A novel nucleoid-associated protein of Mycobacterium tuberculosis is a sequence homolog of GroEL

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ABSTRACT

The Mycobacterium tuberculosis genome sequence reveals remarkable absence of many nucleoid-associated proteins (NAPs), such as HNS, Hfq or DPS. In order to characterize the nucleoids of M. tuberculosis, we have attempted to identify NAPs, and report an interesting finding that a chaperonin-homolog, GroEL1, is nucleoid associated. We report that M. tuberculosis GroEL1 binds DNA with low specificity but high affinity, suggesting that it might have naturally evolved to bind DNA. We are able to demonstrate that GroEL1 can effectively function as a DNA-protecting agent against DNase I or hydroxyl-radicals. Moreover, Atomic Force Microscopic studies reveal that GroEL1 can condense a large DNA into a compact structure. We also provide in vivo evidences that include presence of GroEL1 in purified nucleoids, in vivo crosslinking followed by Southern hybridizations and immunofluorescence imaging in M. tuberculosis confirming that GroEL1: DNA interactions occur in natural biological settings. These findings therefore reveal that M. tuberculosis GroEL1 has evolved to be associated with nucleoids.

INTRODUCTION

Bacterial chromosomes are typically packaged into compact structures, termed nucleoids. The structure and dynamics of nucleoids is determined by several factors like DNA supercoiling, macromolecular crowding and architectural nucleoid-associated proteins (NAP). The NAP not only influence the structure of the chromosomes but are also involved in replication, recombination, repair and transcription (1,2). The protein composition of bacterial nucleoids varies with cell growth conditions and the growth phase (2,3). Due to their role in chromosome compaction, NAPs also affect basic regulatory processes such as transcription. The study of relative interplay of NAPs in nucleoid compaction and their role in global regulation of bacterial transcription is, however, an under-explored area.

More than 12 DNA-binding proteins have been identified in Escherichia coli, that participate in nucleoid formation (1,2). Among the most important members of bacterial nucleoids are HNS (Histone like nucleoid-structuring protein), HU (Histone like protein first isolated from Ribonuclease negative E. coli U93 strain), IHF (Integrative host factor) and Fis (Factor for inversion stimulation) (4,5). Various studies carried out on these proteins have revealed that the DNA-binding affinity of HNS depends on DNA curvature with a distinct preference for A/T rich tracts (6,7). HNS is also a global transcriptional regulator and silences genes involved in virulence and stress response (8–10). HU is a non specific DNA-binding protein, involved in DNA bending, supercoiling and compaction. It binds nicked, gapped, cruciform as well as single-stranded DNA (11,12) and is also involved in replication, recombination and repair (13). HU has also been proposed to counteract the effects of HNS (14). IHF is a sequence homolog of HU, but binds DNA in a sequence specific manner unlike HU. Fis, like HU and IHF can bend DNA upon binding with high affinity (6). The other prominent NAPs are Lrp (leucine-responsive protein), global-transcriptional repressors, CbpA and CbpB (curved DNA-binding protein), StpA, Dps and Hfq (1,2). Apart from the architectural proteins,
DNA polymerase, RNA polymerase, recombination and repair enzymes and several transcription factors also associate with nucleoids in a temporal manner (2). The presence of several NAPs and their antagonistic functions depict heterogeneity and the global regulation of the balance of forces in bacterial nucleoids (6).

*Mycobacterium tuberculosis* (Mt) genome sequence shows remarkable absence of many NAPs. In order to characterize proteins associated with nucleoids in Mt, we have purified some of them and interestingly discovered that a novel NAP in Mt is a sequence homolog of the GroEL chaperonin. We had earlier reported that Mt GroEL is unable to form canonical tetradecamers, and thus are deficient in folding model substrates in vitro (15). Furthermore, one of the copies of the *groEL* genes in Mt was seen to be undergoing rapid divergence, leading to the speculation that it might be acquiring a new biochemical or physiological function (16). We report here that Mt GroEL1 is capable of recognizing nucleic-acid substrates, without sequence specificity, and plays a role in the condensation of DNA in nucleoid formation. We therefore hypothesize that gene duplication in *Mycobacterial groEL* genes might have led to the new biochemical property of GroEL molecules as a result of alterations in the oligomerization of the molecules.

**MATERIALS AND METHODS**

**Purification of GroEL1 (Rv3417c)**

GroEL1 without (His)6 tag was previously cloned in our laboratory in the expression vector pKK233-2 and expressed in Escherichia coli (data not shown). Cell lysates overexpressing GroEL1 from this plasmid were subjected to precipitation with 30% Ammonium Sulphate. The pellet containing GroEL1 was dialyzed against 50 mM Tris–HCl pH 8.0 supplemented with 1 mM EDTA. The dialyzed protein was loaded on the anion exchanger, Q-Sepharose, and was washed with 50 mM Tris–HCl pH 8.0, supplemented with 150 mM NaCl and 1 mM EDTA. Elution was achieved by increasing the salt concentration to 300 mM. Further purification was performed by Size Exclusion Chromatography (SEC) on the Superdex 200 HR 10/30 column (GE Healthcare). GroEL1 eluted out as a dimer in the SEC.

**Biotin streptavidin pull-down purification**

The biotinylated 5′ oligonucleotide, 5′-TATCTAGAGACACGCTGGCAACCAGGAA-3′ and the corresponding antisense oligonucleotide, 3′-ACGGAGGGGCA/TBE (22.25 mM Tris/Borate/8 M LiCl) were annealed by heating equimolar amounts in 0.5x TE (5 mM Tris–HCl pH 8.0, 0.5 mM EDTA, pH 8.0) to 95°C for 5 min and gradually cooling down to room temperature. Biotinylated oligos were coupled to the streptavidin beads (Streptavidin MagneSphere Paramagnetic particles, Promega) by incubating the biotinylated oligos and streptavidin coated beads in 5 mM Tris–HCl (pH 8.0), 0.5 mM EDTA and 500 mM NaCl for 15 min. The cell lysate in buffer (50 mM Tris–HCl pH 8.0, 30 mM NaCl) was allowed to bind to the beads for 1 h at 30°C with slight stirring and washed three times with wash buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl). The protein was eluted with a buffer containing 1M NaCl supplemented with 50 mM Tris–HCl pH 8.0.

**Electrophoretic mobility shift assay (EMSA)**

EMSA were used to study the binding of Mt GroEL1 to different annealed and γ-P32 ATP end-labeled oligonucleotide DNA. DNA was incubated with protein in binding buffer A (10 mM HEPES pH 7.9, 10% Glycerol, 0.1 mM EDTA, 0.2 mM spermidine, 1 μg poly (dI/dC), 10 mM MgCl2, 2 mM DTT) on ice for 1 h. The DNA–protein complexes were fractionated on a native polyacrylamide gel in 0.25 x TBE (22.25 mM Tris/Borate/0.25 mM EDTA), at 150 V, 4°C for 2–3 h. When noted, non (His)6-tagged Mt GroEL1, (His)6-tagged Mt GroEL1, Mt GroEL2, E. coli GroEL, Human Hsp60, M. leprae Cpn60.2 were added at indicated amounts to the 32P-labeled probe.

The different structured oligonucleotides were obtained by first labeling DNA and subsequent annealing and purification. The region of 100–240-bp upstream of *groES* (Rv3418c) was PCR amplified using primer 5′-TATA TATCTAGAGACACGCTGGCAACCAGGAA-3′ and 5′-TATATAGTCGACCGTGGCAACCAGGAA-3′ and used in the EMSA experiments. Similar experiments were also carried out in the binding buffer with 200 mM NaCl. The gels were dried and analyzed by Typhoon Variable Mode Imager and Image Quant software.

**RNA-binding assay**

The binding of GroEL1 to RNA was studied by EMSA. The RNA (UUCUUGCAUCGCAGUAGGGCGAGUGCUA) used in the assay was chemically synthesized (MWG Biotech) and γ-P32 ATP end-labeled. A quantity of 4 nM RNA was incubated with increasing concentrations of protein in binding buffer (10 mM HEPES pH 7.9, 10% Glycerol, 0.1 mM EDTA, 0.2 mM spermidine, 1 μg poly (dI/dC), 10 mM MgCl2, 2 mM DTT). The RNA–protein complexes were fractionated on a native polyacrylamide gel in 0.25 x TBE at 150 V, 4°C for 2–3 h. All the buffers used in the RNA assay were prepared in DEPC treated water.

**DNA major groove and minor groove-binding assay**

To determine the major and minor groove-binding activities of Mt GroEL1 to DNA, 4 nM of 32P-labeled CIRCE24FR probe was pre-incubated with increasing concentrations of Actinomycin D (Gibco Biosciences) and Methyl green (Sigma) for 20 min at 30°C in a reaction volume of 20 μl in buffer A. Mt GroEL1 (1 μM) was added to the reaction, and the incubation further
continued on ice for 1 h. The DNA–protein complexes thus obtained were resolved in a 7% polyacrylamide gel for 2 h at 150 V at 4 °C.

DNA super coiling-protection assay
To demonstrate the ability of the protein to protect super-coiled DNA, 200 ng pBSK plasmid DNA was incubated with 14 μM of Mtb GroEL1 along with 0.4 μM FeCl3, 10 mM DTT, 100 mM ethanol and 2 mM H2O2 in a reaction volume of 15 μl for 30 min at 37 °C. The reaction was terminated by the addition of 10 mM EDTA, followed by phenol extraction of the sample and analyzing on a 1% agarose gel in 1× TAE at 100 V for 30 min at room temperature.

DNA-protection assay
DNA-protection assay was performed as described previously (17), with minor modifications. An amount of 7 μM Mtb GroEL1 was allowed to interact with linear DNA (2 μg of pBSK, 2958 bp linearized with EcoRI) for 1 h at 37 °C in 10 mM NaCl, 0.5 mM EDTA, 10 mM Tris–HCl pH 8.0. Following incubation, the complex was treated with 1 U of DNase I (NEB) for 5 min at 37 °C. Reactions were terminated by incubation at 75 °C for 10 min, followed by treatment with protease K (50 μg), 5 mM MgCl2, 2% SDS and 0.3 M sodium acetate for 1 h at 37 °C. The protein was extracted with phenol; DNA was precipitated with ethanol and 5 μg of glycogen, and loaded on a 1% agarose gel in 1× TAE at 100 V for 30 min at room temperature.

Atomic force microscopy (AFM)
For AFM, freshly cleaved mica discs were used as substrate for immobilizing DNA. Undamaged pBSK, obtained by purification using alkaline lysis method, was diluted to a final concentration of 1 ng/μl in a buffer containing 40 mM HEPES pH 7.0 and 5 mM NiCl2. Mtb GroEL1 was added to a final concentration of 1 ng/μl in the reaction buffer. After 10 min at room temperature, 5 μl of the reaction mixture was adsorbed on mica for 5 min and washed with 200 μl of Milli-Q grade water for three times. During sample preparation, mounting of the AFM and measurements, biomolecules remain in buffer and are never dehydrated, keeping them functional. Bioscope I Atomic Force Microscope using nanoscope IV (DI, Santa Cruz) was used for imaging. All imaging was performed at room temperature with fluid tapping mode AFM. The images were analyzed using NanoScope v6 software.

Isolation of nucleoids from Mtb
A single colony of M. tuberculosis was inoculated in Middlebrook 7H9 medium supplemented with ADC (BD biosciences) and 0.2% Tween 80 and grown to stationary phase, subcultured and allowed to grow. One-percent Glycine was added and further incubated for 2 h at room temperature. The culture was washed thrice with PBS and then resuspended in ice-chilled acetone. The air-dried pellet was resuspended in a buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP40, 20% sucrose, 1mg/ml of lysozyme and protease inhibitor cocktail (Roche), incubated for 2 h, pelleted at 3000 × g and the supernatant loaded on a 12–60% sucrose gradient. This gradient was centrifuged at 5000 rpm in a swing bucket rotor for 15 min and the bottom of the tube punctured to collect fractions. Presence of DNA in the fractions was assayed by determining the OD260. The volume of the fractions corresponding to the sucrose gradient was plotted against the amount of DNA determined. The fractions with highest DNA content were considered to be fractions containing nucleoids from Mtb (18,19).

Mouse monoclonal antibody against purified Mtb GroEL1 was custom generated (Bangalore Genei). The antibody was found to be highly specific. The isolated nucleoids were assayed for the presence of Mtb GroEL1 by western Blot using the monoclonal antibody.

Chromatin immunoprecipitation (ChIP) assay and Southern blot analysis
ChIP assay was performed using Mtb H37Rv strain. Exponentially growing Mtb cells were cross-linked using 1% formaldehyde and the reaction terminated by adding 125 mM Glycine. The cells were washed thrice with PBS and resuspended in buffer containing 0.5 M sucrose, 20 mM HEPES–KOH pH 7.4, 2 mM EDTA and 7 mM β-mercaptoethanol. Protease inhibitor cocktail (Roche Diagnostics, Germany) and 0.5% NP40 were added and cells ruptured in a bead beater and centrifuged at 2000 × g to remove the unlyzed cells. The supernatant was sonicated in a Branson Sonifier (or Sonicator) thrice for 60 s each on ice to ensure that the fragments of size <750 bp were obtained. The supernatant was pre-cleared; anti-Mtb GroEL1 antibody added and kept overnight at 4 °C. The sample was incubated with 40 μl of Protein A/G agarose beads (Santa Cruz Biotechnology, Inc) for 1 h. The pellet washed with 1 ml each of low salt wash buffer (0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0 and 150 mM NaCl), high salt wash buffer (0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0 and 500 mM NaCl), LiCl wash buffer (250 mM LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 20 mM Tris–HCl pH 8.0 and LiCl wash buffer) and twice with TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0) buffer, and eluted with buffer containing 10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0, 1% SDS and 0.1 M NaHCO3. The eluate was reversed cross linked by incubating at 65°C for 6 h and adding 300 mM NaCl. An amount of 1 μg RNase A and 200 μg Proteinase K was further added and incubated for 1 h at 55 °C. The DNA was purified using QIAquick PCR purification kit (Qiagen) and was labeled using Megaprime DNA-labeling systems (GE Healthcare) and γ32P-dCTP as the labeling agent. The probe was subjected to Southern blot hybridization with GE Healthcare Southern blot hybridization with radiolabeled ChIP eluate. The N+ Hybond membrane were hybridized at 65°C overnight in 6 × SSC, 5 × Denhardt’s reagent,
0.5% SDS, 1 mg/ml sheared salmon sperm DNA, 10% Dextran sulphate. The membrane was washed thrice for 30 min with 0.5× SSC and 0.5% SDS at 65°C. The membrane was wrapped and radioactive signals were read on Typhoon 9200 Phosphor imager (GE Healthcare Lifescience).

**Immunofluorescence microscopy**

Indirect immune-fluorescence microscopy was performed as described (20–22) with minor modifications. Mtb H37Rv cells from exponentially growing bacterial cells (OD_{600nm} ~ 0.8) were smeared on slides, air dried and fixed by incubation with 3% paraformaldehyde in 1× PBS for 15 min at room temperature. The slides were then washed three times with 1× PBS and permeabilized by exposing to 2% tolène for 2 min. The slides were again washed thrice with 1× PBS; air dried, dipped in chilled methanol for 5 min and then in chilled aceton for 30 s. These slides were further washed thrice with 1× PBS and blocked with 2% BSA for 2 h at room temperature. The slides were incubated with appropriate dilution of anti-Mtb GroEL1 monoclonal antibody in 1% BSA–PBS. After extensive washing with 1× PBS, the slides were incubated with Goat anti-mouse IgG Alexa Fluor 594 conjugated secondary antibody (Invitrogen) at a dilution of 1:2000, at room temperature for 1 h. The slides were washed thrice with 1× PBS followed by addition of 10 μl of 4′,6-diamino-2-phenylindole (DAPI) (Invitrogen) in mounting media. The cells were visualized on a Leica TCS SP5 confocal microscope. In the controls for assessing specificity of the primary antibody, incubation with the primary antibody was omitted.

**RESULTS**

*Mtb* GroEL1 binds DNA with high affinity in a sequence non-specific manner

The purified recombinant *Mtb* GroEL1 was very homogenous, and confirmed to be a dimer as reported by us earlier (15). The GroEL1: DNA binding was accidentally discovered while studying the binding of heat shock repressor, HrcA to the conserved upstream-regulatory element, CIRCE (Controlling Inverted Repeat for Chaperone Expression). Previous experimental evidence had suggested that *Bacillus subtilis* HrcA is maintained in an active conformation that is able to bind the CIRCE sequences only in association with the GroEL chaperonin (23). We contrarily observed through electrophoretic mobility shift assays (EMSA) that the GroEL1 of *Mtb*, but not HrcA, binds tightly to the CIRCE2FR (TTA GCCGATTGCCATCTAGCACTCTATACATGAGAG TGCTTAGCTAAGGGGC GCCC C) element. This observation was further tested using biotinylated CIRCE2FR oligonucleotides coupled to streptavidin coated paramagnetic beads, to pull down GroEL1 from *Mtb* cell lysates. Analysis of the proteins eluted in these experiments by Mass Spectrometry confirmed the presence of *Mtb* GroEL1 (data not shown).

In order to test if other GroELs bound DNA similarly, we performed EMSA with *Mtb* GroEL-2 and GroELs from other species. In these experiments, whereas *Mtb* GroEL-2, *M. leprae* Cpn60.2, Human Hsp60 and *E. coli* GroEL failed to bind DNA, only non-(His)$_6$ *Mtb* GroEL1 exhibited DNA-binding ability under similar conditions (Figure 1A and Supplementary Figure 1). Since only *Mtb* GroEL1 bound DNA among the five GroELs tested, it appears that *Mtb* GroEL1 might have specifically acquired this property during evolution. Moreover, our result that non-(His)$_6$ *Mtb* GroEL1 binds DNA unlike the (His)$_6$-*Mtb* GroEL1 indicates that the (His)$_6$-tag at the N-terminal end interferes with the DNA-binding activity of *Mtb* GroEL1. The nucleotide binding by GroEL1 revealed an apparent dissociation constant (K_d) of 143 nM (Figure 1B). Thus, these observations clearly show that GroEL1 binds CIRCE DNA with a high affinity.

In order to explore the range of DNA sequences or structures that *Mtb* GroEL1 is able to recognize, we studied the binding of GroEL1 to different sequences. Figures 2A and B depict the binding of GroEL1 to oligonucleotides which are either annealed to their complementary strands forming a double-stranded oligonucleotide (Figure 2A), or simply the oligonucleotides without their complementary strands so that these remain single stranded or form hairpin structures wherever appropriate (Figure 2B). Moreover, as the single-stranded CIRCE2F has an intrinsic propensity to form a stem-loop like structure, we mutated the CIRCE2F sequence in order to generate different single-stranded structures. The mutated sequences were such that the loop between the inverted sequence element was deleted (MutO2), stem part on either side of the inverted repeat was replaced to disrupt the stem loop structure (MutO3 and MutO6), the flanking sequences on both the 3' and 5' ends were removed (MutO4), and the loop between the inverted repeat element was increased in size (MutO5). Lastly randomized sequence of CIRCE maintaining the same nucleotide composition was also tested (MutO7). A pictorial depiction of the mutated oligonucleotides and their sequences is given in Table 1. Surprisingly, GroEL1 bound to all these oligonucleotides suggesting that it might bind DNA in a sequence and structure independent manner. Furthermore, GroEL1 also interacted with nicked and fork shaped DNA structures (Supplementary Figure 2). The calculated pI of the protein was estimated to be ~4.8, and it seems unlikely that it would bind DNA due to its overall basic charge. Therefore, these experiments suggest that *Mtb* GroEL1 binds DNA without any sequence or structure specificity.

The non-specificity of DNA binding led us to explore the interaction of GroEL1 with a longer fragment of DNA. The longer fragment comprised the sequence or structure or both of the DNA. The longer fragment comprised the sequence or structure or both of the DNA.
GroEL1 binds preferentially to single-stranded DNA compared to double-stranded DNA.

In order to further confirm if GroEL1 preferentially binds to double-stranded (dsDNA) or single-stranded DNA (ssDNA), we monitored the gel shift of radiolabeled dsDNA in the presence of increasing known concentrations of unlabeled dsDNA and ssDNA (Figure 3A). As anticipated from the results of experiments shown in Figure 2A and B, GroEL1 binds to both dsDNA and ssDNA (Figure 3B). However, we observe that ssDNA serves as a better competitor than dsDNA (Figure 3B).
GroEL1 thus binds both ssDNA and dsDNA, but has a preference of binding ssDNA over dsDNA. Furthermore, RNA binding was tested with the RNA sequence of CIRCE, a 28-mer (UUCUUGCACUCGGCAUAGGC GAGUGCUA). It was selected to be a complementary probe to the DNA sequence tested. These experiments revealed that GroEL1 also bound RNA, but with a lower affinity than DNA (Figure 4).

**GroEL1 binds to the minor groove of DNA**

In order to test the groove specificity of binding, drugs that specifically interact with the major or minor groove of DNA were evaluated for their ability to compete with DNA binding. There was no formation of the GroEL1: DNA complex in the presence of Actinomycin D, a minor groove-binding drug (Figure 5A). On the other hand, increasing concentrations of Methyl green, a major groove binder did not affect the formation of GroEL1: DNA complex (Figure 5B). The ability of Actinomycin D to compete with GroEL1: DNA complex formation clearly demonstrates that GroEL1 binds to DNA through the minor groove. The minor-groove-binding proteins are typically known to bind DNA with high affinity, exhibit very different global folds and also remodel the DNA conformation in distinct ways (24). It was therefore tempting to explore the role of GroEL1 in maintaining DNA conformation.

**GroEL1 condenses naked DNA into globular structures**

Sequence non-specificity of DNA recognition by GroEL1 suggested that GroEL1 might be involved in the protection of DNA against external damage. We studied the protection of DNA from external damage by two different experiments, firstly, we tested the prevention of formation of nick by Mtb GroEL-1 in supercoiled DNA in the presence of oxidative radicals and secondly, tests were performed to study the protection of DNA from DNaseI digestion. In our experiments, Mtb GroEL1 was seen to

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**Table 1.** Sequences of mutated CIRCE2F oligonucleotide used in this study

| Oligonucleotide | Sequence | Description |
|-----------------|----------|-------------|
| CIRCE2F         | TTAGCGGATTGCCCATTCTACTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Wild-type |
| MutO2           | ATACTTAGCGATTGCCCATTCTACTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | no loop |
| MutO3           | TTAGCGGATTGCCCATTCTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Stem replaced |
| MutO4           | GTGCACTTAGCGATTGCCCATTCTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Reduced stem |
| MutO5           | TTAGCGGATTGCCCATTCTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Increased loop |
| MutO6           | TTAGCGGATTGCCCATTCTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Stem replaced |
| MutO7           | TTAGCGGATTGCCCATTCTACATGAGGTAGCTAGCCTCAAGGGCGCCCTCTT | Randomized CIRCE |
protect supercoiled DNA from damage by hydroxyl radicals generated by the metal catalyzed oxidation system (Figure 6A). GroEL1 was also observed to protect supercoiled plasmid, pBluescript II SK (+), against DNase I digestion (Figure 6B). Thus, these findings suggest that, the non-specific binding to DNA indeed allows GroEL1 to protect DNA against extrinsic damage.

The nature of complexes formed by GroEL1 and DNA were further examined by AFM. AFM images of 2958-bp supercoiled pBluescript II plasmid DNA in absence of GroEL1 showed uniform structures (Figure 6C), whereas GroEL1 alone was globular (Figure 6D). Many of the GroEL molecules appeared as doublets (Figure 6D), reinforcing our earlier observations that the recombinant GroEL1 is dimeric (15). Interestingly, the protein–DNA complexes showed different degrees of condensation running from partial condensation to very condense globular structures (Figure 6E). This condensation of extended, unconstrained DNA molecules into globular particles is comparable with coil-globule transitions induced by neutral polymers. Therefore, the AFM images clearly establish that GroEL1 is capable of condensing a large DNA.

Evidence for in vivo DNA binding and possible functional significance

The in vitro observations of GroEL1 in protection of DNA against extrinsic damage and condensation of plasmids are reminiscent of NAP. We therefore examined the presence of E. coli NAP protein homologs in Mtb (Table 2). Other proteins, namely Dps, Hfq, H-NS and StpA were conspicuous by their absence in the Mtb genome as indicated by their low BLAST scores. Similarly, among the five sequence-specific NAP CbpB, DnaA, Fis, IHF and Lrp, homologs of only DnaA, Lrp and IHF appear to be present in the Mtb genome. Interestingly Dps, which has the function of protecting DNA under starvation conditions in M. smegmatis, was reported to be absent in the Mtb genome (25). Thus, in the apparent absence of a number of NAP and the ability of Mtb GroEL1 to DNA, a possibility arises that Mtb GroEL1 might have adopted nucleoid association function via non-specific DNA binding.

We sought to test the hypothesis that Mtb GroEL1 might be associated with the nucleoid structures by isolating Mtb nucleoids using sucrose gradient centrifugation as described in ‘Materials and methods’ section (Figure 7A). The presence of only HU, CbpA and IciA in Mtb (Table 2). Other proteins, namely Dps, Hfq, H-NS and StpA were conspicuous by their absence in the Mtb genome as indicated by their low BLAST scores. Similarly, among the five sequence-specific NAP CbpB, DnaA, Fis, IHF and Lrp, homologs of only DnaA, Lrp and IHF appear to be present in the Mtb genome. Interestingly Dps, which has the function of protecting DNA under starvation conditions in M. smegmatis, was reported to be absent in the Mtb genome (25). Thus, in the apparent absence of a number of NAP and the ability of Mtb GroEL1 to DNA, a possibility arises that Mtb GroEL1 might have adopted nucleoid association function via non-specific DNA binding.

We sought to test the hypothesis that Mtb GroEL1 might be associated with the nucleoid structures by isolating Mtb nucleoids using sucrose gradient centrifugation as described in ‘Materials and methods’ section (Figure 7A). The presence of GroEL1 in the purified nucleoids was confirmed by SDS–PAGE followed by western blot analysis using anti-GroEL1 mouse monoclonal antibody (Figure 7B and C). As a control, presence of two cytosolic proteins, GroEL-2 and AhpC, was tested in the purified nucleoids by western blotting. However, these were not detected. It is possible that the presence of GroEL1 in the nucleoids is spurious. Alternately, its presence in the nucleoids could be because of its ability to bind to chromosomal DNA. To distinguish between the two possibilities we performed ChIP analysis on the Mtb nucleoids. Formaldehyde cross-linked DNA–protein complex within
the Mtb nucleoids were subjected to immunoprecipitation using GroEL1-specific antibody. The immunoprecipitated DNA was used as a probe in a Southern hybridization experiment where Mtb genomic DNA digested with different restriction enzymes was blotted on a Nylon membrane (see ‘Materials and methods’ section). As can be seen from Figure 7D, anti-GroEL1 immunoprecipitated fraction, when radiolabeled and used as a probe, binds to several fragments of the Mtb genomic DNA. Even though the pattern of hybridization appears as a smear indicating non-specific binding of GroEL1 to the Mtb chromosomal DNA, a few intensely stained bands can also be seen. This suggests that although GroEL1 binds DNA non-specifically, it might bind some DNA sequences with a higher affinity. In a similar ChIP experiment, anti-GroEL-2 antibody did not show binding to chromosomal DNA. No DNA was observed when the GroEL-2 bound fraction was electrophoresed on an agarose gel or by measuring its Optical Density at 260 nm (data not shown). Therefore, the observed presence of GroEL1 in the Mtb nucleoids is a consequence of its ability to bind Mtb chromosomal DNA.

To further determine if the nucleoid association of Mtb GroEL1 is also observed in vivo, Mtb cells were visualized by immunofluorescence using confocal microscopy (Figure 8). Mouse monoclonal antibody against GroEL1 labeled with Alexa-fluor 594 colocalized with the nucleoid DNA stained with DAPI (Figure 8; panel GroEL1 AF594 + DAPI). However, GroEL1 was not found to be colocalized over the entire length of the DNA, but rather in distinct areas, confirming our ChIP–Southern results that there might be regions of Mtb DNA which have higher affinity for GroEL1. These results therefore confirmed that Mtb GroEL1 indeed is a NAP.
DISCUSSION

Prokaryotic GroEL chaperonins are known to facilitate \textit{de novo} folding of 10–15% cytosolic proteins (26,27). The universality of GroEL mechanism and the occurrence of \textit{groES} and \textit{groEL} genes in all organisms are suggestive of the criticality of \textit{groEL} in all life forms. The chaperonin genes in many prokaryotic species, such as Mycobacteria, have been observed to be duplicated, possibly to provide these organisms with adequate redundancy in the protein folding apparatus (16). Intriguingly, some of the paralogous \textit{groEL} genes do not appear to promote refolding of model substrates \textit{in vitro} (15), or \textit{in vivo} as evidenced by lack of functional complementation in the \textit{E. coli} \textit{groEL} Ts allele (28), (Kumar et al., personal communication). Thus, the role of \textit{groEL} duplication in these organisms is not clearly understood.

Gene duplication is an important evolutionary force that provides an organism an opportunity to evolve new functions (29). Gene duplication events in evolution have been suggested to impart selective advantage to organisms by offering differentiated gene regulation or degeneracy of function. Gene duplication has also been suggested to lead to evolution of promiscuous functions, where this serves as a mechanism to acquire varied substrate spectrum (30). Such functional variations obtained as a result of gene duplication provide organisms an adaptive advantage and a better chance of survival. One of the possibilities of \textit{groEL} paralogy in few prokaryotes might therefore be evolution of promiscuous biochemical functions.

Table 2. Comparison of NAPs in \textit{Mt}b and \textit{E. coli}

| S/No. | \textit{E. coli} | \textit{M. tuberculosis} | \textit{e-value} | Remarks |
|-------|-----------------|-------------------------|------------------|---------|
| 1     | HNS             | Rv2477c                 | 7.2              | Absent  |
| 2     | Hfq             | Rv0511                  | 0.55             | Absent  |
| 3     | StpA            | Rv3296                  | 1.4              | Absent  |
| 4     | Dps             | Rv1522c                 | 0.14             | Absent  |
| 5     | HupA            | Rv2986 (HupB)           | 8$e^{-1}$        | Best BLAST hits for all the three proteins of \textit{E. coli} correspond to the same ORF in \textit{Mt}b. |
| 6     | HupB            | Rv2986 (HupB)           | 4$e^{-11}$       |         |
| 7     | IHF-A           | Rv2986 (HupB)           | 3$e^{-14}$       |         |
| 8     | IHF-B           | Rv2986                  | 0.97             | Absent  |
| 9     | CbpA            | Rv0352 (DnaJ1)          | 8$e^{-40}$       | Best BLAST hit corresponds to the same ORF |
|       |                 | Rv2373c (DnaJ2)         | 8$e^{-22}$       |         |
| 10    | Fis             | Rv3077                  | 1.3              | Absent  |
| 11    | Lrp             | Rv3219c                 | 3$e^{-17}$       | Present |

The \textit{e}-values of BLAST scores, when \textit{E. coli} protein sequences are searched in the \textit{M. tuberculosis} genome, are shown. The 11 proteins known to be involved in nucleoid association in \textit{E. coli} were chosen from literature (2).

Figure 7. \textit{In vivo} evidence for the \textit{Mt}b \textit{GroEL1} binding to DNA. (A) Isolation of nucleoid from \textit{M. tuberculosis H37Rv} strain by sucrose gradient centrifugation. (B) The isolated nucleoids separated on a 10% SDS–PAGE (lane 3) along with \textit{Mt}b whole cell lysate (lane 2) and purified \textit{Mt}b \textit{GroEL1}. (C) The presence of \textit{Mt}b \textit{GroEL1} in nucleoids is confirmed by western blotting (lane 2) along with \textit{Mt}b whole cell lysate (lane 1) and purified \textit{Mt}b \textit{GroEL1} (lane 3) as control. (D) Southern blot hybridization with restriction enzyme digested genomic DNA of \textit{Mt}b and ChIP eluted DNA as probe. Lane 1: EcoRI digested \textit{Mt}b genomic DNA, lane 2: BamHI digested \textit{Mt}b genomic DNA.

Figure 8. \textit{Mt}b \textit{GroEL1} is associated with the nucleoid of \textit{Mt}b. Brightfield image of \textit{Mt}b H37Rv is shown. Cells were stained with DAPI to visualize the nucleoid (blue) and Alexa fluor 594 conjugated secondary antibodies against \textit{Mt}b \textit{GroEL1} monoclonal antibody (red) to visualize \textit{Mt}b \textit{GroEL1}. The superimposition of DAPI stained nucleoid and Alexa fluor 594 stained \textit{Mt}b \textit{GroEL1} is shown. The pink color clearly indicates the colocalization of \textit{GroEL1} with \textit{Mt}b nucleoids.
Mtb GroELs have earlier been reported to exist in unusual oligomeric state with a weak chaperonin activity (15). The C-terminal of GroEL1 is rich in histidine residues, which has been reported to be important for biofilm formation in \textit{M. smegmatis} (31). Moreover, Mtb lacking GroEL1 is shown to be defective in granuloma formation (32). Although the Mtb \textit{groEL} mutant has no phenotype in response to addition of H$_2$O$_2$, and that our work shows that GroEL1 protects against oxidative damage, it is likely that other antioxidant proteins such as thioredoxins (33) or AhpC (34) might help protection \textit{in vivo} by free radical scavenging. It is interesting to note that DPS and IHF are present in the closely related \textit{M. smegmatis} but yet absent in \textit{M. tuberculosis}. The significance of the absence of these proteins in \textit{M. tuberculosis}, and whether GroEL might perform their function of DNA protection in \textit{M. tuberculosis} is not clear. Furthermore, the apparent $K_d$ values obtained is comparable to that obtained for other nucleoid-associated DNA-binding proteins. Our observations therefore suggest that the modes of action of Mtb GroEL1 might be similar to the well-studied NAP.

Consistent with the above hypothesis, Mtb GroEL1 has been observed to bind to both ssDNA and dsDNA in an analogous manner as bovine zeta crystalline, ZTA1, which also binds ssDNA as well as dsDNA and has roles in gene expression, growth and differentiation of bovine lens (35). This hypothesis is further supported by the fact that GroEL1 binds DNA through its minor groove which is typically considered to be insufficient for specific recognition. Many of the minor groove-binding proteins usually bend DNA and recruit other proteins through their interactions with hydrophobic patches to effectively act as a DNA chaperone. Mtb GroEL1 therefore might perform similar functions, namely, to compact the chromosomal DNA via minor groove and sequence independent binding, and by recruiting other proteins through its hydrophobic patches.

We hypothesize that Mtb GroEL1 might have changed substrate specificity from polypeptides to polynucleotides via the \textit{E. coli} GroEL-like substrate-recognizing apical domain. Our earlier observations that Mtb GroEL1 exists as a dimer, and not as a tetradecamer, lead us to speculate that subtle mutations are responsible for a change in the oligomeric state of the protein and thereby substrate specificity. It is therefore tempting to suggest that evolutionary tinkering has led to Mtb GroEL1 acquiring the property of nonspecific DNA binding and an ability to act as a DNA chaperone.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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