Mycobacterium tuberculosis DinG Is a Structure-specific Helicase That Unwinds G4 DNA

IMPLICATIONS FOR TARGETING G4 DNA AS A NOVEL THERAPEUTIC APPROACH

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Background: The G4 helicase in M. tuberculosis is so far unidentified. The Mycobacterium tuberculosis genome is GC-rich and contains >10,000 sequences that have the potential to form G4 structures. In Escherichia coli, RecQ helicase unwinds G4 structures. However, RecQ is absent in M. tuberculosis, and the helicase that participates in G4 resolution in M. tuberculosis is obscure. Here, we show that M. tuberculosis DinG (MtDinG) exhibits high affinity for ssDNA and ssDNA translocation with a 5′ → 3′ polarity. Interestingly, MtDinG unwinds overhangs, flap structures, and forked duplexes but fails to unwind linear duplex DNA. Our data with DNase I footprinting provide mechanistic insights and suggest that MtDinG is a 5′ → 3′ polarity helicase. Notably, in contrast to E. coli DinG, MtDinG catalyzes unwinding of replication fork and Holliday junction structures. Strikingly, we find that MtDinG resolves intermolecular G4 structures. These data suggest that MtDinG is a multifunctional structure-specific helicase that unwinds model structures of DNA replication, repair, and recombination as well as G4 structures. We finally demonstrate that promoter sequences of M. tuberculosis, and DinG is a G4 helicase. We implicate G4 structures and DinG as targets for potential therapy.

The significance of G-quadruplexes and the helicases that resolve G4 structures in prokaryotes is poorly understood. The Mycobacterium tuberculosis genome is GC-rich and contains >10,000 sequences that have the potential to form G4 structures. In Escherichia coli, RecQ helicase unwinds G4 structures. However, RecQ is absent in M. tuberculosis, and the helicase that participates in G4 resolution in M. tuberculosis is obscure. Here, we show that M. tuberculosis DinG (MtDinG) exhibits high affinity for ssDNA and ssDNA translocation with a 5′ → 3′ polarity. Interestingly, MtDinG unwinds overhangs, flap structures, and forked duplexes but fails to unwind linear duplex DNA. Our data with DNase I footprinting provide mechanistic insights and suggest that MtDinG is a 5′ → 3′ polarity helicase. Notably, in contrast to E. coli DinG, MtDinG catalyzes unwinding of replication fork and Holliday junction structures. Strikingly, we find that MtDinG resolves intermolecular G4 structures. These data suggest that MtDinG is a multifunctional structure-specific helicase that unwinds model structures of DNA replication, repair, and recombination as well as G4 structures. We finally demonstrate that promoter sequences of M. tuberculosis, and DinG is a G4 helicase. We implicate G4 structures and DinG as targets for potential therapy.

Tuberculosis, resulting from infection with Mycobacterium tuberculosis, continues to be a leading cause of deaths worldwide. It is estimated that one-third of the world’s population is latently infected with this pathogen, and ~10% of those latently infected will develop active disease (1, 2). The emergence of multidrug-resistant and extremely drug-resistant strains coupled with the HIV/AIDS pandemic has further exacerbated the risk of a tuberculosis resurgence (2), underscoring the development of novel therapeutic interventions. Thus, it is important to understand the pathways and the mechanisms that M. tuberculosis adapts for its survival, persistence, and pathogenesis for the identification of novel drug targets.

In addition to the canonical B-form structure, DNA can fold into various other inter- and intramolecular secondary structures, including G-quadruplexes (G4 structures) (3). Mounting evidence points toward these structures existing under physiological conditions and playing functional roles in vivo. The G-rich sequences that fold into G-quadruplex structures (G4 DNA) are conserved throughout evolution and are abundantly present in telomeres, mitotic and meiotic double strand break sites, and transcriptional start sites (3–5). The human genome contains >375,000 G-rich sequence motifs that have the potential to form G4 DNA (6). During replication, unwinding of DNA duplexes provides the opportunity for G-rich sequences to assume G4 DNA structures. In addition, G4 structures that play roles in transcription could be present during DNA replication. Pre-existing G-quadruplexes or those formed during DNA replication block DNA replication and may lead to DNA breakage and cause genome instability (4). Thus, G4 structures need to be resolved for faithful completion of DNA replication, and helicases are implicated in unwinding G4 DNA structures (3,7). Indeed, mutations in RecQ helicases BLM and WRN as well as FANCJ, CHL1, PIF1, and RTE1 helicases that have been shown to resolve G4 DNA are associated with various chromosome instability and cancer susceptibility genetic disorders (4, 7, 8). Similar to the human genome, G4 DNA-forming sequences exist in prokaryotes. A bioinformatics study identified that the Escherichia coli genome contains ~3,000 G4 DNA-forming sequences (9). E. coli RecQ family helicases, such as Saccharomyces cerevisiae-encoded Sgs1 and human BLM and...
WRN helicases, have been shown to catalyze unwinding of G4 DNA in an ATP-dependent fashion (10–12). *E. coli* RecQ helicase also has been shown to resolve G4 structures (13). The genome of *M. tuberculosis* is GC-rich (65%) and possesses ~10,000 G-rich motifs that can fold into G4 structures (9). Notably, the ortholog of *e. coli* recQ is absent in *M. tuberculosis* (14, 15), and currently, it is unclear how this pathogen deals with resolving G4 structures in vivo.

*E. coli* DinG (EcDinG)\(^5\) belongs to the iron-sulfur cluster family of proteins and is an ortholog of mammalian FANCJ helicase (16, 17). *E. coli* DinG is SOS-regulated, and its expression increases in response to DNA damage (18, 19). Deletion or overexpression of dinG causes mild UV sensitivity, and purified protein exhibits helicase activity with a 5′ → 3′ polarity (20). *E. coli* DinG unwinds 5′ flap structures, D-loops, and R-loops but lacks three-way and Holliday junction (HJ) unwinding activity (21). A recent study shows that *E. coli* DinG resolves intermolecular G4 DNA structures (22). Nonetheless, the precise role of DinG helicase in DNA repair, recombination, and replication or G4 DNA metabolism is unclear. Moreover, the role of DinG helicase in other bacteria, including pathogenic *M. tuberculosis* is unknown. Here, we show that *M. tuberculosis* DinG (MtDinG) helicase exhibits structure-specific unwinding activity with a wide variety of DNA substrates that include overhang substrates, flap structures, forked duplexes, replication fork structures, and HJs. Our detailed examination reveals that both *E. coli* and MtDinG translocate unidirectionally on ssDNA with a 5′ → 3′ polarity. DNase I footprinting studies provided mechanistic insights into the helicase activities of DinG. Notably, MtDinG also displayed robust G4 DNA-resolving activity. These results suggest that MtDinG is a multifunctional helicase that may play a crucial role in various DNA metabolic pathways, including G4 resolution, in vivo. Finally, we demonstrate that G4 structure-forming sequences are conserved in the *M. tuberculosis* genome and discuss the implications for targeting these structures as well as DinG helicase for therapy.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Fine chemicals were purchased from Sigma and GE Healthcare. T4 DNA ligase, and restriction enzymes were purchased from New England Biolabs. T4 polynucleotide kinase and DNase I were purchased from Thermo scientific. [γ-\(^{32}\)P]ATP was purchased from Bhabha Atomic Research Center (Mumbai, India). *Haemophilus influenzae* UvrD was purified as described (23).

**Cloning, Overexpression, and Purification of *M. tuberculosis* DinG**—*M. tuberculosis* dinG (Rv1329c) sequence was obtained from the Tuberculist Web server. The gene was amplified using forward (5′-AATGATCATATGAGAAGACTCTGTATT-3′) and reverse primers (5′-TGAATCTCGACTAGTTAGGCCGTTTACGCCTGA-3′). The forward primer contained a restriction site for NdeI and tobacco etch virus protease, whereas reverse primer had an XhoI restriction site. *Mt* dinG was amplified by PCR using *M. tuberculosis* HIS7Ra genomic DNA and cloned into *pET28a* expression vector. Walker A motif K55R mutant MtDinG was generated by PCR using the primers with a desired mutation and cloned into *pET28a* expression vector using NdeI and XhoI restriction sites. The wild-type (WT) N-terminal His-tagged MtDinG was overexpressed in *E. coli* C43 (DE3) cells by transforming *pET28a* MtDinG plasmid. Cells were grown at 37 °C in LB medium supplemented with 50 μg/ml kanamycin. When the *A_{600}* reached 0.6, protein expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 7 h at 24 °C. The cells were collected by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 0.5 mM EDTA, 5 mM 2-mercaptoethanol) and lysed by sonication. The clear lysate was applied onto an Ni\(^{2+}\)-NTA-agarose column pre-equilibrated with buffer A. The column was washed with 10 bed volumes of buffer A containing 0.5 M NaCl and 20 mM imidazole. The bound protein was eluted with a linear gradient of 20–250 mM imidazole in buffer A. The protein fractions were collected, dialyzed against buffer A, and passed onto a heparin-Sepharose column that was pre-equilibrated with buffer A. The protein was eluted with a linear gradient of 100 mM to 1.0 M NaCl in buffer A. The fractions containing MtDinG were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol). Later, it was loaded onto the Sephacryl S-200 column that was pre-equilibrated with buffer B. The peak MtDinG fractions were pooled and concentrated by Amicon ultracentrific (30 kDa), and the aliquots of protein in buffer A with 5 mM DTT were stored at -80 °C. Using a similar protocol, MtDinG K55R mutant was purified and stored at -80 °C.

**Purification of *E. coli* DinG—Nucleotide-deficient *E. coli* AU1115 (ΔrecGsendA), a derivative of BL21pLysS (a kind gift from R. Lloyd), was transformed with *pET30 EcDinG* (a kind gift from Camerini Otero, National Institutes of Health). Cells were grown at 37 °C in LB medium supplemented with 50 μg/ml kanamycin. When the *A_{600}* reached 0.75, protein expression was induced by 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 7 h at 24 °C. The cells were collected by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellet was resuspended in buffer A (50 mM sodium phosphate, pH 8.0, 25% glycerol, 1 M NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol) and lysed by sonication. The clear lysate was applied onto an Ni\(^{2+}\)-NTA-agarose column pre-equilibrated with buffer A. The column was washed with 10 bed volumes of buffer A containing 10 mM imidazole, and the bound protein was eluted with a linear gradient of 10–250 mM imidazole in buffer A. The protein fractions were visualized on 10% SDS-PAGE, and the peak fractions containing EcDinG were pooled, dialyzed against buffer A, and passed onto a heparin-Sepharose column that was pre-equilibrated with buffer A. The protein was eluted with a linear gradient of 100 mM to 1.0 M NaCl in buffer A. The fractions containing EcDinG were pooled and dialyzed against buffer B (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol). Later, it was loaded onto the Sephacryl S-200 column that was pre-equilibrated with buffer B. The peak EcDinG fractions were pooled and concentrated by Amicon ultracentrifugation (30 kDa), and the aliquots of protein in buffer A with 5 mM DTT were stored at -80 °C. Using a similar protocol, EcDinG K55R mutant was purified and stored at -80 °C.
and loaded onto the Sephacryl S-200 column that was pre-equilibrated with buffer C. The peak EcDinG fractions were pooled and concentrated by Amicon ultracentrifugon (30 kDa cut-off) using buffer C containing 100 mM NaCl and 5 mM DTT; later, the aliquots were stored at −80 °C.

UV-visible Spectrum—Purified MtDinG protein was analyzed for the iron-sulfur cluster by absorbance at 403 nm using 100 μl of purified MtDinG in Tris-HCl (pH 8.0) buffer was mixed with 30 μl of concentrated HCl and heat-denatured at 100 °C for 15 min. The denatured protein mix was centrifuged for 5 min at 13,000 rpm at room temperature. The supernatant (100 μl) was transferred to a fresh tube and mixed thoroughly with 1.3 ml of Tris-HCl (pH 8.5). Freshly prepared 5% ascorbic acid (100 μl) and 0.4 ml of 0.1% bathophenanthroline were added sequentially and thoroughly mixed. The reaction was incubated for 1 h at room temperature, and later, absorbance was measured at 535 nm using a 1-cm optical path length quartz cuvette in a UV-visible spectrophotometer (Shimadzu UV-160). Using the molar extinction coefficient of bathophenanthroline 22 (369 mol⁻¹ cm⁻¹), the number of moles of iron bound to MtDinG was calculated.

Preparation of DNA Substrates—All oligonucleotides used in this study were purchased from Sigma-Aldrich and IDT (Table 1). Oligonucleotides were labeled at the 5’ end with [γ-32P]ATP using T4 polynucleotide kinase in the buffer provided by the manufacturers. The radiolabeled oligonucleotides were purified through a 1-ml Sephadex G50 column. Oligonucleotide substrates (Table 2) were prepared by annealing 1:1.5 molar amounts of 5’-γ-32P-phosphorylated and non-phosphorylated oligonucleotides in a 50-μl reaction volume containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1× SSC (3.0 M sodium chloride and 0.3 M sodium citrate). The reaction mixture was heated to 95 °C for 5 min and then cooled slowly in the thermal cycler. The assembled substrates were purified by resolving on 8% native PAGE. All of the G4 DNA substrates were prepared as described previously (11, 12, 24). Briefly, 2 μM or 30 pmol (for tailed G4 structures) of radiolabeled DNA was boiled for 5 min and then allowed to cool gradually over a period of 2 h in a reaction containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA (TE) and 100 mM KCl. For tailed G4 structure preparation, in addition to 100 mM KCl, 100 mM NaCl was used in the reaction buffer. For the preparation of unmolecular G4 DNA from the M. tuberculosis genome, the sequences were retrieved from the PROQUAD G4 DNA database (9), and the formation of G4 DNA was verified using the Non-B DNA database (25) with the criteria of stem size 3–5 and loop length 1–5 nucleotides. From these sequences, G4 DNA was formed using 0.5 μM radiolabeled oligonucleotide in a reaction containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl. The reaction mixture was heated to 95 °C and allowed to cool in thermal cycler over a period of 2 h. The intramolecular G4 formation was identified by the rapid migration on 15% non-denaturing polyacrylamide gels in 1× TBE relative to the unstructured oligonucleotide in the presence or absence of KCl. All of the G4 DNA substrates were resolved and purified from 15 and 18% non-denaturing polyacrylamide gels in 1× TBE containing 100 mM KCl.

Mass Spectrometry and Peptide Mass Fingerprinting—Purified MtDinG (2 mg/ml) was dialyzed in a buffer containing 20 mM Tris-HCl, 1 mM DTT, and 50 mM NaCl and spotted on a MALDI-TOF MS plate using 4-chloro-α-cyanoanodic acid as matrix. MALDI-TOF analysis was performed in an Ultraflex TOF mass spectrometer (Bruker Daltonics) using the positive ion detection mode. Peptide mass fingerprinting of tryptic-digested MtDinG was performed as described (26).

Electrophoretic Mobility Shift Assay—The assays were carried out as described previously (27). Briefly, the reaction mixture (20 μl) containing 25 mM Tris-HCl, 1 mM DTT, 0.1 mg/ml BSA, 5 mM MgCl₂, and 0.5 mM 32P-labeled DNA substrates was incubated with increasing concentrations of MtDinG for 15 min at 37 °C. The DNA-protein complex was resolved on 6% non-denaturing polyacrylamide gels using 0.25× TBE (22.5 mM Tris borate buffer, pH 8.3, and 0.25 mM EDTA) and visualized by a phosphor imager (Fuji FLA-5000, Tokyo, Japan). Reaction products were quantified using Fujifilm MultiGauge software (version 3.0); data were subjected to nonlinear regression analysis and plotted using GraphPad prism (version 5.0; San Diego, CA).

Helicase Assay—The reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mg/ml BSA, 0.5 mM 32P-labeled DNA substrate, 5 mM ATP, and 5 mM MgCl₂. The reactions were initiated by the addition of either EcDinG or MtDinG and incubated at 37 °C for 30 min. Later, the reactions were terminated by the addition of 5 μl of stop buffer (2% SDS, 0.1% (w/v) bromphenol blue, xylene cyanol in 20% glycerol and 5 mg/ml proteinase K) and further incubated for 20 min. The products were separated on an 8–10% non-denaturing polyacrylamide gel in 0.5× TBE at 150 V for 2.5 h at room temperature. For G4 unwinding assays, 10 mM KCl was included in the reaction mixtures as well as in the 1× TBE for resolving the G4 unwound products using 10% non-denaturing polyacrylamide gel. The products were quantified by phosphor imager (Fuji FLA-5000) and analyzed using Fujifilm MultiGauge software.

Streptavidin Displacement Assay—The reaction mixture (20 μl) contained 0.5 nm 32P-labeled biotinylated oligonucleotide in a helicase assay buffer (50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mg/ml BSA) with 5 mM ATP, 5 mM MgCl₂, and 100 nM streptavidin. The reaction mixture was preincubated for 5 min at 37 °C to facilitate streptavidin binding to biotin. The reactions were initiated by the addition of EcDinG or MtDinG and incubated for 15 min at 37 °C. Later, 1 μM free biotin was added before terminating the reaction with 5 μl of stop buffer (2% SDS, 0.1% (w/v) bromphenol blue, xylene cyanol in 20% glycerol and 5 mg/ml proteinase K) and further incubated for 20 min. The products were separated on an 8% non-denaturing polyacrylamide gel in 0.5× TBE at 150 V for 2.5 h at room temperature. The products were quantified by a phosphor imager (Fuji FLA-5000) and analyzed using Fujifilm MultiGauge software.

ATPase Assays—ATPase assay was performed as described previously (28).

DNase I Footprinting—DNase I footprinting was carried out as described previously (28). Reaction mixtures (40 μl) con-
M. tuberculosis DinG Resolves G4 DNA

Cloning and Purification of M. tuberculosis DinG—E. coli

DinG helicase belongs to the iron-sulfur family of helicases that catalyzes unwinding of branched DNA molecules (21). To investigate whether MtDinG can bind to synthetic substrates that resemble intermediates of various DNA metabolic pathways, such as replication, repair, and recombination, we carried out electrophoretic mobility shift assays. The sequences of oligonucleotides and the different substrates used in this study are shown in Tables 1 and 2. Each substrate was prepared using one of the oligonucleotides that was labeled with \( [\gamma-^{32}\text{P}]\)ATP as described under “Experimental Procedures.” The indicated radiolabeled DNA substrates were incubated individually with increasing concentrations of purified MtDinG. The DNA-protein complexes were separated on 6% polyacrylamide gel and analyzed as described under “Experimental Procedures.” MtDinG exhibited robust DNA binding activity with ssDNA; the DNA-protein complex was evident at the tested lowest concentration of protein (25 nm), and the maximum binding was observed at 500 nm MtDinG (Fig. 2A). Similarly, we tested the DNA binding activity of MtDinG with overhang substrates, flap duplexes, and other types of branched DNA molecules. However, compared with ssDNA, MtDinG exhibited relatively weak DNA binding activity with 3’ or 5’ overhang substrates, 3’ or 5’ flap structures, and forked duplex as well as three-strand junctions (Fig. 2, B–G and K). Notably, MtDinG failed to exhibit a significant
binding with replication fork, three-way junction, and blunt end duplex DNA substrates that lack ssDNA tails, indicating that MtDinG requires ssDNA overhangs to bind to different DNA substrates (Fig. 2, H–J). The quantitative data clearly show that MtDinG preferentially binds to ssDNA in contrast to other types of replication/recombination intermediate substrates that lack ssDNA overhangs (Fig. 2K). The data in Fig. 2A show that at 500 nM MtDinG, it was able to form ~100% DNA-protein complexes with 83-mer ssDNA. We also compared the binding affinity of purified K55R MtDinG with ssDNA substrates and overhang substrates and found that Walker motif mutant binds to the DNA as efficiently as WT protein (data not shown).

The Helicase Activity of M. tuberculosis DinG—E. coli DinG has been shown to unwind branched DNA substrates with a 5′ → 3′ polarity (20). To investigate the helicase activity of MtDinG and the substrate preference as well as the polarity of unwinding, we assembled various branched DNA molecules, and MtDinG helicase activity was measured. Radiolabeled blunt-ended double-stranded DNA (dsDNA), 5′ or 3′ ssDNA overhangs, and 5′ or 3′ flap structures were incubated with increasing concentrations of MtDinG (5–250 nM) in the presence of 5 mM ATP and 5 mM MgCl₂. The unwinding activity was measured as described under “Experimental Procedures.” MtDinG failed to unwind 22-mer and 50-mer dsDNA (data not shown) but exhibited robust unwinding activity with 3′ overhang substrates (Fig. 3, B and D). In a parallel study, MtDinG was proficient in unwinding 5′ overhangs, although maximum activity was found at the highest tested concentration of protein (Fig. 3, A and D). The purified MtDinG Walker A motif K55R mutant was devoid of unwinding activity at the highest tested concentration (250 nM), suggesting that MtDinG-mediated helicase activity requires ATP hydrolysis. In parallel reactions, we compared the helicase activity of EcDinG. Consistent with previous studies (20, 21), EcDinG was able to unwind 5′ overhangs efficiently (Fig. 3, A and C). However, in contrast to ear-

TABLE 1

| Oligonucleotide | Length | Sequence (5′ → 3′) | Reference or source |
|-----------------|--------|-------------------|-------------------|
| G               | 22     | GTA CCC GTG CAT   | Ref. 35           |
| H               | 22     | ACT CTA GAG CTA   | Ref. 35           |
| OD3             | 83     | GAT CTG TGG AGC   | Ref. 83           |
| A               | 52     | AAA ATG AGA AAA   | Ref. 84           |
| B               | 51     | GAC GCT CCG AAA   | Ref. 84           |
| C               | 26     | CAA GAT GAG CTC   | Ref. 84           |
| D               | 24     | TCA GAC ACC AGT   | Ref. 84           |
| E               | 50     | CCA AGT AGC TCC   | Ref. 84           |
| F               | 49     | CAA AGT AGC TCC   | Ref. 84           |
| HX12-7          | 49     | GGC TAC AGG CTA   | This study        |
| HX12-8          | 50     | GGC TAC AGG CTA   | This study        |
| HX12-9          | 51     | GGC TAC AGG CTA   | This study        |
| HX12-10         | 50     | TGC CCG CCG TCG   | This study        |
| 5′Biotin Rest25| 54     | GAC(B) TCC GAG CTA| This study        |
| 3′Biotin Rest15| 55     | AAA ATG AGA AAA   | This study        |
| TP              | 55     | TGG ACC AGA CCT   | Ref. 20           |
| B9′-5′-5′       | 55     | TGG ACC AGA CCT   | Ref. 20           |
| OX-1T           | 39     | ACT GTC GAA CTA   | Ref. 11           |
| 3′-Tail d(CGG)J| 28     | GCT CGG CGG CGG   | Ref. 24           |
| 5′-Tail d(CGG)J| 30     | GCT CGG CGG CGG   | Ref. 24           |
| TP              | 49     | TGC ACC AGA CCT   | Ref. 11           |
| TP-B            | 34     | TGC ACC AGA CCT   | Ref. 20           |
| T8              | 65     | TGG ACC AGA CCT   | Ref. 20           |
| B9              | 55     | TGC ACC AGA CCT   | Ref. 20           |
| a               | 50     | GTC GGA TCT CTA   | Ref. 20           |
| b               | 50     | GCT GGA TCT CTA   | Ref. 20           |
| dsT20+          | 20     | CTA GTG AGC TCT   | Ref. 85           |
| dsB20-          | 20     | CTA GTG AGC TCT   | Ref. 85           |
| 5′T5            | 25     | CTCAGGGTCTACGTTCT | Ref. 85           |
| 5′T15           | 35     | CTCAGGGTCTACGTTCT | Ref. 85           |
| 5′T25           | 45     | CTCAGGGTCTACGTTCT | Ref. 85           |
| 5′T35           | 55     | CTCAGGGTCTACGTTCT | Ref. 85           |
| 5′T45           | 65     | CTCAGGGTCTACGTTCT | Ref. 85           |
| 3′T5            | 35     | GTCAGGGTCTACGTTCT | Ref. 85           |
| 3′T25           | 45     | GTCAGGGTCTACGTTCT | Ref. 85           |
| 3′T35           | 55     | GTCAGGGTCTACGTTCT | Ref. 85           |
| 3′T45           | 65     | GTCAGGGTCTACGTTCT | Ref. 85           |

FIGURE 1. Purification and UV-visible spectrum of M. tuberculosis and E. coli DinG. A, protein sequence alignment of Homo sapiens FANCJ, M. tuberculosis DinG, and E. coli DinG using ClustalW2. The red boxes represent the seven helicase motifs, and the red oval indicates the four conserved cysteine residues that form the iron-sulfur domain. B, purification of MtDinG was monitored by SDS-PAGE in 10% gel. Lane 1, uninduced whole cell lysate; lane 2, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside-induced whole cell lysate; lane 3, induced clarified supernatant; lane 4, 3′-NTA fraction; lane 5, heparin-Sepharose column output; lane 6, eluate from Sepharose 200 gel filtration column; lane 7, major protein from MtDinG K55R mutant from Sepharose 200 gel filtration column. M, molecular mass markers. C, mass spectra of purified MtDinG, a.u., absorbance units. D, purification profile of EcDinG on a 10% SDS-polyacrylamide gel. Lane 1, uninduced whole cell lysate; lane 2, 0.5 mM isopropyl 1-thio-β-D-galactopyranoside-induced whole cell lysate; lane 3, induced clarified supernatant; lane 4, 3′-NTA column output; lane 5, SP-Sepharose column output; lane 6, heparin-Sepharose 200 column. M, molecular mass markers. E, UV-visible spectrum of 1 mg/ml purified MtDinG (violet) and EcDinG (red), showing the shoulder peak at 403 nm, indicated by an arrow. BSA was used as a negative control. Inset, purified MtDinG exhibits a brownish yellow color, indicating that MtDinG is an iron-sulfur cluster-containing protein.
**TABLE 2**
DNA substrates used in this study

| S.No | Name of the substrate                          | Oligonucleotide composition | Structure          |
|------|-----------------------------------------------|----------------------------|--------------------|
| 1    | ssDNA                                         | OD3                        | ![ssDNA structure](ssDNA.png) |
| 2    | Blunt end duplex                              | G*, H                      | ![Blunt end duplex structure](Blunt end duplex.png) |
| 3    | 3' Overhang                                   | B*, D                      | ![3' Overhang structure](3 Overhang.png) |
| 4    | 5' Overhang                                   | A*, C                      | ![5' Overhang structure](5 Overhang.png) |
| 5    | Forked duplex                                 | A*, B or a* + b            | ![Forked duplex structure](Forked duplex.png) |
| 6    | 3' Flap                                       | A, B, D*                   | ![3' Flap structure](3 Flap.png) |
| 7    | 5' Flap                                       | A, B, C*                   | ![5' Flap structure](5 Flap.png) |
| 8    | Heterologous replication fork (parental strand labeled) | A*, B, C, D | ![Heterologous replication fork (parental strand labeled) structure](Heterologous replication fork (parental strand labeled).png) |
| 9    | Heterologous replication fork (leading strand labeled) | A, B, C*, D | ![Heterologous replication fork (leading strand labeled) structure](Heterologous replication fork (leading strand labeled).png) |
|   | Description                                      | Examples                  |
|---|--------------------------------------------------|---------------------------|
| 10 | Heterologous replication fork (lagging strand labeled) | A, B, C, D*               |
| 11 | Three way junction                               | A*, B, F                  |
| 12 | Three strand junction                            | A*, B, E                  |
| 13 | Mobile Holliday junction (HJ)                    | HJX12-7, HJX12-8, HJX12-9*, HJX12-10 |
| 14 | Forked duplex with 3'-3' linkage                 | B9+T8 3'-3'               |
| 15 | Forked duplex with 5'-5' linkage                 | T8+B9 5'-5'               |
| 17 | TP-G4 DNA                                        | TP-G4                     |
| 18 | TPB-G4 DNA                                       | TPB-G4                    |
| 19 | OX-1T-G4 DNA                                     | OX-1T-G4                  |
lier studies (20, 21), EcDinG exhibited significant unwinding activity with 3\(^{rd}\) overhangs (Fig. 3, B and C). Subsequently, we tested the ability of MtDinG to unwind forked duplex substrates as well as 5\(^{th}\) flap and 3\(^{rd}\) flap structures. MtDinG and EcDinG displayed significant unwinding activity with forked duplex DNA (data not shown). Notably, consistent with overhang structures, WT MtDinG exhibited more efficient unwinding of 3\(^{rd}\) flap structures than 5\(^{th}\) flap structures (Fig. 3, E, F, and H). Similar to overhang structures, the ATPase-deficient mutant of MtDinG failed to unwind flap structures and forked duplex substrates (Fig. 3, E, F, and H). These results suggest that MtDinG preferentially unwinds 3\(^{rd}\) overhangs or flap structures rather than 5\(^{th}\) substrates. However, at higher concentration, its unwinding activity with 5\(^{th}\) overhangs or flap structures was comparable with that of 3\(^{rd}\) substrates.

To further understand the polarity of MtDinG-catalyzed unwinding, we used two different previously characterized types of substrates (20). The forked duplex substrate was modified to switch the polarity such that the unpaired single-stranded ends contain either 5\(^{th}\) or 3\(^{rd}\) ends. Consistent with overhang substrates and flap structures, MtDinG was able to efficiently unwind substrates with 3\(^{rd}\) ends more robustly than 5\(^{th}\) ends (Fig. 4, A, B, and D). However, at a higher concentration of protein, MtDinG was equally proficient in unwinding substrates with 5\(^{th}\) ends. In agreement with previous data (20, 21), EcDinG displayed robust unwinding with substrates containing 5\(^{th}\) ends, suggesting the preferential unwinding activity with a 5\(^{th}\) → 3\(^{rd}\) polarity (Fig. 4, B and C). However, it exhibited a significant unwinding with modified substrates containing 3\(^{rd}\) ends (Fig. 4, A and C). Together, these data suggest that MtDinG and EcDinG exhibit helicase activities with substrates that contain ssDNA with either 5\(^{th}\) or 3\(^{rd}\) ends. In contrast to DinG helicase, H. influenzae UvrD (HiUvrD) helicase was able to unwind modified forked duplex substrates only with 3\(^{rd}\) ends and not with 5\(^{th}\) ends (Fig. 4E), suggesting that HiUvrD unwinds DNA substrates in a 3\(^{rd}\) → 5\(^{th}\) polar fashion, which is in agreement with an earlier study (23). In all of our helicase assays, we found that EcDinG was able to display optimal activity between 10 and 25 nM protein, but MtDinG required >100 nM protein (substrate/
protein ratio of 1:200). However, it has been shown that mycobacterial enzymes require >100 nM protein for its optimal activity (32–35).

*M. tuberculosis* as Well as *E. coli* DinG Helicases Exhibit ssDNA Translocase Activity with a 5’ → 3’ Polarity—To understand whether a relation exists with respect to the helicase activity of MtDinG with a translocation on ssDNA, we performed a streptavidin displacement assay using biotinylated oligonucleotides. We used two different types of biotinylated oligonucleotides. One set had a biotin at the 5’ end of ssDNA, and the second had a biotin at the 3’ end. Streptavidin forms a strong complex with biotin with high affinity (Kd ~10^-15 M). Using these substrates, streptavidin displacement was monitored with an incubation of increasing concentrations of EcDinG and MtDinG. Interestingly, both EcDinG and MtDinG failed to displace streptavidin from the 5’ biotin-streptavidin complex (Fig. 5, A, C, and D). In contrast, these proteins were efficient in displacing streptavidin from the 3’ biotin-streptavidin complex, suggesting that EcDinG and MtDinG translocates on ssDNA in the 5’ → 3’ direction (Fig. 5, B–D). Notably, the ATPase-deficient mutant of MtDinG failed to exhibit displacement of streptavidin, indicating that the translocase activity of MtDinG requires ATP hydrolysis. In a parallel experiment, we compared the ssDNA translocase activity of HiUvrD helicase and found that in contrast to DinG, HiUvrD was able to translocate only in the 3’ → 5’ direction (Fig. 5, A and B).

Mechanistic Insights into the Helicase Activity of *M. tuberculosis* DinG—Our data with helicase activity and ssDNA translocation with DinG proteins were striking, such that ssDNA translocation was observed only in the 5’ → 3’ direction, but helicase action was observed with substrates containing either 5’ or 3’ ends. Moreover, our DNA binding as well as unwinding studies clearly indicated that MtDinG was able to bind ssDNA with high affinity and also unwind model substrates that con-
M. tuberculosis DinG Resolves G4 DNA

FIGURE 3. M. tuberculosis DinG unwinds overhangs and flap structures. Reactions contained a 0.5 nm concentration of the indicated 32P-labeled DNA substrate in the absence of EcDinG (lane 1) or MtDinG (lane 8) or in the presence of 0.75, 1.5, 3, 6.25, 12.5, and 25 nm EcDinG (lanes 2–7, respectively) or 5, 25, 50, 100, 125, and 250 nm MtDinG (lanes 9–14, respectively) in the assay buffer containing 5 mM ATP and 5 mM MgCl2. Lane 15, reaction with 250 nm MtDinG KSSR. Lane 16, HD, heat-denatured substrate. The filled triangle at the top of the gel image denotes increasing concentrations of EcDinG or MtDinG. A, 5’ overhang; B, 3’ overhang; C, quantitative data for the ability of EcDinG to unwind overhang structures; D, quantitative data for the ability of MtDinG to unwind overhang structures; E, 5’ flap; F, 3’ flap; G and H, quantitative data for the ability of EcDinG and MtDinG, respectively, to unwind flap structures. Data represent the mean of at least three independent experiments with S.D. values indicated by error bars.

tain either 5’ or 3’ ssDNA. These results imply that MtDinG requires ssDNA for loading and unwinding by translocating on ssDNA. These observations led us to investigate the minimum length of ssDNA that is required by MtDinG and EcDinG for its helicase activity. To address this, we prepared 5’ and 3’ overhang substrates with varying lengths of ssDNA overhangs (0, 5, 15, 25, 35, and 45 nucleotides). With our earlier data (Fig. 3, A and B), we noticed that MtDinG was able to unwind 3’ overhangs more proficiently than 5’ overhangs at lower concentrations of proteins. This could be because MtDinG exhibited a higher affinity for 3’ overhang or 3’ flap structures than 5’ substrates (Fig. 2, B, C, E, F, and K). To rule out any sequence bias in the loading of DinG molecules to ssDNA, we designed oligonucleotide substrates to assemble 5’ or 3’ overhangs with different lengths of thymine (poly(dT)) residues in the ssDNA tail region. In all of these substrates, the 20-bp duplex region was identical. These substrates were incubated with increasing concentrations of MtDinG (0–100 nm), and the products were analyzed as described under “Experimental Procedures.” MtDinG failed to exhibit helicase activity with dsDNA or 5’ or 3’ overhang structures containing a 5-nucleotide overhang (Fig. 6, A–D). However, it was able to show significant unwinding activity.
with both 5’ and 3’ overhang structures that contain 15-nucleotide overhangs, and this activity was maximal with 25-nucleotide overhangs (Fig. 6, A–D). Interestingly, a further increase in unwinding was not observed with the substrates that had 35- or 45-nucleotide overhangs (data not shown). These results are in agreement with our DNA binding as well as ATPase activities of MtDinG with these substrates (data not shown), suggesting that 25-nucleotide overhangs provide an optimal length of ssDNA region for efficient binding of MtDinG to 5’ or 3’ overhang structures and for its helicase activity. Notably, in contrast to earlier data with 3’ overhangs, 3’ flap structures, and forked duplex substrates, MtDinG exhibited more robust unwinding with 5’ overhangs than 3’ overhangs, which is consistent with its 5’ → 3’ translocation polarity.

In a parallel study, we also examined unwinding activity of EcDinG with various 5’ and 3’ overhang substrates. Similar to MtDinG, EcDinG failed to unwind dsDNA as well as 5-nucleotide overhang structures. However, EcDinG exhibited significant helicase activity with 5’ overhangs that contain 15-nucleotide ssDNA tails, and this activity was maximal with 25-nucleotide overhangs (Fig. 6, E and G). Interestingly, EcDinG was less efficient with 15-nucleotide 3’ overhangs but displayed significant unwinding when ssDNA tail was increased to 25 nucleotides (Fig. 6, F and H). Together, these results suggest that both MtDinG and EcDinG helicases require ~25-nucleotide ssDNA tails for their efficient loading as well as optimal unwinding activity with 5’ → 3’ polarity.

To gain mechanistic insights into the helicase activity of MtDinG, we carried out DNase I footprinting experiments with 3’ overhang substrates containing a 25-nucleotide (T-25) ssDNA tail. The longer strand was radiolabeled with [32P]ATP, and overhang substrate was prepared as described under “Experimental Procedures.” In the absence of MtDinG, DNase I hypersensitive sites were found at the ssDNA-dsDNA junction. Interestingly, in the presence of MtDinG, DNase I hypersensitive sites were found at the ssDNA-dsDNA junction. Interestingly, in the presence of MtDinG, DNase I hypersensitive sites were found at the ssDNA-dsDNA junction. Interestingly, in the presence of MtDinG, DNase I hypersensitive sites were found at the ssDNA-dsDNA junction.
tide cofactor can influence the binding pattern of DinG proteins to overhang structures, we carried out a DNase I footprinting assay in the presence of ATP and found that the pattern of the DNase I footprint was very similar compared with the data obtained in the absence of ATP (data not shown). These results clearly suggest that MtDinG and EcDinG bind at the junction of ssDNA and dsDNA. Interestingly, our comparative study showed that binding of MtDinG but not EcDinG to the ssDNA-dsDNA junction resulted in specific cleavage at thymine in the ssDNA-dsDNA junction (Fig. 7A, asterisk), implying that the orientation and binding pattern of MtDinG and EcDinG to overhang structures appears to be different. Based on our observation, we propose a model in which binding of MtDinG and EcDinG to the ssDNA-dsDNA junction of 3' overhangs causes destabilization of the shorter complementary strand from the 5' end, which provides a nucleation site for loading of MtDinG as well as EcDinG. Subsequent to nucleation from the 5' end, DinG protein can translocate on the same strand in a 5'→3' polarity and can unwind the opposite strand (Fig. 7B). However, further studies are required to gain more insight into the mechanism of helicase and translocase activities of DinG.

M. tuberculosis DinG Resolves G4 DNA—Spontaneous or damage-induced replication stalling presents a serious threat to genome stability and cell viability (36–43). In E. coli, RuvABC, RecG, and UvrD helicases have been shown to remodel the stalled replication forks to resume normal DNA replication (44–47). Three-way junctions with a gap represent a stalled fork, and we examined whether MtDinG can unwind these structures. We prepared two differ-
ent replication fork structures, and in one case, we labeled leading strand, and in the second case, we labeled lagging strand. Incubation of increasing concentrations of MtDinG (25–750 nM) with a leading strand-labeled replication fork structure resulted in unwinding of lagging strand, and the 5' H11032 flap structure generated was further unwound by MtDinG (Fig. 8A). Similarly, MtDinG was proficient in unwinding lagging strand-labeled replication fork structures (Fig. 8B). In a parallel assay, we measured the ability of EcDinG to unwind replication fork structures. Interestingly, in contrast to MtDinG, EcDinG failed to unwind these structures (Fig. 8, A and B). We also tested the activity of MtDinG with a three-strand junction and a three-way junction without a gap, and MtDinG was proficient in unwinding three-strand junctions but exhibited a weak activity with three-way junctions (data not shown). In a parallel comparison, EcDinG was able to unwind three-strand junctions but failed to exhibit activity with three-way junctions (data not shown). These data suggest that MtDinG but not EcDinG possesses branch migration activity to remodel replication fork structures by unwinding leading as well as lagging strands. Notably, although MtDinG was able to efficiently unwind overhang substrates, flap structures, and forked duplexes in the low-
M. tuberculosis DinG and E. coli DinG binds at the ssDNA-dsDNA junction of 3’ overhang structures. A, reactions contained 5 nM 32P-labeled 3’ overhang substrate having a 20-bp duplex and 25-nucleotide (poly(dT)) overhang in the absence (lanes 1 and 7) or in the presence of 1, 2.5, and 5 μM MtDinG (lanes 2–4) and 1, 2.5, and 5 μM EcDinG (lanes 8–10). Lanes 5 and 6, Maxam and Gilbert T and A + G sequencing ladders. DNase I protected sites are represented by dots, and the hypersensitive site is represented by an asterisk. B, model to explain the unwinding of 3’ overhang structures by MtDinG (see “Results”).

est range of concentration (5–250 nM), it exhibited proficient unwinding activity of branched substrates at 3-fold higher concentration (25–750 nM) (Fig. 8, A and B).

M. tuberculosis DinG, but Not E. coli DinG, Promotes Holliday Junction Branch Migration—HJs are the intermediate structures that are generated during replication fork restart pathways and during recombinational repair processes (40, 41, 48). Because MtDinG was able to efficiently unwind replication fork structures, we tested whether it can unwind HJs. We assembled radiolabeled mobile HJ substrate that contains 12-bp homology at the core of HJ. Interestingly, MtDinG exhibited significant unwinding activity with mobile HJs (Fig. 8 C). In a parallel reaction, we examined the ability of EcDinG to unwind HJs. Consistent with a previous study (21), EcDinG failed to exhibit HJ branch migration activity even at the highest concentration tested (5–250 nM) (Fig. 8 C). To gain insight into the HJ branch migration activity of MtDinG, we compared the efficiency of HJ unwinding with MtRecG. Consistent with a previous study (28), MtRecG exhibited HJ branch migration activity at the tested lowest concentration (5 nM), and this activity was maximal with 10 nM concentration (Fig. 8 D). However, MtDinG displayed weak HJ unwinding activity in the range of ~50 nM, and this activity increased modestly at higher concentrations (250–750 nM) (Fig. 8 D). In order to characterize the rate of HJ unwinding by MtDinG and MtRecG, we measured the kinetics of HJ branch migration by MtDinG in comparison with MtRecG. As shown in Fig. 8, E and F, MtRecG was able to unwind ~40% of HJ within 5 min, and by 30 min, it was able to unwind ~70% of substrates. In a parallel assay, MtDinG exhibited an ~50% lower rate of unwinding compared with MtRecG (Fig. 8, E and F). These data suggest that MtDinG is a less potent HJ-processing helicase compared with MtRecG.

M. tuberculosis DinG Unwinds G-quadruplex (G4) DNA—Germ line mutations in FANCJ cause Fanconi anemia, a rare chromosome instability and cancer susceptibility genetic disorder (49–52). Biochemical studies show that FANCJ and E. coli DinG helicases resolve G4 DNA (22, 24). MtDinG is an ortholog of human FANCJ; however, it is unknown whether MtDinG can unwind G4 DNA structures. To test whether MtDinG can resolve G4 structures, we radiolabeled an oligonucleotide and assembled previously characterized intermolecular TP-G4 structures (11). We incubated TP-G4 structures with increasing concentrations of MtDinG (5–250 nM). Strikingly, MtDinG exhibited a robust unwinding activity with TP-G4 structures, and 100 nM protein was able to unwind almost all of the TP-G4 structures (Fig. 9, A and F). In a parallel reaction, EcDinG also displayed efficient G4-unwinding activity (Fig. 9, A and E). To validate our observation, we have tested the ability of MtDinG as well as EcDinG to unwind other types of G4 DNA substrates that have been reported (11, 24). Similar to TP-G4 structures, both MtDinG and EcDinG were proficient in unwinding TPB-G4 and OX-1T-G4 G4 structures (Fig. 9, B, C, E, and F).
addition to parallel G4 structures, MtDinG was proficient in unwinding anti-parallel OX-1T-G2 tetraplex structures (Fig. 9, D and F). To understand the rate of G4 DNA resolution by MtDinG in comparison with EcDinG, we measured the kinetics of G4 unwinding at different time intervals using TP-G4, TPB-G4, and OX-1T-G4 parallel G4 structures as well as OX-1T-G2 anti-parallel tetraplex structures. With TP-G4 and TPB-G4 substrates, both MtDinG and EcDinG exhibited a robust G4
unwinding with an efficiency of >80% unwinding by 1 min (data not shown). Similarly, EcDinG and MtDinG were able to efficiently unwind OX-1T-G4 with a similar kinetics (data not shown). However, with anti-parallel OX-1T-G2 tetraplex structures, MtDinG exhibited a slower rate of unwinding compared with EcDinG (data not shown).

The G4-stabilizing ligands TMPyP4 and N-methyl mesoporphyrin IX (NMM) have been shown to inhibit the G4–unwinding activity of E. coli RecQ and RecQ orthologs Sgs1 and BLM helicases (13, 53). To determine the specificity of G4 resolution activity of MtDinG, we incubated increasing concentrations of TMPyP4 and compared the TP-G4- and TPB-G4-unwinding activity of MtDinG. Compared with controls, 50 nM TMPyP4 abolished ∼50% of the TP-G4-unwinding activity of MtDinG, and this activity was suppressed to ∼100% at 500 nM TMPyP4 (Fig. 10, A and B). Similarly, 50 μM NMM caused ∼100% inhibition of TP-G4-unwinding activity by MtDinG (data not shown). In a parallel study, we examined the ability of TMPyP4 to mediate inhibition of TPB-G4 unwinding by MtDinG. Consistent with TP-G4 structures, TMPyP4 abrogated the G4 resolution activity of MtDinG (Fig. 10, C and D). Similar to MtDinG reactions, G4 inhibitors were able to suppress the G4-unwinding activity of EcDinG (Fig. 10, A–D). Notably, both TMPyP4 and NMM did not interfere with MtDinG- and EcDinG-promoted forked duplex unwinding (Fig. 10, E and F) (data not shown). These results further suggest that MtDinG resists G4 structures in vitro, and this activity can be specifically inhibited by the G4-stabilizing TMPyP4 and NMM ligands.

* M. tuberculosis DinG Resolves G4 DNA

Unwinds with an efficiency of >80% unwinding by 1 min (data not shown). Similarly, EcDinG and MtDinG were able to efficiently unwind OX-1T-G4 with a similar kinetics (data not shown). However, with anti-parallel OX-1T-G2 tetraplex structures, MtDinG exhibited a slower rate of unwinding compared with EcDinG (data not shown).

The G4-stabilizing ligands TMPyP4 and N-methyl mesoporphyrin IX (NMM) have been shown to inhibit the G4–unwinding activity of *E. coli* RecQ and RecQ orthologs Sgs1 and BLM helicases (13, 53). To determine the specificity of G4 resolution activity of MtDinG, we incubated increasing concentrations of TMPyP4 and compared the TP-G4- and TPB-G4-unwinding activity of MtDinG. Compared with controls, 50 nM TMPyP4 abolished ∼50% of the TP-G4-unwinding activity of MtDinG, and this activity was suppressed to ∼100% at 500 nM TMPyP4 (Fig. 10, A and B). Similarly, 50 μM NMM caused ∼100% inhibition of TP-G4-unwinding activity by MtDinG (data not shown). In a parallel study, we examined the ability of TMPyP4 to mediate inhibition of TPB-G4 unwinding by MtDinG. Consistent with TP-G4 structures, TMPyP4 abrogated the G4 resolution activity of MtDinG (Fig. 10, C and D). Similar to MtDinG reactions, G4 inhibitors were able to suppress the G4-unwinding activity of EcDinG (Fig. 10, A–D). Notably, both TMPyP4 and NMM did not interfere with MtDinG- and EcDinG-promoted forked duplex unwinding (Fig. 10, E and F) (data not shown). These results further suggest that MtDinG resists G4 structures in vitro, and this activity can be specifically inhibited by the G4-stabilizing TMPyP4 and NMM ligands.

* M. tuberculosis DinG Unwinds Both 5'- and 3'-Tailed G4 Structures Efficiently—*To test whether 5’ or 3’ tails influence the G4 resolution activity of MtDinG, we measured the G4-unwinding activity with previously characterized G4 structures that contain either 5’ or 3’ tails (12, 24). In parallel reactions, we compared the activity of EcDinG. Interestingly, EcDinG and MtDinG were able to efficiently unwind 5’-tailed G4 structures (Fig. 11, A and B). Strikingly, MtDinG, but not EcDinG, was proficient in unwinding 3’-tailed G4 DNA substrates (Fig. 11, C and D). These data suggest that, unlike EcDinG, MtDinG can efficiently unwind both 5’- and 3’-tailed G4 structures. To understand the rate of G4 unwinding with tagged substrates, we performed the kinetics of G4 resolution at different time intervals. Compared with EcDinG, MtDinG exhibited a slower kinetics of G4 unwinding with 5’-tailed G4 structures (data not shown). Notably, in contrast to MtDinG, EcDinG failed to unwind 3’-tailed G4 structures (data not shown).

* G4 DNA-forming Sequences Exist in the *M. tuberculosis* Genome—*The *M. tuberculosis* genome is GC-rich (65%), and a bioinformatics study identified that there are >10,000 GC-rich sequence motifs that have the potential to form G4 structures (9). Notably, G4 DNA-forming sequences are conserved and enriched at the promoter regions of human genes (54). However, experimental evidence for whether GC-rich sequences in the *M. tuberculosis* genome can fold into G4 structures is lacking. To test this, using a bioinformatics tool, we identified many G4-forming motifs in the promoters of *M. tuberculosis* genes. To validate some of these sequences experimentally, sequences at the PE_PGRS2, *mce1R*, and *moeB1* genes were used (Fig. 12A) (Table 3). Oligonucleotides corresponding to these sequences were analyzed by CD. Parallel G-quadruplexes show a peak at ∼260–265 nm and a trough at 240 nm, whereas anti-parallel quadruplexes exhibit a peak at 290 nm and a dip at 260 nm. CD spectra were recorded for all of the three oligomers in the presence of 100 mM KCl, where K+ is known to stabilize the G-quadruplex structures. A characteristic peak at ∼265 nm and dip at 240 nm were evident with *mce1R* and PE_PGRS2 sequences (Fig. 12B), indicating that these sequences form a parallel G-quadruplex structure. In contrast, *moeB1* sequence exhibited peak at 290 nm as well as 265 nm (Fig. 12B), suggesting the existence of a G-quadruplex with parallel and anti-parallel conformation.

To validate our observation obtained with CD as well as to determine whether GC-rich sequences located at promoters of PE_PGRS2, *mce1R*, and *moeB1* form intra- or intermolecular G4 DNA structure, these sequences were analyzed by native polyacrylamide gel electrophoresis in the presence or absence of KCl. The mobilities of these oligonucleotides before and after forming G4 structures were compared with known oligonucleotides that do not form G4 DNA. The electrophoresis was carried out in the absence or presence of 100 mM KCl in 1× TBE buffer. As can be seen in Fig. 12C, in the absence of KCl, all of the three gene promoter sequences migrated closely with the control non-G4 DNA-forming sequences. Interestingly, the GC-rich oligonucleotides migrated faster than the control oligonucleotides in the presence of KCl, suggesting the formation of intramolecular G4 structures (Fig. 12C).

The G4 structures also can be analyzed by a DMS protection assay. The N7 positions of guanines that are present in ssDNA...
and dsDNA are susceptible for modification by DMS. However, if the guanines are involved in Hoogsteen base pairing, which is required for G-quadruplex formation, the N7 position is protected from methylation by DMS. To validate further, we performed a DMS protection assay for the three G4-forming sequences in the presence or absence of KCl. Consistent with our CD spectra and data from gel electrophoresis, in the presence of KCl, the guanine residues of PE_PGRS2, mceIR, and moeB1 sequences were protected from methylation by DMS (Fig. 12D). Together, these data clearly show that G4 structure-forming sequences exist in the M. tuberculosis genome.

**DISCUSSION**

The role of DinG helicase in various DNA metabolic transactions and genome maintenance is poorly understood. Here, we demonstrate that M. tuberculosis DinG possesses structure-specific helicase activity. Although MtDinG did not exhibit structure-specific DNA binding activity, it was proficient in unwinding a wide variety of model substrates that resemble intermediates of DNA repair, recombination, and replication.
For the first time, our study demonstrates that, unlike \textit{E. coli} DinG, \textit{MtDinG} unwinds replication fork and HJ structures, indicating the likely role of \textit{MtDinG} in the rescue of stalled replication forks and in the recombinational repair processes. \textit{E. coli} RecQ and its eukaryotic orthologs unwind G4 structures \cite{8, 55}. Notably, RecQ is absent in \textit{M. tuberculosis}, and for the first time, our study shows that \textit{MtDinG} unwinds intermolecular G4 structures, implying that \textit{MtDinG} could replace RecQ at least in part with G4 DNA unwinding activity. Together, these data suggest that \textit{MtDinG} possesses multiple functions, including G4 DNA resolution, to maintain genome integrity. Finally, our \textit{in vitro} studies demonstrate that G4 DNA-forming sequences exist in the \textit{M. tuberculosis} genome and implicate that these structures can be targeted for therapy using small molecule inhibitors.

We find that \textit{MtDinG} exclusively binds to ssDNA and ssDNA-containing substrates and fails to bind linear duplexes or other structures that lack ssDNA. Interestingly, \textit{MtDinG} exhibited efficient unwinding with 3' overhangs and 3' flap structures compared with 5' substrates, probably due to its higher affinity for these substrates. However, at higher concentrations, it was proficient in unwinding 5' overhangs or 5' flap structures. Interestingly, with our ssDNA translocation assay, both \textit{MtDinG} and \textit{EcDinG} displayed translocation activity in the 5'→3' direction, which is in agreement with the XPD family of helicases \cite{56–59}. These data clearly

\begin{figure}
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\caption{G4-specific ligand TMPyP4 specifically inhibits G4 resolution by \textit{M. tuberculosis} as well as \textit{E. coli} DinG. Reactions containing 5 mM ATP, 5 mM MgCl\textsubscript{2}, 0.5 nM \textsuperscript{32}P-labeled TP-G4 (A), TPB-G4 (C), and forked duplex (E) DNA substrates were incubated either in the absence of EcDinG (lane 1) or MtDinG (lane 9) or in the presence of 2 nM EcDinG (lane 2) and 50 nM MtDinG (lane 10) without TMPyP4. In all panels, lanes 3–8 show the reactions carried out in the presence of 2 nM EcDinG with 0.05, 0.1, 0.25, 0.5, 1, and 2 \mu M TMPyP4 inhibitor, respectively. Lanes 11–16 in all panels show the reactions carried out in the presence of 50 nM MtDinG with 0.05, 0.1, 0.25, 0.5, 1, and 2 \mu M TMPyP4 inhibitor, respectively. B, D, and F indicate the percentage inhibition of EcDinG- and MtDinG-promoted TP-G4, TPB-G4, and forked duplex unwinding by TMPyP4, respectively. Data represent the mean of at least three independent experiments with S.D. values indicated by error bars.}
\end{figure}
suggest that MtDinG and EcDinG unwind DNA substrates with a 5’ → 3’ polarity.

Our studies also provided mechanistic insights into translocation and unwinding activity of MtDinG with 3’ overhangs. First, both MtDinG and EcDinG failed to unwind linear duplex DNA. Second, DinG proteins required a minimum of 15-nucleotide 5’ or 3’ overhang structures for its loading onto the ssDNA and helicase activity. Third, DNase I footprinting data showed that DinG proteins bind to ssDNA-dsDNA junctions of 3’ overhangs. Based on these observations, we propose a model in which binding of DinG proteins to ssDNA-dsDNA junctions causes destabilization of the shorter complementary strand from the 5’ end. This destabilization provides the ssDNA platform for the nucleation of DinG proteins from the 5’ end, allowing them to translocate on the same strand in the 5’ → 3’ polarity to unwind the opposite strand (Fig. 7B). Because MtDinG and EcDinG failed to bind and unwind duplex DNA, it is likely that thermal fraying of ends may not be sufficient for the DinG proteins to access the duplex ends for its nucleation. However, our attempts to study the destabilization of the complementary shorter strand at the ssDNA-dsDNA junction with KMnO₄ footprinting failed. This could be because T residues at the destabilized end were not accessible for KMnO₄ modification, possibly due to the nucleation of DinG at the 5’ end. Nonetheless, the ssDNA translocation and unwinding activities of helicases are determined by various factors, such as the oligomeric nature of the protein, rate of unwinding, processivity, step size, and molecules of ATP utilized (59–63). This information is obtained by a combination of structural data, pre-steady-state kinetics, thermodynamic, and single molecule studies. Further studies are required with DinG protein to understand the detailed mechanism of its translocation and helicase functions.

Our biochemical data with replication fork structures and HJ substrates revealed novel functions of MtDinG. Unlike EcDinG, MtDinG was able to proficiently unwind replication fork structures and HJs, albeit at 3-fold higher concentrations compared with the helicase activity with overhangs and flap structures. Surprisingly, although MtDinG did not exhibit binding to replication fork structures, three-way junctions, and HJ substrates (data not shown), it was able to unwind these structures efficiently. These data suggest that MtDinG can recognize replication, repair, and recombination intermediates in a structure-specific manner and unwind these structures, possibly by its dsDNA translocase activity. It is likely that MtDinG interaction with branched DNA substrates is dynamic and transient. The MtDinG K55R Walker motif mutant failed to unwind all of the substrates tested, suggesting that ATP hydrolysis is essential for the translocase as well as helicase activity of MtDinG.

In response to spontaneous or induced DNA damage, replication fork stalls and helicases have been implicated in remodeling the stalled forks to resume replication (38, 40, 41, 64, 65). Our study shows that MtDinG, but not EcDinG, unwinds replication fork structures. It is likely that MtDinG may participate
in the fork reversal activity to restart the replication in vivo. Formation of “chicken foot” structures from the stalled forks is one of the mechanisms by which a cell resumes replication (40, 41, 65, 66). E. coli RuvAB, RecG, and UvrD helicases remodel stalled replication forks to restart the replication (40, 41, 47, 66). In mammalian cells, RecQ helicases are implicated in the recovery of stalled replication forks by their fork reversal activity (7, 38, 55). In the absence of RecQ, it is likely that DinG helicase could have a similar function in M. tuberculosis. Nonetheless, it will be interesting to test whether MtDinG exhibits fork reversal activity to generate chicken foot structures. E. coli RecQ helicase unwinds replication forks to generate gaps and to facilitate RecA loading for SOS signaling and to promote recombination at stalled forks (67, 68). It will be interesting to study whether MtDinG participates in DNA damage signaling through remodeling the replication forks.

Unlike EcDinG, we also noted that MtDinG unwinds HJ substrates, suggesting the possible involvement of MtDinG in recombinational repair through its branch migration activity. We recently showed that MtRecG is a potent helicase that binds...
and unwinds HJ substrates (28). MtDinG may overlap with RecG to unwind replication fork structures and HJ intermediates in vivo. However, compared with MtRecG, MtDinG displayed reduced activity with HJ substrates. *E. coli* SSB modulates the activity of RecQ, PriA, RecG, and DinG helicases (69–72). It is possible that MtDinG may require stimulation by SSB for its HJ branch migration activity.

*E. coli* RecQ and its orthologs unwind G4 DNA structures (10–12), and a recent study shows that EcDinG also resolves intermolecular G4 structures (22). Our study clearly demonstrates that MtDinG unwinds intermolecular G4 structures robustly even at 50 nM concentrations, indicating that MtDinG could be a functional ortholog of *E. coli* RecQ at least in part with G4 DNA unwinding activity. Moreover, our data revealed that, unlike EcDinG, MtDinG proficiently unwound G4 structures with both 5’ and 3’ tails. Recent studies reveal that *S. cerevisiae* as well as human Pif1 helicases also resolve G4 structures (73, 74). Notably, many prokaryotes that encode the Pif1 family of helicases lack both RecQ and DinG (75). The *M. tuberculosis* genome is devoid of both RecQ- and Pif1-encoding genes. In the absence of these helicases, DinG in *M. tuberculosis* could be the sole enzyme that processes G4 structures in vivo. Nonetheless, in addition to G4 resolution, MtDinG was proficient in unwinding replication fork and HJ structures at a ∼3-fold higher concentration. Conceivably, G4 structures are the more physiological substrates for MtDinG.

Targeting G4 structures that are present in the promoters of oncogenes and telomeres is actively under consideration for cancer therapy (76, 77). Interestingly, the *M. tuberculosis* genome is 65% GC-rich with >10,000 G-rich sequences having the potential to form G4 structures. Notably, many of these G4-forming motifs are present in the promoters or in the proximity of promoters of several genes (9). Our study demonstrates the existence of G4 structures in *M. tuberculosis* cells. The genes that are encoded by the *mce1R* operon play a crucial role in the persistence of *M. tuberculosis* in the murine model (78). We demonstrate that G-rich sequences that are present in the promoter region of the *mce1R* gene indeed fold into G4 structure, suggesting that the G4 structure may regulate the expression of *mce1R*. The genes that encode PE_PGRS family proteins are so far found only in the genome of mycobacteria and largely restricted to pathogenic mycobacteria. These proteins have been implicated in *M. tuberculosis* persistence and pathogenesis (79). Our data show that G-rich promoter sequences of PE_PGRS2 folds into G4 structure. The *moeB1* encodes for an enzyme that is involved in synthesis of molybdopterin, which is required as a cofactor for many enzymatic reactions (80). Interestingly, disruption of *moeB1* in *M. tuberculosis* causes growth attenuation in macrophages (81). Our study shows that promoter sequences of the *moeB1* gene also contain G4-forming sequences. These data indicate that G4 structure may play an important role in the mycobacterial gene regulation. Interestingly, a recent study shows that the G4 structure regulates expression of *recF*, *recO*, and *recR* genes in *Deinococcus radiodurans*, which has implications for its radioresistance (82). However, further studies are required to understand the mechanism of G4 DNA-mediated regulation of gene expression in *M. tuberculosis* and also whether such regulation may contrib-

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**TABLE 3**

| Potential G4 DNA-forming sequences from *M. tuberculosis* genome | Pattern | Pattern end | Pattern start | Length | Sequence |
|---------------------------------------------------------------|--------|------------|--------------|--------|----------|
| *mce1R* (Rv0165c) Upstream/Complement | 194,823 | 194,881 | 46 | G 3TCG3CCG3ATTGCCG4ACTTGCCG5CTTGGCG5 |
| *moeB1* (Rv3206c) Upstream/Complement | 3,583,823 | 3,583,850 | 27 | G 3TTAG3AATCG3TAG3CCAG3 |
| PE_PGRS2 (Rv0124) Upstream/Complement | 149,506 | 149,523 | 18 | G 4CG3CG3TTG4 |
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ute to virulence, persistence, and survival of this pathogen in humans.

The abundance of G4-forming sequences in M. tuberculosis provides a great opportunity for identifying inhibitors that target these structures for therapy. Indeed, M. tuberculosis H37Rv cells are sensitive to G4-specific ligand TMPyP4 and exhibit reduced growth.6 It will be interesting to identify the genes that are essential for virulence, persistence, and survival of this pathogen in humans. Targeting such genes that contain G4 structures in their promoters could be a potential therapeutic strategy for combating the infection with this pathogen. In addition to G4 structures, it will be interesting to identify inhibitors that specifically target DinG helicase such that G4 resolution can be potentially blocked at the genome level. Targeting G4 structures in parallel with DinG helicase using specific inhibitors can be a potential synergistic strategy for an effective killing of this pathogen in humans.

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