to, but distinct from, the mitochondria stained with Mitotracker Red (Figure 3A). PfHU signal was also not observed in nuclei that were stained with DAPI. Additionally, PfHU co-localized with apicoplast-targeted GFP in the D10 ACP leader-GFP line with GFP as an apicoplast marker (Figure 3B). These data confirm apicoplast-specific localization of PfHU in P. falciparum as also reported for the HU protein of T. gondii (46).

In order to investigate whether PfHU interacts with P. falciparum apicoplast DNA in vivo, we carried out chromatin immunoprecipitation (ChIP) of total P. falciparum DNA using anti-PfHU antibody. This was followed by PCR-amplification of immunoprecipitated DNA using primer pairs for amplification of a 411 bp internal region of the PfHU gene representing nuclear DNA and a 332 bp fragment (RIII) (11) representing the apicoplast genome. While both primer pairs amplified the corresponding fragments from input DNA (Figure 3C, lanes 2 and 5), amplification of only the apicoplast-specific fragment was observed when DNA precipitated with the anti-PfHU immune serum was used as template (lane 7). Neither fragment was amplified from DNA immunoprecipitated using preimmune serum (lanes 3 and 6). The clear recovery of apicoplast DNA in the ChIP assay indicated that PfHU binds specifically to the apicoplast genome thus...
strengthening its candidature as a key protein in plDNA organization.

**PfHU**<sub>p</sub> binds and condenses DNA

The DNA-binding activity of recombinant PfHU<sub>p</sub> was investigated by electrophoretic mobility shift assays (EMSAs) in agarose gels using supercoiled and linear pBR322. The mobility of the supercoiled form was retarded sharply by PfHU<sub>p</sub> starting at the protein/DNA mass ratio of 0.5 while retardation of linear DNA was clearly visible only beyond the protein/DNA mass ratio of 2 (Figure 4A). This indicated that PfHU<sub>p</sub> exhibits preferential condensation of supercoiled DNA compared to linear DNA. EMSA with increasing concentrations of PfHU<sub>p</sub> and PfHU<sub>ΔC</sub> incubated with supercoiled pBR322 (Figure 4B) revealed a difference in their DNA condensing properties. While PfHU<sub>p</sub> condensed DNA *in vitro* at a concentration of 8 μM (protein/DNA mass ratio ≥10) when nearly all the DNA was retained in the well, complete condensation of DNA by PfHU<sub>ΔC</sub> was observed at 6 μM (protein/DNA mass ratio = 6.2). Additionally, in contrast to PfHU<sub>p</sub>, no intermediate retardation forms were observed with PfHU<sub>ΔC</sub>. A minor retardation with 2 μM PfHU<sub>ΔC</sub> was followed by complete condensation of DNA as the protein was increased from 4 to 6 μM. These results indicate a role of the 42 aa C-terminal domain in influencing the DNA-condensation properties of PfHU<sub>p</sub>.

The effect of salt on DNA–protein interaction was assayed by addition of 50 or 100 mM NaCl to the binding reaction at different PfHU/DNA mass ratios. Partial inhibition of binding was observed with 50 mM NaCl as a much greater amount of PfHU<sub>p</sub> was required to cause the same level of retardation (Figure 4C). Binding was completely inhibited by 100 mM NaCl and no retardation was observed even at the protein/DNA mass ratio of 10 indicating that interaction between PfHU<sub>p</sub> and DNA was primarily electrostatic.

The effect of increasing concentrations of PfHU<sub>p</sub> and PfHU<sub>ΔC</sub> on DNA supercoiling (Figure 4D) showed that the supercoiling ability of both the products was negligible. This contrasts with reports for other HU proteins (47) that hinder relaxation by topoisomerase I and constrain negative supercoils in a concentration-dependent manner.

The effect of over-expression of PfHU<sub>p</sub> on *E. coli* DNA was examined by fluorescence microscopy of DAPI-stained *E. coli* cells that were transformed with the expression vector pQE30 or the PfHU<sub>p</sub> expression vector pQE30-HU<sub>p</sub> + RIG plasmid and induced with IPTG (RIG overcomes codon bias for expression of *P. falciparum* genes in *E. coli*). Compared with control cells, cells expressing PfHU<sub>p</sub> exhibited very slow growth upto 3 h after induction. Additionally, control cells lacking PfHU<sub>p</sub> exhibited uniform DNA distribution while extensive condensation of the *E. coli* nucleoid was visible in cells expressing PfHU<sub>p</sub> (Figure 5). This result indicates that an excess of PfHU<sub>p</sub> causes greater compaction of *E. coli* nucleoids. Similar compaction of *E. coli* nucleoids was also observed with overexpression of PfHU<sub>ΔC</sub> (data not shown).
The minimal binding-site length of PfHU was determined by using double-stranded oligonucleotide probes of 23, 30 and 55 bp in binding reactions resolved in EMSAs on PA gels. A very faint smeared complex was observed with the 23 bp probe (data not shown) while a single clear complex was obtained with the 30 bp probe (Figure 6B). Two complexes were obtained when the 55 bp probe was used (Figure 6A) indicating a binding site of between 24 and 27 bp. The 55 bp probe containing two PfHUp dimer binding sites was also used to determine the active fraction of PfHU by titrating 125 nM of PfHU dimer with increasing concentrations (2.5–160 nM) of the 55 bp DNA probe in EMSA (data not shown). The protein saturated at $\sim 60$ nM DNA indicating that $>95\%$ of the protein was active. The affinity of the PfHU dimer for DNA was determined by calculation of $K_d$ of the single complex obtained with the 30 bp probe (Figure 6C). The $K_d$ value obtained for PfHU was $62.7 \pm 6.5$ nM.

PfHU promotes DNA concatenation

Bacterial HU is capable of mediating DNA ring closure (48) while its dinoflagellate counterpart promotes concatenation of DNA fragments and inhibits DNA circularization (18). The ability of PfHU to promote circularization of short-length DNA fragments as a result of protein-mediated DNA bending was tested in a T4 ligase-mediated DNA ligation assay. Ligation of a 136 bp DNA fragment was carried out in the presence of increasing concentrations of PfHU (lanes 3–6). Lane 1 is free DNA while lane 2 is DNA probe ligated in the absence of PfHU. Ligation reactions were treated with BAL31 nuclease to detect circularized DNA products (lanes 7–10). (B) Ligation of the 136 bp fragment in the presence of increasing concentrations of PfHU (lanes 3–5 and 7–9). Lane 1 is free DNA while lanes 2 and 6 are DNA probe ligated in the presence of HBsu as positive control for DNA circularization. (C) Percentage transformation efficiency of ligation reactions of linear pBR322 carried out in the presence of PfHU. Transformation efficiency was calculated as percentage of that obtained with pBR322 ligated in the absence of PfHU. Mean and SE of repeat determinations is plotted.

Figure 6. Binding site length and affinity of PfHU for DNA. (A) EMSA showing binding of PfHU with end-labeled 55 bp probe. (B) EMSA of PfHU with a 30 bp probe. (C) The $K_d$ of the single complex obtained in (B) was determined from its binding isotherm by curve-fitting using nonlinear regression. The mean $K_d$ value from three repeat experiments was estimated as $62.7 \pm 6.5$ nM.

Figure 7. DNA concatenation by PfHU. (A) DNA ligation of a 136 bp labeled DNA fragment carried out in the presence of increasing concentrations of PfHU (lanes 3–6). Lane 1 is free DNA while lane 2 is DNA probe ligated in the absence of PfHU. Ligation reactions were treated with BAL31 nuclease to detect circularized DNA products (lanes 7–10). (B) Ligation of the 136 bp fragment in the presence of increasing concentrations of PfHU (lanes 3–5 and 7–9). Lane 1 is free DNA while lanes 2 and 6 are DNA probe ligated in the presence of HBsu as positive control for DNA circularization. (C) Percentage transformation efficiency of ligation reactions of linear pBR322 carried out in the presence of PfHU. Transformation efficiency was calculated as percentage of that obtained with pBR322 ligated in the absence of PfHU. Mean and SE of repeat determinations is plotted.
PfHU promoted concatenation of DNA as evident from the increase in exonuclease-sensitive linear multimers of the 136 bp DNA fragment up to PfHU/DNA mass ratio of 20 (Figure 7A). Higher concentrations of protein (PfHU/DNA mass ratio of 40 and 80) inhibited concatenation. Similar results were obtained with PfHU/DNA mass ratio indicating that the C-terminal extension was not responsible for mediating DNA concatenation (Figure 7B). PfHU and PfHU/C1 also promoted concatenation and inhibited circularization of a longer 207 bp DNA fragment in a concentration-dependent manner (data not shown).

PfHU also inhibited DNA circularization as evident from results of the transformation assay (Figure 7C) where linear pBR322 (5 ng) was incubated with PfHU and T4 DNA ligase as in the ligation assay above followed by transformation of E. coli DH5α cells. PfHU exhibited dose-dependent inhibition of DNA circularization. Upto 80% inhibition was observed at PfHU/DNA mass ratio of 20, which corresponds to the protein concentration at which maximum concatenation is seen in Figure 7A.

**AFM studies**

Atomic Force Microscopy of PfHU–DNA complexes at different PfHU-dimer/bp ratios provided further evidence for DNA condensation mediated by PfHU (Figure 8). At lower concentration of protein (dimer/bp ratio 1:750) stiffening of DNA strands was evident (Figure 8B, i). Intermolecular DNA bundling with two DNA strands forming a tight bundle was also observed at this concentration (Figure 8B, ii). PfHU-mediated formation of DNA loops (Figure 8C, ii) was seen at the dimer/bp ratio of 1:500 and assembly of condensed DNA complexes with a small number of foci and extruding DNA loops was initiated (Figure 8C, i and iii). This data indicated that PfHU also forms DNA bridges. Formation of larger PfHU–DNA complexes was seen at higher protein concentration (dimer/bp ratio 1:250) (Figure 8D). These complexes appeared to be formed by the assembly of large DNA bundles/bridges brought together by intermolecular interactions between PfHU subunits (Figure 8D, ii and iii).

**DISCUSSION**

Proteins that mediate organization of the *Plasmodium* organellar genomes are yet to be identified and functionally characterized. While core histones (H2A, H2B, H3 and H4) for nuclear DNA assembly are encoded in the genome together with a few histone variants (50), two nucleosome assembly proteins, PfNAPS and PfNAPL, which preferentially interact with H3–H4 tetramer histones have also been characterized (51). Although the high mobility group (HMG) protein Abf2 is an abundant basic protein that supercoils mitochondrial DNA in yeast (52), the HMG homologs on the *P. falciparum* genome lack predicted mitochondrial-targeting signals. On the other hand, a mitochondrial-targeting sequence is predicted for a putative histone 2B (PF11_0062) encoded by the nuclear genome. Of the 517 nuclear-encoded proteins predicted to target to the apicoplast, PfHU (PF10230c) is the only one with a dsDNA-binding BHL domain similar to HU-like DNA organization proteins of plastids. Our results identify PfHU as a component of the apicoplast and confirm its interaction with apicoplast DNA. The DNA-binding characteristics of PfHU and its effect on bacterial nucleoid condensation are also indicative of its role as a plDNA architecture protein in *P. falciparum*.

Sequence-function analyses of PfHU reveal some interesting features. While the DNA-intercalating proline (P63) and surrounding residues are conserved in most HU proteins, a few exceptions such as bacteriophage SPO1–encoded TF1 and HU from Thermotoga, Thermus, Aquifex, Deinococcus and Mycoplasma have R61 replaced
by V or M residues (53). R61 is replaced by a K residue in P. falciparum while the P63 residue is replaced by L, thus converting the conserved HU RNP motif into a KNL motif. These observed substitutions may be explained by the low G+C content (~75% A + T) of the P. falciparum genome which results in a bias against residues G, P, A and R. Significantly, the T. gondii HU sequence (T. gondii genome has 47% A + T) retains conserved R61 and P63 residues. P63 is implicated in induction and/or stabilization of DNA bending and the absence of this residue in PfHU may explain the inability of the protein to induce DNA circularization. An alternative possibility is that PfHU may bend DNA, but association of multiple PfHU molecules results in out-of-phase bending. HCC3, a likely constituent of permanently condensed crystalline chromosomes of the dinoflagellate Cryptophycodinium cohnii, also lacks P63 and fails to induce DNA circularization in vitro; similar to PfHU, HCC3 promotes DNA concatenation (18). Assaying DNA circularization by PfHU after mutagenesis to restore P63 would be required to confirm the role of L63 in preventing DNA bending by the protein. Analysis of the zP64L mutant of IHF (position corresponding to P63 in HU) that interacts with DNA in a site-specific manner has demonstrated that the substitution affects binding specificity of the protein (54,55).

As opposed to other HU proteins, PfHUp is unable to constrain negative supercoils. The P63 residues in the E. coli HU dimer intercalate into the minor groove of DNA 9bp apart and induce two DNA kinks. These kinks are not co-planar and result in negative supercoiling (underwinding) of ~31° per bp (56). The observed absence of DNA-bending activity of PfHU offers an explanation for its inability to constrain negative supercoils in the DNA double-helix.

HU proteins bind to random DNA sequences with $K_d$ values ranging from 5 to 2500 nM with E. coli HU exhibiting a $K_d$ value of ~200–300 nM (53,57). The $K_d$ value of PfHU–DNA interaction is ~63 nM indicating slightly higher affinity of the protein for DNA compared to E. coli HU. HU proteins have been reported to interact with DNA with binding sites of between 9 and 42 bp (57). It has been suggested that variations in HU-binding site lengths are determined by the presence or absence of amino acids capable of forming salt bridges distal to sites of kinking (58). In HU homologs with shorter binding sites, K3 is proposed to form a salt bridge with D26. In TF1, which lacks D26, K3 contacts DNA 8–9 bp away from the DNA kink leading to a longer binding site of 37 bp. Similarly, the absence of D26 in PfHU may explain its binding site length of 24–27 bp.

There is a difference in patterns of DNA shifts obtained with increasing concentrations of PfHUp and PfHUΔC, with sudden transition to highly condensed DNA observed with the latter. The 42 aa C-terminal domain present in PfHU p has a predominance of acidic residues (net PI of PfHU p is 8.81). PfHUΔC, which lacks the domain, has a PI of ~9.5. The DNA shifts observed in EMSAs with the two proteins indicate that rapid condensation of DNA follows an initial 'nucleation' step with increasing PfHUΔC/DNA ratio, while stepwise assembly of intermediate forms is observed with the full-length PfHU p. Although the precise mode for this is unclear, the negatively charged C-terminal domain unique to PfHU p may directly influence interaction of the protein with DNA. Alternatively, rapid DNA condensation by PfHUΔC may be caused by enhanced cooperativity of binding of PfHUΔC dimers to DNA and/or increased intramolecular attraction between PfHUΔC dimers bound at different sites on DNA. The C-terminal domain also seems to influence stability of PfHU p as removal of the domain results in greater protease-sensitivity. In vivo, the intrinsically unstructured C-terminal domain may interact with other parasite proteins (59) and influence PfHU activity.

AFM analysis of DNA–protein complexes formed with PfHU p showed formation of DNA bundles and bridges. DNA bundling is a property exhibited by the RecA protein (60) and also reported for the dinoflagellate HCC3 (18). DNA bundling by PfHU p may bring the ends of two or more DNA strands closer thus promoting ligase-mediated concatenation observed with PfHU p. The bundling of two strands would be mediated by the interaction between two PfHU p dimers, each of which binds to a single DNA strand. Indeed, formation of such tetrameric PfHU p forms is indicated by chemical-crosslinking of the protein in solution. The clustering of long DNA bundles and bridges to form large complexes observed at high PfHU p concentration could be mediated by intermolecular interactions between PfHU p tetramers. Thick rigid DNA–protein filaments as observed for high concentrations of E. coli HU (1 dimer per 1.8 bp) (28) are seen at much lower concentrations of PfHU p (1 dimer per 750 bp). Unlike E. coli HU, whose AFM analysis shows DNA bending mediated by single dimers at one dimer per 92 bp (28), DNA bending was not observed with PfHU p consistent with our observation of inhibition of DNA circularization with the protein. DNA stiffening and bundling by PfHU p may explain the inhibition of circularization observed with the protein. DNA loop formation and bridging by PfHU p is also clearly seen in AFM images. Formation of DNA bridges by the bacterial nucleoid structuring protein, H-NS, which is structurally distinct from HU, has been reported (61). DNA bridging resulting in formation of loops may have implications not only in DNA compaction mediated by PfHU p but also suggests a possible mechanism by which it may influence transcription processes in the apicoplast.

The origin of apicoplast plastids has been of recent interest and their rhodophyte versus chlorophyte ancestry has been debated (62,63). The presence of a functional HU-like protein in apicoplast plastids provides further support for red algal ancestry of the apicoplast; HU-like proteins are found in plastid genomes of red algal lineage but not in those of green alga (64). Additionally, the sequence of PfHU is closest to red algal plastid HUs of C. merolae and Guillardia theta and the apicoplast and red algal HUs cluster with cyanobacteria HUs (35,65). The presence of a nuclear gene encoding apicoplast-targeted HU in P. falciparum indicates that the gene was acquired by a secondary endosymbiotic event from a red alga with subsequent transfer from the red algal plastid to the host nuclear genome.
Our results provide evidence for the involvement of a HU-like protein in DNA organization and compaction in the plastid of an apicomplexan. Apart from being major components of nucleoids, HU proteins in bacteria also play important roles in initiation of DNA replication and regulation of transcription (21,24,66). Although the specific roles that HU may play in apicoplast DNA replication and/or regulation of transcription remain to be elucidated, the reported missegregation of the T. gondii apicoplast genome upon over-expression of TgHU (67) together with the pLDNA-specific interaction and DNA condensation properties of PIHU described here are indicative of its significance in the process of apicoplast DNA replication and organelar division.

ACKNOWLEDGEMENTS

We thank Dr Samir Sawant and Amol Ranjan for help with confocal microscopy, Anita Mann for AFM analysis, Ashutosh and Dr Amogh Sahasrabuddhe for helpful discussion and J.P. Srivastava for technical assistance. E.V.S.R. and R.N. are recipients of research fellowships from the Council of Scientific and Industrial Research and Department of Biotechnology, respectively. This is CDRI communication no. 7456. Funding by the Council of Scientific and Industrial research (NWP-0038 and NMITLI grant TLP-0010 to SH) is acknowledged. The Open Access publication charges for this article were waived by Oxford University Press.

Conflict of interest statement. None declared.

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