Eukaryotic DNA mismatch repair requires the concerted action of several proteins, including proliferating cell nuclear antigen (PCNA) and heterodimers of MSH2 complexed with either MSH3 or MSH6. Here we report that MSH3 and MSH6, but not MSH2, contain N-terminal sequence motifs characteristic of proteins that bind to PCNA. MSH3 and MSH6 peptides containing these motifs bound PCNA, as did the intact Msh2-Msh6 complex. This binding was strongly reduced when alanine was substituted for conserved residues in the motif. Yeast strains containing alanine substitutions in the PCNA binding motif of Msh6 or Msh3 had elevated mutation rates, indicating that these interactions are important for genome stability. When human MSH3 or MSH6 peptides containing the PCNA binding motif were added to a human MMR extract, mismatch repair activity was inhibited at a step preceding DNA resynthesis. Thus, MSH3 and MSH6 interactions with PCNA may facilitate early steps in DNA mismatch repair and may also be important for other roles of these eukaryotic MutS homologs.

The mutation rate of an organism is reduced by the ability of the general DNA mismatch repair (MMR) system to correct DNA replication errors. In eukaryotes, MMR is initiated when one of two protein complexes binds to mismatches (reviewed in Refs. 1–3). The MutS homologs MSH2 and MSH6 and the MutL homologs MSH2 and MSH6 are involved in the recognition and repair of base-base and small insertion/deletion mismatches. When human MSH3 or MSH6 peptides containing the PCNA binding motif were added to a human MMR extract, mismatch repair activity was inhibited at a step preceding DNA resynthesis. Thus, MSH3 and MSH6 interactions with PCNA may facilitate early steps in DNA mismatch repair and may also be important for other roles of these eukaryotic MutS homologs.

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The abbreviations used are: MMR, DNA mismatch repair; PCNA, proliferating cell nuclear antigen; FEN1, flap endonuclease 1; GST, glutathione S-transferase; y, yeast; h, human; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; replication factor C, and DNA polymerase δ. Working in concert, these proteins complete a DNA excision/resynthesis reaction that specifically corrects errors in the nascent strand (1–3).

Although the signal that directs mismatch repair to the newly replicated strand in eukaryotic cells is unknown, repair of mismatched duplexes in extracts of eukaryotic cells can be directed to one strand by a discontinuity in the DNA backbone. We previously suggested that one-strand discontinuity that might serve as a strand discrimination signal is the primer terminus at the replication fork and that PCNA may link DNA replication and mismatch repair to facilitate recognition and repair of errors in the nascent strand (4). PCNA is the essential sliding clamp that topologically encircles DNA and physically associates with DNA polymerase δ to enhance its processivity (reviewed in Ref. 5). PCNA is required at an early step in DNA mismatch repair that precedes excision of the mismatch (4), as well as for the DNA resynthesis that follows mismatch excision (6). Yeast PCNA has been suggested to interact with Mlh1 in vitro (4), and it interacts with the Msh2-Msh3 heterodimer in vitro (7). Human PCNA can be co-immunoprecipitated with MSH2, MLH1, and PMS2 (6), and a PCNA affinity column binds MSH2 and MSH6 (8). Yeast strains with certain mutant PCNA alleles exhibit a mutator phenotype that is epistatic with mutations in mismatch repair genes (4, 7, 9, 10). One of these alleles, pol30–104, is lethal in combination with a null mutation in RAD52, and this synthetic lethality is suppressed by a mutation in MSH2 (9). This suggests that this mutant PCNA may reduce strand discrimination such that nicking of both strands yields lethal double strand breaks.

Because these data reveal important roles for PCNA in MMR, we are attempting to identify interactions of PCNA with mismatch repair proteins and investigate their functional importance. Here we take advantage of previous studies (reviewed in Refs. 5 and 11) showing that PCNA interacts with several other proteins involved in DNA replication and repair (12–17). These proteins share a common amino acid motif with the consensus sequence Qxxhxaaa (see Fig. 1). A p21 peptide containing this motif binds to the interdomain connector loop of PCNA (18), which is present three times in the trimeric PCNA sliding clamp. The present study was motivated by the observation that the consensus PCNA binding motif is present at the N termini of MSH3 and MSH6. We provide evidence that these motifs mediate physical interactions with PCNA and that these interactions are important for the function of MSH3 and MSH6.

**EXPERIMENTAL PROCEDURES**

**Materials**—N-terminal peptides of hMSH6 and hMSH3 containing wild type or mutant PCNA binding motifs (see Fig. 1B) were synthesized by Research Genetics (Huntsville, AL). Materials for the MMR assays have been described (19).

**Construction of Plasmids**—Bacterial expression plasmids were constructed to produce glutathione S-transferase (GST) fusion proteins containing amino acids 28–47 of yeast Saccharomyces cerevisiae Msh3 (GST-yMsh3), 22–41 of S. cerevisiae Msh6 (GST-yMsh6), 18–37 of human MSH3 (GST-hMSH3), 22–41 of S. cerevisiae Msh6 (GST-yMSH6), and 1–20 of human MSHH and MSH6 with amino acids 28–47 of yeast Saccharomyces cerevisiae Msh3 (GST-yMsh3), 18–37 of human MSH3 (GST-hMSH3), 22–41 of S. cerevisiae Msh6 (GST-yMSH6), and 1–20 of human MSHH with a F10A/F11A substitution (GST-hMSH6, F10A/F11A). These plasmids were made by ligating annealed pairs of oligonucleotides into an EcoRI/XhoI-digested pGEX-4T-1 vector (Amersham Pharmacia Biotech). They each have a stop

2 The sequences of the oligonucleotides used in this study are available upon request.
codon after the last MSH3 or MSH6 codon and a unique HindIII restriction site to facilitate screening during subcloning. The correct sequence was confirmed by DNA sequencing. Plasmids for yeast and human PCNA and GST fusion proteins for human FEN1 and DNA ligase I were as described (13, 16, 20). pGEX-4T-3 without insert was used to produce GSTp.

Assays for PCNA Binding—Proteins were expressed in E. coli strain BL21(DE3) by induction with 0.8 mM isopropyl-β-D-thiogalactopyranoside. Cells were lysed in ice-cold 50 mM Tris-HCl, 150 mM NaCl, 0.2 mg/ml lysozyme, 2 mM EDTA, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (pH 7.4). Binding assay mixtures contained 50 μl of 40% glutathione-agarose beads (Sigma), 300 μl of lysate from cells expressing GST or GST fusion protein, and 400 μl of lysate from cells expressing human or yeast PCNA. Mixtures were incubated for 2 h at 4 °C and then washed five times with 0.8 ml of 50 mM Tris-HCl, 150 mM NaCl (pH 7.4). For binding assays with human PCNA, protein complexes were eluted by heating to 100 °C with 80 μl of 2× Laemmli SDS sample buffer, separated on a 12% SDS polyacrylamide gel, and stained with Coomassie Blue. For binding assays with yeast PCNA, the washed bead mixtures were resuspended in 100 μl of phosphate-buffered saline containing 5 units thrombin (Amersham Pharmacia Biotech) and incubated for 3 h at room temperature. Beads were pelleted by centrifugation, and 6 μl of supernatant was analyzed as above. To characterize proteins associated with the beads after cleavage, the supernatant was removed, beads were washed, and proteins were eluted with 80 μl of 2× SDS sample buffer.

To test interactions with Msh2-Msh6, yeast PCNA was overexpressed in E. coli, purified as described (21), and coupled to Affi-gel 15 beads (Bio-Rad Laboratories) according to the manufacturer’s instructions. Wild type and mutant yMSH2-yMSH6 heterodimer was purified as described.2 20 μg of either Msh2-Msh6 complex was incubated with 20 μl of PCNA beads, or BSA beads as a negative control, for 1 h at 4°C in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.01% Nonidet P-40, and 10% glycerol. Beads were washed 6 times with 20 mM Tris-HCl (pH7.4), 1 mM NaCl, 1 mM DTT, 0.1% EDTA, 0.01% Nonidet P-40, and 10% glycerol. Beads were resuspended in an equal volume of 2× Laemmli loading buffer and subjected to SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane and probed with an antibody to a peptide of amino acids 18–37 of hMSH6 and hMSH6 peptide (amino acids 1–20). This antibody reacts with both yMSH6 and hMSH2.

Construction of Yeast Strains and Measurements of Mutation Rates—A yMSH6 integration vector was constructed by subcloning a 5.3-kb EcoRI/BamHI fragment from the centromeric plasmid pMMR3 (from L. Prakash, University of Texas Medical Branch, Galveston, TX) into the integration vector YIplac211. A yMSH3 integration vector was constructed by subcloning a 4-kb KpnI/PstI fragment from the centromeric plasmid pMM11 (from L. Prakash) into YIplac211. The PCNA binding motifs were altered by site-directed mutagenesis using Pfu turbo polymerase (Stratagene) and mutant oligonucleotides (Genosys). Integration vectors were linearized with AflII and placed into E203 (msH3 and msH6) (22) or SджR838 (23). Integrants were selected as described (24), mutations were verified by DNA sequencing and mutation rates were determined as described (25).

DNA Mismatch Repair— Procedures for measuring MMR activity have been described (19). Repair reactions (25 μl) contained 0–400 μM hMSH3 peptide (amino acids 18–37) or hMSH6 peptide (amino acids 1–20).

RESULTS

MSH3 and MSH6 Contain Consensus PCNA Binding Motifs Not Found in MSH2—Taq MutS protein contains five domains (26), and sequence alignments suggest that human and yeast MSH2, MSH3, and MSH6 proteins share these five domains (26), and sequence alignments suggest that human and yeast MSH2, MSH3, and MSH6 proteins share these five domains (Fig. 1A). However, MSH3 and MSH6 contain distinct N-terminal amino acid sequences not found in Taq MutS or in MSH2 (Fig. 1A). These regions of MSH3 and MSH6 encode the sequence Qxhxhxaxa (Fig. 1B), where h and a are hydrophobic and aromatic amino acids, respectively. Flanking this consensus motif are several charged amino acids that may contribute to binding (reviewed in Ref. 11).

PCNA Interacts with MSH3 and MSH6 Peptides Containing the Binding Motifs—Using glutathione-agarose affinity beads in a pull-down assay previously used to demonstrate PCNA interaction with FEN1, we first examined the ability of human PCNA to interact with human and yeast MSH3 and MSH6 peptides. Human PCNA bound to GST fusion proteins containing short peptide sequences with the putative PCNA binding motifs of hMSH3 (Fig. 2, panel A, lane 5), hMSH3 (lane 7), yMSH3 (lane 9), and yMSH6 (lane 10). These results are similar to PCNA binding to GST fusion proteins containing the PCNA binding motifs of hFEN1 and hDNA ligase I (Fig. 2, panel A, lanes 2 and 3). Substituting alanines for the conserved phenylalanines, which abolishes PCNA binding to the DNA ligase I peptide (lane 4) (16), eliminated PCNA binding by hMSH3 (lane 6) and hMSH6 (lane 8). Next, we examined the ability of yeast PCNA to bind to yeast Msh3 and Msh6 fusion proteins. Because yeast PCNA is 29 kDa and nearly comigrates with the fusion proteins in SDS-PAGE gels, complexes isolated using the pull-down assay were treated with thrombin to release the Msh peptides and any associated PCNA into the supernatant. Analysis of the supernatants (Fig. 2, panel B, left) showed that yeast PCNA bound to fusion proteins of yMSH3 (lane 2), yMSH6 (lane 3), and hFEN1 (lane 4), but not to GST alone (lane 1). Parallel experiments with human PCNA (Fig. 2, panel B, right) confirmed its ability to bind to fusion proteins of yMSH3 (lane 6), yMSH6 (lane 7), and hFEN1 (lane 8) but not to GST alone (lane 5). These experiments also demonstrated that the GST moiety generated by thrombin cleavage did not contribute to the band intensity at 29 kDa in the experiments with yeast PCNA (lanes 1–4). When the bead-associated material after cleavage was analyzed (not shown), the GST moiety was found in approximately equal amounts and no intact fusion proteins were seen, indicating that the cleavage reaction was complete.

PCNA Interacts with the Msh2-Msh6 Heterodimer—We next examined binding of the intact Msh2-Msh6 heterodimer to yeast PCNA attached to Affi-gel beads. Wild type Msh2-Msh6 bound to these beads (Fig. 2C) but not to beads to which BSA had been attached (not shown). This result is similar to a previous demonstration that yPCNA binds to Msh2-Msh3 but not to Msh2 alone (7). PCNA binding was strongly reduced when the heterodimer contained wild type Msh2 and mutant Msh6 with alanine substituted for conserved amino acids in the PCNA binding motif (Fig. 2C).

Alteration of PCNA Binding Motifs Yields Mutators—To examine the functional importance of the yeast Msh3 and Msh6 PCNA binding motifs, we constructed haploid yeast strains with alanine substituted for conserved residues in the motifs

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2 The wild type and mutant Msh2-Msh6 complexes were each highly purified in 1:1 stoichiometry. K. Drotschmann and T. A. Kunkel, manuscript in preparation.
and measured mutation rates \textit{in vivo}. We used a highly sensitive reporter gene that monitors the rate of \textit{Lys} \textsuperscript{+} reversion via single-base deletions in a run of 14 A-T base pairs in the \textit{Lys}2 gene (25). An \textit{msh3} \textit{msh6} double mutant strain had an 11,000-fold higher mutation rate than a wild type strain (Table 1), reflecting inactivation of Msh2-Msh6- and Msh2-Msh3-dependent mismatch repair. However, \textit{msh3} \textit{msh6} single mutant strains had reversion rates that were 16- and 200-fold higher, respectively, than the wild type yeast strain (Table 1). These smaller increases are expected based on the redundancy of these pathways (27) and reflect the contribution of wild type Msh6 and Msh3 to repair. However, substituting alanine for conserved residues in the PCNA binding motif of either \textit{Msh3} or \textit{Msh6} increased reversion rates about 20-fold relative to the respective wild type genes (Table 1). The strain with alanine substitutions in the PCNA binding motif of \textit{Msh3} also had a 10-fold higher rate of reversion at a run of 10 G-C base pairs (23) compared with wild type (Table 1), and the strain with alanine substitutions in the PCNA binding motif of \textit{Msh6} had a mutation rate at the \textit{CAN1} locus that was 2-fold higher than the \textit{msh3}\textit{msh6} yeast strain (Table 1).

\textbf{Inhibition of MMR Activity by Msh Peptides Containing the PCNA Binding Motif—\textit{Next}, we measured MMR activity in extracts of human TK6 cells in the absence or presence of N-terminal hMsh3 and hMsh6 peptides that contain this motif. We reasoned that addition of hMsh3 or hMsh6 PCNA binding peptides to the mismatch repair reaction might compete with native Msh3 or Msh6 for binding to PCNA, thus preventing repair. With the MMR assay used (19), any effect results from inhibition of MMR at a step prior to resynthesis of DNA (4). As shown in Fig. 3, the extract alone efficiently repaired a G-G mismatch and a 2-base insertion mismatch. Addition of hMsh3 or hMsh6 peptides inhibited repair of both mismatches in a concentration-dependent manner. Addition of peptides with alanines replacing the conserved phenylalanines did not inhibit repair.}

\textbf{DISCUSSION}

We have identified consensus PCNA binding motifs in Msh3 and Msh6 and have clearly shown that these N-terminal residues interact with PCNA. Given the presence of this motif, interaction with PCNA was anticipated based on previous demonstrations that several other proteins interact with PCNA via this conserved motif (12–17). Our results with the yeast Msh3 peptide are consistent with a previous study (7) showing an interaction between yeast PCNA and the yeast Msh2-Msh3 heterodimer but not with Msh2 alone. The data with the human Msh6 peptide and intact Msh2-Msh6 are also consistent with hMsh6 binding to a hPCNA affinity column (8). The elevated mutation rates of yeast strains with mutations in these PCNA binding motifs suggest that interactions between PCNA and both Msh3 and Msh6 are important for genome stability. The observed mutator phenotypes may reflect reduced repair of mismatches recognized by Msh2-Msh3 and Msh2-Msh6. This possibility is consistent with the inhibition of strand-specific MMR at a step preceding DNA resynthesis observed when Msh3 or Msh6 PCNA binding peptides are added to MMR reactions catalyzed by an extract of human cells (Fig. 3).

Substitutions in the Msh3 and Msh6 PCNA binding motif that strongly reduced binding to PCNA (Fig. 2) yielded mutation rates that were not as high as in strains completely devoid of Msh3 or Msh6 (Table 1), indicating retention of some MMR function. Precedent for partial repair activity comes from previous studies showing that Fen1 and DNA ligase I participate with reduced efficacy in long-patch base excision repair when similar substitutions are present in their PCNA binding motifs (20, 28, 29). Partial retention of MMR function might reflect residual PCNA interactions with Msh2-Msh3 and Msh2-Msh6 at other sites or with other MMR proteins in a multiprotein complex. For example, yeast two-hybrid analysis indicates that PCNA interacts with Mlh1 (4), and human PCNA co-immunoprecipitates in a complex containing MLH1 and PMS2 (6). Any direct interactions of PCNA with MutL homologues may be at sites other than the interdomain connector loop of PCNA, because we did not find consensus PCNA binding motifs in either Mlh1 or Pms1.

It is also possible that the intermediate mutator phenotypes of strains with substitutions in the Msh3 and Msh6 PCNA binding motifs reflect the importance of PCNA interactions for one form of MMR but not another. Just as replication enzymology differs on the leading and lagging strands, so too might MMR enzymology differ, \textit{e.g.}. at the origin, during chain elongation, or during Okazaki fragment processing. The role of PCNA may also depend on the relative locations of the mismatch and the strand discrimination signal. For example, the nick that can serve as a strand discrimination signal \textit{in vitro} can be either 5' or 3' to the mismatch. The PCNA binding motif of Msh3 and Msh6 interacts with one of three potential binding sites in trimeric PCNA, potentially leaving two other binding sites on PCNA available for binding by other proteins. This may include a subunit of DNA polymerase \(\delta\), the replicative polymerase suggested to participate in DNA resynthesis during MMR (6, 30). Through this physical linkage, mismatches that escape proofreading at the primary terminus may be efficiently recognized just after emerging from the polymerase.

The mismatch may then be removed by a 3'-exonuclease (identity unknown) via excision of a small number of nucleotides in what may be an extended form of proofreading. Alternative and not mutually exclusive models (reviewed in Refs. 1 and 2) are suggested by the fact that the eukaryotic MMR system can
peptides with alanines substituted for the conserved phenylalanines. That repair was indeed strand-specific. All repair values reflect scoring (-) strand. The change in the ratio of colorless to blue plaques indicated the colorless to blue plaque ratio, because the nick directs repair to the in the extract decreases the percentage of mixed plaques and increases E. coli and contains a nick at nucleotide 90 of the LacZ gene. The (+) strand codes for blue plaques whereas the (-) strand encodes colorless plaques, whereas the (-) strand codes for blue plaques and contains a nick at nucleotide 90 of the LacZ gene. The (+) strand is covalently closed and functions in modulating cell survival and spontaneous and induced mutagenesis. To the extent that PCNA participates in these transactions, the ability of MSH3 and MSH6 to bind to PCNA may be important to their recruitment and functions in modulating cell survival and spontaneous and induced mutagenesis. 

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**TABLE I**

| Strain | Mutation rate | 95% Confidence limits | Relative rate |
|--------|---------------|------------------------|--------------|
| Lys * Reversion Rate at Run of 14 A·T Base Pairs | ×10^6 |                          |              |
| Wild Type | 0.14 | 0.12–0.19 | 1 |
| msh3/msh6 | 1500 | 1400–2000 | 11,000 |
| msh3/MSH6 | 2.3 | 1.9–3.3 | 16 (1) |
| msh3/msh6-KQFF | 52 | 19–68 | 370 (23) |
| msh3/msh6 | 28 | 24–36 | 200 (1) |
| msh3-FFa/msh6 | 650 | 560–880 | 4600 (23) |
| msh3-QFF/msh6 | 450 | 380–520 | 3200 (17) |
| Lys * Reversion Rate at Run of 10 G·C Base Pairs | ×10^6 |                          |              |
| Wild Type | 0.42 | 0.34–0.83 | 1 |
| msh3-QFF | 4.4 | 3.7–5.8 | 10 |
| Canavanine Resistance Mutation Rate |                          |              |
| Wild Type | 0.29 | 0.2–0.4 | 1 |
| msh3/msh6 | 5.0 | 4.6–8 | 17 |
| msh3-FFa/msh6 | 0.56 | 0.4–0.7 | 1.9 |
| msh3/msh6-KQFF | 1.1 | 0.9–1.3 | 3.8 |

* The indicated amino acids in the PCNA binding motif were changed to alanine.