The β Sliding Clamp Binds to Multiple Sites within MutL and MutS*

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Francisco J. López de Searo1, Martin G. Marinus1, Paul Modrich8, and Mike O’Donnell4

From 1The Rockefeller University and 2Howard Hughes Medical Institute, New York, New York 10021, the 4Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and the 8Department of Biochemistry, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

The MutL and MutS proteins are the central components of the DNA repair machinery that corrects mismatches generated by DNA polymerases during synthesis. We find that MutL interacts directly with the β sliding clamp, a ring-shaped dimeric protein that confers processivity to DNA polymerases by tethering them to their substrates. Interestingly, the interaction of MutL with β only occurs in the presence of single-stranded DNA. We find that the interaction occurs via a loop in MutL near the ATP-binding site. The binding site of MutL on β locates to the hydrophobic pocket between domains two and three of the clamp. Site-specific replacement of two residues in MutL diminished interaction with β without disrupting MutL function with helicase II. In vivo studies reveal that this mutant MutL is no longer functional in mismatch repair. In addition, the human MLH1 has a close match to the proliferating cell nuclear antigen clamp binding motif in the region that corresponds to the β interaction site in Escherichia coli MutL, and a peptide corresponding to this site binds proliferating cell nuclear antigen. The current report also examines in detail the interaction of β with MutS. We find that two distinct regions of MutS interact with β. One is located near the C terminus and the other is close to the N terminus, within the mismatch binding domain. Complementation studies using genes encoding different MutS mutants reveal that the N-terminal β interaction motif on MutS is essential for activity in vivo, but the C-terminal interaction site for β is not. In light of these results, we propose roles for the β clamp in orchestrating the sequence of events that lead to mismatch repair in the cell.

The process of chromosomal replication can generate small deletions, insertions, or mismatches in newly synthesized DNA. If left unrepaird, post-replicative DNA damage can lead to genomic instability and increased rates of mutation (1, 2). These types of replicative errors are corrected by a set of enzymes that are specialized in performing DNA mismatch repair (MMR)2 and are conserved across all domains of life. In Escherichia coli it is estimated that the fidelity of replication is increased >100-fold by the action of MMR (3). In humans a deficiency in MMR has been linked to increased susceptibility to certain sporadic cancers (4 – 6).

The E. coli MutS protein is an essential component of MMR and it directly binds to abnormal DNA structures to initiate the cascade of events that lead to repair. These events include the removal of a segment of the newly synthesized DNA strand by the combined action of a helicase and one or more nucleases, and the re-synthesis of the resulting gap by a DNA polymerase (1). A second essential component of MMR in E. coli is MutL, which is thought to link the action of MutS with nucleases and a helicase (1, 7). However, although the individual components of MMR in prokaryotes and eukaryotes are mostly well described, many of the details of the repair mechanisms are still poorly defined.

In eukaryotic organisms, MutS homologues MSH3 and MSH6 bind to the processivity factor PCNA (proliferating cell nuclear antigen) (8 – 12), a homotrimeric protein that plays a crucial role in DNA replication, which therefore provides a link between the MMR and replication machineries. Fluorescence microscopy has revealed that MMR enzymes colocalize with active replication centers in the cell (13, 14). In addition, numerous genetic studies in yeast by Kolodner and collaborators show that a distinctive set of mutations in PCNA can result in MMR deficiency (15, 16). Conversely, mutation of the PCNA-binding motif in either MSH3 or MSH6 abolishes the interaction of the clamp and results in yeast strains with elevated mutation rates (11, 12). In bacteria, MutS has been shown to interact with the β clamp, the processivity factor of DNA polymerase III (17).

PCNA and β are ring-shaped proteins that bind to DNA by encircling it, and are generally referred to as “sliding clamps.” Loading of PCNA and β onto DNA requires the action of “clamp loaders” (γ-complex in prokaryotes, replication factor C in eukaryotes), which are multisubunit ATPases that open the ring and place it around primed sites on DNA (18). The β clamp tethers the replicate to DNA by binding it directly and sliding with it along DNA during chain elongation, converting it into a highly processive enzyme (19). In recent years it has become evident that sliding clamps interact with many other proteins involved in all aspects of DNA metabolism. Despite the diversity of the proteins that bind to PCNA or β, the interaction often has a common structure: proteins typically bind to the clamp via N- or C-terminal flexible extensions containing a short motif (20, 21), and the interaction takes place at a hydrophobic pocket near the C terminus of the clamp. Detailed studies of the contact between clamps and their partners show that these interactions are often complex, with involvement of more than one binding surface, and in the case of PCNA even with mechanisms that regulate the interaction by post-translational modifications (22 – 25). In addition, because PCNA is trimeric and β dimeric, it has been speculated that the ring could accommodate more than one ligand at the same time and therefore serve as a mobile platform that coordinates multiple enzymes performing sequential actions on DNA (22, 26).

What is the precise role of processivity clamps in mismatch repair? Because clamps are typically associated with DNA polymerases, and
clamp loaders are integral components of the replication fork, clamps could be used for targeting the MMR machinery to “replication factories” that could therefore contain synthetic errors. A second possibility is that they participate directly in the mechanism of action of mismatch repair. For example, MSH2–MSH6 binds to PCNA in the absence of a mismatch but not in its presence, suggesting that PCNA could help the MSH complex locate the mismatch on DNA (12, 27). In addition, because clamps used by DNA polymerase have a distinct orientation on DNA, they could provide the MMR machinery with the means to discriminate between the parental DNA and the newly synthesized strands (2, 4).

In an effort to gain a deeper understanding of the interplay between MMR and replication, we investigated in greater detail the physical interactions between E. coli MutL, and MutS and the β clamp of DNA polymerase. We find that β binds directly to MutL. Interestingly, MutL only interacts with β in the presence of single-stranded (ss) DNA. Studies herein demonstrate that the site of contact with β is located on a loop in the N-terminal ATP-binding domain of MutL. Mutation of two residues within this loop reduces interaction with β, but does not influence MutL ATPase and function with helicase II. The mutations herein demonstrate that the site of contact with β is located on a loop in the N-terminal ATP-binding domain of MutL. Mutation of two residues within this loop reduces interaction with β, but does not influence MutL ATPase and function with helicase II. Mutation of these two residues abolishes mismatch repair in vivo. In addition we have identified two points of contact on MutS that bind to β, one at the N terminus and another at the C terminus. The C-terminal site has a strong affinity for β, whereas the interaction with the N-terminal site is weak. Interestingly, only the N-terminal site is essential for mismatch repair in vivo. This work provides new insights into the role of β in mismatch repair in the cell, and suggests that the clamp is an essential player. We propose that β helps to order the sequence of events in this multistep repair pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—High pressure liquid chromatography purified N-terminal biotinylated peptides were purchased from Bio-synthesis Inc. All other peptides were purchased from Chiron Mimotopes, which utilizes a synthetic method that leaves a diketopiperidine linker at the C terminus of the peptide. Streptavidin was purchased from Sigma. Labeled nucleotides were from PerkinElmer Life Sciences. Unlabeled nucleotides and AMPPNP were from Amersham Biosciences. Proteins were purified as follows: β as described (28); β containing a C-terminal 6-residue kinase recognition sequence and human PCNA with a 6-residue N-terminal tag were purified and labeled using [γ-32P]ATP as described (29); pol III core was reconstituted from isolated subunits and purified from unbound proteins as described (30).

**MutS and MutL Overexpression and Purification**—The MutS gene was cloned into pET11c (Novagen) to yield pET-mutS28. MutS mutants, MutSn (pET-mutSn) and MutSc (pET-mutSc), were generated using site-directed mutagenesis by the QuikChange method (Stratagene, La Jolla, CA). Constructs were sequenced before use. Purification of MutS and MutL derivatives was based on the procedure described earlier (31). For MutS and its derivatives, plasmids were transformed in E. coli BL21(DE3) and 12L of cells grown at 37 °C in LB media supplemented with 0.2% glucose and 50 μg/ml ampicillin. When the cell culture reached an A600 of 0.6, 1 mM isopropyl 1-thio-β-D-galactopyranoside was added and after a further 2-h incubation the cells were harvested by centrifugation. Cells were suspended in 50 ml of lysis buffer (20 mM KPO4, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM diithiothreitol, 100 mM NaCl) and lysed using a French press at 4 °C. The lysate was clarified by centrifugation and the extract was mixed with a solution of 25% streptomycin sulfate (0.4 ml/1 ml of extract). After stirring, the precipitate was pelleted by centrifugation and the supernatant was treated with solid ammonium sulfate (0.180 g/ml) at 4 °C. After centrifugation, the precipitate was resuspended in buffer A (20 mM KPO4, pH 7.5, 1 mM EDTA, 10 mM diithiothreitol, 25 mM NaCl, 5% glycerol) and dialyzed in the same buffer. This dialysate was applied to a heparin-Sepharose chromatography column (150-ml bed volume) and eluted with a 500-ml linear gradient of NaCl (25–500 mM). Fractions were analyzed on a SDS-polyacrylamide gel and those containing MutS were pooled, dialyzed against Buffer A, and then applied to a 200-ml column of fast-flow S-Sepharose (Amersham Biosciences) equilibrated in Buffer A. The S-Sepharose column was eluted with a 600-ml NaCl gradient (25–500 mM) in Buffer A. MutS-containing fractions were pooled, precipitated with ammonium sulfate, dialyzed against buffer A, and then stored at −80 °C.

The MutL gene was cloned in pET11c to yield pET-mutL. MutL2L in which residues Leu-150 and Phe-151 were replaced by alanine was constructed by site-directed mutagenesis (QuikChange) to yield pET-mutL2L. E. coli BL21(DE3) was transformed with pET-mutL or pET-mutL2L and grown, induced, and harvested as described above for MutS. MutL and MutL2L were purified as described (32) except that a fast-flow Q (Amersham Biosciences) column replaced the hydroxyapatite column.

**Protein Gel Mobility Shift Assays**—Native polyacrylamide gel electrophoresis assays using [32P]β (150 dpn/mmol) were performed as described by López de Saro and O’Donnell (17). Reactions (15 μl) contained 20 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 4% glycerol, 50 μg/ml bovine serum albumin, 100 mM NaCl, 5 mM DTT, and 50 mM [32P]β. Reactions also included peptide, streptavidin, MutS, DNA, MutL, or AMPPNP as indicated in the figure legends. Duplex DNA was a 300-mer duplex generated by PCR and gel purified, and ssDNA was a 90-mer synthetic oligonucleotide (5′-CCCCCTTATTAGGGTTGATCTTCTACTAATACACCGGAAACGACGGACACCAGCCGCTCCCTCAGAGCGCCACCCCT-3′). After incubation at 37 °C for 5 min, 5 μl of the reaction was applied to a 4% native polyacrylamide gel (4% acrylamide-bisacrylamide 29:1, 1× TBE buffer, 5% glycerol). Electrophoresis was performed in 1× TBE buffer (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA) at 19 mA for 90 min (4 °C). Gels were dried and detection of [32P]β was performed using a PhosphorImager. Free and bound forms of [32P]β were quantitated using ImageQuant software (Amersham Biosciences) and the percentage of bound [32P]β obtained using the formula [32P]β_{bound} = [32P]β_{bound} × 100/[32P]β_{total}. Concentrations of the addition of [32P]β ligands are indicated in each figure.

**Superdex 200 Chromatography**—A 25-ml FPLC Superdex 200 column (Amersham Biosciences, separation range 10,000–600,000 Da) was used to analyze MutS and its derivatives. The column buffer contained 50 mM HEPES (pH 7.4), 1 mM EDTA, 100 mM NaCl, and 2 mM DTT. Wild-type MutS, MutS800, and MutS2L were each loaded at a concentration of 25 μM in a volume of 200 μl. Fractions (420 μl) were collected and protein content measured by the Bradford assay (Bio-Rad), using bovine serum albumin as a standard.

**Determination of Mutant Frequency**—The wild-type strain used for the mutagenesis assay was AB1157 (F− thr-1 araC14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpoS396 rpsL31 kgdK51 xylA5 mtl-1 argE3 thi-1) obtained from E. A. Adelberg (Yale University). Strains KM52 and KM75 (from K. C. Murphy, University of Massachusetts Medical School) were derived from AB1157 by replacement of the mutL and mutS coding regions by genes encoding chloramphenicol and tetracycline resistance, respectively (33). Strains were grown at 37 °C to saturation from single colonies in Brain Heart Broth (20 g/liter) supplemented with ampicillin (100 μg/ml) when required. To determine the frequency of rifampicin resistance, aliquots were spread on L plates with or without ampicillin (100 μg/ml) and onto L
DNA was generated using an oligonucleotide that was fully complimentary to mutSc containing the mutSc sequence. Reactions (15 µM MgCl₂, and 3 mM ATP) were incubated for 5 min at 25 °C before loading (4 min) on PEI thin-layer chromatography (TLC) plates to separate ATP from ADP. TLC plates were dried and radioactivity was visualized and quantified using a PhosphorImager (Amersham Biosciences).

**RESULTS**

MutL interacts with the β clamp only in the presence of ssDNA. MutL protein is thought to act as a bridge between the site of DNA damage, recognized by MutS, and enzymes that subsequently process the DNA, like MutH endonuclease, helicases, and exonucleases (1, 7). Structural analysis of MutL indicates that it is a highly flexible molecule with two distinct regions connected by a proline-rich, 100-residue long disordered linker (7, 35). Because the C-terminal domain contains a strong dimerization surface and the N-terminal domains dimerize upon ATP binding, the MutL dimer contains a very large cavity (~100 Å) that is sufficiently large to possibly accommodate 2–4 DNA helices at the same time (7). The N-terminal domain contains the ATP-binding site, and MutL is a weak ATPase that is stimulated by ssDNA; a putative ssDNA-binding site is located at the interface of two N-terminal domains as suggested by structural studies and mutational analysis (36).

Upon binding to ATP, several disordered loops (L1, L2, L3, L45, and the C-terminal domain) become ordered within the N-terminal region of MutL. These loops have been suggested to be critical for the dynamic interactions that take place between the two N-terminal domains during each cycle of ATP binding, hydrolysis, and ADP release (36).

We have reported previously that MutL, in solution does not show an interaction with the β clamp (17). Here we used an assay similar to that used initially to demonstrate the MutS-β interaction, in which MutL complexed with either MutS or DNA polymerase III migrates slower than free β in native polyacrylamide gel electrophoresis (17). In these assays the position of β on the gel is visualized using β labeled with a C-terminal tag that is specifically phosphorylated by a protein kinase (29). Using this method, an interaction between MutL and β is readily apparent in the presence of a single-stranded DNA oligonucleotide (Fig. 1A). A titration of MutL-ssDNA into a reaction containing [³²P]β indicates that they interact with a K₅₀ value of ~250 nM (each as dimer) (Fig. 1B). These results suggest that the interaction of MutL with β may require a conformational change in MutL brought about by ssDNA binding.

Next we tested the effect of the non-hydrolyzable nucleotide analog, AMPPNP, on the binding of MutL to β in the presence of ssDNA (Fig. 1B). Although ATP has no apparent effect on ssDNA binding, ssDNA increases the ATPase rate of MutL, possibly by inducing dimerization of the N-terminal domains of MutL, a pre-requisite for ATP hydrolysis (36). The results show that AMPPNP decreases the affinity of MutL for β in the presence of ssDNA by ~3-fold (Fig. 1B). AMPPNP binding to MutL causes folding of various disordered loops at the N-terminal domains of the MutL dimer (36), and therefore this result suggests that the interaction of MutL with β may be partially regulated by conformational changes in the N-terminal domains of MutL.

To determine whether MutL binds to the same C-terminal face of β as MutS and other proteins, we examined the interaction by the kinase protection assay (Fig. 1C) (17, 37). The rate of phosphorylation of the 6-residue kinase tag attached to the C-terminal face of β was followed in the presence or absence of MutL and the ssDNA oligonucleotide. As a con-
bined phosphorylation of kinase A phosphorylated and PCNA equally in the absence of additional factors, both clamps are phosphorylated equally (lanes 1–3). Addition of MutS-ssDNA prevents labeling of the PCNA control (lanes 4–6), indicating that the 20-mer peptide readily dissociates complexes of pol III core–β and the hydrophilic pocket on β.

In the experiment of Fig. 1D, we used a 20-mer peptide derived from the C terminus (residues 1141–1160) of the α subunit (polymerase) of pol III to challenge the MutL-ssDNA–β complex (Fig. 1D). This peptide binds to the hydrophilic pocket in β that is targeted by the δ subunit of the γ complex as well as all five DNA polymerases of E. coli (38). The result demonstrates that the 20-mer peptide readily dissociates complexes of pol III core–β, MutS–β, and MutL-ssDNA–β, indicating that

![FIGURE 1. MutL interacts with β in the presence of ssDNA. A, native PAGE was used to analyze the interaction of [32P]β (50 nM) with MutL (2 μM) in the presence or absence of dsDNA (10 μM as 300-mer), ssDNA (10 μM as 100-mer), and AMPPNP (1 mM). MutS was included in lanes 8 and 9 to demonstrate that AMPPNP does not affect the ability of MutS to bind β under these conditions. B, a titration of MutL and MutL/LF (0.150–5 μM) into reactions containing 50 nM [32P]β was performed to estimate the affinity of the complexes in the presence of ssDNA and AMPPNP. Complexes were separated by native PAGE and bound and free [32P]β was quantified for each reaction using a PhosphorImager. C, kinase protection assay. Protein kinase labels the C-terminal kinase tags of β that protrude from the face to which DNA polymerase binds these (see diagram). In the absence of additional factors, both clamps are phosphorylated equally (lanes 1–3). Addition of MutL-ssDNA prevents labeling of β but not of the PCNA control (lanes 4–6). D, a peptide derived from the C terminus of the α subunit of DNA polymerase III that binds to the hydrophilic pocket in β displaces the core–β (lanes 2 and 6), MutS–β (lanes 3 and 7), or MutL-ssDNA–β (lanes 4 and 8) complexes, demonstrating that all three proteins interact in a similar fashion with the hydrophilic pocket on β.

![FIGURE 2. Analysis of the MutL β-binding site and influence on ATPase and helicase stimulation. A, alignment of the loop 2 region of MutL in E. coli, with the sequence from Saccharomyces cerevisiae and human MutL homologues (MLH1). Structural elements of the E. coli MutL structure (36) are shown above the sequence. The conserved LF residues that were mutated to alanines in the peptide experiments shown in panel B are boxed. The sequence used to design peptides for experiments described in panel B is underlined. B, biotinylated peptides were attached to an enzyme-linked immunosorbent assay plate coated with streptavidin, and tested for retention of [32P]PCNA (right) as described (38). In the left plate the peptide used was a 20-mer derived from the E. coli sequence in panel A. In the right panel the peptide used was a 20-mer derived from the human MLH1 sequence in panel A. In both mutant peptides the LF sequence (boxed in panel A) was mutated to alanines. C, ATPase assays of MutL and MutL/LF indicate that the mutant retains wild-type activity, and that both are stimulated to a similar extent by ssDNA. D, UvrD helicase is stimulated similarly by MutL (black squares) and MutL/LF (open squares). In lane 2 the helicase substrate (see “Experimental Procedures”) was heated to 100 °C for 4 min prior to loading the gel. UvrD was 0.5 nM and MutL (or MutL/LF) was 0.6 nM (lanes 4 and 9), 1.8 nM (lanes 5 and 10), 5.4 nM (lanes 6 and 11), 17 nM (lanes 7 and 12), and 50 nM (lanes 8 and 13).
**β Clamp Interactions with MutL and MutS**

**TABLE 1**

Mutant frequency of MutL and MutS mutants

| Strain          | Plasmid       | Mutant frequency/10⁸ cells |
|-----------------|---------------|---------------------------|
| MutL            |               |                           |
| KM52 (mutL460::cam) | pET-mutL<sup>a</sup> | 117                        |
| AB1157          | pET15b        | 1                         |
| KM52            | pET-mutL<sup>a</sup> | 110                       |
| KM52            | pET-mutL<sup>a</sup> | 1                        |
| AB1157          | pET-mutL<sup>a</sup> | 101                       |
| MutS            |               |                           |
| AB1157          | pET-mutS<sup>a</sup> | 1                        |
| KM75 (mutS465::tet) | pET-mutS<sup>a</sup> | 112                       |
| AB1157          | pET15b        | 126                       |
| KM75            | pET-mutS<sup>a</sup> | 1                        |
| AB1157          | pET-mutS<sup>a</sup> | 135                       |
| KM75            | pET-mutS      | 1                         |
| AB1157          | pET-mutS      | 1                         |
| KM75            | pET-mutS      | 1                         |
| GM8607 (mutS<sup>a</sup>)<sup>b</sup> |               | 1                        |

<sup>a</sup>GM8607 is AB1157 bearing the mutS<sup>a</sup> mutation in single copy on the chromosome.

MutL likely binds the same hydrophobic site on the β ring that these other proteins bind.

*Conserved Loop L2 of the N-terminal Domain of MutL Is Involved in β Binding*—Inspection of an alignment of diverse bacterial MutL proteins for candidate β-interaction sequences did not reveal an obvious 4- or 5-residue consensus binding motif. However, loop 2 in the N-terminal ATP-binding domain of MutL contains a highly conserved LF motif reminiscent of the one present in the δ subunit of the γ-complex that, despite its deviation from the consensus, binds the hydrophobic pocket in β. We tested β binding to a series of peptides derived from sequences comprising loops L1 and L3 of MutL, with negative results (data not shown), but a 20-mer peptide corresponding to loop 2 (L2) (Fig. 2A) in the N-terminal domain tested positive for binding to β (Fig. 2B, left panel). To examine the peptide for interaction with β, an N-terminal biotinylated 20-mer peptide corresponding to loop 2 of MutL was attached to wells of a microtiter plate coated with streptavidin, and the wells were probed with <sup>32P</sup>β (Fig. 2B). The presence of the peptide resulted in retaining <sup>32P</sup>β in the well. A double mutation to alanine of residues Leu-150 and Phe-151 within the peptide (residue number corresponding to full-length MutL) abolished the interaction. Interestingly, the homologous region in human MLH1 contains a sequence (QXXVXXLF) that may be predicted to bind PCNA (Fig. 2A). A peptide derived from the human MLH1 protein was capable of forming complexes with human <sup>32P</sup>PCNA (Fig. 2B, right panel). Mutation of residues Leu-155 and Phe-156 to alanine in the peptide derived from human MLH1 also abolished the interaction.

These results indicate that loop L2 in MutL is a candidate site for interaction with β and that the homologous residues in human MLH1 could bind PCNA. Mutation of the two residues to alanine renders the MutL mutant inactive in complementing a strain of *E. coli* in which the normal *mutL* gene is inactivated by replacement of the coding sequence with the chloramphenicol acetyltransferase (*cat*) gene (Table 1). The mutant protein was purified (referred to herein as MutL<sup>LF</sup>) and tested for binding to β (Fig. 1B). MutL<sup>LF</sup>-ssDNA showed decreased binding to β with respect to wild-type MutL-ssDNA. This effect may be due to residual binding at the mutated site on MutL<sup>LF</sup>, or may be explained by the presence of a second site of interaction elsewhere in MutL. Interestingly, the affinity of MutL<sup>LF</sup>-ssDNA for β does not decrease in the presence of AMP-PNP and is similar to that of wild-type MutL in the presence of AMP-PNP. To examine whether the double mutation causes a defect in the ATPase rate of MutL, we performed ATPase assays in the presence and absence of ssDNA, and that a site of contact between MutL and ssDNA shows decreased binding to MutL<sup>LF</sup> (Fig. 2D). This study suggests that MutL binds β only in the presence of ssDNA, and that a site of contact between MutL and β is located in the conserved loop L2 on the N-terminal domain of the protein.

**Peptides Derived from N- and C-terminal Sequences of MutS Interact with β**—We have shown previously that *E. coli* MutS interacts with β in solution (17). Many of the proteins that bind to β do so via extreme N- or C-terminal residues (21, 23, 38). To investigate whether MutS utilizes...
N- or C-terminal residues to bind to $\beta$, we synthesized biotinylated 21-mer peptides containing MutS sequences corresponding to the N- and C-terminal residues and probed them for interaction with $\beta$. The assay utilizes $^{32}$P$\beta$ and streptavidin. If the biotinylated peptide binds to $\beta$, the streptavidin, which couples to the biotinylated peptide, shifts the position of $\beta$ in the native polyacrylamide gel. The gel shift requires streptavidin as the peptide alone is not large enough to result in a detectable gel shift of $^{32}$P$\beta$ (compare Fig. 3 lanes 3 and 4 with S and 6). Peptides derived from internal sequences, as well as the peptide derived from the extreme C terminus of MutS ($\text{MutS}_{823-\ldots830}$) did not interact with $\beta$ (data not shown). However, as shown in Fig. 3A, peptides derived from both the N terminus ($\text{MutS}_{1-21}$) and a region very near the C terminus ($\text{MutS}_{802-\ldots820}$) bind to $\beta$ in the native gel assay (Fig. 3A, lanes 5 and 6).

Next, we investigated the effect of the MutS $\beta$-binding peptides on the mobility of preformed MutS-$\beta$ complexes. Interestingly, the results, in Fig. 3B, demonstrate that both the N- and C-terminal MutS peptides disrupt the MutS-$\beta$ complex (Fig. 3B, lanes 1–4), suggesting that both bind to $\beta$ at the same location. We then tested the effect of the two MutS peptides on preformed DNA polymerase III (core)-$\beta$ complexes (Fig. 3B, lanes 5–7). The result shows that the two peptides are both capable of disrupting the pol III core-$\beta$ complex, implying that, despite their lack of sequence similarity, they both bind to the same sites on $\beta$ that are contacted by pol III core.

Studies of the interaction between pol III core and $\beta$ indicate the presence of at least two contact points on $\beta$, possibly the two identical hydrophobic pockets in the two protomers of the $\beta$ dimer (21, 38, 39). Taken together, these results suggest that MutS binds to $\beta$ in a manner similar to that of the pol III core and is consistent with our previous result that MutS binds $\beta$ on the same face of the ring as the $\alpha$ subunit of DNA polymerase III and the $\delta$ subunit of the $\gamma$-complex (17).

The N-terminal $\beta$-Binding Site in MutS Is Essential in Vivo—The studies herein using peptides derived from the MutS sequence suggest that one putative $\beta$-binding site is located in the N terminus, which is a part of the mismatch DNA-binding domain of MutS (Domain 1). To obtain a more detailed view of this interaction, nested peptides were synthesized spanning the first 21 amino acids of MutS (Fig. 4A). As shown in Fig. 4B, peptides corresponding to N-terminal sequences of MutS can efficiently compete the MutS-$\beta$ interaction by binding to $\beta$, except peptide MutS$_{1-11}$. This result defines a particular region of interaction spanning residues Pro-12 to Lys-21. To identify the particular residues in the MutS peptide that are essential for the interaction with $\beta$, residues were mutated one at a time. The results (Fig. 4C) show that certain residues between Met-13 and Arg-19 are important to preserve the interaction with $\beta$. Amino acid replacements Q15A, L18A, and R19A show a greatly reduced binding to $\beta$, whereas M13A, M14A, and Q16A have an intermediate effect. In contrast, peptides with replacements Y17A or L20A bind to $\beta$ similar to wild-type peptide.

Next, we constructed MutS mutants by site-directed mutagenesis of the mutS gene and tested the mutant proteins for binding to $\beta$ in vitro. A double mutant, Q15A/L18A, or a quadruple mutant Q15A/Q16A/L18A/R19A (referred to below as MutS$^\Delta$) did not show a significant decrease in binding to $\beta$, suggesting that the interaction at the N terminus is not necessary for MutS to bind $\beta$ in solution (data not shown). In solution the MutS-$\beta$ interaction may be dominated by the C-terminal region of MutS, which is a strong interaction (see below). It also remains possible that the N-terminal residues identified here could become important for the interaction when MutS and $\beta$ are both bound to DNA. MutS$^\Delta$ binds to heteroduplex DNA containing a G-T mismatch with an affinity similar to wild-type MutS (Fig. 4D), and its ATPase activity is also indistinguishable from wild-type MutS (data not shown).

To determine whether the MutS mutants were functional in vivo, the mutant genes were tested for their ability to complement E. coli KM75, a mismatch repair-deficient strain in which a tetracycline-resistance cassette replaces the chromosomal mutS gene. Plasmids that contain a copy of wild-type MutS restore mismatch repair (Table 1). Consistent
Mutant MutSN gene is unable to complement the repair pathway, suggesting that β interaction at the N-terminal site on MutS is important to function (Table 1).

The C-terminal Domain of MutS Contains a Strong β-Binding Site—Alignment of bacterial MutS sequences from different bacteria allowed Dalrymple and co-workers (21, 40) to identify a putative β-binding motif about 30 residues from the C terminus of E. coli MutS, and they showed that a peptide derived from this region could bind to β. This peptide contains a short sequence, QMSLL, that is related to the β-binding motifs of other E. coli proteins. In light of the experiments described above with peptide MutS<sub>802–820</sub> (Fig. 3), we tested binding of β to two mutant forms of the MutS protein defective in this region near the C terminus. One form of MutS, MutS<sub>ΔC800</sub> (or MutS<sub>800</sub>), contains a 53-amino acid C-terminal truncation and has been described and characterized previously (41, 42). The other contains a 5-residue deletion in the putative β-binding motif (QMSLL) within the C terminus (MutS<sub>Δ812–816</sub>), which will be referred to here as MutS<sup>C</sup>. As shown in Fig. 5A, neither MutS<sub>800</sub> nor MutS<sup>C</sup> interact with β under the conditions of the native polyacrylamide gel-shift assay, suggesting that the motif located at the C terminus is an important site for binding to β in solution.

MutS binds to β<sub>2</sub> with a $K_d$ value of ~250 nM (as dimer) (Fig. 5B), as determined by the native gel electrophoresis assay, and the presence of homo- or heteroduplex DNA, or ATP, did not alter this equilibrium (data not shown). For comparison, a similar experiment was performed using pol III core, which is known to bind β with a $K_d$ of ~250 nM using other methods (43). In solution MutS is a tetramer, but MutS<sub>800</sub> is dimeric, indicating that the missing 53 residues of MutS<sub>800</sub> not only contain a β-binding site, but are also required for tetramerization. We tested the oligomeric state of MutS<sup>C</sup> by gel filtration chromatography (Fig. 5C) but find that MutS<sup>C</sup> co-migrates with wild-type MutS and that therefore MutS<sup>C</sup> appears to be a tetramer. We conclude that the deleted β-binding motif is not a determinant of the oligomeric status of MutS.

Although previous reports have shown that MutS<sub>800</sub> is functional in vivo and can complement a null MutS mutant when expressed from plasmids (41, 44), more recent work has shown that the C-terminal domain of MutS is critical for mismatch repair in vivo when the protein is expressed from a single copy gene in the chromosome (34). However, strain GM8607, containing a deletion of five residues within the C-terminal domain of MutS (MutS<sup>C</sup>), can indeed complement the MutS-null strain when in a plasmid or in single-copy in the chromosome (Table 1). We conclude that, whereas the N-terminal motif seems to be essential for mismatch repair in vivo, the C-terminal one is not.

**DISCUSSION**

To understand the role of processivity clamps in MMR, we have analyzed the E. coli β clamp for interaction and function with the MutL and MutS proteins. We observe an interaction between MutL and β that is only apparent in the presence of ssDNA. Furthermore, binding of ATP modulates the mutL-β interaction. These results indicate that β binds to MutL while MutL is engaged in the process of mismatch repair. The β interaction site in MutL appears similar to that in the δ subunit of the clamp loader, and replacement of 2 amino acids in MutL (MutL<sup>15</sup>) diminishes binding to β, similar to analogous mutations in the δ subunit. The MutL mutant retains ATPase activity and ability to stimulate helicase II. Expression of the MutL<sup>15</sup> mutant fails to complement a MutL defective strain of E. coli, indicating that the interaction of MutL with β is essential to function.

Study of MutS identifies two distinct sites of interaction with β. Analysis of MutS deletion mutants indicate that MutS binds to β most strongly via residues in the C-terminal region of MutS. However, as revealed by peptide analysis, an additional contact site with β is located at the extreme N terminus of MutS. In vivo functional studies reveal that...
MutS, which is mutated in amino acids required for β binding at the N-terminal site, is no longer capable of complementing a MutS-deficient strain of E. coli, indicating that this site is important to mismatch repair in the cell. Overall, the results of the current report highlight the use of processivity clamps in mismatch repair and indicate that they act at more than one step, and possibly coordinating MutS and MutL activities with replication.

Recent evidence has accrued for at least two points of contact between pol IV and the clamp and between pol III and the clamp (23, 39, 45, 46). One may speculate that one contact is strong and allows a partner to bind to the DNA-bound clamp from solution, and that an additional binding site is apparent when the protein is actively engaged in performing its enzymatic activity on DNA. In this regard, the location of clamp interactive residues in the extreme N or C terminus of a protein could make these regions articulated and hinge-like, allowing them to bind the clamp and yet permit rapid association and dissociation from DNA. Surprisingly, to date the interactions of proteins with the clamp and yet permit rapid association and dissociation from DNA. Surprisingly, to date the interactions of proteins with DNA polymerase II C-terminal motif. 

A clue to how MutS may slide on DNA is provided by a second channel (inner channel) apparent in the crystal structure (Fig. 6A). This channel is distinct from the one in which mismatched DNA is bound and its size and surface electrostatic potential is compatible with DNA contact (51). The paradox of a C-terminal interaction of MutS with β can readily be solved if MutS interacts with β when DNA is threaded through the inner channel. An alignment of the channels in both molecules positions the C-terminal and the N-terminal binding sites of MutS at approximately the same distance as exists between the two hydrophobic binding sites of β, ~60 Å apart (Fig. 6A). 

Structural studies of MutS with homoduplex DNA, or of the missing C-terminal extension that interacts with β, will be needed to further evaluate this model. An interesting implication, however, is that upon binding to damaged DNA, the binding surface of MutS to β may disconnect upon the relocation of the DNA duplex to the lower channel (Fig. 6A, left panel). β release from the N-terminal site would be predicted to occur upon binding of the MutS to damaged DNA because the residues implicated in binding form an α-helix when MutS binds mismatched DNA (41). In addition, when MutS binds the mismatch β it would be expected to detach from the C-terminal site as well, because the new distance would now be too great for the C terminus of MutS to interact with β. 

The model in Fig. 6B implicates β in the earliest stages of mismatch repair in which β targets MutS to sites of DNA replication and helps MutS in one-dimensional scanning along DNA for lesions (Fig. 6B, diagrams a and b). In this hypothesis, we propose that DNA threads through the upper chamber in MutS, allowing both MutS sites to bind the β clamp (as described above). MutS binding to the mismatch would reposition the DNA and break both sites of contact with β, ejecting the clamp from the MutS-DNA complex. Previous studies also suggest that

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**FIGURE 6. Model of MutS function with the β clamp.**

A, structure of E. coli MutS bound to a fragment of DNA with a mismatch (1E3M) (left) (41), and of β (2POL) (right) (28). In the case of MutS, the protein used for crystallization lacked the C-terminal 53 amino acids (MutS800). The position of the C terminus in this truncated version of MutS is indicated. Residues Met-13 to Leu-20 from the N-terminal domain of MutS are colored red (Leu-155, Thr-172, Arg-176, Leu-177, and Pro-242), a model for the use of β by MutS during the early stages of mismatch repair. β is loaded onto DNA by the DNA replication machinery and used by the pol II replicase processivity (a). The replicase periodically leaves clamps on DNA that are therefore available for use by MutS to check the newly synthesized DNA for errors (b). Mismatch recognition by MutS results in a conformational change in MutS. This may transfer DNA from the inner channel to the outer channel of MutS, kinking DNA at the damaged site and releasing the clamp (c). C, alignment of the β-binding motifs of E. coli proteins: pol III core (α subunit) internal site (a); pol III core (α subunit) extreme C-terminal motif (b); MutS N-terminal motif (c); MutS C-terminal motif (d); DNA polymerase II C-terminal motif, and DNA polymerase IV C-terminal motif.

MutS is bound to a mismatch. However, the Thermus aquaticus crystal structure of free MutS (without DNA) has shown that the N-terminal domains are highly mobile (44) and suggest that the MutS dimer could possibly form a ring for DNA interaction. This hypothesis is consistent with the behavior that MutS exhibits in a number of studies (49, 50). It has been proposed that the MutS ring may be semi-closed and therefore less stable on DNA compared with β and PCNA (51), but may allow for one-dimensional diffusion on DNA before encountering the mismatch.

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there is a close relationship between mismatch recognition and processivity clamp binding and release (12, 27). The affinity of $\beta$ for MutS (Fig. 5B) is similar to that of pol III core in solution ($\sim 250$ nM (43), Fig. 5B).

Targeting of MutS to $\beta$ could possibly explain the observed reduced mutagenesis rates in the lagging strand versus the leading strand (52, 53), because the mechanism of DNA replication results in the accumulation of processivity clamps on the lagging strand (37). Also, because all processivity clamps loaded at the replication fork have the same orientation with respect to the double helix, $\beta$ could direct repair to the newly synthesized strands.

We show here that the C-terminal site in MutS accounts for the strength of interaction with $\beta$ in solution. Hence, MutS may first bind to $\beta$ via the C-terminal motif and then, as MutS encircles DNA, also via the N-terminal motif. Is there any similarity between the $\beta$ motifs in MutS and pol III? An alignment of the two binding motifs in E. coli pol III $\alpha$ subunit and the two binding motifs in MutS (Fig. 6C) show similarity at positions one and four (Q1XXL2), whereas a hydrophobic residue, either Leu or Phe, has been shown to be accommodated at positions 5 or 6 in some $\beta$ binding motifs (21). This minimal consensus sequence in different ligands of a common binding site reflects the ability of the hydrophobic pocket on the surface of $\beta$ to interact dynamically with several different partners in the cell (38).

The MutL-$\beta$ Interaction Requires ssDNA—The determination of MutL structures by Yang and collaborators (7, 35, 36, 54) has led to exciting new insights into their role in mismatch repair. However, major questions remain regarding the mechanism by which MutL coordinates mismatch repair. For example, the amino acids in MutL that interact with MutS, MutH, and UvrD have not yet been determined, and there is uncertainty as to the mode in which MutL binds to various DNA species. The study presented here adds yet another protein partner to MutL, the $\beta$ clamp processivity factor. Studies of eukaryotic MLH1 also indicate that it binds the PCNA clamp (8, 55–57). Our results show that MutL binding to $\beta$ requires ssDNA. Presumably MutL binding to ssDNA induces a conformational change in the N-terminal domains of the MutL dimer that allow for interaction with $\beta$. It is interesting to note the atomic position of MutL residues Leu-150 and Phe-151 implicated in this study in binding to $\beta$. The structures of MutL with and without nucleotide are shown in Fig. 7A. The $\beta$ interactive residues are exposed in the absence of nucleotide, but are sequestered in its presence. It seems likely that this nucleotide-induced change underlies the observation herein that nucleotide diminishes the ability of MutL to bind $\beta$. We also demonstrate that human PCNA binds to a peptide derived from the corresponding region in human MLH1.

The finding that ssDNA is required for $\beta$ binding by MutL, combined with the fact that $\beta$ slides only on double-stranded DNA, suggests that the interaction between MutL and $\beta$ occurs at a junction between ss- and dsDNA. A ss/dsDNA junction is the natural substrate of DNA polymerases (Fig. 7B), and this structure is also present during the excision step in mismatch repair that involves the combined actions of a helicase and exonuclease to degrade the newly synthesized DNA strand. We show here that the C-terminal site in MutL competes with the pol III helicase and exonuclease to degrade the newly replicated DNA strand (37). Also, because all processivity clamps loaded at the replication fork have the same orientation with respect to the double helix, $\beta$ could direct repair to the newly synthesized strands. We show here that the C-terminal site in MutS accounts for the strength of interaction with $\beta$ in solution. Hence, MutS may first bind to $\beta$ via the C-terminal motif and then, as MutS encircles DNA, also via the N-terminal motif. Is there any similarity between the $\beta$ motifs in MutS and pol III? An alignment of the two binding motifs in E. coli pol III $\alpha$ subunit and the two binding motifs in MutS (Fig. 6C) show similarity at positions one and four (Q1XXL2), whereas a hydrophobic residue, either Leu or Phe, has been shown to be accommodated at positions 5 or 6 in some $\beta$ binding motifs (21). This minimal consensus sequence in different ligands of a common binding site reflects the ability of the hydrophobic pocket on the surface of $\beta$ to interact dynamically with several different partners in the cell (38).

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DNA loop structures have been implicated in mismatch repair to account for the distance between the site of DNA damage and the place where degradation of the damaged DNA strand starts, which can be on the order of kilobases (2, 7, 60). MutL would be well suited for DNA looping as a consequence of the long connector sequences between the N- and C-terminal domains, and the ability of the N-terminal domains to dimerize in an ATP-dependent manner (36).

The models of Figs. 6 and 7 involve a combination of DNA looping and movements of MutS and MutL along the DNA contour that could eventually reconcile some of the current working models of mismatch repair. Further studies will be needed to reveal the exact roles of $\beta$, but the interactions of $\beta$ to both MutS and MutL described in this study indicate that the clamp has multiple functions in mismatch repair.

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DNA: Replication, Repair, and Recombination: The β Sliding Clamp Binds to Multiple Sites within MutL and MutS

Francisco J. López de Saro, Martin G. Marinus, Paul Modrich and Mike O'Donnell

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