Transfer of the MSH2-MSH6 Complex from Proliferating Cell Nuclear Antigen to Mispaired Bases in DNA*

Accelerated Publication

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Proliferating cell nuclear antigen (PCNA) is thought to play a role in DNA mismatch repair at the DNA synthesis step as well as in an earlier step. Studies showing that PCNA interacts with mismatch-binding protein complexes, MSH2-MSH3 and MSH2-MSH6, and that PCNA enhances MSH2-MSH6 mismatch binding specificity suggest PCNA may be involved in mismatch recognition. Here we show that PCNA and MSH2-MSH6 form a stable ternary complex with a homoduplex (G/C) DNA, but MSH2-MSH6 binding to a heteroduplex (G/T) DNA disrupts MSH2-MSH6 binding to PCNA. We also found that the addition of ATP or adenosine 5'-O-(thiotriphosphate) restores MSH2-MSH6 binding to PCNA, presumably by disrupting MSH2-MSH6 binding to the heteroduplex (G/T) DNA. These results support a model in which MSH2-MSH6 binds to PCNA loaded on newly replicated DNA and is transferred from PCNA to mismatched bases in DNA.

The bacterial MutS protein and the eukaryotic heterodimeric complexes of MutS homologue proteins, the MSH2-MSH6 (MutSα) and MSH2-MSH3 (MutSβ) complexes, are mismatch recognition factors that function in MMR† for review, see Refs. 1–4. These proteins have a higher affinity for mismatches as compared with base pairs and form a specific footprint at the site of a mismatch (5–14). The MutS protein recognizes both base/base and insertion/deletion mismatches (5). In contrast, the MSH2-MSH6 complex appears to recognize both base/base and insertion/deletion mismatches, whereas the MSH2-MSH3 complex appears relatively specific for insertion/deletion mismatches (7–11, 14–17). The difference in affinity of these proteins in vitro for different mismatches versus base pairs is small and has generally been reported to be not more than 30-fold (5, 7, 9, 12, 14). When either the MSH2 homodimer, the MSH2-MSH6 heterodimer, or the MSH2-MSH3 heterodimer binds a mismatch, the protein complex forms a ring around the DNA (7, 9, 12, 18–22). On binding ATP, these protein rings undergo a conformational change, are released from the mismatch, and slide along the DNA (7–9, 12, 20–23). A number of potential functions of these sliding clamps have been postulated, although their exact role in MMR is not understood.

The recognition of mismatched bases by the MutS family of proteins in vivo may be more complex than mismatch recognition in vitro. Using MMR-defective mutants in Saccharomyces cerevisiae, one can calculate from spontaneous mutation rates in coding sequences like CAN1 and URA3, or even in rare hypermutable sequences, that MMR likely has the ability to recognize a single mismatched base per genome of ~107 base pairs (4, 9, 15, 16). It seems unlikely that a ~30-fold affinity difference for mismatches versus base pairs can alone account for this in vivo MMR specificity. There are several other mechanisms that may contribute to the specificity of MMR. First, the MutS family of proteins forms complexes with other MMR proteins, and these complexes could have increased mismatch specificity. Examples include the interaction of MSH2 with EXO1 (24), the interaction of the MSH2-MSH6 and MSH2-MSH3 complexes with PCNA (25–27), and the interaction of MutS with the β clamp subunit of DNA polymerase III (28). Second, mismatch binding by the MutS family proteins could drive mismatch-specific conformational changes required for MMR. Examples include the assembly of MutL onto MutS at a mismatch (6, 29), the assembly of the eukaryotic MLH1-containing heterodimeric complexes with either the MSH2-MSH6 or MSH2-MSH3 complexes (30–32), at a mismatch, and even the mismatch-dependent conformational changes induced by ATP binding (7–9, 12, 13, 20–23). Finally, the interaction between the MutS family proteins and either PCNA or the β clamp subunit of DNA polymerase III has suggested that MMR may be coupled to replication (25–28). The observation that hMSH6 and hMSH3 co-localize with PCNA in replication foci (27) and that MutS is targeted to replication regions in bacteria is consistent with this idea (34). Such coupling could occur either by targeting MMR proteins to regions of replicating DNA or by incorporating MMR proteins as part of the replication machinery.

PCNA has been suggested to function in MMR at both an early step and during the DNA resynthesis step (35, 36). One role of PCNA in MMR may be mediated by interactions between PCNA and the MSH2-MSH6 and MSH2-MSH3 complexes through PCNA interaction motifs present in MSH6 and MSH3 (25–27). Mutations that alter amino acids in these PCNA interaction motifs cause a partial MMR defect in vitro and eliminate the in vitro interaction between PCNA and the MSH2-MSH6 and MSH2-MSH3 complexes (25, 26). Similarly, mutations in the gene encoding PCNA that cause MMR defects often eliminate the in vitro interaction between PCNA and the MSH2-MSH6 complex (26, 37). PCNA increases the in vitro mismatch binding specificity of the MSH2-MSH6 complex, and this is eliminated by amino acid substitutions in PCNA that cause MMR defects (26). These results suggest PCNA may play a role in MMR at the mismatch recognition step. To further understand the role of PCNA in mismatch recognition, we investigated the mismatch binding properties of the PCNA-MSH2-MSH6 complex. The results presented here demonstrate that the PCNA-MSH2-MSH6 complex can form a ternary complex with fully base-paired DNA, but when a mismatch is present PCNA is

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‡The abbreviations used are: MMR, DNA mismatch repair; PCNA, proliferating cell nuclear antigen; ATP·S, adenosine 5’-O-(thiotriphosphate).
excluded from the complex. These results suggest an in vitro model of mispair recognition whereby MSH2-MSH6 binds to PCNA on the DNA and is then transferred to the mispair.

MATERIALS AND METHODS

DNAs—The DNA duplexes used in this study have been described previously (9, 12). In brief, the oligonucleotide 5′-ATTCCTCAGCAGA-TAGGAAACATCTGATCAGCAT-3′ (bottom strand) was annealed with either the 5′-GATGCCACGATGAGTTCCTCAGCAGA-3′ (G/C top strand) oligonucleotide or the 5′-GATGCGAGGATGAGTTCCTCAGCAGA-3′ (G/T top strand) oligonucleotide to yield the homoduplex (G/C) or the heteroduplex (G/T) DNAs, respectively. The annealed products were purified by high pressure liquid chromatography using a GEN-PAK FAX column (Waters Corp.) and then 5′-end-labeled with [γ-32P]ATP and T4 polynucleotide kinase if necessary.

Protein Purification—MSH2-MSH6 was purified using a S. cerevisiae overexpression system as described previously (8, 9, 12), and PCNA was purified using an Escherichia coli overexpression system as described previously (38). All protein preparations were greater than 98% pure as judged by SDS-PAGE.

Sedimentation Analysis—Sedimentation analysis was performed as described previously (26, 37). In brief, protein samples (80 µl) were loaded onto the top of 4-ml, 15–30% glycerol gradients and centrifuged at 45,000 rpm at 4 °C for 20 h in a Beckman SW60 rotor. Thirty-one fractions were collected from the bottom of each gradient. The amount of radioactivity present in 15 µl of each fraction was determined by liquid scintillation counting. Aliquots (12.5 µl) of selected fractions were used for subsequent Western blot analysis. Alternatively, 127 µl of each fraction was concentrated using a Microcon YM 30 Centrifugal Filter (Millipore Corp.) and analyzed by SDS-PAGE using 4–15% gradient gels, which were subsequently stained with Coomassie Blue. The following amounts of proteins or DNAs (individually or mixtures thereof) were incubated on ice for 30 min and sedimented through the gradients: 7.5 µg of MSH2-MSH6, 2.5 µg of PCNA, 680 ng of G/C DNA plus 3.7 ng of γ-32P-labeled G/C DNA, 680 ng of G/T DNA plus 3.7 ng of γ-32P-labeled G/T DNA.

Co-precipitation—PCNA was biotinylated according to the ECL protein biotinylation kit (Amersham Biosciences). MSH2-MSH6 (1.5 pmol of heterodimer) and biotinylated PCNA (1.5 pmol of homotrimer) was mixed with either no DNA, G/T DNA, or G/C DNA in buffer A (50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.01% (octylphenoxy)polyethoxyethanol (Sigma)) and incubated on ice for 30 min. The total reaction volume was 40 µl, and the amounts of DNA added are indicated in individual experiments. For each reaction, 80 µl (40 µl of settled resin) of streptavidin-agarose (Novagen) was washed once in buffer A and centrifuged at 500 × g for 1 min at 4 °C. The supernatant was removed, and the reaction mixture was added to the streptavidin-agarose. The mixture was incubated on ice for 30 min with gentle mixing every 5 min. The agarose beads were then washed three times by centrifugation at 500 × g for 1 min at 4 °C followed by resuspension in 40 µl of buffer A. After the last resuspension, SDS loading buffer was added to the mixture, and the mixture was incubated for 10 min at 95 °C. 15 µl of the supernatant was analyzed by SDS-PAGE and Western blotting. For the precipitation experiments with and without nucleotide, MSH2-MSH6 (1.5 pmol of heterodimer) and biotinylated PCNA (1.5 pmol of homotrimer) was mixed with either no DNA, G/T DNA (7.5 pmol), or G/C DNA (7.5 pmol) in buffer A or buffer A supplemented with either ATP (250 µM) or ATPγS (250 µM).

Western Blotting—Protein samples were transferred to immobilized polyvinylidene difluoride membranes (Bio-Rad) in 25 mM Tris base, 192 mM glycine, and 20% (v/v) methanol and analyzed by Western blotting essentially as described previously using polyclonal rabbit antibodies raised against either full-length MSH2, MSH6, or PCNA (26) and the ECL Plus protocol (Amersham Biosciences). The resulting films were quantified by densitometry.

RESULTS

Sedimentation Analysis of MSH2-MSH6-PCNA-DNA Complexes—Using sedimentation analysis, earlier studies showed that the MSH2-MSH6 protein complex interacted with wild-type PCNA but did not interact with several MMR-defective mutant PCNAs to the extent seen with wild-type PCNA (26, 37). Here we studied the interactions between MSH2-MSH6, PCNA, and either homoduplex or heteroduplex DNA. As observed previously (26, 37), the addition of PCNA to MSH2-MSH6 caused MSH2-MSH6 to sediment further into the gradient (lower fraction numbers) than MSH2-MSH6 alone.

FIG. 1. Sedimentation analysis of in vitro interactions between PCNA, MSH2-MSH6, and either homoduplex (G/C) or heteroduplex (G/T) DNA. Reactions containing PCNA, MSH2-MSH6, homoduplex (G/C) DNA, and heteroduplex (G/T) DNA, either individually or in mixtures (as indicated on the right side of the figure), were mixed, sedimented through glycerol gradients, separated into fractions, and analyzed as described under "Materials and Methods." Fraction 1 is the bottom of each gradient. A, Coomassie Blue staining of MSH2 (bottom band) and MSH6 (top band) present in each fraction. B, Coomassie Blue staining of PCNA present in each fraction. C, percentage of radiolabeled homoduplex (G/C) DNA present in each fraction. D, percentage of radiolabeled heteroduplex (G/T) DNA present in each fraction. (Fig. 1A). PCNA alone was found in fractions 17 and above, whereas addition of MSH2-MSH6 caused PCNA to sediment further into the gradient such that PCNA was now observed in the same fractions as the shifted MSH2-MSH6 (Fig. 1B). This shift of MSH2-MSH6 and PCNA to larger sedimentation coefficients is consistent with the formation of a MSH2-MSH6-PCNA complex (26, 37). Addition of either homoduplex (G/C) or heteroduplex (G/T) DNA to MSH2-MSH6 caused both MSH2-MSH6 and the DNA to sediment at a larger sedimentation coefficient than either MSH2-MSH6 (Fig. 1A) or the DNA alone (Fig. 1A, C, and D) (the peak fraction for each DNA sedimented alone was fraction 25). This is consistent with previous observations that MSH2-MSH6 can bind to oligonucleotide duplexes that do or do not contain mispaired bases (7–12, 14). The addition of PCNA to either homoduplex (G/C) or heteroduplex (G/T) DNA did not alter the sedimentation of either PCNA (Fig. 1B) or the DNA (Fig. 1, C, and D). This is consistent with the observation that PCNA does not stably bind to linear DNA (26).

When MSH2-MSH6, PCNA, and the homoduplex (G/C) DNA were combined and sedimented, MSH2-MSH6 (Fig. 1A), PCNA (Fig. 1B), and the homoduplex (G/C) DNA (Fig. 1C) all sedimented further into the gradient than observed with any combination of two. These results suggest that MSH2-MSH6, PCNA, and the homoduplex (G/C) DNA can form a stable complex. Interestingly, the same results were not seen when MSH2-MSH6, PCNA, and the heteroduplex (G/T) DNA were combined and sedimented. Under these conditions, MSH2-MSH6 (Fig. 1A) and the heteroduplex (G/T) DNA (Fig. 1D) co-sedimented to the same position as the preformed MSH2-

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MSH6-heteroduplex (G/T) DNA complex, while all of the PCNA appeared to sediment to the same position as PCNA alone. Moreover, no PCNA was observed in the fractions containing the MSH2-MSH6-heteroduplex (G/T) DNA complex (as observed by staining with Coomassie Blue). The inability to observe co-sedimentation of PCNA with the MSH2-MSH6-heteroduplex (G/T) DNA complex was independent of the order of addition of the proteins and DNA, including preincubation of MSH2-MSH6 with PCNA prior to the addition of the heteroduplex (G/T) DNA (data not shown). These results suggest that when an MSH2-MSH6-PCNA complex binds to the heteroduplex (G/T) DNA, PCNA is excluded from the complex.

To substantiate these findings, Western blotting was used to provide more sensitive detection of PCNA (Fig. 2). After sedimentation and fractionation of reactions that contain PCNA, MSH2-MSH6, and a DNA (either G/C or G/T), peak fractions (fractions 11 and 12) that contain MSH2-MSH6 and DNA were analyzed to detect PCNA. The same fractions from reactions containing PCNA mixed with MSH2-MSH6 or PCNA mixed with either the homoduplex (G/C) or the heteroduplex (G/T) DNA were analyzed as controls. PCNA was present in the two fractions when MSH2-MSH6 or MSH2-MSH6 and the homoduplex (G/C) DNA were incubated with PCNA prior to sedimentation. However, PCNA was not detected when either the homoduplex (G/C) DNA alone, the heteroduplex (G/T) DNA alone, or MSH2-MSH6 with the heteroduplex (G/T) DNA were incubated with PCNA prior to sedimentation. This strengthens the above finding that PCNA forms a complex with MSH2-MSH6 and homoduplex (G/C) DNA but not with MSH2-MSH6 and heteroduplex (G/T) DNA.

**Co-precipitation Analysis of MSH2-MSH6-PCNA-DNA Complexes—**Co-precipitation experiments were performed to analyze the formation of MSH2-MSH6-PCNA-DNA complexes. Biotinylated PCNA was incubated with MSH2-MSH6 and either no DNA or increasing amounts of the homoduplex (G/C) DNA or the heteroduplex (G/T) DNA. The resulting complexes were then precipitated with streptavidin-agarose, and the bound proteins were detected by SDS-PAGE and Western blotting. The addition of the heteroduplex (G/T) DNA caused a large decrease in the amount of MSH2 and MSH6 that co-precipitated with the biotinylated PCNA (Fig. 3A); the amount of MSH2 and MSH6 bound was reduced by ~70% compared with the levels observed when no DNA was added. In contrast, the addition of the homoduplex (G/C) DNA caused a small ~10% decrease in the amount of MSH2 and MSH6 that co-precipitated with the biotinylated PCNA compared with no added DNA (Fig. 3A). These results suggest that the addition of the heteroduplex (G/T) DNA causes MSH2-MSH6 to partition into MSH2-MSH6-DNA complexes thus inhibiting the formation of MSH2-MSH6-PCNA complexes, whereas the addition of homoduplex DNA does not appear to inhibit the formation of MSH2-MSH6-PCNA complexes to the same extent.

Co-precipitation experiments were also used to examine the effect of ATP and ATPγS on the interactions between PCNA and MSH2-MSH6 in the presence or absence of a DNA. When there was no DNA present, the addition of ATP or ATPγS caused only a small ~10% decrease in the amount of MSH2 and MSH6 that co-precipitated with the biotinylated PCNA when compared with samples with no nucleotide added (Fig. 3B). In the presence of the homoduplex (G/C) DNA, the addition of ATP and ATPγS caused a small ~15 or 19% increase, respectively, in the amount of MSH2 or MSH6 bound to PCNA compared with reactions with no nucleotide added (Fig. 3B). In the presence of the heteroduplex (G/T) DNA, the addition of ATP or ATPγS caused a larger decrease, ~70 and ~90%, respectively, in the amount of MSH2 and MSH6 that co-precipitated with the biotinylated PCNA compared with samples with no nucleotide added. It has been shown that addition of ATP or ATPγS to MSH2-MSH6 in the presence or absence of PCNA causes MSH2-MSH6 to dissociate from heteroduplex DNA (7–9, 12, 20, 22, 26). Our results are consistent with the idea that when ATP or ATPγS is added to mixtures containing MSH2-MSH6, PCNA, and the heteroduplex (G/T) DNA, MSH2-MSH6 has a lower affinity for the heteroduplex (G/T) DNA thereby increasing the MSH2-MSH6 available to complex with PCNA.

**DISCUSSION**

In the present study, we observed that MSH2-MSH6 and MSH2-MSH6-PCNA complexes each formed a complex with homoduplex (G/C) DNA and that MSH2-MSH6 formed a complex with heteroduplex (G/T) DNA. In contrast to the results observed with homoduplex (G/C) DNA, when MSH2-MSH6, PCNA, and heteroduplex (G/T) DNA were incubated together, only an MSH2-MSH6-heteroduplex (G/T) DNA complex was obtained. Consistent with this, heteroduplex (G/T) DNA inhibited the formation of a co-precipitable MSH2-MSH6-PCNA complex, whereas homoduplex (G/C) DNA had essentially no
effect on the formation of a co-precipitable MSH2-MSH6-PCNA complex. These results indicate that the MSH2-MSH6-PCNA complex can bind nonspecifically to DNA, but when MSH2-MSH6 binds to a mispair, binding of PCNA to MSH6 is disrupted. This suggests that either binding to a mispair alters the conformation of MSH2-MSH6 such that MSH6 cannot strongly interact with PCNA or that the position of the mispaired DNA within the MSH2-MSH6 ring may exclude PCNA from the complex. To gain further insight into this observation, we are developing approaches to analyze the structure of MSH2-MSH6-PCNA-DNA complexes and are analyzing the effect of other MMR proteins on complex formation. In previous studies using gel mobility shift assays, we observed that addition of PCNA to MSH2-MSH6 increased mispair binding specificity of MSH2-MSH6 but did not alter the mobility of the mispair-MSH2-MSH6 complex, whereas the nonspecific protein-DNA complexes formed often had a somewhat reduced mobility and a more heterogeneous nature (see Fig. 3E of Ref. 26). These results suggest that the conformational changes that occur during the transition between the nonspecific MSH2-MSH6-PCNA-DNA complex and the MSH2-MSH6-mispair complex in which PCNA is excluded may modestly improve mispair binding specificity.

A number of studies have shown that the MSH2-MSH6 and MSH2-MSH3 heterodimers bind to PCNA through a conserved motif found in MSH6 and MSH3 and that these interactions are important for MMR (25, 26). These observations provide molecular insights into how PCNA might function at an early step in MMR and suggest that PCNA can play a role in mispair recognition. Three different types of models have been proposed for how PCNA might function early in MMR (25–27, 35–37). First, it has been suggested that because a PCNA trimer has three distinct protein docking sites, PCNA may assemble a higher order complex containing both MMR and replication proteins at replicating regions of DNA. However, analysis of the interaction of multiple proteins with PCNA has indicated that one PCNA-interacting protein displaces a second from PCNA rather than assembling into a complex with more than one protein bound to PCNA (39–43). Second, it has been suggested that MSH2-MSH6 and MSH2-MSH3 form sliding clamps at mispairs that migrate to the replication DNA polymerase and induce dissociation of the replication complex (27). Constraints on this model are that it often had a somewhat reduced mobility and a more heterogeneous nature (see Fig. 3E of Ref. 26). These results suggest that the conformational changes that occur during the transition between the nonspecific MSH2-MSH6-PCNA-DNA complex and the MSH2-MSH6-mispair complex in which PCNA is excluded may modestly improve mispair binding specificity.
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