Mycobacterial UvrD1 Is a Ku-dependent DNA Helicase That Plays a Role in Multiple DNA Repair Events, Including Double-strand Break Repair*

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Mycobacterium tuberculosis and other bacterial pathogens have a Ku-dependent nonhomologous end joining pathway of DNA double-strand break repair. Here we identify mycobacterial UvrD1 as a novel interaction partner for Ku in a genomewide yeast two-hybrid screen. UvrD1 per se is a vigorous DNA-dependent ATPase but a feeble DNA helicase. Ku stimulates UvrD1 to catalyze ATP-dependent unwinding of 3'-tailed DNAs. UvrD1, Ku, and DNA form a stable ternary complex in the absence of ATP. The Ku binding determinants are located in the distinctive C-terminal segment of UvrD1. A second mycobacterial paralog, UvrD2, is a vigorous Ku-independent DNA helicase. Ablation of UvrD1 sensitizes Mycobacterium smegmatis to killing by ultraviolet and ionizing radiation and to a single-strand break. Ablation of UvrD1 as a novel interaction partner for Ku in a genome-scale two-hybrid screen of M. tuberculosis. Here we present a biochemical and genetic characterization of mycobacterial UvrD1, which reveals a requirement for Ku to activate its latent helicase activity. We provide evidence that Ku and LigD contacts are mediated principally by the LigD polymerase domain (2, 3, 12).

An outstanding issue is whether the bacterial NHEJ apparatus incorporates other components beside Ku and LigD. An important corollary question is whether the actions of Ku or LigD extend beyond NHEJ. To begin to address these problems, we conducted unbiased two-hybrid screens of a Mycobacterium tuberculosis genomic library for LigD and Ku binding partners. The power of the screen was exemplified by the recovery of library plasmid clones encoding Ku when full-length LigD was used as the bait (2). The present study was prompted by the results of a new two-hybrid screen using Ku as the bait, which identified two Ku-binding proteins. One of these is Ku itself, consistent with the fact that mycobacterial Ku is a homodimer (3). A second novel Ku-binding protein is UvrD1, which is one of two mycobacterial homologs of the bacterial UvrD/PcrA helicase clade (13–16). The connection between Ku and UvrD1 is provocative, given that UvrD-like enzymes in other bacteria participate in non-NHEJ pathways of DNA repair. Here we present a biochemical and genetic characterization of mycobacterial UvrD1, which reveals a requirement for Ku to activate its latent helicase activity. We provide evidence that UvrD1, although nonessential for replication, plays a role in the repair of multiple forms of DNA damage, including site-specific chromosomal double-strand breaks.

**EXPERIMENTAL PROCEDURES**

*Yeast Two-hybrid Screen for M. tuberculosis Ku-binding Proteins—The bait plasmid comprised a fusion of the LexA DNA-binding domain (BD) encoded in pEG202 (17) to the N terminus of full-length MtuKu. Expression of the appropriately sized fusion protein in yeast was confirmed by immunoblotting.
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M. tuberculosis Ku (Rv0937c) was PCR-amplified from genomic DNA with primers that introduced a NdeI site at the start codon and a BglII site 3’ of the stop codon. The PCR product was digested with Ndel and BglIII and inserted between the Ndel and BamHI sites in pET16b to generate an expression plasmid encoding the MsmUvrD1 polypeptide fused to an N-terminal His10-tag. The open reading frame encoding M. tuberculosis UvrD1 (Rv0949) was amplified from genomic DNA with primers that introduced an Ndel site at the start codon and a BamHI site 3’ of the stop codon. The PCR product was digested with Ndel and BamHI and inserted into pET16b to generate an expression plasmid encoding the MsmUvrD1 polypeptide fused to an N-terminal His10 tag. Alanine substitution mutations were introduced into the MsmUvrD1 plasmid by PCR amplification with mutagenic primers. The inserts of all UvrD1 plasmids were sequenced to exclude the acquisition of unwanted coding changes during amplification or cloning.

The pET-MsmUvrD1 and pET-MtuUvrD1 plasmids were transformed into Escherichia coli BL21(DE3). Cultures (1 liter) were grown at 37 °C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until the A600 reached ~0.6. The cultures were chilled on ice for 45 min, and the expression of recombinant protein was induced by adjusting the culture to 0.2 mM isopropyl-β-D-thiogalactopyranoside, followed by incubation at 17 °C for 16 h with constant shaking. The cells were harvested by centrifugation, and the pellets were stored at −80 °C. All of the subsequent procedures were performed at 4 °C. Thawed bacteria were resuspended in 50 ml of buffer A (50 mM Tris-HCl, pH 7.5, 0.25 mM NaCl, 10% sucrose). Lysozyme and Triton X-100 were added to final concentrations of 1 mg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation. The soluble extracts were applied to 3-ml columns of nickel-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. The columns were washed with 20 ml of buffer B (50 mM Tris-HCl, pH 8.0, 0.25 mM NaCl, 0.05% Triton X-100, 10% glycerol) and then eluted stepwise with 10-ml aliquots of buffer B containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide compositions of the column fractions were monitored by SDS-PAGE. The His10-UvrD1 polypeptides were recovered predominantly in the 100 and 200 mM imidazole eluates. Fractions containing the UvrD1 protein were pooled and dialyzed against buffer C (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1% Triton X-100, 10% glycerol) containing 150 mM NaCl. The dialysates were adjusted to 250 mM NaCl and then applied to 3-ml columns of DEAE-Sephalac that had been equilibrated with 250 mM NaCl in buffer C. The columns were washed with the same buffer and then eluted stepwise with 500 and 1000 mM NaCl in buffer C. UvrD1 was recovered in the flow-through, which was dialyzed against 150 mM NaCl in buffer C and stored at −80 °C. Protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The yield of wild-type MsmUvrD1 was 5 mg from a 1-liter bacterial culture. The MsmUvrD1-(K45A/T46A) mutant was produced in E. coli BL21(DE3) cells by IPTG induction at 17 °C and purified as described above. The MsmUvrD1-(D235A) mutant was produced in E. coli BL21(DE3) Codon Plus (grown in LB broth containing 0.1 mg/ml ampicillin and 50 μg/ml chloramphenicol) by IPTG induction at 17 °C and purified as described above.

Recombinant Ku —The open reading frame encoding M. tuberculosis Ku was PCR-amplified from genomic DNA with primers that introduced an NdeI site at the start codon and a BamHI site 3’ of the stop codon. The PCR product was digested with Ndel and BamHI and inserted into pET16b (Novagen) to yield a plasmid encoding full-length Ku fused to an N-terminal His10 tag. The insert was sequenced to exclude the acquisition of unwanted coding changes. The pET-MtuKu plasmid was transformed into E. coli BL21(DE3). Ku was produced by IPTG induction at 17 °C and purified from a soluble lysate by nickel-agarose and DEAE-Sephalac chromatography as described above for UvrD1. Ku was recovered in the 100 and 200 mM imidazole eluates during the nickel-agarose step and the flow-through during the DEAE-Sephalac step. The yield of Ku was 30 mg from a 500-ml bacterial culture.

Recombinant UvrD2 —The open reading frame encoding M. smegmatis UvrD2 (MSEMG1952) was PCR-amplified from genomic DNA with primers that introduced BamHI sites over the start codon and 3’ of the stop codon. The PCR product was digested with BamHI and inserted into pET28-His10-Smt3 to generate an expression plasmid encoding the full-length MsmUvrD2 polypeptide fused to an N-terminal His10-Smt3 tag. The D237A coding change was introduced by PCR amplification with mutagenic primers. The inserts of all MsmUvrD2 plasmids were sequenced to exclude the acquisition of unwanted coding changes during amplification or cloning. The expression plasmids were transformed into E. coli BL21(DE3). The wild-type and mutant His10-Smt3-MsmUvrD2 proteins were produced by IPTG induction at 17 °C and purified from soluble lysates by nickel-agarose and DEAE-Sephalac chromatography as described above for UvrD1. His10-Smt3-MsmUvrD2 was recovered in the 1000 mM imidazole eluate during the nickel-agarose step and the flow-through during the DEAE-Sephalac step. The His10-Smt3 tag was then removed by digestion of the preparation with the Smt3-specific protease Ulp1 for 3 h at 4 °C (at a UvrD2:Ulp1 ratio of 1000:1). The tag-free MsmUvrD2 pro-
tein was separated from His10-Smt3 by passage of the digest over a nickel-agarose column. MsmUvrD2 was recovered in the flow-through. The yield of MsmUvrD2 was 1 mg from a 2-liter bacterial culture.

**Velocity Sedimentation**—An aliquot (50 µg) of UvrD1 or UvrD2 was mixed with catalase (50 µg), BSA (50 µg), and cytochrome c (100 µg). The mixture was applied to a 4.8-ml 15–30% glycerol gradient containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5 mM EDTA, 0.05% Triton X-100. The gradient was centrifuged at 50,000 rpm for 18 h at 4 °C in a Beckman SW55Ti rotor. Fractions (−0.2 ml) were collected from the bottom of the tube.

**Nucleoside Triphosphatase Assay**—Reaction mixtures containing (per 10 µl) 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM [γ-32P]ATP or [α-32P]ATP (Perkin-Elmer Life Sciences), 50 ng of salmon sperm DNA, and UvrD1 or UvrD2 as specified were incubated for 5 min at 37 °C. An aliquot (2 µl) of the mixture was applied to a polyethyleneimine-cellulose TLC plate, which was developed either with 0.45 M ammonium sulfate or 0.5 M LiCl, 1 M formic acid. The radiolabeled material was visualized by autoradiography, and 32P, or 32P-ADP formation was quantified by scanning the TLC plate with a Fujix BAS2500 imager. Alternatively, reaction mixtures (20 µl) containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM unlabeled NTP or dNTP, 100 ng of salmon sperm DNA, and UvrD1 or UvrD2 as specified were incubated for 10 min at 37 °C. The reactions were quenched by adding 1 ml of malachite green reagent (BIOMOL Research Laboratories, Plymouth Meeting, PA). Phosphate release was determined by measuring A₆₂₀ and interpolating the value to a phosphate standard curve.

**Helicase Assay**—The 5’ 32P-labeled strand was prepared by reaction of a synthetic oligodeoxynucleotide with T4 polynucleotide kinase and [γ-32P]ATP. The labeled DNA was purified by electrophoresis through a native 18% polyacrylamide gel. The labeled strand was annealed to a 2-fold excess of a complementary DNA strand to form the tailed duplex helicase substrates shown in the figures. Helicase reaction mixtures (10 µl) containing 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 0.5 pmol (50 nM) radiolabeled DNA, and proteins as specified were preincubated for 5 min on ice. The reaction was initiated by adding 1 mM ATP and 5 pmol of an unlabeled oligonucleotide corresponding to the labeled strand of the helicase substrate. The addition of excess of unlabeled strand was necessary to prevent the spontaneous reannealing of the unwound 32P-labeled DNA strand. The reaction mixtures were incubated for 5 min at 37 °C and then quenched by adding 2 µl of a solution containing 2% SDS, 200 mM EDTA, 40% glycerol, 0.3% bromphenol blue. A control reaction mixture containing no protein was run parallel to the test reactions.

**FIGURE 1.** Two-hybrid interaction between Ku and UvrD1. Top panel, the AD-UvrD1-(508–771) fusion isolated in a yeast two-hybrid screen using Ku as bait was retested for galactose-dependent activation of a lacZ reporter in the presence of Ku or the unrelated mycobacterial protein MmaA2. Yeast cells were spotted on agar plates containing X-gal. Bottom panel, the amino acid sequence of MtuUvrD1 is aligned to that of B. stearothermophilus PcrA. NTPase motifs I and II are highlighted in green boxes. The amino acids in the motifs that were subjected to alanine substitution in MsmUvrD1 are indicated by dots. The C-terminal domain of UvrD1 that sufficed for interaction with Ku is highlighted in yellow.
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was heated for 5 min at 95 °C. The reaction products were analyzed by electrophoresis through a 15-cm 15% polyacrylamide gel in 89 mM Tris borate, 2.5 mM EDTA. The products were visualized by autoradiography and quantified by scanning the gel with a Fujix BAS-2500 imaging apparatus.

M. smegmatis uvrD1 Null Mutant—The M. smegmatis ΔuvrD1 mutant was engineered by two-step allelic exchange using suicide vectors and the counterselectable marker sacB (2). The disruption cassette generated an in-frame deletion of the majority of the coding sequence (spanning aa 27–756), thereby avoiding possible polar effects on downstream genes. The gene disruption was confirmed by Southern blotting using flanking DNA sequences as probes. The wild-type M. smegmatis uvrD1 gene (under the control of its native promoter) was inserted into the integrate-based plasmid pMV306-Kan and then introduced by transformation into the chromosomal attB locus of the ΔuvrD1 strain (18). The wild-type M. tuberculosis uvrD1 gene (under the control of the M. smegmatis uvrD1 promoter) was also integrated at the attB locus of a ΔuvrD1 strain.

UV Sensitivity—Aliquots (10 μl) of serial 10-fold dilutions of wild-type and ΔuvrD1 strains of M. smegmatis (A600 = 0.3) were spotted in duplicate on LB agar plates. The plates were irradiated with the indicated UV dose using a Stratalinker 254-nm UV light source. The plates were then incubated in the dark for 3 days at 37 °C. The surviving colonies were counted and normalized to the colony counts from duplicate samples spotted from the same cultures that were not exposed to UV (defined as 100%).

Ionizing Radiation (IR) Sensitivity— Cultures of wild-type and ΔuvrD1 strains of M. smegmatis in log phase (A600 = 0.2–0.3) or stationary phase (A600 = 2.0) were harvested by centrifugation, and the cells were resuspended in phosphate-buffered saline, 0.5% Tween 80. After brief treatment in a water bath sonicator to disperse clumps, aliquots (0.2 ml containing ~10^8 bacteria) were irradiated with a 137Cs source that delivered a dose of 12 grays min^{-1}. Radiation was performed on a rotating platform to ensure equal IR exposure of each cell suspension. Serial dilutions of the irradiated cells were plated on LB agar. IR-surviving colonies were counted with a dissecting microscope and normalized to the colony counts from controls that were not exposed to IR (defined as 100%).

Inducible Chromosome Breakage by Expression of I-SceI Endonuclease—Induction of a site-specific double strand break in the M. smegmatis chromosome was achieved by expression of the rare-cutting I-SceI endonuclease (19) under the control of a tetracycline-regulated promoter (20). The Tet/I-SceI cassette was integrated at the attB locus with or without a neighboring 18-bp recognition site for I-SceI cleavage. Anhydrotetracycline (AHT)-induced I-SceI expression, and cleavage at the target site was verified by Southern blotting of genomic DNA.7 Wild-type and ΔuvrD1 cells (containing the I-SceI cassette and the cleavage site at attB) were grown in the absence of AHT. Aliquots (10 μl) of serial 10-fold dilutions of the cultures were plated in duplicate on LB agar containing 20 μg/ml kanamycin (which selects for the I-SceI cassette) and 50 ng/ml AHT. AHT-surviving colonies were counted with a dissecting microscope and normalized to the colony counts from controls that were plated on LB agar lacking AHT.

RESULTS

Identification of UvrD1 as a Binding Partner for Ku—To identify additional candidate components of the mycobacterial NHEJ pathway, we performed an unbiased yeast two-hybrid screen for mycobacterial gene products that interact with M. tuberculosis Ku. The AD fusion library contains ~1-kilobase pair inserts of M. tuberculosis genomic DNA. An initial round of screening entailed cotransformation of the bait and library plasmids and selection for galactose-dependent leucine prototrophy, followed by secondary screening for galactose-dependent lacZ reporter expression. Plasmids recovered from individual isolates were retested by cotransformation with the BD-Ku plasmid or a control plasmid encoding a BD-MmaA2 fusion (21). Of 67 clones that retested positive for leucine prototrophy

7 N. C. Stephanou, F. Gao, P. Bongiorno, S. Ehrt, D. Schnappinger, S. Shuman, and M. S. Glickman, submitted for publication.
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and lacZ expression, 28 had in-frame fusions to a M. tuberculosis gene. Two contained a fusion between the activation domain and Ku, spanning the segment of Ku from aa 25 to 273. The Ku-Ku interaction in vivo is consistent with biochemical evidence that purified mycobacterial Ku is a homodimer (3).

The novel finding was that 24 of 28 clones recovered from the screen encoded an in-frame AD fusion to a C-terminal fragment of M. tuberculosis UvrD1 (Rv0949). MtuUvrD1 is a 771-aa polypeptide and a putative ortholog of the DNA repair helicases Bacillus stearothermophilus PcrA and E. coli UvrD. The N-terminal segment of UvrD1 contains the canonical motifs I (GXXGXXGKT) and II (DEXX) that comprise the ATP and metal-binding sites of the superfamily I helicases. Indeed, the full suite of amino acids that form the ATPase active site or interact with the 3’-tailed helicase substrate in the DNA co-crystal of PcrA (15, 22) and E. coli UvrD (16) are conserved in MtuUvrD1 (Fig. 1). The primary structures of UvrD1 and PcrA are conserved across their entire lengths, with the notable exceptions of several UvrD1-specific inserts within the C-terminal segment. The 24 AD-UvrD1 clones selected in the Ku interaction screen encoded four different fusions starting from MtuUvrD1 residues 442, 469, 491, and 508 and extending to the C terminus (Fig. 1). The MtuUvrD1 fusions interacted specifically with Ku, and reporter gene activation was abolished by glucose repression of the MtuUvrD1 fusion, indicating that the Ku bait alone did not activate transcription (Fig. 1). These results provide evidence of a physical interaction between mycobacterial Ku and UvrD1 in the absence of other mycobacterial proteins, and they localize the Ku interface of UvrD1 to the distinctive C-terminal portion of the UvrD1 protein.

UvrD1 Ablation Sensitizes Mycobacterium to DNA Damage—M. smegmatis encodes a 783-aa UvrD1 polypeptide (MSMEG5534) that has 637 positions of side chain identity to MtuUvrD1. To gauge the role of UvrD1 in mycobacterial physiology, we deleted the uvrD1 gene of M. smegmatis by removing the bulk of the open reading frame and rejoicing the 5’ and 3’ termini with maintenance of the translation frame. The ΔuvrD1 mutant was generated by a two-step allelic exchange strategy. The ΔuvrD1 cassettes were cloned into mycobacterial suicide plasmids containing hygR and sacB markers, which were then used to transform M. smegmatis mc²155 to hygromycin resistance. The hygR sucrose-sensitive ΔuvrD1 uvrD1 + merodiploid strains were genotyped to verify targeted insertion and then subjected to sucrose counterselection for loss of the parental wild-type strain grew as well as the parental wild-type strain on agar medium and in liquid culture (not shown). The viable ΔuvrD1 strain grew as well as the parental wild-type strain on agar medium and in liquid culture (not shown).

Wild-type and ΔuvrD1 strains were tested for sensitivity to various DNA damaging agents (Fig. 2). The ΔuvrD1 strain was extremely sensitive to killing by UV irradiation, suffering a 1000-fold reduction in survival after exposure to 5–10 J/m², a dose that had virtually no effect on wild-type M. smegmatis (Fig. 2A). Reintroduction of a single copy of wild-type M. smegmatis into the ΔuvrD1 strain by site-specific chromosomal integration at the attB locus restored UV survival to the wild-type level (Fig. 2A), thereby demonstrating that the UV damage repair defect was caused by loss of UvrD1 function. An instructive finding was that introduction of the M. tuberculosis uvrD1 gene at the attB locus also fully complemented the UV repair defect of the M. smegmatis ΔuvrD1 mutant (Fig. 2A).

The ΔuvrD1 strain was 50–440-fold more sensitive than wild-type M. smegmatis to killing by IR in the dose range of
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480–720 grays (Fig. 2B). The IR sensitivity of Δuvrd1 was similar whether the cells were irradiated in log phase or in stationary phase (data not shown). Integration of M. smegmatis or M. tuberculosis uvrD1 at the attB locus of the Δuvrd1 strain restored IR survival (Fig. 2C).

To specifically probe UvrD1 function in the repair of DNA DSBs, we exploited a novel M. smegmatis strain that expresses the rare-cutting yeast mitochondrial I-SceI endonuclease (19) under the control of a tetracycline-inducible promoter (20). I-SceI cleavage at its 18-bp target site generates a staggered DSB overhangs (19). Heterologous expression of I-SceI in mammalian cells containing cleavage elements integrated in the chromosome has afforded a powerful tool to analyze the mechanisms of DSB repair (23). We found that integration of the gene encoding I-SceI into the M. smegmatis attB locus had no impact on growth in the presence of tetracycline, because the bacterial genome has no cognate cleavage site for I-SceI.7 However, co-introduction of the I-SceI gene and a single cleavage site into the chromosome of wild-type M. smegmatis resulted in a significant loss of viability (20% survival) when bacteria were plated on medium containing anhydrotetracycline (Fig. 2D). The Δuvrd1 mutant was 6-fold more sensitive to killing by tetracycline-induced I-SceI expression (3% survival) (Fig. 2D). These experiments demonstrate a function for UvrD1 in the repair of a single DSB in the bacterial chromosome.

UvrD1 Is a DNA-dependent ATPase—To evaluate the enzymatic and physical properties of mycobacterial UvrD1, we produced the MsmUvrD1 protein in E. coli as a His10 fusion and purified it from a soluble extract by nickel-agarose and DEAE-cellulose chromatography. SDS-PAGE revealed a predominant 48-kDa band corresponding to MsmUvrD1 (Fig. 3A). Reaction of purified MsmUvrD1 with 1 mM [γ-32P]ATP in the presence of magnesium and salmon sperm DNA resulted in conversion of nearly all the labeled material to inorganic phosphate (Fig. 3B). A mutated version of the MsmUvrD1 protein was prepared in which Lys45 and Thr46 in motif I (which contact the β phosphate of ATP) were both replaced by alanine. A second mutated version was prepared in which the metal-binding Asp235 in motif II was changed to alanine. The purity of the K45A/T46A and D235A proteins was comparable with that of wild-type MsmUvrD1 (Fig. 3A). The motif I and II mutations abolished the ATPase activity of MsmUvrD1 (Fig. 3B). These results verify that the phosphohydrolase activity is intrinsic to the recombinant protein.

The quaternary structure of MsmUvrD1 was gauged by zonal velocity sedimentation through a 15–30% glycerol gradient. Marker proteins catalase (248 kDa), BSA (66 kDa), and cytochrome c (13 kDa) were included as internal standards. MsmUvrD1 sedimented as a discrete peak in fractions 15–17 overlapping the “heavy” side of the BSA peak (Fig. 3C). The ATPase activity profile peaked in fractions 15−17 and coincided with abundance of the MsmUvrD1 polypeptide (Fig. 3C). A plot of the S values of the three standards versus fraction number yielded a straight line (data not shown). An S value of 5.3 was determined for MsmUvrD1 by interpolation to the internal standard curve. We surmise that MsmUvrD1 is a monomer.

ATP hydrolysis by MsmUvrD1 was optimal at pH 8.0 to 8.5 in Tris buffer and declined sharply as the pH was increased to 9.5 or decreased to <7.0 (data not shown). Kinetic analysis of parallel reactions of MsmUvrD1 with [α-32P]ATP or [γ-32P]ATP revealed that the rate of release of 32P from [γ-32P]ATP was identical to the rate of conversion of [α-32P]ATP to [α-32P]ADP,signifying that UvrD1 catalyzes a single-step conversion of ATP to ADP and P_i (Fig. 4A). From these data, we calculated a turnover number of ~100 s⁻¹. No ATPase activity was evident in the absence of magnesium, which supported ATP hydrolysis over a range of concentrations tested (0.2–5 mM MgCl₂; data not shown). From an ATP titration experiment (not shown), we calculated a K_m of 110 μM ATP and a k_cat of 110 s⁻¹. NTP specificity was examined by colorimetric assay of the release of P_i from unlabeled ribonucleotides ATP, GTP, CTP, or UTP and deoxyribonucleotides dATP, dGTP, dCTP, and dTTP. MsmUvrD1 displayed specificity for hydrolysis of ATP and dATP (Fig. 4B).

The ATPase activity of MsmUvrD1 was strictly dependent on a DNA cofactor. The extent of ATP hydrolysis increased with input salmon sperm DNA in the range of 0.8–25 ng/10-μl
reaction and saturated at ≥50 ng (Fig. 4C). Single-strand oligonucleotides were also effective cofactors for ATP hydrolysis (Fig. 4D). Titration of oligonucleotides of different lengths (44, 18, or 12-mers) revealed that the 44-mer oligonucleotide stimulated the ATPase to a higher level than did the shorter strands and did so at lower concentrations. The oligonucleotide concentrations that supported hydrolysis of 50% of the input ATP were 1, 60, and 150 nM for the 44-, 18-, and 12-mer, respectively.

**UvrD1 Is a Ku-dependent DNA Helicase—**E. coli UvrD1 is a unidirectional 3'-to-5' helicase that utilizes the energy of ATP hydrolysis to unwind duplex DNA after its initial binding to a 3' single-strand segment flanking the duplex (24). To our surprise, MsmUvrD1 displayed extremely feeble strand displacement activity on a canonical 3'-tailed helicase substrate composed of a 24-bp duplex with a 20-nucleotide 3'-dT tail (Fig. 5). Only a trace amount of the 32P-labeled strand was unwound by a 2-fold molar excess of UvrD1 in the presence of 1 mM ATP. The remarkable finding was that the addition of stoichiometric amounts of purified M. tuberculosis Ku, which had no DNA unwinding activity on its own, triggered nearly quantitative displacement of the 32P-labeled strand by MsmUvrD1 (Fig. 5A). Omission of ATP abol-
ished the Ku-dependent helicase activity. Strand displacement was also suppressed by the ATPase-inactivating D235A mutation of MsmUvrD1 (Fig. 5A). Because Ku had no analogous stimulatory effect on the DNA-dependent ATPase activity of MsmUvrD1 (not shown), we surmise that Ku unmasks the potential helicase function of UvrD1 by somehow coupling ATP hydrolysis to movement of UvrD1 through the duplex segment of the helicase substrate.

The extent of unwinding of 50 nM 3'-tailed DNA by 100 nM MsmUvrD1 increased with the amount of added Ku, attaining saturation at 110 nM Ku homodimer (Fig. 5B). Ku elicited a similar concentration-dependent stimulation of DNA unwinding by purified M. tuberculosis UvrD1 (data not shown). These results suggested a stoichiometric functional interaction between Ku, UvrD1, and the helicase substrate, presumably reflecting an underlying physical interaction (e.g. as revealed by the two-hybrid screen). To address this issue, we analyzed by native gel electrophoresis the formation of binary and ternary protein-DNA complexes. The binding reactions were performed in the absence of ATP to preclude unwinding in reaction mixtures containing Ku plus MsmUvrD1. Incubation of the 32P-labeled 3'-tailed duplex with a 2-fold excess of MsmUvrD1 resulted in nearly quantitative formation of a discrete UvrD1-DNA binary complex of retarded electrophoretic mobility (Fig. 5C). In contrast, whereas incubation with Ku also resulted in a nearly quantitative mobility shift of the input DNA, the diffuse nature of the more slowly migrating labeled DNA suggested that the putative Ku-DNA binary complex was prone to dissociate during electrophoresis. This behavior would be consistent with the ability of the ring-shaped Ku dimer (25) to slide off the short duplex segment of the DNA ligand. The instructive finding was that incubation of the DNA together with Ku and MsmUvrD1 resulted in the formation of a novel discrete radiolabeled complex, migrating more slowly than the UvrD1-DNA binary complex, that we surmise is a stable ternary of UvrD1 and Ku bound simultaneously to the 3'-tailed duplex (Fig. 5C). The D235A mutant of MsmUvrD1 also formed a binary complex with the 3'-tailed DNA and a ternary UvrD1-Ku-DNA complex (Fig. 5C), indicating that neither ATP nor the capacity to hydrolyze ATP is required for UvrD1 interaction with DNA or Ku.

Additional experiments showed that Ku stimulation of UvrD1 helicase required the 3'-to-5' single-strand tail and was not apparent when a 5'-tailed duplex substrate (as in Fig. 9) was employed for the helicase assay (not shown). The MsmUvrD1 displays the same 3'-to-5' directionality described for E. coli UvrD and B. stearothermophilus PcrA (13–15). We considered the possibility that the Ku requirement might reflect an inability of mycobacterial UvrD1 to initiate unwinding at a flush duplex/single-strand junction, in which case providing a "forked" duplex with two protruding single strands (3'- and 5'-tails) might enable DNA unwinding in the absence of Ku by bypassing the need to open the strands. E. coli UvrD is proficient at unwinding such forked molecules (26). The forked duplex we employed consisted of the 24-bp duplex segment and 20-nucleotide 3'-dT tail, embellished by a 16-nucleotide 5'-dT tail (Fig. 6). The salient finding was that MsmUvrD1 unwound the forked duplex but still required Ku (Fig. 6A). The extent of unwinding of the forked substrate

**FIGURE 7. Two UvrD paralogs in mycobacteria.** The amino acid sequences of MtuUvrD1 and MtuUvrD2 were aligned by a pairwise blast search. Positions of amino acid side chain identity and similarity are indicated by the amino acid sequences of MtuUvrD1 and MtuUvrD2 were aligned by a pairwise blast search. Positions of amino acid side chain identity and similarity are indicated by the.
increased with the amount of Ku added (Fig. 6B). Higher concentrations of Ku were required to attain optimal unwinding of the forked DNA compared with the 3′/H11032-tailed substrate with the flush duplex-ss junction (compare Figs. 5C and 6B). We suspect this reflects a reduced degree of freedom in the ability of Ku to bind the 24-bp duplex segment (e.g., the fork might act as an impediment to Ku sliding onto the duplex).

A Second Mycobacterial UvrD Paralog: UvrD2—The M. tuberculosis proteome includes a second UvrD paralog (Rv3198) annotated as UvrD2. MtuUvrD2 (700 aa) and its M. smegmatis counterpart (MSMEG1952; 709 aa) contain the canonical helicase motifs. MtuUvrD2 and MsmUvrD2 primary structures are conserved across their entire length and include 539 positions of amino acid identity. UvrD2 homologs (82–84% identity) are present in the proteomes of Mycobacterium avium and Mycobacterium leprae. The conservation of primary structure between Mtu paralogs UvrD1 and UvrD2 embraces the N-terminal 698 aa of UvrD1 and the N-terminal 599 aa of UvrD2, wherein there are 240 positions of side chain identity (Fig. 7). Several UvrD1-specific inserts punctuate the distal half of the UvrD1-UvrD2 alignment. The C-terminal segments of the UvrD1 (aa 699–771) and UvrD2 (aa 599–700) did not align in a pairwise Blast comparison. Thus, UvrD1 and UvrD2 differ principally within the C-terminal domain that, in the case of UvrD1, interacts with Ku.

To assess the activities (if any) of the UvrD2 paralog and compare them to UvrD1, we produced MsmUvrD2 in E. coli as a His10-Smt3 fusion and purified it from a soluble extract by nickel-agarose and DEAE-Sephacel chromatography. The tag was cleaved off by the Smt3-specific protease Ulp1 and tag-free
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MsmUvrD2 (calculated molecular mass, 77 kDa) was purified by a second nickel-agarose step. SDS-PAGE showed that the preparation was highly enriched with respect to the UvrD2 polypeptide (Fig. 8). A mutated version UvrD2-D237A, wherein the motif II aspartate was replaced by alanine, was purified in parallel (Fig. 8A). Wild-type UvrD2 catalyzed vigorous hydrolysis of ATP in the presence of magnesium and salmon sperm DNA (Fig. 8B). From the slope of the titration curve, we estimated a turnover number of 43 s⁻¹. ATP hydrolysis was abolished by the D237A mutation (Fig. 8B). Wild-type UvrD2 per se efficiently unwound the forked duplex helicase substrate (Fig. 8C). The helicase reaction was saturated at a substoichiometric level of input UvrD2 to forked DNA. The D237A mutation abolished the DNA unwinding reaction of UvrD2 (Fig. 8C).

The native size of UvrD2 was assessed by sedimentation through a 15–30% glycerol gradient with internal standards catalase, BSA, and cytochrome c. The ATPase and helicase activity profiles peaked in fractions 13–17 (Fig. 8D) and coincided with abundance of the UvrD2 polypeptide. UvrD2 cosedimented with BSA. We surmise that UvrD2 is a monomer.

ATP hydrolysis by UvrD2 was optimal at pH 6.0–8.0 in Tris buffer (data not shown). Hydrolysis of 1 mM ATP required a divalent cation and was optimal at 1–5 mM MgCl₂ (Fig. 9A). The ATPase activity of UvrD2 was strictly dependent on a DNA cofactor. The extent of ATP hydrolysis increased with input DNA, the duplex was unwound with pseudo-first order kinetics, attaining an end point at between 2 and 5 min (Fig. 10). Ku had no significant impact on the ATPase or helicase activities of UvrD2 (not shown). UvrD2 formed a discrete binary complex with the 3' tailed duplex DNA in the native gel mobility shift assay, but no novel ternary complex was detected in the presence of UvrD2 plus Ku (not shown). We surmise that UvrD2 neither depends on nor interacts with Ku as it performs the DNA unwinding reaction.

**DISCUSSION**

Here we have identified the mycobacterial DEXX box ATPase UvrD1 as a novel binding partner for the NHEJ protein...
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The PcrA bound to DNA in the crystal is a truncated recombinant protein terminating at residue 650; thus, it does not include counterparts of all the potential determinants of Ku binding by UvrD1. Still, it is instructive that the green secondary structure elements form a helical bundle on the protein surface - on the face of the complex from which the “intact” DNA duplex emerges (i.e. to the left of the PcrA-DNA complex in the view shown in Fig. 11A, left panel). We speculate that the Ku dimer, which is a ring-shaped clamp with a central cavity for duplex DNA (25) (modeled in Fig. 11B), binds to the downstream duplex segment in the 3’-tailed helicase substrate and joins a stable Ku-UvrD1-DNA ternary complex by virtue of its contacts to constituents of the “green” Ku-binding surface on UvrD1. The contacts to the helicase at the duplex-ss junction would prevent Ku from spontaneously sliding off the short duplex DNA. Because Ku is not needed for formation of a UvrD1-DNA binary complex or DNA-dependent ATP hydrolysis by UvrD1, we propose that Ku might act as a processivity factor for UvrD1 unwinding, analogous to how the β and proliferating cell nuclear antigen sliding clamps ensure the processivity of the replicative DNA polymerases (27). In this model, Ku might couple ATP hydrolysis to DNA unwinding by maintaining UvrD1 at the duplex-ss junction after each cycle of catalysis and strand displacement, rather than having UvrD1 dissociate from the DNA. The bacterial Ku-UvrD1 connection lends support to previous suggestions that eukaryal Ku plays a β/proliferating cell nuclear antigen-like role in recruiting NHEJ factors to broken DNA ends (38). Clearly, there is much to be done in the way of testing all of the predictions of the model, not the least of which will be to capture a structure of the Ku-UvrD1-DNA complex.

The present study is, to our knowledge, the first documentation of physical and functional interactions between bacterial Ku and a DNA helicase. The findings are of interest in light of reports that eukaryal Ku interacts physically and functionally with the WRN helicase/nuclease and stimulates its exonuclease function (28–34). Eukaryal Ku and its NHEJ partner Lig4 are also reported to interact genetically and functionally with the BLM helicase during DSB repair in vivo (35, 36). WRN and BLM are implicated in maintaining genome integrity and are the affected targets of mutations in human Werner and Bloom disease syndromes, respectively.

A possible mechanism for Ku activation of the UvrD1 helicase is suggested by the crystal structure of the homologous helicase PcrA bound to a 3’-tailed duplex in the presence of AMPPNP and magnesium (15). Two views of the PcrA-DNA substrate complex are shown in Fig. 11A. The helicase is bound at the duplex-single strand junction, such that the 3’-tailed “loading strand” on which the helicase translocates passes through an internal groove in the protein, whereas the trajectory of the displaced strand is outside the protein (15). The helicase covers about 8-bp of duplex flanking the junction. The segment of PcrA that corresponds to the C-terminal Ku-binding domain of UvrD1 is colored green in Fig. 11.

Ku. This interaction, which occurs in vivo in yeast in the absence of other mycobacterial proteins, is mediated by the distinctive C-terminal domain of UvrD1. Although possessed of the typical superfamily I helicase motifs, mycobacterial UvrD1 is a feeble DNA unwinding enzyme on its own, notwithstanding its vigorous DNA-dependent ATPase activity and its ability to form a stable binary complex with the tailed duplex DNA helicase substrate. The latent helicase activity of UvrD1 is revealed in the presence of stoichiometric amounts of mycobacterial Ku, thereby underscoring the functional significance of the Ku-UvrD1 physical interaction discovered in the two-hybrid screen.

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The biochemical connections between mycobacterial Ku and UvrD1 hint at a novel role for UvrD-like proteins in double-strand break repair in bacteria. UvrD is a key participant in mismatch repair and nucleotide excision repair pathways, which entail segmental resections of one strand of the DNA duplex (14). Inactivation of UvrD in *E. coli* or *Salmonella typhimurium* sensitizes these bacteria to killing by UV irradiation (37). Our finding that UvrD1 is critical for repair of UV damage in *M. smegmatis* attests to the contributions of UvrD1 in mycobacterial nucleotide excision repair. Note that UvrD1 function in UV damage repair is apparently independent of Ku, insofar as a Δ*ku* strain of *M. smegmatis* displayed wild-type sensitivity to UV irradiation (7). This is sensible, insofar as single-strand nicks in UV damage repair is apparently independent of Ku, insofar as (37). Our finding that UvrD1 is critical for repair of UV damage sensitizes these bacteria to killing by UV irradiation. This is sensible, insofar as single-strand nicks or gaps are not likely to be accessible to the Ku DNA clamp, which needs a double strand break for ingress to DNA. The question then arises how the latent helicase activity of UvrD1 might be activated during UV repair. It is conceivable that other mycobacterial proteins (perhaps repair pathway-specific) are capable of interacting with UvrD1 and stimulating the UvrD1 helicase.

Our findings that ablation of UvrD1 sensitizes mycobacteria to IR and 1-SceI points to a role for UvrD1 in DSBR repair, a process in which Ku has an imputed role as the lynchpin of the NHEJ pathway. We will report elsewhere that ablation of Ku also sensitizes *M. smegmatis* to 1-SceI (7). These studies provide a foundation for further genetic and biochemical studies of the function of UvrD1 and the determinants of its interactions with Ku. In particular, it will be of interest to isolate separation-of-function mutants of UvrD1 that differentially affect UV repair versus DSBR repair.

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