The *Escherichia coli* MutL protein stimulates binding of Vsr and MutS to heteroduplex DNA

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**ABSTRACT**

Vsr DNA mismatch endonuclease is the key enzyme of very short patch (VSP) DNA mismatch repair and nicks the T-containing strand at the site of a T–G mismatch in a sequence-dependent manner. MutS is part of the *mutHLS* repair system and binds to diverse mismatches in DNA. The function of the *mutL* gene product is currently unclear but mutations in the gene abolish *mutHLS*-dependent repair. The absence of MutL severely reduces VSP repair but does not abolish it. Purified MutL appears to act catalytically to bind Vsr to its substrate; one-hundredth of an equivalent of MutL is sufficient to bring about a significant effect. MutL enhances binding of MutS to its substrate 6-fold but does so in a stoichiometric manner. Mutational studies indicate that the MutL interaction region lies within the N-terminal 330 amino acids and that the MutL minimalization region is at the C-terminal end. MutL mutant monomeric forms can stimulate MutS binding.

**INTRODUCTION**

The hydrolytic deamination of 5-methylcytosine (5-meC), a minor DNA constituent of most organisms, produces DNA thymine residues mismatched opposite guanines. Failure to repair such mismatches is believed to lead to an increased incidence of transition mutations associated with sites of cytosine methylation. Almost one-fourth of all cancer-associated mutations in *E. coli* K-12, the inner cytosine residue of the sequence CCA/TGG carries a 5-meC deamination. Very short patch (VSP) repair acts on T–G mismatches in the context of the Dcm methylation sequence and restores a C/G base pair; the cytosine residue can subsequently be re-methylated. The constraints of sequence context that identify a T–G mismatch as a target for VSP repair, are somewhat relaxed. After a genetic analysis had identified *vsr*, a gene overlapping *dcm*, as essential for VSP repair, the Vsr product was isolated and characterized biochemically as a mismatch- and strand-specific DNA endonuclease (6). VSP repair, as characterized by formal and molecular genetics, reflects in detail the enzymatic properties of Vsr endonuclease, in particular its substrate recognition preferences (6–10).

VSP repair also depends on functional *polA* gene (11) and is greatly reduced by mutations inactivating either *mutL* or *mutS* (12–14). MutL and MutS proteins are essential components of a DNA mismatch repair system which rectifies base–base and insertion/deletion mismatches (15) as well as those produced by recombinational and drug-induced addition (16). In this pathway, repair events are initiated by MutS binding to a mismatch (17,18); the role of MutL is much less well understood. These proteins are thought to interact with each other since MutL expands the footprint of MutS as determined by DNAse I protection (19). MutS-mediated loop growth is stimulated 2-fold by MutL (20); and in yeast the MutL homologue heterodimer, Mlh1/Pms1, can cause a ‘super-shift’ of the corresponding MutS homologue, Msh2–6DNA as measured by migration in non-denaturing polyacrylamide gels (21). MutS and MutL are also required to activate the latent endonuclease activity of MutH, a -GATC specific endonuclease that introduces a nick in the unmethylated strand of a hemimethylated duplex (22). However, there is as yet no catalytic function attributable to MutL. Numerous genes homologous to *E. coli* *mutS* and *mutL* have been found in eukaryotic organisms and predisposition to certain types of hereditary and sporadic cancer have been linked to defects in these genes (23). Surprisingly, VSP repair is not only a mutation avoidance device but it interferes with *mutHLS* DNA mismatch repair with the consequence of active, enzyme-driven mutagenesis, a process that has left pronounced traces in the nucleotide sequence of the *E. coli* K-12 genome (9,24,25). That is during replication T–G mismatches resulting from erroneous incorporation of dGTP opposite to T are converted by VSP repair to C–G. Thus, the evolutionary significance of VSP repair is still subject to

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speculation; but whatever its true selective advantage may be, it seems to be available to other organisms since vsr-like genes have been discovered in Haemophilus parainfluenzae (26), Bacillus subtilis (26,27), Arthrobacter luteus (27,28), Xanthomonas oryzae (29), Norcardia aerocolonigenes (30) and Shigella (4).

In the present study, we employed purified proteins to experimentally address the possible influence of MutL on Vsr and MutS binding to heteroduplex DNA (hDNA).

MATERIALS AND METHODS

Strains and media

Escherichia coli strains: BL21 (λDE3) (ompT- rB mR*) (31); BMH 71-18 [Δ(lac-proAB), thi, supE / F' lacI4, lacZΔM15, proA*B*], source: B. Müller-Hill (32); CC106 [ara, Δ(lac– proB)XH1 / F' lacI*, lacZ*, proB*] (33). Bacteria were grown in either dYT medium (1% Bacto yeast extract, 1.6% Bacto tryptone, 0.5% NaCl) or L broth (1% Bacto tryptone, 0.5% Bacto yeast extract, 1% NaCl). Bacto yeast extract and Bacto tryptone were from Difco. Ampicillin and chloramphenicol (Boehringer Mannheim and Sigma) were added to media at 100 and 25 µg/ml, respectively.

Preparation of oligonucleotide duplexes

100 pmol of the 30mer oligonucleotide KDUP2 (dTACTTGG-7.6, 10 mM EDTA, 5 mM MgCl2 at 70°C) were converted to duplex DNA in the presence of dNTPs and α-32P]ATP by passage through Sephadex G25 (2 ml bed volume; Pharmacia/LKB) and was stored at –20°C.

The Vsr oligonucleotide substrate duplex, HD, was prepared by annealing oligonucleotides from the phage P22 (University of Massachusetts Medical School). The underlined bases are complementary and the mismatched bases are in bold. The DNA sequence of these oligonucleotides is from the phage P22 mnt gene (15). The oligonucleotides were mixed, annealed in 20 mM Tris–HCl, pH 7.6, 10 mM EDTA, 5 mM MgCl2 at 70°C for 10 min, then slowly cooled to room temperature (15). The single-stranded regions were converted to duplex DNA in the presence of dNTPs and [α-32P]ATP (800 Ci/ mmol) and DNA Polymerase I Klenow fragment (New England Biolabs) in reaction buffer supplied by New England Biolabs. Following the reaction, labeled double-stranded hDNA was recovered after passage through a Qiaquick (Qiagen) column.

Preparation of Vsr DNA mismatch endonuclease

Vsr was purified from cell extracts using Chelating Sepharose Fast Flow and heparin Sepharose (HiTrap, Pharmacia Biotech) columns as described by Drotzmann et al. (34).

Preparation of histidine-tagged MutL and MutS

Plasmid pMQ393 is a pACYC184 derivative containing the mutL gene with the T7 promoter and N-terminal polyhistidine sequence from ϕET15b and was constructed and provided by Dr T.-H. Wu (University of Massachusetts Medical School). To prepare MutL-6H from uninduced cells, 1 liter dYT medium supplemented with 25 µg chloramphenicol/ml was inoculated with 30 ml of an overnight culture of E.coli strain CC106 harboring pMQ393 and aerated by shaking at 26°C overnight. Cells were harvested by centrifugation, resuspended in 20 ml buffer A (20 mM HEPES–KOH, pH 7.6, 5 mM β-mercaptoethanol) and lysed by passage through a French pressure cell (138 Mpa). Cell debris was removed by centrifugation. The supernatant was subjected to sonication (Branson sonifier) and re-centrifuged; subsequently it was applied to a 4 ml Chelating Sepharose Fast Flow (Pharmacia Biotech) column loaded with nickel ion. The column was extensively washed with buffer A. MutL was eluted with a step gradient of imidazole (60–300 mM, 4 ml each) in buffer A plus 500 mM NaCl. The 200 and 240 mM fractions contained the bulk of MutL-6H; they were pooled and dialyzed against buffer A. MutL was at least 90% pure as determined by SDS–PAGE. The yield was ~7 mg homogeneous MutL-6H as judged by OD280 and SDS–PAGE.

Alternatively MutL-6H was also prepared from induced cells. Strain BL21 (λDE3) was transformed with ϕET15b plasmids containing the wildtype and mutant mutL genes (35) by selection for ampicillin resistance. MutL protein was isolated from 40 ml transformed cells induced at OD600 =1 with 200 mM IPTG and grown for 2 h at 27°C. Cells were harvested and resuspended in binding buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 5 mM imidazole) and sonicated for 2–3 min followed by centrifugation at 39 000 c.p.m. for 30 min. Binding and elution of protein from the His.Bind (Novagen) resin was as recommended by the manufacturer except that the buffers contained 200 mM NaCl. The column was washed with 80 mM imidazole and the MutL protein eluted with 200 mM imidazole and stored in this buffer. Protein was used within 2 days and was stored at 4°C during this time. MutL protein was at least 90% pure as judged by SDS–PAGE. MutL yield was ~500 µg. Protein concentration was assayed using the Bradford protein assay (Bio-Rad) using bovine serum albumin as a standard.

Histidine-tagged MutL mutant proteins (MutL721, MutL723, MutL725 and MutL726) were prepared as described above. MutS protein was purified using both methods described above except that the cells were induced with 200 µM IPTG for 2 h at 27°C.

Gel filtration

MutL, wildtype and mutant proteins were chromatographed through a Sephacryl S300 (Pharmacia Biotech) column (1 × 60 cm) equilibrated with 25 mM HEPES–KOH (pH 7.4), 100 mM NaCl.
RESULTS

Purification of MutL and MutS

The mutL(pMQ393) and mutS(pMQ395) plasmids used in this study completely complement the mismatch repair mutator phenotype of mutL and mutS mutants (35,36). This indicates that the polyhistidine-tag at the N-terminal end of these proteins imparts no detectable interference with the normal action of the MutL and MutS proteins. Consequently it is unlikely that the effects we describe below are a consequence of the polyhistidine tag.

SDS–PAGE of protein samples from eluates obtained by step-wise elution from chelating Sepharose columns is shown in Figure 1A for MutL and Figure 1B for MutS. Most of the MutL elutes at 200 and 240 mM imidazole (Fig. 1A) while the bulk of MutS elutes at 180 and 200 mM imidazole (Fig. 1B). The proteins were usually dialyzed and used without further purification although dialysis was not necessary to obtain the effects described below.

Influence of MutL protein on Vsr binding

Mutations in either mutL or mutS gene severely impair VSP repair although they do not block it completely (12–14). Since all data collected so far pointed to target binding as the rate-limiting step in Vsr endonuclease action (34), the possible influence of MutL protein on this process was investigated by gel shift assays; to this end, essentially homogeneous MutL and Vsr were prepared as described under Materials and Methods.

MutL by itself does not produce a band shift with the 30mer substrate hDNA (Fig. 2). However, it markedly enhances Vsr binding and it does so in a transient fashion since no ‘supershift’ or intermediate bands were observed. Since the molecular weight of Vsr is 18 kDa and that of MutL is 78 kDa, a supershift should have been easily detected if a stable complex was formed. Note that in Figure 2 Vsr endonuclease is present in a 50-fold excess over hDNA. Vsr has only a low binding affinity for the corresponding homoduplex DNA (34).

If MutL indeed serves the function of a catalyst in the Vsr/hDNA binding reaction, it could possibly be used to relieve the requirement of a large protein excess. In the presence of MutL, five equivalents of Vsr (relative to hDNA) are sufficient to bring about ~50% binding (Fig. 3), which is a 10-fold reduction of Vsr.
Figure 3. Titration of the MutL enhancing effect on Vsr binding to HD hDNA. Vsr (35 equivalents relative to DNA) and the indicated number of equivalents of MutL (relative to Vsr) were present in the assay mixtures. The marker (M) HD hDNA without addition of protein is also shown.

Figure 4. MutS binding to heteroduplex DNA and its inhibition by ATP. Lanes 1 and 10, heteroduplex DNA; lanes 2–7, MutS 40, 60, 80, 100, 120, 200 ng; lane 8, MutS (80 ng) with 2 mM ATP; lane 9, MutS (80 ng) with 2 mM ATP-γ-S.

Since 50 equivalents of MutL relative to hDNA were used in the experiment, non-specific effects of either macromolecular crowding or chemical protection of Vsr endonuclease could not be excluded. Therefore, the amount of MutL required for stimulation of binding at a constant concentration of Vsr (35 equivalents relative to hDNA) was also titrated. As shown in Figure 3, one-hundredth of an equivalent of MutL is sufficient to bring about a significant effect; the requirements for a truly catalytic process are thus fulfilled. In addition, the experiment was repeated with bovine serum albumin (BSA) substituting for MutL at the same molar concentrations. Only at the highest concentrations of BSA could some faint stimulation of binding be observed (data not shown).

Influence of MutL on MutS binding to hDNA

The MutS hDNA substrate was prepared by annealing two synthetic oligonucleotides, which have complementary (except for a G–T mismatch) overlapping sequences at their 3' ends and then filling in the single stranded regions with [32P]-labeled ATP and DNA polymerase I Klenow fragment. This produces a 154 bp DNA with a G–T mismatch centrally located at bp 76. Although a single duplex hDNA product was expected from this reaction, two products were routinely detected by agarose-gel electrophoresis (F1 and F2; Fig. 4, lanes 1 and 10).

MutS binds to hDNA in a concentration-dependent manner (Fig. 4, lanes 2–7) and reaches its maximum at 80 ng (Fig. 4, lane 4) for F2 hDNA. An increase in binding of F1 DNA to MutS occurs at higher protein concentrations and may be the cause of a slower migrating band (B2) above the major bound species (Fig. 4, lanes 4–7). Inclusion of either ATP or ATP-γ-S abolishes apparent binding (Fig. 4, lanes 8 and 9).

Addition of MutL increases the binding of hDNA to 40 ng MutS (compare Fig. 4, lane 2 with Figure 5, lane 2). Densitometric scanning indicates a 3-fold enhancement. Doubling the MutL concentration results in a concomitant doubling in hDNA bound to MutS to reach a maximal 6-fold enhancement at saturation (Fig. 5, lanes 2–4). At these concentrations the molar ratio of MutL to MutS is 3:1. Decreasing this ratio did not result in enhanced binding unlike the situation for Vsr. The MutL concentration-dependent stimulation is less apparent at high MutS concentrations presumably due to saturation (data not shown).

As low concentrations of MutL, F2 hDNA is bound preferentially by MutS (Fig. 5, lane 2) but at higher MutL concentrations F1 hDNA is also retarded (Fig. 5, lanes 3 and 4). MutL at twice the highest concentration used in these experiments shows no apparent binding to hDNA (Fig. 5, lane 1). No hDNA binding by MutS was detected in the presence of MutL plus ATP or ATP-γ-S (data not shown). We have also found that MutL enhancement occurs upon binding to MutS of a duplex 36mer containing a 1 base insertion (data not shown). Stimulation of MutS binding by MutL therefore is independent of the specific mismatches tested or on the length of hDNA.

Addition of MutL to the MutS binding reaction did not produce any detectable ‘supershifted’ or intermediate band in Figure 5 suggesting a transient or unstable association of MutS–hDNA–MutL. This suggestion is supported by the preliminary observation that the bound complexes such as those in Figure 5 contained MutS, but not MutL, as determined by western blotting (data not shown).

In order to determine which part of the MutL protein was responsible for MutS-enhanced binding we used nonsense fragments lacking various amounts of the C-terminal region of MutL. All four nonsense truncated MutL proteins (MutL721, MutL723, MutL725 and MutL726) were able to stimulate MutS binding to the same degree as the wildtype protein (data not shown). The region in MutL responsible for MutS enhanced binding must, therefore, be located in the N-terminal 330 amino acids.
apparent molecular weight of elutes from a Sephacryl S-300 gel filtration column with an MutL protein isolated as described in Materials and Methods MutL multimerization region (19). The protein upon gel filtration was also observed by Grilley three times that of the 68 kDa monomer. Anomalous behavior of (35) elute as monomers (Fig. 6). In partial denaturing SDS–poly- protein contact (16) is an attractive but not yet unequivocally

The biochemistry of MutL action in DISCUSSION Mechanism of action of MutL. The biochemistry of MutL action in mutHLS repair action is much less well understood than that of MutS; its function as an interface between MutS and MutH (16) and participation in stimulating the sequence-specific endonuclease activity of the latter by protein–protein contact (16) is an attractive but not yet unequivocally proven suggestion. Sancar and Hearst (38) have suggested that MutL is an example of the ‘molecular matchmaker’ class of proteins, which causes an ATP-dependent conformational change in one or more DNA binding protein partners to promote protein–DNA interaction. Although this model may be correct, the MutL matchmaking activity would not explain the observations we have reported here with Vsr and MutS since these reactions are transient and ATP-independent.

With the data presented here, we have identified a plausible role for MutL in the molecular mechanism of VSP and mutHLS repair in the sense of MutL mediating complex formation of Vsr endonuclease or MutS with its substrate. MutL stimulation of Vsr binding to hDNA is clearly catalytic and this represents the first demonstration of such an activity for MutL. On the other hand, the enhancement of MutS binding by MutL is not catalytic but stoichiometric. The reason for this difference is unclear; perhaps it is a reflection of the low concentration of Vsr in the cell relative to MutS.

The function of MutL could be exerted by transient association with one or the other binding partner prior to encounter of the second. The notion of DNA–MutL complex formation as the first step was suggested by Bende and Grafstrom (37) and this could operate in a similar fashion in both VSP and mutHLS repair. However, we did not observe binding of MutL to hDNA (Fig. 5) although similar concentrations of MutL were used and the 154 bp hDNA substrate we used is longer than the minimum length required for binding (37). The basis for the discrepancy between these results is not known but could be due to different MutL and/or DNA preparations. If the DNA–MutL complex is the first step in the process, it must be that transient MutL–MutS or MutL–Vsr interaction is required to efficiently load MutS or Vsr onto hDNA. A physical association of Vsr endonuclease with at least one protein component of mutHLS repair was also invoked as the source of the recently discovered mutagenic effect of Vsr overproduction (39). Prolla et al. (40) detected a relatively stable physical interaction between the yeast MutL heterodimer, Mlh1–Pms1, and the hDNA-bound MutS homologue, Msh2, by the appearance of a ‘supershifted’ band on non-denaturing polyacrylamide gels. With E. coli MutL and MutS–hDNA, we did not detect such supershifting (Fig. 5). Prolla et al. (40) did not report a stimulatory effect of the MutL homologs. Whether these results indicate a fundamental difference between the interaction of MutS and MutL proteins in the two organisms remains to be resolved.

Grilley et al. (19) used DNase I protection experiments to show that E.coli MutL, interacted with MutS–hDNA in an ATP (and ATP-γ-S)-dependent manner to enlarge the footprint obtained with MutS alone. Allen et al. (20) found by electron microscopy that MutS and MutL are present at the base of ATP-induced loops although it is not known if they are associated. These results are the only evidence in E.coli for MutL–MutS interaction. The results shown in Figure 5 indicate a transient MutL interaction with MutS–hDNA, which is completely inhibited by ATP or ATP-γ-S. Although these experimental results appear to be contradictory in their nucleotide requirements, the experiments are measuring different end-points. A plausible explanation for the difference in the findings is the length of substrate DNA. The experiments demonstrating loop formation used an ~6 kb substrate DNA whereas the data reported here utilized a 30mer for Vsr and 154 bp oligonucleotide for MutS.

Figure 6. Gel filtration analysis of MutL and mutant proteins. MutL wildtype and mutant proteins (black dots) were chromatographed through a Sephacryl S-300 column equilibrated with 25 mM HEPES–KOH, pH 7.4, 100 mM NaCl. White dots represent the following standards in order of decreasing molecular weight: Apoferritin (440 kDa), Amylase (200 kDa), Alcohol dehydrogenase (150 kDa), Bovine serum albumin (66 kDa) and Ovalbumin (45 kDa). Vo is the void volume and Vr the retained volume.

MutL multimerization region

MutL protein isolated as described in Materials and Methods elutes from a Sephacryl S-300 gel filtration column with an apparent molecular weight of ~200 kDa (Fig. 6) which is about three times that of the 68 kDa monomer. Anomalous behavior of the protein upon gel filtration was also observed by Grilley et al. (19).

In contrast, MutL721 and MutL723 nonsense protein fragments (35) elute as monomers (Fig. 6). In partial denaturing SDS–polyacrylamide gels, the wildtype MutL migrates as two bands corresponding to apparent molecular weights of 210 and 66 kDa (data not shown). In contrast the MutL723 and MutL725 proteins migrated as single bands with apparent molecular weights of 50 and 57 kDa, respectively (data not shown). These data indicate that the MutL multimerization region is located in the C-terminal end of the protein distal to residue 331 and includes or is distal to residue 519.

DISCUSSION

Mechanism of action of MutL.

The biochemistry of MutL action in mutHLS repair action is much less well understood than that of MutS; its function as an interface between MutS and MutH (16) and participation in stimulating the sequence-specific endonuclease activity of the latter by protein–protein contact (16) is an attractive but not yet unequivocally
Two mutant truncated MutL proteins, MutL723 and MutL725, were found to be monomeric in contrast with the wildtype protein which was multimeric (Fig. 6). This result could mean that the multimerization region is at the C-terminal end of MutL. Alternatively, lack of C-terminal amino acids might alter the folding of the truncated proteins such that multimers cannot form. The results of Pang et al. (21) would support the first alternative. They found that the yeast Pmsl–Mlh1 heterodimer interaction domains are located in a 260 amino acid domain near the C-terminus for Mlh1 and in the final 212 amino acids for Pms1. These regions have no amino acid sequence homology with each other or with E.coli MutL suggesting that the location of the multimerization domain in these MutL proteins may be more important than the amino acid sequence per se.

The mutL723 and mutL725 nonsense mutations were isolated as dominant-negative alleles showing elevated spontaneous mutability most likely due to reduced mismatch repair (35). The dominant-negative behavior of these mutants is not entirely explained by their monomeric state since the wildtype MutL should be able to multimerize and act normally. Clearly MutL multimerization is not required for enhanced MutS–hDNA interaction since the mutant proteins are proficient in this process. Therefore a subsequent step in mismatch repair must be affected in which the monomeric mutant proteins block the action of the wildtype multimers.

The results described in this paper suggest that MutL enhances DNA binding of proteins it can associate with. One other such protein is MutH, a -GATC-specific endonuclease which introduces a nick in the unmodified strand of a hemimethylated duplex and which requires MutL and MutS to activate its latent activity. We are currently testing if MutL influences MutH binding and/or nicking activity.

After submission of this manuscript, Habraken et al. (41) reported enhanced yeast Msh2–Msh3 binding to hDNA by yeast Mlh1–Pms1. Their results are qualitatively similar to those reported here for MutS and MutL. No supershifted bands were detected in non-denaturing gels to indicate formation of stable Msh2–Msh3–Mlh1–Pms1 complexes. Inclusion of either Mlh1 or Pms1 antibody during incubation of the Msh2–Msh3 and Mlh1–Pms1 proteins with hDNA resulted in the detection of ternary complexes with retarded mobility.

In conclusion, it will be interesting to determine whether mediating contact between DNA and repair enzyme or protein–protein interaction is a general function of MutL and a common denominator between VSP and mutHLS repair; it is, of course, also possible that MutL serves more than one function and makes different use of these in different pathways of DNA mismatch repair.

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