MutL traps MutS at a DNA mismatch

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DNA mismatch repair (MMR) identifies and corrects errors made during replication. In all organisms except those expressing MutH, interactions between a DNA mismatch, MutS, MutL, and the replication processivity factor (β-clamp or PCNA) activate the latent MutL endonuclease to nick the error-containing daughter strand. This nick provides an entry point for downstream repair proteins. Despite the well-established significance of strand-specific nicking in MMR, the mechanism(s) by which MutS and MutL assemble on mismatch DNA to allow the subsequent activation of MutL’s endonuclease activity by β-clamp/PCNA remains elusive. In both prokaryotes and eukaryotes, MutS homologs undergo conformational changes to a mobile clamp state that can move away from the mismatch. However, the function of this MutS mobile clamp is unknown. Furthermore, whether the interaction with MutL leads to a mobile MutS–MutL complex or a mismatch-localized complex is hotly debated. We used single molecule FRET to determine that Thermus aquaticus MutL traps MutS at a DNA mismatch after recognition but before its conversion to a sliding clamp. Rather than a clamp, a conformationally dynamic protein assembly typically containing more MutL than MutS is formed at the mismatch. This complex provides a local marker where interaction with β-clamp or PCNA could distinguish parent/daughter strand identity. Our finding that MutL fundamentally changes MutS actions following mismatch detection reframes current thinking on MMR signaling processes critical for genomic stability.

Significance

DNA mismatch repair is the process by which errors generated during DNA replication are corrected. Mutations in the proteins that initiate mismatch repair, MutS and MutL, are associated with greater than 80% of hereditary nonpolyposis colorectal cancer (HNPCC) and many sporadic cancers. The assembly of MutS and MutL at a mismatch is an essential step for initiating repair; however, the nature of these interactions is poorly understood. Here, we have discovered that MutL fundamentally changes the properties of mismatch-bound MutS by preventing it from sliding away from the mismatch, which it normally does when isolated. This finding suggests a mechanism for localizing the activity of repair proteins near the mismatch.

DNA mismatch repair | MutS | MutL | FRET

The importance of the nicking activity of MutL homologs is highlighted by the observation that mutations that impair yeast MutLα endonuclease activity cause a significant mutator phenotype and genomic instability (11, 13, 14). Despite the well-established significance of strand-specific nicking in MMR, the mechanism(s) by which MutS and MutL assemble on mismatched DNA to allow subsequent activation of MutL endonuclease activity by β-clamp/PCNA remains elusive. There is general agreement that in both prokaryotes and eukaryotes, after binding a mismatch MutS or MutSr can undergo conformational changes to a mobile clamp state that can move away from the mismatch (6, 15). What happens after this step is mired in controversy. Several disparate models for MutS(α)–MutL(α) mismatch complex formation and the subsequent signaling of repair have been proposed (e.g., see refs. 6, 7, 15–21). One prominent model in the field has MutL(α) joining MutS(α) to form MutS(α)–MutL(α) sliding clamps that diffuse along the DNA to interact with the strand-discrimination signal (β-clamp/PCNA or MutH) (16). Other models include trapping of MutS(α) clamps near the mismatch by MutL(α) followed by DNA looping or, alternately, MutS(α)-induced polymerization of MutL(α) along the DNA to reach the strand-discrimination signal (6, 7, 15, 18, 22). Some degree of localization to the mismatch is suggested by in vitro studies of eukaryotic MMR proteins, indicating that although MutLα can introduce nicks across long stretches of DNA, they occur preferentially in the vicinity of the mismatch (9, 11, 12).

In this study, we have used single molecule fluorescence to demonstrate that in the case of Thermus aquaticus (a MutL-free organism), MutL traps MutS at the mismatch after its ATP-induced endonuclease activity by


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activation but before its conversion into a sliding clamp. The resulting MutS–MutL mismatch complex typically contains more MutL than MutS, with one or two MutS dimers and up to four MutL dimers. MutS exists in a conformationally dynamic state within these complexes, which may be relevant for subsequent steps in MMR. In contrast to a mobile MutS–MutL complex, localization of MutS–MutL at the mismatch can restrict β-clamp/PCNA-activated MutS nicking to the vicinity of the mismatch, thereby enhancing MMR efficiency and limiting excessive excision and resynthesis that can destabilize the genome.

Results and Discussion

MutL Lengthens the Dwell Time of MutS at a DNA Mismatch. We recently used single molecule fluorescence resonance energy transfer (FRET) between donor fluorophore-labeled T. aquaticus (Taq) MutS protein and DNA with an acceptor dye located 9 bp from a T bulge to monitor the interaction of MutS with a mismatch in the presence of ADP or ATP (21). Results from that study revealed that in the presence of ATP, 20% of the mismatch-bound MutS proteins convert into sliding clamps (as reported by transition to FRET 0; Fig. 1 A and B), whereas the other 80%
exhibit simple mismatch binding and dissociation with the same kinetics as seen in the presence of ADP, presumably having undergone ATP hydrolysis prior to mismatch binding (21).

In this study, we examined the effects of MutL on MutS–DNA interactions under different nucleotide conditions. Addition of MutL does not alter the behavior of MutS on mismatched DNA in the presence of ADP, nor of the 80% of MutS in the presence of ATP that behaves the same as with ADP (Fig. S1). In contrast, MutL dramatically alters the behavior of 20% of MutS in the presence of ATP (Fig. 1C), which is the same fraction of MutS that forms ATP-bound sliding clamps in the absence of MutL (21). Decreasing MutL concentration decreases the fraction of MutS proteins that show these altered properties, confirming a MutL-specific effect (Fig. 1H). For this subset, (i) MutL increases the residence time of MutS at the mismatch by ~10-fold, from ~5 s to 40 s (Fig. 1D and E); (ii) MutS rarely exhibits a FRET of 0 before dissociation (or photobleaching), indicating that the MutL-stabilized MutS-mismatch complexes do not form sliding clamps that move away from the mismatch before dissociation (Fig. 1C); and (iii) MutL alters the conformations and dynamics of MutS at the mismatch (discussed later). Notably, experiments with DNA containing a GT mismatch demonstrate that MutL also increases the overall lifetime of ATP-bound MutS at a GT mismatch by ~10-fold (Fig. 1F and G), from tens of seconds to hundreds of seconds, indicating that these findings are not limited to a T bulge (SI Materials and Methods and Fig. S1A–D for details on the MutS-GT DNA complex and SI Materials and Methods).

**MutL Prevents Loading of Multiple MutS on End-Blocked, Mismatch-Containing DNA.** Previous studies, including ours, have shown that ATP-dependent conversion of MutS into a sliding clamp frees up the mismatch site and allows loading of multiple MutS proteins, which get trapped on end-blocked DNA (5, 17, 20, 21). The observation that MutL stabilizes MutS at a mismatch predicts that MutS loading onto end-blocked DNA should be reduced in the presence of MutL. Monitoring the photobleaching of fluorescently labeled MutS on an end-blocked T-bulge substrate in the presence of ATP reveals that without MutL, up to eight MutS dimers can be loaded per DNA with lifetimes greater than 600 s (Fig. 2A–C) (20, 21). In contrast, addition of MutL greatly reduces accumulation of MutS sliding clamps, such that most DNAs are bound by only one or two MutS dimers (Fig. 2D and E). In addition, in the absence of MutL, zero FRET (Fig. 2B) indicates MutS sliding clamps move away from the mismatch, whereas with MutL present, nonzero FRET (Fig. 2D) indicates at least one MutS remains near the mismatch. These results taken together with the FRET data described above (Fig. 1) indicate that MutL traps one or two MutS dimers at or near the mismatch.

**Stoichiometry of MutS–MutL Mismatch DNA Complexes.** Because the dynamic experiments (as in Fig. 1) are limited to concentrations of ~10 nM fluorescent protein, to examine the stoichiometries of MutS–MutL-mismatch complexes in more detail, we (i) incubated Alexa 647-tagged MutS (10 nM or 100 nM) and Alexa 555-tagged MutL (200 nM) with biotinylated T-bulge–DNA at room temperature and 40 °C, (ii) crosslinked the complexes with glutaraldehyde, (iii) captured the crosslinked complexes on a streptavidin surface, and (iv) used single-molecule fluorescence photobleaching to determine the number of Alexa 647-tagged MutS and Alexa 555-tagged MutL proteins in each complex (Fig. 3 and Fig. S2 A–D). In all cases, formation of complexes containing MutL required the presence of mismatched DNA, ATP, and MutS (Fig. 2E–G), and we observed no significant population of excessively large assemblies. Most complexes contain one to two MutS dimers and two to three MutL dimers (Fig. 3D and Fig. S2). This number of MutS dimers is consistent with the number of dimers that we observe in our dynamic experiments with labeled MutS and unlabeled MutL (Fig. 2D and E). In addition, the total number of proteins in the complex is similar to the number of proteins in complexes of yeast MutSα–MutLα detected by surface plasmon resonance (23). The observed excess of MutL over MutS contrasts with the proposed 1:1 stoichiometry in MutS–MutL sliding clamps (16, 24), but agrees with in vivo studies in *E. coli* and yeast, where repair foci contain more MutL than MutS proteins (18, 22), and with early DNA footprinting studies indicating complexes containing multiple MutS and MutL proteins at the mismatch (3, 25). Consistent with the latter observation, additional crosslinking experiments using unlabeled MutL, Alexa 555-tagged MutS and the Cy5–T-bulge–DNA revealed FRET in all complexes, confirming their presence near the mismatch, consistent with our dynamic experiments with uncrosslinked proteins (Fig. 2D). Rather than a sliding MutS–MutL clamp model, our findings suggest a model in which MutL flanks MutS at the mismatch, as first suggested by Modrich and coworkers (6, 7) and more recently by other investigators (18, 22).

**Intermediate Steps During Assembly of MutS–MutL Complexes.** Having characterized the composition of the MutS–MutL complexes at a mismatch, we next sought to elucidate the mechanism of complex formation. To this end, we examined the impact of MutL on the kinetics of MutS mismatch recognition and its subsequent conformational changes in solution, in real time (Fig. 4). Our previous experiments (21) showed that conversion of MutS into a sliding clamp involves at least two steps wherein MutS first binds to the mismatch (resulting in FRET of 0.65) and then undergoes a conformational change (resulting in FRET 0.45) before forming a clamp that diffuses away from the mismatch (resulting in loss of the donor signal) (Figs. 1B and 4B).
As expected, MutL does not alter the FRET of the initial MutS mismatch recognition complex (0.65); however, it dramatically changes subsequent conformational transitions. The dwell-time distributions of the first FRET state (0.65) in the presence or absence of MutL exhibit clear rise and decay (Fig. 4D and G), indicating two rate-limiting steps between FRET 0.65 and the next FRET state (26, 27) and therefore the existence of two states with a FRET of 0.65 (which we designate 0.65 and 0.65*) (Fig. 4F). Fitting these data (SI Materials and Methods) (Fig. 4D and G, red lines) yields similar rates in the absence of MutL (1.1 ± 0.67 s⁻¹ and 0.45 ± 0.02 s⁻¹) and in its presence (0.56 ± 0.14 s⁻¹ and 0.20 ± 0.05 s⁻¹). Given that the rates of both transitions are slower than the estimated rate of ATP-induced ADP dissociation measured in ensemble studies (28), we propose that the first step (0.65→0.65*) requires ADP release followed by rapid ATP binding (10⁹ M⁻¹ s⁻¹ and >10¹⁰ s⁻¹ at 2 mM ATP) (28, 29), and the second step (to FRET 0.45 without MutL; Fig. 4C and D) is a conformational change of the doubly ATP-ligated state (Fig. 4H), consistent with previous suggestions (15, 17). Notably, although MutL does not dramatically impact the FRET levels or kinetics of the initial MutS conformational change (0.65→0.65*), it alters the subsequent conformation, which exhibits FRET 0.45 without MutL, but FRET 0.3 with MutL. These results indicate that MutL interacts with MutS after the ADP–ATP exchange, as suggested by previous studies (15, 17), but before MutS transitions to FRET 0.45, demonstrating that MutL binding to MutS immediately after its ATP binding-induced conformational change traps it at the mismatch (Fig. 4H). This latter finding provides an explanation for the observation that yMutL can interact with an ATPase-site mutant of yMutS that does not form a sliding clamp (30). Interestingly, a recent study monitoring DNA bending with small angle X-ray scattering in solution (31), suggests that, for E. coli proteins, MutL interacts with MutS after an ATP-dependent conformational change from a bent DNA state to an unbent DNA state. If E. coli and Taq MMR follow the same pathway (discussed later in Conclusions), then extrapolating this result suggests that our FRET 0.45 state (between protein and DNA) involves unbent DNA (Fig. 4F) (32, 33).

In the absence of MutL, MutS in the 0.45 FRET state transitions to a sliding clamp with FRET of 0 and ultimately slides off the free DNA end (no donor fluorescence) (Fig. 4B). In contrast, in the presence of MutL, MutS remains at the mismatch and fluctuates rapidly between FRET of 0.3 and 0.6 before eventually dissociating directly from the mismatch (or photobleaching) without transitioning to 0 FRET (Figs. 1C and 4E). The narrowness of the FRET 0.3 and 0.6 histograms (Fig. 4F) confirms that MutL-stabilized MutS remains at or very near the mismatched base, because movement of MutS even a few nucleotides from the mismatch would broaden the FRET distributions. In addition, we only observe these two interconverting states (FRET 0.3 and 0.6) in the presence of MutL (Fig. 4F vs. C), strongly suggesting that MutL is present and is influencing the conformation of MutS. To understand the nature of the rapid transitions, we also monitored intraprotein FRET between donor and acceptor fluorophore-tagged mismatch binding domains 1 of MutS dimers bound to unlabelled DNA (Fig. S3 A–D). The data show that these domains alternate between two conformational states with the same kinetics as the FRET transitions seen between MutS and the DNA (Fig. 4F). Taken together, these results indicate that MutL traps MutS at (or very near) the mismatch site, but that MutS mismatch binding domains remain mobile. It is notable that MutS domains I switch between conformationally mobile and static states depending on its ligand-bound form (e.g., mobile in free MutS, static in mismatch-bound MutS and then mobile again in ATP-, mismatch- and MutL-bound MutS) (21). A specific role for MutS domain I dynamics in signaling downstream events after mismatch recognition remains to be determined.

Conclusions

In summary, by directly monitoring assembly of individual MutS–MutL complexes at DNA mismatches, we have observed initial events in the repair mechanism following mismatch recognition. This observation that MutL can trap MutS at the mismatch before it forms a sliding clamp raises the question of what function might be served by sliding clamps. It may be the means by which MutS clears the mismatch site if MutL does not arrive in a timely manner to initiate repair. Our study also does not rule out the possibility that mobile MutS–MutL signaling complexes may form and complement the functions of stationary MutS–MutL mismatch complexes in DNA repair, e.g., for long-range search of a strand-discrimination signal when one is not available near the mismatch (5, 16). In MutH-dependent methyl-directed MMR (as in E. coli), localized assembly of MutS–MutL at the mismatch alone cannot account for orientation-dependent loading of the appropriate 5′-to-3′ or 3′-to-5′ excision system at the nick made by MutH at a d(GATC) site (34), because the mismatch can be up to a kilobase from the break (35) and the helicase loading process must involve signaling along the helix contour. The apparent requirement for mobile MutS–MutL complexes in methyl-directed repair may reflect fundamental differences from MutH-independent repair, such as in Taq and eukaryotes. In particular, early steps in methyl-directed repair are β/PCNA clamp-independent and the MutL homolog lacks endonuclease activity (34), whereas, in MutH-independent repair, MutL has latent endonuclease activity that is activated by β/PCNA at an early step. In the latter system, interactions between β/PCNA clamps, which are loaded onto primer-template DNA junctions in a specific orientation, and MutS–MutL complexes trapped at the mismatch site could direct MutL nicking
activity to the nascent strand in the vicinity of the mismatch. This constraint would in turn limit the extent of strand excision and resynthesis and increase the efficiency of DNA mismatch repair (9, 11, 14, 36).
Materials and Methods

Taq MutS was expressed in E. coli, purified, and dye labeled at the M88C position as described (21). Taq MutM was cloned from Taq strain YT-1 (ATCC) genomic DNA into expression vectors either with or without His-tags, expressed in E. coli and purified by affinity, ion-exchange, and gel-filtration chromatography. A cysteine was inserted between the 6His-tag and MutL sequence for labeling, when indicated. The 550-bp DNA substrates are similar to those described previously (21), except an unintended internal flap overhang was corrected. Lipid passivated, streptavidin surfaces were used to immobilize biotinylated/digoxin-labeled DNA substrates, which could be blocked at the 5′ tethered end by antidigoxin binding as described previously (21), smFRET was measured in a prism-type total internal reflection fluorescence microscope with a dualview image splitter before an emCCD and analyzed as described previously (21, 37). Experiments to determine complex stoichiometry were performed by mixing biotinylated DNA, ATP, MutS, and MutL in solution 10 min before adding glutaraldehyde to 0.8% final concentration for 1 min, diluting 46-fold with Tris buffer (20 mM Tris HCl, 100 mM NaOAc, 5 mM MgCl₂, pH 7.8) to quench crosslinking, flowing over an imaging surface coated with streptavidin islands and lipid bilayer passivation. This surface captured complexes via biotinylated DNA for single molecule fluorescence imaging and photo-bleaching step counting. All imaging was performed in imaging buffer (20 mM Tris acetic acid, pH 7.8, 100 mM NaOAc, 5 mM MgCl₂, 2% glucose (w/v) with oxygen scavenging/triplet state quenching additives, 100 units/mL glucose oxidase, 1,000 units/mL catalase, 0.05 mg/mL cyclooctatetraene, and 14 mM 2-mercaptoethanol). Additional details are available in SI Materials and Methods.

Note Added in Proof. A recently published study demonstrated that addition of E. coli MutL greatly reduces the rate at which MutS slides off mismatched DNA, consistent with our findings (41).

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