MutS homolog sliding clamps shield the DNA from binding proteins

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ABSTRACT

MutS homolog (MSH) proteins initiate mismatch repair (MMR) by recognizing mispaired nucleotides and forming stable sliding clamps that randomly diffuse along the DNA. The MSH sliding clamps then load MutL homolog (MLH/PMS) proteins that form a second stable sliding clamp, which together are capable of interacting with downstream MMR components and/or the excision-initiation site that may be hundreds to thousands of nucleotides distant from the mismatch. The binding of specific or non-specific proteins to the DNA between the

mismatch and the distant excision-initiation site could conceivably obstruct the free diffusion of these MMR sliding clamps, inhibiting their ability to initiate repair. We employed bulk biochemical analysis, single-molecule fluorescence imaging, and mathematical modeling to determine how sliding clamps might overcome hindrances along the DNA. The results suggest a simple mechanism where the thermal motion of MSH sliding clamps alters the association kinetics of DNA binding proteins over long distances. These observations appear generally applicable for any stable sliding clamp that forms on DNA.

INTRODUCTION

Sliding clamps on DNA have been conserved throughout evolution and play essential roles in coordinating DNA replication, repair and the cellular damage response (1-3). Among notable examples are the MutS homolog (MSH) proteins, which operate as a homo- or hetero-dimer and specifically recognize mismatched nucleotides, lesions or structures within the DNA (4). MSH proteins bind and hydrolyze ATP (5,6). Importantly, DNA mismatch or structure recognition provokes ATP binding by MSH proteins that ultimately results in the formation of a stable sliding clamp (5-8). These MSH sliding clamps move by 1-dimensional (1D) thermal (Brownian) diffusion with a lifetime of up to 10 min while in intermittent contact with the DNA backbone (9-12). The MSH sliding clamps that function in mismatch repair (MMR) act as a platform to load MutL homolog (MLH/PMS) proteins, which then form a second extremely stable freely-diffusing ATP-bound sliding clamp that communicates mismatch recognition along the DNA to an excision-initiation site, which may be hundreds to thousands of nucleotides distant from the mismatch (4,13).

Unrelated DNA binding proteins may become roadblocks if they bind between the mismatch and the downstream excision-initiation site. Single-molecule imaging of molecular motors that use the energy of ATP hydrolysis to move along the DNA have demonstrated that these proteins can actively remove roadblocks (14). Alternatively, some proteins that rely on thermal fluctuation-driven motion may hop over roadblocks on DNA (15). Remarkably, MSH sliding clamps that rely entirely on 1D thermal diffusion to move along the DNA have been shown to disassemble nucleosomes (16). The mechanism of MSH-dependent nucleosome disassembly was not immediately obvious since the histone octamer-DNA interactions that comprise a nucleosome are numerous and would appear to require significant energy to release (17).

One hypothesis is that multiple randomly diffusing MSH sliding clamps might increasingly occupy nucleosomal DNA that is transiently unwrapped as a result of thermal fluctuation (18). Such 1D thermal motion of multiple MSH sliding clamps was projected to provide an explicit mechanism for influencing the binding of other proteins over distances that are larger than the total DNA footprint of the MSH particles (16,18). Here we have used bulk analysis and single-molecule fluorescence imaging to examine the properties of MSH sliding clamps on DNA. We found that increasing the numbers of MSH sliding clamps on a DNA decreased LacI repressor association to its cognate LacO promoter. We also show that the number of MSH sliding clamps already bound to the DNA affected the loading of additional MSH sliding clamps at a mismatch. Together these observations appear consistent with the ability of MSH sliding clamps to affect the association kinetics of additional DNA binding proteins.

RESULTS

ATP Bound MutS Homologs Reduce LacI Binding to its Cognate LacO Site

The random diffusion characteristics of MSH sliding clamps on DNA suggested that they might influence the association of heterologous DNA binding proteins over distances greater than their footprint. To address this possibility we examined the effect of three different MSH proteins, *Thermus aquaticus* TaMutS, *Escherichia coli* EcMutS and human (*Homo sapien*) HsMSH2-HsMSH6, on the binding of the *E.coli* transcription factor LacI to its cognate LacO site using Surface Plasmon Resonance (SPR; **Fig. 1*a-c***, **left panels**; **Supplemental Fig. *S1***). In this system the proximal end of a 98 bp mismatched DNA was attached to the SPR surface utilizing a biotin-streptavidin linkage. The mismatch was located 15 bp from the surface and the 21 bp LacI LacO site (19-21) was located 12 bp from the distal end that was blocked by digoxigenin-antidigoxigenin (dig-antidig). This DNA substrate could retain up to three MSH sliding clamps that engender a footprint of ~26 bp in the presence of ATP (6,9,22-25). However, there is only enough space for LacI to bind if there is less than three MSH sliding clamps are present on the DNA. Following subtraction of the intrinsic MSH dissociation kinetics, we find that the association rate of LacI ($k_{on\bullet LacI}$) decreased with increasing MSH concentration in the presence of ATP (**Fig. 1*a-c***, **middle and right panels**). As a control we show that increasing MSH concentration in the absence of ATP does not affect LacI binding (**Supplementary Fig. *S2***). These observations are consistent with the conclusion that $k_{on\bullet LacI}$ is inhibited with increasing ATP-bound MSH sliding clamps on the DNA.

The Binding of LacI is altered by the number of MutS Sliding Clamps on DNA

SPR studies are unable to determine how the MSH sliding clamps affect the ability of LacI to bind the mismatched DNA. We consider at least two possibilities: 1.) MSH sliding clamps saturate the DNA effectively obscuring the LacO site, or 2.) subsaturating MSH sliding clamps affect LacI binding at a distance. We used a single molecule total internal reflection fluorescent (smTIRF) microscope system to determine the number of MSH sliding clamps on the mismatched DNA and their effect on LacI binding. Cy3-labeled TaMutS was utilized in these studies since we have previously demonstrated specific mismatch binding and the formation of stable ATP-bound sliding clamps on defined mismatched DNA substrates (9,12). The 98 bp DNA was attached to a passivated surface via a biotin-neutravidin linkage and modified to contain a +dT mismatch 15 bp from the proximal end with a Cy5 fluorophore 9 bp distal of the mismatch. The LacO site was located similarly to the SPR mismatched DNA substrate (**Fig. 2*a***; **Supplementary Table *S1*).** The Cy5 location was previously shown to have no influence on mismatch binding activity or the formation of TaMutS sliding clamps (9,12). The position of the LacO insured that when bound by LacI the TaMutS sliding clamps may access only 65 bp of the 98 bp mismatched DNA.

LacI binding was determined by observing the time-averaged FRET produced by Cy3-TaMutS on the Cy5-labeled LacO-mismatched DNA (9,12). FRET is inversely correlated with the length of accessible DNA and thus depends on the presence or absence of bound LacI. We calculate time-averaged FRET efficiencies of Cy3-TaMutS on the full- length 98 bp LacO-mismatched DNA of E_1 ~ 0.32, E_2 ~ 0.34 and E_3 ~ 0.30 for one, two and three single fluorophore labeled sliding clamps, respectively (Figure 2; **Supplementary Fig. S3**). The binding of LacI to the LacO-mismatched DNA reduces the effective length that Cy3-TaMutS sliding clamps may occupy, resulting in a calculated increase in the time-averaged FRET efficiency of $E_{1\bullet LacI}$ ~ 0.60 and $E_{2\bullet LacI}$ ~ 0.46 for one and two single-labeled Cy3-TaMutS sliding clamps, respectively. The site size of three TaMutS sliding clamps on the LacO-mismatched DNA is expected to fully

exclude LacI binding.

The number of TaMutS sliding clamps on single LacO-mismatched DNA molecules was determined by counting the number of Cy3 photobleaching steps (**Fig. 2*b*; Supplementary Figure S3**). These were separated into single (E_1), double (E_2) and triple (E_3) fluorophores with associated FRET efficiency (E). Based on the labeling efficiency we expected 30% of the TaMuS dimers might contain two Cy3 fluorophores, potentially influencing the sliding clamp count. However, in the absence of LacI the binned FRET efficiencies fit to normal curves with means of E_1 = 0.31 ± 0.08, E_2 = 0.30 ± 0.12 and E_3 = 0.21 ± 0.08, which closely correlated with the calculated time-averaged FRET efficiencies (**Fig. 2*a***: **Supplementary Fig. S3**). Prebinding LacI to the LacO-mismatched DNA followed by loading Cy3-TaMutS sliding clamps resulted in DNA molecules that contained one- or two- fluorophores with FRET efficiencies that correlated extremely well with the calculated time averaged FRET efficiencies (see: **Fig. 2*a*** compared to **Fig. 2*b*** for one fluorophore**,** $E_{1\cdot LacI}$ = 0.60 ± 0.10; or compared to **Supplementary Fig. S3** for two fluorophores, $E_{2\cdot LacI}$ = 0.47 ± 0.07). We conclude that simple Cy3 fluorophore counting provides a reasonable approximation of the number of TaMutS sliding clamps, and that time-averaged FRET efficiency is a practical indicator of the presence or absence of LacI on the LacO-mismatched DNA.

We noted that the distributions of binned FRET efficiency with and without LacI overlapped for both the one- or two-fluorophore cases. A stringent criterion for distinguishing LacI bound DNA is to identify molecules with a FRET efficiency that is greater than two standard deviations from the mean ($E_{1\cdot LacI}$ > 0.4; $E_{2\cdot LacI}$ > 0.33). We then examined 40 μm x 80 μm fields containing ~60 well-resolved molecules and found that when one Cy3 fluorophore was associated with the Cy5-labeled LacO-mismatched DNA, 55% of the molecules displayed a time-averaged FRET efficiency consistent with LacI bound to the DNA ($E_{1\cdot LacI}$ > 0.4; **Fig. 2*c***). When two Cy3 fluorophores were associated with the Cy5 LacO- mismatched DNA, 37% of the molecules displayed a time-averaged FRET efficiency consistent with LacI bound to the DNA ($E_{2\cdot LacI}$ > 0.33; **Fig. 2*c***). As expected, we did not observe any molecules that displayed altered time-averaged FRET when three or four Cy3 fluorophores were associated with the Cy5 mismatched DNA, although these events were relatively rare (**Fig. 2*c***). We note that with the four Cy3 fluorophore case at least one of the TaMutS sliding clamps must contain two fluorophores. These results support the conclusion that increased numbers of MSH sliding clamps exclude LacI transcription factor binding to DNA.

MutS Sliding Clamps on the DNA affect the k_{on} of LacI

To determine whether MutS sliding clamps affect the on- or off-rate of LacI binding kinetics, we developed a single molecule protein-induced fluorescence enhancement (smPIFE) system (26). A Cy3 fluorophore was placed 2 bp distal to the LacO site on the 98 bp mismatched DNA (**Fig. 3*a***; **Supplementary Table S1**). LacI binding induced a PIFE signal (**Supplementary Fig. S4*a***) in which the $\tau_{off\cdot LacI}$ was dependent upon protein concentration and could be used to determine the on-rate ($k_{on\cdot LacI}$; **Supporting Information; Supplementary Fig. S4*b***) and off-rate ($\tau_{on\cdot LacI}$ = 1/ $k_{off\cdot LacI}$) in order to calculate the equilibrium dissociation constant ($K_{D\cdot LacI}$ = $k_{off\cdot LacI}$ / $k_{on\cdot LacI}$ = 119 ± 22 pM), which was similar to historical reports (19). To count the number of ATP-bound sliding clamps we labeled TaMutS or EcMutS with Alexa647 as previously described (9,12).

For these studies the $\tau_{off\cdot LacI}$ was determined with 1 nM LacI, which was then followed by counting the number of Alexa647 photobleaching events to determine the number of fluorophores on single LacO-mismatched DNA molecules (**Fig. 3b**; **Supplementary Fig. S5**;

Supplementary Information). The resulting $\tau_{off\text{•}LacI}$ histograms were individually binned for one- and two-fluorophore photobleaching events (12). To account for the possibility of unlabeled and double-labeled MutS dimers the $\tau_{off\text{•}LacI}$ histograms were fit to a double exponential (**Fig. 3*c***). We then normalized the total probability density to 1 and based on the experimentally determined labeling efficiency, fixed the ratio of double fluorophore bleaching events to single fluorophore bleaching events to reduce the number of variables to two (**Fig. 3*b***; **Supplementary Fig. S5**; **Supporting Information**). These fits could either be used directly or subjected to maximum likelihood estimation to garner the $\tau_{off\text{•}LacI}$ when one ($\tau_{off\text{•}LacI\text{-}1}$) or two ($\tau_{off\text{•}LacI\text{-}2}$) MutS sliding clamps were present on the LacO-mismatched DNA (**Supplementary Table *S3***). These τ_{off} values were then used to calculate the $k_{on\text{•}LacI}$ in the presence of 1 MutS sliding clamp or 2 MutS sliding clamps and displayed individually with standard deviation error for experimental comparison (**Fig. 3*c***). These results demonstrate that increased numbers of MutS sliding clamps decrease the k_{on} of LacI association to its cognate LacO binding site.

Reduction in LacI binding is consistent with a predictive model for freely diffusing MSH sliding clamps on DNA

We developed a model that quantitatively describes the reduction of $k_{on\text{•}LacI}$ as a result of any number of sliding clamps and DNA lengths. This model treats MSH sliding clamps as particles that engage in free one-dimensional (1D) diffusion along the DNA, with the only requirement that their footprints cannot overlap (18). A similar model was originally described by Lewi Tonks (27) and is generally recognized as a 1D Tonks gas model. Perhaps more importantly, the properties of such a 1D Tonks Gas/Sliding Clamp can be systematically calculated (**Supporting Information, Model for Freely Diffusing MutS Sliding Clamps**).

In order to test the application of the Tonks Gas/Sliding Clamp model in the absence of additional proteins and to establish its main parameter, the footprint size σ of the MutS sliding clamps, single molecule fluorescence imaging was used to count the number of MutS sliding clamps on a DNA containing a single mismatch (**Fig. 4*a*; Supplementary Table S1**). In these studies a 95 bp DNA was linked at the proximal end to a passivated surface via 5'-biotin-NeutrAvidin and the distal end was blocked by 3'-digoxigenin-anti-digoxigenin antibody (dig-antidig; **Fig. 4*a***). Blocking both ends (surface and dig-antidig) effectively retains freely diffusing MutS sliding clamps on the mismatched DNA as previously described (9,12).

An extrahelical +dT mismatch was positioned at 15 bp from the proximal end and the DNA was labeled 7 bp from the distal end with Cy5 (**Fig. 4*a***). Cy3-labeled TaMutS was used in this analysis since we have previously demonstrated specific mismatch binding and the formation of stable ATP-bound sliding clamps on similar mismatched DNA substrates (9,12). We first determined that the location of the Cy5 does not influence TaMutS mismatch binding or the formation of an ATP-bound sliding clamp (**Supplementary Fig. S8**). These results suggest the ATP-bound TaMutS sliding clamps loaded onto the DNA are the consequence of genuine mismatch recognition events.

Our previous studies showed that ATP-bound Cy3-TaMutS sliding clamps rapidly diffuse along the length of short oligonucleotides producing a time-averaged FRET with the Cy5-labeled DNA (**Fig. 4*b***) (9,12). These observations indicated that the number of ATP-bound TaMutS sliding clamps could be unequivocally determined by monitoring the FRET signal that results from Cy3-TaMutS located on the Cy5-mismatched DNA and then counting the number of Cy3 photobleaching events over a 100 sec observation (**Fig. 4*b***; **Supplementary**

Fig. S7).

We found that the fraction of TaMutS sliding clamp-associated fluorophores depended on protein concentration (**Fig. 4*c***, data points; **Supplementary Fig. S9**). The Tonks Gas/Sliding Clamp model makes a specific prediction regarding the reduction of the effective on-rate for binding a new TaMutS clamp that is related to the number of MutS sliding clamps already diffusing on the DNA. This prediction allows for calculation of the concentration-dependent distribution of MutS sliding clamp-associated fluorophores (**Supporting Information**). The general agreement with the measured distribution provides further support for the Tonks Gas/Sliding Clamp model (**Fig. 4*c***, lines). Moreover, the interpolated footprint size of σ = 26 bp generally agrees with previous biochemical determinations (28).

The Tonks Gas/Sliding Clamp model may also be used to predict the reduction in $k_{on\cdot LacI}$ for a DNA containing a LacO binding site (see **Fig. 3**). This is because LacO is only accessible to LacI for a fraction of the total binding time since the freely diffusing MSH sliding clamps may transiently occlude at least part of the LacO site (**Supporting Information**). Moreover, this model allows the calculation of the on-rate for LacI ($k_{on\cdot LacI}$) at different DNA lengths and numbers of MSH sliding clamps bound to the DNA (**Fig. 4*d***). The ratio of $k_{on\cdot LacI}$ with zero MSH sliding clamps ($k_{on\cdot LacI\text{-}0}$) to that with N number of MSH sliding clamps on the DNA ($k_{on\cdot LacI\text{-}N}$) provides a convenient measure of the reduction in LacI binding (**Fig. 4*d***). For example, this model predicts at least a 2-fold reduction in $k_{on\cdot LacI\text{-}N}$ ($k_{on\cdot LacI\text{-}0}/k_{on\cdot LacI\text{-}N}$ = 2) when two MutS sliding clamps are confined to 160 bp or less, and when three MutS sliding clamps are confined to 235 bp or less. At least a 10-fold reduction in $k_{on\cdot LacI\text{-}N}$ ($k_{on\cdot LacI\text{-}0}/k_{on\cdot LacI\text{-}N}$ = 10) is predicted when two MutS sliding clamps are confined to 100 bp or less, and three MutS sliding clamps are confined to 140 bp or less.

For the specific DNA used in **Fig. 3** we calculated a distribution of predictions that resulted in a mean with upper and lower quartile for $k_{on\cdot LacI\text{-}0}/k_{on\cdot LacI\text{-}N}$ using several hypothetical MSH footprints (σ = 24, 25 or 26 bp), LacI footprints (σ = 21 or 25 bp) and assuming that either the 5'-biotin-NeutrAvidin or dig-antidig linkages increased the DNA length by an additional 2 bp (**Fig. 4*e***). For two MutS sliding clamps this calculated ratio, employing the average of the $k_{on\cdot LacI\text{-}2}$ presented in **Fig. 3*c***, is in excellent agreement with the mean predicted by the Tonks Gas/Sliding Clamp model ($k_{on\cdot LacI\text{-}0}/k_{on\cdot LacI\text{-}2}$ = 9.6 ± 2.0 s.d.; **Fig. 4*e***, red star in Two MutS). For one MutS sliding clamp, we noted a larger effect on the ratio than predicted by the Tonks Gas/Sliding Clamp model ($k_{on\cdot LacI\text{-}0}/k_{on\cdot LacI\text{-}1}$ = 2.6 ± 0.9 s.d.; **Fig. 4*e***, red star in One MutS). The model calculations assume that the DNA is a stiff rod and the MutS proteins are inelastic spheres with defined site size. However, small changes on the effective DNA length that may be occupied by a MutS sliding clamp and/or the site size of either the MutS or the LacI proteins (elasticity or hydration) could easily account for the differences in experimental and predicted effects of one MutS sliding clamp on $k_{on\cdot LacI}$.

DISCUSSION

Our results provide consistent evidence that stable freely diffusing ATP-bound MSH sliding clamps can influence the association of other binding proteins with the DNA. The major effect of the multiple MSH sliding clamps is to reduce the k_{on} of a DNA binding protein. This would effectively increase the K_D of a DNA binding protein ultimately reducing its equilibrium binding as well as its localization to the region occupied by stable sliding clamps. There appeared to be little effect on the k_{off} of LacI when 1 MSH sliding clamp was present, although the number of observations was low as a result of the pM K_D of LacI. A unique effect on k_{on} implies that the exclusion effect introduced by multiple MSH sliding clamps would only affect unbound DNA binding proteins or previously bound DNA

binding proteins immediately following equilibrium dissociation.

These observations support a previous hypothesis that the disassembly of nucleosomes by HsMSH2-HsMSH6 was the result of MSH sliding clamps inhibiting nucleosome DNA re-wrapping kinetics around the histone octamer (16,18). This concept also explains the synergistic effect of HsMSH2-HsMSH6 catalyzed nucleosome disassembly when histone post-translational modifications that reduced nucleosome DNA wrapping stability were present (16,18). Recent studies from our group demonstrated that MSH sliding clamps load MLH/PMS proteins onto the DNA in a cascade of extremely stable ATP-bound sliding clamps (13). It seems possible that these two sliding clamps together may synergistically reduce the localized association of proteins that could inhibit communication and/or excision processes during mismatch repair.

There are a number of other stable sliding clamps that associate with DNA in all organisms. In eukaryotes these include the replicative processivity factor PCNA and the DNA damage response complex RAD9-HUS1-RAD1 (9-1-1 complex) that are ubiquitous in dividing cells and are essential for genome maintenance and stability (1,2). It is likely that biological evolution has selected for these stable sliding clamp complexes based at least in part on the useful physical property that they may freely diffuse over long distances and may ultimately exhibit the properties of a 1D Tonks gas.

EXPERIMENTAL PROCEDURES

Proteins and DNA

Thermus aquaticus TaMutS(C42A,T469C) was expressed, purified, and labeled with Cy3 or Alexa647 as previously described with a labeling efficiency per monomer of 54.5% or 45%, respectively (9,12). *E.coli* EcMutS(D835R,R840E) containing a C-terminal Formylglycine Generating Enzyme (FGE) hexa-amino acid recognition sequence and a hexa-histidine tag was expressed, purified and labeled with Alexa647 by Hydrazinyl-Iso-Pictet-Spengler (HIPS) ligation as previously described (29,30) with a labeling efficiency per monomer of 26%. The human HsMSH2-HsMSH6 heterodimer was purified as previously described (31). Mismatch binding and ATP-dependent sliding clamp formation was determined by Surface Plasmon Resonance (SPR), as previously described (5,32). PAGE gel purified DNA oligonucleotides (**Supplementary Table S1**) were purchased from Midland Reagents (Midland, TX) or IDT (Coralville, IA). Fluorophore labeling of DNA and purification by reverse phase HPLC on a C18 column (Agilent) was performed as previously described (9,12). Complementary oligonucleotides were annealed and purified by HPLC on a Gen-Pack ion exchange column (Waters) as previously described (33).

Surface Plasmon Resonance (SPR) binding kinetic analysis

A 98 bp mismatched DNA containing a 5'-biotin at one end and a 5'-dig at the other end was attached to a streptavidin coated SPR chip (Biacore) as previously described (**Supplementary Table S1**; (32). MSH protein binding at indicated concentrations was performed in SM Buffer (20 mM Tris-HCl pH 7.8, 100 mM NaCl, 5 mM MgCl2, 0.1 mM DTT. 0.1 mM EDTA, 0.5 mM ATP) and minus glucose, GOD/CAT and Trolox, but with 0.005% P20 (GE healthcare, BR100054), 0.2mg/ml Acetylated BSA (Promega, R3961) and 25 nM antidig (Roche) at 10 μl/min and 23°C (EcMutS and HsMSH2-HsMSH6) or 35°C (TaMutS). For studies that examined the effect of MSH sliding clamps on LacI binding, following MSH binding in the presence of ATP, LacI (0.5 nM for TaMutS; 1.5 nM for EcMutS and human HsMSH2-HsMSH6) was injected and binding compared to the absence of LacI. The MSH dissociation curve in the absence of LacI was subtracted from the rate curve in the presence of LacI to obtain

the LacI association curve (**Supplementary Fig. S1**). The LacI association curve was fit to a single component binding exponential to obtain the $k_{on\text{•}LacI}$ at each concentration of initial MSH binding/loading.

Single molecule PIFE

We assembled a prism-type laser excitation total internal reflection fluorescence (TIRF) microscopy system (9,12) containing an Olympus IX-71 with water-type 60X objective (N.A. 1.2), a Photometrics DV2 two channel dual color separation system and a Princeton Instruments ProEM 512 Exelon charge-coupled device recorder. The DNA substrate was identical to the SPR substrate except it was labeled by Cy3 near the LacO binding site that when bound by LacI induced protein-induced fluorescence enhancement (PIFE; **Supplementary Table S1**; (26). Fluorescent images were analyzed using IDL (ITT VIS) and MATLAB (The MathWorks) scripts following 532 nm (PIFE) and 635 nm (MSH fluorophoe photobleaching and counting) DPSS laser excitation (Crystal Laser, 50 mW) at 250-400 ms time resolution. The τ_{off} (period of protein-free DNA) and τ_{on} (period of protein-bound DNA) were determined by measuring fluorescence intensity changes upon LacI binding (**Supplementary Fig. S4*a*)**, where the cumulative distributions at several concentrations of LacI were fit to a single exponential decay (**Supplementary Fig. S5*b***). The $k_{on\text{•}LacI}$ $[(\tau_{off\text{•}LacI})^{-1}]$ and $k_{off\text{•}LacI}$ $[(\tau_{on\text{•}LacI})^{-1}]$ were found to be linear and used to obtain the $K_{D\text{•}LacI}$ (**Supplementary Fig. S4*b* and 4*c***). We note that two measures for τ_{off}, the time between the initial injection to the first LacI binding event or the time between LacI binding dissociation and reassociation, gave nearly identical k_{on} between 0-1 nM LacI (compare **Supplementary Fig. 4*b* with 4*c***). Above 1 nM LacI the τ_{off} was increasingly short relative to our time resolution and fluorophore PIFE intensity fluctuations, thus escalating the measurement error.

The loading of MSH sliding clamps followed by LacI protein binding was carried out in SM Buffer with the Alexa647-TaMutS (250nM, or 315nM) or Alexa647-EcMutS (30 nM, or 40 nM) added to the flow cell for 10 min; followed by a 10x volume wash with SM Buffer to eliminate free MSH protein; followed by LacI (1 nM) injection. The τ_{off} in the presence of MSH sliding clamps was obtained as the time between the initial LacI injection and the first LacI binding event PIFE induced by LacI binding. The number of Alexa647 flurophores was obtained from the number of photobleaching steps as described previously (9,12). The Methods utilized for data analysis may be found in Supplementary Data.

Single Molecule FRET and Photobleaching Analysis

The indicated concentration of Cy3-TaMutS was incubated with indicated Cy5-DNA (**Supplementary Table S1**) for 5 min in SM Buffer to load TaMutS sliding clamps. After washing free MutS with SM Buffer plus oxygen scavaging system [OSS; 0.8% w/v D-glucose, 146 U/ml Glucose Oxydase (GOD), 2170 U/ml Catylase (CAT), 2 mM Trolox], time-averaged Förster resonance energy transfer (FRET) was detected as previously described using a prism-type laser excitation smTIRF microscopy system (9,12) containing an Olympus IX-71 with water-type 60X objective (N.A. 1.2), a Photometrics DV2 two channel dual color separation system and a Princeton Instruments ProEM 512 Exelon charge-coupled device recorder. The number of Cy3 fluorophore photobleaching steps were counted as described previously (9,12) and compared to the number and distribution of fluorophores predicted based on the TaMutS monomer labeling efficiency. Briefly, laser intensity may be increased and the resulting step loss of fluorophore signal(s) until zero fluorescence is an indicator of the number of fluorophores on a single molecule. Fluorescent images and

FRET following 532 nm DPSS LASER excitation were analyzed using IDL (ITT VIS) and MATLAB (The MathWorks) scripts. Data analysis methods may be found in Supporting Information.

SUPPORTING INFORMATION

Supporting Information available on-line

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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FIGURE LEGENDS

Figure 1. SPR analysis of LacI binding in the presence of MSH sliding clamps. A 5'-biotin 98 bp duplex DNA (**Supplementary Table S1**) containing a +dT (TaMutS) or G/T (EcMutS and HsMSH2-HsMSH6) mismatch was anchored to the surface of the streptavidin coated Surface Plasmon Resonance (SPR, Biacore) chip and the remaining end blocked by dig-antidig as previously described (32). Each curve is color-coded and comes in pairs ± LacI injection. Association curves were processed as described in **Supplementary Fig. 1** and **Supporting Information** to extract $k_{on\bullet LacI}$. **a)** TaMutS (0 nM, 20 nM, 50 nM, 100 nM, 200 nM, 500 nM, and 800 nM) ± LacI (0.5 nM). Subtracted LacI association curve in the presence of TaMutS (middle) and calculated $k_{on\bullet LacI}$ plotted against TaMutS concentration (right). **b)** EcMutS (0 nM, 10 nM, 30 nM, 60 nM, 100 nM, and 200 nM) ± LacI (1.5 nM). LacI association curve in the presence of EcMutS (middle) and calculated $k_{on\bullet LacI}$ plotted against EcMutS concentration (right). **c)** HsMSH2-HsMSH6 (0 nM, 10 nM, 30 nM, 60 nM, 100 nM, and 200 nM) ± LacI (1.5 nM). LacI association curve in the presence of HsMSH2- HsMSH6 (left) and calculated $k_{on\bullet LacI}$ plotted against HsMSH2-HsMSH6 concentration (right).

Figure 2. The frequency of LacI binding events is reduced with the number of Cy3-TaMutS fluorophores on a mismatch DNA. a) An illustration of the mismatched DNA substrate. The 95 bp substrate contains a mismatch at 15T+ and a Cy5 fluorophore at the 24th nt from the 5'-biotin bound to the smTIRF flow cell surface with the remaining end blocked by dig-antidig (**Supplementary Table S1**). Binding of Cy3-TaMutS to the mismatch results in a high FRET signal (E ~ 0.8) that in the presence of ATP resolves into a sliding clamp with time-averaged FRET (E ~ 0.3) as previously described (12). The binding of LacI and/or multiple sliding clamps alter the time-averaged FRET efficiency as shown below the middle and right molecules and as described in the text. **b)** Representative trace showing the anti-correlation between Cy3-TaMutS (green) and Cy5-DNA (red) intensities caused by time-averaged FRET (blue) in the absence of a bound LacI (left) and in the presence of a bound LacI (right) as well as a single photobleaching step of Cy3-TaMutS emission indicating the number of fluorophores. Binned histogram insets fit to normal distributions indicate mean FRET efficiency for one-fluorophore +LacI and -LacI with indicated number (N) of events. **c)** The frequency of LacI binding. Total observations are shown above the bar graph from two separate experiments of a 40 μm x 80 μm field of view containing ~60 well-resolved molecules. LacI binding events were distinguished by their characteristic time-averaged FRET value and then binned with the number of photobleached fluorophores (see text).

Figure 3. The number of MSH sliding clamps affects the rate of LacI binding association. The binding kinetics of LacI (1 nM) to LacO was directly measured by smPIFE as previously described (26) and correlated with the number of TaMutS (500 nM) and EcMutS (200 nM) sliding clamps stably bound to the mismatched DNA after washing unbound MutS. **a)** An illustration of the mismatched DNA substrate. The 98 bp DNA containing a 15T+ mismatch was similar to **Fig. 2** except the DNA contained a Cy3 fluorophore located 11 nt from the dig-antidig capable of PIFE upon LacI binding, and the TaMutS and EcMutS were labeled with Alexa647 as described (**Supporting Information**). **b)** Representative trace showing PIFE in the Cy3 channel upon LacI binding (τ_{off} indicated) and the number of Alexa647 fluorophore photobleaching events associated with the same DNA for one-fluorophore (top) and two- fluorophores (bottom). **c)** The $k_{on\bullet LacI}$ for LacI only, for LacI in the presence of 1 or 2 TaMutS sliding clamps, and for LacI in the presence of 1 or 2 EcMutS sliding clamps (± s.d.). The $k_{on\bullet LacI}$ was determined from the $\tau_{off\bullet LacI}$ data shown in **Supplementary Table S3** and color-coded similarly as follows: red hash, least squares calculation using one fluorophore data; blue hash, least squares calculation using two fluorophore data; green hash, maximum likelihood calculation using one fluorophore data; purple hash, maximum likelihood calculation using two fluorophore data (**Supporting Information**).

Figure 4. Concentration dependence of loaded TaMutS sliding clamps compared to theoretical Tonks Gas/Sliding Clamp model predictions. a) An illustration of the substrates utilized to count TaMutS sliding clamps on the DNA. A 95 bp duplex DNA was anchored to the smTIRF flow cell surface by a biotin-NeutrAvidin at one end while the other end was blocked by a dig-antidig complex. Cy5 was linked to the 7th base from the dig-antidig and the mismatch was located at the 15th bp position from the 5'-biotin end. **b)** Representative traces showing the anti-correlation between Cy3-TaMutS and Cy5-DNA intensities caused by time-averaged FRET as well as photobleaching steps of Cy3-TaMutS emission. **c)** Comparison between the fraction of TaMutS predicted by the Tonks Gas/Sliding Clamp model (solid line) and experimental number of TaMutS (circles ± s.e.) determined at three different TaMutS concentrations (10 nM,50 nM, and 300 nM). **d)** The reduction in the ratio of LacI on-rate with zero MutS sliding clamps over the LacI on-rate with N number of MutS sliding clamps ($k_{on\bullet LacI\text{-}0}/k_{on\bullet LacI\text{-}N}$) with different DNA lengths. **e)** The mean (line) and upper and lower quartile (boxes) for $k_{on\bullet LacI\text{-}0}/k_{on\bullet LacI\text{-}N}$ was determined for a 98 bp mismatched DNA (**Fig. 3)** using individual assumptions for the MSH footprint (σ = 24, 25 or 26 bp), LacI footprints (σ = 21 or 25 bp) or the possibility that either the 5'-biotin-NeutrAvidin or dig-antidig linkages increased the DNA length by an additional 2 bp. The red stars indicate the experimentally observed ratio from **Fig. 3*b***.