Functional Interaction of MutY Homolog with Proliferating Cell Nuclear Antigen in Fission Yeast, Schizosaccharomyces pombe*

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The MutY homolog (MYH) is responsible for removing adenes misincorporated on a template DNA strand containing G or 7,8-dihydro-8-oxoguanine (8-oxoG) and thus preventing G:C to T:A mutations. Human MYH has been shown to interact physically with human proliferating cell nuclear antigen (hPCNA). Here, we report that a similar interaction between SpMYH and SpPCNA occurs in the fission yeast Schizosaccharomyces pombe.

Binding of SpMYH to SpPCNA was not observed when phenylalanine 444 in the PCNA binding motif of SpMYH was replaced with alanine. The F444A mutant of SpMYH expressed in yeast cells had normal adenine glycosylase and DNA binding activities. However, expression of this mutant form of SpMYH in a SpMYHΔ cell could not reduce the mutation frequency of the cell to the normal level. Moreover, SpMYH interacted with hPCNA, and SpPCNA interacted with hMYH but not with F518A/F519A mutant hMYH containing mutations in its PCNA binding motif. Although the SpMYHΔ cells expressing hMYH had partially reduced mutation frequency, the F518A/F519A mutant hMYH could not reduce the mutation frequency of SpMYHΔ cells. Thus, the interaction between SpMYH and SpPCNA is important for SpMYH biological function in mutation avoidance.

Oxidation damage to DNA can induce mutagenesis and lead to degenerative diseases. One of the most stable products of DNA damage resulting from reactive oxygen species is 7,8-dihydro-8-oxoguanine (8-oxoG) or GO. If not repaired, GO lesions in DNA can produce A/G mismatches during DNA replication (1) and result in G:C to T:A transversions (2–5). In Escherichia coli, MutT, MutM, and MutY have been identified in humans (15–21). These three enzymes (hMTH1, hOGG1, and hMYH), like their E. coli homologs, are proposed to function in the reduction of 8-oxoG in the human genome (22). Although no mutY homolog has been found in the budding yeast Saccharomyces cerevisiae, a mutY homologous (SpMYH) gene of fission yeast Schizosaccharomyces pombe was identified and cloned (23). The SpMYH gene encodes a 461-amino acid protein, which displays 28 and 31% identity to E. coli MutY and hMYH, respectively. Expression of SpMYH cDNA in an E. coli mutY mutant cell is able to reduce the mutation frequency. As with the E. coli MutY, the SpMYH protein has both adenine glycosylase and weak AP lyase activities on A/G and A/GO mismatches (23). An SpMYH knockout strain (SpMYHΔ) of S. pombe has been shown to be a mutator (24). Disruption of SpMYH also causes increased sensitivity to H2O2 but not to UV irradiation. Thus, MutY homolog plays an important role in defense against oxidative stress in eukaryotes.

We have shown that hMYH is directly associated with human apurinic/apyrimidinic endonuclease (hAPE1), proliferating cell nuclear antigen (hPCNA), and replication protein A (hRPA), suggesting that hMYH plays a role in the long patch base excision repair pathway (25). It has been suggested that hMYH repair is coupled to DNA replication through docking with hPCNA and hRPA (25, 26). The coupling to DNA replication may provide a signal to target the MYH repair to the daughter DNA strands. In such a model, MYH can remove adenes on the daughter strands mismatched with guanines or 8-oxoG as a result of DNA replication errors but cannot excise the adenes on the template strands. Here, we provide direct evidence that the interaction between SpMYH and SpPCNA of S. pombe is important for SpMYH biological function in mutation avoidance. A mutant form of SpMYH, which has normal glycosylase activity but cannot interact with SpPCNA, is partially defective in vivo. In addition, interactions between MYH and PCNA proteins from both S. pombe and humans are interchangeable. S. pombe will be an excellent model system to study mammalian MYH repair.

EXPERIMENTAL PROCEDURES

Construction of Mutant SpMYH, Wild-type hMYH, and Mutant hMYH in a Yeast Expression Vector—The cDNA of hMYH was cloned into the S. pombe expression vector pREP41X (American Type Culture Collection) by the PCR method. XHO-5-MYH and XMA-3-MYH were used as primers, and pET11a-hMYH (27) was used as the template to amplify the cDNA by Pfu DNA polymerase (Stratagene, La Jolla, CA) of hMYH gene. The cDNA of SpMYH containing the F444A mutation and cDNA of hMYH containing the double mutation F518A/F519A were constructed by the PCR method. 5′ Primer (XHO-5-SP) and mutation...
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**Table I**

| Name                  | Sequence                                                                 | Purpose                                    |
|-----------------------|--------------------------------------------------------------------------|--------------------------------------------|
| XHO-5-SP              | 5′-GCATCTGCTCAGGATGCAGATTCAAAATCAT-3′                                   | 5′ Primer SpMYH in pREP41X                 |
| F444A                 | 5′-GGGCTGAGCGATTATGCAAGTACTCTCTATTTTTC-3′                                | Mutant SpMYH in pREP41X                   |
| XHO-5-MYH             | 5′-GGATCGCTTGGAGATCCCGCAGGGTTGCTG-3′                                     | 5′ Primer hMYH in pREP41X                 |
| XMA-3-MYH             | 5′-TGTCGGTCCGCCGCTGCCAGGGCTTCTC-3′                                      | 3′ Primer hMYH in pREP41X                 |
| XMA-3-AA              | 5′-TGATCGGGCGGCTACTCGGCTGATCTGCTG-3′                                    | Mutant hMYH in pREP41X                    |
| PCNA-5-NDE            | 5′-AGATCTGACAGTTATGCAAGTACTCTCATTATTTTAC-3′                              | His-SpPCNA in pET21a and pET28b           |
| PCNA-5-ECO            | 5′-GGGCTGATATGCTCCATCACTTTCACCAATTTTAC-3′                                | His-SpPCNA in pET21a                      |
| PCNA-5-BAM            | 5′-GGGCTGATATGCTCCATCACTTTCACCAATTTTAC-3′                                | His-SpPCNA in pET28b                      |
| CHANG143              | 5′-TATTTAGGATAGTGGCGCCGGCTGTTG-3′                                       | Δ231-hMYH in pOX-4T-2                     |
| CHANG143              | 5′-TATTTAGGATAGTGGCGCCGGCTGTTG-3′                                       | Δ231-hMYH in pOX-4T-2                     |
| CHANG219              | 5′-GGGATATAGTCAGTGCCATTTACCATTTTAC-3′                                   | 5′ Primer SpMYH in pOX-4T-2               |
| CHANG220              | 5′-GGGATATAGTCAGTGCCATTTACCATTTTAC-3′                                   | 3′ Primer SpMYH in pET28b                 |
| CHANG220              | 5′-GGGATATAGTCAGTGCCATTTACCATTTTAC-3′                                   | MYH substrate                             |
| CHANG220              | 5′-GGGATATAGTCAGTGCCATTTACCATTTTAC-3′                                   | MYH substrate                             |
| CHANG220              | 5′-GGGATATAGTCAGTGCCATTTACCATTTTAC-3′                                   | MYH substrate                             |

* Mutated bases are italicized.  
° Represents 8-oxo-G.  
\[ \text{Mismatched base is underlined.} \]

SpPCNA protein was purified as a His-tagged protein from the BL21-star cell harboring plasmid pET28-SPCNA, and then the tag was removed similar to the procedures described above for SpMYH (F444A) mutant protein. Recombinant SpMYH expressed in E. coli was purified according to the procedures described by Lu and Fawcett (23). Human PCNA expressed in E. coli was from Dr. Mike O'Donnell (Rockefeller University and Howard Hughes Medical Institute).

**Nickel-agarose Affinity Binding**—The His-tagged SpPCNA from the BL21-star cell harboring plasmid pET21-SPCNA was bound to nickel-agarose (Qiagen Inc.) according to the manufacturer’s procedures. Purified SpMYH and SpMYH (F444A) (200 ng) expressed in E. coli were added to beads and incubated 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, the bound proteins were eluted by buffer N containing 250 mM imidazole. The unbound and eluting fractions were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The affinity-purified SpMYH polyclonal antibodies (28) were used for Western blotting analysis.

**GST-hMYH Pull-down Assay**—Expression, immobilization of GST-hMYH constructs, and GST-pull-down assay were similar to the procedures described previously (25). Purified hPCNA and SpPCNA (200 ng) expressed in E. coli were added to the GST-hMYH or GST-hMYH constructs (300 ng) immobilized on glutathione-Sepharose 4B (Amersham Biosciences, Inc.). The pellets (P) and supernatants (S, 30 µl) were fractionated on a 10% SDS-polyacrylamide gel, and Western blot analyses for hPCNA and SpPCNA were performed with antibody against hPCNA (Calbiochem-Novabiochem Corp.) (29). A control was run concurrently with immobilized GST alone.

**Assays of SpMYH Binding and Glycosylase Activities in Yeast Extracts**—The binding and glycosylase assays for SpMYH in yeast cell extracts with an A/8-oxoG-containing DNA were described previously (23, 24) except using 9 mM EDTA. The DNA substrate was a 20-mer duplex DNA containing an A/8-oxoG mismatch (see Table I) that was labeled at the 5′-end of the mismatched A-containing strand.

**Measurement of Mutation Frequency**—Five independent yeast colonies were grown to late log phase in Emm containing 0.1 mg/ml uracil. Additional amino acids were supplemented for the wild-type strain (0.1 mg/ml Leu and His) and the SpMYH strain (0.1 mg/ml Leu). Each culture (0.2 ml) was plated onto Emm agar plates containing 1 mg/ml 5-fluoro-orotic acid (FOA) and 0.1 mg/ml uracil. FOA-resistant colonies were counted after 5 days of growth. The cell titer was determined by plating 0.1 ml of a 10⁻⁴ dilution onto plates without FOA. The mutation frequency was calculated as the ratio of FOA-resistant cells to the total cells. The measurement was repeated more than three times.

**RESULTS AND DISCUSSION**

**SpMYH Physically Interacts with SpPCNA**—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 (30, 31). Generally, this motif contains a glutamine (Gln) at position 1, an aliphatic residue such as leucine (Leu), isoleucine (Ile), or methionine (Met) at position 4, and a pair of aromatic residues (Phe or Tyr) at

**Expression and Purification of the Recombinant Proteins**—CHANG219 and CHANG220 were used as primers, and pREPSPY-A was used as a template to amplify SpPCNA DNA containing the F444A mutation. The PCR product was cleaved by NdeI and BamHI and ligated into the NdeI-BamHI-digested vector. Two clones containing the SpPCNA gene (pET21-SPCNA and pET28-SPCNA) were confirmed by DNA sequencing.

**Expression and Purification of the Recombinant Proteins**—Expression, immobilization of GST-hMYH constructs, and GST-pull-down assay were similar to the procedures described previously (25). Purified hPCNA and SpPCNA (200 ng) expressed in E. coli were added to the GST-hMYH or GST-hMYH constructs (300 ng) immobilized on glutathione-Sepharose 4B (Amersham Biosciences, Inc.). The pellets (P) and supernatants (S, 30 µl) were fractionated on a 10% SDS-polyacrylamide gel, and Western blot analyses for hPCNA and SpPCNA were performed with antibody against hPCNA (Calbiochem-Novabiochem Corp.) (29). A control was run concurrently with immobilized GST alone.

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(A) PCNA binding motifs

| Protein | Organism | Position | Motif |
|---------|----------|----------|-------|
| hMYH    | H. sapiens | 509      |       |
| mMYH    | M. musculus | 490     |       |
| SpMYH   | S. pombe  | 454      |       |

(B) SpMYH binds to SpPCNA

| SpMYH | SpPCNA | BSA |
|-------|--------|-----|
| 1     | U      | B   |
| 2     | U      | U   |
| 3     | U      | B   |
| 4     | U      | U   |

![Figure 1](image.png)

The physical interactions between MYH and PCNA proteins from both S. pombe and humans are interchangeable. Because SpMYH and hMYH have different PCNA binding motifs (Fig. 1A), we speculate whether SpMYH can bind to hPCNA or SpPCNA can bind to hMYH. The affinity binding experiments with GST fusion proteins of SpMYH and hMYH were performed with purified hPCNA and SpPCNA, respectively. Human PCNA was detected in the GST-SpMYH pellets (Fig. 2A, lanes 1 and 2) but not in the GST beads (Fig. 2A, lanes 3 and 4). Conversely, SpPCNA was detected in the pellets of Δ231-hMYH fused to GST (Fig. 2B, lanes 1 and 2) but not in the GST beads (Fig. 2B, lanes 5 and 6). Resides Phe-518 and Phe-519 of hMYH have been identified as being essential for hPCNA binding (25). When both Phe-518 and Phe-519 of hMYH were mutated to Ala residues, GST-hMYH could not bind SpPCNA (Fig. 2B, lanes 3 and 4). Thus, the interactions between MYH and PCNA proteins from both S. pombe and humans are interchangeable.

The physical interactions between MYH and PCNA proteins from both S. pombe and humans are consistent with the high homologies of these proteins. The PCNA proteins from both organisms have 51% identity whereas SpMYH displays 31% identity to hMYH. The anti-hPCNA antibody directed toward residues 112-121 can also cross-react with SpPCNA (Fig. 2B). Several proteins have been shown to bind to a hydrophobic pocket consisting of the interdomain connector loop (residues 118-135) and loops on the C termini of the PCNA trimer (34, 35). A ClustalW alignment indicates that amino acid sequences in this pocket are very conserved between SpPCNA and hPCNA.

F444A Mutant of SpMYH Had Normal Adenine Glycosylase and DNA Binding Activities—The SpMYH protein is highly homologous to E. coli MutY and hMYH. The N-terminal domain of E. coli MutY has the catalytic activity (36-40); however, the C-terminal domain is important for GO recognition (36, 37, 40, 41). The PCNA binding motifs located at the C-terminal ends of eukaryotic MYH are not conserved in bacterial MutY proteins. To test that Phe-444 is not at the active site of SpMYH, mutant SpMYH (F444A) was generated and expressed in the SpMYHΔ yeast cell. Because wild-type and F444A mutant SpMYH from plasmids were expressed about 30-60-fold higher than that from chromosomal gene (Fig. 3, lanes 1, 3, and 5), different amounts of cell extracts were used in the SpMYH assays. When the extract from the SpMYH (F444A) mutant was assayed for SpMYH activities, the glycosylase (Fig. 4A, lane 4) and DNA binding (Fig. 4B, lane 4) activities of SpMYH with an A/GO-containing DNA substrate could be detected. The glycosylase activity of F444A mutant protein was similar to that of the expressed wild-type SpMYH (Fig. 4A, compare lanes 3 and 4) because the F444A mutant was slightly higher expressed in yeast cells than that of wild-type enzyme (Fig. 3, compare lanes 3 and 5). The substrate binding activity of expressed F444A mutant was slightly weaker than that of the expressed wild-type enzyme (Fig. 4B, compare lanes 3 and 4) but was higher than that of the SpMYH from the chromosomal gene (Fig. 4B, compare lanes 1 and 4). Thus, Phe-444 of SpMYH is not involved in the catalytic activity but is essential for binding to SpPCNA.

Expression of F444A Mutant of SpMYH in the SpMYHΔ Cells Is a Mutator—We have shown that a SpMYHΔ S. pombe strain, JSP303-7Y (SpMYHΔ), is a mutator (24). Expression of wild-type SpMYH in the SpMYHΔ can reduce the mutation frequency to the same level as wild-type cells. Because the nmt1 promoter controls the expression of SpMYH cDNA in...
Fig. 2. A, SpMYH physically interacts with hPCNA. GST-SPMYH (lanes 1 and 2) or GST alone (lanes 3 and 4) were immobilized on beads and bound to purified hPCNA. Western blot was probed with antibody against hPCNA. S, supernatant; P, pellet. B, hPCNA physically interacts with SpMYH but not with mutant hMYH(F518A/F519A). Lanes 1 and 2 used GST fusion protein containing residues 232–535 of hMYH, while lanes 3 and 4 used GST fusion protein containing mutant (M) Δ231-hMYH(F518A/F519A), and lanes 5 and 6 used GST alone. Western blotting to detect SpPCNA was performed with antibody against hPCNA due to their high homology. S, supernatant; P, pellet.

Fig. 3. Expression levels of SpMYH and hMYH in yeast cells. The S. pombe extracts (lanes 1–5) and purified SpMYH from E. coli (23) were fractionated by 10% SDS-polyacrylamide gel electrophoresis, and SpMYH protein was detected by Western blotting with polyclonal antibodies against purified SpMYH. Lane 1, extract of wild type JSP303 (WT, 30 μg of total protein); lane 2, SpMYH extract (Δ, 30 μg of total protein); lane 3, SpMYH complemented with wild-type SpMYH (Δ + WT) in media without thiamine (5 μg of total protein); lane 4, SpMYHΔ complemented with wild-type SpMYH in media containing 5 μg/ml thiamine (Δ + WT + t, 30 μg of total protein); lane 5, SpMYHΔ complemented with F444A mutant SpMYH (Δ + FA) in media without thiamine (5 μg of total protein). Lanes 6–8, 5.6, 11.2, and 56 ng of purified SpMYH from E. coli, respectively. The amounts of SpMYH in lanes 1, 3, and 5 were estimated as 0.1, 3, and 6 ng per μg of total protein. Extracts of S. pombe expressing wild-type or F518A/F519A mutant hMYH (lanes 9 and 10, respectively, 80 μg of total protein) and partially purified hMYH from E. coli (lane 11, about 60 ng of hMYH protein) (27) were fractionated by 8% SDS-polyacrylamide gel electrophoresis, and hMYH protein was detected by Western blotting with polyclonal antibodies (a516) against a peptide of hMYH (25). The amounts of hMYH in lanes 9 and 10 were estimated as 4 and 6 ng per μg of total protein.

Fig. 4. Mutant SpMYH(F444A) has normal glycosylase and DNA binding activities. A, DNA glycosylase activity of SpMYH with an A/GO-containing DNA. Lane 1, used wild type extracts (WT, 0.5 μg of protein); lane 2, used SpMYH knockout extracts (Δ, 0.5 μg of protein); lane 3, used extracts from SpMYHΔ expressing wild-type SpMYH (Δ + WT, 0.1 μg of protein); lane 4, used extracts from SpMYHΔ expressing mutant SpMYH(F444A) (Δ + FA, 0.1 μg of protein). The DNA samples were heated at 90°C for 2 min and analyzed on 14% polyacrylamide, 7 M urea sequencing gels. The gel was then autoradiographed. Arrows indicate the intact DNA substrate (I) and the cleaved DNA fragment (N). B, DNA binding assay of SpMYH with an A/GO-containing DNA. Reaction products after incubation with cell extracts (same order and amounts as in A) were fractionated on an 8% native polyacrylamide gel. The gel was then autoradiographed. Arrows indicate the free DNA substrate (F) and the protein-DNA complex (C). pREP41X, SpMYH protein expression can be regulated by varying concentrations of thiamine in the minimal medium. At 5 μg/ml thiamine, the expression of SpMYH is almost completely suppressed (24) (Fig. 3, lane 4). Therefore, cells growing in minimal media containing 5 μg/ml thiamine provide good controls for mutation frequency measurement. As shown in Table II, the SpMYHΔ cell expressing wild-type SpMYH protein in media without thiamine had a mutation frequency similar to that of the wild type (compare lines 1 and 3) although the expression level of SpMYH from plasmid is about 30-fold higher than that of the SpMYH from chromosomal gene (Fig. 3, lanes 1 and 3). It appears that the SpMYH protein amount from the chromosomal gene is sufficient to maintain the genome stability. When the expression of SpMYH was inhibited by 5 μg/ml thiamine, the cell’s mutation frequency was much higher than that of the wild type (Table II, compare lines 1 and 4).

To test whether interaction with SpPCNA is important for the SpMYH function in vivo, SpMYHΔ yeast cell expressing mutant SpMYH (F444A) was tested for mutation frequency. The expression level of F444A mutant protein in yeast cells was slightly higher than that of wild-type enzyme in the absence of thiamine (Fig. 3, compare lanes 3 and 5). The mutation frequencies of SpMYHΔ yeast cells expressing mutant SpMYH were about 25-fold higher than that of the wild type and was slightly lower than that of the parental SpMYHΔ strain (Table II, compare lines 5 and 6 with lines 1 and 2). Thus, the F444A mutant SpMYH could not complement the chromosomal SpMYH mutation. Therefore, a mutant SpMYH that retains substrate binding and glycosylase activities but cannot interact with SpPCNA is nearly defective on oxidative DNA repair. These results provide direct evidence that the interaction be-
between SpMYH and SpPCNA is important for SpMYH biological function in mutation avoidance.

As shown above, the physical interactions between both MYH and PCNA proteins from *S. pombe* and humans are interchangeable. We then expressed hMYH and mutant hMYH (F518A and F519A) in the SpMYHΔ yeast cell and tested their in *vivo* complementation activities. When wild-type hMYH was expressed in the SpMYHΔ cells, the mutation frequency was 8-fold higher than that of the wild type but was 5-fold lower than that of the parental SpMYHΔ strain (Table II, compare lines 7 and 8 with lines 1 and 2). Therefore, hMYH is partially functional in *S. pombe* cells. However, when the F518A/F519A mutant hMYH was expressed in the SpMYHΔ cells, the mutation frequency was the same as that of the parental SpMYHΔ strain (Table II, compare last two lines with line 2). As shown in the Western blot in Fig. 3, the expression levels of both wild-type and mutant hMYH (lanes 9 and 10) from the plasmids were similar. Additionally, the expression levels of hMYH and SpMYH in yeast cells were comparable when they were compared to known amounts of recombinant hMYH (Fig. 3, compare lanes 9 and 10 with lane 11) and SpMYH (Fig. 3, compare lanes 3 and 5 with lanes 6–8) expressed in *E. coli*, respectively. Therefore, partial rescue of the mutator phenotype of SpMYHΔ cells by wild-type hMYH is not due to the low expression levels of hMYH. It is possible that hMYH may not act as SpMYH in the yeast cells because hMYH does not interact well with other enzymes involved in the SpMYH repair pathway such as AP endonuclease or single-stranded DNA-binding protein. It has been shown that mouse MYH activity can be stimulated by human AP endonuclease (32) and that hMYH physically interacts with hRPA (25).

It has been suggested that hMYH base excision repair is coupled to DNA replication through docking with hPCNA and hRPA (25, 26). PCNA may be important to position MYH protein to discriminate between the parental and daughter strands. In such mechanism, MYH excises misinserted A from template GO but does not act on template A when the DNA polymerase misinserts GO. However, some in vitro MYH function is retained in the F444A mutant SpMYH. Thus, the role of PCNA is not absolutely essential. This partial retention of the function of mutant SpMYH(F444A) may reflect that the mutant protein can still act on A/G mismatches on daughter strands through interaction with other replication proteins such as RPA. It is also possible that DNA polymerase has a higher frequency of inserting A on the GO template than inserting GO on the A template.

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