Contribution of Human Mlh1 and Pms2 ATPase Activities to DNA Mismatch Repair*

Guy Tomer‡, Andrew B. Buermeyer§, Megan M. Nguyen‡, and R. Michael Liskay‡

From the ‡Department of Molecular and Medical Genetics, Oregon Health and Science University, Portland, Oregon 97201 and the §Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, Oregon 97331

MutLo, a heterodimer composed of Mlh1 and Pms2, is the major MutL activity in mammalian DNA mismatch repair. Highly conserved motifs in the N termini of both subunits predict that the protein is an ATPase. To study the significance of these motifs to mismatch repair, we have expressed in insect cells wild type human MutLo and forms altered in conserved glutamic acid residues, predicted to catalyze ATP hydrolysis of Mlh1, Pms2, or both. Using an in vitro assay, we showed that MutLo proteins altered in either glutamic acid residue were each partially defective in mismatch repair, whereas the double mutant showed no detectable mismatch repair. Neither strand specificity nor directionality of repair was affected in the single mutant proteins. Limited proteolysis studies of MutLo demonstrated that both Mlh1 and Pms2 N-terminal domains undergo ATP-induced conformational changes, but the extent of the conformational change for Mlh1 was more apparent than for Pms2. Furthermore, Mlh1 was protected at lower ATP concentrations than Pms2, suggesting Mlh1 binds ATP with higher affinity. These findings imply that ATP hydrolysis is required for MutLo activity in mismatch repair and that this activity is associated with differential conformational changes in Mlh1 and Pms2.

Mismatch repair (MMR) helps to protect the genome from replication errors caused by DNA polymerases. Its pivotal role as caretaker of genome stability is exemplified by HNPCC, a hereditary predisposition to colon, rectal, and other cancers as caretaker of genome stability is exemplified by HNPCC, a hereditary predisposition to colon, rectal, and other cancers

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The MutL-like ATPase domain is highly conserved, suggesting that eukaryotic MutL homologues also possess ATPase activity. Indeed, the N-terminal fragment of human Pms2 has been shown recently to have an ATPase activity catalyzed by the conserved glutamic acid (33). Studies in bacteria and yeast indicate the importance of the ATPase domain in MMR (34, 35).

In this report, we present results demonstrating the importance of the ATPase domains of human MutL during MMR. Furthermore, the results suggest an asymmetry within human MutL as reflected by differential conformational changes in response to adenine nucleotides.

**EXPERIMENTAL PROCEDURES**

**Preparation of MP-1 Cell Line—**Mouse embryonic fibroblasts deficient for both Mlh1 and Pms2 (MP-1) were derived by crossing doubly heterozygous animals in a C57BL/6 genetic background. Embryos were harvested from timed pregnancies, homogenized, and placed in culture as described (36). Immortalized clones arising spontaneously after senescence were pooled and expanded. Genotyping to identify the Mlh1-/−, Pms2-/− cells was performed by using a PCR assay (37, 38) with high molecular weight DNA harvested from surplus tissue and subcloned in plasmid vectors.

**Preparation of Extracts—**Cytoplasmic extracts were prepared as described (39). Cells were grown in up to 40 P-150 plates; trypsinized; washed once with PBS, once with isotonic buffer (20 mM HEPES, pH 7.9, 250 mM sucrose, 5 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride); and then with hypotonic buffer (20 mM HEPES, pH 7.9, 5 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride); resuspended in hypotonic buffer at a density of 7 × 10⁷ cells/ml; and allowed to swell on ice for 15 min. The swollen cells were lysed in a Dounce homogenizer using pestle B and then incubated on ice for 30 min and centrifuged at 2000 × g for 10 min at 4 °C. The supernatant was recentrifuged at 12,000 × g for 10 min at 4 °C. The supernatant from this step, which is the cytoplasmic extract, was aliquoted and frozen in liquid nitrogen.

**Preparation of DNA Substrates—**All substrates were prepared from phage DNA kindly provided by Dr. Paul Modrich. A 5′ nick G-T mismatch DNA substrate was prepared from φM13 phage ssDNA and φM13 phage dsDNA linearized with Sau96I (40). The nick is located 125 bp 5′ to the mismatch on the complementary strand. Repair of the nicked (complementary) strand requires the substrate to be nicked (underlined) in the complementary strand.

To prepare a 3′ nick one-base IDL substrate, a 150-bp PCR fragment site was amplified using PBlueScript as a template and the primers 5′-GTGACTCTAGAGAGTTAGCTCACTCATTAGG-3′ and 5′-CTGACTCTAGAGAGTTAGCTCACTCATTAGG-3′. The fragment that contained an EcoRI site provided by the upstream primer (underlined) was digested with XbaI and cloned into the unique HindIII site in both φM13R2 and φM13R2II, generating φM13R2I and φM13R2RII, respectively. The φN6I site is located 10 bp from the mismatch and on the other side of it relative to Sau96I. φM13R2RII dsDNA was linearized with EcoRI and then mixed with φM13R2RII phage ssDNA. In this substrate, the nick is located 130 bp 3′ to the mismatch on the complementary strand. Sensitivities to restriction enzymes are dependent upon whether the mismatch is an unmatched A in the complementary strand. Sensitivities to restriction enzymes are dependent upon whether the mismatch is an unmatched A in the complementary strand.

The glutamic acid to alanine substitution has been made using the QuikChange site-directed mutagenesis kit (Stratagene). Two 36-mer mutagenic oligonucleotides containing the desired mutation (underlined, from GAG to GCC) in their middle (5′-GGACCTCGTGAATGAGCTAGTGAAGAACGCTCTG-3′ and 5′-CCAGACGTGGTCTAATAGGCGCTTATCCAGCGATGTCG-3′) were annealed on the same sequence on opposite strands of a plasmid containing the human Pms2 cDNA. Similarly, two 38-mer mutagenic oligonucleotides (5′-CGGCCAGCAGCTAAATGCTAAACGCAGATGGAGACTG-3′ and 5′-CATGTCCTACATGCTGTTTAGATACAGTACGTCG-3′) were annealed on opposite strands of the same plasmid. PCR was carried out with PfX DNA polymerase using 16 cycles of 95 °C for 30 s, 55 °C for 5 min, and 68 °C for 12 min each. The products, which are nicked circular strands, were incubated with DpnI to digest the methylated, nonmutated parental DNA template and were used to transform DH10B E. coli cells. Plasmids were retrieved from transformants and sequenced to confirm the presence of the mutation. Fragments spanning the mutation were cut out and replaced by the wild type fragments in Mlh1 or Pms2 cDNAs.

**Expression and Purification of Human MutL—**The Bac-to-Bac baculovirus (Invirogen) expression system was used to express MutLα in Spodoptera frugiperda (SB) cells infected with recombinant baculovirus. Human Pms2 cDNA was cloned into pFastBac DUAL in two steps. A 760-bp fragment from the 5′-end was PCR-amplified using an upstream primer (5′-ATACGCTGGGTATCGGATCATCTACATCATCACATCGAGCGACGTGAGCTCGAG-3′) encoding six consecutive histidines in front of the second amino acid and 5′-GGGGAGCTGAGCAGAGAAAGG as downstream primer. The amplified fragment was digested with BamHI and PvuII and subcloned into pFastBac DUAL between BstXI and PvuII unique sites. This plasmid was cleaved with PvuII and ligated to the 3′ fragment of Pms2. Mlh1 cDNA was subcloned into the resulting plasmid between BstXI and EcoRI in the other multicloning site under the polyhedrin promoter. Recombinant pFastBac DUAL plasmids were used to transform DH10B E. coli cells (Invirogen) that contain baculovirus shuttle vector (bacmid). Transformants in which the expression cassettes containing the cloned cDNAs from the
The column was washed with 10 volumes of buffer H plus 20 mM "human/mouse complementation of extracts from a MEF cell line (MP-1) generated after expression in insect cells (Fig. 1). 7.5, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA)." The conserved glutamic acid residue that has been substituted to alanine in this study is shown in italics. The conserved glutamic acid residue because it was clearly implicated in the catalysis of an ATPase motif I in the GHL superfamilies. The aligned conserved glutamic acid residue that has been substituted to alanine in this study is shown in boldface capital letters.

RESULTS

Human MutLα Complements Extracts from MMR-deficient Mouse Embryonic Fibroblasts (MMFs) — Human MutLα, purified after expression in insect cells (Fig. 1A), was tested for complementation of extracts from a MEF cell line (MP-1) genetically deficient in both Mlh1 and Pms2. The validity of this "human/mouse" approach is supported by reports showing that a human Mlh1 cDNA can complement the "MMR" phenotypes of Mlh1-deficient MEFS (42) and that human chromosome 2 contains the MSH2 allele complements mouse Msh2/− cells (43). These results indicate the high degree of conservation of the mammalian MMR system.

We used mismatch-containing plasmid substrates as previously described (44) with the modification of using cytoplasmic extracts instead of nuclear. As shown in Fig. 2, A (lane 1) and B (lane 1), MP-1 cytoplasmic extract was inefficient in the repair of a 5′ G-T and a 5′ one-base IDL (insertion deletion loop) heteroduplexes, respectively. The addition of recombinant human MutLα restored repair activity (Fig. 2, A (lane 3) and B (lane 2)). These repair levels were comparable with those of MMR-proficient MEF and HeLa cell cytoplasmic extracts (data not shown) and were in the range reported for HeLa cell nuclear extracts (10, 44). This repair activity meets other criteria characteristic of in vitro MMR including sensitivity to aphidicolin (data not shown) and being limited to the nicked strand (Fig. 2A, compare lanes 3 and 4).

Conserved Glutamic Acid Residues in the N Terminal of Mlh1 and Pms2 Are Each Required for in Vitro MMR — To test the role of the putative ATPase domain of human MutLα in MMR, we mutated a conserved glutamic acid residue in the N terminus of Mlh1 and Pms2 (Fig. 1B). We chose to mutate this residue because it was clearly implicated in the catalysis of ATP hydrolysis in human Pms2 (33) as well as E. coli MutL (28) and gyrase B (30) and has been shown to be essential for in vitro MMR in E. coli (45) and yeast (35).

We have purified human MutLα expressed in insect cells, in

FIG. 1. Wild type and mutant MutLα proteins. A, shown is a Coomassie-stained SDS-PAGE gel. Lane 1, SF9 cells were infected with recombinant baculovirus encoding wild type human MutLα at a multiplicity of infection of 3 for 48 h. Lysate was prepared as described under "Experimental Procedures" and loaded on a nickel column (Ni2+-nitrilotriacetic acid; Qiagen). Proteins that bound the column in the presence of 20 mM imidazole were eluted with 500 mM imidazole. Lanes 2–5, the eluate shown in lane 1 and similar eluates from purifications of the three mutant MutLα proteins were further purified using a ResourceQ column (Amersham Biosciences), as described under "Experimental Procedures." B, an alignment of ATPase motif I in the GHL superfamily. The conserved glutamic acid residue that has been substituted to alanine in this study is shown in boldface capital letters.

pFastBac DUAL plasmids were transferred by transposition into the bacmid were isolated. Recombinant bacmid preparations from these transformants were used to transfect SF9 cells. 150–300 ml of logarithmic phase SF9 cells grown in Sf-900IISFM medium (Invitrogen) supplemented with 20 mM imidazole, was loaded on an Ni2+-nitrilotriacetic acid column (Qiagen) equilibrated with buffer H (25 mM Tris, pH 7.9, 100 mM KCl, 1% Nonidet P-40, 5 mM 2-mercaptoethanol, 5 mM phenylmethylsulfonfonyl fluoride, one EDTA free protease inhibitor mixture tablet (Roche Molecular Biochemicals)). The supernatant, supplemented with 20 mM imidazole, was loaded on an Ni2+-nitrilotriacetic acid column (Qiagen) equilibrated with buffer H (25 mM Tris, pH 7.9, 0.5 mM NaCl, 10% glycerol, 5 mM 2-mercaptoethanol) plus 20 mM imidazole. The column was washed with 10 volumes of buffer H plus 20 mM imidazole, and the bound protein was eluted with buffer H plus 500 mM imidazole. The eluate from the nickel column was diluted to 100 mM NaCl and loaded on a 1-ml Resource Q column (Amersham Biosciences), washed with buffer T (50 mM Tris, pH 7.8, 10% glycerol, 0.01% Nonidet P-40) plus 100 mM NaCl, and eluted with a 0.1–1 M NaCl gradient in buffer T. Fractions containing MutLα that eluted at ~0.3 M NaCl were pooled and concentrated using a Centricon 30 concentrator (Amicon), and the buffer was exchanged to MutL storage buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM EDTA).

RESULTS

Human MutLα Complements Extracts from MMR-deficient Mouse Embryonic Fibroblasts (MMFs)—Human MutLα, purified after expression in insect cells (Fig. 1A), was tested for complementation of extracts from a MEF cell line (MP-1) genetically deficient in both Mlh1 and Pms2. The validity of this "human/mouse" approach is supported by reports showing that

FIG. 2. Agarose gel analysis of products from in vitro MMR assays. A, a cytoplasmic extract of MP-1 cells was incubated with a 5′ nicked G-T heteroduplex (schematic illustration shown to the right) alone (lanes 1 and 2), in the presence of 0.1 μg of wild type (lanes 3 and 4) or mutant MutLα proteins (lanes 5–10). The DNA from each reaction was purified and was split to two reactions. One half was digested with HindIII to probe repair of the complementary (nicked) strand of the DNA substrate (C). The other half was digested with Clai plus XhoI to probe repair of the viral (continuous) strand (V). The arrows indicate unrepaired DNA (6.4-kb band) and repaired DNA (3.3- and 3.1-kb bands). The intermediate band (3.2-kb) is a cleavage product of Sau96I that denotes the cleavage site of the phage dsDNA used to prepare the heteroduplex. This is also the location of the 5′-nick in the heteroduplex. B, a similar in vitro repair assay was carried out with a 5′ nicked one-base IDL heteroduplex, with the DNA digested with Clai plus XcmI to probe repair of the complementary (nicked) strand.

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which the glutamic acid residue was substituted to alanine in either Mlh1 at position 34 (MutL<sup>A</sup>mE34A), Pms2 at position 41 (MutL<sup>A</sup>mE41A), or both (MutL<sup>A</sup>mE34A/pE41A) (Fig. 1A). Studies showed that heterodimer formation is dependent primarily on the C termini of Mlh1 and Pms2 (46). Therefore, not surprisingly, these mutations did not affect heterodimer formation as evidenced by similar ratios of the two protomers in both the wild type and mutant dimers (Fig. 1A). Moreover, the expression levels and protein yields were similar to wild type MutL<sup>A</sup> proteins, residual repair of the G-T mismatch was limited to the nicked strand (Fig. 2A).

FIG. 4. Limited V8 proteolysis analysis of wild type human MutL<sup>A</sup>. In the Western blot shown, wild type human MutL<sup>A</sup> was preincubated with or without 5 mM ATP for 15 min, followed by the addition of V8 protease (Promega). Samples (20 μl) were taken after 1, 2, 5, 10, and 20 min after the addition of the protease, immediately boiled for 8 min in the presence of SDS-PAGE sample buffer, separated by SDS-PAGE, blotted on Immobilon-P membranes, and probed with either anti-Mlh1 antibody (A) or anti-Pms2 antibody (B) against the N termini of the proteins, as described under "Experimental Procedures." ATP-dependent Conformational Changes in Wild Type MutL<sup>A</sup> Studied by Limited Proteolysis—We next asked whether the putative ATPase activity of MutL<sup>A</sup> drives conformational changes similar to MutL, and asked what is the contribution of the individual protomers. Limited proteolysis analysis is often used to detect conformational changes in proteins (48). Previous analysis of yeast MutL<sup>A</sup> has suggested ATP-dependent conformational change in Mlh1 (35). ATP-dependent conformational changes have been reported recently for an N-terminal fragment of yeast Pms1 (49) but not in the context of the full-length MutL<sup>A</sup>.

Here, we performed limited proteolysis followed by Western blotting, using specific antibodies to the N termini of Mlh1 or Pms2, to study the effect of ATP on possible conformational changes in each protomer of human MutL<sup>A</sup>. Because the epitope for the Mlh1 and Pms2 antibodies is located at the N terminus of both protomers (residues 1–20), all of the detected fragments in Fig. 4, A and B, should contain the intact N termini of Mlh1 and Pms2, respectively, but have different C termini.

Using V8 protease, Mlh1 was digested from the full-length size of 80 kDa to fragments of 49, 40, and 30 kDa and smaller sizes (Fig. 4A). In the presence of 5 mM ATP, the 40- and 30-kDa N-terminal fragments were protected, compared with continued degradation without ATP. Pms2 was cleaved by V8 protease from the full-length size of 100 kDa to a 47-kDa N-terminal fragment and subsequently to a 40-kDa N-terminal fragment (Fig. 4B). ATP (5 mM) retarded the degradation slightly, as shown by the presence of an 18-kDa fragment seen only without ATP. However, there was no detectable protection of the 47- and 40-kDa fragments by ATP.

Trypsin, which has different substrate specificity than V8, digested MutL<sup>A</sup> more rapidly, and ATP-induced protection of both Mlh1 and Pms2 was more apparent (Fig. 5). The protec-
tion of ATP, we repeated the assay but with various ATP concentrations (Fig. 6A). We also used a higher trypsin/protein ratio (1:4 compared with 1:10 in the previous experiment) in order to facilitate quantitation of Pms2 protected fragments.

Similar to the previous conditions, we could not detect the N-terminal fragments of Mlh1 in the absence of ATP (Fig. 6A, lanes 1–3). In contrast to the previous conditions, the N-terminal fragments of Pms2 were not detected without ATP, reflecting the higher trypsin concentration in this assay. The degradation rate of Mlh1 and Pms2 was more rapid than in the previous assay (compare Fig. 6C with Fig. 5E), again consistent with higher trypsin concentrations in this assay. Here, again, Mlh1 was more resistant than Pms2 (Fig. 6C).

Mlh1 and Pms2 N termini were protected by ATP in a concentration-dependent manner (Fig. 6A; quantitation in Fig. 6B). We calculated $K_{1/2}$ values for Mlh1 and Pms2. These are the concentrations of ATP that give the half-maximal effect on proteolysis and are expected to reflect dissociation constants or the affinities for the nucleotide. Assuming that at 10 mM ATP Mlh1 or Pms2 are saturated and therefore maximally protected, $K_{1/2}$ for the Mlh1 30-kDa fragment occurred at $<0.1$ mM ATP, whereas for the Pms2 40-kDa fragment $K_{1/2}$ occurred between 0.1 and 0.5 mM ATP (Fig. 6B).

**Effect of Glutamic Acid Substitutions on ATP-induced Conformational Changes**—We next studied the effects of altering the conserved glutamic acid residues on the trypsin digestion patterns of the N-terminal domains of human MutLα using essentially the same assay as described above. Similar to wild type MutLo, the three altered forms were protected by ATP in a concentration-dependent manner (Fig. 6, D, G, and J).

The limited proteolysis results for Mlh1 and Pms2 N termini in MutLo mE34A (Fig. 6D) were similar to that of wild type MutLo, both in terms of the $K_{1/2}$ values (Fig. 6E) and stability of the N-terminal fragments (Fig. 6F). The digestion pattern of the Mlh1 N terminus in MutLo pE41A (Fig. 6G) was similar to Mlh1 in wild type MutLo. In contrast, the Pms2 N terminus cleavage pattern in MutLo pE41A was different than in wild type MutLo in that a higher ATP concentration was required for protection. Specifically, the $K_{1/2}$ value for Pms2 occurred at 0.5–10 mM ATP (Fig. 6H) compared with 0.1–0.5 mM for wild type MutLo (Fig. 6B) and MutLo mE34A (Fig. 6E). One explanation for this difference is that the Pms2 E41A mutation may reduce ATP binding in addition to abolishing ATP hydrolysis. This explanation would be consistent with the finding that the equivalent mutation in MutL, E29A, reduced ATP binding (28).

The proteolytic pattern of the double mutant MutLo mE34A/pE41A was different from that of the other three forms of MutLo (Fig. 6J) in that the Mlh1 and Pms2 N termini were more protected by ATP, as evident by the slower degradation rate (Fig. 6L, compare with Fig. 6, C, F, and I). The Mlh1 30-kDa fragment was essentially stable in the double mutant. Interestingly, the $K_{1/2}$ value of Mlh1 N-terminal domain in the double mutant protein was higher (0.1–0.5 mM) than that of the same protomer in the single mutant protein, MutLo mE34A ($<0.1$ mM) (Fig. 6, compare K with E). Thus, Mlh1 carrying the E34A substitution showed different apparent affinities to ATP, depending on Pms2 status. In contrast, Pms2 carrying the E41A substitution showed an “inherent” ATP binding defect with a $K_{1/2}$ in the range of 0.5–10 mM ATP, similar to the same Pms2 protomer in the single mutant protein, MutLo pE41A. Therefore, the E34A substitution by itself may not affect ATP binding to Mlh1.

**Limited Proteolysis of Wild Type MutLo with Nonhydrolyzable ATP Analogs**—The protection from proteolysis of mutant MutLo proteins predicted to be deficient in ATP hydrolysis suggests that ATP binding but not hydrolysis is necessary for this effect. To test this possibility further, we studied the ef-
FIG. 6. Limited trypsin proteolysis analysis of wild type and mutant MutLo proteins as a function of ATP concentration. Left column, wild type MutLo (A) or mutant MutLo proteins (D, G, and J) were preincubated for 5 min with varying ATP concentrations for 5 min prior to trypsin addition. Subsequently, samples (20 μl) were taken at 5, 10, and 20 min, immediately boiled for 8 min in the presence of SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by Western blotting with antibody against the N terminus of either Mlh1 or Pms2. Only protected bands are shown (40-kDa Pms2 fragment and 30-kDa Mlh1 fragment). The blots were quantitated as described for Fig. 5. The graphs on the right quantitate the corresponding blot on the left column (e.g. B and C represent the blot shown on A). The schematic diagram on the left represents the MutLo proteins in each row: Mlh1 (M), Pms2 (P), and glutamic acid substitution (ε). For each Western blot shown, there are two readings: the extent of conformational change as reflected by the stability of the protected band at a given ATP concentration (right column, Stability) and affinity for ATP-binding/ATP-dependent protection (middle column, ATP titration). Middle column, proteolysis pattern as a function of ATP concentration. Quantitation of band intensities at 5 min is shown. For each blot (A, D, G, and J) in the left column, band intensity in lane 10 (10 mM ATP) was taken as 100% relative to reactions with lower ATP concentrations (lanes 1, 4, and 7). Stippled bars, Pms2; black bars, Mlh1. Right column, stability of the Mlh1 and Pms2 N termini in the presence of 10 mM ATP. For each blot shown in the left column, the band intensity at 5 min (lane 10) was taken as 100% relative to 10- and 20-min time points (lanes 11 and 12, respectively). Stippled bars, Pms2; black bars, Mlh1.

Effects of a nonhydrolyzable ATP analog on limited proteolysis patterns of wild type MutLo. As shown in Fig. 7, AMP-PNP protected N-terminal fragments of both Mlh1 (Fig. 7C) and Pms2 (Fig. 7A) from proteolysis. These fragments were of similar size to those protected by ATP (compare lanes 6–8 with lanes 10–12 in Fig. 7, A and C). Interestingly, AMP-PNP had a differential protection effect on MutLo. It protected Pms2 better than ATP (Fig. 7B), whereas ATP protected Mlh1 better than AMP-PNP, when these nucleotides were at 10 mM (Fig. 7D). Thus, the protection of wild type MutLo by AMP-PNP is not similar to the protection of the double mutant MutLo by ATP (Fig. 6), although *a priori* the former should imitate the hydrolysis mutant. The reason for this may be explained by different affinities of Pms2 and Mlh1 for AMP-PNP. As shown in Fig. 7E, Mlh1 and Pms2 N termini were protected by AMP-PNP in a concentration-dependent manner. However, calculation of the $K_{\text{diss}}$ values (Fig. 7G) suggests that Mlh1 binds AMP-PNP very poorly ($K_{\text{diss}}$ of 0.5–10 mM for AMP-PNP compared with 0.1 mM or less for ATP), whereas Pms2 binding is less affected (Fig. 7F). Using the same assay, Mlh1 showed a similar “binding” defect for ATP/βS, whereas Pms2 had no defect compared with ATP (data not shown). The results with nonhydrolyzable ATP analogs suggest that ATP binding is sufficient for protection of MutLo from proteolysis.

**Limited Proteolysis of Wild Type and the Double Mutant MutLo with ADP**—To study further whether ATP binding is sufficient to drive a conformational change in MutLo, we performed limited trypsin proteolysis of wild type MutLo in the presence of ADP (Fig. 8A). ADP addition resulted in some protection of both Mlh1 and Pms2 (compare lanes 5 and 9 with lane 1). However, the protection of Mlh1 by ADP was negligible compared with ATP (Fig. 8C, ATP/ADP intensity ratio >10 at 10 mM) and was apparent only at 10 mM. In contrast, the protection of Pms2 by ADP was similar to that of ATP (Fig. 8C, ATP/ADP intensity ratio 0.9 at 10 mM) and was apparent at lower nucleotide concentrations. Because wild type MutLo is expected to hydrolyze ATP to ADP, the differential protective effect of Pms2 by ADP might result from ADP generated during hydrolysis. To explore this possibility, we tested the double mutant MutLo mE34A/pE41A, which is predicted not to hydrolyze ATP. The analysis shows (Fig. 8B) that both Pms2 and Mlh1 in the double mutant are protected by ADP, as was shown for the wild type protein (Fig. 8B, compare lanes 9 and 12 with lane 1). However, in contrast to the wild type MutLo, ATP protected Pms2 in the double mutant to a higher extent than ADP (Fig. 8C, ATP/ADP intensity ratio 2.8 at 0.5 mM and 4.6 at 10 mM). Similar to the wild type protein, ATP protected Mlh1 in the double mutant >10-fold more than ADP. These results suggest that Mlh1 and Pms2 differ in their ADP binding properties and/or ADP-induced conformational changes and that the major nucleotide that drives these conformational changes is ATP.

**DISCUSSION**

In this report, we have studied the role of the N-terminal ATPase domains of human Mlh1 and Pms2, which comprise MutLo, in MMR. We compared wild type MutLo with forms in
FIG. 7. Limited trypsin proteolysis of wild type MutLo with ATP and AMP-PNP. A–D, wild type MutLo was preincubated without nucleotide or with 10 μM of either ATP or AMP-PNP for 15 min. Samples were taken just prior to (lanes 1, 5, and 9) or 5, 10, and 20 min following trypsin addition (1:4 trypsin/protein ratio) and were processed as described for Fig. 6. A, limited proteolysis of Pms2 N terminus. B, stability of the 40-kDa Pms2 fragment in A. The band intensity at lane 6 was taken as 100% relative to lanes 7–12. C, limited proteolysis of Mlh1 N terminus. D, stability of the 30-kDa Mlh1 fragment in C. The band intensity at lane 6 was taken as 100% relative to lanes 7–12. E, wild type MutLo was preincubated without nucleotide or with various concentrations of AMP-PNP for 10 min. Reactions started by the addition of trypsin. The samples were processed as described in the legend of Fig. 6. The digestion of Pms2 N terminus (top) or Mlh1 N terminus (bottom) is shown as a function of AMP-PNP concentration. F, relative protection of the N terminus of Pms2, at 5 min, as a function of AMP-PNP or ATP concentrations. For the top blot in E, band intensity in lane 10 (10 μM AMP-PNP) was taken as 100% relative to reactions with lower AMP-PNP concentrations (lanes 1, 4, and 7). The data for Pms2 protection by ATP were taken from Fig. 6B. G, relative protection of the N terminus of Mlh1, at 5 min, as a function of AMP-PNP or ATP concentrations. For the bottom blot in E, band intensity in lane 10 (10 μM AMP-PNP) was taken as 100% relative to reactions with lower AMP-PNP concentrations (lanes 1, 4, and 7). The data for Mlh1 protection by ATP was taken from Fig. 6D.2

which Mlh1 and/or Pms2 were altered at conserved glutamic acid residues, predicted to be crucial for ATP hydrolysis (28). Using an in vitro complementation assay with recombinant forms of MutLo, we have shown that these conserved residues were important for MMR activity. We have used these full-length biologically active proteins in limited proteolysis assays and showed that in the presence of ATP both Mlh1 and Pms2 were more resistant to protease digestion, suggesting that ATP binding induces conformational changes. We also reported differential nucleotide binding and conformational changes between Mlh1 and Pms2, suggesting an asymmetry within MutLo.

The finding that the double mutant MutLo lacked detectable in vitro MMR activity suggests that ATP hydrolysis by human MutLo is essential for its function in MMR and is in agreement with genetic and biochemical studies of MutL proteins in E. coli and S. cerevisiae (28, 35). The data in Fig. 3 suggest that whereas repair by wild type MutLo was saturated early in the MMR reaction, repair by the single mutants continued to increase, suggesting that total repair might approach wild type values with longer incubation times. This suggests that ATP hydrolysis by one protomer may be sufficient for continuing the cycling of MutLo, albeit at a slower rate. In vivo, the time window allowing repair may be limiting, therefore rendering a more severe phenotype to the single mutants. Indeed, Mlh1 null mouse cells expressing human Mlh1E34A show a mutator phenotype indistinguishable from their uncomplemented parent cells.

Based on previous in vitro studies, mismatch repair in mammalian cells has bidirectional capability to initiate repair from nicks located either 5′ or 3′ to the mispair (50). As proposed for E. coli MutL, the ATPase activity of MutLo may coordinate the downstream steps in MMR. A priori, the directionality of repair may depend on inherent asymmetry within MutLo. Using substrates containing either 5′ or 3′ nicks with the single mutants, our results suggest that the ATPase activities of Mlh1 and Pms2 are both important for 5′ and 3′ in vitro MMR. Thus, they are in apparent contrast to previous studies suggesting that Pms2 was more important for 5′ to 3′ repair and Mlh1 was more important for 3′ to 5′ repair (47). Whether other functions of MutLo will show additional effects on the directionality of repair remains to be seen. We also observed that the single mutants partially repaired two mispairs, G-T and one-base IDL. Taken together, our findings suggest that the lack of ATPase activity in MutLo results in a general defect in MMR.

The protection of Mlh1 and Pms2 from proteases in the presence of ATP suggests that the N-terminal domains of both protomers undergo conformational changes. In theory, conformational changes may also render a protein more sensitive to proteases. The finding that each protomer became more resistant to protease digestion, suggesting that ATP binding may coordinate the downstream steps in MMR.

2 A. B. Buermeyer and R. M. Liskay, unpublished data.
protected Mlh1 far better than ADP in both wild type and the double mutant better than ADP (Fig. 8). In contrast, ATP protected Mlh1 and Pms2 from proteolysis.

The finding that the glutamic acid substitutions did not block ATP binding, and the full extent of conformational change de- 
estensive ATP-induced conformational change in Mlh1 compared with Pms2. A less extensive conformational change in human Pms2 is consistent with recent studies on the structure of the N terminus of human Pms2 (33), in which three of the above mentioned five loops are ordered in the monomeric Pms2 N-terminal fragment in the absence of ATP and do not change conformation further with ATP. Although the structure of the N terminus of human Mlh1 has not been reported, we predict, based on our limited proteolysis studies, that more residues become ordered in Mlh1 upon ATP binding than in Pms2.

Another differential effect detected in the limited proteolysis studies was that Mlh1 was protected at lower ATP concentrations than Pms2, suggesting a higher affinity of Mlh1 for ATP, a finding that is consistent with a recent study of N-terminal domains that contain the ATP binding sites are in light gray (for Pms2) and dark gray (for Mlh1). A, the N termini do not interact in the absence of bound nucleotide. The circular shapes represent the presumed “unordered” conformation that is sensitive to trypsin. B, ATP binding induces conformational changes in the N-terminal domains of both proteins, depicted schematically as transition to rectangular shapes. ATP causes a conformational change, possibly rendering residues in the N terminal “ordered” and resistant to trypsin. C, ATPinduced conformational changes cause dimerization of the N termini. Although the conformational changes depicted in B and the resulting dimerization may occur simultaneously, for illustration purposes these events are separated into discrete steps. The interface between the protomers may impart further resistance to trypsin digestion. D, hydrolysis of ATP. Based on the proteolysis data, Mlh1 appears to bind ADP with less affinity than Pms2. Thus, ADP release from Mlh1 may precede that of Pms2. D to A, ADP release cycles MutLb back to the nucleotide-free form.

The significance of the differential binding of nucleotides and conformational changes for Mlh1 and Pms2 to in vivo MMR is not clear. One possible scenario is that Mlh1, which has higher affinity to ATP, binds the nucleotide first and undergoes conformational change. Next, Pms2 binds a second ATP molecule, changes conformation, and hydrolyzes ATP to ADP. The presence of ADP or the conformational change for Pms2 may elicit ATP hydrolysis by Mlh1, which in turn may facilitate ADP release by Pms2. This putative highly ordered cycling between nucleotide-free and -bound forms may regulate the timing of protein-protein interactions during different stages of mismatch repair. A simple model for the ATPase cycle MutL that shows nucleotide binding and the associated conformational

Fig. 8. Limited trypsin proteolysis analysis of MutL with ADP versus ATP. A, wild type human MutL was preincubated either without nucleotide or with varying concentrations of ATP or ADP for 5 min, followed by the addition of trypsin (1:4 trypsin:protein ratio). Samples were taken 5 min later and processed as described for Fig. 6. B, MutL mE34A/pE41A was preincubated without nucleotide or with 0.5 and 10 mM ATP or ADP for 5 min, prior to the addition of trypsin. Samples (20 μl) were taken at 5, 10, and 20 min and processed as described in the legend of Fig. 6. C, relative intensity of protected bands with ATP compared with ADP. The graph shows that for wild type Pms2, ATP-dependent protection was similar to ADP, whereas in the double mutant, ATP resulted in better protection of Pms2 than ADP. As shown, for both wild type and the double mutant, Mlh1 was protected >10-fold more by ATP compared with ADP. The data was extracted from the gels in A and B.

Fig. 9. A model for N-terminal conformational changes in human MutLα during the ATPase cycle. Pms2 and Mlh1 are dimerized through their C-terminal domain throughout the cycle. The N-terminal domains that contain the ATP binding sites are in light gray (for Pms2) and dark gray (for Mlh1). A, the N termini do not interact in the absence of bound nucleotide. The circular shapes represent the presumed “unordered” conformation that is sensitive to trypsin. B, ATP binding induces conformational changes in the N-terminal domains of both proteins, depicted schematically as transition to rectangular shapes. ATP causes a conformational change, possibly rendering residues in the N termini “ordered” and resistant to trypsin. C, ATP-induced conformational changes cause dimerization of the N termini. Although the conformational changes depicted in B and the resulting dimerization may occur simultaneously, for illustration purposes these events are separated into discrete steps. The interface between the protomers may impart further resistance to trypsin digestion. D, hydrolysis of ATP. Based on the proteolysis data, Mlh1 appears to bind ADP with less affinity than Pms2. Thus, ADP release from Mlh1 may precede that of Pms2. D to A, ADP release cycles MutLb back to the nucleotide-free form.

Interestingly, ADP conferred similar protection from proteolysis to wild type Pms2 as ATP, but ATP protected Pms2 in the double mutant better than ADP (Fig. 8). In contrast, ATP protected Mlh1 far better than ADP in both wild type and the mutant protein. One possible explanation for this differential protection effect of Pms2 versus Mlh1 is that ADP formed during hydrolysis stays bound to Pms2 while dissociating faster from Mlh1. This is based on the observation that wild type Mlh1 required 10 mM ADP to show protection, whereas Pms2 was protected at lower ADP concentrations (Fig. 8A). Furthermore, Pms2 may hydrolyze ATP faster than Mlh1, as has been suggested recently for yeast Pms1 (49). Taken together, the nucleotide binding site of Pms2 may be occupied with ADP for longer periods of time than Mlh1, thus preventing rebinding of ATP. Although ADP can provide protection from proteolysis, the conformational change it elicits in the protein is smaller compared with ATP, as was suggested for bacterial MutL (28). In contrast to the wild type protein, the double mutant does not hydrolyze ATP; hence, ADP is not present to block ATP binding, and the full extent of conformational change in Pms2 is detected.
changes in Mlh1 and Pms2 is shown in Fig. 9.

The double glutamic acid mutant showed increased resistance of both Mlh1 and Pms2 proteins to trypsin relative to the other three MutLo proteins. One interpretation is that the ATP-induced conformational changes result in dimerization of the N termini of Mlh1 and Pms2, which in turn increase protection from protease digestion. Protein-protein interaction can protect from proteolysis; for example, heterodimerization of the α and γ subunits of a G protein protected the former from tryptic digestion (51). Evidence for N-terminal dimerization has been reported for *E. coli* MutL (28), yeast MutL (35), and other members of the GHL superfamily, including Hsp90 (27) and bacterial gyrase B (26). We suggest that this dimerization occurs in the wild type and the single mutant forms as well, but the dimer intermediate of these forms is more transient. ATP hydrolysis ultimately results in disruption of the dimer as the ATPase cycle proceeds (see Fig. 9). However, when both protomers cannot hydrolyze ATP due to mutation, the N termini are trapped in the dimer state, as suggested for gyrase B (52). Although not necessary for the ATPase activity of human Pms2 (33), N-terminal dimerization is likely to be required for mediating downstream events in MMR, possibly by generating new interfaces for protein-protein interactions, as was suggested for Hsp90 and *E. coli* MutL (53, 54). In turn, ATP hydrolysis would ensure these interactions are transient by resetting the repair cycle intermediates.

Although the conformational changes detected in the limited proteolysis assay probably reflect the inherent characteristics of Mlh1 and Pms2, each protomer may allosterically or physically affect its partner by virtue of C-terminal or N-terminal interactions, thus adding to the complexity level of the assay. For example, the apparent affinity of Mlh1E34A to ATP seems to be influenced by the ability of Pms2 to cycle between ATP-bound and free forms (Fig. 6). Therefore, a more thorough analysis will be required to elucidate the repair cycle intermediates.

Genetic studies with *S. cerevisiae* suggest a functional asymmetry within MutLo in that mutations in the conserved glutamic acid residues of MLH1 had a more severe mutant phenotype than the equivalent mutations in *PMS1* (35). In our study, we did not however observe a difference in repair activity in *vitro* between the single mutant MutLo forms. The basis for this apparent discrepancy may reflect differences between ATP binding properties of yeast and human MutLo. For example, the *Km* for ATP of yeast Pms1 N-terminal fragment was 19-fold higher than that of a similar human Pms2 fragment (33, 49), although an N-terminal deletion in the former might account for such a difference. Alternatively, the yeast-human difference may reflect the comparison of *in vitro* versus *in vivo* assays. The MMR machinery in *in vivo* may be coupled to DNA replication, possibly by proliferating cell nuclear antigen, as suggested by several groups (17–23). This coupling might facilitate strand discrimination during MMR, a process to which Mlh1 or Pms2 may contribute in an asymmetric manner. Therefore, the current *in vitro* MMR assay that operates independent of DNA replication may not fully represent repair as it occurs *in vivo*. Further studies in *in vivo* and *in vitro* are required to more fully delineate the contributions of Mlh1 and Pms2 to MMR in mammalian cells.

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