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Conservation of Functional Asymmetry in the Mammalian MutL α ATPase

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Abstract

The DNA mismatch repair (MMR) protein dimer MutL α is comprised of the MutL homologues MLH1 and PMS2, which each belong to the family of GHL ATPases. These ATPases undergo functionally important conformational changes, including dimerization of the NH₂-termini associated with ATP binding and hydrolysis. Previous studies in yeast and biochemical studies with the mammalian proteins established the importance of the MutL α ATPase for overall MMR function. Additionally, the studies in yeast demonstrated a functional asymmetry between the contributions of the Mlh1 and Pms1 ATPase domains to MMR that was not reflected in the biochemical studies. We investigated the effect of mutating the highly conserved ATP hydrolysis and Mg²⁺ binding residues of MLH1 and PMS2 in mammalian cell lines. Amino acid substitutions in MLH1 intended to impact either ATP binding or hydrolysis disabled MMR, as measured by instability at microsatellite sequences, to an extent similar to *MLH1* null mutation. Furthermore, cells expressing these *MLH1* mutations exhibited resistance to the MMR-dependent cytotoxic effect of 6-thioguanine (6-TG). In contrast, ATP hydrolysis and binding mutants of PMS2 displayed no measureable increase in microsatellite instability or resistance to 6-TG. Our findings suggest that, *in vivo*, the integrity of the MLH1 ATPase domain is more critical than the PMS2 ATPase domain for normal MMR functions. These *in vivo* results are in contrast to results obtained previously *in vitro* that showed no functional asymmetry within the MutL α ATPase, highlighting the differences between *in vivo* and *in vitro* systems.

1. Introduction

DNA mismatch repair (MMR) is a highly conserved cellular function that corrects replication errors, signals apoptosis in response to certain DNA damaging agents, and suppresses homeologous recombination [1]. In cells compromised for MMR, mutation rates can increase by as much as 1000-fold [2]. In humans, germline mutations in certain MMR genes can cause increased cancer susceptibility, notably hereditary nonpolyposis colorectal cancer [3].

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Conflict of interest

The authors declare that there are no conflicts of interest.

The process of mismatch repair is best understood in the bacterium *Escherichia coli*, where a minimal system was reconstituted *in vitro* [4]. MMR is initiated when a homodimer of the MutS protein recognizes and binds to a mismatch [5,6]. Next, MutS recruits the MutL homodimer, which has been termed a “molecular matchmaker” as it couples mismatch recognition to downstream repair events, including strand discrimination, excision, and resynthesis [7].

Multiple MutL homologues exist in eukaryotes, each functioning as heterodimers [8,9]. The predominant heterodimer involved in MMR is MutL α , which consists of MLH1 and PMS2 (PMS1 in yeast) [9]. Similar to MutL, MutL α interacts with multiple MMR components, including MutS homologues, the replication processivity factor PCNA, and the exonuclease EXO1 [10–12].

The first described biochemical activity assigned to MutL and its homologues was an ATPase activity. Sequence analysis and determination of the crystal structure of the N-terminus assigned MutL to the GHL ATPase family, which also includes gyrase B and Hsp90 [13,14]. All GHL members contain an ATPase domain composed of four highly conserved N-terminal motifs necessary for protein dimerization and function [14]. MutL was shown to undergo an ATPase cycle whereby ATP binding caused a conformational change leading to dimerization of the N-termini [13]. It was subsequently shown that ATP binding was necessary for recruitment and activation of downstream MMR components, including MutH and UvrD [15].

Unlike bacterial MutL, MutL α in eukaryotes exists as a heterodimer. The first study to address the consequences of individually mutating the components of MutL α supported a functional asymmetry *in vivo* in the contributions of the Mlh1 and Pms1 ATPase domains to MMR [16]. Together with a second *in vivo* study [17], the alanine substitutions impacting the ATPase domain of Mlh1 had a much greater impact on MMR-dependent mutation avoidance than the corresponding Pms1 mutations. In stark contrast, ATP binding and hydrolysis mutants of either human MLH1 or PMS2 both strongly compromised MutL α activity as determined by *in vitro* MMR assays [18,19].

The apparent lack of functional asymmetry within MutL α suggested by the human *in vitro* studies is at odds with the asymmetry observed in budding yeast. This discrepancy could reflect a functional difference between the MutL α ATPase of yeast and human cells. Another explanation is that the yeast studies were *in vivo* while the human studies were conducted *in vitro*. To further probe the discrepancy, we stably expressed exogenous human WT or mutant forms of MLH1 or PMS2 in mouse cells null for either MLH1 or PMS2. We then assayed for repair efficiency by microsatellite instability at mono- and dinucleotide runs. In addition, we compared the MMR-dependent response to the methylation mimetic drug 6-thioguanine among the cell lines. We found that whereas ATP binding or hydrolysis mutants of MLH1 strongly impacted both MMR-dependent spell-checking and damage response functions, the equivalent mutants of PMS2 had no detectable impact on MMR. These results support a functional asymmetry between the ATPase domains of MLH1 and PMS2 and highlight the level of functional conservation between yeast and mammalian MutL α .

2. Materials and methods

2.1 Cell lines and media

MC2 *Mlh1*^{-/-} and WT complemented cell lines were derived as described [20]. *MLH1*-E34A and *MLH1*-N38A cells were created by transfecting plasmids expressing *MLH1*-E34A and *MLH1*-N38A human *MLH1* cDNA off the CMV promoter into *Mlh1*^{ko/ko} mouse

embryonic fibroblasts (MEFs). 20 μ g linearized DNA was electroporated into 1×10^7 cells at 0.32 kV and 500 μ F. Stably expressing transfectant colonies were selected using neomycin at 200 μ g/mL.

C18 *Pms2*^{-/-} and WT complemented cells were derived as described [21]. *PMS2-E41A*, and *PMS2-N45A* cells were created as above, only using *PMS2-E41A* and *PMS2-N45A* human *PMS2* cDNA containing plasmids transfected into *Pms*^{ko/ko} MEFs. Stably expressing transfectant clonal lines were selected using puromycin at 2 μ g/mL.

All cell lines were maintained in DMEM supplemented with 10% BCS, 1xNEAA, and 50 μ g/mL gentamycin sulfate and grown in a humidified incubator with 5% CO₂.

For microsatellite analysis, cell lines were subcloned, passaged at 1:10 for two weeks, and then 300 cells were plated on 150 mm plates for colony formation. For each cell line, at least ten clones stably expressing the various *MLH1* and *PMS2* expression vectors were expanded and harvested for genomic DNA.

2.2 Plasmid construction

MLH1-E34A, and *PMS2-E41A* were constructed as described [18], only the cDNA containing the mutation was cloned into the expression vectors pCMV-neo (for *MLH1*) and pCMV-puro (for *PMS2*). *MLH1-N38A* and *PMS2-N45A* were created by site-directed mutagenesis (Stratagene) of pBluescript plasmids containing the *MLH1* or *PMS2* cDNA, according to manufacturer's protocol. Primers containing the desired mutations were used for PCR with the plasmids as template:

5'CAAAGAGATGATTGAGGCCTGTTTAGATGCAAAATCC3' and 5'GGATTTTGCATCTAAACAGGCCTCAATCATCTCTTTG3' for *MLH1-N38A* and 5'GGTAAAGGAGTTAGTAGAAGCCAGTCTGGATGCATGCTGG3' and 5'CCAGCATGCATCCAGACTGGCTTCTACTAACTCCTTTACC3' for *PMS2-N45A*. The cDNAs were cloned into either pCMV-neo or pCMV-puro and mutations confirmed by sequencing.

2.3 Western blots

1×10^6 cells were lysed in 1x SDS sample buffer, heated at 95° for 5 min, and 50 μ g total protein run on an 8% SDS-PAGE gel. The protein was transferred to a PDVF membrane and probed with mouse anti-human MLH1, PMS2, and MSH6 antibodies (BD Biosciences) at 1:1000, 1:500, and 1:2500. Membranes were probed with goat anti-mouse HRP secondary antibody (Jackson ImmunoResearch) at 1:1000. Bands were visualized using enhanced chemiluminescence (Perkin Elmer).

2.4 DNA preparation

Genomic DNA was prepared by trypsinizing cells, washing in PBS, and resuspending in DNA lysis buffer containing 10mM Tris pH 7.5, 10mM EDTA pH 8, 10mM NaCl, and 0.5% sarkosyl, plus 1 mg/mL proteinase K. Lysates were incubated overnight at 55° and the proteinase K was heat inactivated at 95° for 5 min.

2.5 Microsatellite instability analysis

Six loci containing dinucleotide (CA)_n repeats of repeat length n=17–34 were utilized. DNA was diluted 1:10 and subjected to PCR using the following primers:

D4MIT27-F:5'GCACGGTAGTTTTTCCAGGA3'

D4MIT27-R:5'TGGTGGGCAGGCAATAGT3'

D19MIT41-F:5'AGCCCTCCACCCAGTTTC3'
 D19MIT41-R:5'TCTGGGGAAAAAGGATGAGA 3'
 D13MIT67-F:5'TTTCATGGAGTCGAGTATTTTGG 3'
 D13MIT67-R:5'ATCTTGCATAGAACCTTTGGATG3'
 D16MIT4-F:5'AGTTCCAGGCTACTTGGGGT3'
 D16MIT4-R:5'GAGCCCTCATTGCAAATCAT3'
 D17MIT123-F:5'CACAAGGAGGGAGCCTGTAG3'
 D17MIT123-R:5'CACCGTAAGAGTCTAATAATAAGGGG3'
 D13MIT139-F:5'AGAATAAGTCAAGGCTATGATGTGG3'
 D13MIT139-R:5'TTGTTTGTGTTTGAAGTAGAACG 3'

PCRs were conducted using a Robocycler under the following conditions: 95°C 5 min, 64° 1 min, 72° 1 min, 95° 1 min, 62°/60°/58° 1 min, 72° 1 min, 95° 1 min, 56°/52° 1 min, 30 cycles of 95° 1 min, 52° 1 min, 72° 1 min, with a final extension of 72° 3 min. PCR products were loaded onto a 7% denaturing acrylamide gel. The DNA was transferred to a nitrocellulose membrane and hybridized using a biotin-labeled (CA)¹⁰ oligodeoxynucleotide. P-values were calculated using Fisher's exact t-test on Prism v. 3.0 software.

Six loci containing (A)_n runs of repeat length n=23–37 were analyzed. A universal system of fluorescently tagged M13 primers was used to label PCR products for analysis [22]. All forward primers contained a 5' M13 tail of the following sequence: 5'CACGACGTTGTAAAACGAC3'. Fluorescently labeled M13 forward primer was included to produce a 3-primer reaction resulting in amplification of the mononucleotide loci with a fluorescently labeled M13 end. PCR was carried out using the following primers to amplify the A run loci:

uPAR-F:5'CACGACGTTGTAAAACGACGCTCATCTTCGTTCCCTGTC3'
 uPAR-R:5'GTTTCTTCATTTCGGTGGAAAGCTCTGA3'
 JH116-F:5'CACGACGTTGTAAAACGACCTCTGGGCATACCAGCTACTG3'
 JH116-R:5'CTCCATCCTGTGAGGTTAAACACAT3'
 27A-F:5'CACGACGTTGTAAAACGACCTATTGGATAAGTATGAGGTACTG3'
 27A-R:5'GTTTCTTCACCATAGTGCTAGCAATCAAGTGG3'
 23A-F:5'CACGACGTTGTAAAACGACTTGCTGAATTGGTGAGCTTC3'
 23A-R:5'GTTTCTACGTCAAAAATCAATGTTAGG3'
 M-BAT 26-F:5'CACGACGTTGTAAAACGACTCACCATCCATTGCACAGTT3'
 M-BAT 26-R:5'GTTTCTCTGCGAGAAGGTACTCACCC3'
 M-BAT 37-F:5'CACGACGTTGTAAAACGACTCTGCCCAAACGTGCTTAAT3'
 M-BAT 37-R:5'GTTTCTCCTGCCTGGGCTAAAATAGA3'

PCR conditions were as follows: 95° 5 min, 20 cycles of 94° 15 sec, 65° 20 sec (–1° per cycle), 72° 20 sec, 35 cycles of 92° 15 sec, 50° 15 sec, 72° 15 sec, and a final extension of 72° for 10 min. PCR products were subjected to fragment size analysis by the OHSU MMI Sequencing Core using an ABI Prism Sequencer and analyzed using Peak Scanner software.

2.6 6-TG colony formation assay

Cells were plated out onto 100 mm plates at a density of 300 or 500 cells per plate. After 24 hours, 6-thioguanine (Sigma) was added for 24 hours, the cells were washed and fresh media was added. 7–10 days after addition of 6-TG the media was removed and the plates were fixed in 25% ethanol, 50% methanol, 0.25% methylene blue to stain the colonies. Colonies with greater than 50 cells were counted. For each dose of 6-TG, three plates were analyzed, and the experiment was repeated three times. Results represent the average of three experiments.

3. Results and discussion

3.1 Generation of *MLH1* and *PMS2* ATPase mutant cell lines

To assess the effect of alanine substitutions within the ATPase domain of *MLH1* and *PMS2* on the function of mammalian MutL α , stable cell lines were generated by transfecting WT or mutant *MLH1* or *PMS2* human cDNA into spontaneously established *Mlh1*^{-/-} or *Pms2*^{-/-} mouse embryonic fibroblasts (MEFs) and selecting for stably expressing colonies. Two alanine substitution mutations were examined, one at a residue critical for ATP hydrolysis (*MLH1-E34A* and *PMS2-E41A*) and one at a residue important for Mg²⁺ binding, which is necessary for coordination of ATP binding (*MLH1-N38A* and *PMS2-N45A*) [18,19]. To ensure similar levels of protein expression among the cell lines, quantitative Western blots were performed using the MMR protein MSH6 as a loading control. We selected cell lines that expressed the ATPase mutants at a level similar to that of the corresponding WT protein expressing lines that showed full complementation. As seen in Figure 1, the *MLH1* and *PMS2* ATP hydrolysis and Mg²⁺ binding mutant proteins are expressed at approximately the same level as the corresponding exogenously expressed WT proteins.

3.2 Asymmetric microsatellite instability phenotypes of MutL α ATPase mutants

Once equal expression levels between the cognate cell lines were determined, we performed microsatellite instability assays. Cells were subcloned, passaged for two weeks, and plated out to establish individual clones. At least 10 clones per cell line were expanded, again tested for stable protein expression, and DNA prepared for PCR analysis. PCR was conducted using primers specific for di- or mononucleotide runs that had previously been established to show high instability in MMR-null cell lines [20,23].

For the dinucleotide repeat analysis, the PCRs were run on a 7.5% acrylamide gel and analyzed for shifts. As seen in Table 1, the knockout cell lines for both *Mlh1* (MC2) and *Pms2* (C18) had a significantly higher frequency of dinucleotide repeat mutation than the WT complemented lines, with 11.1% for MC2 *Mlh1*-null cells and 2.2% for the *MLH1* WT-expressing cells ($p=0.001$, Fisher's t-test) and 12.9% and 1.5% for the C18 *Pms2*-null and *PMS2* WT-expressing cells, respectively ($p=0.001$). These results are consistent with previous reports showing an increase in microsatellite instability when MMR is compromised [24]. Of interest, both *MLH-E34A* and *MLH1-N38A* showed a frequency of mutation that was equivalent to the MC2, *Mlh1*^{-/-} cells. *MLH1-E34A* showed a frequency of 6.5% ($p=0.015$ vs WT) and *MLH1-N38A* had a frequency of 9% ($p=0.003$ vs WT). On the other hand, *PMS2-E41A* and *PMS2-N45A* had a frequency of mutation that was equal to the C18 + WT *PMS2* complemented cell line (3% and 3.8%, $p=0.68$ and $p=0.43$ vs WT). These results suggest that at dinucleotide runs, the *MLH1* ATPase mutants impact repair to a greater degree than the *PMS2* mutants.

Next, for mononucleotide analysis, the PCR products were fluorescently tagged and subjected to fragment length analysis as described in Materials and Methods. As shown in Table 2, the MC2 *Mlh1*^{-/-} and C18 *Pms2*^{-/-} cells had a frequency of mononucleotide

mutation significantly different than the WT complemented cells (19.7% and 13% vs 1.4% and 3.5%, $p=0.002$ and 0.01). *MLH1-E34A* had a frequency of slippage that was equivalent to the MC2 *Mlh1*^{-/-} cells (12.5% $p=0.007$ vs WT), while the *PMS2-E41A* and *PMS2-N45A* cells had a mutation frequency that was similar to the *PMS2* WT complemented line (2.1% $p=0.69$ and 3.6% $p=1$). The *MLH1-N38A* cells, however, displayed an “intermediate” phenotype, with 8.3% mutation. The mutation frequency of the *MLH1-N38A* cells was statistically different from both the complemented ($p=0.05$) and MC2 parent cell lines ($p=0.02$). Overall, the *MLH1* ATPase mutants displayed a greater degree of instability at mononucleotide runs than the corresponding *PMS2* mutants.

Taken together, the mono- and dinucleotide MSI data showed that mutations disabling either the *MLH1* ATP binding or hydrolysis functions impacted MMR-dependent mutation avoidance *in vivo* to a greater extent than the equivalent *PMS2* mutants.

3.3 Asymmetric cytotoxic response to 6-thioguanine of MutL α ATPase mutants

MMR has been shown to be necessary to signal apoptosis in response to the S_N1-type DNA methylation mimetic drug 6-TG [25]. To assess whether the ATPase mutations of *MLH1* and *PMS2* disrupt this MMR-dependent response, we compared 6-TG resistance between the *MLH1* and *PMS2* parental, WT complemented, and mutant expressing cell lines. As shown in Figure 2, the *Mlh1*-null MC2 cells showed increased resistance to 6-TG when compared to the WT complemented cells at all drug doses examined except the highest dose. Interestingly, the ATP hydrolysis and Mg²⁺ binding mutants of *MLH1* displayed a 6-TG resistance level similar to the MC2 cells. At only the highest dose of 6-TG did the mutant the cells form colonies at a frequency equal to WT. It was previously shown that high doses of methylating agents trigger a cellular response independent of MMR [26].

Similar to MC2, C18 cells were more resistant to 6-TG than *PMS2* WT complemented cells, as shown in Figure 2. In contrast to the *MLH1* ATPase mutants, the *PMS2* ATP hydrolysis and Mg²⁺ binding mutants were indistinguishable from the WT complemented cells at all 6-TG doses. Therefore, the alanine substitutions of *PMS2* that were intended to disrupt either ATP binding or hydrolysis had no effect on the MMR-dependent response to 6-TG.

The 6-TG response results for the *MLH1* and *PMS2* cell lines are consistent with the MSI results presented above. Both the *MLH1* ATP hydrolysis and Mg²⁺ binding mutants disabled repair *in vivo* as measured by increased microsatellite instability. In addition, the *MLH1* mutant cell lines were resistant to the cytotoxic effect of 6-TG, providing further evidence that mutations disabling the *MLH1* ATPase domain disrupt overall MMR function. The *PMS2* ATP hydrolysis and Mg²⁺ binding mutant cell lines did not display increased microsatellite instability, and showed no increased resistance to the cytotoxic effects of 6-TG, suggesting that, *in vivo*, *PMS2* ATPase domain function is less critical for MMR function than *MLH1* ATPase domain function.

3.4 Discussion

Previous work in yeast demonstrated an asymmetry in the contribution of the ATPase domains of Mlh1 and Pms1 to MMR function. Mutations impacting ATPase domain of Mlh1 impacted MMR-dependent mutation avoidance to a greater degree than equivalent the mutations of Pms1 [16,17]. In contrast to yeast, mutations in the ATPase domain of human *MLH1* and *PMS2* affected repair equally, as measured by an *in vitro* repair assay [18,19]. However, when we stably expressed equivalent ATP binding and hydrolysis mutations in mouse cells, we obtained results consistent with a functional asymmetry in the contribution of the ATPase domains of *MLH1* and *PMS2* to MMR. Specifically, our findings suggest that ATP binding and hydrolysis by *MLH1* are more critical for MMR than ATP binding

and hydrolysis by PMS2. Differential binding of ATP by MLH1 and PMS2 could account for this asymmetry, as it has been shown that yeast Mlh1 has a >10-fold higher affinity for ATP than does Pms1, and that Mlh1 appears to bind ATP before Pms1 [17]. In turn, because MutL α is responsible for recruiting downstream factors such as EXO1 and PCNA [11,12], the ATPase domain of MLH1 may be more critical for this recruitment than the ATPase domain of PMS2.

Interestingly, the findings presented here and those of others [16–19] show that the ATP binding and hydrolysis residues of PMS2 are critical for full MMR activity *in vitro* but dispensible *in vivo*. One possible explanation is that a factor, which can compensate for the ATPase activity of PMS2 *in vivo*, is not active in *in vitro* extracts. Another possibility is that the PMS2 ATPase is necessary for assembling an active MMR complex *in vitro*, whereas that complex is normally assembled *in vivo*, perhaps by the close association of MMR with the replication apparatus [11].

While the PMS2 ATPase domain does not appear to be critical for MMR-dependent mutation avoidance or the 6-TG damage response *in vivo*, it may be critical for certain MMR-dependent recombination functions. Previous analysis with yeast Pms1 ATPase mutants demonstrated that the Pms1 ATPase domain is more critical for repair of recombination intermediates than for repair of replication errors [27]. It would be interesting to analyze the contribution of the mammalian PMS2 ATPase domain to processing recombination intermediates. Further investigation into the MLH1 and PMS2 ATPase domains of MutL α should provide more insight into the biochemical basis for the observed functional asymmetry described here.

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Figure 1. Protein expression in MC2 and C18 parent and complemented cell lines

Western blots of MC2 (*Mlh1*^{-/-}) cells expressing WT MLH1, *MLH1*-E34A, and *MLH1*-N38A in the left panel and C18 (*Pms2*^{-/-}) cells expressing WT PMS2, *PMS2*-E41A, and *PMS2*-N45A in the right panel. The MSH6 signal was used as a loading control. MC2 cells display reduced PMS2 expression, as PMS2 depends on MLH1 for protein stability [23].

Figure 2. 6-Thioguanine response in MLH1- and PMS2-expressing cells

Cells were plated out and exposed to varying doses of 6-thioguanine for 24 hours. Colonies were stained with methylene blue and counted 8–10 days after plating. A) MC2, *MLH1*-WT, *MLH1*-E34A, or *MLH1*-N38A cells. B) C18, *PMS2*-WT, *PMS2*-E41A, or *PMS2*-N45A cells.

Table 1

Microsatellite instability analysis at (CA)_n repeats in *MLH1* and *PMS2* expressing cell lines.

Cell line	#slips/total	Frequency (%)	p-value vs. WT	p-value vs. null
MC2 <i>Mlh1</i> ^{-/-}	23/224	11.1	0.001	
MC2 WT	8/360	2.2		0.001
<i>MLH1-E34A</i>	15/230	6.5	0.015	0.176
<i>MLH1-N38A</i>	12/132	9	0.003	0.58
C18 <i>Pms2</i> ^{-/-}	48/372	12.9	0.001	
C18 WT	2/126	1.5		0.001
<i>PMS2-E41A</i>	4/132	3	0.68	0.007
<i>PMS2-N45A</i>	6/156	3.8	0.43	0.001

Table 2

Microsatellite instability analysis at (A)_n repeats in *MLH1* and *PMS2* expressing cell lines.

Cell line	Slips	Frequency (%)	p-value vs. WT	p-value vs. null
MC2 <i>Mlh1</i> ^{-/-}	17/86	19.7	0.0002	
MC2 WT	1/74	1.4		0.0002
<i>MLH1-E34A</i>	16/128	12.5	0.007	0.2
<i>MLH1-N38A</i>	10/120	8.3	0.05	0.02
C18 <i>Pms2</i> ^{-/-}	16/124	13	0.01	
C18 WT	4/114	3.5		0.01
<i>PMS2-E41A</i>	2/96	2.1	0.69	0.005
<i>PMS2-N45A</i>	4/110	3.6	1	0.017