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The Human Mismatch Recognition Complex hMSH2-hMSH6 Functions as a Novel Molecular Switch

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Summary

The mechanism of DNA mismatch repair has been modeled upon biochemical studies of the *E. coli* DNA adenine methylation–instructed pathway where the initial recognition of mismatched nucleotides is performed by the MutS protein. MutS homologs (MSH) have been identified based on a highly conserved region containing a Walker-A adenine nucleotide binding motif. Here we show that adenine nucleotide binding and hydrolysis by the human mismatch recognition complex hMSH2-hMSH6 functions as a novel molecular switch. The hMSH2-hMSH6 complex is ON (binds mismatched nucleotides) in the ADP-bound form and OFF in the ATP-bound form. These results suggest a new model for the function of MutS proteins during mismatch repair in which the switch determines the timing of downstream events.

Introduction

The most widely accepted model for post-replication mismatch repair is based on work with the DNA adenine methylation (Dam)–instructed pathway of Escherichia coli (reviewed by Modrich, 1989, 1991, 1997; Modrich and Lahue, 1996). In this model, the MutS protein recognizes and binds mispaired nucleotides that result from polymerase misincorportation errors. MutS mismatch binding is followed by interaction with the MutL protein, which appears to accelerate a proposed ATP-dependent translocation of the MutS-MutL complex (Allen et al., 1997) to a hemimethylated GATC Dam site that is bound by the MutH protein. The MutS-MutL complex then stimulates an intrinsic endonuclease activity of MutH; this results in a strand scission on the unmethylated DNA strand that directs one of three single-stranded exonucleases (RecJ, Exo I, ExoVII) to degrade the newly replicated strand that appears to be resynthesized by the PolIII holoenzyme complex. The net result is a strand-specific mismatch repair event that can be bidirectional.

Homologs of the prokaryotic MutS and MutL proteins have been identified in eukaryotes (reviewed by Fishel and Kolodner, 1995; Fishel and Wilson, 1997). However, outside of gram-negative bacteria, there do not appear to be homologs of MutH. Thus, the mechanism of strand discrimination in even close relatives of E. coli, the gram-positive bacteria, remains a mystery. Furthermore, multiple MutS and MutL homologs have been identified in yeast and human cells that individually participate in such diverse activities as nuclear and organelle mismatch repair as well as distinct meiotic functions.

Germ-line mutations of the *h*uman *M*ut*S* and *M*ut*L* *H*omologs, hMSH2, hMLH1, and hPMS2, are associated with the common cancer predisposition syndrome, hereditary nonpolyposis colorectal cancer (HNPCC) (Fishel et al., 1993; Bronner et al., 1994; Nicolaides et al., 1994). Furthermore, the yeast and human MutS and MutL homologs appear to exist primarily as heterodimeric proteins (Prolla et al., 1994; Drummond et al., 1995; Li and Modrich, 1995; Acharya et al., 1996; Marsischky et al., 1996). Thus, hMSH2 protein associates with hMSH3 or hMSH6 proteins, and hMLH1 protein associates with hPMS2 protein. In addition, the hMSH2-hMSH3 and hMSH2-hMSH6 protein complexes appear to possess overlapping and redundant mispair binding activities, at least partially explaining the lack of mutations in hMSH3 and hMSH6 in HNPCC (Acharya et al., 1996; Risinger et al., 1996).

Classification of MutS and MutL homologs is based on the recognition of highly conserved regions of amino acid identity. The most highly conserved region of the MutS homologs is confined to approximately 150 amino acids that encompass a helix-turn-helix domain associated with a Walker-A adenine nucleotide and magnesium-binding motif (Walker et al., 1982). This adenine nucleotide–binding domain constitutes more than 80% of the identifiable homology between MutS homologs (Fishel and Wilson, 1997). Both purified bacterial and yeast MutS homologs have been found to possess an intrinsic low-level ATPase (Haber and Walker, 1991; Chi and Kolodner, 1994; Alani et al., 1997). This ATPase is likely to be important for the function of the MutS homologs because mutation of conserved amino acid residues in the adenine nucleotide–binding domain results in a dominant mutator phenotype in both bacteria and yeast (Haber and Walker, 1991; Wu and Marinus, 1994; Alani et al., 1997). A central role for the adenine nucleotide–binding domain is consistent with the ATP-dependent translocation model of mismatch repair proposed by Modrich and colleagues (Allen et al., 1997).

Genetic and biochemical studies of the human mismatch repair process indicate that it is similar to bacterial mismatch repair (Miller et al., 1976; Glazer et al., 1987; Holmes et al., 1990; Thomas et al., 1991). Purified hMSH2 protein binds mismatched nucleotides and DNA lesions (Fishel et al., 1994a, 1994b; Mello et al., 1996), and the specificity as well as the affinity of that recognition is enhanced by association with hMSH3 or hMSH6 (Drummond et al., 1995; Acharya et al., 1996; Palombo et al., 1996). Here, we have used G/T mismatch recognition by the hMSH2-hMSH6 protein complex as a model for human mispair binding. We show that, like the bacterial and yeast proteins, hMSH2-hMSH6 possesses an intrinsic ATPase activity. However, our data indicate that this ATPase activity, and the associated adenine nucleotide–binding domain, functions to regulate mismatch binding as a molecular switch. The hMSH2-hMSH6 molecular switch is ON (bound to the mismatch)

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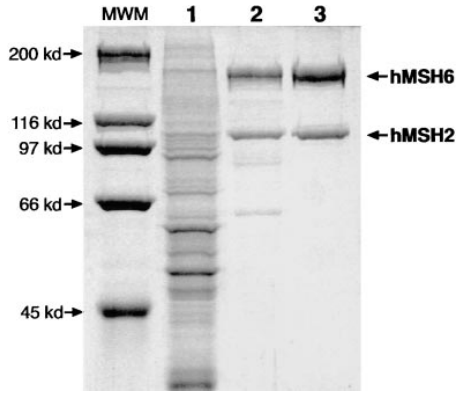

Figure 1. Purification of the hMSH2-hMSH6 Complex

A Coomassie-stained gel following the two-step purification of the protein complex. Molecular weight standards (Biorad) are shown. (Lane 1) Crude extract from insect cells infected with a dual expression virus containing hMSH2 and hMSH6; (lane 2) peak fractions from a nickel affinity column eluted with an imidazole gradient; (lane 3) peak fractions from a PBE anion exchange column eluted with an NaCl gradient. Arrows indicate hMSH2 (104.7 kDa) and hMSH6 (152.8 kDa).

in the ADP-bound form and OFF in the ATP-bound form. Hydrolysis of ATP results in the recovery of mismatch binding, while ADP→ATP exchange results in mismatch dissociation. These results support a model for timing/triggering of mismatch repair that is based on the association and dissociation of hMSH2-hMSH6 from mismatched nucleotides, which are controlled by γ-phosphate hydrolysis and adenine nucleotide exchange. The similarity of hMSH2-hMSH6 to G protein switches is striking (Bokoch and Der, 1993) and suggests that there may be associated protein activities similar to those found with GTPases, where both accelerators of the GTPase (GAP or RGS) (Dohlman and Thorner, 1997; Tocque et al., 1997) and guanine nucleotide exchange factors (GNEF) have been described (Quilliam et al., 1995).

Results

Overexpression and Purification of the hMSH2-hMSH6 Protein Complex

hMSH2 and hMSH6 proteins were overexpressed and purified from Sf9 insect cells using a dual expression baculovirus vector (Figure 1). Purification of hMSH2 and hMSH6 resulted in a stable heterodimer composed of a 1:1 molar ratio of these subunits as judged by quantitative densitometry of Coomassie-stained gels. Our purification methodology resulted in >95% homogeneous protein that was highly active and free of any detectable contaminating nucleic acid and nucleotides.

G/T Mismatch Binding by hMSH2-hMSH6 Is a Model for Mispair Recognition

The hMSH2-hMSH6 protein complex has been shown to bind to the eight possible mismatched nucleotide combinations as well as a subset of single nucleotide insertion/deletion mismatches (Hughes and Jiricny, 1992; Drummond et al., 1995; Acharya et al., 1996; data not shown). We have chosen the G/T mismatch as a model

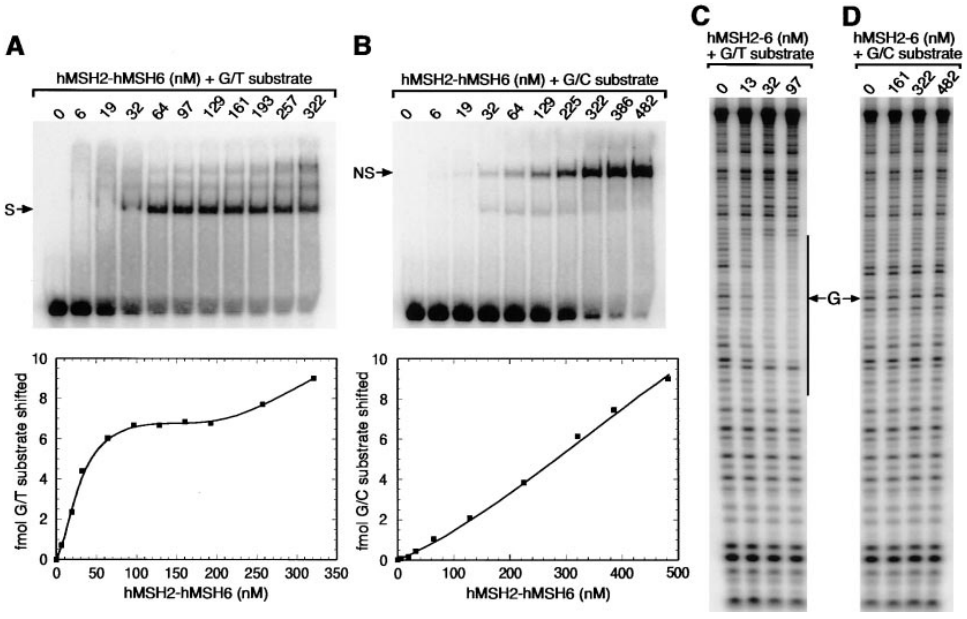

Figure 2. hMSH2-hMSH6 Binds G/T Mismatch DNA Specifically and Homoduplex DNA Nonspecifically

(A and B) Gel mobility shift assays of hMSH2-hMSH6 protein with 9 fmol of an 81 base pair G/T mismatch (A) or homoduplex (B) oligonucleotide. Protein concentration (nM) is indicated above each lane and the specific (S) and nonspecific (NS) mobility shift is indicated by the arrow (A and B).

(C and D) DNase I footprinting of hMSH2-hMSH6 with G/T mismatch DNA (C) or homoduplex DNA (D). The DNase I protection region on the G/T mismatch DNA is indicated by the vertical line to the right of (C). The position of the G residue of the G/T mismatch (C) or the G/C homoduplex DNA (D) is indicated with arrows.

for quantitative binding analysis of hMSH2-hMSH6 because of its relatively high recognition specificity (Figure 2). The apparent dissociation constant (K_d) in a simple buffer system (see Experimental Procedures) without adenine nucleotide or magnesium was determined for binding to the 81 bp fully duplex DNA and to an identical DNA containing a G/T mismatch at nucleotide 41. Both gel shift (Figure 2A) and DNase I footprint (Figure 2C) analysis revealed the $K_{d\cdot G/T}$ for a G/T mismatch to be 20 $\pm$ 5 nM, while binding was not saturable above 400 nM for homoduplex DNA (Figure 2B). Gel mobility shifts performed with a 39-mer oligonucleotide containing a G/T mismatch or a buffer containing 2 mM $MgCl_2$ yielded similar results (data not shown). The hMSH2-hMSH6 complex appears to bind a G/T mismatch in multiple slower-migrating forms at concentrations above 200 nM, suggesting alternate binding mechanisms. The significance of these multiple binding forms is unknown.

DNase I footprint analysis indicates that hMSH2-hMSH6 forms a specific binding complex that asymmetrically protects 25 nucleotides on both DNA strands of an 81 bp DNA containing a G/T mismatch (Figure 2C; data not shown). There appear to be two separate protection domains: one centered on the G/T mismatch and the other adjacent to this protected region, separated by a single DNase I–sensitive (unprotected) nucleotide. These data are qualitatively similar to those found for the E. coli and Thermus aquaticus MutS proteins (Su and Modrich, 1986; Biswas and Hsieh, 1997). While a shifted complex could be detected with homoduplex DNA, no specific DNase I footprint could be identified (Figure 2D). Lack of saturability and lack of a specific footprint are consistent with the ability of hMSH2-hMSH6 to associate weakly along the length of homoduplex DNA.

The shifted complex observed for the homoduplex DNA and the G/T mismatch DNA was found to migrate differently (Figure 2): the homoduplex DNA or nonspecific (NS) shift migrated more slowly than the G/T mismatch DNA-specific (S) shift. The binding specificity of these gel-shifted complexes was confirmed by excising the S- and NS-shifted complex and performing DNase I footprint analysis, which appeared similar to those shown in Figures 2C and 2D (data not shown). These results may suggest that the hMSH2-hMSH6 bound to the homoduplex DNA adopts a different conformation than hMSH2-hMSH6 bound to the G/T mismatch DNA or, alternatively, that there is more hMSH2-hMSH6 complex bound to the homoduplex DNA. Interestingly, no NS shift was observed with a 39-mer homoduplex (data not shown), suggesting that a minimum number of base pairs may be necessary to form the NS complex.

The hMSH2/hMSH6 Complex Converts ATP to ADP in the Presence of Mismatched DNA

Both bacteria and yeast MutS homologs have been shown to possess an intrinsic low-level ATPase (Haber and Walker, 1991; Chi and Kolodner, 1994; Alani et al., 1997). However, there are conflicting reports regarding the stimulation of these intrinsic ATPases by mismatched heteroduplex and/or homoduplex DNA (Chi and Kolodner, 1994; Alani et al., 1997). We have found

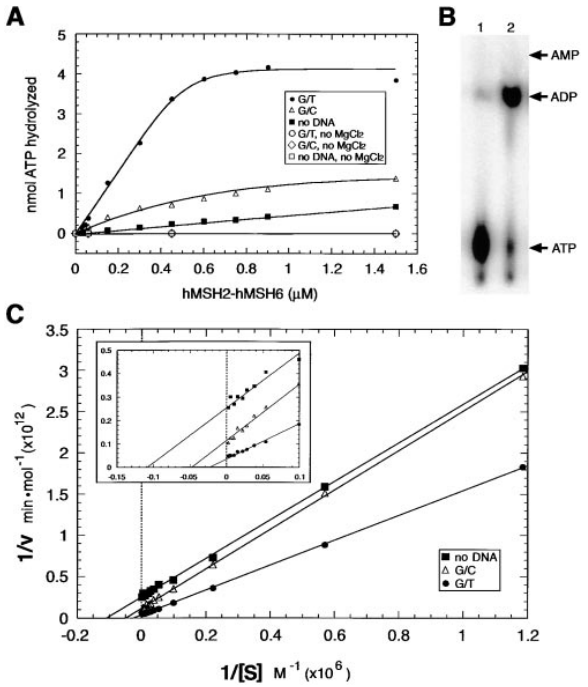

Figure 3. hMSH2-hMSH6 Hydrolyzes ATP to ADP in the Presence of G/T Mismatch DNA

(A) ATPase activity of hMSH2-hMSH6 without DNA and in the presence of 240 nM homoduplex DNA or G/T mismatch DNA.

(B) TLC analysis of hMSH2-hMSH6 ATPase in the presence of G/T mismatch DNA: (lane 1) no protein and (lane 2) 60 nM hMSH2-hMSH6.

(C) Lineweaver-Burk analysis of the steady-state hMSH2-hMSH6 ATPase (Dixon and Webb, 1979). Assays were performed with 60 nM hMSH2-hMSH6 (in the presence of homoduplex and G/T mismatch DNA) and 200 nM hMSH2-hMSH6 (without DNA) and varying ATP. (Inset) A magnification of the 1/v and 1/[S] (ATP) intercepts. The ATPase activity in the presence of G/T mismatch DNA ($V_{max\cdot ATP\cdot G/T} \approx$ 31 pmol/min; $K_{m\cdot ATP\cdot G/T} \approx 46$ μM), homoduplex ($V_{max\cdot ATP\cdot G/C} \approx$ 8.9 pmol/min; $K_{m\cdot ATP\cdot G/C} \approx 23$ μM), or no DNA ($V_{max\cdot ATP} \approx$ 3.6 pmol/min; $K_{m\cdot ATP} \approx$ 10 μM) was calculated from the intercept of 1/v and 1/[S].

that hMSH2-hMSH6 has an intrinsic steady-state, DNA-dependent ATPase that absolutely requires the presence of magnesium as a cofactor (Figure 3A). Thin layer chromatography (TLC) revealed that the hMSH2-hMSH6 ATPase uniformly converts ATP to ADP plus inorganic phosphate (Pi) (Figure 3B). Using Lineweaver-Burk (Figure 3C) and Eadie-Hofstee analysis (not shown), we find that the hMSH2-hMSH6 steady-state ATPase is most active in the presence of a G/T mismatch ($k_{cat\cdot ATP\cdot G/T} \approx 26$ min^{-1}; $K_{m\cdot ATP\cdot G/T} \approx 46$ μM), substantially less active in the presence of homoduplex DNA ($k_{cat\cdot ATP\cdot G/C} \approx 7.4$ min^{-1}; $K_{m\cdot ATP\cdot G/C} \approx 23$ μM), and largely inactive in the absence of DNA ($k_{cat\cdot ATP} \approx 0.9$ min^{-1}; $K_{m\cdot ATP} \approx 10$ μM). There is no difference in stimulation of the ATPase when comparing a 39-mer, 81-mer, or 2.9 kb homoduplex or comparing a 39-mer or 81-mer G/T mismatch (data not shown), suggesting that there is no DNA-length dependence of the hMSH2-hMSH6 ATPase. The inverse relationship between k_{cat} and K_m for these three reactions is intriguing and suggests that, while the rate of hydrolysis is increased in the presence of a mismatch (Figure 3A), the affinity for ATP decreases. These results are qualitatively

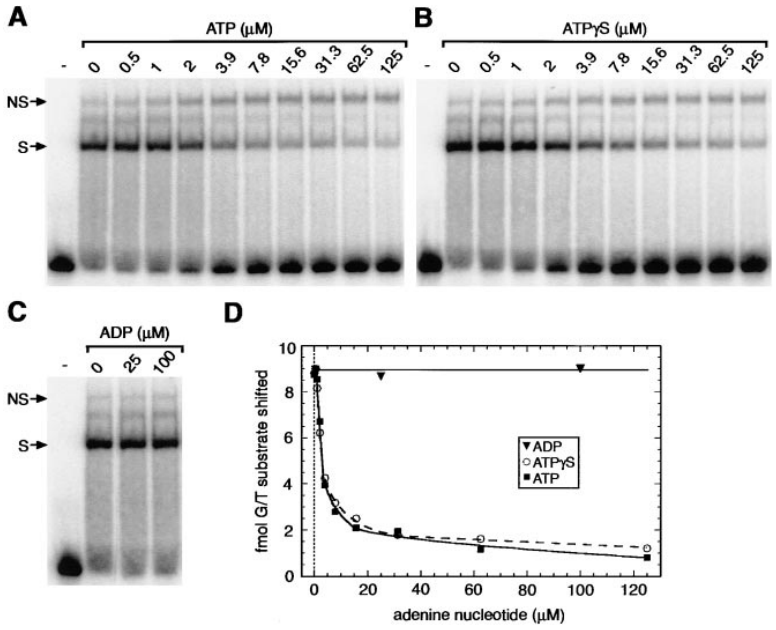

Figure 4. ATP and ATPγS Abolish G/T Binding by hMSH2-hMSH6 while ADP Is Refractory

The effect of varying amounts of ATP, ATPγS, and ADP on G/T mismatch binding under nonhydrolyzing conditions (no magnesium) is shown in (A), (B), and (C), respectively. The amount of adenine nucleotide (μM) in each reaction is indicated at the top of each gel. The G/T specific shifts were quantitated using a phosphorimager and displayed with respect to the nucleotide concentration (D). In all panels, (-) indicates substrate in the absence of protein.

similar to the phenomenon of uncompetitive inhibition, which may be ascribed to the presence of independent binding sites as well as compulsory ordered binding mechanisms (Dixon and Webb, 1979). Interestingly, ssDNA also stimulates the hMSH2-hMSH6 ATPase (data not shown), which may help to explain conflicting reports regarding the ATPase activities for related MutS homologs.

hMSH2/hMSH6 Mispair Binding Is Abolished by ATP in the Absence of Hydrolysis

It has been reported that both bacterial and eukaryotic MutS homologs fail to form a specific complex with a mismatch oligonucleotide in the presence of ATP (Grilley et al., 1989; Drummond et al., 1995; Alani et al., 1997). These results are the foundation of the ATP-dependent translocation model for MutS protein function during bacterial mismatch repair (Modrich, 1989, 1991, 1997; Modrich and Lahue, 1996; Allen et al., 1997). A suggestion that ATP hydrolysis was required for the mispair release is based on the observation that a nonhydrolyzable analog of ATP, Adenylyl-imidodiphosphate (AMP-PNP), does not alter mispair binding (Drummond et al., 1995; Alani et al., 1997; Allen et al., 1997). Similarly, we find that the hMSH2-hMSH6 complex is released from a G/T mismatch in the presence of ATP (Figures 4A and 4D). The IC_{50} required for ATP-dependent release of a G/T mismatch by hMSH2-hMSH6 was calculated to be approximately 3 μM. Furthermore, the poorly hydrolyzable analog Adenosine-5′-O-(3-thiotriphosphate) (ATP-γ-S) (Yu and Egelman, 1992) produced a similar release from a G/T mismatch as ATP, also with a calculated IC_{50} of 3 μM (Figures 4B and 4D). The addition of ADP to the mismatch binding reaction resulted in a slight increase in binding affinity (Figures 4C and 4D). The release of hMSH2-hMSH6 from the G/T mismatch does not appear to require hydrolysis of the γ-phosphate because (1) the release occurs in the absence of exogenous magnesium (Figure 4A), which is absolutely required for hMSH2-hMSH6-dependent ATP hydrolysis (Figure 3); and (2) the poorly hydrolyzable analog of ATP, ATP-γ-S, produces a similar release with or without magnesium (Figure 4B, unpublished data). Furthermore, the NS binding of hMSH2-hMSH6 to homoduplex DNA is insensitive to the addition of exogenous ATP (data not shown). Thus, the alteration of hMSH2-hMSH6 binding induced by ATP is confined specifically to the recognition of mismatched nucleotides.

The addition of dATP to a binding reaction also results in release of the G/T mismatch substrate from hMSH2-hMSH6 in the same manner as ATP and ATP-γ-S (Figure 5). No other nucleotide was found to stimulate the release of the G/T mismatch by hMSH2-hMSH6 (Figure 5). Interestingly, two other nonhydrolyzable analogs, AMP-PNP and Adenyl (β,γ-methylene)-diphosphonate (AMP-PCP), did not release hMSH2-hMSH6 from a G/T mismatch (Figure 5). Equilibrium competition between these analogs and ATP suggests that they bind hMSH2-hMSH6 and appear to behave similarly to ADP (data not shown). These observations suggest that nonhydrolyzable adenine nucleotide analogs do not necessarily provide a clear indication of the role of hydrolysis in the formation of an active mismatch binding complex. It is possible that the hMSH2-hMSH6 ATPase functions similarly to the Ras GTPase, where donation of a hydrogen bond to the beta-gamma bridging oxygen of GTP is thought to contribute to catalysis (Maegley et al., 1996). Failure of AMP-PNP and AMP-PCP to stimulate release of mismatched DNA suggests that the conformational switch controlling release of the G/T mismatch DNA depends on an interaction with the beta-gamma bridging oxygen.

Following addition of ATP, approximately 15% of the S-shifted material gradually becomes associated with the DNA in the form of an NS-shifted complex (Figures 4A and 4B). This fraction is consistent with the amount of NS binding observed for homoduplex DNA at this

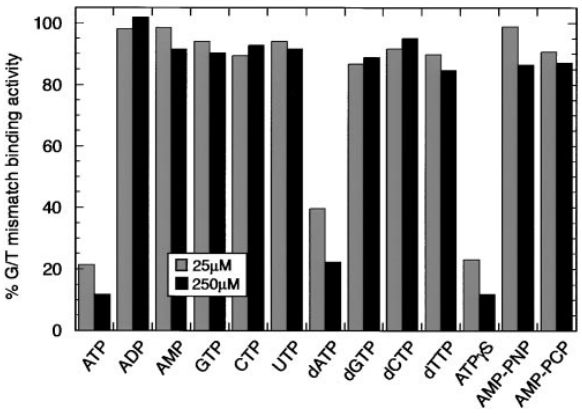

Figure 5. The Effect of Nucleotides and Analogs on hMSH2-hMSH6 Mismatch Binding Activity

Two concentrations, 25 μM (gray) and 250 μM (black) were assessed for each nucleotide under nonhydrolyzing conditions (absence of magnesium) with 80 nM hMSH2-hMSH6. Biological nucleotides and analogs (ATPγS, AMP-PNP, AMP-PCP) are shown. Shifted complexes were quantitated using a phosphoimager. Activity was measured as a percentage relative to a binding reaction containing no nucleotide.

concentration of hMSH2-hMSH6 (Figure 2B). These results suggest that some of the hMSH2-hMSH6 that dissociates from the mismatch can reassociate with the duplex arms or the ends of the mismatch-containing oligonucleotide similar to that found with homoduplex DNA (Figure 2B).

ATP Hydrolysis by hMSH2-hMSH6 Results in Recovery of Mispair Binding Activity

To determine the role of ATP hydrolysis in mismatch recognition, an experimental system was designed in which ATP or ATP-γ-S was introduced into an hMSH2-hMSH6 mismatch binding reaction in the absence of magnesium. As demonstrated in Figures 4 and 5, such conditions result in release from the mismatch in the absence of hydrolysis. At time zero, magnesium was added to the reaction at 37°C, and the G/T mismatch binding activity of hMSH2-hMSH6 was followed over time (Figures 6A–6C). In the reaction containing ATP, nearly 70% of the specific mismatch binding function of hMSH2-hMSH6 was recovered after 10 min, while the remaining activity (>95% total) was recovered by 50 min. The reaction containing ATP-γ-S recovered substantially less (~22%) of its mismatch binding activity, suggesting that efficient hydrolysis is essential for mismatch binding recovery. Substitution of ATP with dATP produced quantitatively similar recovery of mispair binding activity (data not shown). These results suggest that the intrinsic ATPase associated with the human MutS homologs hMSH2-hMSH6 is required for recovery from mismatch release induced by binding to and/or exchange with ATP.

Complete recovery of the mispair binding activity of hMSH2-hMSH6 was also regained by competing ATP with ADP (Figures 6D and 6E). In this competition experiment, a fixed concentration of ATP (0.2 mM) was introduced with magnesium and the concentration of ADP was varied (0–3.2 mM) in separate binding reactions. We found that a 2- to 3-fold excess of ADP resulted in half-reversal of the ATP-dependent hMSH2-hMSH6 release from a G/T mismatch. Complete recovery from mispair release occurred with a 16-fold excess of ADP. A qualitatively similar, though functionally opposite, result was obtained when the competition was performed by introducing a fixed concentration of ADP and varying the concentration of ATP (data not shown). Thus, ADP and ATP are nearly equivalent in their ability to associate with hMSH2-hMSH6, while at the same time eliciting an opposite functional effect on mismatch binding. Hence, ADP, the product of ATP hydrolysis, is likely to be responsible for mispair binding recovery.

Taken together, these observations support a model in which hMSH2-hMSH6 functions as a molecular switch where the ATP bound state is OFF (released from the G/T mismatch) and the ADP-bound state is ON (bound to a G/T mismatch) (Figure 7A).

ATP Hydrolysis and ADP Exchange Determine Mispair Binding Functions of hMSH2-hMSH6

The steady-state analysis of an ATPase reflects the rate-limiting step of the reaction, which could be either γ-phosphate hydrolysis or adenine nucleotide exchange (Figure 7A). To understand the mechanism of the hMSH2-hMSH6 ATPase and to further define the rate-limiting steps, we examined both the γ-phosphate hydrolysis and nucleotide exchange steps directly. Single turn-over γ-phosphate hydrolysis was performed by preloading [γ-^{32}P]-ATP (0.2 μM) onto hMSH2-hMSH6 in the absence of magnesium, and at time zero magnesium cofactor and an excess of cold ATP were added to start the reaction. We found that hMSH2-hMSH6 rapidly hydrolyzed ATP in the presence or absence of DNA (Figure 7B). These results suggest that the γ-phosphate hydrolysis step is unlikely to be rate limiting in the steady-state hydrolysis experiments (Figure 3).

In addition, the total extent of hydrolysis (equivalent to the total number of preloaded hMSH2-hMSH6) appeared to depend on the amount of G/T mismatch DNA (Figures 7B and 7C). As the concentration of the G/T mismatch DNA added to the prebinding reaction exceeded the apparent $K_{d\text{-}G/T}$ for G/T mismatch DNA (20 nM), the extent of total hydrolysis decreased (Figure 7C). These results suggest that if hMSH2-hMSH6 binds to a mismatch first, it may be largely resistant to subsequent binding by ATP. This observation is consistent with the pseudo-uncompetitive behavior described in the steady-state ATPase experiments (Figure 3B) (Dixon and Webb, 1979).

Adenine nucleotide exchange was examined by preloading [^{3}H]-ADP onto hMSH2-hMSH6 in the presence of magnesium (Figure 7D). At time zero, an excess of cold ATP was added to the reaction, and the amount of ADP that remained bound to hMSH2-hMSH6 was determined over time. In the absence of DNA, we found very little ADP nucleotide exchange during a 5 min reaction period ($t_{1/2} >> 300$ s) (Figure 7D). These results demonstrate that in the absence of DNA nucleotide exchange is the rate-limiting step for the hMSH2-hMSH6 ATPase. However, in the presence of a G/T mismatch, nucleotide exchange was significantly more rapid ($t_{1/2} <$

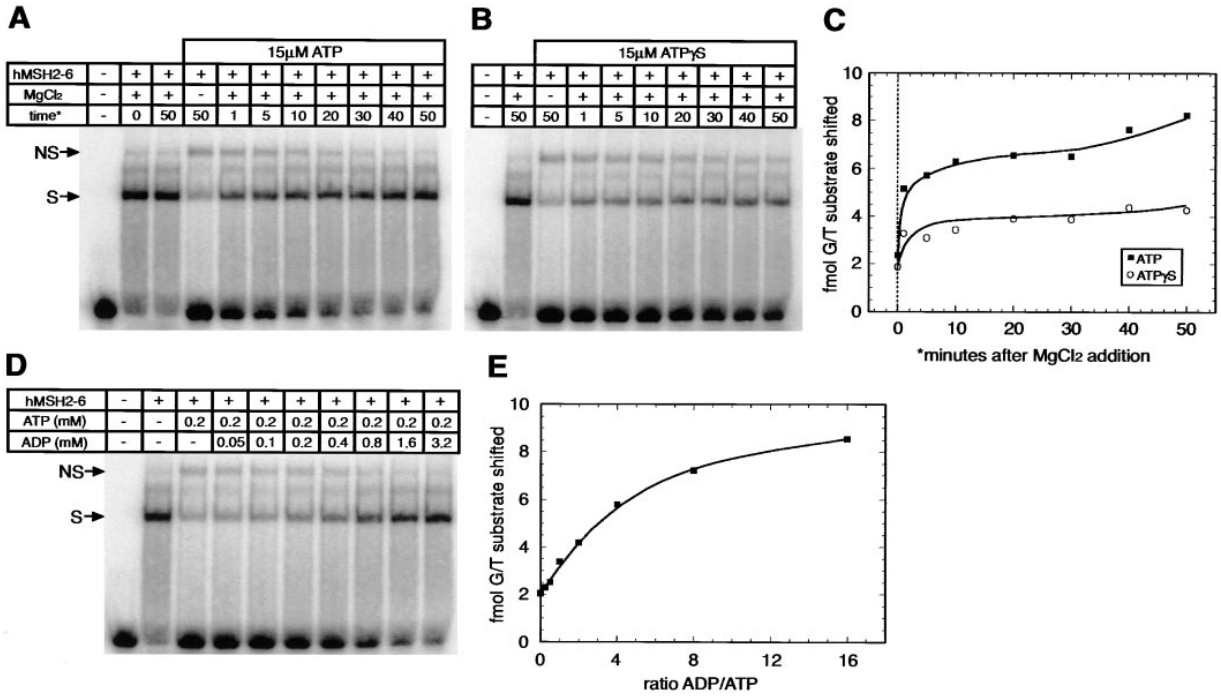

Figure 6. ATP Hydrolysis or ADP Binding by hMSH2-hMSH6 Restores G/T Mismatch Binding

Binding reactions of hMSH2-hMSH6 and a G/T substrate were equilibrated with 15 μM ATP (A) or 15 μM ATPγS (B) under nonhydrolyzing conditions (absence of magnesium) for 15 min. $MgCl_2$ (1 mM) was then added at time zero to activate ATP hydrolysis. Reactions were stopped by the addition of EDTA (5 mM).

(C) The G/T specific shifts shown in (A) and (B) were quantitated with a phosphoimager and displayed with respect to time of incubation. In the absence of adenine nucleotide, hMSH2-hMSH6 mismatch binding activity was reduced by 13% following the 50 min incubation period at 37°C (A).

(D) Competition of ATP-induced mispair release by ADP (in the presence of 1 mM $MgCl_2$). The concentration of ATP was constant at 0.2 mM and the ADP concentration was varied between 0 and 3.2 mM.

(E) The G/T specific shifts shown in (D) were quantitated with a phosphoimager and displayed with respect to the ratio of ADP/ATP.

2 s). Taken together with the single turn-over γ-phosphate hydrolysis experiments, these results imply that in the absence of mismatch DNA, hMSH2-hMSH6 is capable of a single ATP hydrolysis that produces an ADP-bound form. Binding of hMSH2-hMSH6 to a G/T mismatch stimulates the exchange of ADP→ATP, which results in release from the mismatch and recycling of the protein complex. These results further support the hypothesis that hMSH2-hMSH6 is a molecular switch and suggest that it is likely to be controlled by the exchange of ADP.

The Release of hMSH2-hMSH6 from a G/T Mismatch Occurs by Simple Dissociation

Current models of mismatch recognition have implicated ATP-dependent translocation and/or treadmilling on DNA as mechanisms for mismatch association/dissociation (Modrich, 1989, 1991, 1997; Modrich and Lahue, 1996; Allen et al., 1997). Common to all of these models is a time-dependent unidimensional tracking on both duplex and mismatched DNA. The ability to distinguish the NS and S binding of hMSH2-hMSH6 to the 81 bp G/T mismatch DNA (see Figure 4) provided an opportunity to examine the dissociation mechanism of hMSH2-hMSH6 from a G/T mismatch as well as from homoduplex DNA. In these experiments, the G/T mismatch substrate was bound by hMSH2-hMSH6, and at time zero an excess of an unlabeled competitor DNA and/or ATP was introduced. If a tracking or sliding mechanism were operable for hMSH2-hMSH6 dissociation, we would expect time-dependent loss of the S-shifted band and coincident gain of the NS band as observed in Figures 4A and 4B. If a simple dissociation mechanism were operable, then direct loss of the S-shifted band would occur in the presence of excess unlabeled competitor homoduplex DNA (as secondary reassociation with the arms or ends of the labeled G/T mismatch substrate would be precluded under these conditions).

To address the mechanism of hMSH2-hMSH6 dissociation from the G/T mismatch substrate, we performed three experiments (Figure 8). In the first, the stability of hMSH2-hMSH6 bound to the G/T mismatch substrate was interrogated by introducing a 400-fold excess of unlabeled competitor DNA (only) at time zero (Figure 8C). We found the S-shifted complex was not reduced significantly over a 10 min incubation period ($t_{1/2} >>$ 600 s). These results demonstrate that hMSH2-hMSH6 bound to a G/T mismatch is normally stable in the presence of an excess of unlabeled competitor DNA. In the second experiment, ATP (only) was added at time zero to hMSH2-hMSH6 bound to the G/T mismatch (Figure 8A). We observed the gradual loss of the S-shifted band ($t_{1/2} \approx$ 20 s) coincident with a gradual gain of the NS-shifted band that is predictable based on Figure 2B (64

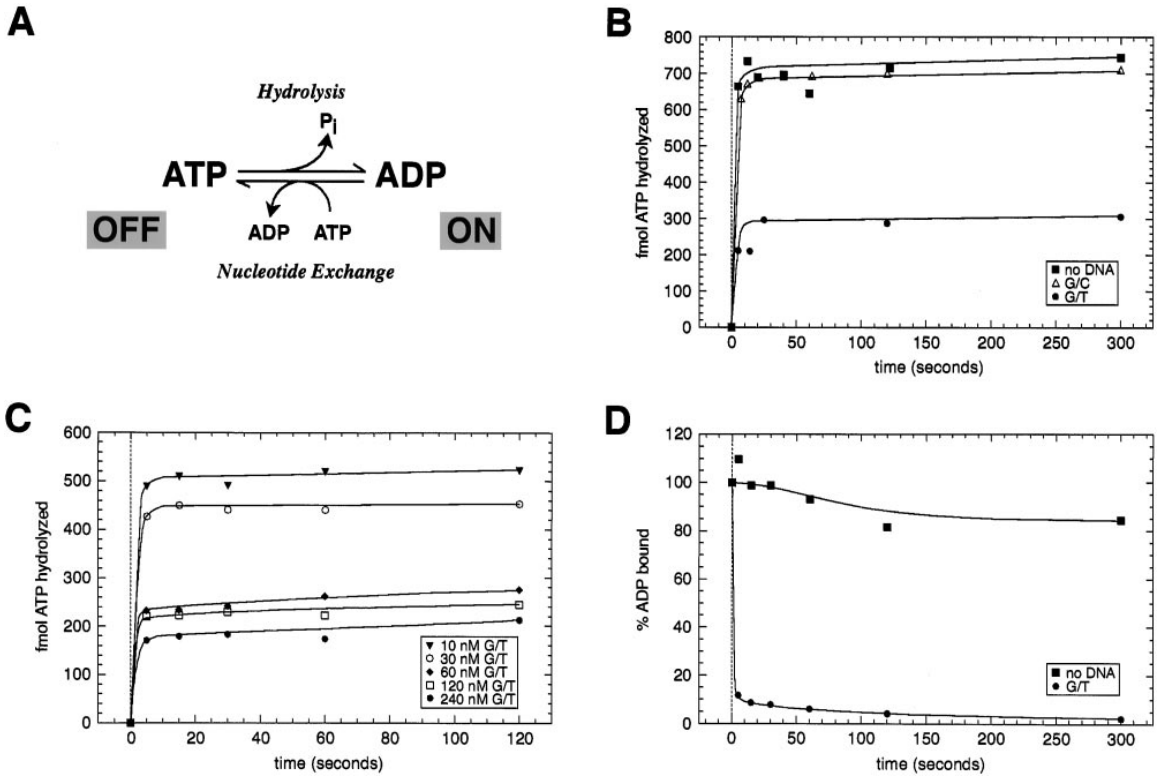

Figure 7. The hMSH2-hMSH6 Molecular Switch and Its Analysis by Single-Step γ-Phosphate Hydrolysis and ADP→ATP Exchange

(A) Representation of an ATP/ADP molecular switch. The ATP-bound form of hMSH2-hMSH6 is OFF for mismatch binding, while the ADP-bound form of hMSH2-hMSH6 is ON for mismatch binding.

(B) Single step γ-phosphate ATP hydrolysis assays performed in the presence of homoduplex (240 nM), G/T mismatch (240 nM), or no DNA (see Experimental Procedures).

(C) Single step γ-phosphate hydrolysis reactions performed with varying amounts of G/T mismatch DNA.

(D) The ADP→ATP exchange rate with no DNA or in the presence of G/T mismatch DNA (240 nM) (see Experimental Procedures). Aliquots were removed at the indicated time points. The relative percentage of ADP remaining bound to the protein was plotted with respect to time of incubation.

nM hMSH2-hMSH6). These results suggest that ATP induces a time-dependent dissociation of hMSH2-hMSH6 from the G/T mismatch and that some of the hMSH2-hMSH6 is transformed into NS-shifted material.

In order to distinguish between tracking/sliding and simple dissociation/reassociation, we performed a third experiment in which both ATP and a 400-fold excess of unlabeled competitor DNA were introduced at time zero (Figure 8B). Again, we observed the gradual loss of S-shifted material ($t_{1/2} \approx 20$ s) consistent with ATP-induced dissociation from the G/T mismatch. However, under these conditions no NS-shifted material was formed over the entire time period. These results suggest that in the presence of excess unlabeled competitor DNA the dissociation of hMSH2-hMSH6 from the G/T mismatch does not proceed through any NS intermediate. When excess unlabeled competitor DNA is added to the homoduplex NS shift (see Figure 2B), all shifted material is absent from the zero time point (Figure 8D). These results suggest that even at 4°C the NS-bound DNA is exceedingly unstable and dissociates rapidly.

We conclude that translocation or treadmilling by hMSH2-hMSH6 is unlikely because (1) no intermediate or NS-shifted material was observed during ATP-induced dissociation from G/T mismatch DNA in the presence of excess unlabeled competitor DNA, (2) dissociation of shifted material from homoduplex DNA is rapid even at low temperatures, and (3) ATP hydrolysis is not required for any of the dissociation processes, since these experiments were performed in the absence of magnesium. We obtained qualitatively identical results in the presence of magnesium for both the hMSH2-hMSH6 dissociation from a G/T mismatch as well as the rapid low-temperature dissociation from homoduplex DNA (data not shown).

Discussion

We have described a well-defined system designed to interrogate the mechanism of mispair recognition by the human MutS homologs hMSH2-hMSH6. Quantitative analysis of binding and site recognition size have been detailed. In addition, we have described the intrinsic ATPase activity associated with hMSH2-hMSH6 and found that ATP disrupts mispair binding by hMSH2-hMSH6. However, the mechanism of this disruption and the implications of its role in human mismatch repair appeared to be contrary to prevailing models describing

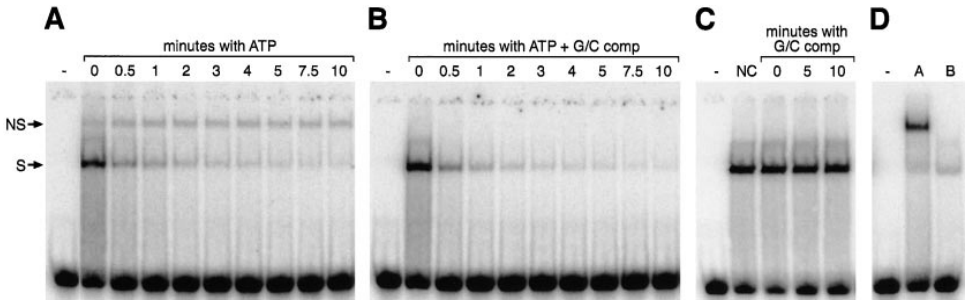

Figure 8. Dissociation of hMSH2-hMSH6 from a G/T Mismatch

(A) The dissociation of hMSH2-hMSH6 from G/T mismatch DNA in the presence of ATP (1 mM).

(B) The dissociation of hMSH2-hMSH6 from G/T mismatch DNA in the presence of ATP (1 mM) and a 400-fold excess of unlabeled homoduplex DNA.

(C) The dissociation of hMSH2-hMSH6 from G/T mismatch DNA in the presence of a 400-fold excess of unlabeled homoduplex DNA. After an initial binding reaction at 37°C for 15 min with hMSH2-hMSH6 (70 nM) in the absence of magnesium, homoduplex unlabeled competitor or ATP was added at time zero and incubation continued. The time of continued incubation following addition of unlabeled competitor and/or ATP is indicated above each lane in (A), (B), and (C).

(D) The dissociation from homoduplex DNA. For (D), hMSH2-hMSH6 (322 nM) was incubated with homoduplex DNA probe for 15 min at 37°C (lane A), the reaction cooled to 4°C, a 1100-fold excess of unlabeled competitor homoduplex DNA was added (lane B), and the gel loaded immediately.

Lane NC (C): no unlabeled homoduplex competitor. In all panels, (-) indicates substrate in the absence of protein.

the role of other MutS homologs (Allen et al., 1997; Modrich, 1997).

hMSH2-hMSH6 Mispair Recognition Constitutes a Molecular Switch

The proposal that the hMSH2-hMSH6 complex functions as a molecular switch is based on several interconnected observations: (1) ADP and ATP have opposing effects on hMSH2-hMSH6 mispair binding; (2) mispair release by hMSH2-hMSH6 does not require ATP hydrolysis; (3) the hydrolysis of ATP by hMSH2-hMSH6 results in recovery of mispair binding activity; (4) γ-phosphate hydrolysis is not rate limiting for hMSH2-hMSH6 steady-state ATPase activity; (5) ADP→ATP exchange appears rate limiting for hMSH2-hMSH6 steady-state ATPase activity; (6) ADP→ATP exchange by hMSH2-hMSH6, and apparently not γ-phosphate hydrolysis, is accelerated by a G/T mismatch; and (7) ATP-dependent mispair release by hMSH2-hMSH6 occurs rapidly and by simple dissociation. These observations can be accommodated by a model for hMSH2-hMSH6 mispair binding in which the functions of γ-phosphate hydrolysis and ADP→ATP exchange act in determining whether hMSH2-hMSH6 binds or is released from a mismatched nucleotide. The recognition of hMSH2-hMSH6 as a switch suggests that its role in mismatch repair may be as a trigger for determining the timing of the subsequent excision repair event.

Implications for Mismatch Repair

Integrating the observation that hMSH2-hMSH6 constitutes a molecular switch into a mechanism for mismatch repair appears to simplify existing models. We estimate that the number of hMSH2-hMSH6 molecules in the nucleus of a proliferating cell exceeds 1000 (Drummond et al., 1995; Wilson et al., 1995; Meyers et al., 1997). A calculated $K_d \approx 20$ nM for mismatched DNA implies that a single mismatched nucleotide in a human cell is likely to be efficiently recognized and bound with high affinity. In the presence of ADP, this high-affinity binding is likely to be nearly irreversible. Thus, the problem does not appear to be binding the mismatch but, instead, dissociating from the mismatch in order to allow a subsequent excision repair event. We would propose that this tight binding acts as a flag for the assembly or nearby localization of the excision repair machinery. When the complete system is assembled, then the exchange of ADP→ATP would be triggered and hMSH2-hMSH6 released from the mismatch, thus allowing a concerted excision and resynthesis event at the region containing the mismatched nucleotide.

Once released from the mismatched nucleotides, the intrinsic ATPase of hMSH2-hMSH6 would then hydrolyze the ATP, resulting in a form that is once again competent for mispair binding. As a free protein complex, hMSH2-hMSH6 does not efficiently exchange the ADP with ATP, providing a long-term mismatch recognition–competent molecule.

There are still two interesting conundrums surrounding the mechanism of the hMSH2-hMSH6 molecular switch. The first revolves around the enhanced ability of hMSH2-hMSH6 to exchange ADP→ATP in the presence of a G/T mismatch. While the mechanism of altered nucleotide exchange proficiency is itself interesting, this observation appears to suggest that hMSH2-hMSH6 might be oscillating between the mismatch recognition–competent ADP-bound form and the mismatch-released ATP-bound form while continually hydrolyzing ATP. Such a system would seem energetically wasteful and unlikely to occur in vivo. This concern is somewhat diminished when one compares the k_{cat} for robust ATPases (100–1000 min^{-1}) to the k_{cat} of hMSH2-hMSH6 in the presence of a G/T mismatch (26 min^{-1}) (Graves Woodward and Weller, 1996; Jiang et al., 1997). However, there would appear to be at least two other possibilities: (i) while the ADP exchange rate is clearly faster in the presence of a G/T mismatch than in the absence of DNA, it is still slow relative to downstream mismatch

repair events; or (ii) the ADP-bound form is stabilized by other proteins that provide the ultimate trigger for ADP→ATP exchange during the course of a mismatch repair event. Regardless, it is clear that hMSH2-hMSH6 binding to a G/T mismatch elicits a change in the protein such that it is now competent to exchange ADP→ATP, where in the absence of DNA it was refractory.

The second puzzle surrounds the question of which MutS homolog, hMSH2 or hMSH6, is actually catalyzing the ATPase reaction and which is performing the mismatch binding function. While there is no a priori reason to exclude hMSH2 or hMSH6 from either of these functions, it is interesting to note that there is greater conservation of the adenine nucleotide–binding domain between the known MSH2 homologs (61% identity between 5 homologs) as compared to the known MSH6 homologs (42% identity between 4 homologs). Experiments to define the individual contributions of hMSH2 and hMSH6 are in progress.

Generality of MutS Function

Our studies with the human hMSH2-hMSH6 mispair binding reaction are consistent with genetic studies in both bacteria and yeast in which mutation of the adenine nucleotide–binding and hydrolysis domain(s) result in a dominant mutator phenotype (Haber and Walker, 1991; Wu and Marinus, 1994; Alani et al., 1997). We would further propose that there may be two opposing functional alterations of MutS homologs that may result in such a dominant mutator phenotype: (1) alterations in the ability to bind and/or exchange ADP for ATP, and (2) alterations in the ability to hydrolyze ATP. An inability to bind and/or exchange ADP would result in a permanently mispair-bound form of MutS that would preclude the repair machinery from the mismatch site. An inability to hydrolyze ATP would result in a permanently released form of MutS that would be unable to recruit the appropriate repair machinery to the site of the mismatch. Both these conditions would result in increased mutation rates as a consequence of unrepaired mismatched nucleotides (Wu and Marinus, 1994). Furthermore, preliminary studies with purified E. coli MutS protein suggest that it also functions as a molecular switch (S. A., S. G., and R. F., unpublished data). Thus, it is likely that the function of MutS proteins as molecular switches that determine the timing of mismatch repair is universal.

Similarity to G Protein Switches

The hMSH2-hMSH6 molecular switch is remarkably similar to G protein switches (Bokoch and Der, 1993). G proteins have been known for some time to trigger translocation events in protein synthesis (Laalami et al., 1996), cascade events in cell signaling (Wiesmuller and Wittinghofer, 1994), and ligand-binding signals from membrane receptors (Spiegel, 1987). Many of these G proteins are associated with regulators that stimulate both the GTP hydrolysis reaction (Tocque et al., 1997) as well as GDP→GTP exchange (Quilliam et al., 1995; Dohlman and Thorner, 1997). Likewise, we would suggest that timing of mismatch repair may be regulated by stimulation of the ATPase (AAP) or ADP→ATP exchange (ANEF) activities. The latter could occur by either stabilizing the ADP-bound form of hMSH2-hMSH6 or stimulating ADP→ATP exchange to effect release of hMSH2-hMSH6 from the mismatch. Obvious candidates for such a regulatory role are the human MutL homologs.

Experimental Procedures

Overexpression and Purification of hMSH2-hMSH6

hMSH2 and hMSH6 clones have been previously described (Fishel et al., 1993; Acharya et al., 1996). hMSH2 and His6-tagged-hMSH6 were overexpressed in SF9 insect cells using the pFastBac dual expression vector (GIBCO-BRL). SF9 cells were harvested and suspended in buffer A (300 mM NaCl, 20 mM imidazole, 25 mM HEPES-NaOH [pH 7.8], 10% glycerol, and protease inhibitors [0.5 mM PMSF, 0.8 μg/ml pepstatin, and 0.8 μg/ml leupeptin]). All purification procedures were carried out at 4°C. Cells were passed through a 25G needle and the crude extract cleared by ultracentrifugation. The supernatant was loaded onto a nickel-NTA Superflow (Qiagen) column, washed with buffer A, and eluted with a linear gradient of imidazole from 20 mM to 200 mM. The hMSH2-hMSH6 complex eluted at approximately 70 mM imidazole. Peak fractions were loaded directly onto a PBE 94 (Pharmacia) column equilibrated with buffer B (300 mM NaCl, 25 mM HEPES-NaOH [pH 7.8], 1 mM DTT, 0.1 mM EDTA, 10% glycerol, and protease inhibitors), washed with buffer B, and eluted with linear gradient of NaCl from 300 mM to 1 M. hMSH2-hMSH6 eluted from the PBE 94 column at approximately 575 mM NaCl. Peak fractions were dialyzed against 100 mM NaCl, 25 mM HEPES-NaOH (pH 7.8), 1 mM DTT, 0.1 mM EDTA, and 20% glycerol. Aliquots were frozen with liquid nitrogen and stored at −80°C for several months with no detectable loss of activity. hMSH2-hMSH6 was compared to a BSA standard (Boehringer Mannheim) on a Coomassie-stained 6% SDS PAGE to calculate protein concentration. Band intensities were measured using a Biorad Gel Doc and Molecular Analyst software.

Preparation of 39 and 81 Base Pair Oligonucleotide Probes

The sequences of the 39-mer and 81-mer oligonucleotides were: 5′-CCT GGT ACC TCG AGC GAT C**G**A GCT TGG TGG AAT TCG CCG-3′ and 5′-AAA GCT GGA GCT GAA GCT TAG CTT AGG ATC ATC GAG GAT C**G**A GCT CGG TGC AAT TCA GCG GTA CCC AAT TCG CCC TAT AGT-3′. A homologous DNA substrate was made by annealing a complementary oligonucleotide, and a G/T mismatch was made by annealing an oligonucleotide containing a T across from the bolded G. End-labeled ^{32}P DNA oligonucleotides were annealed to a 10-fold excess unlabeled complementary DNA (or DNA creating a G/T mismatch). Benzoylated naphthoylated DEAE cellulose (BND cellulose, Sigma) was used to remove single-stranded DNA. We found no detectable single-stranded oligonucleotide present when these substrates were examined by native PAGE. Unlabeled duplex substrates were prepared similarly.

Gel Mobility Shift and Footprint Assays

The standard assay was performed with 9 fmol of labeled 81 bp DNA substrate in a buffer containing 50 mM NaCl, 25 mM HEPES-NaOH (pH 7.8), 1 mM DTT, 0.01 mM EDTA, 15% glycerol, and 10 ng/μl poly dI·dC (Pharmacia). Concentrations of adenosine nucleotides or unlabeled competitor DNA were made as indicated in the figure legends. $MgCl_2$ when present was 1 mM, and when absent, 5 mM EDTA was introduced into the reaction mix. The reactions were carried out in 20 μl and incubated at 37°C for 15 min unless otherwise indicated. Upon completion, reactions were immediately placed on ice and separated on a 4% polyacrylamide (29:1bis), 4% glycerol gel with a TAE buffer (40 mM Tris Acetate, 1 mM EDTA). Gels were dried and quantitated using a phosphoimager (Molecular Dynamics). DNAse I Footprinting and Maxam-Gilbert sequencing reactions were performed by standard protocol (Ausubel et al., 1994).

ATPase Assays

The ATPase activity was measured in a reaction buffer (20 μl) consisting of 40 mM HEPES (pH 7.8), 75 mM NaCl, 10 mM $MgCl_2$, 1.75 mM DTT, 0.075 mM EDTA (pH 8.0), 15% glycerol, 75 μg/ml

acetylated BSA (Promega) (buffer P), 500 μM unlabeled ATP (except where indicated), and 16.5 nM [γ-^{32}P]-ATP. Steady-state reactions were performed using 60 nM hMSH2-hMSH6 with 240 nM of a 39 bp homoduplex or G/T mismatch DNA or without DNA, as indicated. Reactions were incubated at 37°C for 30 min and stopped by the addition of 400 ml of 10% activated charcoal (Sigma) containing 1 mM EDTA. The charcoal was pelleted and duplicate 100 μl aliquots of the supernatant were counted by liquid scintillation. Initial velocity assays were performed by incubating the protein for 10 min at 25°C in buffer P containing 200 nM cold ATP and 16.5 nM [γ-^{32}P]-ATP, in the absence of $MgCl_2$. To start the reaction, an equal volume of buffer was added, which raised the $MgCl_2$ and ATP concentrations to 10 mM and 500 μM, respectively. Aliquots were removed at the indicated time points and processed as described above. The zero time point control was removed and processed before the addition of buffer P containing $MgCl_2$ and ATP.

ADP Exchange Assays

The ADP exchange rate was determined in buffer E (25 mM HEPES [pH 7.8], 75 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 15% glycerol, 75 μg/ml acetylated BSA [Promega]) containing 2.3 μM [^{3}H]-ADP. hMSH2-hMSH6 (60 nM) was incubated with the ADP-containing buffer for 10 min at room temperature. DNA (240 nM G/T mismatch) was then added and the incubation continued for another 10 min in a final volume of 10 μl. The order of addition of DNA and/or ADP did not affect the kinetic results. An equal volume of buffer E containing 1 mM ATP was added at 25°C to start the reaction, which was then stopped at indicated times by dilution into 4 ml of ice-cold stop buffer (25 mM HEPES [pH 7.8], 100 mM NaCl, 10 mM $MgCl_2$). The solution was immediately filtered on a Millipore HAWP nitrocellulose membrane and washed thrice with 4 ml of ice-cold stop buffer. Filters were air dried, incubated overnight in scintillation fluid, and the amount of radioactivity retained on the filters quantitated using a Beckman counter. A zero time point control was removed and processed before starting the reaction. The amount of [^{3}H]-ADP retained for the zero time point was taken as 100% ADP bound.

Thin Layer Chromatography (TLC) Analysis

An ATPase reaction in the presence of a G/T mismatch (described above) containing 15 μM ATP and 0.01 μM [α-^{32}P]-ATP was performed for 20 min at 37°C. Thin layer chromatography was performed as previously described (Fishel et al., 1988).

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