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RESEARCH LETTER

Mycobacterium smegmatis histone-like protein Hlp is nucleoid associated

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Abstract

Eubacteria encode proteins that are required for nucleoid organization and for regulation of DNA-dependent processes. Of these histone-like proteins (Hlps), *Escherichia coli* HU has been shown to associate with the nucleoid and to regulate processes such as DNA repair and recombination. In contrast, the divergent HU homologs encoded by mycobacteria have been variously identified as involved in the physiology of dormancy, in the response to cold shock, or as laminin-binding proteins associated with the cell envelope. We show here, contrary to previous reports that the HU-related Hlp from *Mycobacterium smegmatis* associates with the nucleoid *in vivo*. Using indirect fluorescent antibody microscopy we show that cold shock causes Hlp to accumulate in the cytoplasm of *M. smegmatis*. No evidence of surface-associated Hlp was found in *M. smegmatis* cells treated for cell wall permeabilization. Quantitative Western blots indicate that exponentially growing cells contain *c.* 120 molecules per cell, with upregulation of Hlp after cold shock estimated to be *c.* 10-fold. That Hlp associates with the nucleoid *in vivo* suggests functions in DNA metabolism, perhaps in adaptation to environmental stress.

Introduction

Histone-like proteins (Hlps), such as *Escherichia coli* HU, are involved not only in nucleoid organization, but also in regulating DNA-dependent processes (Dame, 2005). HU was first characterized in 1975 as a Hlp isolated from *E. coli* strain *U* 13 (hence the name HU; Rouvière-Yaniv & Gros, 1975). HU, along with other small basic proteins such as H-NS and Fis, contribute to the organization and compaction of the bacterial genomic DNA, creating what has been termed bacterial chromatin. In their association with the genomic DNA, these proteins generate a much more dynamic structure compared with eukaryotic chromatin, which functions intimately in regulating gene expression. Further, bacterial Hlps are so categorized only based on their ability to compact the genomic DNA and to constrain supercoils, as they share no sequence or structural similarity with eukaryotic histones. Mycobacteria encode two-domain Hlp in which the N-terminal domain shares significant homology with HU proteins and the C-terminal domain exhibits numerous sequence motifs resembling those found in eukaryotic histone H1 (Prabhakar *et al.*, 1998; Mukherjee *et al.*, 2008a). The C-terminal proline-, alanine- and lysine-rich repeats also resemble those found in the unique N-terminal extension of the *Deinococcus radiodurans*-encoded HU homolog, where they direct the protein to a unique position on four-way junction DNA (Ghosh & Grove, 2006). The *Mycobacterium tuberculosis*-encoded Hlp homolog binds DNA, and a potential interaction with the *M. tuberculosis* chromosome was suggested (Prabhakar *et al.*, 1998). In contrast, Hlp homologs from *Mycobacterium smegmatis* and *Mycobacterium leprae* have instead been suggested to serve as laminin-binding proteins and to be associated with the cell surface (Pethe *et al.*, 2001b; Soares de Lima *et al.*, 2005). However, there are several unanswered questions associated with the latter function, not least of which the mechanism by which Hlp becomes associated with the mycobacterial extracellular surface (the sequence of Hlp contains no predicted signal peptide) and the observation that Hlp binds DNA with high affinity *in vitro* (Mukherjee *et al.*, 2008a).

Stressful environmental conditions can cause bacterial populations to enter a dormant or latent state from which they are able to return to a metabolically active state upon return of favorable conditions. *Mycobacterium tuberculosis* is notorious for its ability to cause latent infection (Oliver, 2005). *Mycobacterium smegmatis* is considered largely as a nonpathogenic model of *M. tuberculosis* because the physiological behaviors of these two species under anaerobiosis are very similar, both capable of entering a dormant state and of resuming growth when environmental conditions improve (Dick *et al*., 1998; Hutter & Dick, 1998). Notably, when *M. smegmatis* experiences oxygen starvation or cold shock, its Hlp homolog is upregulated and dormancy is induced (Lee *et al*., 1998; Shires & Steyn, 2001). The *hlp* knock-out mutants are not able to resume growth after cold shock, perhaps because they are unable to adapt metabolically (Shires & Steyn, 2001). However, it has also been reported that the viability of anaerobic dormant cultures is unaltered after disruption of the *hlp* gene (Lee *et al*., 1998).

Here we show, contrary to previous reports, that *M. smegmatis* Hlp accumulates intracellularly, and that it can associate with the bacterial nucleoid *in vivo*. Because eubacterial HU homologs, such as *Thermotoga maritima* HU (TmHU) and *Bacillus subtilis* HU, can cause compaction of genomic DNA when overexpressed in *E. coli* (Mukherjee *et al*., 2008a), we also overexpressed Hlp in *E. coli* for comparison against genomic DNA compaction by TmHU. Quantitative Western blot analyses indicate a 10-fold upregulation of *M. smegmatis* Hlp in response to cold shock, and that exponentially growing cultures contain an average of 120 molecules per cell, a much lower cellular concentration than that estimated for other HU homologs.

Materials and methods

Bacterial strains and protein preparations

Mycobacterium smegmatis (MC^2 155 strain from American Type Culture Collection) was grown in Middlebrook 7H9 broth (Difco Laboratories) containing albumin and dextrose enrichment. The cells were grown at 37 °C and 250 r.p.m. until $OD_{600\,nm}$~0.1; for cold shock, cells were then transferred to a 10 °C prechilled enclosed shaking water bath at 250 r.p.m. for 24 h to induce cold shock (Shires & Steyn, 2001) and then again grown for 1 h at 37 °C. For growth on solid medium, Middlebrook 7H10 bacto agar was used (Difco Laboratories) as per the manufacturer's instructions. Hlp and truncated variants were prepared and characterized as described (Mukherjee *et al*., 2008a). TmHU was obtained as described (Grove & Lim, 2001).

Western blotting

For detection of Hlp, monoclonal antibody 3A10 was used that was originally generated against *Notophthalmus viridescens* histone H1 (DiMario & Gall, 1990). Perchloric acid was used to extract H1 from enriched newt liver nuclei, followed by gel purification and partial sequencing to confirm purity of the antigen. Mouse monoclonal antibodies were generated and characterized as described (DiMario & Gall, 1990). For Western blot analysis, protein samples were obtained from 2-mL *M. smegmatis* cell culture pellets before and after cold shock by resuspending them in 50-μL Laemmli sample buffer followed by boiling at 90 °C for 30 min (shorter times of incubation resulted in incomplete lysis). Samples were electrophoresed along with dilutions of recombinant Hlp on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane for 4 h at 100 V. The polyacrylamide gel was stained with Coomassie blue after transfer to ensure complete transfer of proteins. The PVDF membrane was blocked with 5% nonfat dry milk dissolved in 1 × phosphate-buffered saline (PBS) with 0.5% Tween 20 (PBST) for 1 h at room temperature. The membrane was then incubated overnight at 4 °C with 1 : 5000 dilution of mouse anti-histone H1 antibody (3A10; DiMario & Gall, 1990) followed by three washes with 1 × PBST and incubation with 1 : 100 dilution of goat anti-mouse immunoglobulin G (IgG) (H+L) horseradish peroxidase-conjugated secondary antibody (BioRad) at room temperature for 1 h. The membrane was washed three times with 1 × PBST and incubated with a colorimetric substrate, Opti-4CN (BioRad), as per the manufacturer's protocol. Concentrations of Hlp were calculated as a function of the integrated density of the bands using ALPHAIMAGER 2200 (Alpha Innotech Corporation). The amount of Hlp in *M. smegmatis* samples was calculated against recombinant Hlp standard curve. Recombinant Hlp was produced in *E. coli* and purified as described (Mukherjee *et al*., 2008a). Only the full-length recombinant monomeric Hlp was considered in the calculations. Bacterial morphology was evaluated under × 100 magnification on a Leica DM RXA2 deconvolution microscope and cells were counted with a hemocytometer. The number of Hlp molecules per cell was estimated by multiplying the moles of Hlp measured in the *M. smegmatis* sample by Avogadro's number and dividing by the estimated number of cells in the sample volume loaded on the gel.

Immunofluorescence microscopy

Indirect fluorescent antibody microscopy was performed as described, with modifications (Cimino *et al*., 2006; Dasgupta *et al*., 2006). Cells were harvested from exponentially growing bacteria ($OD_{600\,nm}$~0.2) and resuspended in 1/3–1/6 volume of 4% paraformaldehyde in 1 × PBS and fixed by incubation for 15 min at room temperature followed by

40 min on ice and 5 min at room temperature with 50 mM NH_4Cl. Alternatively, cells were fixed in the media using 2% (v/v) paraformaldehyde, but no differences were seen in Hlp protein localization. Cells were washed three times in 1 × PBS and resuspended in 150 μL 1 × PBS; for permeabilization, lysozyme was added to a final concentration of 2 mg mL^{-1} and EDTA to 5 mM, and the suspension was incubated at 37 °C for 20 min, followed by 5-min room temperature incubation with Triton X-100 (final concentration 0.1%). Cells were washed three times with 1 × PBS and blocked with 2% bovine serum albumin (BSA) in 1 × PBS at room temperature for 30 min. Incubation with mouse anti-histone H1 antibody 3A10 was carried out in 1% BSA in 1 × PBS at 1 : 100 dilution at 4 °C overnight. After three washes with 1 × PBS the cells were incubated with goat anti-mouse IgG Alexa Fluor 546-conjugated secondary antibody (Invitrogen) at 1 : 200 dilution at room temperature for 1 h. The cells were washed three times in 1 × PBS and resuspended in a final volume of 20 μL 1 × PBS followed by addition of 7 μL 4′,6-diamino-2-phenylindole (DAPI) (Invitrogen) and soaked overnight at 4 °C. Cells were mounted on a slide and DAPI, Alexa Fluor 546 fluorescence and differential interference contrast images were viewed with a × 100 oil immersion objective on a Leica DM RXA2 deconvolution microscope with a SensiCam camera (The Cooke Corporation) and images were captured using SLIDEBOOK 4.1 digital microscopy software (Intelligent Imaging Innovations Inc.). Slides were prepared from three separate cultures, and at least 750 cells were examined from numerous microscopic fields.

Escherichia coli BL21(DE3)pLysS were transformed with pHlp carrying the Hlp gene and grown in Luria–Bertani media at 37 °C, 250 r.p.m. to an $OD_{600\,nm}$∼0.2 and induced with isopropyl-β-D-thiogalactopyranoside (final concentration 1 mM) for 1 h and fixed as previously described. Cells were soaked overnight in DAPI (Invitrogen) and the nucleoid morphology was observed after mounting the cells on a slide and viewing DAPI fluorescence and bright field images under 100 × oil immersion objective on a Leica DM RXA2 deconvolution microscope with a SensiCam camera and compared with the noninduced *E. coli* BL21(DE3)-pLysS. Slides were prepared from two separate cultures, and several microscopic fields were evaluated, totaling *c.* 120 cells.

Results

Association with genomic DNA

Sequence analysis reveals two distinct domains in mycobacterial Hlp homologs (Fig. 1). The N-terminal HU-homologous domain is predicted to adopt the characteristic fold, and conserved residues include proline 63, positioned within the β arms of HU proteins to intercalate between the DNA base pairs during DNA binding (Rice *et al.*, 1996; Swinger *et al.*, 2003). The lysine-rich C-terminus of mycobacterial Hlp (Fig. 1) is similar to eukaryotic histone H1, which contains several S/TPKKA motifs reported to be responsible for DNA condensation (Bharath *et al.*, 2002; Ellen & van Holde, 2004). The predicted conservation of the HU fold as well as sequence motifs in the C-terminal domain, seen to engage DNA in other proteins, suggests a role in DNA-dependent activities.

Several HU homologs have been shown to contribute to organization of genomic DNA *in vivo* (Kellenberger & Arnold-Schultz-Gahmen, 1992; Köhler & Marahiel, 1997; Kobayashi *et al.*, 2002; Mukherjee *et al.*, 2008b). To evaluate whether Hlp may likewise participate in genomic DNA compaction, Hlp was overexpressed in *E. coli*, following which cells were DAPI stained, and their nucleoids were visualized using fluorescence microscopy. TmHU, which was previously shown to compact the *E. coli* nucleoid (Mukherjee *et al.*, 2008b), was also expressed. While the nucleoids of *E. coli* cells not expressing any heterologous protein are somewhat disperse (Fig. 2a), significant DNA compaction is seen on expression of TmHU (Fig. 2b). Similarly, nucleoids of *E. coli* cells expressing Hlp are more compact as compared with wild-type cells (Fig. 2c). Evidently, Hlp retains the ability of other HU homologs to affect nucleoid organization. Whether this effect is due only to Hlp-DNA interaction causing DNA compaction or if it is in part due to coupled effects on transcription–translation processes is unclear (Woldringh, 2002); this caveat notwithstanding, these data indicate that Hlp, like other HU homologs, associates with the genomic DNA *in vivo*.

We further localized Hlp in *E. coli* by immunofluorescence. We have previously used a monoclonal antibody (3A10) against eukaryotic histone H1 to detect the HU homolog from *D. radiodurans* (DiMario & Gall, 1990; Ghosh & Grove, 2006). What histone H1, *D. radiodurans* HU and mycobacterial Hlp have in common is a lysine-rich domain; in H1, a number of S/TPKKA repeats are found, while in *D. radiodurans* HU and in Hlp, PAKKA repeats are found (Fig. 1). As expected, the epitope recognized by this antibody in *D. radiodurans* HU maps to the PAKKA repeats (Ghosh & Grove, 2006). We therefore anticipated that antibody 3A10 would likewise recognize mycobacterial Hlp. Immunofluorescence was therefore performed using the mouse anti-histone H1 antibody 3A10 followed by Alexa Fluor 546-conjugated secondary antibody. *Escherichia coli* cells carrying the plasmid harboring the *Hlp* gene, but not induced to express Hlp, revealed only a few red patches, presumably due to leaky expression of the protein (data not shown). In contrast, overexpression of Hlp resulted in marked cytoplasmic staining (Fig. 2d), confirming that the antibody indeed recognizes Hlp *in vivo*. Colocalization of

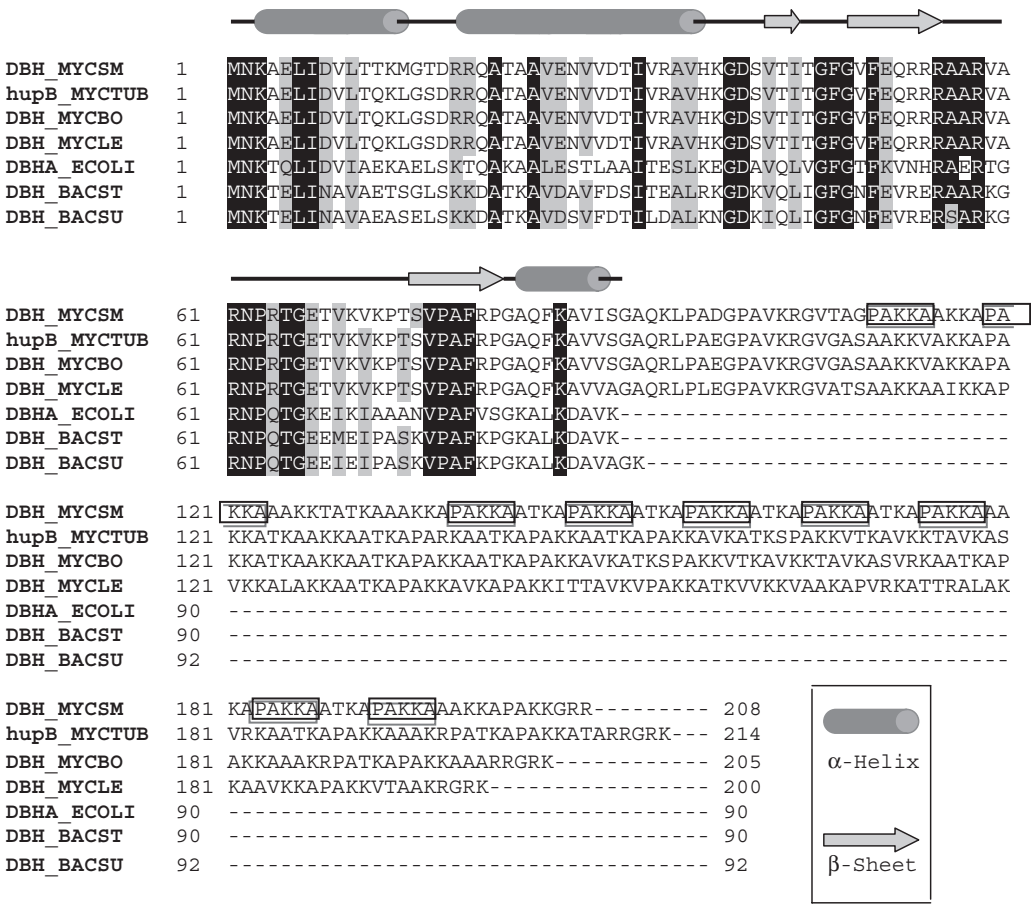

Fig. 1. Multiple sequence alignment of HU proteins [55]. For the HU fold, 80% identical residues are shaded in black and 80% conserved residues are shaded in gray. The sequences in the alignment are, from top to bottom: Hlp from *Mycobacterium smegmatis* (DBH_ MYCSM), *Mycobacterium tuberculosis* (hupB_MYCTUB), *Mycobacterium bovis* (DBH_MYCBO), *Mycobacterium leprae* (DBH_MYCLE), HupA from *Escherichia coli (*DBHA_ECOLI), HU from *Bacillus stearothermophilus* (DBH_BACST), HU from *Bacillus subtilis (*DBH_BACSU). The secondary structures are shown as α-helices or β-sheets above the sequences, based on the *B. stearothermophilus* HU structure (Tanaka *et al.*, 1984). Conserved PAKKA repeats within the *M. smegmatis* Hlp sequence are boxed.

Hlp with the nucleoid appears modest, perhaps due to a failure of Hlp to associate with the surface of the compacted nucleoids and/or an inability of the antibody to interact with Hlp associated with the compacted DNA.

Cellular concentration of Hlp in *M. smegmatis*

Having shown that anti-histone H1 antibody 3A10 recognizes recombinant Hlp *in vivo* (Fig. 2d), we wanted to confirm its reactivity with the lysine-rich C-terminal domain of Hlp. As expected, the antibody recognizes full-length Hlp as determined by Western blotting (Fig. 3a, lanes 3–5). To verify its reactivity with the lysine-rich repeats, Hlp variants were used representing the C-terminal repeat region as well as Hlp truncated for either the entire C-terminal domain or just the lysine-rich repeats. Antibody 3A10 recognizes the protein representing the C-terminal repeats (Fig. 3b, lane 1), but not either of the C-terminal truncations, confirming its reactivity with the lysine-rich repeat region of Hlp.

Antibody 3A10 evidently recognizes recombinant Hlp, both in Western blots as well as *in vivo*. Noting that cold shock has been previously shown to result in upregulation of Hlp, which was then sufficiently abundant to allow its isolation from SDS-PAGE (Shires & Steyn, 2001), there must also be sufficient cellular Hlp content for detection by Western blotting. As shown in Fig. 3a (lane 2), a single band of the expected molecular weight of Hlp (21 230 Da) is seen when Western blotting is performed with *M. smegmatis* cells that have been exposed to cold shock ($OD_{600\,nm}$∼0.1), suggesting specific recognition. Hlp is also detected in cells grown at 37 °C ($OD_{600\,nm}$∼0.25), but in very low abundance (Fig. 3a, lane 1). Lanes 3–5 show increasing amounts of recombinant Hlp; the faster migrating species are likely in

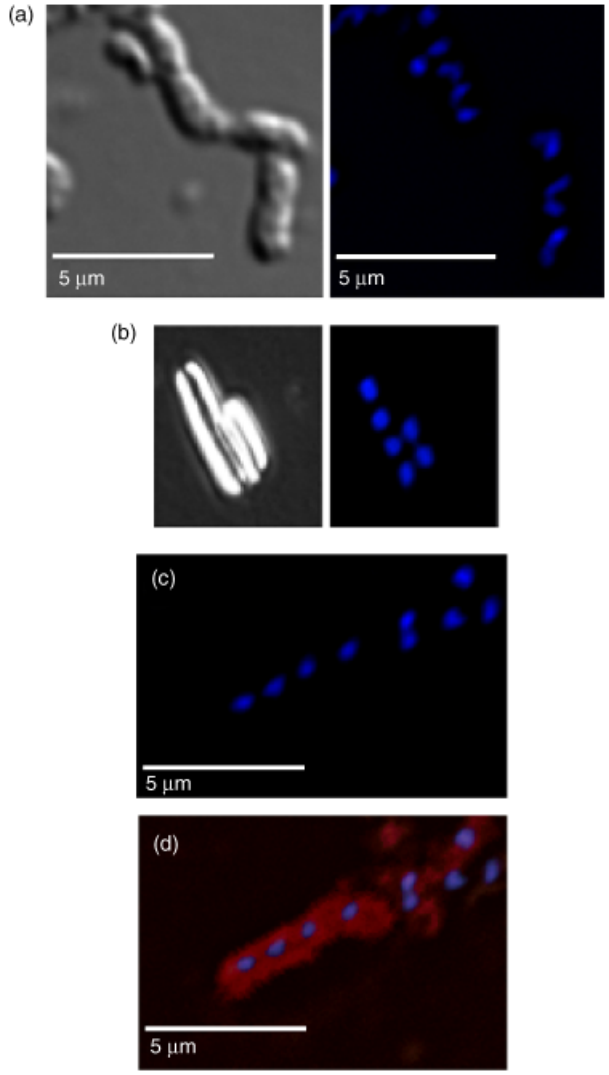

Fig. 2. *Mycobacterium smegmatis* Hlp compacts *Escherichia coli* genomic DNA. (a) Brightfield image of *E. coli* and DAPI-stained (blue) nucleoids of the same field. (b) Brightfield image of *E. coli* expressing TmHU and DAPI-stained nucleoids (blue) (c) DAPI-stained nuclei (blue) of *E. coli* after overexpression of recombinant *M. smegmatis* Hlp. (d) Overexpressed recombinant *M. smegmatis* Hlp identified by anti-histone H1 antibody and Alexa Fluor 546-conjugated secondary antibody (red) and nucleoids stained with DAPI (blue).

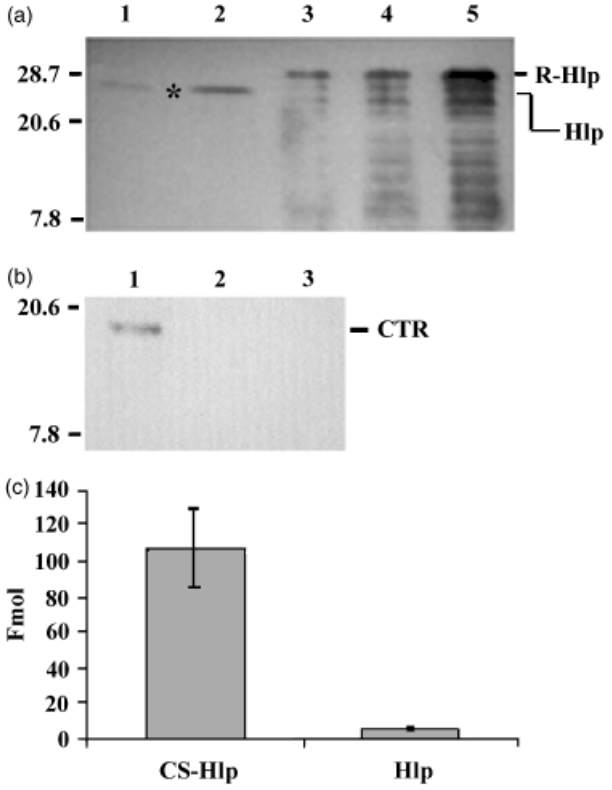

Fig. 3. *Mycobacterium smegmatis* Hlp is upregulated by cold shock. (a) Western blot showing upregulation of Hlp. Position of marker proteins (kDa) shown at the left. Lane 1, Hlp from cells before cold shock ($OD_{600\,nm}$~0.25); lane 2, after 24 h of cold shock ($OD_{600\,nm}$~0.1); lane 3, 1.25 pmol of recombinant Hlp (R-Hlp); lane 4, 2.5 pmol of recombinant Hlp; and lane 5, 5 pmol of recombinant Hlp. R-Hlp migrates higher in the gel due to its 6 × His tag, and both native and recombinant Hlp migrate slower than their calculated molecular mass due to their positive charge. The positions of both native (Hlp) and recombinant Hlp (R-Hlp) are indicated at the right, and an asterisk identifies the Hlp band in lane 1. Truncated species were not included in the densitometric quantitation. (b) Western blot showing only the C-terminal repeat region (CTR) of Hlp is identified by the anti-histone H1 antibody. Position of marker proteins (kDa) shown on the left. Lane 1, CTR (10 pmol; the position of the band is identified by CTR at the right; CTR is lacking the N-terminal 107 residues); lane 2, repeat-less Hlp (10 pmol; residues 1–118) and lane 3, tail-less Hlp (10 pmol; residues 1–97). Note that blots in panels (a) and (b) were developed independently and cannot be quantitatively compared. (c) Quantitation of cellular Hlp per 10 000 *M. smegmatis* CFU for cold shock-induced (CS-Hlp) cells and uninduced cells (Hlp). Error bars represent SD from two experiments.

part produced due to incomplete transcription by T7 RNA polymerase of the numerous $d(C)_n \cdot d(G)_n$ repeats of the gene sequence encoding the C-terminal domain (Krasilnikova *et al*., 1998), which in turn produced truncated protein fragments, and partly due to degradation during overexpression.

The cellular concentration of Hlp was estimated from densitometric comparisons against known concentrations of recombinant Hlp. Upregulation of intracellular Hlp per CFU showed an estimated 20-fold increase as compared with noncold shock-induced cells (Fig. 3c). Under culture conditions, however, *M. smegmatis* forms cell clusters of different sizes, and CFU does not represent the actual cell count. The morphology of cell clusters before and after cold shock was therefore examined and found to be similar in

appearance and pattern. The number of cells was therefore also estimated by counting the average number of cells per cluster, where five or more cells clumped together was counted as a cluster. The semi-quantitative determination of cell count notwithstanding, an estimated average of 117 molecules was found in each exponentially growing *M. smegmatis* cell, with an average of 1112 monomer molecules per cell after 24 h of cold shock, which reflects a 10-fold upregulation in intracellular protein level. This is consistent with the previously reported increase in Hlp mRNA following cold shock (Shires & Steyn, 2001).

Localization of Hlp in *M. smegmatis*

To determine the cellular localization of Hlp, *M. smegmatis* cells were subjected to cold shock to upregulate the Hlp, which was visualized by immunofluorescence using antibody 3A10 followed by Alexa Fluor 546-conjugated secondary antibody. Nucleoids were visualized by DAPI staining. Significant cytoplasmic staining was seen, along with fluorescent patches attributable to the presence of Hlp associated with the nucleoids (Fig. 4a). Notably, staining of the cell surface was not observed. Omission of the anti-histone H1 antibody shows no nonspecific binding of the secondary antibody detectable in the cell or at the cell surface (Fig. 4b). When captured stacks of images (Fig. 5a) were subjected to a 3D best quality volume view using the SLIDEBOOK 4.1 digital microscopy software, the cytoplasmic cellular localization of Hlp and its partial association with the nucleoid can be clearly seen. The DAPI-stained blue nucleoids (Fig. 5b) and Hlp (Fig. 5c) colocalize (Fig. 5d). When the same image was subjected to a 3-D surface view, no red patches were seen (data not shown), indicating the absence of surface-exposed epitopes recognized by the antibody.

Hlp was also localized in cells not subjected to cold shock and was again seen to be cytoplasmic and partly associated with the nucleoid, but the red patches were smaller and had lower intensities (data not shown) due to the lower number of available molecules (Fig. 3). When cold shock-induced *M. smegmatis* cells were subjected to only fixation and no cell wall treatment for permeabilization and processed for immunofluorescence, bright red patches were seen at the cell wall surface; however, this staining was entirely due to nonspecific binding of IgG (Alexa Fluor 546-conjugated secondary antibody) to cell wall proteins (Invitrogen) (data not shown). This is in striking contrast to previous reports indicating that mycobacterial Hlp is associated only with amorphous material surrounding the cell surface (Pethe *et al.*, 2001b; Soares de Lima *et al.*, 2005).

Discussion

Mycobacterium smegmatis Hlp is nucleoid associated

To cause infection, pathogenic mycobacteria must first adhere to their target cells within the host. For *M. tuberculosis*, a heparin-binding hemagglutinin adhesin (HBHA), exposed on the bacterial surface, is involved in adherence to epithelial cells (Menozzi *et al.*, 1996; Pethe *et al.*, 2001b). HBHA contains lysine-rich repeats akin to those of the Hlp C-terminal domain. The saprophytic *M. smegmatis* also encodes an HBHA homolog that is associated with cell wall fractions, but this HBHA homolog does not appear to play a role in epithelial cell adherence; indeed, *M. smegmatis* exhibits reduced adherence to epithelial cells (Biet *et al.*, 2007). That *M. smegmatis* Hlp is surface-associated was inferred from immunoelectron microscopy using antibody

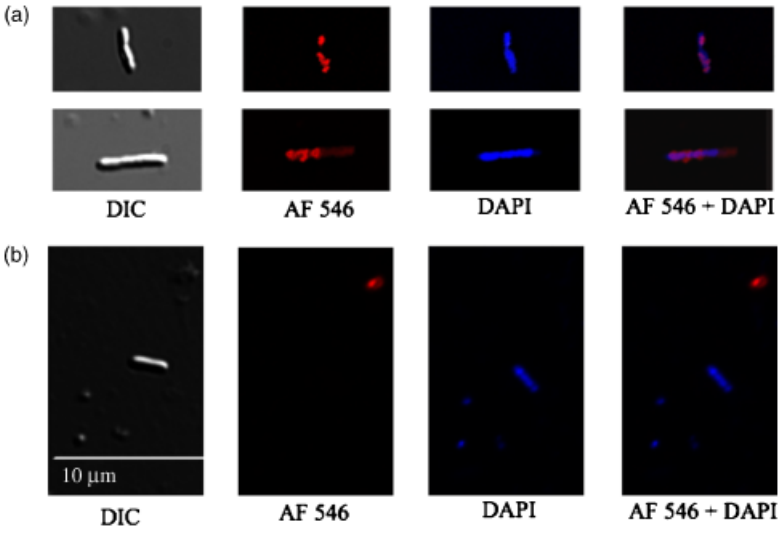

Fig. 4. Immunofluorescence microscopy of cold-shocked *Mycobacterium smegmatis* shows intracellular localization of Hlp. Entire cells are visualized through differential interference contrast (DIC) images. Cells are stained with DAPI to visualize the nucleoid (blue) and Alexa Fluor 546-conjugated secondary antibody (AF 546; red) to visualize Hlp after treating with (a) or without (b) mouse anti-histone H1 primary antibody. Superimposition of DAPI-stained nucleoid and red patches of Alexa Fluor 546-conjugate is shown in the rightmost panels. The red dots in the upper right corner in panel (b) are due to nonspecific binding of Alexa Fluor 546-conjugated secondary antibody to cell debris.

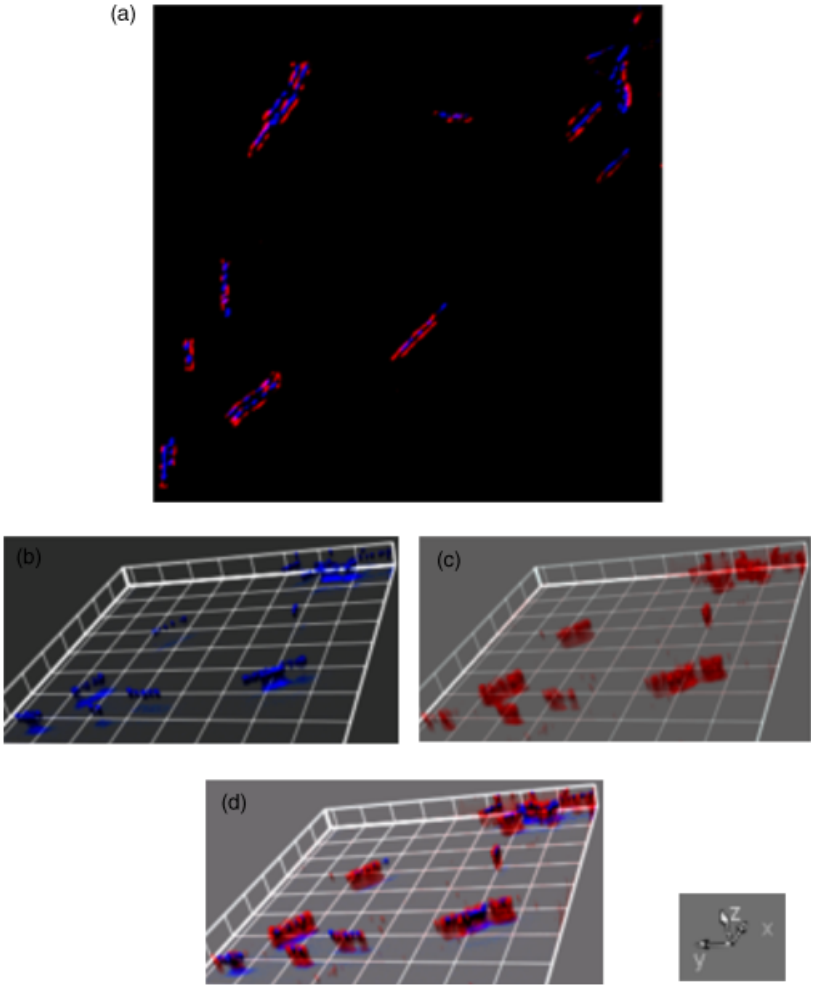

Fig. 5. 3-D volume view of *Mycobacterium smegmatis*. (a) DAPI-stained nucleoids (blue) associated with Alexa Fluor 546-tagged Hlp (red). (b) 3-D volume view of DAPI-stained nucleoid of the field shown in (a). (c) 3-D volume view of Hlp-stained red with Alexa Fluor 546-conjugate. (d) Overlay of (b) and (c). The grids shown in (b), (c) and (d) are 5 μm.

to the lysine-rich C-terminal domain of *M. tuberculosis* HBHA, which cross-reacted with the C-terminal repeats of Hlp; antibody labeling was reported to be associated only with amorphous material surrounding the *M. smegmatis* cells (Pethe *et al*., 2001b). Notably, cells were not permeabilized, and identification of intracellular Hlp was not attempted.

For Hlp to be surface-associated is unexpected given its homology to HU proteins whose functions are in nucleoid organization and DNA metabolism. Our data show that Hlp, when expressed in *E. coli*, organizes the genomic DNA, as evidenced by the formation of more compacted nucleoids (Fig. 2c). However, levels of Hlp expression in *E. coli* are higher than in *M. smegmatis* (compare Figs 2d and 4a), and excess Hlp accumulates in the cytoplasm (Fig. 2d). Notably, a similar cellular localization is observed in *M. smegmatis*, where Hlp accumulates in the cytoplasm and can be seen to colocalize partially with the DAPI-stained nucleoids (Fig. 4).

We note that antibody to histone H1 has been previously reported to recognize mycobacterial Hlp. Histone H1 has a tripartite domain structure in which the C-terminal domain contains a number of S/TPKKA repeats that resemble the recurring PAKKA repeats characteristic of the *M. smegmatis* Hlp C-terminal domain (Fig. 1). The C-terminal histone H1 domain appears to exist as a random coil in solution, likely due to charge repulsion between lysine residues (Clark *et al*., 1988). Association with nucleic acids was speculated to neutralize the excess positive charge, thereby also inducing helical structure. Analogously, we surmise that the Hlp C-terminal domain is unstructured in solution. Notably, the predominant serum marker of ulcerative colitis, an antibody termed pANCA, was reported to recognize an epitope within the C-terminal domain of histone H1 associated with the lysine-rich motifs and to cross-react with mycobacterial Hlp (Cohavy *et al*., 1999; Eggena *et al*., 2000). This would correspond to the epitopes also recognized by antibody 3A10, which recognizes the lysine-rich

repeats in both Hlp (Fig. 3) and *D. radiodurans* HU (Ghosh & Grove, 2006). Further, autoantibodies to nucleolin associated with systemic lupus erythematosus cross-react with histone H1; nucleolin also contains several characteristic lysine-rich repeats (Jarjour *et al.*, 1992).

Mycobacterium smegmatis Hlp was reported to bind laminin and to promote mycobacterial adherence to human pneumocytes and macrophages (Pethe *et al.*, 2001a). Further, exogenously added *M. leprae* Hlp was seen to enhance cellular adherence to a human Schwann cell-derived cell line, indicating a bridging function of the Hlp in target cell adhesion (Soares de Lima *et al.*, 2005). It follows from this observation that surface-associated Hlp need not originate from the bacterial cell to which it adheres. Regardless of the mechanism by which Hlp may become associated with the extracellular surface, we conclude that the permeabilization steps used in our protocol must have removed any surface-associated Hlp, as no surface staining was detectable (Figs 4 and 5). Cells that were not treated for permeabilization showed intense surface staining that was entirely attributable to nonspecific binding of the Alexa Fluor 546-conjugated secondary antibody. In addition to the previously suggested role of mycobacterial Hlp in mediating cell adhesion, the intracellular localization of Hlp reported here strongly suggests that Hlp participates in DNA metabolism.

Mycobacterium tuberculosis can enter a drug-resistant latent stage due to gradual oxygen depletion, and it can resume growth under favorable conditions to cause disease at a later time. Under stressed conditions, cellular metabolism and cellular transcription profiles change and many proteins are upregulated, including *M. smegmatis* Hlp, which is up-regulated under cold shock and when adapting to dormancy (Lee *et al.*, 1998; Shires & Steyn, 2001). Delineating the role of Hlp in DNA-dependent processes may further our understanding of molecular processes that define dormancy.

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