Functional characterization of two human MutY homolog (hMYH) missense mutations (R227W and V232F) that lie within the putative hMSH6 binding domain and are associated with hMYH polyposis

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ABSTRACT

The base excision repair DNA glycosylase MutY homolog (MYH) is responsible for removing adenines misincorporated into DNA opposite guanine or 7,8-dihydro-8-oxo-guanine (8-oxoG), thereby preventing G:C to T:A mutations. Biallelic germline mutations in the human *MYH* gene predispose individuals to multiple colorectal adenomas and carcinoma. We have recently demonstrated that hMYH interacts with the mismatch repair protein hMSH6, and that the hMSH2/hMSH6 (hMutSα) heterodimer stimulates hMYH activity. Here, we characterize the functional effect of two missense mutations (R227W and V232F) associated with hMYH polyposis that lie within, or adjacent to, the putative hMSH6 binding domain. Neither missense mutation affects the physical interaction between hMYH and hMSH6. However, hMYH(R227W) has a severe defect in A/8-oxoG binding and glycosylase activities, while hMYH(V232F) has reduced A/8-oxoG binding and glycosylase activities. The glycosylase activity of the V232F mutant can be partially stimulated by hMutSα but cannot be restored to the wild-type level. Both mutants also fail to complement *mutY*-deficiency in *Escherichia coli*. These data define the pathogenic mechanisms underlying two further hMYH polyposis-associated mutations.

INTRODUCTION

Cells possess several repair pathways for dealing with the many different types of lesions in DNA. Reactive oxygen species (ROS) are the most prevalent source of DNA lesions in aerobic organisms, and oxidative damage to DNA can result in mutagenesis and lead to degenerative diseases. One of the most abundant and highly mutagenic forms of oxidative damage to DNA is 7,8-dihydro-8-oxo-guanine (8-oxoG or GO). GO lesions are repaired primarily by the base excision repair (BER) pathways. The human 8-oxoG glycosylase (hOGG1) protein, a functional eukaryotic homolog of *Escherichia coli* MutM, can remove both ring-opened purine lesions and mutagenic GO adducts if they are paired with cytosines (1–4). When C/GO is not repaired by hOGG1, adenines are frequently incorporated opposite GO bases during DNA replication (5,6) which, after a second round of replication, lead to G:C to T:A transversion mutations (6–9). Both the human MutY homolog (hMYH) and MutS homologs (hMSH2/hMSH6 or hMutSα) are involved in defending against the mutagenic effects of A/GO mispairs.

The mismatch repair (MMR) system enhances the fidelity of DNA replication and genetic recombination. Inherited deficiencies in the human MMR genes cause hereditary non-polyposis colorectal cancer, an autosomal dominant disorder characterized by early-onset colorectal cancer and other extra-colonic cancers, notably endometrial cancer and cancers of the stomach, small bowel, ureter and renal pelvis (10). The hMSH2 protein forms a heterodimer with hMSH6 to recognize base–base mispairs and short insertion-deletion loops or forms

a heterodimer with hMSH3 to recognize larger insertion-deletion loops [reviewed in (11–13)]. The MMR system is also involved in repair of oxidative DNA damage, and mouse embryonic stem cells carrying a defective *Msh2* allele accumulate oxidized bases in their DNA (14,15). Recently, Msh2p/Msh6p of yeast *Saccharomyces cerevisiae* and human hMSH2/hMSH6 have been shown to bind A/GO mismatches (16,17).

The human homolog of bacterial MutY, hMYH, encodes a DNA glycosylase that excises adenines misincorporated opposite GO or G through the BER pathway [reviewed in (18,19)], thereby preventing G:C to T:A transversions (20–23). Germline mutations in the *hMYH* gene cause autosomal recessive colorectal adenomatous polyposis ('hMYH polyposis'), which is characterized by multiple adenomas, some of which progress to cancer (24–28). Tumors from affected patients contain somatic G:C to T:A mutations in the adenomatous polyposis coli (*APC*) gene, a key gene that controls cellular proliferation in the colon. We have shown that hMYH interacts with hMutSα via hMSH6 and that hMYH activity is enhanced by hMutSα (29). These observations suggest that hMSH6 and hMYH may cooperate with each other in defending against the mutagenic effects of GO lesions (29).

In this report, we characterize the functional effect of two pathogenic hMYH missense mutations (R227W and V232F) that lie close to, or within, the putative hMSH6 binding domain (29). We show that although neither of the missense mutations affected binding to the hMSH2/hMSH6 complex, both had impaired A/GO binding and glycosylase activities and failed to complement MutY-deficiency in *E.coli*. These results provide evidence that the characteristic patterns of somatic G:C to T:A mutations found in colorectal tumors from hMYH polyposis patients are due to a reduced ability of mutant hMYH to recognize and repair A/GO mismatches.

MATERIALS AND METHODS

hMYH missense mutations

R227W was identified in combination with G382D in a 42-year-old patient (patient C103) with four colorectal adenomas and rectal cancer (30), and V232F was identified in combination with Y165C in a 70-year-old male (patient 18) with between 100 and 1000 colorectal polyps (27).

Bacteria

E.coli mutY⁻ mutant strain PR70 (Su- *lacZ X74 galU galK* Smʳ *micA68*::Tn*10*kan) was obtained from M. S. Fox. The strain CC104 containing a *lacZ* mutation at residue 461 of β-galactosidase and its derivative CC104 *mutM*::mini-kan *mutY*::mini-Tn*10* were obtained from J. H. Miller. DE3 lysogenic strains were constructed according to the procedures described by Invitrogen.

Construction of the hMYH mutants

The *hMYH* gene in pGEX-4T-hMYH (31) was isolated as a BamHI–XhoI digested fragment and transferred into pET21a (Novagene) to obtain the clone pET21a-hMYH. Mutant *hMYH* genes were constructed by site-directed mutagenesis of pET21a-hMYH and pGEX-4T-2-hMYH. Complementary

oligonucleotides containing the appropriate mismatches for R227W (679 C→T) and V232F (694 G→T) were designed using the QuikChange Primer Design program (http://labtools.stratagene.com/QC/QCprimers) (R227W, 5′-GGCA-ACGTAGCA**T**GGGTGCTGTGCCGT-3′ and 5′-ACGGC-ACAGCACCC**A**TGCTACGTTGCC-3′; V232F, 5′-GGGT-GCTGTGCCGT**T**TCCGAGCCATTGGTGCTG-3′ and 5′-CAGCACCAATGGCTCGGA**A**ACGGCACAGCACCC-3′). Site-directed mutagenesis reactions were carried out according to the manufacturer's instructions (Stratagene). The entire *hMYH* open reading frame was sequenced to confirm the presence of the desired mutation and to ensure that no unwanted mutations had been introduced.

Wild-type and mutant *hMYH* genes were transferred from pET21a-hMYH into pGEV1 (obtained from M. Clore at NIH) by PCR. PCRs were performed with the templates pET21a-hMYH, pET21a-hMYH(R227W), pET21a-hMYH(V232F) and the primers Chang 226 (5′-GCTATCGCTAGCATGA-CACCGCTCGTCTCCCGC-3′) and Chang 398 (5′-TATT-CTCGAGCTGGGCTGCACTGTTGA-3′). The PCR products were digested with NheI and XhoI, ligated into the NheI–XhoI-digested pGEV1 vector and expressed to produce fusion proteins with streptococcal protein G (GB1 domain) at their N-termini and a 6-His tag at their C-termini. The generated plasmids were named pGEV-hMYH, pGEV-hMYH(R227W) and pGEV-hMYH(V232F). A similar approach was used to transfer the wild-type and mutant *hMYH* genes into pKK223-3 (Amersham Pharmacia) using the primers Chang 402 (5′-GCCGAATTCATGACACCGCTCGTCTCCCGCCTG-3′) and ChangXMA-3-hMYH (5′-TGCGTTCCCGGGTCACTG-GGCTGCACT-3′), to allow expression of untagged proteins from the *tac* promoter. Products were digested with EcoR1 and XmaI before ligating into EcoR1–XmaI digested pKK223-3 to generate plasmids pKK-hMYH, pKK-R227W and pKK-V232F.

Protein expression and purification

Mutant and wild-type pGEV1-hMYH constructs were transformed into *E.coli* PR70/DE3 (*mutY* mutant) cells containing the pRARE2 vector (Novagen) to enhance the expression of the hMYH protein. The cells were grown at 37°C in Luria–Bertani (LB) medium containing appropriate antibiotics. The host PR70/DE3 cell has a transposon insertion at the chromosomal *mutY* gene and does not have MutY activity. Protein expression was induced at an OD_{600} of 0.6 by adding isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.2 mM and incubating at 20°C for a further 16 h before harvesting the cells. Recombinant hMYH was detected by 10% SDS–PAGE.

His-tagged hMYH fusion proteins were bound to nickel agarose according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). The cell extracts were incubated with the beads at 4°C for 1 h. After washing with buffer N [50 mM potassium phosphate (pH 8.0), 300 mM NaCl] containing 50 mM imidazole, bound proteins were eluted by increasing imidazole concentration in buffer N to 250 mM. Partially purified proteins were visualized by 10% SDS–PAGE and fractions containing hMYH were pooled, dialyzed with buffer A [20 mM potassium phosphate (pH 7.4), 0.1 mM EDTA, 10% glycerol, 0.5 mM DTT and 0.1 mM phenylmethlysulfonyl fluoride], divided into small aliquots and stored at −80°C.

Western blot analysis

Cell paste from 0.5 ml of the culture was resuspended in 0.1 ml of cracking dye containing 60 mM Tris–HCl (pH 6.8), 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.01% bromophenol blue and boiled for 10 min. Cell lysates (30 µl) or partially purified hMYH proteins (2 µl) were fractionated by 10% SDS–PAGE and transferred to a nitrocellulose membrane (32). The hMYH protein was detected by western blotting with polyclonal antibodies against a polypeptide of hMYH (residues 344–361). The antibody was affinity purified by binding to a peptide affinity column and further purified by absorption to a nitrocellulose membrane containing immobilized C-terminal hMYH. The membrane was subjected to the enhanced chemiluminescence analysis system from Amersham Pharmacia International according to the manufacturer's protocol. To estimate the concentrations of partially purified GB1-hMYH, a western blot including known amounts of GB1-tagged *E.coli* MutY was assayed with an antibody to annexin-2 (BD Bioscience, San Diego, CA) and quantitated.

GST–hMYH pull-down assay

GST–hMYH fusion proteins were immobilized on glutathione–sepharose 4B (Amersham Parmacia) and incubated with purified hMSH2/hMSH6 (hMutSα) heterodimers as described previously (29). GST–hMYH–hMutSα complexes were pelleted and detected by SDS–PAGE and western blotting with antibodies against hMSH6 (BD Biosciences). Concentrations of mutant and wild-type proteins bound to beads were estimated from Coomassie-stained gels, and similar levels of all GST–hMYH proteins in the assay were confirmed by western blotting with antibodies against hMYH.

Oligonucleotide substrates

The DNA substrates used in this study were

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44mer  5′  AATTGGGCTCCTCGAGGAATTA–
               GCCTTCTGCAGGCATGCC        3′
       3′       CCCGAGGAGCTCCTTAAY–
               CGGAAGACGTCCGTACGGGGCC  5′
20mer  5′  CCGAGGAATTXGCCTTCTG   3′
       3′  GCTCCTTAAYCGGAAGACG   5′
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(where X = A or C and Y = G or GO).

The top strand was labeled at the 5′ end with [γ-^{32}P]ATP and polynucleotide kinase and was then annealed with the bottom strand. The single-stranded overhangs were filled in with the Klenow fragment of DNA polymerase I and unlabeled deoxynucleotide triphosphates as described by Lu *et al.* (33). Radioactive-labeled 20mer and 44mer DNA substrates were used for gel mobility shift and glycosylase assays, respectively.

hMYH glycosylase activity assay

The glycosylase assay was carried out in a 10 µl reaction volume containing 25 mM HEPES (pH 7.0), 5 mM EDTA, 1.5% glycerol, 50 µM ZnCl$_2$, 1.8 fmol of ^{32}P-labeled 44mer duplex DNA containing an A/GO mismatch and partially purified hMYH. The reaction was incubated at 37°C for 1.5 h before adding 2 µl of 1 M NaOH and incubating at 90°C for 30 min. Samples were lyophilized, resuspended in 3 µl formamide dye (90% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue) and heated to 90°C for 2 min before loading onto a 14% polyacrylamide gel containing 7 M urea.

hMYH-DNA binding assay

The ability of hMYH to bind A/GO containing DNA was assayed by gel retardation as described by Lu (34) with some modifications. The reaction mixture contained 10 mM Tris–HCl (pH 7.6), 0.5 mM DTT, 0.5 mM EDTA, 1.5% glycerol, 20 ng poly(dI–dC) with 1.8 fmol ^{32}P-labeled 20mer duplex DNA (29) and partially purified hMYH in a total volume of 20 µl. After incubation at 37°C for 30 min, the reaction was supplemented with 2 µl of 50% glycerol and analyzed on a 6% polyacrylamide gel in 50 mM Tris borate (pH 8.3), 1 mM EDTA and 2.5% glycerol.

Measurement of mutation frequency

Mutant and wild-type pKK223-3-hMYH constructs were transformed into *E.coli* CC104*mutYmutM* and single colonies cultured in LB medium. Protein expression was induced at OD$_{600}$ 0.6 by adding IPTG at a final concentration of 0.2 mM and incubating at 20°C for a further 16 h. Four independent overnight cultures were plated on both LB agar (10^{-6} dilution) and LB agar containing 0.1 mg/ml rifampicin (Rif). The mutation frequency was calculated from the ratio of the colony number on the Rif-plate (Rif-resistant, Rif^R) to the colony number on the LB plate (total viable cells). The averages and standard deviations were obtained from more than three experimental data.

RESULTS

Wild-type and mutant hMYH physically interact with hMSH6

We have previously defined the hMSH6 binding domain to residues 232–254 in hMYH (Figure 1) (29). Two hMYH missense mutations (R227W and V232F) have previously been characterized in patients with hMYH polyposis (27,30) and lie close to, or within, the hMSH6 binding domain (Figure 1).
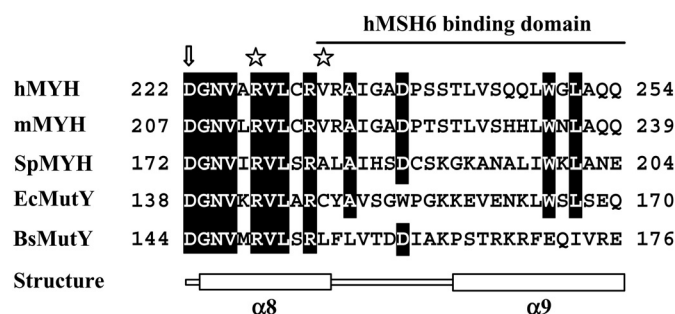

Figure 1. Evolutionary conservation of the putative hMSH6 binding domain in hMYH and the mutations analyzed in this study. Alignment of human (hMYH, U63329), mouse (mMYH, AY007717), fission yeast (SpMYH, AF053340), *E.coli* (EcMutY, P17802) and *B.stearothermophilus* (BsMutY, 1RRQ_A) MYH homologs was carried out using Clustal W (v.1.7). The putative hMSH6 binding domain is marked by a line and the highly conserved residues are shaded. The hMYH missense mutations R227W and V232F are at conserved and partially conserved residues, respectively (indicated by stars). The conserved Asp at the active center of the glycosylases is marked by an arrow. The secondary structural elements of BsMutY (46) are shown at the bottom.

We examined whether these two hMYH mutants were defective in binding with hMSH2/hMSH6 (hMutSα) using glutathione *S*-transferase (GST) pull-down assays. As shown in Figure 2, wild-type GST–hMYH proteins immobilized on glutathione sepharose beads could pull down hMutSα, confirming the physical interaction between the two proteins. Both hMYH(R227W) and hMYH(V232F) retained their ability to interact with hMutSα (Figure 2).

Impaired glycosylase activities of the hMYH(R227W) and hMYH(V232F) proteins

To further characterize the hMYH(R227W) and hMYH(V232F) mutant proteins, we expressed the proteins
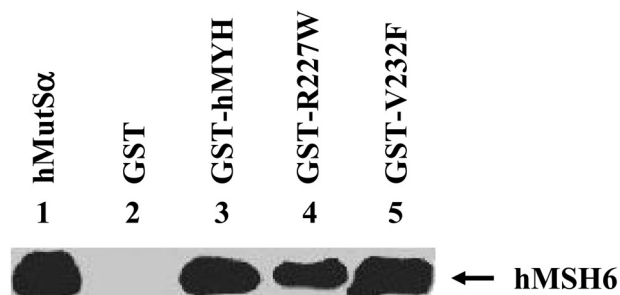

Figure 2. Physical interaction between hMutSα and wild-type and mutant hMYH. Purified hMutSα (hMSH2/hMSH6 complex) pulled down by GST–hMYH beads was fractionated by a 10% SDS–polyacrylamide gel followed by western blot analysis using antibodies against hMSH6. Equivalent concentrations of wild-type and mutant GST–hMYH proteins were used in the assay. Purified hMutSα (0.2 μg) was applied to lane 1. For control, hMutSα was applied to beads containing GST alone (lane 2). Wild-type, hMYH(R227W) and hMYH(V232F) all bound strongly to hMutSα.

as fusion proteins tagged by both streptococcal protein G (GB1 domain) and 6-His in an *E.coli* PR70/DE3/pRARE host. The GB1 domain increased the solubility of hMYH and the His-tag allowed affinity purification. The plasmid pRARE contains rare tRNA genes for improved hMYH protein translation. After passing through a nickel-agarose column, the partially purified hMYH proteins (2 μl) were applied to 10% SDS–PAGE for Coomassie blue staining and western blotting against hMYH to determine their purity and concentrations (Figure 3A and B). hMYH proteins were partially purified to ∼15% homogeneity after passing through a nickel-agarose column (Figure 3A). Surprisingly, the amounts of protein indicated by western blotting were not proportional to that indicated by Coomassie blue staining. There are two possibilities for this discrepancy: (i) the band of the correct size for GB1-hMYH probably contains other protein(s) and (ii) the mutant and wild-type proteins do not react to the hMYH antibody equally. Since the concentrations of partially purified hMYH proteins cannot be precisely determined from the Coomassie blue staining, we determined the amounts of proteins by comparing them with known amounts of GB1-MutY on western blotting (data not shown). The protein concentrations of wild-type, hMYH(R227W) and hMYH(V232F) were estimated to be 44, 32 and 59 μg/ml, respectively.

Glycosylase activities were assayed using a 44 bp duplex containing an A/GO mismatch with partially purified proteins. hMYH(R227W) protein had no detectable glycosylase activity with A/GO mismatches (Figure 4A, lane 2), and hMYH(V232F) displayed a reduced ability to excise adenine (cleaving only 7% of the DNA substrates as compared with 55% by wild-type hMYH; Figure 4A, lanes 1 and 3). After adjusting for the protein concentrations, hMYH(R227W) and
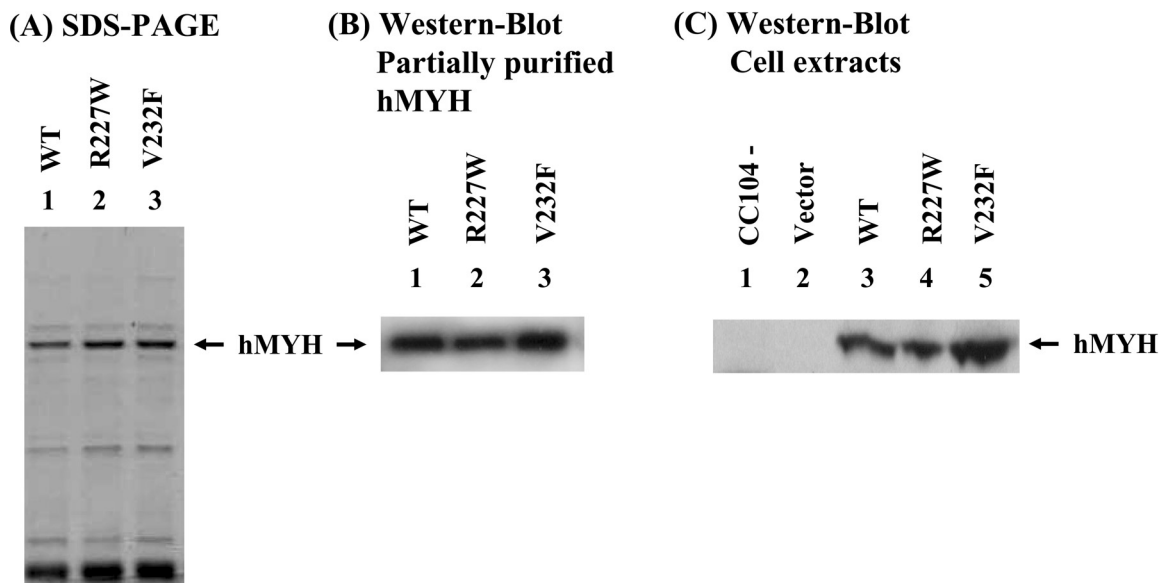
Figure 3. Analyses of hMYH mutant proteins. (**A**) Partially purified GB1-hMYH-His fusion proteins detected by Coomassie blue staining. Wild-type and mutant proteins were expressed in *E.coli* PR70/DE3 and partially purified by nickel-agarose affinity chromatography. An aliquot of 2 μl of each protein was applied to 10% SDS–PAGE for staining with Coomassie blue. (**B**) Partially purified GB1-hMYH-His fusion proteins detected by western blotting. Samples are similar to (A) but detected by western blotting with polyclonal antibodies against hMYH residues 344–361 purified by peptide affinity column. (**C**) Expression of wild-type and mutant hMYH proteins in *E.coli* CC104*mutYmutM*. hMYH proteins in cell lysates were detected by western blotting with polyclonal antibodies against hMYH residues 344–361 purified by peptide affinity column and membrane-bound hMYH. Lane 1, CC104− (*mutYmutM*); lanes 2–5, CC104− (*mutYmutM*) expressing pKK223-3 vector alone, wild-type hMYH, hMYH(R227W) and hMYH(V232F), respectively. V232F mutant hMYH protein was expressed ∼2-fold higher than those of wild-type and R227W hMYH proteins *in vivo*.

(A) Glycosylase

(B) DNA binding
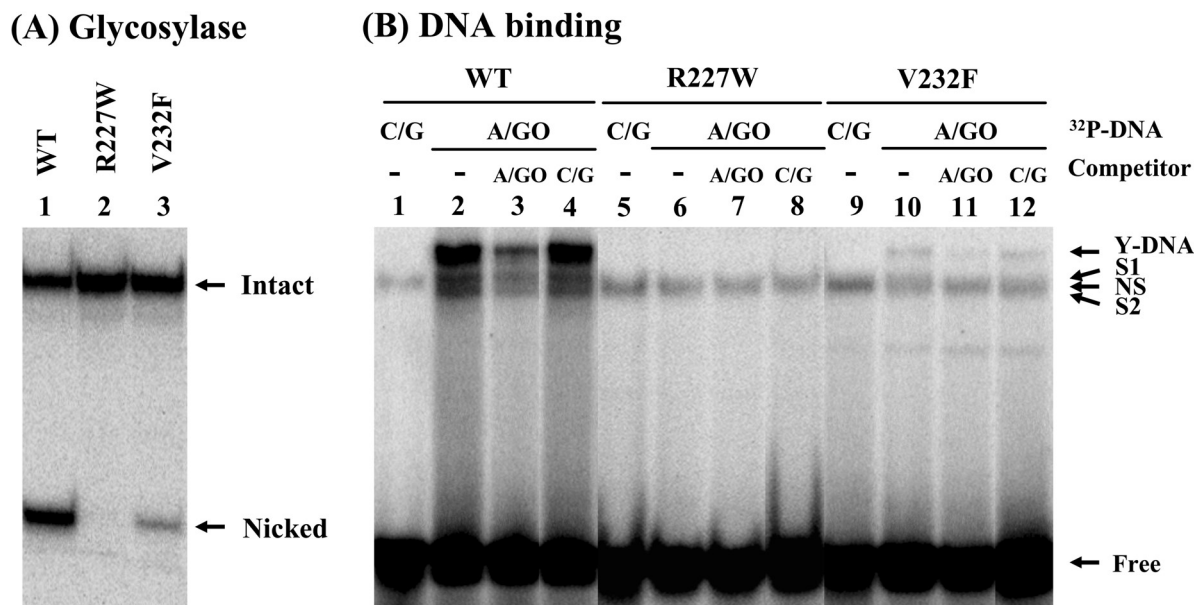
Figure 4. (**A**) Glycosylase activities of partially purified GB1-hMYH-His mutants. Lane 1, wild-type (WT) hMYH; lane 2, hMYH(R227W); and lane 3, hMYH(V232F). 5′ end labeled 44mer DNA duplex (1.8 fmol) containing an A/GO mismatch was incubated with partially purified wild-type (48 ng), R227W (32 ng) and V232F (59 ng) GB1-hMYH for 1.5 h at 37°C before cleavage of the phophodiester backbone by NaOH. The reaction products were fractionated on a 14% polyacrylamide sequencing gel containing 7 M urea. Data presented are a PhosphorImager image. (**B**) DNA-binding activities of GB1-hMYH mutants. The ^{32}P-labeled DNA (20mer) containing the C/G base pair (lanes 1, 5 and 9) and A/GO mismatch (lanes 2–4, 6–8 and 10–12) were incubated with partially purified wild-type (48 ng), R227W (32 ng) and V232F (59 ng) GB1-hMYH at 37°C for 30 min, and products were run on non-denaturing 6% polyacrylamide gels. Lanes 1–4, 5–8 and 9–12 are reactions performed with hMYH (WT), hMYH (R227W) and hMYH (V232F), respectively. Lanes 3, 7 and 11 contain a 50-fold excess of unlabeled A/GO-DNA (competitor) in addition to the labeled substrate. A 50-fold excess of unlabeled homoduplex (C/G-DNA) was added to lanes 4, 8 and 12. Data presented are a PhosphorImager image. The arrows mark the major specific hMYH–DNA complex (Y-DNA), two minor specific hMYH–DNA complexes (S1 and S2), non-specific protein–DNA complex (NS) and free DNA substrate (free).

hMYH(V232F) had only ∼0 and 11% of the wild-type glycosylase activity toward A/GO-containing DNA substrates.

DNA-binding affinities of mutant hMYH proteins

Using gel retardation assays, we compared the A/GO-DNA binding activities of hMYH(R227W) and hMYH(V232F) with wild-type hMYH. Three DNA–protein complexes were observed with the tagged wild-type hMYH (Figure 4B, lane 2), two DNA–protein complexes were observed with the tagged hMYH (V232F) (Figure 4B, lane 10) and only one DNA–protein complex was observed with the tagged hMYH (R227W) (Figure 4B, lane 6). To verify the specific complex of hMYH with the A/GO substrate, binding reactions were carried out with a ^{32}P-labeled C/G-containing 20mer substrate (Figure 4B, lanes 1, 5 and 9). Only one protein–DNA complex was observed with the wild-type and mutant hMYH proteins and its mobility is the same as that of the complex in the hMYH (R227W) reaction but is different from the three DNA–protein complexes observed with wild-type hMYH. In addition, competitive binding experiments were performed, in which ^{32}P-labeled A/GO-containing DNA was incubated with a 50-fold excess of unlabeled A/GO and C/G substrates. All three DNA–protein complexes observed with wild-type hMYH (Figure 4B, lane 3) and the upper band in binding reaction of hMYH(V232F) (Figure 4B, lane 11) were weakened upon the addition of excess unlabeled A/GO substrate. The unlabeled homoduplex (C/G 20mer) had little effect on the hMYH binding to labeled A/GO substrate (Figure 4B, lanes 4 and 12). Thus, the three DNA–hMYH complexes

observed with wild-type hMYH appear to be specific (marked with Y-DNA, S1 and S2 complexes). The DNA–protein complex shown in the hMYH (R227W) binding reactions and the lower DNA–protein complex in the hMYH (V232F) binding reactions were not changed by adding either excess unlabeled A/GO-containing DNA or C/G homoduplex (Figure 4B, lanes 7, 8, 11 and 12). Therefore, this complex is non-specific (marked by NS). For binding capacity of the mutant hMYH proteins, only the top complex (marked by Y-DNA) was taken into consideration. Approximately 12% of the DNA substrates were bound by the wild-type enzyme (Figure 4B, lane 2); the hMYH (R227W) protein exhibited no DNA binding toward A/GO mismatches (Figure 4B, lane 6); and hMYH (V232F) has an A/GO DNA-binding activity (Figure 4B, lane 10) ∼2% compared with the wild-type enzyme.

The glycosylase activities of wild-type hMYH and V232F hMYH can be stimulated by hMutSα

We have previously shown that the binding and glycosylase activities of hMYH with A/GO mismatch are enhanced by hMutSα (30). Because the hMYH missense mutants (R227W and V232F) can physically interact with hMutSα (Figure 2), we examined whether their function can be stimulated by hMutSα. When wild-type hMYH (10 nM) was supplemented with hMutSα (0.25 or 2 nM) in the glycosylase reactions, we observed a 2.4-fold increase of glycosylase activity (Figure 5, lanes 1–3). However, no glycosylase activity was restored to the hMYH(R227W) mutant (109 nM) by adding hMutSα (Figure 5, lanes 4–6). The glycosylase activity of
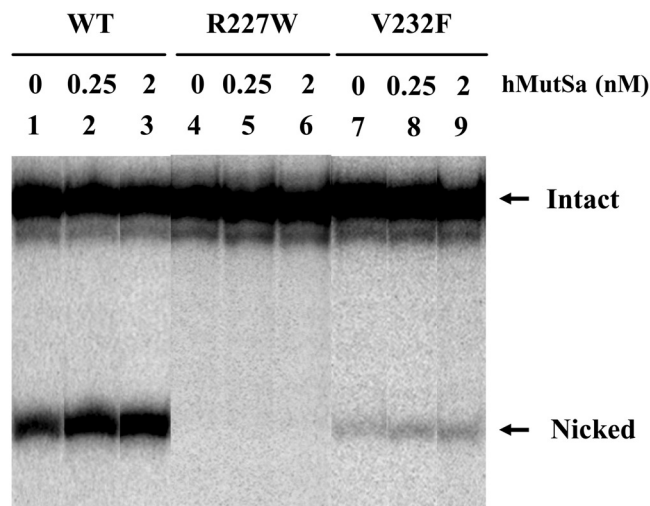
Figure 5. Functional interaction between hMutSα and wild-type and mutant hMYH proteins. The glycosylase assay was performed as in Figure 4A, except that the reactions contained different amounts of hMYH proteins and 0.25 or 2 nM of hMutSα. Lane 1–3, reactions contained 10 nM of wild-type hMYH with 0, 0.25 and 2 nM of hMutSα, respectively; lanes 4–6, reactions contained 109 nM of hMYH(R227W); and lanes 7–9, reactions contained 40 nM of hMYH(V232F). The arrows indicate the intact DNA substrate and nicked product.

hMYH(V232F) mutant protein (40 nM) could be partially stimulated by hMutSα (Figure 5, lanes 7–9), but could not be restored to the wild-type level.

The reaction conditions under which we observe hMYH binding to A/GO mismatches in the presence of hMutSα contain 30 mM NaCl and use 44mer DNA (30). Under these conditions, the non-specific protein–DNA complex (NS) (Figure 4B) is more dominant. We were unable to detect significant enhancement of A/GO-DNA binding activity of partially purified GB1- and His-tagged hMYH by hMutSα (data not shown).

In vivo complementation activity of the hMYH(R227W) and hMYH(V232F) mutants

E.coli cells with a single mutation in *mutY* or *mutM* genes are moderate mutators; however, the *mutYmutM* double mutant is a strong mutator because it fails to remove GO lesions and correct replication errors (35,36). In the absence of functional MutY and MutM, a high level of mutations in the Rif-binding site of RNA polymerase renders the cell resistant to Rif. To study the *in vivo* complementation activity of the hMYH(R227W) and hMYH(V232F) mutants, the mutant genes were expressed under the control of the *tac* promoter as untagged proteins in CC104*mutYmutM* cells. Western blot analysis revealed that V232F mutant hMYH protein (Figure 3C, lane 5) was expressed ∼2-fold higher than those of wild-type and R227W hMYH proteins (Figure 3C, lanes 3 and 4). CC104*mutYmutM* cells with vector pKK223-3 had a lower mutation frequency than the cells without the vector (Table 1, compare rows 2 and 3), the reason for this is unclear, although we have seen this anomaly before (37). Expression of wild-type hMYH significantly lowered the mutation frequency in *E.coli* CC104*mutYmutM* (Table 1, compare rows 3 and 4) ($P < 0.007$); however, the expression of hMYH(R227W)

Table 1. Mutation frequencies of *mutYmutM E.coli* expressing mutant hMYH proteins

Strains[a]	Mutation frequency[b] of Rif^R colonies/10^8 cells	Increase (fold)[c]
CC104+ (wild type)	0.7 ± 0.2	1
CC104− (*mutYmutM*)	667 ± 48	953
CC104−/pKK223-3 (vector)	223 ± 28	319
CC104−/pKK-hMYH (wild type)	142 ± 29	203
CC104−/pKK-R227W (mutant)	272 ± 68	389
CC104−/pKK-V232F (mutant)	187 ± 36	267

[a]*E.coli* strains CC104+ and CC104− contain wild-type and mutated *mutY* and *mutM* genes, respectively.
[b]The *in vivo* activities of wild-type and mutant hMYH were measured by the frequency of Rif-resistant colonies (average of at least three separate experiments, with errors reported as the standard deviations).
[c]Fold increase compared with the wild-type strain.

mutant protein did not (Table 1, compare rows 3 and 5). Expression of hMYH(V232F) reduced the mutation frequency slightly, but not significantly ($P < 0.17$) (Table 1, row 6), when compared with vector alone (Table 1, row 3).

DISCUSSION

ROS are the most prevalent source of DNA lesions in aerobic organisms, and mammalian cells have developed several repair pathways to cope with the resultant oxidative DNA damage. The BER protein hMYH is essential in protecting against such damage and inherited defects in hMYH predispose to colorectal tumors in humans (24–28). Furthermore, although Myh knockout mice are cancer-free (38,39); combined Myh and Ogg1—deficiency predisposes mice to lung and ovarian tumors and lymphomas (40). In this study, we have confirmed a direct interaction between hMSH2/hMSH6 and hMYH and provide further support that a reduced ability of mutant hMYH to recognize and repair A/GO mismatches underlies the mechanism of pathogenesis in hMYH polyposis. Additional studies are now warranted to determine whether disruption of the hMYH–hMSH6 interaction and/or uncoupling of the MMR and BER pathways also contributes to colorectal tumourigenesis.

To date, the biochemical effects of only the two most common hMYH mutations in Caucasians, Y165C and G382D, have been defined (41–43). The Y165C of hMYH and the equivalent Y82C of *E.coli* MutY are defective in DNA glycosylase activity, while hMYH(G382D) expressed in *E.coli* is inactive (43) and the equivalent EcMutY(G253D) is only partially inactive (41,42). Here, we show that two additional variants of hMYH (R227W and V232F) associated with hMYH polyposis are also functionally compromised, although both retain the ability to physically interact with hMSH6. Similar to the Y165C mutation (43), the R227W mutation severely reduces the ability of hMYH to bind an A/GO mispair and catalyze adenine excision from A/GO mismatches. In addition, the R227W mutant also displays an impaired function to complement the phenotype of *E.coli mutY*. hMYH(V232F) is also deficient in DNA binding and glycosylase activities, although the biochemical differences between the mutant and wild-type enzymes are less pronounced than for hMYH(R227W). The glycosylase activity

of the V232F mutant can be partially stimulated by hMutSα but cannot be restored to the wild-type level. This mutant enzyme may be able to recognize mispairs and catalyze some reactions as observed for the human G382D homolog (44). This could result in the 'partially repaired' site being exposed to inappropriate and potentially mutagenic activities by enzymes, such as hOGG1 (44).

The deleterious effects of the R227W and V232F mutations in hMYH polyposis may be explained by the bacterial MutY structures (45,46). Both R227 and V232 are located at an alpha helix containing the conserved Asp at the active center (Figure 1). The R227 residue of hMYH is conserved in the MutY family, and the corresponding residues of *E.coli* and *Bacillus stearothermophilus* MutY are R143 and R149, respectively (Figure 1). According to the recent X-ray structure of BsMutY–DNA complex (46), this conserved Arg is within 3 Å to the phosphate group 5′ to the mismatched adenine. A mutation of this conserved Arg to Trp will decrease the positive charge and may affect the interaction between MutY and DNA by reducing their electrostatic interaction. The Val232 residue of hMYH is less conserved in the MutY family and the corresponding residues of *E.coli* and *B.stearothermophilus* MutY are Cys148 and Leu154, respectively (Figure 1). Cys148 of EcMutY (45) and Leu154 of the BsMutY–DNA complex (46) are within 4 Å to the iron–sulfur cluster which is ligated by four conserved cysteines. The residues spacing the four conserved cysteines are dominated by positively charged amino acids and are important for substrate recognition. The bulky side chain of the substituted Phe in V232F may disrupt the proper alignment of the iron–sulfur cluster loop and thus affect DNA binding and catalysis.

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