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The endonuclease domain of MutL interacts with the β sliding clamp

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Abstract

Mismatch repair corrects errors that have escaped polymerase proofreading enhancing replication fidelity by at least two orders of magnitude. The β and PCNA sliding clamps increase the polymerase processivity during DNA replication and are important at several stages of mismatch repair. Both MutS and MutL, the two proteins that initiate the mismatch repair response, interact with β. Binding of MutS to β is important to recruit MutS and MutL to foci. Moreover, the endonuclease activity of human and yeast MutLα is stimulated by PCNA. However, the concrete functions of the processivity clamp in the repair steps preceding DNA resynthesis remain obscure. Here, we demonstrate that the C-terminal domain of MutL encompasses a bona fide β-binding motif that mediates a weak, yet specific, interaction between the two proteins. Mutation of this conserved motif correlates with defects in mismatch repair, demonstrating that the direct interaction with β is important for MutL function. The interaction between the C-terminal domain of MutL and β is conserved in both *B. subtilis* and *E. coli*, but the repair defects associated with mutation of this β-binding motif are more severe in the former, suggesting that this interaction may have a more prominent role in methyl-independent than methyl-directed mismatch repair systems. Together with previously published data, our work strongly suggests that β may stimulate the endonuclease activity of MutL through its direct interaction with the C-terminal domain of MutL.

Keywords

DNA mismatch repair; MutL; β-sliding clamp; PCNA; endonuclease

INTRODUCTION

Mismatch repair (MMR) corrects errors that have escaped polymerase proofreading, thereby enhancing replication fidelity [1]. Mutations inactivating mismatch repair proteins lead to genome instability and, in humans, a predisposition to sporadic and hereditary cancers [2].

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In the prototypical Gram-negative bacterium *Escherichia coli*, three proteins work together to identify the mismatch and target the newly synthesized strand for repair. MutS recognizes a mismatched base pair or a small insertion/deletion loop and recruits a molecular matchmaker, MutL, which in turn activates the latent endonuclease MutH. MutH cleaves the unmethylated DNA strand at hemimethylated GATC sites transiently generated during DNA replication. Subsequently, helicase II (UvrD), exonucleases (ExoI and ExoX or RecJ and ExoVII, depending whether the nick is generated 3′ or 5′ to the mismatch), single-strand binding protein (SSB), DNA polymerase III holoenzyme and DNA ligase are recruited to excise the newly synthesized strand slightly past the mismatch and re-synthesize it correctly (reviewed in [1,3]).

Mismatch repair proteins are evolutionary conserved and homologues of MutS and MutL have been identified in all organisms. Although most bacteria and all eukaryotes do not encode a MutH homolog, a pre-existing nick is sufficient to activate mismatch repair in a system reconstituted from purified proteins [4]. It has been shown that certain human and yeast homologues of MutL (hPMS2, hMLH3, yPMS1 and yMLH3) encompass a PCNA-dependent endonuclease activity [5,6]. PCNA, and its bacterial counterpart the β subunit of DNA polymerase III (β), are ring-shaped structures that enhance polymerase processivity by creating a topological link with the DNA template and enabling sliding during chain elongation [7], hence they are often referred to as sliding clamps. Beyond replication, sliding clamps are also important for exchanging polymerases when the replication fork encounters damaged DNA and in orchestrating post-translational repair [1,8]. Most proteins that interact with PCNA and β do so through extended sequences at their N- or C-termini. However, while the PCNA-interacting motif (PIP box, QxxLxxFF) has a strictly defined consensus sequence, the β-interacting motif (QLxLF) is poorly conserved and absent in a number of β-binding proteins.

PCNA interacts with eukaryotic MutSα (hMSH2-hMSH6) and MutLα (hMLH1-hPMS2) [9-11]. It has also been shown to interact with exonuclease I (EXOI) and to co-localize with EXOI at DNA replication foci [12]. PCNA plays a clear role in DNA re-synthesis and it is presumed to function at earlier steps of mismatch repair, however its role remains unclear. Similarly, bacterial MutS has two binding sites for β, a weak site at the N-terminus, within the mismatch binding domain, and a stronger site at its C-terminus [13,14]. In the Gram-positive bacterium *Bacillus subtilis*, the C-terminal site is necessary to recruit MutS to mismatches and to activate the MMR response [14]. One putative β-interacting site has been identified within the ATPase domain of *E. coli* MutL [13]. Interaction with β mediated through this β-binding site is regulated by conformational changes induced by nucleotide- and single-stranded DNA binding to MutL [13]. However, mutation of this motif only reduces the interaction with β, suggesting that additional β-binding sites may be present.

The recent crystal structure of the endonuclease domain of *B. subtilis* MutL (BsMutL-CTD) has revealed that three conserved motifs (ACR, C(P/N)HGRP and FXR) cluster around the endonuclease motif ($DQHA(X)_2E(X)_4E$) to define a unique active site [15]. Sequence analysis also revealed the presence of an additional conserved motif within the C-terminal domain of MutL that is unrelated to the endonuclease activity of the protein [16]. The consensus sequence of this motif loosely resembles that of the β-binding motif and its location in the structure of BsMutL-CTD suggests that it could mediate protein-protein interactions [15]. Here we present evidence demonstrating that this conserved motif is a bona fide β-binding motif and that it indeed mediates the interaction between the C-terminal domain of MutL and β. Disruption of this motif abrogates the interaction between MutL and β in both *E. coli* and *B. subtilis*, suggesting that this interaction is conserved in both methyl-directed and methyl-independent mismatch repair systems. However, the mutator phenotype associated with the disruption of this motif is milder in *E. coli* than in *B. subtilis*, implying

that the interaction between MutL and β may have a more prominent role in methyl-independent mismatch repair systems.

MATERIALS AND METHODS

Cloning of MutL variants

BsMutL-CTD (pAG8188, residues 433 to 627) was cloned as described earlier [15]. The BsMutL-CTD* variant (pAG8350, encompassing a ^{487}QEMIVP mutated to ^{487}AEMAAP) was generated by QuikChange (Stratagene) using pAG8188 as template and the regulatory subdomain of BsMutL, BsMutL-RGD (pAG8313, residues 471 to 574), was subcloned into the pProExHTa vector using the NcoI and XhoI restriction sites. The pET15b plasmids encoding full-length (EcMutL, pTX418) and the C-terminal domain of *E. coli* MutL (pWY1295, residues 432 to 615) were a kind gift from Dr. Wei Yang. The EcMutL-CTD* variant (pAG8417, ^{482}QPLLIP to ^{482}ASAAAP) was generated by overlap PCR and subcloned in pET15b between the NdeI and BamHI restriction sites. The regulatory subdomain of EcMutL, EcMutL-RGD (pAG8442, residues 466 to 569), was subcloned into the pProExHTa vector using the NcoI and XhoI restriction sites. For the *in vivo* mismatch repair assays, three variants of full-length EcMutL were generated by overlap PCR and subcloned in pET15b: pAG8472 (EcMutL-Q482A), pAG8480 (EcMutL-L485A) and pAG8477 (EcMutL*, ^{482}QPLLIP to ^{482}ASAAAP). All plasmids were verified by DNA sequencing (MOBIX, McMaster University).

Protein expression and purification

All MutL variants were overproduced and purified as described earlier with minor modifications [15,17,18]. Purified proteins were stored in 20 mM TRIS pH 8, 100 mM KCl, 1 mM DTT and 5% glycerol (storage buffer). The over-expression plasmids encoding *E. coli* β (residues 1-366) and *B. subtilis* β (residues 1-378) were generous gifts from Dr. Mike O'Donnell and Dr. Lyle A. Simmons. Ecβ was overproduced in *E. coli* BL21 (DE3) Star cells and Bsβ in BL21 (DE3) recA^{-} cells as described earlier [14,19]. Cell pellets containing Ecβ were resuspended in buffer A (20 mM Tris pH 8.0, 1 mM EDTA, 5 mM DTT, 0.05 M NaCl, and 5% glycerol), incubated with 0.6 mg/ml lysozyme for 30 minutes on ice, and cells disrupted by sonication. Lysates were clarified by centrifugation at 39,000 g and loaded onto a heparin column connected in tandem to a Q-sepharose column equilibrated with buffer A. Purified Ecβ was eluted from the Q-sepharose column using a salt gradient to 0.5 M NaCl. The sample was further purified over a MonoQ 5/50 column (GE Healthcare).

Cell pellets containing Bsβ were resuspended in 50 mM Tris pH 7.0 and 10% sucrose and lysed by freeze-thaw in 0.5 M NaCl, 20 mM $SpCl_3$, and 0.45 mg/ml lysozyme. Cell lysates were clarified by centrifugation as above and the soluble fraction containing Bsβ was loaded onto a HiTrap nickel-chelating column equilibrated with 20 mM Tris pH 7.6, 0.5 M NaCl, 1.4 mM 2-mercaptoethanol, 45 mM imidazole, and 15% glycerol and eluted with 0.24 M imidazole. The sample was subsequently purified by ionic exchange using a Q-sepharose column pre-equilibrated with 20 mM Tris pH 7.6, 1 mM EDTA, 5 mM DTT, 0.15 M KCl, and 15% glycerol using a salt gradient to 0.4 M KCl. All subsequent experiments were performed with His-tagged Bsβ.

Protein Cross-linking with BS^3

Frozen protein samples were thawed and loaded onto a Superdex-S200 (GE Healthcare) equilibrated in 20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM DTT, and 5% glycerol (reaction buffer) to exchange the buffer, as well as, to remove protein aggregates caused during freezing/thawing. MutL variants (20 μM) or β (10 μM) were incubated with 0.4, 0.8, 1.0, and 1.2 mM Bis(sulfosuccinimidyl)suberate (BS^3, Sigma) in reaction buffer for

30 minutes at 22°C. Crosslinking reactions (10 μL) were quenched with 30 mM Tris pH 7.5 for 15 minutes at 22°C and resolved on 10% SDS polyacrylamide gels stained with Coomassie blue. Optimal crosslinking for both proteins was observed at BS^3 concentrations of 0.8 mM and hence all subsequent controls using the individual proteins were done at this crosslinker concentration. MutL and β were incubated at 2:1 molar ratios to account for the possibility that two MutL dimers were bound to a dimer of β. Mixtures of MutL:β (2:1) were incubated with 0.4, 0.8, 1.0, and 1.2 mM BS^3 and the reaction was quenched after 30 minutes as described above. The reaction products were resolved on 4-15% SDS polyacrylamide gels (BioRad) stained with Coomassie blue. The identity of the bands was confirmed by mass spectrometry (Bioanalytical and Spectrometry Laboratory, McMaster University).

Determination of Mutation rates

The CC107 *mutL::miniTn10* strain was constructed by transducing CC107 [20] to Tet^r with a P1 *vir* lysate grown on strains carrying a mini*Tn10* insertion in the *mutL* gene (Miller et al, unpublished data). CC107 *mutL::miniTn10* cells were then transformed with either pET15b, pTX418, pAG8472, pAG8477 or pAG8480. 1 or 2 ml Luria-Bertani broth cultures containing 100 μg/ml ampicillin were seeded with 100-1,000 transformed cells and grown overnight. Samples were plated on LB plates containing 100 μg/ml rifampicin, and dilutions were plated on LB plates with ampicillin to determine the titer. The frequencies of rifampicin resistant (Rif^r) mutants were determined, and the median frequency (*f*) from a set of cultures was used to calculate the mutation rate per replication by the method of Drake, using the formula $\mu = f/\ln N\mu$, where *N* is the number of cells in the culture [21]. Ninety-five percent confidence limits were determined according to Dixon and Massey [22].

RESULTS

Putative β-binding motif in MutL-CTD

Sequence analysis has identified a conserved motif with a consensus sequence of Qxφ[L/I]xP (where φ is a hydrophobic residue) within the C-terminal domain of MutL. This motif is found in prokaryotic and eukaryotic MutL homologues harboring an endonuclease activity, but also in those that do not (Figure 1A and [16]). The structures of the C-terminal domains of *E. coli* and *B. subtilis* MutL reveal that this chiefly hydrophobic motif resides in the regulatory subdomain of the C-terminal region and it is conspicuously exposed to the solvent (Figure 1B-C and [15, 18]). Superimposition of the regulatory subdomains of BsMutL and EcMutL returns root mean square deviations of 2 Å for 89 Cα-atoms (Figure 2A). This value is twice that obtained when superimposing the dimerization subdomain of both proteins. However, the conformation of the conserved Qxφ[L/I]xP motif (^{487}QEMIVP in BsMutL and ^{482}QPLLIP in EcMutL) is virtually identical in both structures (r.m.s.d. of 0.27 Å), suggesting that it could mediate specific interactions between MutL and other repair factors.

Since the endonuclease activity of human and yeast MutLα is highly stimulated by PCNA [5], we suspected that this ubiquitous motif could mediate the interaction between MutLα and PCNA, or between bacterial MutL and β [15]. Indeed, the Qxφ[L/I]xP motif loosely resembles the consensus β-binding motif (QLxLF) [23]. In all the β structures bound to peptides or small protein domains encompassing a β-binding motif, the motif adopts an extended conformation that lines the C-terminal end of β. Two critical residues, glutamine at position one and leucine at position four, anchor this interaction. Additional hydrophobic and bulky residues at positions five or six strengthen the interaction, but they are less conserved and adopt variable conformations (Figure 2B). The putative β-binding motifs in BsMutL-CTD (^{487}QEMIVP) and EcMutL-CTD (^{482}QPLLIP) contain the conserved Gln and

Leu/Ile at positions one and four. Superimposition of these motifs onto several β-binding motifs bound to β (or PIP motifs bound to PCNA) returned root mean square deviations smaller than 0.4 Å, strongly suggesting that this loop could mediate the interaction between the C-terminal domain of MutL and β. Indeed, the first four residues of the motif adopt the same extended conformation seen in other bona fide β-binding motifs with the side chains of the conserved Gln and Leu/Ile occupying the major binding pockets on β and PCNA (Figure 2C). At position five, the main chain changes direction, in a similar manner to the consensus PIP motif in FEN-1 (PDB 1RXM), and it is followed by a sharp kink at the following proline that projects the MutL away from the ancillary binding pockets in the β-clamp (Figure 2C).

Bacillus subtilis MutL-CTD interacts specifically with β

We wanted to probe whether this conserved motif in MutL could indeed mediate the interaction with β. However, we could not detect the interaction by either size exclusion chromatography or fluorescence anisotropy (data not shown), suggesting that the interaction between BsMutL-CTD and Bsβ is probably transient. Since transient interactions can often be detected using chemical crosslinkers, we treated BsMutL-CTD and Bsβ with Bis(Sulfosuccinimidyl) suberate (BS3), a crosslinker that contains two amine reactive NHS-esters separated by an 11.4 Å spacer. Treatment of BsMutL-CTD (22,664 Da) with increasing concentrations of BS3 resulted in two crosslinked products (Figure 3). The first one migrated on SDS-polyacrylamide gels faster than the BsMutL-CTD monomer and could result from the intramolecular crosslinking of the dimerization and regulatory subdomains. The second product migrated at a molecular weight consistent with the formation of BsMutL-CTD dimers (45,328 Da). Similarly, treatment of the Bsβ (42,103 Da) with increasing concentrations of BS3 resulted in the formation of intra- and inter-molecular crosslinks, consistent with the formation of β dimers (84,206 Da).

When BsMutL-CTD and Bsβ (2:1) were pre-incubated prior to treatment with BS3, a new product appeared with an apparent molecular weight consistent with a BsMutL-CTD monomer bound to a Bsβ monomer (Figure 3, top). The presence of the two proteins in this crosslinked product was confirmed by mass spectroscopy (data not shown). The equivalent product was also seen when the regulatory subdomain of BsMutL (BsMutL-RGD, 12,136 Da) was preincubated with Bsβ (Figure 3, center). This region of MutL lacks the dimerization subdomain and, therefore, only monomers of BsMutL-RGD were found when the protein was incubated with BS3. The presence of an additional crosslinked product in the samples containing both BsMutL-RGD and Bsβ indicates that the regulatory subdomain of MutL mediates the interaction with β. Since the conserved ^{487}QEMIVP motif resides in this region of MutL, we subsequently tested whether mutation of this sequence abrogated their interaction. When Bsβ and the BsMutL-CTD* variant (^{487}QEMIVP-^{487}AEMAAP, 22,537 Da) were treated with BS3, only crosslinking products corresponding to MutL-MutL or β-β interactions were detected (Figure 3, bottom), demonstrating that the ^{487}QEMIVP motif mediates the interaction between the C-terminal domain of BsMutL and Bsβ.

Escherichia coli MutL-CTD interacts specifically with β

Since this motif is highly conserved, we next asked whether the interaction between β and MutL was conserved in other species. To this end, we assayed for a specific interaction between *E. coli* MutL-CTD (EcMutL-CTD, 20,158 Da) and *E. coli* β (Ecβ, 40,587 Da). Similarly to Bsβ, we found that crosslinking of Ecβ only resulted in the formation of dimers (81,174 Da). However, EcMutL-CTD formed dimers, as well as, larger oligomers (Figure 4A). This finding was not unexpected since the crystal structure of EcMutL-CTD had initially suggested that dimerization occurred by the association of the regulatory subdomains (Figure 4B [18]), but ensuing analysis of the quaternary structure of EcMutL-

CTD proved that dimers formed through the dimerization subdomain of the protein as depicted in Figure 1C and [24]. Given the presence of multiple interaction surfaces, dimers (40,316 Da), trimers (60,474 Da), tetramers (80,632 Da) and larger oligomers were indeed expected following crosslinking with BS3 (Figure 4A).

Similarly to the *B. subtilis* proteins, a new crosslinking product also appeared when EcMutL-CTD and Ecβ (2:1) were pre-incubated prior to treatment with BS3 (Figure 4C). This product migrated on SDS-polyacrylamide gels at a molecular weight consistent with the formation of a complex between MutL-CTD and β (60,745 Da, Figure 4C). However, this product co-migrated in SDS-polyacrylamide gels with the EcMutL-CTD trimer (60,474 Da). Crosslinking experiments conducted at higher MutL:β ratios revealed that the band migrating at this molecular weight was indeed a doublet, suggesting the presence of both EcMutL-CTD trimers and EcMutL-CTD/Ecβ complexes (see bands labeled 1 and 2 on the Figure 4C inset). We verified the presence of both, MutL and β, in this crosslinked product using mass spectrometry (data not shown). As we had seen with the proteins from *B. subtilis*, the regulatory subdomain of EcMutL also supported the interaction with Ecβ (Figure 4D). We then assayed whether a variant of EcMutL-CTD encompassing a mutated ^{482}QPLLIP motif (EcMutL-CTD*, ^{482}QPLLIP-^{482}ASAAAP, 19,965 Da) could support the interaction and found that this variant of the protein did not form a complex with Ecβ (Figure 4E). Therefore, we concluded that the ^{482}QPLLIP motif mediates the interaction between the C-terminal domain of EcMutL and β.

It has been previously shown that the polC subunits from *Staphylococcus aureus*, *Streptococcus pyogenes* and *B. subtilis* can use *E. coli* β as their processivity subunit, while *E. coli* DnaE cannot use β from other species [25-27]. Therefore, we assayed whether the interaction between heterologous MutL and β could be detected. Indeed, we detected specific interactions between BsMutL-CTD and Ecβ, as well as, between EcMutL-CTD and Bsβ (Figure 5). These results reinforce the idea that the conserved motif in the C-terminal domain of MutL likely binds to the conserved pocket in β in a species-independent manner.

The MutL-CTD/β interaction is important for efficient mismatch repair

We have previously shown that mutations on the conserved ^{487}QEMIVP motif of BsMutL impair mismatch repair in *B. subtilis* [15]. Therefore, we next assayed whether the integrity of the ^{482}QPLLIP motif in EcMutL was also important for mismatch repair in *E. coli*. To this end, we generated the EcMutL-Q482A, EcMutL-L485A and EcMutL-(^{482}QPLLIP-^{482}ASAAAP) variants and measured both the frequencies (*f*) and mutation rates (μ) per cell per replication (Table 1). As a control, we measured the mutation rates obtained for a mismatch repair deficient strain, as well as, this strain complemented with a plasmid encoding wild-type MutL, which were virtually identical to our previously published values [18,28]. Mutation of the entire ^{482}QPLLIP motif clearly impaired MutL function, but not to the same extend as the *mutL*-deficient strain (Table 1). This finding was interesting, because disrupting the ^{487}QEMIVP motif in BsMutL impairs mismatch repair to a level comparable to the *mutL*-deficient strain [15]. Accordingly, the EcMutL-Q482A and EcMutL-L485A variants also had milder phenotypes than the corresponding BsMutL variants (Table 1 and [15]). In fact, mutation of Gln482 resulted in a MutL variant that behaved similar to wild-type MutL by this particular assay. Collectively, this data suggests that the interaction between MutL and β is important for MutL function across species, but it has a more prominent role in methyl-independent than methyl-directed mismatch repair systems.

DISCUSSION

We have shown that the C-terminal domains of *B. subtilis* and *E. coli* MutL interact specifically with β through a conserved motif that resides within the regulatory subdomain

of the protein. The motif that mediates this interaction (Qxφ[L/I]xP) partially resembles the consensus sequences of the β-binding motif (QLxLF) and the PIP-box (QxxLxxFF). These three motifs have two strictly conserved residues at positions one (Gln) and four (Ile/Leu). In the structures of β and PCNA bound to peptides encompassing β-binding motifs or PIP-boxes, respectively, these conserved residues occupy two well-defined binding pockets (Figure 2 and [29,30]). Accordingly, mutation of the conserved Gln and Ile/Leu residues in either BsMutL-CTD or EcMutL-CTD abrogated binding to β and affected mismatch repair function.

Most proteins that interact with either β or PCNA do so through extended sequences at their C-terminus. However, the β-binding motif in MutL is embedded in the regulatory subdomain and thus its conformation is restricted by the tertiary structure of the protein. The conserved proline at the C-terminus of the motif likely restrains the loop on an exposed conformation, indirectly enhancing the interaction to β. In the BsMutL-CTD* (^{487}QEMIVP to ^{487}AEMAAP) and EcMutL-CTD* (^{482}QPLLIP to ^{482}ASAAAP) variants this proline residue is intact and yet they have lost the ability to interact with β (Figures 3 and 4). Furthermore, mutation of this Pro492 in BsMutL to alanine does not affect mismatch repair *in vivo*, whereas mutation of the conserved Gln487 and Ile490 does [15], reinforcing the idea that this proline may help present the interacting motif but does not interact directly with the sliding clamp. Interestingly, Gly and Pro residues are often found immediately following internal β-binding motifs [23], suggesting that an increased flexibility or a restrained conformation may assist at presenting these internal binding sites to β.

In vitro, PCNA stimulates the endonuclease activity of MutLα in a mismatch independent manner [5,6] and, by virtue of its loading orientation, seems to determine the strand direction of MutLα incision in a mismatch-dependent manner [31]. Therefore, this interaction must be tightly regulated to ensure that the endonuclease activity of MutLα is only stimulated when a mismatch has been encountered. The lack of aromatic residues at the C-terminus of the MutL β-binding motif could result in a weaker interaction with β than other β-binding factors. A suboptimal β-binding motif could potentially be out competed by other β-binding proteins encompassing canonical β-binding sites, thereby providing an additional level of regulation. Indeed, the interaction between yeast MutLα and PCNA appears to be mediated by yMLH1 rather than yPMS1 supporting the idea that the interaction with a canonical PIP-box (as the one found in yMLH1) is stronger than that with a suboptimal site such as the motif present in MutL homologues encompassing an endonuclease activity (Figure 1 and [10]).

Both MutS and MutL have multiple β-binding sites. MutS has a strong β-binding site located at its C-terminal domain that mediates the MutS-β interaction in solution and aids the localization of MutS to mismatches in *B. subtilis*. The integrity of this site is not essential for mismatch repair in *E. coli*, although it is critical for mismatch repair in *B. subtilis*. Additionally, a weaker β-binding site is found in the mismatch-binding domain of MutS. Mutation of this site does not disrupt the MutS-β interaction in solution, but it confers a mutator phenotype [13,32]. Initially, one β-binding site was identified in MutL, located in the ATPase domain of the protein. Interaction with β mediated by this site only occurs in the presence of single stranded DNA and is weakened by nucleotide binding, presumably due to the dimerization of the ATPase domains of MutL. Mutation of this β-binding site weakens, yet does not abolish, the MutL-β interaction suggesting that other β-binding sites may be present [13]. The β-binding site of MutL characterized in the present work also mediates a specific interaction with β.

It is plausible that the two β-binding sites found in MutL orchestrate its multiple functions in mismatch repair. For instance, binding of β to the N-terminal site could aid in localizing

MutL to mismatches, while binding of β to the C-terminal site could tether the endonuclease domain of MutL to DNA. The latter scenario would suggest that β, or its eukaryotic analog PCNA, stimulate the endonuclease activity of MutL by bypassing the inability of its C-terminal domain to bind DNA [15], thereby drawing a parallel with the way that PCNA simulates the activity of FEN-1 [33]. This idea is in agreement with a recent study by Modrich and co-workers proposing that the interaction with PCNA determines the strand bias and the strand direction of MutLα incision [31]. Defects in this motif confer a strong mutator phenotype in *B. subtilis* [15], but only a mild mutator phenotype in *E. coli* (Table 1), likely reflecting the different roles of this interaction in methyl-directed and methyl-independent mismatch repair systems. Since MutL homologues that do not encode an endonuclease activity also interact with β through this C-terminal β-binding motif, tethering of the C-terminal domain of MutL to DNA may also enhance other steps of the repair process. Indeed, both the N- and C-terminal domains of MutL are necessary to interact and activate the helicase activity of UvrD in a DNA-dependent manner [18]. Therefore, the MutL-β interaction could potentially influence activation of UvrD in *E. coli*. While this idea needs further testing, it suggests that the interaction between the C-terminal domain of MutL and β could regulate several steps in the mismatch repair process, or help MutL recruit additional repair factors to the damaged site. This, in turn, would explain why mutation of this β-binding site is more deleterious in some organisms than others and reinforces the idea that β, and its eukaryotic analog PCNA, orchestrate the sequence of events that lead to mismatch repair in the cell.

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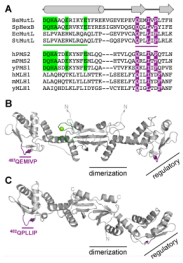

Figure 1. Conserved putative β-binding motif in MutL

(A) Sequence alignment of the putative β-binding motif (highlighted in purple) found in MutL and its location with respect to the endonuclease motif when present (highlighted in green). The top group includes *Bacillus subtilis* MutL (BsMutL) and *Streptococcus pneumoniae* HexB (SpHexB) that contain the conserved endonuclease motif, as well as, *Escherichia coli* MutL (EcMutL) and *Salmonella typhimurium* MutL (StMutL) that do not have endonuclease activity. The bottom group includes the eukaryotic *Homo sapiens* PMS2 (hPMS2), *Mus musculus* PMS2 (mPMS2), *Saccharomyces cerevisiae* PMS1 (yPMS1), as well as, eukaryotic MutL homologues that do not encompass an endonuclease motif: *Homo sapiens* MLH1 (hMLH1), *Mus musculus* MLH1 (mMLH1), *Saccharomyces cerevisiae* MLH1 (yMLH1). **(B)** Ribbon diagram of the C-terminal domain of *Bacillus subtilis* MutL (PDB ID: 3KDK) with the endonuclease motif shown in green and the β-binding motif shown in purple. The structural Zn^{2+} metal ion found at the endonuclease site is depicted as a green sphere. The N- and C-termini, the β-binding motif and the dimerization and regulatory subdomains are labeled for clarity. **(C)** Ribbon diagram of the C-terminal domain of *Escherichia coli* MutL (PDB ID: 1X9Z) shown as in (B).

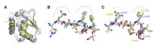

Figure 2. Structural comparison of the β-binding motifs found in MutL-CTD and other clamp-binding proteins

(A) Superimposition of the regulatory subdomains of *B. subtilis* MutL (blue) and *E. coli* MutL (yellow) shown as ribbon diagram. The N- and C-terminal domain boundaries are labeled. **(B)** Superimposition of the β-binding motif from polIV/DinB (green, PDB ID: 1UNN) and the PIP-box from FEN-1 (tan, PDB ID: 1RXM). Dotted lines depict the polar (left) and hydrophobic (right) pockets occupied by the conserved Gln and Leu residues. **(C)** Superimposition of the β-binding motifs of *B. subtilis* MutL (blue) and *E. coli* MutL (yellow) onto the structure of FEN-1 bound the PCNA (tan, PDB ID: 1RXM). Conserved residues are labeled.

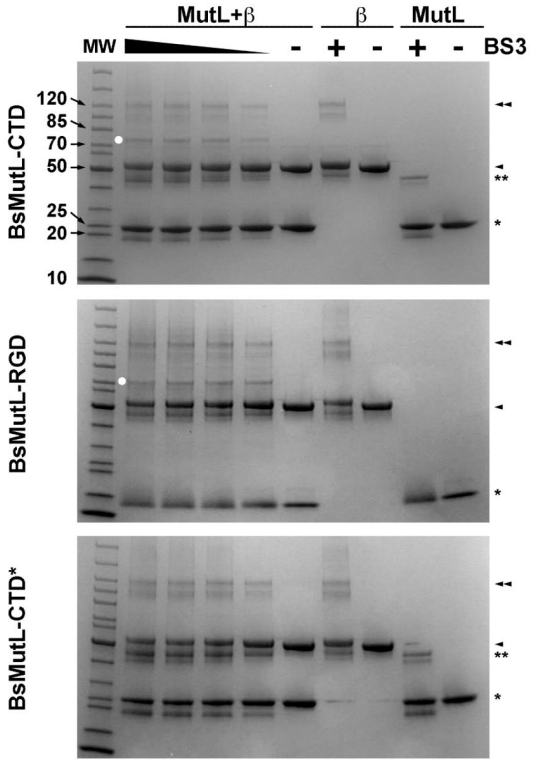

Figure 3. Complex formation between the endonuclease domain of *B. subtilis* MutL and β

Interaction between the C-terminal domain of BsMutL (BsMutL-CTD, top), the regulatory domain of BsMutL (BsMutL-RGD, center) or the C-terminal domain of BsMutL encompassing a mutated β-binding motif (BsMutL-CTD*, ^{487}QEMIVP-AEMAAP, bottom) with the *B. subtilis* β (β). The proteins were incubated in the presence/absence of BS3 and the reaction products were resolved by SDS-PAGE. From left to right, the gels show molecular weight markers (MW), mixtures of MutL (0.02 mM) and β (0.01 mM) incubated with decreasing concentrations of BS3, Bsβ incubated in the presence (+) or absence (−) of BS3, BsMutL variant (as indicated) incubated in the presence (+) or absence (−) of BS3. Monomers and dimers of BsMutL are indicated with one or two asterisks, and monomers and dimers of the β are indicated with one of two arrowheads, respectively. The presence of crosslinked products corresponding to the interaction of BsMutL with β is indicated with a white dot. Incubation of BsMutL-CTD* and β in the presence of BS3 does not result in the formation of this crosslinked product, indicating that the integrity of the β-binding motif is necessary to maintain the interaction.

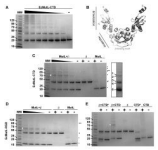

Figure 4. Complex formation between the C-terminal region of *E. coli* MutL and β

(A) 4-15% SDS-PAG showing the BS3-crosslinked products of the C-terminal domain of EcMutL (EcMutL-CTD). White asterisks indicate the migration of EcMutL-CTD oligomers. From left to right, the gel shows molecular weight markers (MW), EcMutL-CTD (0.04 mM) incubated with decreasing concentrations of BS3 (1.2, 1.0, 0.8, 0.5, 0.4, 0.3 0.2 and 0.1 mM) and EcMutL-CTD alone (−). **(B)** Alternative dimer interface found in the EcMutL structure (PDB ID: 1X9Z). The crystallographic dimer is maintained by the interaction of the regulatory subdomains, whereas the physiological dimer shown in Figure 1 is maintained by the interaction of the dimerization subdomains. **(C)** Mixtures of EcMutL-CTD and *E. coli* β were incubated in the presence or absence of BS3 and the reaction products were resolved by gradient SDS-PAGE. The protein and crosslinker concentrations, the gel layout and the labels of the bands are the same as Figure 3. The inset corresponds to the same experiment performed with excess EcMutL-CTD (0.2 mM, #5) and shows that some of the oligomeric forms of EcMutL-CTD (trimers #2 and dimers #4) migrate similarly to the β monomer (#3) and the crosslinked EcMutL-CTD/β product (#1). **(D)** Crosslinking products between the regulatory subdomain of EcMutL (EcMutL-RGD) and Ecβ obtained in the presence of BS3. The gel is shown and labeled as panel (C). **(E)** Comparison of the crosslinked products obtained with EcMutL-CTD and EcMutL-CTD* encompassing a mutated β-binding motif (^{482}QPLLIP-^{482}ASAAAP) and β. From left to right, the gels show molecular weight markers (MW), mixtures of EcMutL-CTD* and Ecβ, EcMutL-CTD and Ecβ, Ecβ, EcMutL-CTD* and EcMutL-CTD incubated in the presence (+) or absence (−) of BS3. For all reactions containing BS3 a final concentration of 1.2 mM was added.

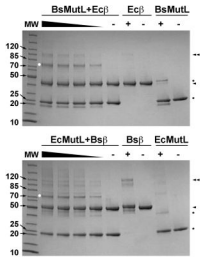

Figure 5. Interaction between heterologous MutL-CTD and β

Interaction between BsMutL-CTD and Ecβ (top) and EcMutL-CTD and Bsβ (bottom). From left to right, the gels show: molecular weight markers (MW), mixtures of MutL-CTD and β as indicated incubated with decreasing concentrations of BS3, β and MutL-CTD in the presence (+) or absence (−) of BS3.

Table 1

Frequencies (*f*) and rates (μ) of mutations in *rpoB* of a *mutL::miniTn*10 strain transformed with plasmids encoding variants of EcMutL.

	Empty vector (pET15b)	**EcMutL (pTX418)**	**EcMutL-Q482A (pAG8472)**	**EcMutL-L485A (pAG8480)**	**EcMutL-^{482}ASAAAP (pAG8477)**
$f \times 10^8$ *	910 (600-1,400)	3.3 (2.1-8.9)	2.7 (0.9-7.2)	272 (210-380)	66 (34-88)
$\mu \times 10^8$ **	110 (77-160)	0.9 (0.64-2.0)	0.9 (0.41-1.9)	39 (30-52)	11 (6.1-14)

* The *rpoB* mutation frequency was calculated by dividing the median number of mutants by the average number of cells in a series of cultures. Values in parentheses are the 95% confidence limits [22].

** The mutation rate per cell per replication (μ) was determined by the method of Drake [21]. Values in parentheses are 95% confidence limits.