

Physical and functional interactions between *Escherichia coli* MutL and the Vsr repair endonuclease

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ABSTRACT

DNA mismatch repair (MMR) and very-short patch (VSP) repair are two pathways involved in the repair of T:G mismatches. To learn about competition and cooperation between these two repair pathways, we analyzed the physical and functional interaction between MutL and Vsr using biophysical and biochemical methods. Analytical ultracentrifugation reveals a nucleotide-dependent interaction between Vsr and the N-terminal domain of MutL. Using chemical crosslinking, we mapped the interaction site of MutL for Vsr to a region between the N-terminal domains similar to that described before for the interaction between MutL and the strand discrimination endonuclease MutH of the MMR system. Competition between MutH and Vsr for binding to MutL resulted in inhibition of the mismatch-provoked MutS- and MutL-dependent activation of MutH, which explains the mutagenic effect of Vsr overexpression. Cooperation between MMR and VSP repair was demonstrated by the stimulation of the Vsr endonuclease in a MutS-, MutL- and ATP-hydrolysis-dependent manner, in agreement with the enhancement of VSP repair by MutS and MutL *in vivo*. These data suggest a mobile MutS–MutL complex in MMR signalling, that leaves the DNA mismatch prior to, or at the time of, activation of downstream effector molecules such as Vsr or MutH.

INTRODUCTION

Genome stability requires continual repair of DNA damage and mismatches prior to replication. Most

mismatches that result from DNA polymerase errors during replication are the target of the post-replicative DNA mismatch repair (MMR) system. Base damage can also generate mismatches, e.g. oxidation of G results in 8-oxoG:A mismatches after replication, which are recognized by either MMR or MutY/OGG2 (1,2), or methylation of G which results in O⁶-methylguanine:C mismatches that are the target of MMR or 6-O-methylguanine-DNA methyltransferases (MGMT) (3). In addition to replication errors, T:G mismatches can arise via deamination of 5-methylcytosine which is used by many organisms as a chemical tag that allows the distinguishing of foreign DNA from organism or viruses lacking this modification (4). T:G mismatches can also be repaired by specialized enzymes such as the ubiquitous T:G DNA glycosylases or the very-short patch (VSP) repair system found in many bacteria (5,6). In *E. coli* post-replicative MMR is initiated by MutS recognizing the mismatch. MutS recruits MutL to form a ternary complex that coordinates subsequent repair steps. This complex activates the latent endonuclease MutH, which nicks the erroneous DNA daughter strand at hemimethylated GATC-sites that can up to 1000 bp from the mismatch (7). The nicked strand is removed in a MutSL-dependent manner by action of UvrD helicase, single-strand-binding protein (SSB) and one of several exonucleases. Repair is completed by the DNA polymerase III holoenzyme and DNA ligase I (7). T:G mismatches are targeted by the VSP repair system when they occur within 5'-CTWGG/CCWGG (W is A or T) sequences, which arise from deamination of 5'-C^{5me}CWGG/CCWGG, methyl group addition resulting from the action of the *E. coli* DNA cytosine methyltransferase (Dcm) (3,8). Thus while the principal biological function of VSP repair is to prevent ^{5me}C to T mutations, its overall effect on the bacterial genomes is to maintain Dcm sites.

Several experimental observations have led to the conclusion that VSP repair has evolved a well-nuanced

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relationship with the general MMR proteins. Strains without a *vsr* gene are completely deficient in VSP repair, and hence, have a high frequency of C→T mutations at 5-methylcytosines (9,10). VSP repair is reduced (11), but not eliminated, in cells which are unable to produce MutS or MutL (12–14). Overexpression of Vsr leads to a mutator phenotype in *E. coli* which can be attenuated by simultaneous overexpression of MutL or MutH but not MutS (15,16). The two repair pathways seem to be separated in time, MMR being active mainly in the exponential phase whereas VSP repair operates in the stationary phase (17). Levels of MutS and MutH are down regulated as *E. coli* cells enter stationary phase whereas the amount of MutL does not alter (18,19). Finally, a physical and functional interaction and between MutL and Vsr has been demonstrated using bacterial- and yeast-two hybrid analysis (20), and the observed stimulation of Vsr DNA binding and DNA cleavage by MutL (21,22). A mutant Vsr protein lacking the N-terminal 14 amino acids (Vsr-Δ14) has diminished endonuclease and VSP repair activity, but interacts with MutL as strongly as the wild type in a bacterial two-hybrid assay (20). However, the endonuclease activation of Vsr-Δ14 by MutL was eliminated (21). Recently, based on *in vivo* data, it has been suggested that MMR MutS and MutL collaborate with Vsr endonuclease in the repair of O⁶-methylguanine by methyltransferases, i.e. Ada and Ogt (23). However, little biochemical data is available that directly demonstrates competition or synergism between Vsr and the MMR-protein MutS *in vitro*.

Here we present a detailed biochemical and biophysical analysis of the physical and functional interaction between MutL and Vsr. For the first time we have investigated the functional interaction between MutS, MutL and Vsr in an *in vitro* assay using a long circular DNA substrate containing a T:G mismatch in the sequence context of the Vsr recognition sequence (see above). Our data suggest that the activation of MutH and Vsr by MutL follow a similar mechanism involving a mobile MutS–MutL complex.

MATERIALS AND METHODS

Strains, plasmids, enzymes and reagents

E. coli K12 strains CC106 (P90C [*ara*Δ[*lac-proXIII*] [*F'*lacIZ *proAB*⁺]]) (23), TX2929 (CC106 *mutS201::Tn5;Km^r*) and the pET-15b (Novagen) derived plasmids pTX412 and pTX418 containing the *mutS* and *mutL* genes, respectively, under control of the T7 promoter were kindly provided by Dr M. Winkler (18). Expression plasmid for MutL-E29A was a kind gift of Dr. W. Yang (24). Single-cysteine variants of MutL have been described before and are named according to the position of the unique cysteine residue, e.g. MutL-314 is a variant with cysteine at position 314 introduced into the cysteine-free background (25). Plasmid pMQ402 (His₆-MutH), a pBAD18 derivative, was a kind gift of Dr M. Marinus (26). Plasmids coding for Vsr (pRSETB-Vsr), His-Vsr (pDV111) or His-VsrΔ14 (pDV114) have been described before (21,27,28). Assays for complementation of the mutator phenotype were carried out as reported

elsewhere (29). MutS, MutL and Vsr proteins were expressed using *E. coli* strain HMS174 (λDE3) (Novagen); for MutH *E. coli* strain XL1 blue MRF' (Stratagene) was used. Protein purification and analytical size-exclusion chromatography was performed as described previously (29). Untagged Vsr protein was expressed using pRSETB-Vsr and purified as described before (30).

Analytical ultracentrifugation sample preparation

Wild-type Vsr was stored in 25 mM HEPES (pH 8.0) 100 mM NaCl, 0.5 mM DTT (buffer A), whereas histidine tagged MutL–NTD was stored in 10 mM HEPES (pH 8.0) 500 mM KCl, 1 mM EDTA, 1 mM DTT. Since sedimentation velocity experiments can be affected by the presence of high concentrations of KCl, 500 mM KCl was replaced with 300 mM KCl, via extensive dialysis. The concentrations of purified proteins were determined by absorbance spectroscopy at 280 nm using calculated extinction coefficients of 32 400 and 26 030 M⁻¹ cm⁻¹, respectively. Dimerisation of MutL–NTD and formation of the MutL–NTD–Vsr complex were promoted by addition of MgCl₂/AMPPNP to a final concentration of 5 mM to the sample prior to analytical ultracentrifugation (AUC) experiment.

AUC and the data treatment

Sedimentation velocity experiments were carried out in a Beckman Coulter (Palo Alto, CA, USA) ProteomeLab XL-I analytical ultracentrifuge using both absorbance at 280 nm and interference optics. All runs were carried out at the rotation speed of 48 000 r.p.m. and experimental temperature of 4°C. The sample volume was 400 μl, and the sample concentrations ranged between 0.2 and 1.9 mg/ml. The weight average sedimentation coefficient was calculated by integrating the differential sedimentation coefficient distribution (31) and value of molecular mass from sedimentation velocity data was calculated as described before (32). Details for the quantitative analysis of the sedimentation velocity data are given in Supplementary Data.

Circular heteroduplex DNA substrates

Circular DNA substrates (5708 bp) containing a single T:G mismatch at position 169 and a hemimethylated GATC site at position 356 bp were generated using a derivative of pET-15b and a procedure similar to that described before (33). Plasmid pET-15b-XhoI (34) was used to generate the plasmid pET-15b-Vsr by PCR mutagenesis (35). Four new sites for the nicking endonuclease Nt.Bpu10I (underlined) were introduced using the oligodeoxynucleotides MP-Bpu10I-I (5'-CGT CAT CCT CGG CTC AGG CAC CCT GGG TGC TGA GGG CAT AGG CTT-3') and MP-Bpu10I-II (5'-GCC GCG CCT GAG CCA TAT GCT CGA GGA TCC CTC AGC TAA CAA AGC-3'). Substrates containing a T:G mismatch in the sequence context 5'-CTTGG-3'/3'-GGAC^{mec}C-5' were generated using a procedure similar to that described before (33). Briefly, the Dcm and Dam methylated plasmid (350 ng/μl; 100 nM) was nicked by Nt.Bpu10I (CC[^]TNAGC; 0.05 U/μl; 0.14 U/μg) for 16 hr

at 37°C, followed by denaturation and re-annealing in the presence of the 5'-phosphorylated oligodeoxynucleotides Nt-G:T (5'-TCA GCA CCT AGG GTG CC-3') and Nt-GATC (5'-TGA GCC ATA TGC TCG AGG ATC CC-3') in 50-fold molar excess. After ligation with T4 DNA ligase (0.1 U/μl) for 8 h at 25°C, the reaction mixture was treated with (0.04 U/μl) exonuclease I (Fermentas) and (0.05 U/μl) exonuclease III (New England Biolabs) at 37°C for 16 h to remove any nicked and linear DNA fragments. Optionally, the DNA was methylated at the hemimethylated GATC-site by *dam* methyltransferase (New England Biolabs). Finally, the DNA was precipitated with one volume of isopropanol and 1/10 volume of 3 M sodium acetate. The resulting covalently-closed circular DNA, containing a single hemimethylated Dcm site with a T:G mismatch and an additional hemimethylated GATC site, was resuspended in 50 μl 10 mM Tris-HCl, pH 7.9.

Thrombin-cleavage of MutL

Full-length MutL was cleaved with 20 units of thrombin (Sigma) per mg of MutL for 30 min at 37°C giving two defined protein fragments, MutL-NTD (comprising residues 1–375) and MutL-CTD (comprising residues 376–615). Once cleavage of MutL was complete, as judged by SDS-PAGE, MutL-NTD was separated from MutL-CTD by gel filtration using a L-6200A Merck-Hitachi HPLC system with a Superdex 75TM column equilibrated with 10 mM HEPES/KOH pH7.9, 200 mM KCl, 1 mM EDTA and 1 mM DTT. MutL-NTD and MutL-CTD aliquots were snap-frozen in liquid nitrogen and stored at -70°C.

Site-directed mutagenesis of Vsr

The gene encoding the C139S Vsr variant was generated using the oligodeoxynucleotide 5'-CGA TCT GCG CGC TGG CCC CTT CGC CGC TGA TCC-3' in the mutagenesis protocol described before (25) with pDV111 as the template (27). The whole *vsr* gene was sequenced.

Mismatch-provoked activation of MutH

Ten nanomolars of heteroduplex DNA substrate (484 bp) containing a T:G mismatch at position 385 and a single unmethylated GATC site at position 210 (34) of DNA was incubated with 200 nM MutH, 1 μM MutL, 400 nM (monomer equivalents) MutS and the indicated concentration of Vsr in 10 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 1 mM ATP, 50 μg/ml BSA and 125 mM KCl at 37°C. MutH endonuclease activity was scored by the appearance of cleaved products (analyzed by 6% polyacrylamide gel electrophoresis). Alternatively, a circular heteroduplex DNA containing a single hemimethylated GATC sites and a single T:G mismatch in the sequence context CC^{5me}AGG/CTTGG was used.

Photocrosslinking

Single-cysteine MutL variants (10 μM) were pre-incubated in the presence or absence of 1 mM ATP, ADP or AMPPNP for 30 min on ice in 10 mM Tris-HCl pH 7.5,

10 mM MgCl₂ and 125 mM KCl (crosslinking buffer). Either single-cysteine MutL variants or Vsr were modified with 100 M excess of benzophenone-4-maleimide (BPM) for 30 min at room temperature. The modification reaction was stopped with 5 mM DTT. Modified proteins were mixed with the respective interaction partners in a 1:1 molar ratio (e.g. 2.5 μM Vsr/5 μM MutL monomers) in 25 μl and irradiated for 30 min on ice at 366 nm with a handheld UV lamp (Bachofer, Reutlinger) at a distance of 5 cm. After addition of 6.25 μl of 160 mM Tris-HCl pH 6.8, 5% (v/v), 2% (w/v) SDS, 40% (v/v) glycerol (SDS-loading buffer) the reaction mixture was subjected to SDS-PAGE.

Thiol-thiol crosslinking

Vsr and single-cysteine MutL variants were pre-incubated on ice at 2.5 μM each for 30 min in the presence or absence of ATP or AMPPNP in crosslinking buffer. Methanethiosulfonate MTS (methanethiosulfonate) crosslinkers of varying spacer arm length were purchased from Toronto Research Chemicals. Stock solutions of the crosslinkers were made in DMSO and stored at -20°C. Crosslinkers were diluted in DMSO and added to the proteins resulting in an indicated molar excess of crosslinker:protein and a final concentration of 10% DMSO. After 5 min, reactions were quenched with 1 mM N-ethylmaleimide prior to addition of SDS-loading buffer. Samples were analyzed as described above for the photocrosslinking procedure.

A scheme of all the crosslinking experiments is shown in Supplementary Data.

RESULTS

AUC of the MutL-NTD and Vsr

The molecular matchmaker MutL coordinates MMR and crosstalk with other repair processes by interacting with a variety of proteins. For the interaction between MutL and Vsr, the N-terminal domain (NTD) of MutL had been demonstrated to be sufficient *in vivo* (20). However, it is not clear whether this interaction requires nucleotide binding by MutL, as observed for the interaction between MutL and MutH (24). To answer this question the interactions between MutL and Vsr were assessed in an AUC sedimentation velocity experiment. Vsr distributed in the AUC cell mainly as a species with sedimentation coefficients of 1.9 S (Figure 1A) which is in good agreement with the sedimentation coefficient of 1.93 S calculated from the crystal structure for a Vsr monomer with HYDROPRO (36); a second minor species of 2.9 S was observed, however, the identity of this species could not be assigned (see Supplementary Data for additional information). The addition of AMPPNP, a non-hydrolysable analogue of ATP to Vsr had minimal effect on the distribution of the species: Vsr remained basically a monomer (Figure 1B) although the distribution became broader. MutL-NTD was a single species with the peak centred around 3.1 S (Figure 1C) indicative of a monomeric form. The addition of 5 mM AMPPNP resulted in the formation of second species with sedimentation coefficient

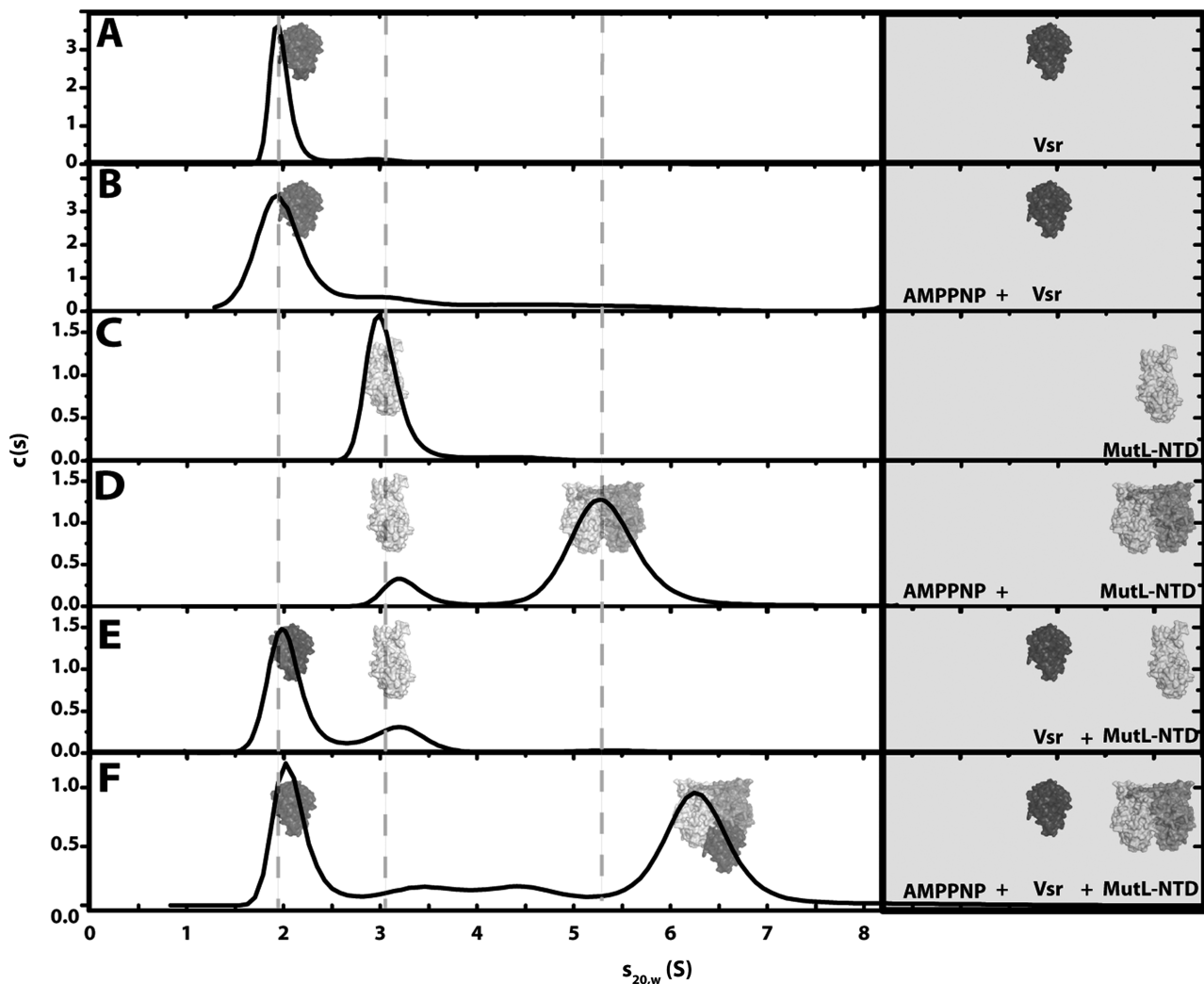


Figure 1. Nucleotide-dependent interaction between Vsr and the N-terminal domain of MutL. General size distributions (expressed in $s_{20,w}$) of Vsr, MutL-NTD and complexes of Vsr and MutL-NTD (2:1 molar ratio) as revealed by AUC. (A) Vsr, (B) Vsr with AMPPNP, (C) MutL-NTD, (D), MutL-NTD with AMPPNP, (E) Vsr and MutL-NTD, and (F) Vsr and MutL-NTD with AMPPNP. See ‘Materials and Methods’ section for details.

of 5.2S indicative of the formation of the MutL-NTD dimer (Figure 1D). In the absence of nucleotide, Vsr and MutL-NTD did not form any significant amount of complex (Figure 1E). However, in the presence of AMPPNP an additional species with sedimentation coefficient of about 6.1 S was observed concomitant with the disappearance of the MutL-NTD dimer. This species could be interpreted as complex of a MutL-dimer with one or two Vsr molecules (Figure 1F). In summary, our sedimentation velocity analysis demonstrated that the interaction between MutL-NTD and Vsr is nucleotide dependent, as described previously for the MutL-NTD MutH interaction (24,25).

Photocrosslinking MutL to Vsr

In order to gain insights into the structural arrangement of the MutL-Vsr complex, we employed a strategy used previously for analyzing the MutL-MutH complex (25). To this end, we modified a series of single-cysteine MutL variants (Figure 2) with benzophenone-4-maleimide

(BPM), (Figure 3A) and subsequently tested the benzophenone-modified proteins for photocrosslinking to Vsr (Figure 3B). Only MutL-314 was able to form a photocrosslink with Vsr in an ATP-dependent manner (Figure 3B); this was verified by in-gel tryptic digestion and mass spectrometry analysis (data not shown). Interestingly, MutL-314 had been shown before to form a photocrosslink to MutH suggesting that the binding sites of MutL for MutH and Vsr are overlapping or in close proximity (25). Next we tested, whether MutL-NTD is also sufficient to allow the formation of the photocrosslink with Vsr. To this end, MutL-314 was cleaved with thrombin which removes the N-terminal His-tag and cleaves the protein between Pro-364 and Arg-375 resulting in an N-terminal (MutL-NTD-314) and a C-terminal (MutL-CTD) fragment (37). After gel filtration purification MutL-NTD-314 was modified with BPM followed by photocrosslinking to Vsr in the absence or presence of ATP or AMPPNP essentially as described for full-length MutL (Figure 3C). In the absence of

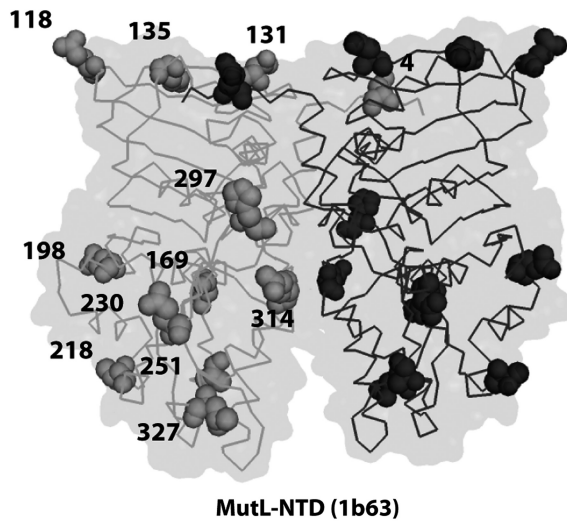


Figure 2. Location of cysteine residues in MutL. Location of cysteine residues in single-cysteine MutL variants mapped to the dimeric structures of the MutL-NTD (25) (pdb code 1b63). All molecular graphics were produced with PyMOL (www.pymol.org).

nucleotide, no photocrosslink formation was observed (Figure 3C, lane 1). However, MutL-NTD-314 incubated with either ATP or AMPPNP was able to form a photocrosslink product with Vsr (Figure 3C, lanes 2 and 3). In the absence of Vsr, no photocrosslink product was observed regardless whether MutL-NTD-314 was incubated in the absence or presence of nucleotide (data not shown). These results are in agreement with the AUC data (Figure 1), indicating that the C-terminal domain of MutL is not required for the physical interaction with Vsr.

Photocrosslinking Vsr to MutL

Vsr contains five cysteine residues, of which three are involved in zinc binding (Cys-66, 73 and 117), one is buried (Cys-59) and only one is surface exposed (Cys-139) (Figure 4A). Chemical modification experiments using PEG-5000 maleimide indicated that one to two cysteine residues can be modified (Figure 4B). Notably, the variant Vsr-C139S, in which the surface exposed cysteine residue was exchanged to serine, displayed a significantly reduced but still observable labelling. This variant retained endonuclease activity and its ability to inhibit the MutL mediated mismatch-provoked activation of MutH, similar to wild type Vsr (data not shown), indicating that the mutation has not impaired the interaction with either DNA or MutL. In order to test whether any cysteine residue of Vsr is in close proximity to MutL, we attempted to crosslink benzophenone-modified Vsr and Vsr-C139S to full-length MutL and MutL-NTD. In contrast to the photocrosslinking, the benzophenone-modified MutL-NTD to Vsr, benzophenone-modified Vsr/Vsr-C139S did not form a photocrosslink with MutL-NTD (Figure 4C, lane 2). Crosslinked complexes to full-length MutL were observed with Vsr but not with Vsr-C139S (Figure 4C, lanes 4 and 8) suggesting that Cys-139 is involved in the photocrosslinking reaction to MutL.

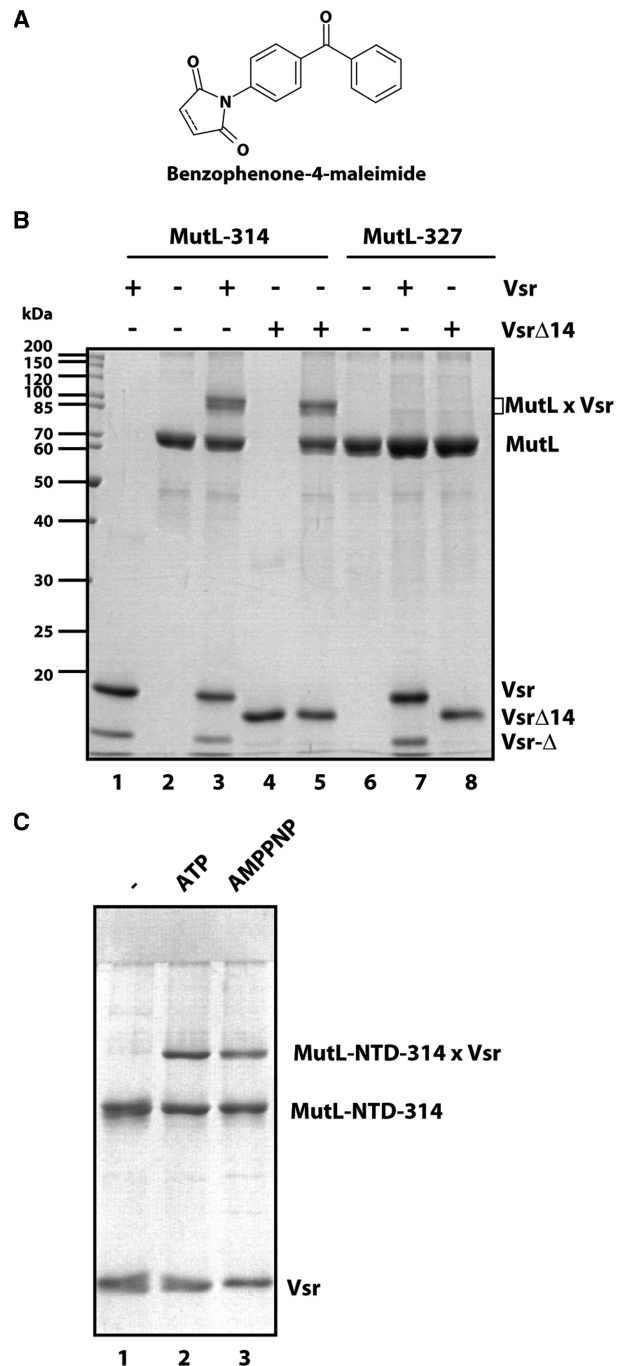


Figure 3. Photocrosslinking MutL-314 to Vsr. (A) Structure of the benzophenone-4-maleimide (BPM). (B) Single-cysteine MutL variants (2.5 μM) modified with benzophenone at cysteine 314 or 327, respectively, were photocrosslinked in the presence of ATP to Vsr or VsrΔ14 (2.5 μM) by irradiation at 366 nm for 30 min. Reaction mixtures were analyzed by SDS-PAGE. The identity of the crosslinked species (L-Vsr) was verified by in-gel tryptic digest and mass spectrometry analysis (data not shown). Photocrosslinks were observed only with MutL-314. MutL-327 is shown as an example for a MutL-variant, not forming a photocrosslink with Vsr. (C) Nucleotide dependence of photocrosslinking MutL-NTD-314 to Vsr. MutL-NTD-314 was incubated in the absence or presence of the indicated nucleotide for 30 min prior photocrosslinking to Vsr essentially as described above.

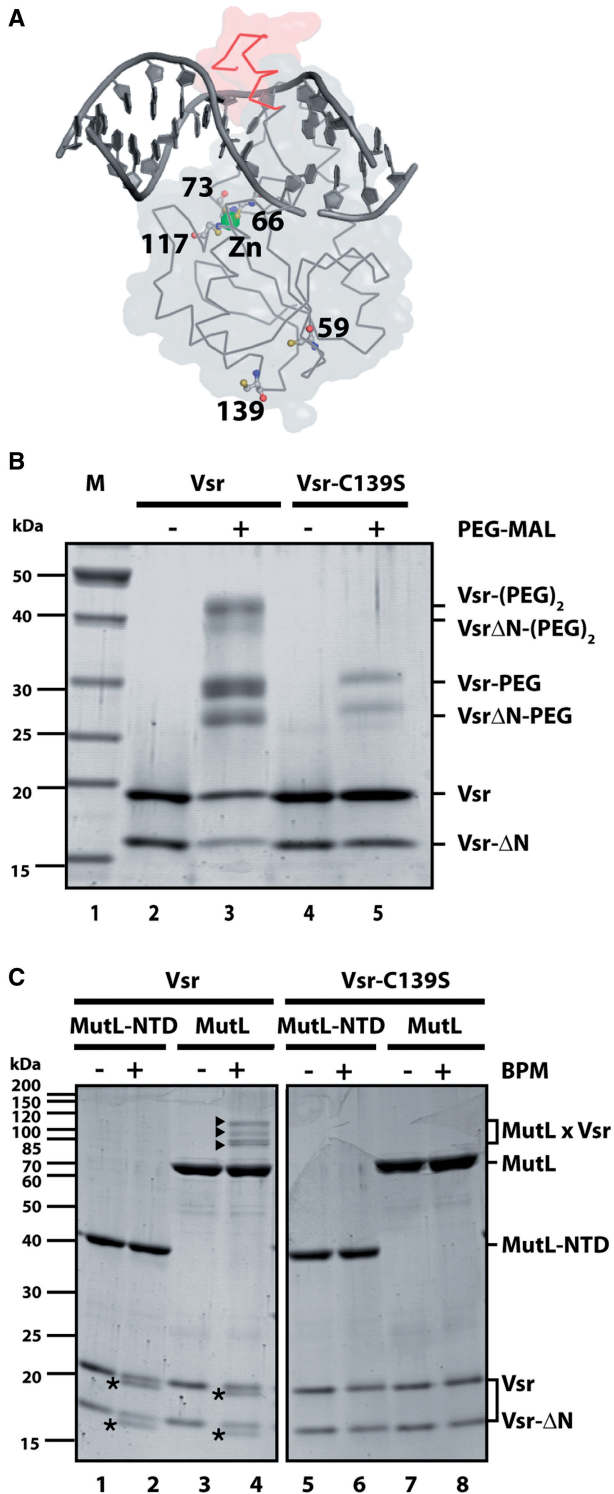


Figure 4. Photocrosslinking of Vsr to MutL. (A) Structure of Vsr in complex with DNA-product (pdb code 1cw0) (52) showing the location of cysteine residues and the structural Zn-ion. The N-terminal residues missing in Vsr-Δ14 are highlighted in red. (B) Vsr and Vsr C139S were modified with a 25-molar excess of PEG-MAL and analyzed by SDS-PAGE (see ‘Materials and Methods’ section). (C) Vsr and Vsr C139S were modified with benzophenone-4-maleimide (BPM) and analyzed for photocrosslinking to MutL-NTD or full-length MutL in the AMPPNP-bound form essentially as described in Figure 3. Note the intermolecular crosslink within Vsr (labelled with asterisks) and the crosslink between Vsr and MutL (triangles).

Following photocrosslinking of benzophenone-modified Vsr to a full-length MutL variant, labelled with a fluorophore at residue 297 in the NTD, limited proteolysis demonstrated that the photocrosslink is formed to residues of NTD (Supplementary Data). The absence of the photocrosslink with MutL-NTD may be due to subtle structural changes in the orientation of Vsr bound to MutL in the absence of the MutL-CTD thereby preventing the photocrosslink formation. Taken together our data support the idea that in complex with MutL the surface exposed Cys-139 of Vsr is in proximity to residues of the NTD of MutL.

Chemical crosslinking MutL and Vsr

In order to get additional information about the orientation of Vsr and MutL, we applied the cysteine-cysteine crosslinking approach. We tested Vsr for crosslinking to selected single-cysteine variants of MutL in the presence of AMPPNP using the long-range homobifunctional crosslinker MTS-11-MTS (Figure 5A). Variant MutL-327 formed a crosslink with both Vsr and the truncated form (Vsr-ΔN), indicating that the loss of N-terminal amino acids did not influence the interaction between MutL and Vsr (Figure 5A, lane 3). A quantitative analysis of the crosslinking yields revealed that MutL-327 formed significantly more crosslinked products to Vsr than any other MutL variant tested (Figure 5B). Similar to the physical interaction analysis and the photocrosslinking experiments, chemical crosslinking was dependent on the presence of nucleotide (ATP, AMPPNP or ADP) and was not observed in the absence of nucleotide (data not shown). These results suggest that one or more cysteine residues clustering in two regions of Vsr, are in close proximity to residue 327 of MutL. Since Cys-139 was important for the formation of a photocrosslinked product from benzophenone-modified Vsr to MutL, we asked whether this residue is also important for chemical crosslinking. In contrast to the photocrosslinking reaction, chemical crosslinking was still possible between MutL-327 and Vsr-C139S indicating that Cys-139 is not essential for formation of the chemical crosslink (Figure 5A, lane 5). We concluded that one of the exposed residues from the zinc-binding site is involved in the chemical crosslink reaction. This assumption is supported by the fact that similar results were obtained with the double mutant Vsr-C59S/C139S and that chemical crosslinking between MutL-327 and either Vsr or Vsr-C139S was strongly impaired upon addition of 100 μM ZnCl₂ (data not shown).

Vsr inhibits mismatch-provoked activation of MutH by MutS and MutL

Overexpression of plasmid-borne Vsr in *E. coli* has been shown to be mutagenic (38), an effect attenuated by co-overexpression of MutL or MutH but not MutS (15). We asked whether the physical interaction between Vsr and MutL is sufficient to explain the inhibitory effect on MMR *in vivo*. Here, we tested the influence of Vsr on the initial steps in DNA MMR, i.e. the mismatch-provoked activation of the MutH endonuclease by MutS and MutL. To this end, we tested the cleavage of a linear DNA

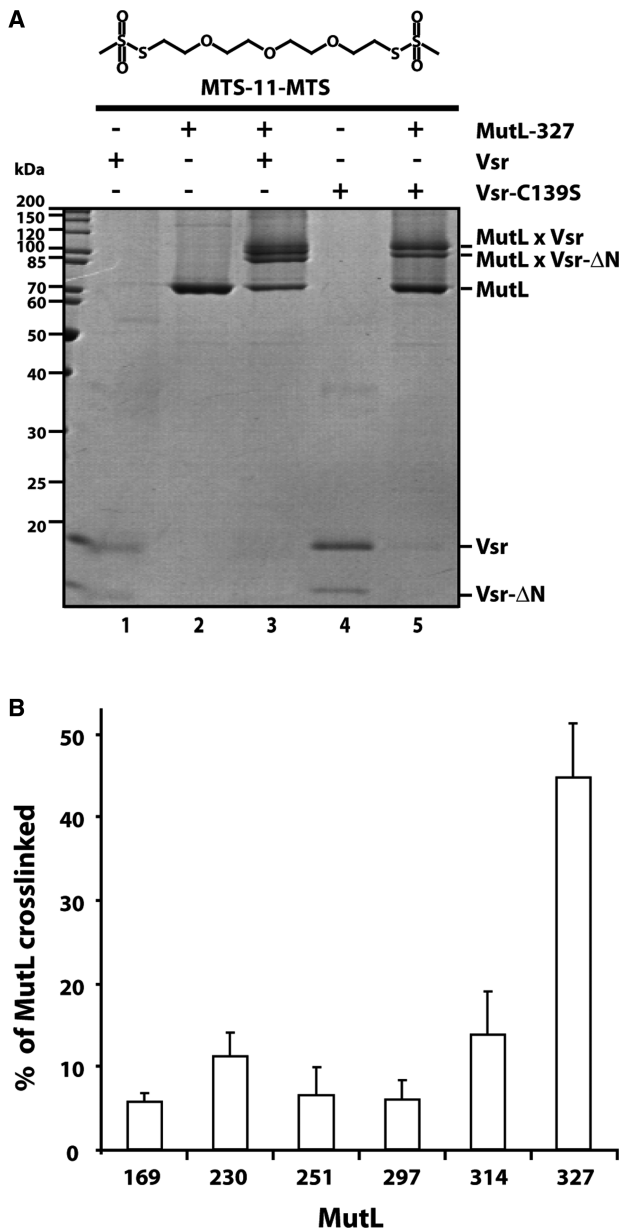


Figure 5. Thiol-thiol crosslinking: (A) The homobifunctional crosslinker MTS-11-MTS was used to crosslink MutL-327 in its AMPPNP form to Vsr or Vsr-C139S, respectively. (B) Quantitative analysis of crosslinking the indicated single-cysteine MutL variants and Vsr with MTS-11-MTS in the presence of AMPPNP. Mean crosslink yields \pm SE are based on three independent experiments.

substrate containing a single unmethylated GATC site and a T:G mismatch in a sequence context not related to the Vsr-recognition sequence (Figure 6A). Upon incubation with MutH (200 nM), MutL (1 μ M) and MutS (400 nM), this DNA substrate is nicked twice in both unmethylated DNA strands resulting in two linear products (Figure 6B). Under the conditions used, the DNA cleavage rate by MutH was >20-fold lower in the absence of MutS, MutL, or the T:G mismatch (data not shown). Addition of about 200 nM levels of Vsr inhibited the reaction by 50% and Vsr in excess over MutL almost completely abolished DNA cleavage (Figure 6B). Similar results were

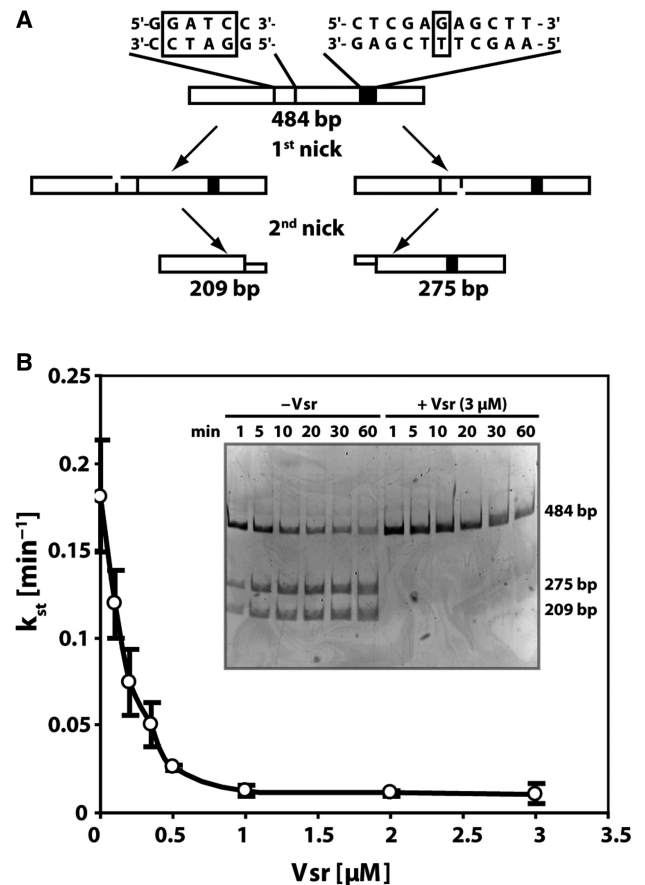


Figure 6. Inhibition of mismatch-provoked MutS/MutL-dependent activation of MutH by Vsr: (A) Scheme for double nicking of a T:G mismatch DNA containing a single unmethylated GATC-site (484 bp) by MutH resulting in two cleavage products (209 and 275 bp). (B) T:G mismatch DNA (484 bp, 10 nM) containing a single unmethylated GATC-site was incubated with 200 nM MutH, 400 nM MutS and 1 μ M MutL in the presence of the indicated concentration of Vsr. Apparent first-order rate constants (k_{st}) for the appearance of the cleavage products are plotted against concentration of Vsr.

obtained using a circular heteroduplex DNA containing a single T:G mismatch and a hemimethylated GATC-site (data not shown). Next we tested the accessibility of the GATC site in the presence of MutS, MutL and Vsr by monitoring the DNA cleavage at the GGATCC site by BamHI. No inhibitory effect on BamHI cleavage by Vsr was observed, ruling out blocking of the GATC-site by non-specific DNA binding of Vsr (data not shown). Using a different circular heteroduplex DNA substrate with a BamHI-site only 4 bp away from the T:G mismatch binding of MutS is blocking the action of BamHI whereas Vsr is not, indicating that Vsr is not strongly binding to this mismatch. However, Vsr is inhibiting the mismatch-provoked activation of MutH on this substrate similar as for the linear heteroduplex DNA shown in Figure 6 (Supplementary Data). Finally, we tested the inhibitory action of Vsr on the MutS and mismatch-independent activation of MutH by MutL that is observed only at low ionic strength (25,37,39). Under the conditions used, the rate of DNA cleavage by MutH (500 nM) is 0.24 min^{-1} . Upon addition of 2 μ M Vsr this rate decreased

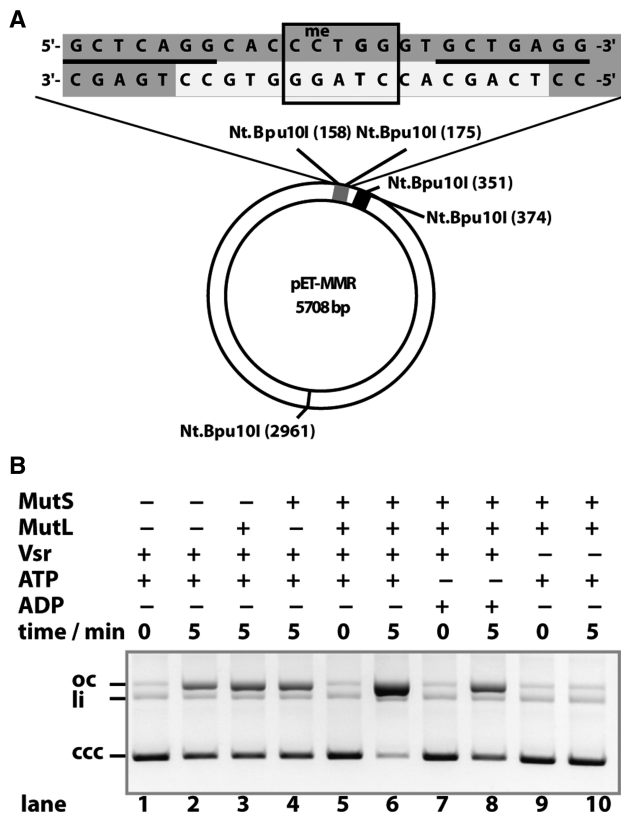


Figure 7. Stimulation of the Vsr endonuclease activity requires MutS, MutL and ATP-hydrolysis: (A) A covalently closed circular (ccc) DNA containing a T:G mismatch (shown in bold) was generated as described in Materials and Methods. The recognition sequence of Vsr is boxed. (B) DNA nicking was monitored after incubation 15 nM DNA for 5 min at 37°C in the absence or presence of the indicated compounds: Vsr (75 nM), MutS (400 nM), MutL (400 nM) and ATP or ADP (1 mM). ccc, covalently closed circle; oc, open circle; li, linear.

almost 10-fold to 0.03 min^{-1} (data not shown). In conclusion, a competition between Vsr and MutS for mismatch binding is not necessary for the interference by Vsr of MutH mediated DNA cleavage, which is a consequence of competition between Vsr and MutH for binding to MutL.

Vsr endonuclease is stimulated by MutS and MutL

The efficiency of Vsr repair *in vivo* is influenced by the presence of both MutL and MutS (40). The present and previous studies have provided evidence for a physical and functional interaction between Vsr and MutL, but little *in vitro* data is available for the role of MutS. Therefore, we analyzed the role of MutS and ATP hydrolysis on the activity of Vsr using a covalently closed circular DNA substrate containing a single T:G mismatch in the recognition sequence of Vsr (Figure 7A). Our analysis demonstrates that the endonuclease activity of Vsr is greatly stimulated in the presence of MutS, MutL and ATP (Figure 7B). Under the experimental conditions containing near physiological salt concentrations (125 mM KCl) neither MutL nor MutS alone were able to stimulate the Vsr endonuclease (Figure 7B, lanes 3 and 4). Moreover, the stimulation was dependent on the presence of ATP

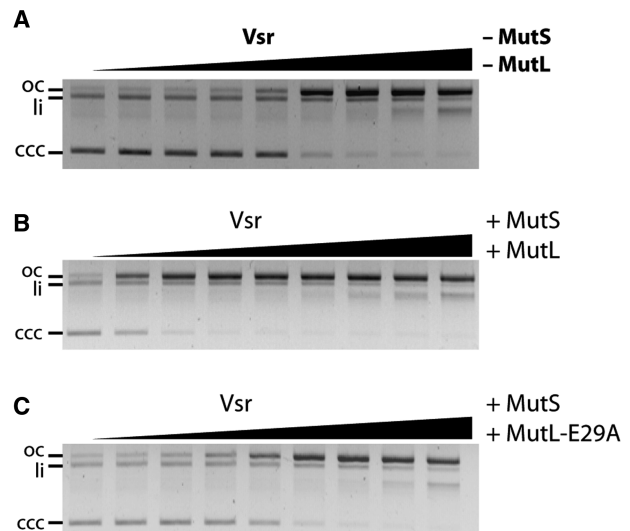


Figure 8. Stimulation of the Vsr endonuclease activity requires ATP hydrolysis by MutL: Nicking of 15 nM covalently closed circular DNA containing a T:G mismatch within the Vsr recognition sequence (see Figure 7) was monitored after incubation for 5 min at 37°C in the presence of increasing concentrations of Vsr (0, 1, 3, 9, 27, 81, 243, 729, 2187 nM), 1 mM ATP in the (A) absence or presence of MutS (100 nM) and (B) MutL (200 nM) or (C) MutL-E29A (200 nM).

which cannot be substituted by ADP (compare lanes 6 and 8). In the absence of Vsr, no cleavage was observed with MutS, MutL and ATP (lane 10). Furthermore, DNA substrates without a mismatch or a mismatch in a different sequence context were not cleaved by Vsr indicating the observed DNA nicking was specific for Vsr (data not shown). AUC and crosslinking revealed that the interaction of Vsr and MutL required nucleotide binding but not hydrolysis. Therefore, we asked whether the MutS-dependent stimulation of Vsr requires the ATPase activity of MutL. To this end the ATP-binding proficient but ATPase impaired variant MutL-E29A was tested for its ability to stimulate the Vsr endonuclease (Figure 8) (24). In contrast to the results obtained with wild-type MutL little or no stimulation of DNA nicking by Vsr was observed with this variant suggesting that ATP-binding and ATP-hydrolysis are required for a functional interaction between MutL and Vsr under physiological salt concentrations.

DISCUSSION

Many DNA repair systems have overlapping substrate specificities, giving rise to the need to co-ordinate their activities. The repair of T:G mismatches by the MMR and the VSP systems falls into this category. MMR is inhibited by excess Vsr, reflecting a physical and functional interaction between MutL and Vsr, as shown by several studies (20–22,40). Our *in vitro* data, using sedimentation velocity analysis (Figure 1), clearly show the formation of a protein–protein complex between MutL and Vsr, supporting the *in vivo* observations. Indeed, using photochemical and chemical crosslinking

(Figures 3–5) we could map the interaction site of MutL for Vsr to a site overlapping with the binding site for MutH (25,41). This finding supports a model of competition for a common binding site on MutL between the MMR proteins MutH and the VSP protein Vsr, explaining the inhibitory effect of Vsr overexpression on MMR *in vivo*, which is reversed by overproduction of either MutL or MutH (37). Indeed, we could demonstrate that Vsr does not influence initial steps in DNA MMR, i.e. ternary complex formation between DNA, MutS and MutL (data not shown). However, Vsr efficiently inhibits the mismatch-provoked MutS and MutL-dependent activation of MutH (Figure 6 and Supplementary Data), suggesting that the mutagenic effect of Vsr arises as a direct consequence of competition between MutH and Vsr for binding to MutL, postulated before (40). As expected, MutH inhibited the activation of Vsr by MutS and MutL (Supplementary Data).

The stimulatory effect of both MutS and MutL on VSP repair has been puzzling, especially since structural analysis of the MutS-DNA and Vsr-DNA complexes clearly revealed that MutS and Vsr cannot be bound simultaneously to the same DNA mismatch (40). In the present study we demonstrated that the physical interaction between MutL and Vsr is important but not sufficient for an enhancement of Vsr activity under physiological ionic strength. Our analysis revealed that both MutL and MutS are necessary and sufficient for the stimulation of Vsr endonuclease and that this functional interaction requires ATP hydrolysis (Figure 7), similar to the mismatch-provoked activation of MutH (42). Whereas the physical interaction between MutL and Vsr could be observed in the absence of ATP-hydrolysis and with the N-terminal domain of MutL (Figure 1), the functional interaction, resulting in activation of Vsr, requires ATP-hydrolysis, as the ATPase deficient MutL-E29A has almost completely lost its stimulatory ability (Figure 8). Similarly, MutL-E29A is not able to support the mismatch-provoked activation of MutH at physiological ionic strength (125 mM KCl). Our conclusions are at odds with experiments conducted at low ionic strength (20 mM NaCl), suggesting a role for ATP-hydrolysis by MutL only for steps after DNA incision by MutH and loading of DNA helicase II (43). However, the conclusions are fully consistent with data reporting the inability of MutS to further increase the MutH activation by MutL-E29A at higher ionic strength (44). Notably, at lower salt concentration (e.g. 50 mM KCl) MutL-E29A is able to activate MutH in a MutS independent manner as similarly described for MutL wild type (data not shown) (44).

Several conclusions can be drawn from the present study. MutL shares a common or overlapping interaction site for two of its effector proteins, MutH and Vsr. The interaction site for MutH is located between the N- and C-terminal domains reminiscent to the position of the client binding site of Hsp90 which shares structural homology to MutL in the ATPase domain (45,46). Although binding of the effector proteins (Vsr or MutH) to the MutL-NTD is dependent on nucleotide binding but not hydrolysis, this is not true for the functional interaction. At physiological ionic strength, the activation of Vsr

and also MutH (data not shown) in a mismatch and MutS-dependent manner requires ATP-hydrolysis by MutL (Figure 8). Finally, since mismatch binding by MutS and Vsr is mutually exclusive, only models involving a mobile rather a stationary MutS that does not remain bound at the mismatch are consistent with the observed MutS–MutL-dependent activation of Vsr (7,47). Similarly, MMR has been reported to be efficient even if the mismatch and the GATC-site are separated by only 4 bp, a distance which is too short to allow simultaneous binding of MutS at the mismatch and MutH at the GATC-site (48).

We envision at least two possible ways that MutS and MutL could allow access of Vsr to the T/G mismatch, or MutH to adjacent GATC sites. One possibility is that MutS and MutL leave the site of the mismatch before the arrival of the effector proteins, as has been proposed previously (40). The only way to explain the stimulatory effect of the MutS–MutL complex is to postulate that it changes the conformation of the DNA to make it easier to bind. Our data show that Vsr and MutL interact, which makes this scenario unlikely. The other possibility is that MutS and MutL act as damage sensors, recruiting any one of a number of effectors to the site. The resulting ATP-dependent interactions between sensor(s) and effector(s) both displace the sensors and activate the effectors. Such a model is supported by recent *in vivo* studies showing that MutL recruits the Vsr and MutH endonucleases in response to DNA damage (49). If the recruited protein is able to mediate repair, the MutS–MutL complex would dissociate. If the recruited protein is unable to mediate repair, the MutS–MutL complex would remain, allowing subsequent recruitment of another effector. Such a model would account for the fact that MutS and MutL enhance Vsr activity, but are not required for VSP repair.

The inhibitory effect of Vsr on MMR explains why regulation of Vsr expression is required for proper MMR function (and vice versa for MutH in stationary phase although). Indeed, MutH is inhibiting the activation of Vsr by MutS and MutL (Supplementary Data). However, beside this, it is possible that Vsr is kept low in growing cells not because it interferes with MMR but because it can stimulate CTAGG-to-CCAGG mutations, as shown before (38).

The question of when and how Vsr or MutH enter and leave the repair pathway needs to be addressed before the mechanistic details of competition and cooperation between various proteins interacting with MutL can be understood. The observed similarities between the stimulation of Vsr and MutH by MutS and MutL offers new routes for future in-depth analysis of the activation of effector proteins in the MMR pathways in a comparative manner. Only one Vsr homolog from another organism has been analyzed but not in the context of MMR (50). Since homologs of a Vsr endonuclease are present in 198 bacterial species [REFSEQ 01-23-09 (51)] from across the bacterial kingdom in contrast to MutH homologs that are almost exclusively found in γ -proteobacteria (currently 130 in REFSEQ), it will be important to understand whether the observed physical and functional interaction between Vsr and MutL in *E. coli* is a general DNA repair

pathway used also in other bacteria. The functional assay involving the activation of Vsr by MutS and MutL described in the present study might be exploited for the mechanistic analysis of DNA MMR pathways in bacterial systems.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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