Biochemical Analysis of the Human Mismatch Repair Proteins hMutSα MSH2*G674A-MSH6 and MSH2-MSH6*T1219D

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This article contains supplemental Figs. S1 and S2.

Background: MutSα, a heterodimer of MSH2 and MSH6, is essential for DNA mismatch repair.

Results: hMSH2*G674A-hMSH6wt and hMSH2wt-hMSH6*T1219D mutant proteins fail to efficiently license mismatch-provoked, nick-directed excision.

Conclusion: Different defects underlie the apparently similar repair deficiency of these two mutants.

Significance: Findings provide new insights into the mechanism of mismatch repair with implications for cancer predisposition and the apoptotic response to DNA damaging agents.

The evolutionarily conserved DNA mismatch repair (MMR) system has a key role in correcting errors generated during DNA replication and recombination, and it contributes substantially to maintenance of genetic fidelity and stability (3–6). Loss of MMR greatly increases the spontaneous mutation rate, and Lynch syndrome, a cancer susceptibility syndrome of the colon and other organs, is caused by inherited defects in MMR. In addition, the acquired loss of MMR through epigenetic silencing or somatic mutations is associated with a large subset of sporadic cancers. MMR proteins are also required for activation of cell cycle checkpoints and apoptotic responses to several classes of DNA damaging agents (4, 7, 8).

MMR can recognize and correct not only base mispairs, such as G:T, but also insertion/deletion loops that give rise to frameshift mutations (9–11). In eukaryotes, the first step of MMR is the recognition of mismatches by one of two highly conserved proteins, MutSα, a heterodimer of MSH2 and MSH6 (Fig. 1), or MutSβ, a heterodimer of MSH2 and MSH3. MutSα targets both base mispairs and small insertion/deletion loops, whereas MutSβ targets small and large insertion/deletion loops (12, 13). Both MutSα and MutSβ proteins are members of the ABC transporter family of ATPases, and their ability to bind and hydrolyze ATP is essential for MMR function (4–6). MutSα contains two composite ATPase sites located at the C termini of MSH2 and MSH6, with conserved residues from each subunit contributing to the active site in the other. The two sites are non-equivalent, with the site in MSH6 having higher affinity for ATP and the site in MSH2 having higher affinity for ADP (14–16). MutSα has been proposed to function as a “molecular switch” involving communication between the mismatch recognition and the nucleotide binding sites (17, 18). In this model mismatch recognition by MutSα mediates a rapid exchange of
ADP for ATP, which in turn leads to conformational changes that alter MutSα interactions with DNA. In vitro, MutSα forms a clamp-like structure that diffuses along the DNA; this effect requires ATP binding but not ATP hydrolysis (17–19). ATP binding by MutSα is also required for its interaction with MutLα, a heterodimer of MLH1 and PMS2, that mediates initiation of excision targeted at the error-containing daughter strand (5, 20).

Consistent with the central role of MutSα in detecting and signaling responses to mismatched and damaged DNA, the loss of MSH2 or MSH6 activity results in accumulation of somatic mutations in tumor cells and resistance to the genotoxic effects of many DNA damaging agents (4, 21). Edelmann and co-workers generated two knock-in mouse strains harboring Msh2<sup>G674A</sup> or Msh6<sup>T1217D</sup> alleles and found that the mice developed cancer, exhibited microsatellite instability, and yielded embryonic fibroblasts that displayed impaired MMR in vitro. Their reports also indicated these are separation-of-function alleles in that the MMR response is abrogated, but the apoptotic response to DNA-damaging agents is retained (1, 2).

The corresponding Saccharomyces cerevisiae mutant alleles encoding Msh2<sup>G693A</sup>-Msh6<sup>wt</sup> and Msh2<sup>wt</sup>-Msh6<sup>G1067D</sup> confer a dominant mutator phenotype (22, 23). Biochemical studies of Msh2<sup>wt</sup>-Msh6<sup>G1067D</sup> suggest that the mutant protein retains mismatch binding activity but fails to properly couple mismatch recognition with nucleotide binding and hydrolysis (16, 24, 25). Relatively little is known about the corresponding human MutSα proteins hMSH2<sup>G674A</sup>-hMSH6<sup>wt</sup> and hMSH2<sup>wt</sup>-hMSH6<sup>T1219D</sup>. Residue Gly-674 is located in the Walker A ATP binding motif within the conserved C-terminal ATPase domain of hMSH2, and Thr-1219 is at the hMSH2-hMSH6 heterodimer interface in close proximity to the ABC ATPase “signature motif” of hMSH6 and the P loop of the hMSH2 ATP binding site (Fig. 1) (26). These residues have functional significance in human MMR, as Lynch syndrome alleles encode the MSH2<sup>T1219I</sup> mutation (27), and a mutant hMSH2 protein having a Gly-674 to Arg substitution is defective for MMR in vitro (28).

Here we present a detailed characterization of hMSH2<sup>G674A</sup>-hMSH6<sup>wt</sup> and hMSH2<sup>wt</sup>-hMSH6<sup>T1219D</sup> mutant proteins using a battery of in vitro assays to understand the underlying basis for their MMR defect. We confirm that both mutants fail to carry out MMR in vitro despite being proficient in mismatch recognition. Steady-state and pre-steady-state analysis of h MutSα-DNA interactions and ATPase activity reveal that hMSH2<sup>G674A</sup>-hMSH6<sup>wt</sup> and hMSH2<sup>wt</sup>-hMSH6<sup>T1219D</sup> proteins, although retaining mismatch recognition and intrinsic ATP hydrolysis activities, fail to license a robust excision step. Instead, the mutant hMutSα proteins remain bound to the mismatch. Our findings provide a more detailed characterization of the human mutant proteins particularly with regard to excision, which has not previously been examined, provide a molecular basis for the observed phenotype of the heterozygous T1217D mouse, highlight differences in the molecular defects of the G674A and T1219D mutant MutSα proteins, and provide a basis for thinking about how they might mediate the apoptotic response to certain DNA damaging agents.

### EXPERIMENTAL PROCEDURES

**Protein Purification**—Recombinant hMutSα and hMutLα were expressed in insect cells using the baculovirus system and purified over a 6-ml Resource<sup>TM</sup> Q anion exchange column (GE Healthcare), 5-ml HiTrap<sup>TM</sup> Heparin affinity column (GE Healthcare), and HiLoad 16/60 Superdex 200 sizing column (GE Healthcare) as described (29). In the final chromatographic step, wild type hMutSα and hMSH2<sup>G674A</sup>-hMSH6<sup>wt</sup> were eluted in buffer A (25 mM HEPES, pH 7.5, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 1× Complete protease inhibitor mixture (Roche Applied Science); 0.1% PMSF) containing 100 mM KCl, whereas MSH2<sup>wt</sup>-MSH6<sup>T1219I</sup> was eluted in buffer A containing 300 mM KCl. hMutLα was eluted in buffer A containing 200 mM KCl. Concentrations of MutSα and MutLα were determined with a modified Bradford protein assay (Bio-Rad) using BSA as standard.

For LacI, a fragment containing the ORF of lacI and C-terminal termination codon was amplified by PCR from pDM1.1 plasmid (30) (gift from Dr. Sankar Adhya, NCI) and inserted into pBAD/Myc-His vector (Invitrogen) at NcoI and EcoRI.
sites. LacI/pBAD was transformed into OneShot TOP10 cells (Invitrogen). 1 ml of 0.5% w/v arabinose was added to a 1-liter culture at optical density 0.5. Cells were collected 4 h after induction, resuspended in lysis buffer (25 mM HEPES, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 10% glycerol) plus Complete protease inhibitor (Roche Applied Science), and lysed by sonication. The lysate was passed over a 6-ml Resource Q column (GE Healthcare), and the flow-through was loaded on a 5-ml HiTrap-Heparin HP column (GE Healthcare); LacI eluted between 300 and 500 mM KCl in lysis buffer. The eluate was loaded on a 120-ml Superdex 200 column (GE Healthcare) equilibrated in lysis buffer containing 200 mM KCl. Fractions containing LacI were stored in lysis buffer plus 200 mM KCl. Final yield was ~8 mg/liter culture.

Mismatch Repair and Excision Assays—Mismatched DNA substrates were derived from pSCW01 and pSCW02 as described previously (29). The mismatched DNA substrate was purified by CsCl/ethidium bromide equilibrium centrifugation before use. Nicked substrates were prepared by incubation with either of Nt.BbvCI (5′-nick) or Nt.BspQI (3′-nick) (New England Biolabs) followed by phenol-chloroform extraction and ethanol precipitation.

HeLaS3- and hMSH2-deficient LoVo cells (American Type Culture Collection) were maintained at 37 °C in DMEM (Invitrogen) with 10% fetal bovine serum (Invitrogen), 50 units of penicillin, and 50 μg/ml streptomycin (Invitrogen) in 5% CO2, humidified atmosphere. Nuclear extracts were isolated exactly as described previously (29). The final nuclear supernatant was concentrated to 5–8 mg/ml using Amicon Ultracel-10K (Millipore).

In vitro MMR assays were performed in a 40-μl volume containing 75 fmol (100 ng) of nicked heteroduplex DNA, 100 μg of nuclear extract, 20–40 nm hMutSα proteins, 0.1 mM each of four dNTPs, 20 mM Tris-HCl, pH 7.6, 1.5 mM ATP, 1 mM glutathione, 5 mM MgCl2, 50 mg/ml BSA, and 110 mM NaCl. The reactions were assembled on ice, incubated at 37 °C for 15 min, and terminated by proteinase K addition (New England Biolabs). The restored DNA was digested either with PstI and AseI and annealed with a32P-labeled oligomer to a5′-TTG GAG CAG ACC TAC ACC GA-3′ (AflII digestion); 5′-CCT GGC TTA TCC CCT GT TGT-3′ (PciI digestion); 5′-CCA GTA GTG ATA ACA CTG C-3′ (PvuII digestion); 5′-TGC TTC AAT AAG CTG TGC-3′ (Xhol digestion). Membranes containing reaction products were analyzed using Fuji phosphorimaging BAS-2500 and ImageGauge V4.22.

DNA Binding Electrophoretic Mobility Shift Assays (EMSA) and Fluorescence Anisotropy—EMSA assays were as described previously with minor modifications utilizing gel-purified, 5′-32P-radiolabeled 35- to 350-base-pair duplex substrates containing a G:T mispair or corresponding homoduplex A:T control (33). The sequences were 5′-CCGG ATC ATC GAG GTG ATA ACA CTG C-3′ (the mismatched base is underlined), and then loaded onto a 6% native polyacrylamide gel (Invitrogen) that was pre-electrophoresed for 10 min. Samples were subjected to electrophoresis at room temperature at 220 V in 1× Tris borate-EDTA buffer and visualized by Fuji phosphorimaging BAS-2500 after drying.

DNA substrates for fluorescence anisotropy assays were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), and their sequences were 5′-TAC CTC ATC TCG AGC GTG CCG ATA-TAMRA-3′ and the complement 5′-TAT CGG CAC GTT CCA GAT GAG GTA-3′ (the mismatched base is underlined). The strands were annealed in 20 mM Tris-HCl, pH 7.8, 100 mM sodium acetate, 5 mM magnesium chloride in 1:1 molar ratio by heating at 70 °C for 20 min and slow cooling to room temperature. DNA binding was measured by titrating the proteins into a solution of 5 or 10 nM DNA in the annealing buffer in the absence or presence of 1 mM ADP or ATP. TAMRA anisotropy was measured at λex 553 nm and λem 574 nm in a Horiba Jobin Yvon Fluorolog-3 at 23 °C. Binding data were fit as described previously (34).

Surface Plasmon Resonance Analysis—Surface plasmon resonance (SPR) measurements were performed on a BLACore 3000 (GE Healthcare). Control homoduplex (A:T) and mismatched (G:T) 238-base-pair DNA substrates were prepared from PCR fragments using a modification of a previously described method (18). The T base of G:T was 106 nt from the 5′ end of the biotinylated strand. The A:T substrate was made by PCR amplification of pSCW02 plasmid with a PstI site at position 186 (186CTGCAG; bold denotes the position of A:T; numbering refers to pSCW02 (29)) using a biotinylated forward primer (5′-GGA TAC ATA TTT GAA GAT TGT ATT TAG AAA AAT...
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AAA CAA ATA GGG G) and a nonphosphorylated reverse primer (5′-TCA CAC ATC aat tgt tat cgc ctc aca att CGC TCT GCC GCA GCC GAA CGA CGC) in which lowercase denotes a lacO operator sequence. A:T substrates were confirmed to be PstI+ and FauI-.

The G:T substrate was made from two different PCR fragments. The T strand is from the A:T PCR fragment (PstI+ and FauI-) described above except the reverse primer containing a lacO operator sequence was 5′-phosphorylated. The G strand is derived from a PCR fragment of a second plasmid template, pSCW02_CG, produced by replacing T with C in pSCW02, resulting in loss of the PstI site and gain of a new FauI site. The primers were as used for the A:T substrate, except the forward primer was 5′-phosphorylated and had no biotin. The reverse primer containing a lacO operator sequence was unphosphorylated. These two intermediate PCR fragments were incubated with λ exonuclease (New England Biolabs) to remove the 5′-phosphorylated strand of double-stranded PCR fragment. The biotinylated T strand and the G strand were annealed to each other by heating at 95°C and slow cooling (~2 h) to create a new double-stranded G:T substrate that was resistant to cleavage with either PstI or FauI. The 238-bp A:T or G:T DNA was immobilized on a streptavidin-coated SA Bia-core chip (GE Healthcare).

Interactions of hMutSα and hMutSα-hMutLα ternary complex with DNA were measured as described with minor modification (33, 35). Briefly, 25 nM hMutSα was flowed over the DNA in running buffer containing 20 mM Tris-Cl, pH 7.6, 1 mM DTTP, 0.005% surfactant P20, 5 mM MgCl2, 110 mM KCl at a flow rate of 20 μl/min. After equilibration was reached, dissociation kinetics were measured by co-injecting either running buffer alone or running buffer with 1 mM ATP or ADP. SA sensor ChIPs were regenerated by a 20-μl injection of 0.5% SDS.

hMutSα dissociation from the end-blocked DNA substrate was measured by including 50 nM LacI in the running buffer during the equilibration and hMutSα binding phases. After hMutSα association, running buffer with either 1 mM ATP plus 50 nM LacI or 1 mM ATP plus 1 mM IPTG was flowed over the SA sensor chip to monitor dissociation.

Binding of hMutLα and hMutSα on the DNA substrate could be measured only in the presence of ATP. Briefly, 25 nM hMutSα was flowed over DNA in the running buffer containing 1 mM ATP for 10 min or first for 5 min then coinjected with 25 nM hMutLα for another 5 min. All experiments were performed at 25°C, and samples were maintained at 4°C before injection.

Steady-state ATPase Assay—hMutSα steady-state ATPase activity was measured at 30°C by the malachite green assay as described (36) with some modifications. The reaction contained 0.5 μM wild type or mutant hMutSα, 0.5 mM ATP in the absence or presence of 1 mM 35-bp A:T or G:T DNA in 30 mM Tris-Cl, pH 7.5, 5 mM MgCl2, 110 mM KCl, 0.1 mg/ml BSA. The DNA sequences were the same as described above in EMSA assays. Reactions were initiated with hMutSα, 30-μl aliquots were quenched with equal volumes of ice-cold 0.6 M perchloric acid at various times, and the inorganic phosphate was analyzed by the malachite green assay.

hMutSα-DNA Association/Dissociation Kinetics—hMutSα binding to DNA was monitored by changes in fluorescence anisotropy of the 35-bp DNA labeled with 5-(and 6-) carboxytetramethylrhodamine (TMRA; Invitrogen) at the 3′ end (G′:T_TMRA). 0.8 μM wild type or mutant proteins were mixed rapidly in 1:1 ratio with 0.16 μM G′:T_TMRA in steady-state ATPase buffer on a stopped-flow instrument (KinTek Corp., Austin, TX), and fluorescence anisotropy was monitored over time (polarized λEX = 550 nm and λEM > 570 nm) at 30°C. hMutSα dissociation from DNA was measured under the same conditions by mixing preformed hMutSα-G′:T_TMRA complex (0.8 μM hMutSα, 0.16 μM DNA) ± 0.2 mM ADP in 1:1 ratio with an excess of unlabeled 35-bp G′:T DNA (12 μM) ± an excess ADP or ATP (1–4 mM). Three or more kinetic traces were averaged for each experiment and fit empirically to single or double exponential functions to estimate the rate constants.

hMutSα-2′-(or-3′)-O-(N-methylanthraniloyl)adenosine 5′-diphosphate (MantADP) Association/Dissociation Kinetics—hMutSα binding to mantADP (Invitrogen) was measured by stopped-flow experiments at 30°C. 0.4 μM hMutSα ± 1.2 μM G′:T DNA was mixed rapidly in 1:1 ratio with 20 μM mantADP in 25 mM Hepes-NaOH, pH 7.5, 10 mM MgCl2, 100 mM KCl, 0.1 mg/ml BSA, and fluorescence was monitored over time (λEX = 352 nm and λEM > 420 nm). MantADP dissociation from hMutSα was measured under the same conditions by mixing preformed hMutSα-mantADP complex (0.4 μM hMutSα, 20 μM mantADP) ± 1.2 μM G′:T DNA in 1:1 ratio with excess ATP (8 mM). Three or more kinetic traces were averaged for each experiment and fit empirically to single or double exponential functions to estimate the rate constants.

RESULTS

hMutSα Mutants Fail to Carry Out MMR in Vitro—Recombinant wt hMSH2-hMSH6, hMSH2G674A-hMSH6 (denoted as G674A), and hMSH2T1219D-hMSH6 (denoted as T1219D) proteins (supplemental Fig. S1) were assessed for their ability to support MMR in an in vitro assay using nuclear extracts from LoVo cells missing hMutSα. Correction of a G:T mispair to G:C in a 5′-nicked pSCW01_GT mismatched substrate restores a cryptic PstI cleavage site. Thus, after MMR, the corrected substrate yielded 0.8- and 1.2-kb fragments when cleaved with PstI and Asel. The uncorrected G:T substrate linearized by Asel yielded a 2-kb fragment. As indicated in Fig. 2A, there was little if any MMR activity in LoVo cell nuclear extracts, whereas HeLa cell nuclear extracts yielded close to an 80% correction of the G:T mispair. As noted previously, repair does not approach 100% due in part to ligation of the nicked substrate by ligases in the nuclear extracts, rendering the covalently closed plasmid resistant to MMR (37). The addition of recombinant hMutSα WT protein restored MMR activity in LoVo extracts, with ~50% of the substrate being repaired. The G674A hMutSα mutant had low MMR activity, with 10% of the substrate being repaired, and the T1219D mutant had no detectable repair activity. With respect to the low level of MMR observed above in the case of the G674A mutant (Fig. 2A, lanes 7 and 8), it is possible that random nuclease activity present in LoVo extracts (see below; Fig. 3, B and C, lane 2) could contribute to this apparent MMR. Nevertheless, for reasons outlined below, we...
believe that the G674A mutant retains very low levels of bona fide MMR activity.

hMSH2WT-hMSH6WT (G674A) and hMSH2WT-hMSH6T1219D (T1219D) were also deficient in 5'-nick-directed repair of the pSCW01_GT DNA substrate, as shown in Fig. 2B. In control experiments, HeLa cell nuclear extracts yielded 20% repair, and LoVo extracts supplemented with recombinant wild type hMutSα corrected 16% of the substrate. Although 3'-directed mismatch repair, which depends on the endonuclease activity of MutLα, was not as robust in vitro as 5'-directed MMR, as noted previously (38, 39), it was apparent that the G674A mutant exhibited very limited MMR activity (4%), and the T1219D mutant failed to promote any detectable 3'-directed MMR.

MMR activity was assayed with a second DNA substrate, pSCW02_GT, containing a G:T mispair. Correction of the G:T mispair to G:C introduces a FauI cleavage site. Thus, MMR was monitored by the generation of 0.8- and 1.2-kb fragments after digestion with FauI and AseI (29). Both G674A and T1219D mutants failed to carry out either 5'- or 3'-nick-directed MMR with the pSCW02_GT DNA substrate (data not shown). Our in vitro observations are consistent with the previous knock-in mouse models demonstrating that MutSα G674A and T1219D mutants are defective for MMR activity in vivo (1, 2).

The question of whether the G674A and T1219D mutants can suppress endogenous MMR activity, i.e. whether they exhibit dominant negative behavior, was addressed by carrying out MMR with HeLa nuclear extracts in the presence of recombinant wild type hMutSα proteins as indicated. Lane 1, control, cofacially closed pSCW01 homoduplex and 5'-nicked pSCW01_GT in the absence of nuclear extract used for quantitation. Lane 2, control, cofacially closed pSCW01 homoduplex in the absence of nuclear extract. Lane 3, control, 5'-nicked pSCW01_GT in the absence of nuclear extract. Lane 4, MutSα G674A and T1219D mutants are defective for 3'-nick-directed MMR. Lane 5, 3'-nick-directed MMR restores PstI cleavage.

MMR activity was assayed with a second DNA substrate, pSCW02_GT, containing a G:T mispair. Correction of the G:T mispair to G:C introduces a Faul cleavage site. Thus, MMR was monitored by the generation of 0.8- and 1.2-kb fragments after digestion with Faul and AseI (29). Both G674A and T1219D mutants failed to carry out either 5'- or 3'-nick-directed MMR with the pSCW02_GT DNA substrate (data not shown). Our in vitro observations are consistent with the previous knock-in mouse models demonstrating that MutSα G674A and T1219D mutants are defective for MMR activity in vivo (1, 2).

The question of whether the G674A and T1219D mutants can suppress endogenous MMR activity, i.e. whether they exhibit dominant negative behavior, was addressed by carrying out MMR with HeLa nuclear extracts in the presence of recom-
TABLE 1  Nucleotide dependence of hMutSα WT, G674A, and T1219D mutants on binding to a 24-base pair G:T heteroduplex DNA

Dissociation constants (Kd) were determined for hMutSα and mutants with 24-bp G:T heteroduplex DNA substrates by fluorescence anisotropy (see “Experimental Procedures”) in 100 mM NaOAc in the absence or presence of 1 mM ATP or ADP. Errors are the S.D. of three independent experiments.

| Nucleotide | ATP  | ADP  |
|------------|------|------|
| No mutant  | nM   | nM   |
| hMutSα WT  | 7.5 ± 7 | 107.9 ± 26 | 8.9 ± 8.8 |
| hMutSα G674A | 15.4 ± 14 | 6.6 ± 4 | 6.2 ± 8 |
| hMutSα T1219D | 5.2 ± 1 | 6.3 ± 3 | 10.8 ± 3 |

MutSα Mutants Can Recognize G:T Mispair—The ability of MutSα G674A and T1219D mutant proteins to recognize a G:T DNA mismatch, the initial step in MMR, was examined by EMSA and fluorescence anisotropy. Qualitative assessment of mismatch recognition by EMSA revealed that the G674A and T1219D mutants retained mismatch binding activity and the ability to discriminate between G:T mispairs and homoduplex DNA (supplemental Fig. S2, A and B). Quantitative assessment by fluorescence anisotropy revealed high affinity binding to G:T by all the proteins in the absence of nucleotides or in the presence of ADP (Table 1). As expected, wild type hMutSα affinity for G:T was substantially reduced in the presence of ATP, in agreement with a previous report (40). In contrast, mismatch binding by the G674A and T1219D mutants was insensitive to ATP (Table 1).

MutSα Mutants Block Excision—Mismatch recognition by MutSα is followed by an excision step directed to the newly synthesized strand containing the error. The ability of hMutSα G674A and T1219D mutants to signal excision was assayed by MMR assays in which dNTPs were omitted. After incubation of the G:T MMR substrate containing a 5'-nick with LoVo extract and either wild type, G674A, or T1219D proteins, the DNA was linearized by Asel and annealed to 32P-labeled oligonucleotide probes complementary to sequences at varying distances from the initiating nick. Probe 1 is adjacent to the 5'-nicking site, and probe 2 spans the mismatch site. As shown in Fig. 3A, random nuclease activity initiating at the nick was observed in LoVo extract alone and supplemented with wild type or mutant hMutSα proteins. Similar activity was seen at the nick in an A:T homoduplex substrate and was largely independent of hMutSα. Probe 2 revealed that the addition of wild type hMutSα resulted in significant excision in the vicinity of the G:T mismatch over the background level. In contrast, the G674A mutant quenched background level excision near the mismatch, and this effect was more pronounced in the case of the T1219D mutant. These data reveal that G674A only weakly supports extensive excision of the mispaired base, whereas T1219D is completely deficient for this step. Both mutants can block excision whether it be EXO1-mediated or spurious in nature.

To map more precisely the extent of excision that results in gapped DNA intermediate(s), we performed Southern blot experiments using two probes complementary to the nicked strand. Excision assays were performed with nicked G:T or A:T homoduplex substrate that was incubated with LoVo extract and recombinant wild type, G674A, or T1219D hMutSα and then linearized by AflIII. For 5'-nick-directed excision, the probe was located 664 nt from the Nb.BbvCl nick site. Excision is predicted to generate a family of shorter fragments; e.g. excision to the mismatch site would generate a 127-nt gap and a 537-nt radiolabeled product. As shown in Fig. 3B, all samples yielded a low background level of random excision with the nicked homoduplex (A:T). LoVo extract alone (Fig. 3B, lanes 2 and 8) yielded similar patterns of excision fragments for both G:T and A:T DNA substrates, reflecting random excision that is not mismatch-directed. With the addition of wild type hMutSα, excision was restored to the level observed with HeLa nuclear extract, and the gap was extended up to several hundred nucleotides beyond the mismatch, consistent with prior observations (5, 32). The G674A mutant promoted limited excision beyond the mismatch, yielding a pattern of excision that resembles that of wild type MutSα, and is distinct from LoVo extract alone. The prominent presence of a fragment larger than 537 nt indicates that, for the most part, excision terminated prematurely before reaching the mismatch site. The T1219D mutant was also unable to promote excision to the mismatch, and a strong termination position was detected upstream of the mismatch site. These data indicate that 5'-directed excision can initiate with low frequency in the case of the G674A mutant (compared lanes 2 and 4 in Fig. 3B) but is not supported by the T1219D mutant. Moreover, spurious exonu-
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cleolytic degradation is largely blocked by these two mutants that remain tightly bound to the mismatch.

Fine mapping of excision tracts was carried out using the PciI cleavage site, which is 296 nt away from the 5′-nick site, and yields a 169-nt radiolabeled product if excision reaches the mismatched base. In the case of LoVo extract supplemented with wild type hMutSα or for the HeLa nuclear extract control, a family of DNA fragments that reflected excision tracts extending 50 nt or more beyond the mismatch was generated. The addition of G674A or T1219D to the LoVo reaction resulted in strong excision stops just 5′ to the mismatch site (Fig. 3C). Therefore, in the presence of these two mutant proteins, excision initiated at the nick but was terminated before reaching the mismatch. These results suggested that the G674A and T1219D hMutSα mutants remained bound to their target site and blocked excision.

We also explored the ability of wild type MutSα and the G674A and T1219D mutants to support 5′-nick-directed excision of an O6-methylguanine (O6-meG):T mispaired DNA substrate, a cytotoxic mismatch resulting from DNA alkylation by the S′1 class of DNA alkylating agents. As indicated in the right-hand panels of Fig. 3B-C, a low level of background excision was observed in the presence of LoVo extract alone. The addition of wild type hMutSα restored excision to levels similar to that of HeLa nuclear extract. The G674A mutant supported limited excision beyond the mismatch, whereas the T1219D mutant failed to promote excision to the mismatch, resulting in a strong termination position upstream of the mismatch site. These data revealed that the MutSα G674A and T1219D mutants behaved similarly in vitro with respect to 5′-directed excision in the case of O6-meG:T and G:T mismatches.

The G674A and T1219D mutants were also evaluated for their ability to promote 3′-nick-directed excision in an analogous assay in which excision is directed from a Nt.BspQI site 50 nt 3′ to the mismatch on the top strand of the G:T pSCW02 substrate. 3′-Nick-directed excision requires the endonucleolytic activity of MutLα, which incises the newly synthesized strand on both sides of the mismatch providing a substrate for 5′-→3′-mediated excision by EXO1 (38, 39). The distance from the PvuI cleavage site to the Nt.BspQI nick is 600 nt (Fig. 3D). The G:T mispair, very low levels of nonspecific excision were observed in reactions containing LoVo extract alone. In the presence of recombinant WT hMutSα, excision was restored to a similar level as in the HeLa nuclear extract control, and gaps extended to ~450 nt in length reflecting endonucleolytic cleavage by MutLα up to several hundred nucleotides upstream of the mismatch followed by EXO1 processing. The G674A mutant promoted much weaker excision, whereas the T1219D mutant failed to support any significant excision.

Digestion of 3′-nick-directed excision products with XhoI located 216 nt away from the nick site and 166 nt from the mispair confirmed that the G674A mutant supported limited 3′-nick-directed excision, whereas the T1219D mutant did not support any detectable excision (Fig. 3E). Experiments with 3′-nicked homoduplex A:T DNA show very low background levels of nonspecific 3′ to 5′ excision in LoVo and HeLa extracts. Close examination of the excision tracts revealed that the G674A mutant, but not the T1219D mutant, supported initial endonucleolytic incisions by MutLα on both sides of the mismatched top strand that give rise to incised DNAs that could be further processed by EXO1. These excision intermediates constitute a family of DNA fragments denoted by the bracket in Fig. 3E. The percentage of these MutLα cleavage intermediates as a function of total DNA substrate was quantified. HeLa cell extracts yielded conversion of 29% that of starting substrate to excision intermediates for the G:T mismatch; the addition of recombinant hMutSα WT protein to LoVo extracts resulted in cleavage of 21% of the substrate. G674A supported nucleolytic processing of 11% of the mismatched DNA. Comparison of G674A with WT reveals the presence of a 10-fold faster rate of cleavage by MutLα than wild type protein in the experimental condition (data not shown).

The excision assays suggest that there is a significant difference regarding the molecular defects of G674A and T1219D mutants. T1219D fails to support either 5′- or 3′-nick-directed excision, the latter consistent with its inability to productively interact with MutLα (see below). G674A can trigger mismatch-dependent endonucleolytic excision by MutLα, although to a lesser extent than wild type MutSα, but subsequent EXO1 processing appears to be substantially diminished.

ATP Modulation of Mismatch Binding—Because ATP binding and hydrolysis by MutSα is critical for MMR, the mutant proteins were tested for their ATP hydrolysis activity and the effects of nucleotide binding on their interaction with DNA. Gly-674 resides in the P loop of the ATPase active site of hMSH2 that is critical for ATP binding. Thr-1219 resides at the ABC transporter ATPase signature motif that is critical for ATP hydrolysis.

As shown by the kcat values in Table 2, wild type hMutSα hydrolyzes ATP at a slow steady-state rate in the absence of DNA, which is stimulated ~20-fold in the presence of G:T mismatched DNA. Surprisingly, both G674A and T1219D mutants exhibit ~5-fold faster kcat than wild type protein in the absence of DNA. Moreover, in contrast to wild type hMutSα, the steady-state ATPase rates of the mutants are severely suppressed in the presence of G:T mismatched DNA (and

| No DNA | A:T | G:T |
|--------|-----|-----|
| hMutSα WT | 0.03 ± 0.001 | 0.47 ± 0.005 | 0.68 ± 0.010 |
| hMutSα G674A | 0.26 ± 0.003 | 0.42 ± 0.005 | 0.05 ± 0.002 |
| hMutSα T1219D | 0.39 ± 0.015 | 0.89 ± 0.033 | 0.02 ± 0.001 |
Mismatch undergoes rapid mantADP binding and release when rate from DNA in the absence of nucleotide (Fig. 4C). Wild type hMutSα and the G674A and T1219D mutants bind DNA rapidly (Fig. 4A) and dissociate at a slow rate from DNA in the absence of nucleotide ($k_{off} = 0.002 \text{s}^{-1}$; Fig. 4B). ATP stimulated rapid dissociation of wild type hMutSα (>1000-fold increase in $k_{off}$) but had a much smaller effect on G674A (5-fold increase in $k_{off}$) and no significant effect on the T1219D mutant (Fig. 4C). Wild type hMutSα dissociates more rapidly from a G:T mismatch in the presence of ADP than in the absence of nucleotides (Fig. 4D), consistent with previous reports (18, 19, 41, 42). The two mutants also exhibit slightly faster dissociation in the presence of ADP. Adding ATP to a ternary complex of ADP-hMutSα-G:T-TAMRA further stimulated dissociation of wild type hMutSα from DNA (>7.5-fold increase over $k_{off}$ with ADP alone) but had a smaller effect on G674A and no effect on T1219D (Fig. 4E). These results demonstrate that the G674A and T1219D mutants do not couple mismatch recognition and nucleotide binding like wild type hMutSα.

The above finding led us to test whether the mutants can undergo the mismatch binding-induced ADP-to-ATP exchange and sliding clamp formation associated with an activated state of MutS that can license the excision step. Stopped-flow experiments measuring mantADP binding and dissociation kinetics showed that wild type hMutSα bound to G:T mismatch undergoes rapid mantADP binding and release when challenged with ATP ($k_{off} = 1 \text{s}^{-1}$) (Fig. 5, A and B). G:T-bound G674A exhibited similar mantADP binding and release kinetics as wild type protein, although the fraction of bound mantADP was lower in the absence of DNA (Fig. 5, C and D). T1219D also exhibited lower mantADP binding and release compared with wild type protein (Fig. 5, E and F). Thus, the G674A mutant and especially the T1219D mutant do not bind and exchange ADP for ATP upon mismatch binding as effectively as wild type hMutSα. This nucleotide exchange is critical for the formation of a MutSα sliding clamp, and the lack of this exchange is consistent with a significant fraction of the mutants remaining tightly bound to the mismatch site.

**hMutSα Mutants Are Defective for Sliding Clamp Formation**—The ability of WT, G674A, or T1219D hMutSα to form sliding clamps on mismatched DNA was tested directly by SPR. These studies utilized a 238-bp G:T mismatch heteroduplex or the corresponding A:T homoduplex having one biotinylated end for immobilization on the streptavidin-coated sensor chip; the distal DNA end contained a lacO binding site that could be blocked by introduction of LacI repressor protein and subsequently unblocked by introduction of IPTG (18). hMutSα WT, G674A, and T1219D proteins bound unblocked DNA rapidly in the absence of nucleotides, and the extent of binding to G:T mismatch DNA was significantly greater than to A:T homoduplex DNA (Fig. 6A, compare solid and dotted lines). Dissociation from DNA in the absence of nucleotide was slow in all cases.

In the presence of ATP, WT hMutSα dissociated rapidly from the unblocked DNA (Fig. 6B), as expected for a sliding clamp of hMutSα that diffuses along the duplex. Consistent with the fluorescence anisotropy results (Table 1), ATP only modestly accelerated dissociation of the G674A mutant from DNA and had little discernible effect on T1219D mutant dissociation. ADP accelerated the dissociation of wild type hMutSα compared with the absence of nucleotides, but dissociation by G674A and T1219D appeared to be largely resistant to modulation by ADP (Fig. 6C). ADP is known to reduce DNA binding and increase the specificity of mismatch recognition by MutS and MutSα (18, 19, 41, 42). Inclusion of ADP during the SPR association phase resulted in reduced wild type hMutSα binding to both G:T and A:T DNA (data not shown). Subsequent addition of ATP greatly enhanced WT hMutSα dissociation from DNA. G674A and T1219D failed to exhibit any discernible effects of ADP on DNA binding or dissociation, and ATP only modestly stimulated dissociation of the G674A mutant from the G:T DNA.

Our findings predicted substantial defects in the ability of the G674A and T1219D mutants to form an ATP-dependent sliding clamp. This finding was confirmed in SPR experiments performed in the presence of LacI protein that blocks the distal DNA end. As shown in Fig. 6, D and E, WT, G674A and T1219D hMutSα proteins remained bound to the G:T DNA substrate in the presence of LacI. When LacI binding was reversed by the addition of 1 mM IPTG, wild type hMutSα protein rapidly dissociated from DNA in the presence of ATP, consistent with formation of a sliding clamp. G674A exhibited gradual dissociation, suggesting that this mutant protein can assume a sliding clamp conformation in the presence of ATP but with greatly reduced efficiency compared with wild type. The T1219D mutant showed little dissociation under the same conditions. Together these data reveal that hMutSα G674A and T1219D mutants are differentially compromised for effective nucleotide exchange and sliding clamp formation after binding to mismatched DNA.

**hMutSα Mutants Are Defective in Ternary Complex Formation with hMutLa**—Because G674A and T1219D exhibited defective coupling of nucleotide and mismatched DNA binding activities, which is required for the productive interaction of MutSα and MutLa, we asked whether these mutant proteins could form a ternary complex with hMutLa. As shown in Fig. 7, A–C, wild type MutSα bound to a G:T mismatch readily formed a ternary complex with MutLa in the presence of ATP. In contrast, G674A was much less proficient, and T1219D was completely deficient in ternary complex formation with MutLa. MutLa by itself exhibited no detectable binding to mismatched DNA under these conditions (data not shown).

**DISCUSSION**

Biochemical studies of two mutant hMutSα proteins, hMSH2
g674α hMSH6wt and hMSH2wt hMSH6T1219D, corre-
**FIGURE 4.** MutSα mutants are defective in ATP binding-induced dissociation from G:T mismatched DNA. DNA binding and dissociation by wild type (red), G674A (blue), and T1219D (green) proteins were monitored by fluorescence anisotropy of G:TTAMRA in stopped-flow experiments. Single or double exponential fits of the data are solid lines through averaged traces in the same colors. A, DNA binding was measured by mixing MutSα with DNA in the absence of nucleotide. Final reactions contained 0.4 μM MutSα, 0.08 mM G:TTAMRA, and DNA. B and C, DNA dissociation was measured by mixing MutSα-G:TTAMRA complex with excess unlabeled G:T trap DNA in the absence (B) or presence (C) of ATP. Final reactions contained 0.4 μM MutSα + 0.08 μM G:TTAMRA, 6 μM unlabeled G:T ± 0.5 mM ATP. D and E, the effect of ADP was assessed by incubating ADP with MutSα-G:TTAMRA complex and then mixing with excess unlabeled G:T DNA and ADP (D) or ATP (E). Final reactions contained 0.4 μM MutSα + 0.1 mM ADP + 0.08 μM G:TTAMRA, 6 μM unlabeled G:T + 2 mM ADP or ATP.
Human MutSα Mismatch Repair Mutants

The figure shows experiments on MutSα mutants, specifically the T1219D mutant, which exhibit defects in nucleotide binding and exchange.

The figure comprises six panels, labeled A to F, each illustrating the binding and release of mantADP by MutSα proteins.

Panel A: Shows mantADP binding to MutSα WT.

Panel B: Shows mantADP release from MutSα WT.

Panel C: Shows mantADP binding to MutSα GA.

Panel D: Shows mantADP release from MutSα GA.

Panel E: Shows mantADP binding to MutSα TD.

Panel F: Shows mantADP release from MutSα TD.

The study demonstrates that corresponding to murine alleles that confer MMR deficiency, cancer, and shortened lifespan in mice, reveal that these human mutant proteins recognize mismatches but fail to support the excision step of MMR. Although both mutants are capable of hydrolyzing ATP, normal communication between the mismatch and nucleotide binding domains is disrupted.
mutant proteins behave as dominant inhibitors of MMR in vitro, most likely because they fail to efficiently form an ATP-induced sliding clamp upon mismatch recognition, remain trapped at the mismatch site, and fail to license excision of the mismatch.

Despite similar MMR deficiencies for these two mutant hMutSα proteins and similar phenotypes of mutant mice harboring corresponding single amino acid substitutions in murine MSH2 and MSH6, careful examination of hMSH2G674A-hMSH6wt and hMSH2wt-hMSH6T1219D proteins reveals that they differ from each other in the molecular basis of their MMR defect. Whereas T1219D is essentially unable to support mismatch repair steps subsequent to mismatch recognition, G674A exhibits a low level of functional activity in mismatch repair assays. This residual activity is reflected in the ability of G674A to form a small population of sliding clamps and productive ternary complexes with MutLα that support low levels of both 5′- and 3′-directed excision and repair. In support of this notion, heterozygous Msh2wt/Msh6T1219D mice have similar rates of survival compared with wild type mice probably reflecting residual MMR activity (1).

In contrast, hMSH2wt-hMSH6T1219D suffers from severe disruption of normal communication between the nucleotide binding and mismatch binding domains of MutSα despite possessing composite ATP binding sites that retain ATP hydrolysis activity. Nucleotide-induced conformational changes that normally occur when MutSα binds to a mismatch fail to take place in T1219D. Thus the mutant protein fails to assume a sliding clamp conformation, remains frozen at the mismatch site, and loses its ability to recruit and activate MutLα to carry out 5′- or 3′-directed excision. This severe MMR deficiency and its dominant mode of action likely explain why heterozygous Msh6TD/+ mice have a low survival frequency, intermediate between that of Msh6+/+ and Msh6TD/TD mice (2). Identification of similar dominant alleles may have important implications for heterozygous carriers in the human population.

Earlier studies investigating the formation of ternary E. coli MutS and MutL complexes on mismatched DNA revealed that ATP binding but not hydrolysis by MutS is required for ternary complex formation (33, 45, 46). The S. cerevisiae MSH2wt-MSH6G1067D mutant failed to form ternary complexes despite apparently normal ATP binding to the MSH6 subunit (24).

FIGURE 6. MutSα G674A (GA) and T1219D (TD) fail to assume a sliding clamp conformation on mismatched DNA. A–C, MutSα G674A and T1219D failed to undergo rapid ATP-induced dissociation from a G:T mismatch. DNA binding was monitored by SPR using an unblocked 238-bp G:T heteroduplex (157 response units) and a 238-bp A:T homoduplex (156 response units). 25 nM MutSα protein was injected for 5 min followed by running buffer containing no nucleotide (A), 1 mM ATP (B), or 1 mM ADP (C). D and E, MutSα G674A and T1219D fail to assume a sliding clamp conformation in the presence of ATP. An SPR assay was performed as above, but the 238-bp G:T heteroduplex (167 response units) was blocked at the distal end by the inclusion of LacI in all phases. After binding of MutSα proteins, the dissociation buffer contained either 1 mM ATP and LacI (D) or 1 mM ATP and 1 mM IPTG (E). Binding of LacI alone and subsequent release by the addition of 1 mM IPTG is indicated in orange.
These findings suggest that events downstream of ATP binding to MSH6 in mismatch-bound MutSα are critical for recruitment of MutLα to a mismatch. Our results support this mechanistic scheme for human MutSα.

Although our data clearly point to an inability of T1219D mutant to assume an ATP-dependent conformation at a mismatch that licenses subsequent steps of MMR, the structural dynamics underlying this change in MutSα are not yet clear. X-ray crystallographic studies of bacterial MutS-mismatch complexes with ADP-beryllium fluoride or ATP revealed small conformational changes in both ATP binding and DNA binding domains as well as adjacent domains (43, 44). However, both structures represent intermediates that do not reveal all the structural transitions required for an activated state of MutS at a mismatch, presumably due to constraints imposed by crystal contacts. Thr-1219 is unlikely to participate directly in ATP binding and hydrolysis, as we observe a robust intrinsic ATPase rate for the T1219D mutant. Instead, we speculate that this Thr residue may play an important role in stabilizing and/or promoting the ATP-activated conformation of MutSα consistent with the observation that an HNPCC allele encoding an MSH6 T1219I mutation confers loss of MMR in vivo.

MutSα can also target mispairs involving damaged or modified bases such as O6-meG that result from exposure to many DNA alkylating agents (7, 8). Two models have been proposed to explain the MMR-dependent DNA damage response: futile cycle and direct signaling. The “futile cycle” model proposes that DNA polymerase replicates with low fidelity past the alkylated base, incorporating thymine instead of cytosine across the resulting base, incorporating thymine instead of cytosine across the 6-meG:T mismatch and can block 5'-nick-directed excision in the case of G674A or random excision in the case of T1219D. As discussed above, the resulting short regions of gapped DNA may be the direct signal that induces checkpoint activity. It is also possible that the persistence of hMutSα G674A and T1219D proteins on DNA impedes key cellular processes such as replication by analogy to the excision block observed in MMR assays. In any case, the mechanism of damage signaling mediated by hMutSα G674A and T1219D is unlikely to result from futile cycles of MMR.

The data presented here highlight the utility of a careful biochemical analysis of the various steps of MMR by mutant MMR proteins, especially hereditary non-polyposis colorectal cancer alleles. Moreover, although different mutants may appear qualitatively similar, detailed functional profiling can aid in identi-
fication of mutants that can be exploited to address unsolved problems, for example, the conformational changes in MutSα induced by mismatch and nucleotide binding in MMR and the basis for the apoptotic response to DNA damaging agents in MMR-proficient mammalian cells.

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