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Functional Analyses of *Escherichia coli* MutS-β Clamp Interaction In Vitro and In Vivo

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Abstract *Escherichia coli* MutS is a highly conserved mismatch repair (MMR) protein that plays a key role in recognizing DNA mismatches and the early steps of MMR. Previous studies revealed an interaction between MutS and the replicative protein β clamp, but it remains unclear whether the interaction functions during the process of MMR. In order to provide insight into the significance of this interaction, Far Western, Surface plasmon resonance and cell survival/mutagenesis assays were used to determine its possible influences on the in vitro and in vivo properties of MutS. The results show that a quintuple mutation of MutS residues 812–816 ($MutS^{\beta C}$), or single alanine substitution mutation of MutS residues M813 or L815 completely blocks binding of MutS to β clamp. Wild type β clamp interferes with DNA binding by MutS. When treated with the base analog 2-aminopurine, $MutS^{\beta C}$ confers more mutations and less cellular growth rate in the *mutS*-deficient strain than the wild type MutS. These data indicate that the MutS-β interaction has functional consequences during MMR in *E. coli*.

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Introduction

DNA mismatch repair (MMR) is the primary mechanism for correcting mismatches and insertion/deletion loops generated during DNA replication [1]. MMR is highly conserved from bacteria to human cells. In humans, defects in MMR are associated with increased risk of developing cancer [8].

Escherichia coli MutS plays a critical role in the early steps of MMR, because it recognizes and binds to mismatches or insertion/deletion loops, and recruits MutL to form a ternary complex with the DNA lesion. The MutL–MutS-DNA complex recruits downstream MMR proteins, which correct DNA mismatches to maintain high fidelity DNA replication [1]. β clamp is a processivity factor for replicative DNA polymerases, and it also acts as a scaffold protein that interacts with various proteins which plays an important role in coordinating DNA metabolic pathways in *E. coli* [5]. Recent studies demonstrate that *E. coli* MutS interacts with β clamp and that this interaction involves two putative regions of MutS: an N-terminal region involving residues 15–18 and a C-terminal region involving residues 812–816 [10], which initially implicate MutS-β clamp interaction in MMR. But no evidence demonstrates that the MutS-β clamp interaction has any roles in MMR.

In order to gain insight into the role of this interaction, this study conducted its detailed characterizations. The results demonstrate for the first time that mutation of MutS residues 812–816 ($MutS^{\beta C}$), which completely blocks binding of MutS to β clamp, confers a mutator phenotype in *mutS*-defective cells treated with 2-aminopurine. Furthermore, wild type β clamp, but not Q299 β clamp mutant, interferes with DNA binding by wild type MutS. Our results indicate the involvement of the MutS-β interaction in MMR and their implications for the function of the interaction during MMR are discussed.

Materials and Methods

Plasmid Construction and Site-Directed Mutagenesis

The plasmids pQE30-*β clamp-sbp* and pQE30-*mutS-sbp* were constructed as previously described for pQE30-*mutL-sbp* [7]. Briefly, gene was C-terminally tagged by a streptavidin-binding peptide (SBP, MDEKT TGWRG GHVVE GLAGE LEQLR ARLEH HPQGQ REP) coding sequence and a linker peptide SGSGSGSGSG coding sequence was inserted between the gene and SBP-tag. Derivatives of the pQE30 or pET15b plasmids encoding *E. coli mutS* and *β clamp* mutants were constructed by overlap PCR. The structure of all clones was confirmed by DNA sequencing.

Protein Expression and Purification

Recombinant proteins were expressed and purified from *E. coli* M15 transformed with pQE30 derivatives using Ni^{2+}-NTA column chromatography as described [7]. The His-tag and SBP-tag recombinant proteins showed normal MMR activity and efficiently complement MMR-deficient strains [4, 7].

Far Western Assay

The target proteins (wild type or mutant MutS) were loaded onto a nitrocellulose membrane, followed by the SBP-tagged protein (*β* clamp-SBP) at a final concentration of 800 nM. Binding of the SBP-tagged protein to the target protein was measured by adding alkaline-phosphatase conjugated strepavidin (Promega) and alkaline-phosphatase substrate (Amercisco) to the membrane. Colorimetric analysis of the alkaline-phosphatase reaction product was used to quantify binding of the SBP-tagged protein to its target.

Surface Plasmon Resonance Assay

Surface plasmon resonance (SPR) was performed with the BIAcore 3000 (BIAcore AB, Uppsala, Sweden) at 25°C. In order to measure DNA–protein interactions, 5′biotinylated dsDNA was immobilized on a streptavidin (SA)-coated sensor chip at a density that corresponds to 300 response units (RU). Binding to homologous and mismatched dsDNA substrates was measured on flow cells 2 and 3 of the four flow cell SA chip, respectively. The biotinylated homologous dsDNA was made by annealing oligonucleotide 1 (5′-biotin-gtgtc cagat ccgtc tacct gagaa ctatt-3′) and 2 (5′-aatag ttctc aggta gacgg atctg gacac-3′). The heterologous dsDNA was made by annealing oligonucleotide 1 and 3 (5′-aatag ttctc aggtg gacgg atctg gacac-3′) with a GT mispair at position 15. In order to measure the MutS/*β* clamp interaction, MutS-SBP protein was immobilized on a SA chip at a density corresponding to approximately 3,000 RU. Samples were injected at different concentrations at a flow rate of 30 µl/min for 2 min. Data were corrected by subtracting the response units of flow cell 1, which did not have any immobilized biotinylated DNA or SBP-tagged protein. All experiments were repeated independently, and representative data were shown.

Mutation Frequency Assay

The frequency of accumulation of spontaneous mutations to rifampicin resistance in cell populations was designed to determine the bioactivity of MMR as described previously [7]. In brief, bacterial cells were cultured for 24 h at 37°C with shaking. Cultures were plated on LB with 100 µg/ml ampicillin to determine the total number of viable cells, and plated on LB containing 100 µg/ml ampicillin and 100 µg/ml rifampicin to determine number of rifampicin-resistant cells. Mutation frequency was expressed as the ratio of rifampicin-resistant colonies to viable colonies.

2-Aminopurine Mutagenesis

2-Aminopurine (2-AP) is a mutagenic nucleotide analog that is incorporated into DNA as 2-AP • T and 2-AP • C mispairs, which are recognized by MMR proteins [12]. Mutagenesis by 2-AP was carried out as described [4]. Cells from an overnight culture were diluted 1:100 with fresh LB containing the desired concentration of 2-AP, then grown for 7 h at 37°C and plated on LB with 100 µg/ml ampicillin to compare the number of viable cells between different samples, or grown for 24 h and plated in the absence and presence of rifampicin to determine mutation frequency.

Statistical Analyses

Mutation frequencies or colony-forming units ratios (CFU ratios) were processed by variance analysis (ANOVA). All data are expressed as means ± SD of triplicate experiments. Statistical significance was determined using the Tukey test. P values of <0.05 were considered statistically significant.

Results

Mutations in the C-Terminal Binding Motif of MutS Block MutS/*β* Clamp Interaction In Vitro

The putative MutS/*β* clamp interface has been mapped to the two regions of MutS [10]. To confirm this result at higher resolution, residue substitutions and Far Western assay were used to assess the contribution of individual residues of MutS to its interaction with *β* clamp. The result

showed that MutS$^{\beta C}$ (C-terminal quintuple mutant Q812G/M813S/S814G/L815S/L816G) had a strong defect in binding to β clamp while MutS$^{\beta N}$ (N-terminal quadruple mutant Q15A/Q16A/L18A/R19A) had little or no impact on the ability of MutS to bind β clamp.

Fine mapping of the C-terminal β clamp binding region showed that MutS M813A and L815A failed to bind β clamp, even at the highest concentration tested (Fig. 1). Thus, MutS M813 and L815 play a crucial role in the interaction between MutS and β clamp in vitro.

β Clamp Inhibits MutS DNA Binding Activity

Surface plasmon resonance (SPR) studies were carried out to examine whether binding to β clamp influences the interaction between MutS and DNA. For this purpose, SPR experiments were performed with immobilized DNA, increasing concentrations of β clamp and a constant concentration of MutS (50 nM). Under standard assay conditions, 50 nM MutS was injected and binding of MutS to immobilized DNA was recorded as response units (RU) 120 s after injection. A signal of 438 RU was observed with mismatched DNA in the absence of β clamp. This signal decreased in the presence of 50 or 200 nM β clamp to 402 or 311 RU, respectively (Fig. 2a). β clamp also reduced binding of MutS to homologous DNA (Fig. 2b).

When β clamp was injected alone, almost no response was observed (even for 500 nM β clamp, only 14.3 RU on homoduplex DNA and 13.8 RU on mismatched DNA). A recent paper also demonstrated that β clamp weakly binds to the short blunt duplex DNA, which may slide off the linear DNA rapidly [3]. Thus, the decreased RU in the presence of β clamp suggests that binding of β clamp inhibits binding of MutS to DNA. This idea was tested by carrying out SPR experiments with β clamp mutants carrying alanine substitution mutations in β clamp residues involved in its interaction with MutS, including an R176A/L177A double mutant and a Q299A single mutant.

Binding of 100 nM Q299A β clamp mutant to immobilized MutS was 20-fold or twofold lower than binding of 100 nM wild type β clamp or R176A/L177A β clamp mutant, respectively (Fig. 2c), indicating that the Q299A β clamp mutant has very low affinity for MutS. Consistent with these results, even at a high concentration (200 nM), Q299A β clamp mutant did not inhibit binding of MutS to immobilized DNA (Fig. 2a). These results suggest that wild type β clamp has a functional interaction with MutS, and this interaction inhibits the MutS DNA binding activity.

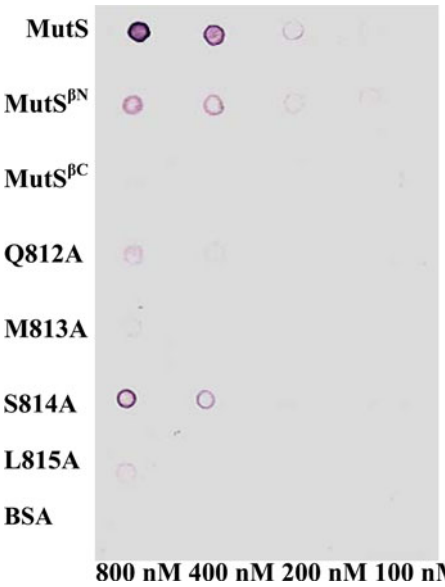

Fig. 1 Effect of mutations in β clamp binding motifs of MutS on the MutS/β clamp interaction. Far western analysis of the interactions between wild type or mutant MutS and β clamp was conducted as described in Materials and methods. MutS$^{\beta C}$ is a C-terminal quintuple mutant (Q812G/M813S/S814G/L815S/L816G) of MutS and MutS$^{\beta N}$ is an N-terminal quadruple mutant (Q15A/Q16A/L18A/R19A). The indicated amount of wild type or mutant MutS were applied to a nitrocellulose membrane, followed by incubation of the membrane with 800 nM β clamp-SBP (streptavidin-binding peptide). MutS was set as positive control and BSA was set as negative control. The purple color indicated a positive reaction

Requirement for MutS C-Terminal β Clamp Binding Motif for Resistance to High Mutagenic Stress

The mutant MutS$^{\beta C}$ was transformed into the *mutS*-deficient strain KM75 (kindly provided by Dr K.C Murphy, University of Massachusetts Medical School) and rifampicin resistance mutation frequency of the transformed cells was designed to determine its MMR bioactivity. The KM75 was a mismatch repair-deficient strain derived from *E. coli* AB1157 [11] and a tetracycline-resistance cassette replaced the chromosomal MutS gene. KM75 does not contain the gene coding T7 RNA polymerase, thus expression of MutS gene carried by pET15b depends on the adventitious use of an RNA polymerase promoter on the plasmid as it was described [13].

In KM75 cells expressing wild type recombinant MutS, the mutation frequency (6.1 ± 0.9 for KM75/pET15b-MutS per 10^8 cells) was 100-fold lower than in KM75 cells expressing empty vector (713 ± 127 for KM75/pET15b per 10^8 cells), indicating that the plasmid carrying MutS complements the MMR defect in KM75 cells. KM75 cells expressing MutS$^{\beta C}$ showed no statistically different mutation frequency than the cells expressing wild type MutS, even though Far Western data indicate a strong defect in binding of MutS$^{\beta C}$ to β clamp (Table 1; Fig. 1). This result suggests that MutS$^{\beta C}$ supports a normal level of MMR under a low load of mismatches.

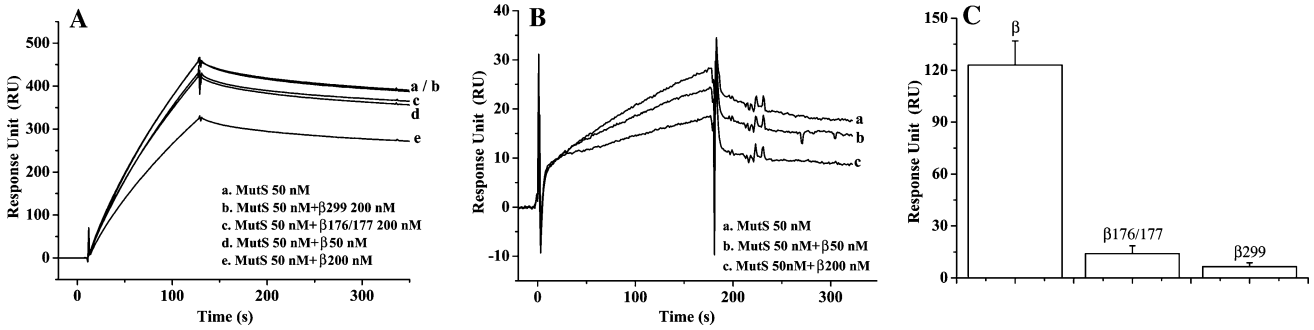

Fig. 2 Effect of β clamp on the DNA binding activity of MutS. **a** and **b** SPR analysis of the interaction between MutS and DNA in the presence of the indicated concentrations of wild type or mutant β clamp (an R176A/L177A double mutant and a Q299A single mutant). SPR sensor chip carried 300 RU mismatched dsDNA (**a**) or homologous dsDNA (**b**). **c** The interaction between 100 nM wild type or mutant β clamp and immobilized MutS was quantified by SPR assay. RU values were recorded 120 s after injection. The error bars represent the standard deviation of triplicate experiments

In order to examine the importance of the MutS-β clamp interaction in cells carrying a high load of induced mismatches, KM75 cells expressing wild type MutS or MutS$^{\beta C}$ were grown in the presence of various concentrations of 2-AP, and the frequency of mutation to rifampicin-resistance was examined. In KM75 cells expressing wild type MutS, the mutation frequency was 21.1-fold higher in the presence of 0.375 mg/ml 2-AP than in its absence. When the 2-AP concentration was increased two or fourfold, the mutation frequency increased to two to threefold (Table 1). In KM75 cells expressing MutS$^{\beta C}$, a comparable increase in mutation frequency was observed in the presence of 0.375 mg/ml 2-AP. However, a greater mutation frequency was observed in the presence of 0.75 or 1.5 mg/ml 2-AP in cells expressing MutS$^{\beta C}$ than in cells expressing wild type MutS. These results suggest that MutS$^{\beta C}$ is associated with a partial MMR defect in cells under high concentration of 2-AP.

Cellular growth was also compared in KM75 cells expressing wild type MutS or MutS$^{\beta C}$ in the presence of 2-AP. The results showed that low or high concentrations of 2-AP inhibited cell proliferation more strongly in cells expressing MutS$^{\beta C}$ than in cells expressing wild type MutS (Table 2). This result suggests that 2-AP-induced DNA lesions accumulate in cells carrying MutS$^{\beta C}$, which impairs cell proliferation [14]. In addition, in the presence of 0.375 mg/ml 2-AP the mutator phenotype of MutS$^{\beta C}$ was statistically significant in the case of growth but not in mutation frequency, which seems to be at odds with the fact that cell proliferation is impaired by DNA lesions. A possible explanation is that only a small proportion of the overall DNA lesions finally lead to antibiotic-resistance mutations.

Based on the mutator phenotype of MutS$^{\beta C}$ in the presence of 2-AP, we conclude that the interaction between MutS and β clamp has functional significance in cells subject to mutagenic stress.

Discussion

The results presented here show that mutation of M813 or L815 in the C-terminal β clamp binding site abolishes the MutS-β clamp interaction, but a compound mutation in the N-terminal β clamp binding site (MutS$^{\beta N}$) results in a moderate decrease in β clamp binding. Therefore, we conclude that the C-terminal region of MutS stabilizes binding to β clamp.

Table 1 Mutation frequency ($\times 10^{-8}$) of the MutS$^{\beta C}$ and MutS strain in response to 2-AP

2-AP (mg/ml)	KM75/pET15b-MutS	KM75/pET15b-MutS$^{\beta C}$
0	6.1 ± 0.9	15.3 ± 4.2
0.375	129 ± 17	138 ± 18
0.75	233 ± 11	279 ± 11*
1.5	390 ± 24	589 ± 32*

* Indicates $P < 0.05$ significant difference in mutation frequency compared to the KM75/pET15b-MutS group given the same dose f 2-AP

Table 2 CFU ratio* of MutS$^{\beta C}$ and MutS in response to 2-AP

2-AP (mg/ml)	KM75/pET15b-MutS	KM75/pET15b-MutS$^{\beta C}$
0	1	1
0.375	0.76 ± 0.06	0.44 ± 0.08**
0.75	0.40 ± 0.10	0.16 ± 0.05**
1.5	0.17 ± 0.06	0.07 ± 0.02

* CFU ratio is the percentage of colony-forming units of cells cultured in 2-AP for 7 h against cultured in the absence of 2-AP for each strain

** Indicates $P < 0.05$ significant difference in CFU ratio compared to the KM75/pET15b-MutS group given the same dose f 2-AP

Biochemical analysis of MutS and β clamp by SPR presented here demonstrates for the first time that β clamp modulates binding of MutS to heterologous and homologous DNA (Fig. 2). The exact interpretation of this result is not clear, but it indicates possibly complex functional roles for MutS residues in the MutS-β clamp interface. Lopez et al. [9] have reported that the α subunit of DNA polymerase III holoenzyme also contains two β clamp binding sites: the internal site is critical for interaction with β clamp, while the τ subunit and β clamp compete for interaction with the C terminus of the polymerase α subunit during DNA synthesis. The crystal structure of MutS shows that the N-terminal β clamp binding motif lies in an α-helix which is adjacent to a β-sheet structure that interacts specifically with DNA mismatches [6]. This spatial distribution indicates that the MutS N-terminal β clamp binding motif may play a direct role in DNA binding. Therefore, we interpret our results as: β clamp may compete with DNA for binding to the N terminus of MutS, and binding of β clamp could sterically block binding of DNA to MutS. This hypothesis is contrary to the function of MutS/β clamp interaction in *Bacillus subtilis*, which was reported to stabilize MutS at a mismatch [15]. Replacement of the β clamp interaction motif in *Bacillus subtilis* MutS resulted in increase in mutation frequency [15], but *E. coli* KM75 cells expressing $MutS^{\beta C}$ showed no statistically different mutation frequency than the cells expressing wild type MutS except in the presence of high concentration of 2-AP (Table 1). This difference presumably arises because the functions of MutS/β clamp interaction precede mismatch discrimination by MutS in *E. coli* MMR.

Since $MutS^{\beta C}$ only confers a mutator phenotype in cells exposed to mutagenic stress (this study), it is supposed that MutS/β clamp interaction may have a regulatory effect during the MMR in the presence of high mutational load.

Based on these analyses, we speculate that the role of this interaction during MMR is as follows: the C-terminal β clamp binding site of MutS enables it to co-localize with β clamp in the context of a crowded intracellular environment, then competition between DNA and β clamp for MutS promotes dissociation of the MutS/β clamp complex. β clamp is an essential component of the replication machinery. Thus, the local concentration of MutS in the vicinity of replicating DNA would be enriched through the above process, which could facilitate genome scanning by MutS. As the cellular amount of MutS remains unchanged in response to increased mismatched base pairs [2], the above effect would permit more mismatches to be corrected in the presence of high mutational load. This possibility would be useful for maintaining genomic fidelity and cell survival in adverse living circumstances.

In summary, the results from this study validate the involvement of MutS-β clamp interaction in MMR and suggest that this interaction may regulate MMR which provides a novel basis for further studies of the roles of the interaction in MMR.

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