Activation of \textit{Saccharomyces cerevisiae} Mlh1-Pms1 Endonuclease in a Reconstituted Mismatch Repair System*

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Background: Biochemical analysis of \textit{S. cerevisiae} MMR mutants has been limited by a lack of reconstituted MMR reactions.

Results: 3’ nick-directed Mlh1-Pms1-dependent endonuclease and reconstituted MMR reactions were developed.

Conclusion: 3’ nick-directed MMR required the Mlh1-Pms1 endonuclease and was eliminated by mutations inactivating Exo1-independent MMR.

Significance: The reconstituted MMR reactions facilitated analysis of uncharacterized MMR mutants and the mechanism of Exo1-independent MMR.

Previous studies reported the reconstitution of an Mlh1-Pms1-independent 5’ nick-directed mismatch repair (MMR) reaction using \textit{Saccharomyces cerevisiae} proteins. Here we describe the reconstitution of a mispair-dependent Mlh1-Pms1 endonuclease activation reaction requiring Msh2-Msh6 (or Msh2-Msh3), proliferating cell nuclear antigen (PCNA), and replication factor C (RFC) and a reconstituted Mlh1-Pms1-dependent 3’ nick-directed MMR reaction requiring Msh2-Msh6 (or Msh2-Msh3), exonuclease 1 (Exo1), replication protein A (RPA), RFC, PCNA, and DNA polymerase 6. Both reactions required Mg$^{2+}$ and Mn$^{2+}$ for optimal activity. The MMR reaction also required two reaction stages in which the first stage required incubation of Mlh1-Pms1 with substrate DNA, with or without Msh2-Msh6 (or Msh2-Msh3), PCNA, and RFC but did not require nicking of the substrate, followed by a second stage in which other proteins were added. Analysis of different mutant proteins demonstrated that both reactions required a functional Mlh1-Pms1 endonuclease active site, as well as mispair recognition and Mlh1-Pms1 recruitment by Msh2-Msh6 but not sliding clamp formation. Mutant Mlh1-Pms1 and PCNA proteins that were defective for Exo1-independent but not Exo1-dependent MMR \textit{in vivo} were partially defective in the Mlh1-Pms1 endonuclease and MMR reactions, suggesting that both reactions reflect the activation of Mlh1-Pms1 seen in Exo1-independent MMR \textit{in vivo}. The availability of this reconstituted MMR reaction should now make it possible to better study both Exo1-independent and Exo1-dependent MMR.

DNA mismatch repair (MMR)$^2$ plays a critical role in maintaining genome stability by excising nucleotides that are mispair-corrupted as a result of DNA replication errors. Because MMR targets repair to the newly synthesized DNA strands, it reduces the frequency of mutations that occur as a result of these DNA replication errors (1–5). As a consequence, mismatch repair defects underlie the development of cancers associated with high rates of base substitution and frameshift mutations, with the latter often occurring in microsatellite sequences, resulting in a diagnostic phenotype called microsatellite instability (6–9). MMR also corrects mispaired bases in heteroduplex recombination intermediates, thus playing a role in gene conversion, and MMR helps to prevent recombination between divergent sequences preventing genome rearrangements formed by nonallelic homologous recombination (10–15). Whether MMR defects lead to genome rearrangements that play a role in the development of cancer is not clear, although MMR defective cancer cell lines have been described that have both microsatellite instability and increased genome rearrangements, suggesting that it does play a role (16).

The process of eukaryotic MMR initiates with the identification of mispairs in the genome by two different heterodimeric protein complexes that are homologs of the bacterial MutS dimer protein complex, the Msh2-Msh6 (\textit{MutS homolog}) (sometimes called MutS\textalpha) and Msh2-Msh3 (sometimes called MutS\textbeta) complexes (5, 17–22). These two complexes have distinct but overlapping mispair binding specificities and are partially redundant (5, 17, 18, 22–26). After mispair recognition, ATP binding by the Msh2-Msh6 and Msh2-Msh3 complexes induces a conformational change, which converts these complexes to a clamp form that slides along the DNA and licenses them to recruit a complex that is related to the bacterial MutL dimer protein complex (27–34). In \textit{Saccharomyces cerevisiae}, the major MutL-related complex that functions in MMR is Mlh1-Pms1 (Mlh1-Pms2 in humans, sometimes called MutL\textalpha) (35, 36). Two other MutL-related complexes exist (37–39): the first, Mlh1-Mlh3, can substitute for Mlh1-Pms1 to a very lim-

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$^‡$ The abbreviations used are: MMR, mismatch repair; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication protein A; Exo1, exonuclease 1.
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Experimental Procedures

Protein Purification—Mutations resulting in the Pms1-A99V or Pms1-G19D amino acid substitutions were introduced into the Pms1 expression vector pRD1099 LEU2 GAL10-PMS1-FLAG by standard site-directed mutagenesis methods, and the expected sequences of the mutant PMS1 genes were verified by DNA sequencing essentially as previously described (44). The methods for purifying Exo1, DNA polymerase δ, RPA, Msh2-Msh6, Msh2-Msh3-Msh6-F337A, Msh2-Msh3, RFC-Δ1N, and PCNA have been described previously (57) and in some cases were adapted from methods described in other studies (24, 61–66); many of the preparations of these proteins used were those described previously (57). Mlh1-Pms1, Mlh1-Pms1-A99V, Mlh1-Pms1-G19D, Mlh1-Pms1-E707K, and Mlh1-Pms1-C848S were purified as described previously (33, 44), and many of the protein preparations used were those described previously (33, 44). Msh2-Msh6-FF33AA was the protein preparation described in Ref. 61, and Msh2-Msh6-S1036P and Msh2-Msh6-G1142D were the protein preparations described in Ref. 33.

Nick-directed Endonuclease Assays—Mlh1-Pms1 endonuclease assays were performed in 40-μl reactions containing 20 mM HEPES-KOH, pH 7.6, 140 mM KCl, 5 mM MgCl₂, 0.5 mM MnSO₄, 2 mM ATP, 1 mM DTT, 0.2 mg/ml BSA, 1.2% (w/v) glycerol, 195 fmol of Msh2-Msh6, 145 fmol of Mlh1-Pms1, 110 fmol of RFC-Δ1N, 145 fmol of PCNA (PCNA trimers), and 100 ng (52 fmol) of a pBS-SK-derived +1 (+T) mispaired plasmid substrate with a nick in the +T strand at the AflIII site constructed as previously described (57, 67). For one experiment, a homoduplex DNA containing an AT base pair with the A in the strand containing the nick at the AflIII site was constructed as previously described (57, 67). All of the reaction components including the substrate DNA were combined in a master mix followed by addition of the proteins and any reaction components that were varied in individual experiments. The reactions were incubated at 30 °C for 30 min and then terminated by addition of 30 μl of 0.35% SDS, 0.3 mg/ml proteinase K, 400 mM NaCl, 0.3 mg/ml glycerol, and 13 mM EDTA followed by incubation at 55 °C for 15 min. The DNA present in the samples was then purified by phenol extraction and ethanol precipitation and linearized by digestion with Scal. The digestion products were then analyzed by electrophoresis through a 1% denaturing agarose gel followed by Southern blotting with probes specific for either the nicked or continuous strands of the substrate DNA (41).

Denaturing Agarose Gels—To perform denaturing agarose gel electrophoresis (41), a melted 1.1% agarose gel was first made in distilled water. The liquid agarose was cooled to ~55 °C; NaOH and EDTA were added to final concentrations of 50 and 2 mM, respectively, resulting in a final agarose concentration of 1%; and the solution was poured into an appropriate slab gel. Once solidified, the gels were equilibrated in running buffer consisting of 50 mM NaOH and 2 mM EDTA for 30
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Ten nanograms of DNA from the endonuclease reaction was then combined with denaturing agarose gel loading dye consisting of 200 mM NaOH, 40 mM EDTA, 10% Ficoll (w/v) and 0.1% brom cresol blue and loaded onto the gel, which was then subjected to electrophoresis at 25 V for 3 h. Next, the gels were neutralized using a buffer consisting of 1.5 M NaCl and 0.5 M Tris, pH 7, for 30 min. DNA from the neutralized gels was then transferred to nylon membranes and probed with singly biotinylated probes for either the nicked (5'-attatccctagtgacctcgggcaagagcaactcgcgtcgcggcatacct) or continuous strand (5'-aggtatgcggacgacctgtcttgctgcggcgccatcgggataat), which hybridized to the substrate DNA adjacent to the Scal site, on the Nael side of the Scal site following a previously published procedure (41) (see Fig. 1). Visualization of the Southern blots was performed using a Thermo Scientific chemiluminescent nucleic acid detection module, and the chemiluminescent signal was detected in the linear range using a Bio-Rad ChemiDoc MP imaging system. The percentage of nicked product DNA was calculated as the amount of nicked product DNA that was smaller than 1.55 kb (nicked strand) or 2.92 kb (continuous strand) divided by the combined signal of both the nicked and linear DNAs. In many experiments, the amount of nicked product observed was normalized to the amount of nicked product observed in the complete wild-type protein reaction. In these experiments, 100% nicking ranged from 4% (2 fmol) to 34% (18 fmol) of the substrate nicked (for an example of an individual experiment, see Figs. 3 and 7). The standard error was calculated from the results of three or more independent experiments and is indicated by the error bars in individual figures.

Reconstituted Mlh1-Pms1-dependent MMR Assays—Reactions were performed essentially as previously described (57) with the modification that the reactions were performed in two stages. In the first stage, 195 fmol of Msh2-Msh6, 145 fmol of Mlh1-Pms1, 110 fmol of RFC-Δ1N, and 145 fmol of PCNA were incubated for 10 min at 30°C with 100 ng (52 fmol) of +T mispaired substrate with a strand discontinuity at the AflIII site (57) in a final volume of 5 μl. The proteins and DNA were combined in 2.5 μl and mixed with 2.5 μl of a master reaction buffer mix containing 33 mM Tris, pH 7.6, 75 mM KCl, 2.5 mM MgCl2, 1.66 mM glutathione, 8.3 mM MgCl2, 80 μg/ml BSA, 200 μM dNTPs, and 1 mM MnSO4. Following the 10-min incubation period at 30°C, 195 fmol of Msh2-Msh6, 145 fmol of Mlh1-Pms1, 110 fmol of RFC-Δ1N, 145 fmol of PCNA (PCNA trimers), 40 fmol of DNA polymerase δ, 2.1 fmol of Exo1, and 900 fmol of RPA were added to the initial 5 μl along with H2O as required and 2.5 μl of a modified version of the above master reaction buffer mix lacking MnSO4, bringing the final reaction volume to 10 μl followed by a 2-h incubation period at 30°C. The first stage reaction contained final concentrations of 0.5 mM MnSO4 and 4.2 mM MgCl2, and the second stage reaction contained final concentrations of 0.25 mM MnSO4 and 4.2 mM MgCl2; we did not add additional MnSO4 to the second stage reaction because the MnSO4 present in the first stage reaction was sufficient to support full activity. In reactions with Zn2+, ZnSO4 was substituted for MnSO4 at the same concentration as indicated above for MnSO4. Note that because KCl was present in the different protein dilutions, the final KCl concentration in the repair reaction was 100 mM. Repair was monitored by measuring the extent of restoration of a PstI site located at the site of the mispair by agarose gel electrophoresis as previously described. In many experiments, the amount of repair observed was normalized to the amount of repair observed in the complete reaction. In these experiments, 100% repair ranged from 7.7% (4.1 fmol) to 29% (15.3 fmol) of the substrate repaired (for an example of an individual experiment, see Fig. 1). The S.E. was calculated from the results of three or more independent experiments and is indicated by the error bars in individual figures.

Results

Mlh1-Pms1-dependent MMR Catalyzed by Purified S. cerevisiae Proteins—In a previous study, we demonstrated that a combination of six purified S. cerevisiae proteins including Msh2-Msh6 (or Msh2-Msh3), Exo1, RPA, DNA polymerase δ, PCNA, and RFC-Δ1N (or RFC) catalyzed mismatch-dependent repair of a mispaired substrate containing a nick at either an Nael site 343 bp 5’ or at an AflIII site 442 bp 3’ (i.e. 2,479 bp 5’) from the mispair (57). Both repair reactions were mediated by 5’→3’ excision catalyzed by Exo1, and addition of the Mlh1-Pms1 endonuclease to either reaction had no effect on the efficiency or extent of repair (57). In the present study, we aimed to develop an Mlh1-Pms1 endonuclease-dependent MMR reaction using a mispaired substrate containing a +1 (+T) insertion mispair and a nick at the AflIII site 3’ to the mispair. To achieve this, three reaction parameters were investigated including: 1) reducing the amounts of Exo1 to prevent mispair-dependent 5’→3’ excision initiating at the AflIII site from reaching the mispair, which would mask any repair initiating from nicks introduced by Mlh1-Pms1; 2) separating the reaction into two reaction stages to allow for the interaction of Mlh1-Pms1 with substrate DNA; and 3) inclusion of Mn2+ in the reactions at the concentrations previously used to support the Mn2+-dependent nicking activity of Mlh1-Pms1 (or human Mlh1-Pms2) (41, 42, 44) in addition to Mg2+, to achieve optimal Mlh1-Pms1 endonuclease activation. This facilitated the development of an optimal reaction in which a 10-min first stage reaction containing Mlh1-Pms1, Msh2-Msh6, PCNA, RFC-Δ1N, Mn2+, Mg2+, ATP, and substrate DNA was then added to a second stage reaction mixture containing Msh2-Msh6, PCNA, RFC-Δ1N, Exo1, RPA, DNA polymerase δ, Mg2+, and ATP followed by incubation for different times. Mlh1-Pms1-dependent repair of the nicked strand of the substrate DNA occurred over a 2-h period and was detected by cleavage with PstI, whose recognition sequence in the discontinuous strand was restored by MMR at the mispair site and Scal to produce a diagnostic pair of 1.1- and 1.8-kb fragments (Fig. 1).

To further evaluate the reaction conditions, the requirement of different proteins and divalent cations in the first stage reaction were examined (Fig. 2). The +T mispair was repaired to varying but significant levels when Mlh1-Pms1 alone, Mlh1-Pms1, and Msh2-Msh6 or Mlh1-Pms1, PCNA, and RFC-Δ1N were present in the first stage reaction, but the level of repair in each case was below that observed when Mlh1-Pms1, Msh2-Msh6, PCNA, and RFC-Δ1N were present in the first stage reaction (Fig. 2A). A lower but significant amount of repair above that seen in reactions without Mlh1-Pms1 was observed when no protein was present in the first stage reaction, and all
seven proteins were present in the second stage reaction (Fig. 2A), indicating that the two-stage experimental design results in maximum levels of repair but is not absolutely required for Mlh1-Pms1-dependent repair to occur. Interestingly, the requirement for divalent cations in the first stage reaction depended on whether ATP was also present in the first stage reaction (Fig. 2B). In the presence of ATP in the first stage reaction, a divalent cation was also required in the first stage reaction; adding either Mn\(^{2+}\) or Mg\(^{2+}\) to first stage reactions containing ATP supported repair provided both Mn\(^{2+}\) and Mg\(^{2+}\) were ultimately present in the second stage reaction. In the absence of ATP in the first stage reaction, no divalent cation was required in the first stage, provided Mn\(^{2+}\), Mg\(^{2+}\), and ATP were present in the second stage reaction. Although significant levels of repair were observed when only Mg\(^{2+}\) (or Mn\(^{2+}\)) was present in the repair reactions, the highest level of repair was observed in reactions that contained ATP, Mg\(^{2+}\), and Mn\(^{2+}\) in the first stage and additional Mg\(^{2+}\) in the second stage of the reaction, and therefore these conditions were used in the standard repair reaction in this study. We also found that Zn\(^{2+}\) would fully substitute for Mn\(^{2+}\) (Fig. 2B).

**Efficient Activation of Nick-directed Mlh1-Pms1 Endonuclease Activity Requires Msh2-Msh6, RFC-Δ1N, PCNA, Mg\(^{2+}\), and Mn\(^{2+}\)**—To investigate the relationship between the Mlh1-Pms1-dependent repair reaction and the introduction of nicks by the Mlh1-Pms1 endonuclease, we developed an assay to measure endonuclease activity on the nicked strand of the AffIII mispaired substrate similar to assays described in previous studies (41, 42, 46). This endonuclease assay was similar to the first stage of the repair assay except that the reaction volume was 40 μl, the final salt concentration was 140 mM KCl, and the enzymatic activity was monitored by Southern blotting using a probe that hybridized to the nicked strand of the mispaired substrate adjacent to the unique Scal site (Fig. 1). This allowed detection of smaller molecular weight species resulting from nicks on the 1.55-kb mispair-containing fragment between the Scal and AffIII sites. Using this method, we observed the accumulation of up to 18 fmol of nicked product (Fig. 3C, inset) in reactions containing Mlh1-Pms1, Msh2-Msh6, PCNA, RFC-Δ1N, ATP, Mn\(^{2+}\), and Mg\(^{2+}\). Mn\(^{2+}\) was used at the concentration that was previously shown to support the Mn\(^{2+}\)-dependent nicking activity of Mlh1-Pms1 (or human Mlh1-Pms2) (41, 42, 44). No smaller molecular weight species were observed when the reaction products were hybridized with a probe specific for the continuous strand of the substrate DNA, indicating that nicking only occurred on the strand containing the pre-existing nick (Fig. 3C). In addition, nicking of the already nicked strand was only observed above background when the substrate contained a mispair and was not observed when the substrate did not contain a mispair (Fig. 3D).

To further characterize the endonuclease activity, we tested the protein and divalent cation requirements for the activation of the Mlh1-Pms1 endonuclease. The endonuclease activity of Mlh1-Pms1 was completely dependent on the addition of Msh2-Msh6, RFC-Δ1N, and PCNA. Mlh1-Pms1 alone, Mlh1-Pms1 together with Msh2-Msh6, or Mlh1-Pms1 with RFC-Δ1N and PCNA did not not have endonuclease activity (Fig. 2B).
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34). Msh2-Msh3, which was not previously tested for its ability to activate the Mlh1-Pms1 endonuclease but is known to activate the hMlh1-Pms2 endonuclease (43), was able to completely substitute for Msh2-Msh6 (see Fig. 6A). Finally, although a significant level of endonuclease activity was observed when either Mg2+ or Mn2+ alone was added to the reactions, higher levels of endonuclease activity were observed when both Mn2+ and Mg2+ were added to the reactions (Fig. 3B), and hence we included Mn2+ and Mg2+ in our standard reaction conditions.

Mlh1-Pms1 and PCNA Mutants That Are Defective in Exo1-independent MMR Have Defects in Activating the Mlh1-Pms1 Endonuclease and in Reconstituted MMR Reactions—To gain insights into the protein requirements and mechanisms of Mlh1-Pms1-dependent MMR in vitro, we investigated the effects of a series of mutations in different MMR genes on the ability of the respective MMR proteins to support MMR and activation of the Mlh1-Pms1 endonuclease in vitro. All of the experiments investigating MMR in vitro utilized the two-stage repair reaction described above in which the first stage contained Msh2-Msh6, Mlh1-Pms1, PCNA, RFC-Δ1N, ATP, Mg2+, and Mn2+ and the second stage contained Msh2-Msh6, PCNA, RFC-Δ1N, Exo1, RPA, DNA polymerase δ, ATP, and Mg2+.

We initially investigated whether four different mutant Mlh1-Pms1 proteins could support MMR and Mlh1-Pms1 endonuclease activation in vitro. The Mlh1-Pms1-E707K and Mlh1-Pms1-C848S proteins that have amino acid substitutions that inactivate the endonuclease active site (44) were completely defective for endonuclease activation (Fig. 4A) and were as defective for repair as omitting Mlh1-Pms1 (Figs. 1C and 4B), indicating that the Mlh1-Pms1 endonuclease activity is required for MMR in vitro. We also tested the Mlh1-Pms1-A99V and Mlh1-G19D-Pms1 proteins, which have amino acid substitutions that cause defects in Exo1-independent MMR but not Exo1-dependent MMR (52). The Mlh1-Pms1-A99V and Mlh1-G19D-Pms1 proteins were partially defective for Mlh1-Pms1 endonuclease activation (Fig. 4A). Consistent with this result, substituting the Mlh1-Pms1-A99V and Mlh1-G19D-Pms1 proteins for Mlh1-Pms1 in the reconstituted MMR reaction resulted in modestly reduced repair but not to the extent observed in the endonuclease active site mutants (Fig. 4B); this difference likely reflects the fact that Mlh1-Pms1-A99V and Mlh1-G19D-Pms1 are only partially defective for endonuclease...
activation and given the longer incubation times used in the reconstituted MMR reactions likely contribute enough nicking to allow significant levels of repair to occur.

Because the mutant Mlh1-Pms1 proteins that have defects in Exo1-independent but not Exo1-dependent MMR had reduced activity in both mispair-directed Mlh1-Pms1 endonuclease activation and reconstituted MMR reactions, we next analyzed two mutant PCNA proteins, PCNA-E143K and PCNA-C81R. PCNA-E143K was originally identified in genetic screens for pol30 mutations causing defects in Exo1-independent but not Exo1-dependent MMR (52), and PCNA-C81R was originally identified in a screen for pol30 mutations causing general MMR defects (68). The pol30-C81R mutation was later found to cause much stronger defects in Exo1-independent MMR compared with Exo1-dependent MMR (45, 69). These two mutants were previously shown to have altered interactions with Msh2-Msh6 but to be fully proficient for supporting Mlh1-Pms1-mediated nicking of supercoiled homoduplex DNA substrates in reactions containing Mlh1-Pms1, RFC-D1N, and PCNA (45). The PCNA-E143K mutant was severely, but not completely, compromised in Mlh1-Pms1 endonuclease activation, whereas the PCNA-C81R mutant was almost completely defective in Mlh1-Pms1 endonuclease activation (Fig. 5A). Consistent with this observation, the PCNA-E143K mutant was partially defective in supporting MMR in vitro, whereas the PCNA-C81R showed an even greater defect in supporting MMR in vitro, but neither was completely defective for supporting MMR in vitro, even when the level of PCNA present in the repair reactions was reduced to 25% of the amount of PCNA present in the standard repair reaction (Fig. 5B). A control experiment showed that these mutant PCNA proteins were proficient for supporting repair of the 5′ nicked substrate whose repair does not require Mlh1-Pms1 (57), indicating that these mutant PCNA proteins are proficient in the PCNA-dependent gap filling reaction (Fig. 5C). This indicates that the PCNA-E143K and PCNA-C81R defects specifically reduced the ability of PCNA to support the activation of the Mlh1-Pms1 endonuclease on nicked mispaired substrates.

**Mispair Recognition and Mlh1-Pms1 Recruitment but Not Sliding Clamp Formation or Interaction with PCNA by Msh2-Msh6 Are Required for Mlh1-Pms1 Endonuclease Activation and MMR in Vitro**—We next investigated a series of mutations affecting the Msh2-Msh6 complex for their effects on activation of Mlh1-Pms1 endonuclease activity and Mlh1-Pms1-dependent MMR by analyzing the following four mutant Msh2-Msh6 complexes: 1) the Msh2-Msh6-FF33AA mutant that is defective for interacting with PCNA through the Msh6 PIP Box motif (61, 70); 2) the Msh2-Msh6-F337A mutant that has defects in mispair recognition (19, 57); and 3) two dominant mutant proteins including Msh2-Msh6-S1036P that has a defect in binding ATP at the Msh2 ATP-binding site and that affects the Msh2-Msh6 complex; and 4) Msh2-Msh6-F337A that resulted in a loss of activation of the Mlh1-Pms1 endonuclease and a significant reduction of repair in the reconstituted Mlh1-Pms1-dependent MMR reaction (Fig. 6). In addition, consistent with results obtained with human Msh2-Msh6 (MutSa) (71, 72), eliminating the ability of Msh6 to interact with PCNA (Msh2-Msh6-FF33AA) did not reduce activation of the Mlh1-Pms1 endonuclease or cause a defect in MMR in vitro (Fig. 6). Msh2-Msh3 was able to substitute for Msh2-Msh6. Omission of Msh2-Msh6 or substitution of Msh2-Msh6 with the mispair recognition defective Msh2-Msh6-F337A resulted in a loss of activation of the Mlh1-Pms1 endonuclease and a significant reduction of repair in the reconstituted Mlh1-Pms1-dependent MMR reaction (Fig. 6). Msh2-Msh3 was able to substitute for Msh2-Msh6 in both the Mlh1-Pms1 endonuclease activation and the reconstituted Mlh1-Pms1-dependent MMR reactions consistent with previous analysis of the reconstituted 5′ nick-driven MMR reaction and studies on the activation of the hMlh1-Pms2 endonuclease (43, 59) (Fig. 6).

The two dominant Msh2-Msh6 mutants had distinctly different behaviors in the Mlh1-Pms1 endonuclease activation.

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**FIGURE 5. Effects of pol30 (PCNA) mutations that cause defects in Exo1-independent MMR on activation of the Mlh1-Pms1 endonuclease and Mlh1-Pms1-dependent MMR in vitro.** A, four-protein Mlh1-Pms1 endonuclease reactions with the +1 (+T) substrate containing a 3′ nick at the AflIII site, 0.5 mM MnSO4, and 5 mM MgCl2 were performed for 30 min as described in Fig. 3A and under “Experimental Procedures.” In all cases, the level of nicking obtained was normalized to the level of nicking observed in the complete, standard reaction. The presence of wild-type or mutant PCNAs containing the indicated amino acid substitutions in the reactions is indicated by the key below the histogram. B, two-stage repair reactions with the +1 (+T) substrate containing a 3′ nick at the AflIII site were performed for 2 h as described in Fig. 1 and under “Experimental Procedures.” In all cases, the level of repair obtained was normalized to the level of repair observed in the complete, standard repair reaction. The presence of wild-type or mutant PCNAs containing the indicated amino acid substitutions at either the normal concentration or 25% of the normal concentration is indicated by the key above the histogram. C, repair reactions with the +1 (+T) substrate containing a 5′ nick at the NaeI site were performed for 3 h exactly as previously described (57). The presence of wild-type or mutant PCNAs containing the indicated amino acid substitutions is indicated by the key below the gel image, and the percentage of repair is indicated above each lane. MW, molecular weight markers.
and reconstituted MMR reactions. The Msh2-Msh6-S1036P mutant was highly defective for activating the Mlh1-Pms1 endonuclease, whereas the Msh2-Msh6-G1142D was proficient for activating the Mlh1-Pms1 endonuclease, suggesting that Mlh1-Pms1 recruitment but not sliding clamp formation is required for activation of the Mlh1-Pms1 endonuclease (Fig. 6A). The Msh2-Msh6-S1036P and Msh2-Msh6-G1142D complexes both fully supported MMR in vitro (Fig. 6B), which in the case of the Msh2-Msh6-S1036P mutant was surprising because this mutant was highly defective in activating the Mlh1-Pms1 endonuclease. We confirmed that the reconstituted MMR reaction containing the Msh2-Msh6-S1036P complex required the Mlh1-Pms1 endonuclease and was significantly reduced when the endonuclease active site mutant Mlh1-Pms1-E707K was substituted for wild-type Mlh1-Pms1 (Fig. 7A). After examining the differences between the Mlh1-Pms1 activation assay and the in vitro MMR assay, we found that increasing the KCl concentration in the reconstituted MMR reaction from 100 to 140 mM, which is the KCl concentration in the Mlh1-Pms1 endonuclease activation assays, reduced the amount of repair in the presence of Msh2-Msh6-S1036P but not wild-type Msh2-Msh6 (Fig. 7B). Additionally, lowering the KCl concentration in the Mlh1-Pms1 endonuclease activation assay from 140 to 100 mM restored activation of the Mlh1-Pms1 endonuclease in the presence of the Msh2-Msh6-S1036P protein (Fig. 7C). This indicates that Msh2-Msh6-S1036P has a salt-sensitive defect in the activation of the Mlh1-Pms1 endonuclease.

Discussion

A key challenge in elucidating MMR mechanisms is linking the biochemical properties of purified MMR proteins and reconstituted MMR reactions to MMR mechanisms in vivo. Achieving this would allow determining whether the biochemical properties of MMR proteins and reconstituted MMR reactions can account for MMR in vivo and allow identification of features of MMR that have not yet been reconstituted in vitro. To facilitate these efforts, in the present study we have reconstituted Mlh1-Pms1-dependent 3’ nick-directed MMR reactions, as well as Msh2-Msh6-dependent, Mlh1-Pms1 endonuclease reactions using S. cerevisiae proteins. Critical to this effort have been the development of a two-stage MMR reaction and the identification of an essential divalent cation requirement for MMR in vitro. The availability of this reconstituted MMR reaction now makes it possible to exploit the wealth of MMR-defective mutations identified in genetic studies to explore the relationships between the biochemical properties of MMR proteins and MMR mechanisms in vivo.

We observed that a mixture of Msh2-Msh6 (or Msh2-Msh3), Mlh1-Pms1, Exo1, RPA, PCNA, RFC, DNA polymerase δ, ATP, Mg²⁺, and Mn²⁺ repaired a mispaired plasmid substrate containing a +1 (+T) mispair and a 3’ nick located 442 bp from the mispair in an Mlh1-Pms1-dependent, Msh2-Msh6 mispair recognition-dependent reaction. We also observed a mispair-dependent, mispair-recognition-dependent, nick-directed nicking reaction catalyzed by Msh2-Msh6 (or Msh2-Msh3), Mlh1-Pms1, PCNA, and RFC under essentially the same reaction conditions. Three modifications of our previ-
ously reported reconstituted 5′-nick-directed MMR system were required to observe Mlh1-Pms1-dependent MMR in vitro (57). First, it was necessary to reduce the levels of Exo1 to limit long-patch 5′-nick-directed MMR from the AflIII nick that potentially obscures Mlh1-Pms1-dependent repair; however, it should be noted that this modification possibly reduces the efficiency of excision from nicks introduced by Mlh1-Pms1. Second, it was necessary to add both Mg²⁺ and Mn²⁺ to the reactions to observe optimal Mlh1-Pms1-dependent nicking and Mlh1-Pms1-dependent MMR in vitro. This is in contrast to previous studies of MMR reconstituted with human proteins and mispair-dependent nicking of DNA catalyzed by human Msh2-Msh6 (or Msh2-Msh3), Mlh1-Pms2 (MutL φ), PCNA, and RFC or S. cerevisiae Msh2-Msh6, Mlh1-Pms1, PCNA, and RFC, which only required Mg²⁺; however, these studies did not appear to test whether addition of Mn²⁺ or Zn²⁺ would stimulate the reactions reported (41, 42, 46, 58, 59). The most likely explanation for this is that the divalent cation bound at the Mlh1-Pms1 endonuclease active site was lost during our purification of Mlh1-Pms1, creating a requirement for Mn²⁺ or Zn²⁺, because this metal binding site is not expected to bind Mg²⁺ (41, 44, 73, 74). Finally, it was necessary to develop a two-stage reaction where at a minimum, the first stage reaction contained Mlh1-Pms1 and substrate DNA, and the second stage reaction additionally contained all of the remaining reaction components. The endonuclease activity of Mlh1-Pms1 was required in the reconstituted MMR reaction and a combination of Msh2-Msh6 (or Msh2-Msh3), PCNA, and RFC was required to activate the Mlh1-Pms1 endonuclease to nick the mispaired substrate on the nicked strand under the reaction conditions used. The most likely mechanism of MMR under these conditions involves nicking of the substrate on the nicked strand close enough on the 5′ side of the mispair for the previously characterized short patch repair reaction to excise and resynthesize the nicked DNA strand in the vicinity of the mispair as previously shown for human MMR (41).

Optimal Mlh1-Pms1-dependent MMR required a first stage reaction containing Msh2-Msh6 (or Msh2-Msh3), Mlh1-Pms1, PCNA, RFC and substrate DNA, whereas inclusion of only Mlh1-Pms1 and substrate DNA in the first stage reaction resulted in ~50% (Fig. 2A) of maximal activity. The inclusion of ATP alone did not support a functional first stage reaction, whereas the inclusion of ATP plus a divalent cation or divalent cations alone supported functional first stage reactions. Because the Mlh1-Pms1 endonuclease was inactive under several of the functional first stage reaction conditions (Mlh1-Pms1 alone with substrate DNA under any condition; Msh2-Msh6, Mlh1-Pms1, PCNA, RFC, and substrate DNA without ATP and divalent cations including Mn²⁺), the first stage reaction does not likely involve nicking of the substrate DNA but rather likely provides an opportunity for Mlh1-Pms1 to interact with the substrate DNA.

The MutL N-terminal domains and the equivalent Mlh1 and Pms1 N-terminal domains are known to associate on binding ATP, resulting in a ring-like conformation in which the associated N-terminal domains are joined to the dimerized C-terminal domains of MutL or Mlh1 and Pms1, respectively, by two unstructured linkers (73, 75–80). The N-terminal domains then dissociate from each other upon hydrolyzing ATP, which requires a divalent cation, and ADP release (76–79). Interestingly, the only conditions where the first stage reaction is functional are when the ATP binding and hydrolysis-driven Mlh1 and Pms1 N-terminal domain association and dissociation cycle is active (ATP and Mg²⁺ or Mn²⁺) or when the N-terminal domains of Mlh1 and Pms1 are not able to interact with each other (−ATP). In contrast, conditions that support stable association of the N-terminal domains of Mlh1 and Pms1 (ATP without Mg²⁺ or Mn²⁺) did not support a functional first stage reaction. This suggests that either the free N-terminal domains of Mlh1 and Pms1 or a region of one or both of the Mlh1 and Pms1 linkers that is blocked by the associated N-terminal domains of Mlh1 and Pms1 is critical for the activity of Mlh1-Pms1 in the first stage reaction.

Consistent with previously published studies (41, 42, 46), a combination of Msh2-Msh6 (or Msh2-Msh3), Mlh1-Pms1, PCNA, and RFC was found to promote nicking of the nicked strand of the mispaired substrate under essentially the same reaction conditions as the complete MMR reaction, provided the reactions contained ATP, Mg²⁺, and Mn²⁺. This is in contrast to previous studies of MMR reconstituted with human proteins (57). First, it was necessary to reduce the levels of Exo1 to limit nick-directed MMR system to the Exo1-independent and Exo1-dependent MMR pathways that have been identified in genetic studies, we investigated four mutant proteins that are defective in Exo1-independent MMR but are proficient in Exo1-dependent MMR (44, 45, 52). Two mutant PCNA proteins, PCNA-E143K and PCNA-C81R, which have altered interactions with Msh2-Msh6 but are fully proficient in PCNA- and RFC-dependent activation of the Mlh1-Pms1 endonuclease on supercoiled DNA substrates (45), were significantly but not completely defective in activating the Mlh1-Pms1 endonuclease on nicked mispaired substrates in the presence of Msh2-Msh6 and RFC. These results raise the possibility that some type of interaction between Msh2-Msh6
and PCNA is important for the activation of the Mlh1-Pms1 endonuclease, even though the interaction between the Msh6 PIP Box and PCNA is not required for activation of the Mlh1-Pms1 endonuclease. Similarly, two mutant Mlh1-Pms1 complexes having amino acid substitutions in the N-terminal domains of either Mlh1 or Pms1 that only compromise Exo1-independent MMR (52) were significantly but not completely defective in activation of the Mlh1-Pms1 endonuclease in the four-protein nicking reaction. Little is known about the specific biochemical defects caused by these two amino acid substitutions, although because of their locations (52) they could affect interactions between ATP and the N-terminal domains of Mlh1 and Pms1 (78, 79). Overall, these results support the hypothesis that higher levels of activation of the Mlh1-Pms1 endonuclease are required for Exo1-independent MMR compared with the levels of Mlh1-Pms1 endonuclease activation required to support Exo1-dependent MMR (44, 45).

The biochemical properties of the reconstituted Mlh1-Pms1-dependent MMR reaction generally corresponded to those of the Msh2-Msh6 (or Msh2-Msh3), PCNA, RFC, and mispair-dependent activation of the Mlh1-Pms1 endonuclease. These include the defects caused by the Msh6 mispair recognition-defective mutation, the Mlh1-Pms1 endonuclease active site mutations, the Exo1-independent MMR-specific mutations affecting PCNA and Mlh1-Pms1, and the defect caused by the Msh6-S1036P amino acid substitution at high KCl concentrations, as well as the lack of an effect of the Msh6 PIP Box-PCNA interaction-defective mutation and the Msh6-G1142D defect. Overall, these results suggest that the reconstituted Mlh1-Pms1-dependent MMR reaction corresponds to a reaction in which Mlh1-Pms1 endonuclease activation, potentially at the higher levels corresponding to that required for Exo1-independent MMR (44, 45), is coupled to a 5′ mispair-dependent excision reaction requiring Exo1 in vitro (50, 57, 84).

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