## Structure of the Endonuclease Domain of MutL: Unlicensed to Cut

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Structure of the Endonuclease Domain of MutL: Unlicensed to Cut

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SUMMARY

DNA mismatch repair corrects errors that have escaped polymerase proofreading, increasing replication fidelity 100- to 1000-fold in organisms ranging from bacteria to humans. The MutL protein plays a central role in mismatch repair by coordinating multiple protein-protein interactions that signal strand removal upon mismatch recognition by MutS. Here we report the crystal structure of the endonuclease domain of Bacillus subtilis MutL. The structure is organized in dimerization and regulatory subdomains connected by a helical lever spanning the conserved endonuclease motif. Additional conserved motifs cluster around the lever and define a Zn^{2+}-binding site that is critical for MutL function in vivo. The structure unveils a powerful inhibitory mechanism to prevent undesired nicking of newly replicated DNA and allows us to propose a model describing how the interaction with MutS and the processivity clamp could license the endonuclease activity of MutL. The structure also provides a molecular framework to propose and test additional roles of MutL in mismatch repair.

INTRODUCTION

DNA mismatch repair (MMR) maintains genomic stability by correcting errors that have escaped polymerase proofreading (Kunkel and Erie, 2005). MMR proteins are also implicated in a variety of other cellular processes such as DNA damage signaling, apoptosis, meiotic and mitotic recombination, and somatic hypermutation (Modrich, 2006). Mutations in MMR genes are associated with an increased mutation rate and microsatellite instability, the hallmark of human nonpolyposis colorectal cancer (Peitomaki, 2005).

Initiation of MMR depends on the coordinated action of three proteins. MutS recognizes a mismatched base pair or a small insertion/deletion loop and recruits MutL in an ATP-dependent manner. Subsequently, the newly synthesized strand is marked for repair. In Escherichia coli, strand discrimination is achieved by mismatch-provoked activation of the MutH endonuclease, which cleaves the unmethylated DNA strand at hemimethylated GATC sites transiently generated during DNA replication. Although most bacteria and all eukaryotes do not encode a MutH homolog, a pre-existing nick is sufficient to activate MMR in a system reconstituted from purified proteins (Zhang et al., 2005). It has been shown that MutL homologs from species lacking a MutH endonuclease harbor an intrinsic latent nicking endonuclease activity that is vital for its function in MMR (Erdeniz et al., 2007; Kadyrov et al., 2006, 2007, 2008).

MutL is composed of two structurally conserved domains connected by a variable flexible linker (Guarne et al., 2004). The N-terminal region encompasses an ATPase domain of the GHL ATPase superfamily that is conserved from bacteria to humans (Ban et al., 1999; Guarne et al., 2001). Conversely, the sequence conservation in the C-terminal dimerization region of MutL is low. The structure of the C-terminal domain of E. coli MutL (EcMutL) reveals that this region is organized into two distinct subdomains (Guarne et al., 2004; Kosinski et al., 2005).

While prokaryotic MutL homologs form homodimers, their eukaryotic counterparts form heterodimers. In humans, there are four paralogs of MutL (hMLH1, hPMS2, hPMS1, and hMLH3) that form three heterodimers by association of hMLH1 with hPMS2 (hMutLα), hPMS1 (hMutLβ), and hMLH3 (hMutLγ) (Li and Modrich, 1995; Lipkin et al., 2000; Raschle et al., 1999). hMutLα is necessary for MMR function, and hMutLγ has a role in meiotic recombination; however, the function of hMutLβ is unknown (Kunkel and Erie, 2005). The C-terminal regions of hPMS2 and hMLH3 encompass a conserved DQHA(X)_2E(X)_4E motif that is required for endonuclease activity. Based on sequence analysis and molecular modeling, three additional conserved motifs (ACR, C[P/N]HGRP, and FXR) have been predicted to form a single active site with the endonuclease motif (Kosinski et al., 2008). Analysis of the reconstituted human MMR system indicates that the endonuclease activity of MutLα provides a loading site for MutSα-activated exonuclease I (Kadyrov et al., 2006).
Here we present the structure of the C-terminal dimerization domain of *Bacillus subtilis* MutL (BsMutL) harboring the endonuclease activity of the protein. The structure reveals the conserved three-dimensional organization of the endonuclease site of MutL and exposes the presence of a regulatory Zn$^{2+}$-binding site that is important for the MMR function of BsMutL in vivo. The structure allows for us to propose a model describing how the association of MutS and the DNA polymerase III processivity clamp (b clamp) with MutL could license nicking of a newly synthesized DNA strand.

**RESULTS AND DISCUSSION**

**Crystal Structure of BsMutL-CTD**

Three crystal forms of the C-terminal domain of BsMutL (BsMutL-CTD) were obtained. Crystal form I was used to determine the structure of BsMutL-CTD by multiwavelength anomalous diffraction using crystals grown with Sel-Met substituted protein (Table 1). This crystal includes four independent monomers (molecules A–D) in the asymmetric unit that associate through crystal symmetry to form the functional BsMutL-CTD dimer. Crystal forms II and III contained a single dimer in the asymmetric unit. In the three structures, the N- and C-terminal ends of BsMutL-CTD (residues 433–461/580–627) define the dimerization subdomain, while residues 474–573 define an external subdomain that protrudes to the solvent, herein referred to as the regulatory subdomain (Figures 1A and 1B). The subdomains are connected by helix $\alpha$A (residues 463–473), encompassing the conserved endonuclease motif, and the linker connecting helices $\alpha$D–$\alpha$E (residues 575–581), which is disordered in our structures (Figure 1A). However, the relative orientation between subdomains varies from one crystal form to another (see Movie S1 available online).

The three complimentary conserved motifs associate with the endonuclease motif cluster around helix $\alpha$A to delineate a single catalytic site with the conserved endonuclease motif ($462^\text{DQHA}(\chi_2)^{\text{E}(\chi_4)^{\text{E}}}$) (Figures 1B and 1C). $604^\text{CPhGRP}$ resides in the $\alpha$E-$\beta$8 loop, $572^\text{SKC}$ (consensus sequence ACR) is the last turn of the $\alpha$D helix, and $623^\text{FKR}$, at the C terminus of the protein, reaches the active site of the other protomer (Figure 1B). Except for the $572^\text{SKC}$ motif, contributed by the regulatory subdomain, all motifs reside in the dimerization subdomain.

Even though the C-terminal regions of EcMutL and BsMutL have very low sequence similarity, their structures have nearly identical topologies (Figure S1). However, key differences exist. Superimposition of the EcMutL-CTD monomer onto the BsMutL-CTD monomer returned root-mean-square deviations (rmsds) of only 1 Å for the dimerization subdomains but >2 Å for the

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**Data Refinement**

| Resolution (Å) | 30–2.5 | 38.9–2.0 | 32.8–2.3 |
| Reflections (work) | 31,772 | 60,020 | 41,004 |
| Reflections (test) | 1,631 | 3,049 | 2,113 |
| Atoms refined | 6,122 | 3,294 | 3,120 |
| Solvent atoms | 105 | 190 | 74 |
| Zn atoms | – | – | 4 |
| R $(\text{Res})$ (%) | 21.7 (26.8) | 19.1 (22.8) | 21.4 (26.9) |
| Rmsd in bonds (Å) | 0.003 | 0.005 | 0.004 |
| Rmsd in angles (°) | 0.609 | 0.875 | 0.745 |
| Mean B values (Å$^2$) | 44.1 | 45.6 | 43.9 |

$^a$Data in the highest-resolution shell are shown in parentheses.
regulatory subdomains, reflecting the increased divergence of this region. This is intriguing because the regulatory subdomain contributes minimally to the endonuclease site. The most striking difference between the two structures is the organization of the secondary structure elements surrounding helix a, which would preclude the formation of a functional endonuclease site even if EcMutL had the conserved DQHA(X)E(X)4E motif (Figure S1). Notably, the extended a-E-b loop in BsMutL, rather than the additional helix seen in EcMutL, brings the 604CPHGRP motif closer to helix a and secludes the endonuclease site. The dimerization interfaces are also remarkably different. While the BsMutL dimer buries 1065 Å², the EcMutL dimer only conceals 910 Å². Interestingly, the reorientation of the apposing b sheets in the BsMutL-CTD dimer allows the 623FKR motif to reach the adjacent endonuclease site.

**BsMutL Has Weak Endonuclease Activity**

Similarly to other MutL orthologs, BsMutL had a weak endonuclease activity dependent on Mn²⁺ (Figure 2A). Both a point mutation in the endonuclease motif (D462N) and deletion of the ATPase domain virtually abolished the endonuclease activity of BsMutL (Figure 2A). Notably, the extended a-E-b loop in BsMutL, rather than the additional helix seen in EcMutL, brings the 604CPHGRP motif closer to helix a and secludes the endonuclease site. The dimerization interfaces are also remarkably different. While the BsMutL dimer buries 1065 Å², the EcMutL dimer only conceals 910 Å². Interestingly, the reorientation of the apposing b sheets in the BsMutL-CTD dimer allows the 623FKR motif to reach the adjacent endonuclease site.

Figure 1. Crystal Structure of BsMutL-CTD

(A) Ribbon diagram of the BsMutL-CTD monomer. Secondary structure motifs are labeled and colored blue (helices) and yellow (strands) with the connecting loops in light green. The endonuclease and the endonuclease-associated motifs are shown in purple, while the additional conserved motifs are shown in orange.

(B) Ribbon diagram of the BsMutL-CTD dimer with one protomer shown as in (A) and the other one as gray ribbons.

(C) Sequence alignment of the C-terminal regions of BsMutL, hPMS2, and EcMutL. Secondary structure elements of BsMutL-CTD are shown as arrows (strands) and cylinders (helices). The five conserved motifs are highlighted in purple and underlined. Conserved hydrophobic residues are highlighted in yellow. The conserved 487QEMIVP motif is highlighted in orange. See also Figure S1.

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Addition of 0.5 mM ATP stimulated the nicking activity of BsMutL, but higher concentrations of ATP (5 mM) inhibited the nicking activity, presumably due to excess nucleotide chelating Mn²⁺ ions away (Figure 2B, lanes 5 and 6). Unexpectedly, addition of ATP and/or Mg²⁺ stimulated a second cut on the nicked DNA to yield a linear product. The cut of the two strands at nearby points could be due to the presence of two endonuclease sites in the BsMutL homodimer or a consequence of the high-ion concentrations used in the experiment. We favor the former because incubation with 10 mM Mn²⁺ did not cause nicking of the two strands (data not shown), but addition of only 1 mM of a second metal ion such as Zn²⁺ or Co²⁺ yielded a linear product (Figure 2D). Interestingly, Mg²⁺ did not support double nicking under these conditions, suggesting that BsMutL may have higher affinity for Zn²⁺ or Co²⁺ than Mg²⁺.

We then characterized the ATPase activity of BsMutL (K_m = 0.4 mM and k_cat = 0.3 min⁻¹) and found that it is a weaker ATPase than other MutL homologs (Ban et al., 1999; Guarné et al., 2001; Hall et al., 2002). Given the slow ATP-hydrolysis rate, the stimulation of the endonuclease activity of BsMutL was likely due to ATP binding rather than ATP hydrolysis. In good agreement with this idea, ADP did not stimulate the endonuclease activity of BsMutL (Figure 2B, lanes 9 and 10).

However, two known nonhydrolyzable analogs of ATP, AMPPnP and ATPγS, did not stimulate the endonuclease activity of BsMutL beyond the levels observed when both Mn²⁺ and Mg²⁺ were present (Figure 2B, compare lanes 4, 7, 9, and 11).

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Figure 2. Endonuclease Activity of BsMutL
(A) Nicking activity of BsMutL (left) and BsMutL-CTD (center) in the presence of Mg2+, Zn2+, Mn2+, or Cd2+ as indicated. Comparison of the nicking activity of BsMutL and BsMutL-D462N in the presence of Mn2+ (right). Migration of either. Conversely, the BsMutL-CTD dimer found in the asymmetrical unit of crystal form III contained two Zn2+ ions bound to each protomer; a fully occupied Zn2+ ion (Zn2+) was coordinated by the side chains of residues Glu468, Cys604, His606, and a well-ordered water molecule, and a partly occupied site (Zn2+) coordinated by the side chains of residues His464, Glu468, Cys573 and a water molecule (Figure 3A). The nature of the metal ion was confirmed on the anomalous difference electron density maps from diffraction data collected at the Zn2+ absorption edge (Table 1).

It had been previously reported that the putative Zn2+-binding site in hPMS2 could be related to the regulatory metal-binding site found in the iron-dependent repressors from the DtxR/MntR family (Kosinski et al., 2008). A structural comparison revealed that, while motifs 462DQHAX2EX4E, 572SCK, and 604CGHGRP from BsMutL could be superimposed to the regulatory metal-binding sites of IdeR or MntR, the residues coordinating the metal ion differed. However, another Mn2+-dependent repressor from the same family (ScaR, PDB 3HRU) encompasses a regulatory metal-binding site identical to the fully occupied Zn2+ site in BsMutL-CTD.

To probe whether the two metal-bound sites found in our structure were true Zn2+-binding sites, we measured the affinity of purified BsMutL-CTD and various BsMutL-CTD variants for zinc. To this end, we measured the fluorescence of increasing concentrations of Zn2+ bound to the fluorescence indicator FluoZin-3. In the absence of protein, fluorescence increased exponentially, reaching maximum values at around 1.5–2 μM ZnCl2. The sigmoidal response observed for wild-type and the D462N, H464S, and E473K variants was characteristic of zinc binding by the protein (Figure 3B), suggesting that these BsMutL-CTD variants still retained the ability to bind zinc. Addition of the sulfhydryl-modifying agent methyl methanethiosulfonate (MMTS) resulted in an increase of fluorescence to the level detected in the absence of protein, indicating that one or more cysteine residues within the C-terminal domain of BsMutL were important for Zn2+ binding (data not shown). Conversely, fluorescence profiles of E468K, C604A, H606S, and C604A/H606S mutants did not have a sigmoidal response (Figure 3B), revealing that these BsMutL-CTD variants had lost the ability to bind zinc. These results confirmed that Glu468, Cys604, and His606 define the Zn2+-binding site in BsMutL (Figure 3A).

Integrity of the Conserved Motifs Is Important for Mismatch Repair In Vivo
We presumed that the integrity of the Zn2+-binding site in MutL would be important for proper MMR. Therefore, we measured the MMR efficiency of BsMutL variants encompassing point mutations in the conserved residues involved in the endonuclease or Zn2+-binding sites. Mutation of D462A, H464A, E468K, or H606A completely inactivated MMR in vivo (Figure 3C), underscoring the importance of these residues. Similarly, the equivalent mutations in hPMS2 also conferred a strong mutator phenotype (Kosinski et al., 2008). Conversely, the BsMutL-Q463A and BsMutL-E473K variants had similar MMR efficiency to wild-type BsMutL, suggesting that not all the conserved residues within these motifs play essential roles in MMR.

We next analyzed the importance of other conserved motifs found in the C-terminal region of MutL, namely the 487QEMIVP
motif. Mutation of Ile490 almost completely inactivated MMR in vivo, whereas mutation of Pro492 was without effect (Figure 3C). A BsMutL-Q487A variant conferred approximately a 50-fold mutator phenotype about 5-fold lower than a mutL null strain. Replacement of residues 487QEMIV with five alanine residues also abrogated MMR in vivo (Figure 3C). This conserved loop is conspicuously exposed and loosely resembles the consensus \( \beta \)-binding motif (Dalrymple et al., 2001). Most notably, its conformation is nearly identical to that seen in the structures of other peptides bound to the \( \beta \) clamp (Figure 3D). Superimposition of the 486VQEMIVPL sequence from BsMutL onto the structures of \( \beta \) clamp bound to peptides from polymerase II, FEN-1, and clamp loader. Additionally, MutS and MutL would thus require a significant conformational change or additional repair factors to mask helix xC, since some of the negatively charged residues in helix xC are conserved in other MutL homologs harboring the endonuclease motif (Figure 1C and Kosinski et al., 2008). Licensing the endonuclease activity of MutL would thus require a significant conformational change or the interaction with other repair factors to overcome the DNA repulsion in the vicinity of the endonuclease site. We presume that a conformational change like that induced in MutL\( \alpha \) upon nucleotide binding could allow DNA bound at the ATPase domain to reach the endonuclease site (Sacho et al., 2008). However, additional repair factors are likely required to mask helix xC.

The endonuclease activity of MutL\( \alpha \) is greatly stimulated by the presence of PCNA and RFC (Kadyrov et al., 2006; Kadyrov et al., 2007), the eukaryotic homologs of the \( \beta \) clamp and the clamp loader. Additionally, MutSz and PCNA form a stable complex (Iyer et al., 2008). Human MutL\( \alpha \) interacts with MutSz through its ATPase domain (Plotz et al., 2006), but the region of MutSz that interacts with MutL\( \alpha \) is not known. Conceivably, the three proteins could form a ternary complex involved in strand discrimination; however, whether MutSz and MutL\( \alpha \) can interact simultaneously with PCNA is controversial (Dzantiev et al., 2004; Lee and Alani, 2006). Bacterial MutS has two binding...
sites for the β clamp (Lopez de Saro et al., 2006; Simmons et al., 2008). In B. subtilis, the C-terminal site is necessary to recruit MutL to mismatches and to activate the MMR response (Simmons et al., 2008). We presume that the ATPase domain of MutL could interact with MutS, while its C-terminal domain interacts with the β clamp (Figure 4B). This model is supported by the presence of the β-binding-like motif (487QEMIV) within the C-terminal domain of MutL and the fact that a PCNA-binding sequence has been identified in the dimerization region of yeast MLH1 (Lee and Alani, 2006).

Collectively, our data pose an attractive model in which the endonuclease activity of MutL is repressed by impaired DNA binding. Based on these data, the simplest mechanism would harness MutL and β clamp, allowing for DNA binding and licensing of the endonuclease activity. Consequently, the structure provides a platform for future mechanistic studies of MutL-MutS-β at the early steps of MMR.

EXPERIMENTAL PROCEDURES

Cloning, Purification, and Crystallization

Full-length BsMutL was amplified from genomic DNA and cloned into the pProEXHTa expression vector (Life Technologies). His-tagged BsMutL was purified using a Ni2+-chelating affinity column equilibrated with 20 mM Tris (pH 8), 0.5 M NaCl, 1.4 mM β-mercaptoethanol, 5% glycerol, and 100 mM PMSF. BsMutL was eluted using 300 mM imidazole and subsequently injected on a hydrophobic column equilibrated with 20 mM Tris (pH 8), 1 M KCl, 1 mM DTT, and 5% glycerol. BsMutL was further purified by ionic exchange and size exclusion chromatography (MonoQ 5/50 and Superdex-S200, GE Healthcare) equilibrated with 20 mM Tris (pH 8), 100 mM KCl, 1 mM DTT, and 5% glycerol (storage buffer). Guided by a structure-based sequence alignment, we subcloned the C-terminal fragment of BsMutL (BsMutL-CTD, residues 433–627). BsMutL-CTD was purified similarly to BsMutL with an additional ionic exchange purification step after His-tag cleavage with TEV-protease. Mutants of BsMutL and BsMutL-CTD were generated by QuikChange (Stratagene) and verified by DNA sequencing (MOBIX, McMaster University).

Crystal form I was grown in 25% PEG-monomethyl ether 550, 0.1 M MgCl2, 0.1 M Tris (pH 9), and 5% PEG 400. Two additional crystal forms were obtained when the protein was supplemented with 50 mM ZnCl2 and 50 mM CoCl2. Crystal form II was grown in 25% PEG 3350, 0.15–0.2 M NaCl, and 0.1 M Tris (pH 7). Addition of 0.5 mM ZnCl2 to this crystallization solution yielded crystal form III. PEG 400 (10%) was added to all crystallization conditions prior to flash freezing in liquid nitrogen.

Data Collection and Structure Determination

For crystal form I, a three-wavelength MAD data set was collected at X29B in NSLS, Brookhaven National Laboratory (Upton, NY). Data were indexed, processed, and merged using HKL2000 (Otwinowski and Minor, 1997). Twenty-seven out of thirty-six Selenium sites were found and refined using SOLVE (Terwilliger and Berendzen, 1999). A native data set to 2.5 Å was used for subsequent manual building and refinement, which was done using standard protocols in phenix.refine and COOT (Afonine et al., 2005; Emsley and Cowtan, 2004). Complete data sets of crystal forms II and III were collected at the X25 beamline in NSLS. Data were collected at a wavelength corresponding to the Zn2+-absorption edge (Table 1), as measured using fluorescence scans. All final models have more than 92% of the residues within the most favored regions in the Ramachandran plot and none in disallowed regions. Figures depicting molecular structures were generated using PyMol (DeLano, 2002).

Endonuclease and DNA-Binding Assays

BsMutL nicking activity was assayed as previously described (Kadyrov et al., 2006), with minor modifications described in the Supplemental Information. To assess DNA binding by BsMutL, supercoiled DNA (5 nM) was incubated with BsMutL, variants (100 nM, dimer) in endonuclease buffer for 90 min at 37°C. Reaction mixtures (20 μL) were resolved on 1% TAE agarose gels and quantified using ImageJ (http://rsbweb.nih.gov/ij/). Data were presented as the mean of three independent measurements, and the error bars correspond to the standard errors of the mean (SEM = s/n, where s is the average and n the sample size).

Zinc-Affinity Fluorescence Assay

Wild-type and variants of BsMutL-CTD (2 μM) were incubated with 1 μM Fluo-Zin-3 (Invitrogen) in 10 mM HEPES (pH 6) and 200 mM KCl buffer treated with 1% Chelex-100 (Fluka). A calibration fluorescence curve was generated using buffer including increasing concentrations of ZnCl2 (0.25–3 μM) in the absence of protein. Spectra (500–600 nm) were recorded at an excitation wavelength of 494 nm (corrected for buffer effects). Innerfilter effects were neglected because a linear fluorescence intensity response up to stoichiometric amounts
of Zn²⁺ was seen when using 4 μM FluoZin-3. Data were presented as the mean of three independent measurements, and the error bars correspond to the SEM.

**Mismatch Repair Assays of BsMutL Variants**

MMR assays were performed largely as described (Simmons et al., 2008). See the Supplemental Information for a more complete description of the methods.

**ACCESSION NUMBERS**

Atomic coordinates and structure factors of BsMutL-CTD have been deposited in the Protein Data Bank under accession codes 3GAB, 3KDG, and 3KDK.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, one movie, and one table and can be found with this article at doi:10.1016/j.molcel.2010.06.027.

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