Human Homolog of the MutY Repair Protein (hMYH) Physically Interacts with Proteins Involved in Long Patch DNA Base Excision Repair*

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The human MutY homolog (hMYH) is a DNA glycosylase involved in the removal of adenines or 2-hydroxyadenines misincorporated with template guanines or 7,8-dihydro-8-oxodeoxyguanines. hMYH is associated in vivo with apurinic/apyrimidinic endonuclease (APE1), proliferating cell nuclear antigen (PCNA), and replication protein A (RPA) in HeLa nuclear extracts as shown by immunoprecipitation and Western blotting. However, binding of hMYH to DNA polymerases β and δ was not detected. By using constructs containing different portions of hMYH fused to glutathione S-transferase, we have demonstrated that the APE1-binding site is at a region around amino acid residue 300, that the PCNA binding activity is located at the C terminus, and that RPA binds to the N terminus of hMYH. A peptide consisting of residues 505–527 of hMYH contains a conserved PCNA-binding motif binds PCNA, and subsequent amino acid substitution identified Phe-518 and Phe-519 as essential residues required for PCNA binding. RPA binds to a peptide that consists of residues 6–32 of hMYH and contains a conserved RPA-binding motif. The PCNA- and RPA-binding sites of hMYH are further confirmed by peptide and antibody titration. These results suggest that hMYH repair is a long patch base excision repair pathway.

Base excision repair (BER) is the major mechanism that removes damaged bases by specific DNA glycosylases leaving behind an apurinic/apyrimidinic (AP) site as an intermediate. Several DNA glycosylases have associated lyase activities that cleave the 3’ side of the AP site (1–3). Human MutY glycosylase homolog (hMYH) is involved in the removal of adenines or 2-hydroxyadenines mismatched with guanines or 7,8-dihydro-8-oxodeoxyguanines (8-oxoG) that arise through DNA replication errors and DNA recombination (4–8). Together with 8-oxoG glycosylase (hOGG1) (9–12) and human MutT homolog (13–15), the hMYH protein protects the cell from the mutagenic effects of 8-oxoG, the most stable product known caused by oxidative damage to DNA. The formation of 8-oxoG in DNA, if unrepaired, can lead to misincorporation of adenines opposite the 8-oxoG lesions resulting in C:G to A:T transversions (16–18). To complete the repair after glycosylase action, the AP site is further processed by an incision step, DNA synthesis, an excision step, and DNA ligation. There are two alternative pathways to repair AP-containing DNA as follows: a short patch BER pathway and a long patch BER pathway (19–21).

The short patch BER pathway has a repair patch of only 1 nucleotide and requires four proteins as follows: AP endonuclease (APE1 also called HAP1 or Ref1), DNA polymerase β (polβ), and DNA ligase III/XRCC1 heterodimer (22). The long patch BER pathways with a repair patch of 2–6 nucleotides can be reconstituted with six proteins as follows: APE1, replication factor C, proliferating cell nuclear antigen (PCNA), flap endonuclease 1 (FEN1), DNA polymerases δ (polδ) and ε (polε), and DNA ligase I (21, 23). However, the long patch BER reaction can be stimulated by polβ and trimeric replication protein A (RPA, a single-stranded DNA-binding protein consisting of 11-, 34-, and 70-kDa subunits) (24–26).

The DNA glycosylase remains bound to the mutagenic AP site until other enzymes in the BER pathway displace it. Protein-protein interactions have been reported among the enzymes involved in BER. APE1 has great affinity for the AP site, cleaving the DNA 5’ to the baseless site, and can displace both thymine- and uracil-DNA glycosylases presumably through interaction with the glycosylases (27–29). When bound to DNA, DNA polymerase β interacts with APE1 for repair synthesis (30) and with the noncatalytic scaffold protein XRCC1 (subunit of the XRCC1-Ligase III heterodimer) (31) to complete the short patch BER. For the long patch BER, PCNA has been found to interact with a nuclear form of uracil DNA glycosylase (UDG2), DNA polymerases δ and ε, replication factor C, FEN1, and DNA ligase I (32, 33). In addition, PCNA also interacts with enzymes involved in nucleotide excision repair, mismatch repair, branch structure processing, cell cycle control, and chromatin assembly (32, 34). Thus, PCNA may act as a molecular adaptor, coordinating and regulating the actions of DNA rep-
llication, DNA repair, and cell cycle control (35). UDG2 has been demonstrated to colocalize to the replication fork through interactions with PCNA and RPA, where BER is initiated to repair misincorporated uracils (36). RPA is also involved in the latter stages of long patch BER by stimulation of FEN1 activity, whereas repair of 8-oxoG by hOGG1 and repair of T or U opposite G by TDG are mainly via the short patch pathway (37). Little is known regarding the repair patch size and the cleaved DNA fragment (N). Lanes 4–6 are from a nonconcurrent experiment.

It has been shown that the type of DNA glycosylase that recognizes the lesion can determine the appropriate patch-size repair pathway (42, 43). The repair by 3-methylenedine DNA glycosylase in HeLa extracts is by both short and long patch pathways, whereas repair of 8-oxoG by hOGG1 and repair of T or U opposite G by TDG are mainly via the short patch pathway (42, 43). Little is known regarding the repair patch size and protein-protein interactions involved in the hMYH repair pathway. In this paper, we demonstrate that hMYH binds to APE1, PCNA, and RPA, through the use of coimmunoprecipitation as well as affinity binding to glutathione S-transferase hMYH (GST-hMYH) fusion protein constructs and hMYH peptides. This suggests that hMYH catalyzes base excision repair via a PCNA-dependent long patch pathway.

EXPERIMENTAL PROCEDURES

Cloning of hMYH Gene and the GST-hMYH Protein Constructs—The hMYH gene was cloned from a HeLa cDNA library (Invitrogen), using the primers 5′-GCTGATTAATATGACACCGCTCGTCTCCCGC-3′ (CHANG 262) and 5′-GCTAACCTAGCTACAGGCTGCTCTCCCGC-3′ (CHANG 227). The polymerase chain reaction product was digested with AseI and BamHI and ligated into the NdeI-BamHI-digested pET11a vector (Novagen). The sequence of the cloned hMYH cDNA was confirmed to be the same sequence as published (5). The hMYH gene and deletion constructs were subsequently cloned into a pGEX-4T-2 vector (Amersham Pharmacia Biotech) to express fusion proteins of glutathione S-transferase and hMYH (GST-hMYH) using the primers and restriction enzymes listed in Table I.

Expression and Immobilization of GST-hMYH Constructs—Escherichia coli (BL21/DE3) cells harboring the expression plasmids of the GST-hMYH constructs were grown in LB broth containing 100 mg/ml ampicillin at 25 °C. Protein expression was induced at an A600 of 0.6 by the addition of isopropylthiogalactoside to a final concentration of 0.4 m M, and the cells were harvested 2 h later by centrifugation at 10,000 × g for 20 min. The cell paste, from a 500-ml culture, was resuspended in 9 ml of buffer G (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA) and treated with lysozyme (1 mg/ml) for 30 min at room temperature. After adding 0.2% Triton X-100 and 2 mM dithiothreitol, the solution was then centrifuged at 10,000 × g for 20 min. To the supernatant (10 ml), 1 ml of a 50% slurry of glutathione-Sepharose 4B in buffer G was added and incubated overnight at 4 °C. The GST-hMYH fusion proteins bound to the beads were pelleted at 1000 × g for 5 min and incubated with 5% bovine serum albumin in buffer G overnight at 4 °C. The beads were pelleted at 1000 × g for 5 min and washed four times with 5 ml of buffer G. The beads were suspended in buffer G containing chymostatin, pepstatin, leupeptin (5 μg/ml), 0.1% sodium azide, and a protease inhibitor mixture (Sigma) to form a 50% slurry and were stored at 4 °C.

HeLa Nuclear Extract—HeLa S3 cells, grown in Joklik’s minimum Eagle’s medium with 5% newborn calf serum, 2 g/liter NaHCO3, 1% nonessential amino acids, 4 mM l-glutamine, and 1% penicillin/streptomycin were purchased from The Cell Culture Center (Minneapolis, MN). HeLa nuclear extracts were prepared as described (8, 44). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad).

Coimmunoprecipitation and Immunoblots—HeLa nuclear extract (1.0 mg) was diluted 2-fold to 1 ml in buffer G containing Sigma protease inhibitor mixture, 5 μg/ml leupeptin, pepstatin, and chymotrypsin, and precleared by adding 30 μl of protein G-agarose (Life Technologies, Inc.) for 1–4 h at 4 °C. After centrifugation at 1000 × g, the supernatant was incubated with 1 μg of anti-PCNA (Calbiochem), 1 μg of anti-RPA 70 (the 70-kDa subunit) (45), 1 μg of anti-APE1 (Santa Biotechnology), 1 μg of anti-PCNA, and GST-hMYH (GST 516) were incubated with anti-PCNA, anti-APE1, and polβ, respectively, and Western blotting with polβ antibody 516 was performed to detect hMYH. Lane 1 represents the control HeLa nuclear extract (N) (40 μl), lane 2 is a fraction (~2%) of the supernatant (S), and lane 3 is the pellet (P). Controls with no first antibody were run concurrently. B, binding of APE1, RPA, and PCNA in HeLa nuclear extracts to GST-hMYH or GST bound to glutathione-Sepharose. Western blot analysis for APE1, PCNA, and RPA34 was performed as described under “Experimental Procedures.” Lanes 2 and 3 are derived from binding with GST-hMYH and lanes 4 and 5 are controls from binding with GST alone and are from nonconcurrent experiments. C, adenine glycosylase activity of the immunoprecipitants. Human MYH was immunoprecipitated using preimmune serum of hMYH antibodies (C, lane 1), hMYH peptide antibody 516 (Y1, lane 2), hMYH peptide antibody 344 (Y2, lane 3), anti-APE1 (A, lane 4), anti-PCNA (P, lane 5), or anti-RPA70 (R, lane 6). The washed pellets were resuspended in 40 μl of glycosylase buffer and assayed for adenine glycosylase activity with DNA containing an A/G-oxyG mismatch labeled at the 3′-end of the mismatched A-containing strand. The sequencing gel containing the fractionated products was autoradiographed. Arrows indicate the intact DNA substrate (I) and the cleaved DNA fragment (N). Lanes 4–6 are from a nonconcurrent experiment.
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(A)

GST-hMYH

GST

DNase I

S

P

S

P

S

P

S

P

S

P

S

P





1. APE1

2. PCNA

3. RPA

(B)

NatCl (M)

0

0.15

0.3

0.6

1.0

2.0





1

2

3

4

5

6

7

8

9

10

11

12

1. APE1

2. PCNA

3. RPA

Fig. 2. APE1, PCNA, and RPA bind directly to hMYH in vitro. A, pull down of purified APE1, PCNA, and RPA by GST-hMYH bound to glutathione-Sepharose is independent of DNA. Purified human APE1 (100 ng, expressed in E. coli), PCNA (100 ng, expressed in E. coli), or trimeric RPA (100 ng, expressed in the baculovirus expression system in insect Sf9 cells) was added to bind GST-hMYH (300 ng) immobilized on glutathione-Sepharose 4B as described under "Experimental Procedures." Samples in lanes 3 and 4 were treated with 4.5 units of DNase I, and samples in lanes 1 and 2 were not treated with DNase I. Lanes 5 and 6 are controls from binding with GST alone and are from nonconcurrent experiments. The pellets (P) and a fraction (40%) of supernatant (S) were fractionated by a 10% SDS-polyacrylamide gel followed by Western blot analysis for APE1, PCNA, and RPA. A4344 performed with respective antibodies. B, salt dependence of hMYH complexes. Human MYH complexed with APE1, RPA, and PCNA was produced as described in lanes 1 and 2 of A except the reactions were performed in buffer containing different NaCl concentrations as labeled.

DNA Glycosylase Assay—The DNA cleavage activity (DNA glycosylase activity followed by heating) of hMYH was assayed similarly as E. coli MutY glycosylase as described (46, 50, 51) except different buffer and incubation times were used. The DNA substrate is a 20-mer duplex DNA containing an A/8-oxoG mismatch (5'-CCGAGGATTTGCGCCCTTCTGC-3' and 3'-GGCTCCTTAAAGCGGAAAGCGC-5', where O represents 8-oxoG) that is labeled at the 3′-end of the mismatched A-containing strand. Human MYH was immunoprecipitated from HeLa nuclear extracts as mentioned above and washed with buffer G. The pellet was then washed five times with 800 µl of glycosylase reaction buffer containing 10 mM Tris-HCl, pH 7.6, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 15% (v/v) glycerol and resuspended in 40 µl of the same buffer. To the reaction mixture was added 1.8 fmol of 3′-end-labeled 20-mer duplex containing the A/8-oxoG DNA mismatch. After 60 min of incubation at 37 °C, the reaction mixture was boiled for 5 min and centrifuged at 10,000 × g, and the supernatant was extracted with phenol and precipitated by ethanol. The pellets were dissolved in 3 µl of loading dye containing 90% (v/v) formamide, 10 mM EDTA, 0.1% (v/v) xylene cyanol, and 0.1% (w/v) bromphenol blue. The DNA samples were heated at 90 °C for 2 min and analyzed on 14% polyacrylamide, 7 M urea sequencing gels (52). The gel was then autoradiographed.

GST-hMYH Pull-down Assay—Purified recombinant APE1 (100 ng) expressed in E. coli, PCNA (100 ng) expressed in E. coli, or trimeric RPA (100 ng) expressed in the baculovirus expression system in insect Sf9 cells (45) were added to the appropriate GST-hMYH constructs (Table I) (300 ng) immobilized on glutathione-Sepharose 4B (see above), and incubated overnight in 50 µl of buffer G containing 0.1% Nonidet P-40 at 4 °C. For RPA, the Nonidet P-40 was omitted from all steps. After centrifugation at 1000 × g, the supernatant was saved, and the pellets were washed five times with 800 µl of buffer G containing 0.1% Nonidet P-40 at 4 °C. The pellets and supernatants (20 µl) were fractionated on a 10% SDS-polyacrylamide gel, and Western blot analyses for APE1, PCNA, and RPA were performed as described (49). A control was run concurrently with immobilized GST alone. For coprecipitation of APE1, PCNA, or RPA with GST-hMYH from HeLa nuclear extracts, 200–300 ng of GST-hMYH or GST alone was added to HeLa nuclear extract (1.0 mg) and incubated overnight at 4 °C. After centrifugation at 1000 × g, the supernatant (∼2% of total volume) and the pellet was treated as described above.

DNase I Treatment of hMYH Complexes—Glutathione-Sepharose 4B beads immobilized with GST-hMYH were complexed with APE1, PCNA, and trimeric RPA as described above except that after washing five times with 800 µl of buffer G, the complexes were washed five times with 800 µl of DNase I reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and suspended in 50 µl of buffer G). To the reaction mixture, 4.5 units of DNase I (Sigma) were added and incubated for 30 min at 37 °C. After centrifugation at 1000 × g, the supernatant was saved, and the pellets were washed five times with 800 µl of DNase I reaction buffer at 4 °C. The samples were analyzed as mentioned above. Controls were run concurrently without DNase I and with GST alone.

Peptide-Sepharose Pull-down Assay—The peptides CRKKPRMGQQVLNDFFRHSHDTA (p505), CRKKPRMGQQVLNDARAAHSTDA (p505FF→AA), and CRSLRRLWAIMRKRPAAVGSGHR9QAS (p6) were synthesized (University of Maryland Biopolymer Laboratory) and immobilized on activated CNBr-Sepharose 4B (Amersham Pharmacia Biotech) by methods described previously (53) with the addition of an extra incubation for 48 h at 4 °C in 65 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 300 mM glycine. The method employed for the peptide pull-down assay was similar to the GST-hMYH pull-down assay, except APE1, PCNA, or RPA (100 ng), and human APE1, PCNA, and trimeric RPA as described above. Controls with Sepharose-immobilized BSA were run concurrently.

RESULTS

Active hMYH Is Found in Complexes Containing APE1, PCNA, and RPA in HeLa Nuclear Extracts—To investigate the hMYH-interacting proteins in vivo, an antibody against an hMYH peptide (α516) was used to immunoprecipitate proteins from HeLa nuclear extracts that were subsequently analyzed by Western blotting to identify the interacting proteins. Antibodies against several BER proteins were utilized in these experiments. We were able to detect APE1, PCNA, and RPA but not polβ and polδ in the immunoprecipitants (Fig. 1A, panels 1–5). In the reciprocal experiments, hMYH was detected in the immunoprecipitants using anti-APE1, anti-PCNA, and anti-RPA70 as the first antibodies (Fig. 1A, panels 6–8). Human MYH was not detected in the immunoprecipitants using anti-polβ and anti-polδ (Fig. 1A, panels 9 and 10). These results suggest a physical interaction exists between hMYH and APE1, PCNA, and RPA (but not polβ and polδ) in vivo. Controls without the primary antibody were run concurrently, and hMYH, APE1, PCNA, and RPA were not detected in these immunoprecipitants (data not shown). In addition, a control was detected in the immunoprecipitants using anti-APE1 antibodies (data not shown). Because hMYH, APE1, PCNA, and RPA were detected in both the supernatant and the immunoprecipitants (Fig. 1A, compare lanes 2 and 3 in panels 1–3 and 6–8), some of the nuclear hMYH, APE1, PCNA, or RPA may remain free or may be associated with other unrelated proteins.

To demonstrate further an interaction between hMYH and APE1, PCNA, and RPA, GST-hMYH immobilized on glutathio-
one-Sepharose was added to HeLa nuclear extracts (Fig. 1B) to pull down the interacting proteins. The 535-residue hMYH was fused to GST and expressed in E. coli. This form (type 1) of hMYH has been shown to be transported into the mitochondria of E. coli. (7, 54) but has been shown to localized to the nuclei by Tsai-Wu et al. (55). The ability of APE1, PCNA, and RPA to bind hMYH was demonstrated by detection of these proteins to hMYH was observed up to 1.0 M NaCl but was abolished in buffer containing 2.0 M NaCl (Fig. 2D). GST pull-down assays were employed using various GST-hMYH constructs (Table I) to determine the binding regions of APE1, PCNA, and RPA. Controls were run concurrently with immobilized GST alone. Western blot analyses of the pellets (P) and a fraction (~40%) of supernatants (S) were performed similarly as in Fig. 2A. B, graphic depiction of GST-hMYH constructs and the results of APE1, PCNA, and RPA binding to the hMYH fusion proteins. + indicates a positive interaction, and – indicates no binding.

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**FIG. 3.** Determination of regions of hMYH involved in APE1, PCNA, and RPA binding. A, the GST pull-down assays were employed using various GST-hMYH constructs (Table I) to determine the binding regions of APE1, PCNA, and RPA. Controls were run concurrently with immobilized GST alone. Western blot analyses of the pellets (P) and a fraction (~40%) of supernatants (S) were performed similarly as in Fig. 2A. B, graphic depiction of GST-hMYH constructs and the results of APE1, PCNA, and RPA binding to the hMYH fusion proteins. + indicates a positive interaction, and – indicates no binding.

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**TABLE I**

| GST-hMYH constructs Residues | Primers used | Name | Cloning sites |
|-------------------------------|--------------|------|---------------|
| I, intact 1–535 | 5′-TATTAGGATCCATGACACCCTGCTGTC-3′ | GU3 | BglI-XhoI |
| II, ∆N1 65–535 | 5′- AAATGAGATCCATGACACCCTGCTGTC-3′ | GU2 | BglI-XhoI |
| III, ∆N2 295–535 | 5′- TATTTCGAGATGCTGCTGTC-3′ | CHANG317 | BglI-XhoI |
| IV, ∆N3 319–535 | 5′- TATTTCGAGATGCTGCTGTC-3′ | CHANG328 | BglI-XhoI |
| V, ∆C1 3–318 | 5′- TATTATGATCCATGACACCCTGCTGTC-3′ | GU2 | BglI-SalI |
| VI, ∆C2 1–504 | 5′- TATTTCGAGATGCTGCTGTC-3′ | CHANG316 | BglI-SalI |
| VII, ∆N4 33–535 | 5′- TATTTCGAGATGCTGCTGTC-3′ | CHANG325 | BglI-XhoI |

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against APE1, PCNA, and RPA70 all had adenine glycosylase activity (Fig. 1C, lanes 4–6). Thus, this suggests that active hMYH is present in complexes with APE1, PCNA, and RPA proteins in the HeLa nuclear extracts.

**Human MYH Binds Directly to APE1, PCNA, and RPA—** Since APE1, PCNA, and RPA interact with many different proteins (Tables II–IV and Refs. 20 and 21), it is possible that an indirect association between hMYH and these three proteins may occur via intermediate proteins. To demonstrate that a direct interaction exists between hMYH and APE1, PCNA, and RPA, we performed affinity binding experiments with full-length GST-hMYH (residues 1–535) and individual homogeneously purified APE1, PCNA, and RPA. All three proteins were detected in the GST-hMYH pellets (Fig. 2A, lanes 1 and 2, panels 1–3) but not in the GST beads (Fig. 2A, lanes 5 and 6, panels 1–3). It is worth noting that the binding reaction between hMYH and APE1 is more complete than that between hMYH and PCNA or RPA. Thus, there are direct associations between hMYH-APE1, hMYH-PCNA, and hMYH-RPA.

Because APE1, PCNA, and hMYH are all DNA-binding proteins, we questioned whether their interactions could be mediated by sequences of DNA in the nuclear extract. DNase I treatment of the hMYH complexes failed to remove APE1, PCNA, and RPA from the GST-hMYH pellets (Fig. 2A, lane 4, panels 1–3). The amount of DNase I used was sufficient to digest 5 μg of a plasmid into low molecular weight fragments (data not shown), hence proving the association of hMYH with APE1, PCNA, and RPA is independent of DNA. The binding of these proteins to hMYH was observed up to 1.0 M NaCl but was abolished in buffer containing 2.0 M NaCl (Fig. 2B). These observations are characteristic of hydrophobic interactions (56).

**Identification of APE1, PCNA, and RPA Binding Domains in hMYH—** By using constructs containing different portions of hMYH fused to GST (Table I), we determined the hMYH interaction regions with APE1, RPA, and PCNA. The APE1 binding domain was localized to the region including residues 295–318 of hMYH because construct III retained binding activity (Fig. 3A, panel 1, lane 6). None of the constructs bound to immobilized GST alone (Fig. 1B, panels 1–3, lane 5). Actin, in the extracts, was not bound to GST-hMYH beads (data not shown). To determine whether the hMYH present in the immunoprecipitates is active, we assayed the adenine glycosylase activity toward dS-oxoG-containing DNA. Fig. 1C shows that the immunoprecipitates with both hMYH peptide antibodies (a344 and a516) possesses adenine glycosylase activity (lanes 2 and 3). Furthermore, the immunoprecipitates with antibodies against APE1, PCNA, and RPA70 all had adenine glycosylase activity (Fig. 1C, lanes 4–6). Thus, this suggests that active hMYH is present in complexes with APE1, PCNA, and RPA proteins in the HeLa nuclear extracts.

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with PCNA, we compared the hMYH amino acid sequence with the consensus PCNA-binding motif (33). It has been shown that PCNA can interact with many proteins involved in DNA replication and repair and that these PCNA-binding proteins share a common motif (32, 33) (Table II). Generally, this motif contains a glutamine at position 1, an aliphatic residue such as leucine, isoleucine, or methionine at position 4, and a pair of aromatic residues (Phe or Tyr) at positions 7 and 8 (Table II). The residues flanking the conserved motif also show a preponderance of proline and basic residues. A potential PCNA-binding motif, QQVLDNFF, was found at residues 512–519 of hMYH. To demonstrate that hMYH binds to PCNA through this motif, construct VI of GST-hMYH (Table I) without the putative PCNA-binding site was created and used to assess PCNA binding. Fig. 4A (middle panel) showed that deletion of residues 505–535 of hMYH resulted in a complete loss of PCNA binding. A synthetic polypeptide containing residues 505–527 (p505) of the hMYH sequence was immobilized on Sepharose 4B and was used to pull-down PCNA in an affinity binding assay. As shown in Fig. 4B, PCNA bound to the peptide Sepharose (upper panel, lane 6) but not to the control Sepharose containing immobilized BSA (upper panel, lane 2). It has been reported that substitution of the two aromatic residues at positions 7 and 8 of the PCNA-binding motif in DNA polymerase δ, methyl-5'-cytosine DNA methyltransferase, FEN1, and DNA ligase 1 abolished their interactions with PCNA (32, 33, 40, 57). To validate further this PCNA-binding domain of hMYH, the two conserved phenylalanines (Phe-518 and Phe-519 of hMYH) were substituted with two alanine residues in a synthetic polypeptide, p505FF3AA. PCNA was unable to bind to the mutated peptide p505FF3AA that has been immobilized on Sepharose 4B (Fig. 4A, upper panel, lane 8). These results demonstrate that the motif QQVLDNFF is indeed a true PCNA binding domain and that the two phenylalanine residues are

### Table II

| Protein | Organism | Position | Motif | GenBank No. |
|---------|----------|----------|-------|-------------|
| polδ    | S. cerevisiae | 151 | CNV TGFVNYLY VPAPN SSDA | X61920 |
| pol δ   | S. pombe | 130 | VHV VGEPYFY VKAVGF RP | LO7734 |
| pol δ   | H. sapiens | 139 | CHI HGAPFY TPAPGF GP | M81735 |
| pol 32  | S. cerevisiae | 335 | LKK GTDDFF KKRK | Z49543 |
| FEN1    | H. sapiens | 334 | GST QGQDPPF KVTGSSSA | P39748 |
| DNA ligase I | H. sapiens | 1 | M ORSMFF HPKEKKAR | M36067 |
| MCMT    | H. sapiens | 158 | STR QTTS FF AKGPAKRP | X63629 |
| Tigger Cds2 | H. sapiens | 86 | LMR QTSLSVF KKLQPCPQ | U49937 |
| Cdc27   | S. pombe | 359 | KFP QKTSMSFG GK | M74062 |
| p57     | H. sapiens | 266 | KLS GPDSDFF AKTMRAP | U48869 |
| p21Waf1/Cip1 | H. sapiens | 141 | KRK QTSMSDF HSKRRFLS | P38936 |
| Rad2    | S. pombe | 335 | TIF QGQFDPPF KVFPRSPR | X77071 |
| RAD27   | S. cerevisiae | 337 | SGI QGQDPPF QVQPTEQ | P26793 |
| XPG     | H. sapiens | 987 | QLT QGQDPPF KLAQKKE | P28715 |
| UDG2    | S. cerevisiae | 18 | KRK QTDDPF GTKSTNE | K04470 |
| UDG2    | H. sapiens | 1 | MGQ KTLFP SFSPKRHH | X89398 |
| hMYH    | H. sapiens | 509 | RMQ QQVLDNFF ASHIDTA | U63329 |
| mMYH*   | M. musculus | 490 | PSLG QQVLDPPF QRHIETDPK | AA409964 |
| SpMYH*  | S. pombe | 435 | KRRK QTTSNLK EPK | Z69240 |
| hMHS3   | H. sapiens | 18 | PAR QAVGSPF QSTGLKSTD | J04810 |
| hMHS6   | H. sapiens | 1 | MRR QSTYFP FKSPALSDA | P52701 |

The eight residue PCNA binding motif is numbered 1–8 on the top. Conserved residues are boxed in black at positions 1, 4, 7, and 8. Proline and basic residues are boxed in gray. Sequences marked by * contain putative motifs. UDG2, nuclear uracil DNA glycosylase; MCMT, methyl 5’- cytosine DNA methyltransferase. SpMYH, S. pombe MYH; h, human; XPG, X. pigmentosum group G protein.
show that hMYH binds PCNA and RPA through its C-terminal (residues 505–527) and N-terminal (residues 6–32) motifs, respectively.

**Inhibition of Formation of hMYH Complexes by Antibodies**—Zhang and coworkers (59) reported that the interaction between DNA polymerase δ and PCNA was inhibited by the presence of an anti-PCNA antibody directed toward residues 121–135 since these residues overlap the binding site of many PCNA-binding proteins. By using a similar approach, we showed that binding of hMYH to PCNA was inhibited by pre-incubation of PCNA with an anti-PCNA antibody directed toward residues 112–121 (Fig. 5A, lanes 3 and 4). Since some PCNA-binding proteins such as FEN1 and p21 have been shown to bind PCNA at the front of the PCNA trimer (60), we suggest that hMYH also binds to the same region of PCNA, a hydrophobic pocket. However, the association of APE1 and hMYH was not affected by the presence of the anti-APE1 peptide (residues 299–318) antibody, thus implying that this region at the C terminus of APE1 is not involved in the hMYH interaction (Fig. 5A, lanes 7 and 8).

To determine further which subunit of the trimeric RPA is involved in the interaction with hMYH, we preincubated RPA trimer separately with anti-RPA11, -RPA34, and -RPA70 antibodies and then assessed its binding to GST-hMYH. Incubation with the antibody against RPA34 (Fig. 5B, lanes 5 and 6) but not against RPA11 (Fig. 5B, lanes 3 and 4) or against RPA70 (Fig. 5B, lanes 7 and 8) decreased the binding of RPA to GST-hMYH. These data, showing that hMYH interacts with trimeric RPA through subunit RPA34, correlate well with the suggestion that the N terminus of hMYH contains a RPA-binding motif for RPA34 (Fig. 4 and Table III).

**DISCUSSION**

The human MYH is involved in immediate post-replicative removal of adenines or 2-hydroxyadenines misincorporated with template G and 8-oxoG bases (4). Its function in increasing fidelity of DNA replication is similar to that of UDG2 that removes misincorporated uracils during replication (36). Similar to the finding with UDG2 (36), we demonstrate here that hMYH physically interacts with PCNA and RPA as well as APE1 both in vivo and in vitro. However, binding of hMYH to DNA polβ and polδ was not detected. By using various GST constructs and peptide affinity assays, we defined the amino acid sequences responsible for the interaction of these three proteins with hMYH (Fig. 6). The APE1-binding site is at a region around amino acid residue 300, and the PCNA binding activity is located exclusively at the C terminus (residues 505–527), and RPA binds to the N terminus (residues 6–32) of hMYH. This is the first biochemical demonstration that hMYH interacts with proteins in the long patch BER pathway and thereby supports the hypothesis that it may exist as a multicomponent complex.

Residues 505–527 of hMYH contain the motif QXQXLXFF that is a well characterized PCNA-binding motif (32, 33) (Table II and Fig. 6). This PCNA-binding motif is also conserved in the corresponding MYH sequences from mouse and Schizosaccharomyces pombe (61) (Table II). Mouse and S. pombe MYH both contain the conserved leucine at position 4 as well as a phenylalanine at position 8. Although Chuang et al. (62) reported that the conserved glutamine is essential for the interaction between methyl-5'-cytosine DNA methyltransferase and PCNA, several PCNA-binding proteins lack this conserved glutamine (Table II). It has yet to be demonstrated whether the same interaction occurs in the yeast system. The PCNA sliding clamp also interacts with several mismatch repair proteins (65–67), and the
A conserved PCNA-binding motif can be found in the MSH3 and MSH6 sequences (Table II). It has been suggested that PCNA may act as a molecular adaptor, coordinating and regulating the actions of DNA replication, DNA repair, and cell cycle control. However, the mechanism by which PCNA selects the appropriate partners remains unclear. It has been reported that checkpoint proteins, Rad1, Rad9, and Hus1, are related to PCNA (68–70). It remains to be determined whether or not these PCNA-binding proteins bind to PCNA-related proteins.

Residues 6–32 of hMYH bind the 34-kDa subunit of trimeric RPA and share a common motif present in other RPA-binding proteins such as UDG2, XPA, and RAD52 (48, 58, 71) (Table III). Alignment of the p6 sequence with the N-terminal sequence of mouse MYH also suggests a putative RPA-binding site in mouse MYH (Table III). However, no RPA-binding motif was found in S. pombe MYH sequence. Several forms of hMYH have been reported (7, 54). The form of hMYH used in this study of protein-protein interactions is the type 1 (residues 1–535) as defined by Takao et al. (7) and as published by...

FIG. 5. Inhibition of hMYH binding to PCNA, APE1, and RPA. A, inhibition of hMYH interaction with PCNA and APE1 by anti-PCNA and anti-APE1 antibodies, respectively. PCNA (100 ng) was preincubated with 1 μg of anti-PCNA antibody (lanes 3 and 4) and APE1 (100 ng) with 1 μg of anti-APE1 antibody (lanes 7 and 8) before being added to GST-hMYH (300 ng) in the absence of the antibodies. The pull-down procedures are similar as described in Fig. 2A. B, hMYH binding to RPA is inhibited by the antibody raised against RPA34. Trimeric RPA (100 ng) was preincubated with 1 μg of antibody raised against RPA11 (the 11-kDa subunit, lanes 3 and 4), RPA34 (the 34-kDa subunit, lanes 5 and 6), or RPA70 (the 70-kDa subunit, lanes 7 and 8) for 4 h before being added to GST-hMYH (300 ng). For control, RPA (100 ng) was added to GST-hMYH (300 ng) in the absence of the antibodies (lanes 1 and 2). Western blot analyses against anti-RPA34 of the pellets (P) and a fraction (40%) of supernatants (S) were performed similarly as in Fig. 2A.

FIG. 6. Schematic summary of APE1, PCNA, and RPA-binding sites on hMYH. Conserved residues are boxed in black.

| Protein | Organism | Position | Motif | GenBank No. |
|---------|----------|----------|-------|-------------|
| hMYH    | H. sapiens | 8        | 123456789012345678901234 | U63329 |
| mMYH*   | M. musculus | 12       | KQPANHKRRTRALSSQAKPSLD | AA409964 |
| UDG2    | H. sapiens | 67       | SAEQLDRITQKAPALRRAAR | P13051 |
| UDG2*   | M. musculus | 60       | SAEQLDRITQKAPALRRAAR | AAF76936 |
| XPA     | H. sapiens | 23       | SVRASVRORQALRORALR | P23025 |
| XPA*    | M. musculus | 25       | AVRASVRORQALRORALR | S41948 |
| XPA*    | X. laevis | 22       | AVRATKRRORQALRORALR | P27088 |
| RAD52   | H. sapiens | 251      | SEATHORKRQOLQOFQPERMEK | NM002879 |

The 24-residue RPA binding motif is numbered on the top. Sequences are aligned according to the NMR structural basis (58) and the residues of human UDG2 involved in intermolecular NOE are boxed in black. Basic and aliphatic residues flanking the K/R-X-K/R sequence are boxed in gray. Sequences marked by * contain putative motifs. UDG2, nuclear uracil DNA glycosylase; XPA, Xeroderma pigmentosum group A protein.
Slupska et al. (5). Type 1 hMYH has been shown to be present in the mitochondria by Takao et al. (7, 54) but has been shown to localize to the nuclei by Tsai-Wu et al. (55). Takao et al. (7) reported that type 2 hMYH (residues 15–535) is the nuclear form. Our data showing that type 1 interacts with RPA in the HeLa nuclear extracts is consistent with the results of Tsai-Wu et al. (55) because only the type 1 contains the intact RPA-binding site.

The interaction of APE1 with hMYH requires residues 295–318 that share conserved residues with the binding site of DNA polymerase b, the only protein shown to interact with APE1 (30) (Table IV). However, direct binding of APE1 to the region consisting of residues 295–318 has not been demonstrated. The interaction of hMYH with APE1 appears to be independent of DNA, whereas interaction between pol β and APE1 requires bound DNA (30). The putative APE1-binding site contains a conserved motif SGXYDV where X and Y are any of the amino acids. Residues 1, 2, and 5 of this 6-amino acid motif are conserved, whereas position 6 may require a nonpolar aliphatic amino acid. It is also evident from the alignment that positively and negatively charged residues flank this putative APE1-binding site.

The interactions of hMYH with APE1, PCNA, and RPA suggest the hMYH repair pathway is a long patch BER. The successive players in this repair pathway may be assembled in a complex to perform concerted actions. Upon removal of the mismatched adenines, hMYH may recruit APE1 so that the cytotoxic and mutagenic AP site can be completely processed. After the incision by APE1, pol β and FEN1 may replace hMYH through their interaction with PCNA to synthesize and remove an oligonucleotide containing the AP site. Alternatively, APE1 may recruit DNA polymerase β to complete BER similar to the previous findings that APE1 displaces thymine- and uracil-DNA glycosylases from AP-containing DNA (27–29) and interacts with DNA polymerase β for repair synthesis (30). The docking of hMYH onto PCNA and RPA couples base excision repair to DNA replication. This may explain the paradox that hMYH can be directed to repair the misincorporated adenosines on the daughter strand but not the parental strand.

RPA is involved in the long patch but not the short patch BER. The interaction of RPA with hMYH, which is involved in the first step of BER to recognize the A/8-oxoG mismatch, may place RPA in a position to participate in the early and later stages of long patch BER. RPA is also involved in nucleotide excision and recombination repair (58). It has been suggested that RPA plays an architectural role in assembly of the repair complexes (58). A plausible theory is that PCNA-bound hMYH may recruit RPA so that factors essential for DNA repair and replication are localized to the site of DNA damage. The function of RPA in the hMYH repair pathway may be to protect the single-stranded DNA region and to stimulate the FEN1 activity (37). It is noteworthy that UDG2 interacts with PCNA and RPA, and these proteins colocalize to the replication foci where BER is initiated to repair misincorporated uracils. It will be interesting to show whether hMYH colocalizes to the replication foci with PCNA and RPA. Since protein-protein interactions can modulate the enzyme activities, e.g. PCNA can enhance the processivity of DNA polymerase δ and FEN1 activity (35, 72, 73), experiments to study the effects of APE, PCNA, and RPA on hMYH glycosylase activity are underway.

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| Protein | Organism | Position | Motif | GenBank No. |
|---------|----------|----------|-------|-------------|
| hMYH    | H. sapiens | 295      | 12345678901234567890123 | U63329 |
| mMYH    | M. musculus | 278      | 12345678901234567890123 | AA409964 |
| pol β   | H. sapiens | 178      | 12345678901234567890123 | P06746 |
| pol β   | R. norvegicus | 178     | 12345678901234567890123 | P06766 |
| pol β   | X. laevis   | 178      | 12345678901234567890123 | O57383 |
| pol β   | C. fasciculata | 185     | 12345678901234567890123 | Q23687 |
| pol β   | L. infantum | 185      | 12345678901234567890123 | Q9U6N3 |

The 23-residue APE1 binding motif is numbered at the top. Residues conserved with the hMYH sequence are boxed in black.
