Clamp Directs Localization of Mismatch Repair in Bacillus subtilis

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INTRODUCTION

Replicative DNA polymerases are responsible for duplicating entire genomes with high fidelity (for review see Johnson and O’Donnell, 2005). Despite the high fidelity of this process (e.g., Kool, 2002), replication errors still occur. Correction of these errors requires the enlistment of mismatch repair (MMR) proteins to restore the proper coding sequence (for review see Kunkel and Erie, 2005). MMR is an important, highly conserved repair pathway that is found in both prokaryotes and eukaryotes (Culligan et al., 2000).

In bacteria, deletion of MMR genes results in a several hundred-fold increase in mutation frequency (Cox et al., 1972). In hu-
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SUMMARY

MutS homologs function in several cellular pathways including mismatch repair (MMR), the process by which mismatches introduced during DNA replication are corrected. We demonstrate that the C terminus of Bacillus subtilis MutS is necessary for an interaction with β clamp. This interaction is required for MutS-GFP focus formation in response to mismatches. Reciprocally, we show that a mutant of the β clamp causes elevated mutation frequencies and is reduced for MutS-GFP focus formation. MutS mutants defective for interaction with β clamp failed to support the next step of MMR, MutL-GFP focus formation. We conclude that the interaction between MutS and β is the major molecular interaction facilitating focus formation and that β clamp aids in the stabilization of MutS at a mismatch in vivo. The striking ability of the MutS C terminus to direct focus formation at replisomes by itself, suggests that it is mismatch recognition that licenses MutS’s interaction with β clamp.

In eukaryotes, a heterodimer of MutS homologs (Saccharomy-
ces cerevisiae MSH2-MSH6 [MutSx]; [Prolla et al., 1994b] and hMSH2-hMSH6 in humans [Acharya et al., 1996]) is responsible for the recognition of most mismatches and small insertion-deletion loops (Drummond et al., 1995). These proteins recruit heterodimeric MutL homologs, (S. cerevisiae MLH1-PMS1 [MutLx], and human hMLH1-hPMS2 [Prolla et al., 1994a]), which function analogously to E. coli MutL and appear important for coordinating the actions of the remaining proteins in the pathway. The purified human proteins have been used to reconstitute MMR in vitro (Dzantiev et al., 2004). These studies have revealed that MMR excision from a 5’ strand break requires MutSx, an exonuclease (EXO1), and single-stranded binding protein (RPA). MMR excision from a strand break 3’ to the mismatch requires the proteins mentioned above in addition to MutLx, the clamp loader (RFC), and the replication processivity clamp (PCNA). These experiments indicate that the protein assemblies and mechanisms required for excision and repair are different depending on the directionality of the strand break relative to the mismatch. Theses studies also demonstrate the dependence on a processivity clamp for 3’ directed repair.

In B. subtilis, both MutS and MutL fused to green fluorescent protein (GFP) localize as discrete foci in cells exposed to the...
mismatch-inducing agent 2-aminopurine (2-AP) (Smith et al., 2001). A new challenge in understanding MMR is to determine the protein interactions that allow for the coordinated assembly of MMR foci in vivo. *B. subtilis* serves as a particularly useful system for addressing this issue because *B. subtilis* is the only bacterium in which MMR proteins have been visualized in vivo. In addition, like eukaryotes and most bacteria, *B. subtilis* uses a DNA methylase-independent mechanism for strand discrimination indicating that *B. subtilis* serves as a valuable model system for understanding this process.

Experiments in several systems have shown that MutS homologs interact with replication processivity clamps (β clamp in bacteria and PCNA in eukaryotes) (Gu et al., 1998; Kleczkowska et al., 2001; Lau and Kolodner, 2003; Lee and Alani, 2006; Lopez de Saro et al., 2006; Umar et al., 1996). Disruption of the interaction between MSH6 and PCNA in *S. cerevisiae* results in mild mutator phenotypes in vivo (for review see Schofield and Hsieh, 2003). The only modifications shown to result in strong mutator phenotypes encompass large deletions of the N-terminal domain that forms a flexible linker to PCNA (Shell et al., 2007). In *E. coli*, internal deletion of a β clamp binding motif renders MutS refractory to β clamp binding in vitro, yet in vivo the corresponding mutS allele confers a wild-type MMR phenotype (Lopez de Saro et al., 2006). Collectively, MutS homologs from several organisms have been shown to bind their resident processivity clamps; however, the in vivo significance for this interaction is unclear.

We examined the mechanism that governs the focus formation response of MutS to mismatches in *B. subtilis*. We studied an as-

Figure 1. MutS Interacts with the β Clamp through the C-Terminal 58 Amino Acids

The peptide array and far-western blot were probed with β clamp bearing a single Myc tag (β-Myc). (A) Shown are 10-mer peptides of interest from the MutS N terminus that failed to bind β or the C-terminal 58 amino acids that bound β-Myc. The amino acid sequence is indicated, and the β clamp binding motif or N-terminal motif is highlighted in gray. Ponceau staining of the peptides is shown in the left-most panel. (B) β-Myc, MutS, MutS800, and His-DnaE probed with β-Myc.

(C) Interaction between MutS or MutS800 with β clamp covalently linked to a sensor chip was completed using surface plasmon resonance as measured with a BiAcore biosensor (Experimental Procedures). Representative SPR traces for MutS 1.0 μM (blue) and 4.0 μM (red), and MutS800 2.0 μM (green) and 10.0 μM (purple) are shown.

sortment of MutS variants altered in their C terminus and found that interaction between MutS and β clamp is required for MutS-GFP focus formation. Furthermore, we found that the C-terminal 58 amino acids are not only necessary but also sufficient for focus formation and that these foci exhibit the same subcellular distribution as the replisome. We also found that MutS deleted for this C-terminal region is reduced for binding to β clamp, although this purified mutant protein retains the ability to bind a mismatch in vitro. Taken together, we conclude that interaction between MutS and β is the major molecular interaction that facilitates focus formation and that β clamp aids in the stabilization of MutS at a mismatch in vivo.

RESULTS

The C Terminus of MutS Is Required for Interaction with β Clamp

Several lines of evidence indicate that processivity clamps play an important role in MMR (Kleczkowska et al., 2001; Lopez de Saro et al., 2006; Umar et al., 1996). *B. subtilis* MutS contains a putative β clamp binding motif (806QLSFF810). It has been hypothesized that β clamp binding motifs modulate the interaction between a variety of proteins and the β clamp (Dalrymple et al., 2001). In vitro studies of *E. coli* MutS have shown that both its N- and C-terminal regions interact with β clamp (Lopez de Saro et al., 2006). The N-terminal MutS motif is necessary for MMR in *E. coli*, while the C-terminal MutS motif appears to be dispensable (Lopez de Saro et al., 2006) in vivo.

As a first step toward determining whether β clamp contributes to mismatch-dependent MutS-GFP focus formation in *B. subtilis*, we used a peptide array approach to identify MutS sequences capable of interacting with the β clamp. Unlike *E. coli*, we found that peptides containing the conserved N-terminal motif (QQYI12) failed to bind β (Figure 1A). Instead, we found that...
The C Terminus of MutS Is Required for MMR and Focus Formation In Vivo, but Not Mismatch Binding In Vitro

We show that at least two nonoverlapping 10-mer peptides in the C-terminal 58 amino acids have the capability to bind β clamp. We also found that MutS800 is significantly reduced for binding to β, suggesting that the entire C-terminal region might be involved in interaction with β and thus might influence MMR and focus formation. Therefore, we constructed a set of mutS-gfp alleles that successively removed segments of this 58 amino acid C-terminal region from MutS within the MutS-GFP fusion context (Figure 2A). All of these truncated mutS alleles were present at the normal mutS gene location on the chromosome and provide the only source of MutS protein in the cell. Because integration of these alleles disrupts mutL expression, mutL was ectopically expressed from the amyE locus (Experimental Procedures).

All of the C-terminal mutS deletion mutants exhibited profound deficiencies for MMR as assayed by mutation frequency (Figure 2B). Strikingly, truncation of only the last 8 amino acids of MutS (MutS850-GFP) resulted in nearly a complete loss of MMR activity in vivo (Figure 2B). Furthermore, all of the MutS deletion proteins accumulated to levels similar to those observed for wild-type protein in vivo (Figure 2B). To ensure this result was not influenced by the GFP component of MutS-GFP, we also replaced the native mutS gene with each corresponding mutS truncation allele lacking the GFP fusion. We found that all of these mutS C-terminal deletion alleles resulted in profound MMR deficiencies (Figure S2).

All of the MutS-GFP derivatives with truncation of the MutS C-terminus exhibited major defects in focus formation when cells were treated with 2-AP to introduce replication errors (Figures 3C and 3F). These results suggest that interaction between MutS and β clamp through the MutS C terminus might contribute to stabilizing MutS at mismatches in vivo. Another explanation could be that the MutS C-terminal truncation mutants are unable to bind mismatches. To discriminate between these possibilities, we tested MutS800 and MutS850 for mismatch binding in vitro. We found that mismatch binding by purified MutS800 and MutS was nearly indistinguishable (Figure 3D). Because MutS850 only truncated the last eight amino acids, we examined this protein for binding to β clamp by far-western blotting. We found that MutS850 was reduced for binding β but was not completely defective, and this protein also bound a mismatch in vitro (Figures S1B and S1C). Taken together, the only biochemical difference that we have identified between MutS and MutS800 (or MutS850) is interaction with β clamp.

In E. coli MutS, removal of the C-terminal region (53 amino acids) causes defects in tetramer formation and mismatch binding (Bjornson et al., 2003), and the truncated protein is defective in vivo when expressed from the native locus (Calmann et al., 2005). Whether or not tetramer formation by E. coli MutS provides an in vivo function is unclear (Mendillo et al., 2007; Miguel et al., 2007). Our finding that MutS800 and MutS850 bind a mismatch in vitro suggests that our deletion mutants do not suffer from the same biochemical defects as the E. coli protein. These data support the model that removal of C-terminal residues from MutS disrupts interaction between MutS and β clamp, which uncovers a defect in the cellular response to mismatches in vivo, but not mismatch binding in vitro. We conclude that, in vivo,
interaction between MutS and β clamp helps stabilize MutS at mismatches and stimulate focus formation.

The Putative β Clamp Binding Motif Is Important for MMR and MutS Focus Formation In Vivo

To examine the importance of the putative β clamp binding motif to MMR, we performed comprehensive alanine scanning mutagenesis of this penta-amino acid motif (Figure 2C). We found that substitution of the second to last amino acid position (806QLSF810) in this motif with an alanine indicated that it was important for MMR activity in vivo. We also found that substituting the first two positions (806QL810) or the last two positions (806QLSSF810) with alanines conferred an increase in mutation frequency similar to replacing the entire motif with alanines. Even so, replacement of the entire motif with alanines (MutS5A) resulted in only an 8-fold increase in mutation frequency (Figure 2C) compared with 40-fold for the ΔmutS strain when both are normalized to the mutS-GFP strain.

These results suggest that, although this motif contributes to MutS function in vivo, considerable MMR can occur in its absence. We asked whether a correlation exists between the mild mutator phenotype observed for MutS5A and a decrease in focus formation. We challenged our MutS5A-GFP strain with 2-AP and observed a 2-fold (MutS5A-GFP 21%, n = 1102; MutS-GFP 42%, n = 1309) reduction in focus formation (Figures 3B and 3F) indicating that MutS5A is less proficient for focus formation than MutS-GFP in response to replication errors. We also determined the subcellular position of MutS5A-GFP relative to MutS-GFP. We found that the position of MutS5A-GFP and MutS-GFP were nearly identical, indicating that the frequency of MutS5A-GFP focus formation is altered, but not the subcellular position at which foci form (Figure 3G).

Immunoblot analysis of these MutS-GFP β clamp binding motif mutants revealed that the levels of these proteins were indistinguishable from wild-type MutS fused to GFP in vivo (Figure 2C). Finally, as an additional control we constructed fusions of MutS, MutS5A, and MutS800 to a monomeric version of GFP and found that the localization properties of these proteins were almost identical to the more commonly used GFP-MUT2 employed in the above analyses (Table S2). We conclude that the β clamp binding motif of B. subtilis MutS contributes to both MutS focus formation and MMR; however, the system can function moderately well in the absence of the motif.

MutS800 and MutS5A Are Reduced for MutL Recruitment

Previous experiments examining MutS-GFP and MutL-GFP localization in B. subtilis demonstrated that 2-AP treatment failed to stimulate MutL-GFP focus formation in strains lacking the mutS gene (Smith et al., 2001). Taken together with biochemical studies from E. coli demonstrating that MutL directly binds to MutS (Wu and Marinus, 1999), these observations indicate that MutS-GFP foci must form prior to MutL-GFP foci in vivo. We therefore asked whether our C-terminal MutS variants impaired for their own focus formation and MMR were also impaired for supporting MutL-GFP focus formation.
To this end, we utilized strains in which the mutS800 and mutS5A alleles were located at their native chromosomal locus. The mutL-gfp allele was integrated at an ectopic locus (amyE) and placed under control of an IPTG-inducible promoter. We used this strategy because the integration of the mutS alleles disrupts expression of the mutL gene directly downstream (Smith et al., 2001). MutL-GFP forms foci in response to 2-AP treatment from an ectopic locus at the same frequency as MutL-GFP expressed from the native locus (Smith et al., 2001). The mutS800 allele supported only a small frequency of MutL-GFP focus formation (C ≥ 3%, n = 2866), a level nearly identical to the DmutS allele (C ≥ 2% , n = 1042), indicating that mutS800 is completely defective for recruiting MutL-GFP to mismatches (Figures 4C, 4D, and 4F). The MutL-GFP foci that formed in a mutS5A genetic background were the same, qualitatively, as those that formed in a mutS+ background. We conclude that localization of MutL-GFP to mismatches requires an MMR-proficient mutS allele.

Biochemical studies have revealed that E. coli MutS homodimers form clamp-like structures that can slide away from a mismatch along the DNA (Acharya et al., 2003). This led to the demonstration that a single mismatch could stimulate the loading of multiple MutS homodimers (Acharya et al., 2003). Nevertheless, we asked whether the C terminus of MutS is not only necessary but also sufficient for MutS focus formation. We used a strain expressing yellow fluorescent protein (YFP) from an ectopic locus using an IPTG-inducible promoter. As expected, YFP alone was diffuse in the cell and failed to show any specific localization pattern (Figure 5A). However, when we linked YFP to the C-terminal 58 amino acids of MutS (MutSC-YFP), we found that this protein localized as foci in virtually every cell in a wild-type background in the absence of 2-AP addition. A similar result was obtained in a strain deleted for both mutS and mutL (Figure 5B). Thus focus formation by the MutSC-YFP protein does not require interaction with intact MutS or MutL. The foci formed in the mutSL+ strain are qualitatively more intense, suggesting that intact MutS may increase the number of MutSC-YFP in each focus even though intact MutS is not absolutely required (Figure 5). MutSC-YFP formed foci in C ≥ 82% of cells (n = 326) in a DmutSL strain, indicating that in vivo the C terminus of MutS is sufficient to localize as a focus. This result was very intriguing because it suggests that DNA binding by MutS is not required for focus localization.

Figure 3. The C Terminus of MutS Is Required for Focus Formation
All samples shown are representative. Cells were treated with 600 μg/ml 2-AP to induce mismatches. Shown are (A) MutS-GFP foci, (B) MutS5A-GFP, and (C) MutS800-GFP. (D) MutS, MutS800, and (-) control (no protein) were incubated with homoduplex DNA (T/A) or mismatched heteroduplex DNA (T/G) as described (Biswas and Hsieh, 1996). MutS bound to DNA was retained on the nitrocellulose membrane and exposed to film. (E) Percent of cells with foci untreated. (F) Percent of cells with foci 45 min following addition of 2-AP. (G) Comparison of the cellular position of MutS-GFP single foci (black bar) and MutS5A-GFP foci (white bar) following 2-AP addition. The number of cells scored for untreated and 2-AP treated in (D) and (E) are as follows: MutS-GFP (n = 837, n = 1309); MutS5A-GFP (n = 1044, n = 1102); MutS800-GFP (n = 396, n = 958); MutS810-GFP (n = 567, n = 410); MutS830-GFP (n = 382, n = 944); MutS840-GFP (n = 595, n = 1022); MutS850-GFP (n = 771, n = 801). The cell membrane is stained with FM4-64 and is colored in red, while MutS-GFP foci are in green. The white bar indicates 2 μm. White dots are adjacent to MutS-GFP foci.

To this end, we utilized strains in which the mutS800 and mutS5A alleles were located at their native chromosomal locus. The mutL-gfp allele was integrated at an ectopic locus (amyE) and placed under control of an IPTG-inducible promoter. We used this strategy because the integration of the mutS alleles disrupts expression of the mutL gene directly downstream (Smith et al., 2001). MutL-GFP forms foci in response to 2-AP treatment from an ectopic locus at the same frequency as MutL-GFP expressed from the native locus (Smith et al., 2001). The mutS800 allele supported only a small frequency of MutL-GFP focus formation (C ≥ 3%, n = 2866), a level nearly identical to the DmutS allele (C ≥ 2% , n = 1042), indicating that mutS800 is completely defective for recruiting MutL-GFP to mismatches (Figures 4C, 4D, and 4F). The MutL-GFP foci that formed in a mutS5A genetic background were the same, qualitatively, as those that formed in a mutS+ background. We conclude that localization of MutL-GFP to mismatches requires an MMR-proficient mutS allele.

The MutS C Terminus Is Necessary and Sufficient to Localize YFP to β Clamp In Vivo
Biochemical studies have revealed that E. coli MutS homodimers form clamp-like structures that can slide away from a mismatch along the DNA (Acharya et al., 2003). This led to the demonstration that a single mismatch could stimulate the loading of multiple MutS homodimers (Acharya et al., 2003). This mechanism provided a facile explanation for the MutS focus formation we had observed (Smith et al., 2001). Nevertheless, we asked whether the C terminus of MutS is not only necessary but also sufficient for MutS focus formation. We used a strain expressing yellow fluorescent protein (YFP) from an ectopic locus using an IPTG-inducible promoter. As expected, YFP alone was diffuse in the cell and failed to show any specific localization pattern (Figure 5A). However, when we linked YFP to the C-terminal 58 amino acids of MutS (MutSC-YFP), we found that this protein localized as foci in virtually every cell in a wild-type background in the absence of 2-AP addition. A similar result was obtained in a strain deleted for both mutS and mutL (Figure 5B). Thus focus formation by the MutSC-YFP protein does not require interaction with intact MutS or MutL. The foci formed in the mutSL+ strain are qualitatively more intense, suggesting that intact MutS may increase the number of MutSC-YFP in each focus even though intact MutS is not absolutely required (Figure 5). MutSC-YFP formed foci in C ≥ 82% of cells (n = 326) in a DmutSL strain, indicating that in vivo the C terminus of MutS is sufficient to localize as a focus. This result was very intriguing because it suggests that DNA binding by MutS is not required for focus localization.
formation, but instead interaction with β clamp is sufficient for focus formation. It has been shown in both *B. subtilis* and *E. coli* that β clamp fused to GFP formed discrete foci, suggesting that β clamp is concentrated to sites of replication in vivo (Kongsuwan et al., 2002; Meile et al., 2006). We performed a similar analysis, except that β-GFP was expressed from its native promoter at the native chromosomal locus, and this fusion protein also formed discrete foci in vivo (Figure 5E).

The simplest explanation is that MutSC-YFP binds to the β clamp, and it is this interaction that allows for visualization of foci in vivo. The Subcellular Position of MutSC and β Clamp Is Consistent with the Replisome

We asked whether both β-GFP and MutSC-YFP formed foci at cellular positions consistent with the replisome (defined as replicative DNA polymerase and associated proteins at the replication fork). Indeed, both β-GFP and MutSC-YFP localization for single-focus cells was indistinguishable from the subcellular position of DnaX-GFP (replisome) (Figure 5F). An alternative explanation for the MutSC-YFP focus is that the C terminus is highly multimerizing, which allows for us to visualize this protein fragment as a focus. Because MutSC-YFP shows the same localization pattern as the replisome (DnaX-GFP), we find this explanation unlikely. Our observation that the frequency of MutSC-YFP foci in cells untreated with a mismatch-inducing agent was so high (82%) relative to the frequency of focus formation by MutS-GFP (5%) was of interest. These data suggest that, in the absence of a mismatch, MutS conformation is such that interaction with β clamp is weak or nonexistent. In contrast, in the presence of a mismatch, MutS undergoes a conformational change licensing the MutS C terminus to interact with β clamp.

A Mutant β Clamp Is Reduced for Supporting MutS-GFP Focus Formation and MMR In Vivo

The data presented above are consistent with a MutS/β clamp interaction providing an important step in MMR. In support of this hypothesis, we have determined that a previously isolated allele (*dnaN5*) encoding a mutant form of β confers a mutator phenotype (Figure 6), and this allele confers a reduction in MutS-GFP focus formation (see below).

The *dnaN5* allele was isolated because it confers a temperature-sensitive replication phenotype at 49°C (Karamata and Gross, 1970). Sequencing of the *dnaN5* allele revealed that it encodes a G73R missense mutation (Ogasawara et al., 1986). We found that strains bearing G73R had elevated mutation frequencies (Figures 6A and 6B). The G73R conferred an ∼10-fold mutator at 30°C and ∼25-fold at 37°C (Figures 6A and 6B). An epistasis analysis demonstrated that the mutation...
In this study, we found that the C terminus of MutS is required for MMR and localization as a focus in response to replication errors. We have shown that this region of MutS is also required for interaction with β clamp. Furthermore, the C terminus of MutS is also sufficient for focus formation, even in the absence of mismatches. We interpret these results to mean that interaction between MutS and β is the major molecular interaction that facilitates focus formation and that β clamp aids in the stabilization of MutS at mismatches in vivo. This conclusion is supported by experiments demonstrating that a mutant form of MutS impaired for focus formation is a mutator in vivo, and this protein still binds a mismatch in vitro. These data strongly argue that, in a living cell, mismatch binding and focus formation are more complicated than MutS simply recognizing and binding to the error and recruiting several more MutS dimers to the site of the mismatch. These data also reinforce the idea that focus formation is physiologically important for MMR activity in vivo as we find a distinct correlation between mutS alleles that are defective for MMR activity and focus formation.

In a reciprocal approach, we show that a mutant form of the β clamp is impaired for MMR and MutS-GFP focus formation. This observation strengthens our argument that β clamp is critical for MMR and MutS-GFP focus formation. We also show that a ΔmutSL allele is epistatic to our β clamp mutant, indicating these proteins function in the same pathway.

Our findings that the C terminus of MutS is critical for interaction with β and MMR activity in vivo are in contrast to observations in E. coli. In E. coli, the C-terminal β clamp binding motif of MutS is important for interaction with β clamp in vitro, although the N-terminal interaction site is required for MMR activity in vivo (Lopez de Saro et al., 2006). The differences in β clamp binding observed between these two systems could reflect the use of methylation-directed repair by E. coli. Taken together, we conclude that β clamp helps recruit MutS into a focus at mismatches in B. subtilis.

β Clamp Recruits MutS-GFP into a Focus in Response to Mismatches

Because we deleted the entire domain structure of MutS responsible for DNA binding and still observed foci, we propose that the
MutS C terminus has an intrinsic ability to bind β and localize as a focus. We propose that, in the absence of a mismatch, intact MutS assumes a conformation that negatively controls the interaction between MutS and β clamp. Once MutS binds a mismatch, MutS assumes a conformation that licenses interaction with β clamp through the MutS C terminus. We propose that this conformational change takes place in MutS and not β clamp because our MutS C-terminal fragment appears to bind β, indicating that β clamp is constitutively able to interact with the MutS C terminus.

Several models have been proposed suggesting that MutS recognizes and then relocates or diffuses away from the mismatch (Allen et al., 1997; Gradia et al., 1999). Placing these observations into context with ours, we propose that formation of the MutS•β clamp complex is a required step to stimulate the repetitive loading of MutS dimers at a mismatch in a living cell. Another interpretation could be that MutS is bound to β clamp during replication and it dissociates from β once a mismatch is detected, resulting in the recruitment of more MutS dimers and focus formation. We find this explanation unlikely. We show that β clamp forms discrete foci during normal growth. If MutS constitutively associates with β during replication, we should be able to visualize MutS-GFP foci in the absence of mismatches by virtue of the interaction with β clamp. To the contrary, we only observe MutS foci after inducing mismatches or by expression of the MutS C terminus and thus bypassing the requirement for a mismatch to visualize foci.

strand were used to direct the repair system (Lacks et al., 1982). Examination of MMR using purified components from the human system has demonstrated both 3’ and 5’ repair from a single-strand break (Dzantiev et al., 2004; Zhang et al., 2005). Considered together with ours, these results indicate that interaction between MutS and β clamp might help localize MutS to the site of replication, bringing MutS into close proximity with the 3’ terminus or the 5’ end of an Okazaki fragment at a replication fork, allowing for the system to direct mismatch removal from these sites.

Recently, it was shown that human MutL homolog hPMS2 contains an endonuclease activity that is required for MMR in vitro from a 3’ strand break (Kadyrov et al., 2006). The endonuclease active site is conspicuously conserved among many organisms that lack methyl-directed MMR, including B. subtilis MutL (Kadyrov et al., 2006). Moreover, in human, S. cerevisiae, E. coli, and now B. subtilis, MutS homologs and some MutL homologs have been shown to interact with their cognate processivity clamps (PCNA or β) (Kieczkowski et al., 2001; Lee and Alani, 2006; Lopez de Saro et al., 2006; Shell et al., 2007). Together with our localization results, these data collectively support the model that MMR proteins assemble at mismatches located at sites of replication, stimulating the formation of MutS and MutL repair complexes through the direction of β clamp. We propose a model where MutS is stabilized by β clamp at mismatches in vivo located at or near the site of
DNA replication. MutS then recruits MutL, where MutL could incise a nick to help direct removal and repair of the mismatch.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions, Medium, and Mutagenesis Experiments**

For microscopy all strains were grown at 30°C in S750 minimal medium. The S750 medium was supplemented with 1% glucose, 0.1% glutamate, 40 μg/ml tryptophan, and 40 μg/ml phenylalanine or 0.5% casamino acids for Figure S. Antibiotic concentrations were the same as previously described (Smith et al., 2001). Mutagenesis experiments were accomplished as described (Smith et al., 2001) except that for the data in Figure 2, 600 μg/ml 2-AP was added for 1 hr prior to harvesting and plating of the cells.

**Construction of *B. subtilis* Strains Expressing Mutant Forms of *mutS***

All of the mutS alleles were constructed using plasmid pBTS240 that has been previously described (Smith et al., 2001). To verify that the intended mutS missense mutations were properly integrated, we PCR amplified the 3’ end of the full-length mutS gene fused to gfp from the indicated strains and sequenced the PCR product (data not shown). The mutS alleles encoding the C-terminal deletion mutants and the mutSSA allele were cloned into plasmid pJL74 (LeDeaux and Grossman, 1995) using restriction sites EcoRI and XhoI. These alleles were integrated into the chromosome by transformation at the normal mutS gene location placing expression under the native mutS promoters.

**Cell Treatment and Live Cell Microscopy**

Microscopy and cell treatment for microscopy were done essentially as described (Smith et al., 2001). Briefly, cells were grown in S750 minimal medium at 30°C. Where indicated, cultures were split and one was treated with 2-AP for 1 hr in mid-log phase (OD600 0.2–0.4), while the control culture was untreated. Aliquots (300 μl) of cells were extracted from each culture and treated with the vital membrane stain FM4-64 (Molecular Probes) and DAPI to visualize the DNA. Images were captured with a Nikon E800 microscope and OpenLab software (Improvision). Images were adjusted, colored, and merged using OpenLab software.

**Protein Purification, Far-Western Blotting, Peptide Array Analysis, and Immunoblotting**

MutS, MutS800, MutS850, and β-Myc were cloned into pETT11T and overexpressed (primer sequences available upon request) in E. coli BL21omp3 cells using standard procedures. Cell pellets containing β-Myc, MutS, MutS800, and MutS850 were resuspended in buffer A (0.02 M KHP04 [pH 7.4], 50 mM KCl, 10% glycerol, 0.1 M EDTA, and 0.1 M DTT) + 150 mM KCl, 20 mM SpCl3, 1 mM AEBSF, and 0.3 mg/ml lysozyme. For each protein, cells were ruptured by freeze/thaw lysis after incubation on ice for 20 min. The lysate was clarified by centrifugation prior to dilution with buffer A and application to a monoQ column for purification. An elution gradient of 0.1–1 M KCl was used with each protein eluting near 700 mM KCl. Purity of proteins was determined to be greater than 90% as judged by SDS-PAGE. Purified His6-ΔNΔV was from Dr. Neil Brown (University of Massachusetts, Worcester).

Far-western blotting was done with slight modification from a procedure already described (Ludlam et al., 2001). Briefly, equal amounts of the indicated proteins were immobilized onto a nitrocellulose membrane (Pierce). Peptide arrays were performed as described (Beuning et al., 2006), and immunoblots were done as described (Simmons et al., 2007). Primary antibodies used to detect β clamp were raised against β and are from Dr. C. Lee (Massachusetts Institute of Technology). The primary was used in a 1:5000 dilution, and the goat anti-rabbit HRP-conjugated secondary antibody was used in a 1:200 dilution.

**Surface Plasmon Resonance**

A BIAcore 3000 biosensor was used for our analysis of MutS or MutS800 binding to β clamp-Myc. Standard research-grade CM5 sensor chips were used for the immobilization of 10,200 resonance units of β clamp. Immobilization of β was accomplished using the amine coupling kit (BIAcore) following the manufacturer’s recommendations. β clamp was immobilized by using 10 mM sodium acetate buffer (pH 4.0). MutS and MutS800 were diluted in binding buffer prior to injection of the blank or β clamp-coupled surfaces.

**Supplemental Data**

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, three tables, and three figures and can be found with this article online at http://www.molecule.org/cgi/content/full/29/3/291/DC1/.

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