Interaction of Checkpoint Proteins Hus1/Rad1/Rad9 with DNA Base Excision Repair Enzyme MutY Homolog in Fission Yeast, Schizosaccharomyces pombe*

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The DNA glycosylase MutY homolog (MYH) is responsible for removing adenines misincorporated opposite DNA strands containing guanine or 7,8-dihydro-8-oxo-guanine by base excision repair thereby preventing G:C to TA mutations. MYH has been shown to interact with the proliferating cell nuclear antigen (PCNA) in both human and fission yeast Schizosaccharomyces pombe systems. Here we show that S. pombe (Sp) MYH physically interacts with all subunits of the PCNA-like checkpoint protein heterotrimer, SpRad9/SpRad1/SpHus1, in yeast extracts and when the individual subunits are expressed in bacteria. The SpHus1 and SpPCNA binding sites are located in discrete regions of SpMYH. Immunoprecipitation assays reveal that the interaction between SpHus1 and SpMYH increases dramatically after hydrogen peroxide treatment, and this increase in the SpHus1-SpMYH interaction correlates with the presence of SpHus1 phosphorylation. In contrast, the interaction between SpPCNA and SpMYH after hydrogen peroxide treatment remains nearly unchanged. SpMYH associates with SpHus1 in a complex of ~450 kDa, the reported native molecular mass of the SpRad9/SpRad1/SpHus1-MYC complex. A larger portion of SpMYH shifts to the 150–500-kDa regions after hydrogen peroxide treatment in comparison with untreated extracts. SpHus1 phosphorylation is substantially reduced in SpMYH cells after hydrogen peroxide treatment. These data suggest that MYH may act as an adaptor to recruit checkpoint proteins to the DNA lesions.

Cell cycle checkpoints are surveillance mechanisms that monitor chromosome structure and coordinate DNA repair during cell cycle progression (1–3). Defects in proper checkpoint controls are associated with a number of human pathologies (4, 5). The checkpoint controls are highly conserved from yeast to humans. In Schizosaccharomyces pombe, six genes known as the rad checkpoint genes (rad9, rad1, hus1, rad17, rad3, and rad26), are required for proper functioning of the checkpoints and DNA replication under stress (6). Each of these checkpoint genes has a mammalian homolog. It has been reported that Rad9, Rad1, and Hus1 form a heterotrimer complex (referred to as the proliferating cell nuclear antigen (PCNA)1-like 9-1-1 complex) and are related in structure to the PCNA sliding clamp (6–8). The S. pombe (Sp) Rad17 protein is homologous to the largest subunit of replication factor C, the clamp loader. SpRad3, a phosphatidylinositol 3-kinase-related protein, is homologous to human ATM (ataxia telangiectasia-mutated protein) and ATR (ATM- and Rad3-related protein) kinases. SpRad3 plays a central role in cell cycle checkpoint regulation. It acts to initiate cell cycle arrest by transducing the DNA damage signal through phosphorylation of SpChk1 and SpCds1 kinases in a SpRad9/SpRad1/Hus1 and SpRad17-dependent manner. SpHus1 is also phosphorylated by SpRad3 in response to DNA damage (9). The SpRad26 protein is the homolog of human ATR-interacting protein and forms a complex with SpRad3.

Because myriad forms of DNA lesions are caused by endogenous and environmental factors, the mode through which checkpoint proteins recognize these various forms of DNA damage is still poorly understood. Human ATM/human ATR/SpRad3 and Rad17 are proposed to act at an early step to sense DNA damage (10). It has been suggested that these checkpoint proteins may detect a common intermediate, such as single-stranded DNA coated by replication protein A, which is processed by various DNA repair pathways (11), or they may require a series of “adaptors” to recognize DNA damage. Potential candidates for such adaptor proteins are the DNA damage recognition proteins. Recently, a few DNA damage recognition proteins involved in mismatch repair, nucleotide excision repair, and double-strand break repair have been shown to interact with checkpoint proteins (12–15). These reports support a hypothesis that DNA repair proteins recognize the lesions and provide signals for checkpoint factors. In this work, we investigate the possible association of S. pombe checkpoint proteins with the base excision repair protein MutY homolog (MYH).

Cells possess several DNA repair pathways for dealing with the many different types of DNA lesions. Reactive oxygen species are the most prevalent source of DNA lesions in aerobic organisms. Oxidative damage to DNA can result in mutagenesis, in some cases leading 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), which causes G:C to A:T transversions. Although the eukaryotic mis-

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1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; 9-1-1 complex, Rad9, Rad1, and Hus1 heterotrimer complex; ATM, ataxia telangiectasia-mutated protein; ATR, ATM- and Rad3-related protein; GO or 8-oxoG, 7,8-dihydro-8-oxo-guanine; GST, glutathione S-transferase; h, human; HA, hemagglutinin; MSH, MutS homolog; OGG1, 8-oxoG glycosylase; MYH, MutY homolog; PBS, phosphate-buffered saline; Sc, S. cerevisiae; Sp, S. pombe.
match repair protein MSH2/MSH6 (MutS) heterodimer can recognize A/GO mismatches (16, 17), the GO lesions in DNA are repaired mainly by base excision repair pathways. The 8-oxoG glycosylase (OGG1) protein, a functional eukaryotic 8-oxoG glycosylase (OGG1) protein, a functional eukaryotic  

| Name        | Sequence                                                                 | Purpose                        |
|-------------|---------------------------------------------------------------------------|--------------------------------|
| Leu-5-Bgl   | 5'-ATGTAGACGTTACCAGGATGCAATCTAGGGAAACAA-3'                               | 5' primer of leu2              |
| Leu-3-Anti  | 5'-ATCCAGGACGTGACCGTACTTCAAGCTGCTGCACATC-3'                               | 3' primer of leu2              |
| MYH-Xho-5   | 5'-ACGTACAGAGATGCGATATCTACAGGAAATGC-3'                                    | 5' primer of SpMYH in pSCF172  |
| MYH-Bam-3   | 5'-GTCTGCTCGAGATGTCGGATTCAAATCATTCT-3'                                     | 3' primer of SpMYH in pSCF172  |
| Hus-5-Xho   | 5'-CATGGCTCAGATGGAATTCACTTAAACACAGTTA-3'                                   | 5' primer of SpRad1 in pSCF172 |
| Hus-5-HA    | 5'-CATGGCTCAGATGGAATTCACTTAAACACAGTTA-3'                                   | 3' primer of SpRad1 in pET21a  |
| Rad1-5-Xho  | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| Radl-3-HA   | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| Rad-5-Nde   | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| Hus-3-Hind  | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| RAD1-5-NDE  | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| RAD1-3-BAM  | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| Rad-5-Nhe   | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| Rad-3-PET   | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| BAM-5-SP    | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| SP-3-443    | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| SP-3-230    | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| BAM-5-245   | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |
| CHANG220    | 5'-GTCTGCGATGGAAGTAAATCTGATAGCAAGC-3'                                     | 3' primer of SpRad1 in pET21a  |

**TABLE I**

**Oligonucleotides used**

*Yeast Cells—Fission yeast S. pombe strains hus1-MYC (501 h- leu1-32, ura4-D18, ade6-706, his1-MYC, with the SpMYH gene tagged with 13 MYC epitopes inserted at its C terminus) (6) and Δhus1 (h- hus1+: leu1-32 ura4-D18) were obtained from Dr. Antony Carr (Medical Research Council Cell Mutation Unit, UK) through Dr. Teresa Wang (Stanford University). S. pombe 201402 (h- ura4-D18 leu1-31 ade6-M210 can1-1) was purchased from ATCC.

**Construction of hus1-MYC/SpMYH**—The his3 gene in the plasmid pSMYH19 containing the SpMYH:his3 insert (pSMYH-his) (23) was replaced by leu2 gene. The leu2 gene was amplified by PCR from S. pombe, and cloned into pET21a (Stratagene, La Jolla, CA) primers listed on Table I. The PCR product was purified, digested with BglII, and inserted into the BglII-cleaved plasmid pSMYH-his. The plasmid with SpMYH:leu2 was amplified in bacterial cells, linearized with restriction enzyme AatII, and then transfected into S. pombe hus1-MYC (501 h- leu1-32, ura4-D18, ade6-706, his1-MYC) by electroporation. The yeast genomic SpMYH was replaced with leu2 interrupted SpMYH cDNA (SpMYH:leu2) by homologous recombination. The transformed cells with leu+ phenotype were selected on the YNB plates (0.67% yeast nitrogen base without amino acids, 2% glucose, 1.5% agar) supplemented with 0.1 mg/ml adenine and 0.1 mg/ml of uracil. An interruption of SpMYH gene in the yeast chromosome was verified by PCR.

**Transformation of hus1-MYC/SpMYH Δ Cells with Wild-Type SpMYH cDNA**—The entire open reading frame of SpMYH cDNA from pSMYH19 (22) was amplified by PCR with primers listed on Table I. The product was digested with XhoI and BamHI and then inserted into pSCF172 (American Type Cell Culture). The obtained plasmid pSCF172-SpMYH was transformed into hus1-MYC/SpMYH Δ cells by electroporation. Transformed cells were selected with ura+ phenotype on the YNB agar plates supplemented with adenine (33). The expression of SpMYH protein was confirmed by Western blotting analysis with polyclonal antibodies against SpMYH.

**Construction of Hemagglutinin (HA)- and His-tagged SpHus1, SpRadl, SpRad9 Fusion Proteins**—The cDNA of SpHus1, SpRad1, and SpRad9 were amplified by PCR from an S. pombe cDNA library in pGADGH (kindly provided by D. Beach, Cold Spring Harbor Laboratory) using FsuI DNA polymerase (Stratagene) and the appropriate primers (listed in Table I). To construct HA-tagged proteins, the SpHus1 and SpRad1 cDNA were amplified with the primer pairs Hus-5-Xho/Hus-3-HA and Radl-5-Xho/Rad1-3-HA, respectively. The PCR products were digested with XhoI and BamHI, cloned into pSP172, and transformed into S. pombe 201402 by electroporation. Transformed cells with the ura+ phenotype were selected on Edinburgh minimal medium plates supplemented with 0.1 mg/ml leucine and adenine (33). To produce His-tagged proteins, SpHus1, SpRad1, and SpRad9 cDNA were cloned into pET21a using the primer pairs Hus-5-Nde/Hus-3-Hind, Rad1-5-NDE/RAD1-3-BAM, and Rad9-5-Nhe/Rad9-3-PET, respectively. The PCR products were digested with appropriate restriction enzymes, cloned into pET21a, transformed into E. coli BL21-Sr cells (Invitrogen), and selected via ampicillin resistance. All clones were confirmed by DNA sequencing.

**Construction of Truncated Glutathione S-Transferase (GST)-SpMYH Proteins**—The plasmid pGEXSMYH expressing full-length SpMYH tagged with GST has been previously described (24). The truncated SpMYH cDNA fragments were PCR-amplified using appropriate primers (listed in Table I) from the template pGEXSMYH and were ligated
into pGEX-4T-2 at the BamHI site and transformed into E. coli BL21-Star cells (Invitrogen). The sequences of the cloned genes were confirmed through DNA sequencing.

**GST pull-down—**GST pull-down assays were performed in a manner similar to previously described procedures (25). E. coli (BL21StarDE3) cells (Stratagene, La Jolla, CA) harboring the expression plasmids were cultured in Luria-Bertani broth containing 100 mg/ml ampicillin at 25 °C. Protein expression was induced by an A_{sub} of 0.6 by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.4 mm, and the cells were harvested 16 h later by centrifugation at 10,000 × g for 20 min. The cell paste from a 1-liter culture was resuspended in 10 ml of phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_{2}HPO_{4}, and 1.4 mM KH_{2}PO_{4}). After sonication, the solution was centrifuged at 10,000 × g for 20 min. To the supernatant (10 ml), 1 ml of a 50% slurry of glutathione-Sepharose 4B (Amersham Bioscience) in PBS was added and incubated for 2 h at 4 °C. The GST fusion protein-bound to the beads were pelleted at 1000 × g for 5 min and then washed 5 times with 5 ml of PBS. The beads were suspended in PBS containing 0.1% sodium azide and 0.1% of a protease inhibitor mixture (Sigma-Aldrich) to form a 50% slurry and stored at 4 °C. GST constructs (300 ng) immobilized on glutathione-Sepharose 4B were incubated with 5% bovine serum albumin in PBS in 1 h at 4 °C, washed with PBS, then incubated with 500 μg of yeast extracts prepared as described by Chang et al. (25) overnight in 200 μl of PBS at 4 °C. After centrifugation at 1000 × g for 2 min, the supernatant was saved, and the pellets were washed 5 times with 1 ml of PBS. The pellets and supernatants were fractionated on a 10% SDS-polyacrylamide gel followed by Western blot analysis (34). A control was run concurrently with immobilized GST alone.

**Nickel-agarose Affinity Binding—**The His-tagged SpHus1 expressed in E. coli (BL21StarDE3) cells was bound to nickel-agarose (Qiagen Inc., Valencia, CA) according to the manufacturer’s procedures. Purified SpMYH (200 ng) expressed in E. coli (BL21) 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, the bound proteins were eluted by buffer N containing 250 mM imidazole. The unbound and eluting fractions were fractionated on a 10% SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Affinity-purified SpMYH polyclonal antibodies (35) were used for Western blotting analysis.

**Treatment of S. pombe with H_{2}O_{2}—**Fission yeast (hus1-MYC) and (SpMYH/Hus1-MYC) cells (250 ml) were grown in YEPD medium (20 g of pepton, 10 g of yeast extract, 20 g of dextrose/liter) to an optical density (OD) of 0.6. Hydrogen peroxide was added to the culture at a final concentration of 6 mM. After shaking at 30 °C for 1 h, cells were spun down and transferred to fresh YEPD medium without hydrogen peroxide. After various recovery time intervals, cells were harvested and resuspended in PBS for protein extraction via sonication.

**λ-Phosphatase Treatment—**For λ phosphatase treatment, 10 μg of yeast (hus1-MYC) cell extract was added to a reaction mixture containing 1 μg of λ-poly(A)5′-dithiothreitol, 2 μM MnCl_{2}, 0.01% Brij35. The dephosphorylation reaction was carried out with 2 μl (800 units) of λ-phosphatase (New England Biolabs, Beverly, MA) at 30 °C for 1 h. Dephosphorylation of SpHus1 protein was determined by Western blot analysis using a c-Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Co-immunoprecipitation—**Extracts of yeast (hus1-MYC) in PBS containing Sigma protease inhibitor mixture were prepared as described by Chang et al. (25) and preclarified by the addition of 30 μl of protein A-Sepharose (Amersham Biosciences) for 1–4 h at 4 °C. After centrifugation at 1000 × g for 2 min, the supernatant was incubated with 1 μg of anti-SpMYH antibody (35) overnight at 4 °C. Protein A-Sepharose (30 μl) was added and incubated for 4–12 h at 4 °C. After centrifugation, the supernatant was saved, and the pellet was washed 5 times with 800 μl of PBS. The pellet was then reconstituted in 1× SDS loading buffer (30 mM Tris-HCl, pH 6.8, 5% (v/v) glycerol, 1% (w/v) SDS, 0.5 mM b-mercaptoethanol). The pellet fractions were resolved on a 10% polyacrylamide gel containing SDS and transferred to a nitrocellulose membrane. Western blot analysis was performed using an antibody against the HA peptide. As shown in Fig. 1 A, SpHus1 and GST-tagged intact SpMYH were detected, whereas GST alone was not.

**Size Fractionation—**Yeast cells were grown to log phase in 1 liter of YEPD medium. Hydrogen peroxide treatment was performed as described above followed by cell recovery for 2 h in fresh YEPD medium lacking hydrogen peroxide. After being harvested by centrifugation, cells were resuspended in 10 ml of PBS and sonicated for 6 cycles of 10 s sonication followed by 20 s of rest. After centrifuging at 10,000 rpm in a SS34 rotor for 30 min at 4 °C, the supernatant (approximate 15 ml) was treated with 65% ammonium sulfate. The protein precipitant was resuspended in buffer S (20 mM potassium phosphate, pH 7.4, 150 mM KCl, 0.1% IGEFA-C AL-630 (Nonidet P-40), 0.5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and dialyzed against the same buffer for 3 h. The dialyzed protein sample was centrifuged, and the supernatant (1 mg of protein in 0.5 ml) was loaded onto a 24-mL Superose 12 HR 10/30 column (Amersham Biosciences) that had been equilibrated with buffer S. The flow rate was set to 0.25 ml per min, and 0.25-ml fractions were collected. The column was calibrated using size markers (blue dextran, thyroglobin, apoferritin, β-amyelase, bovine serum albumin, oval albumin, and carbonic anhydrase) from SigmaAldrich.

**RESULTS**

**Physical Interaction between SpMYH and SpHus1 in S. pombe—**The PCNA sliding clamp has been shown to interact with many proteins containing a common motif (36, 37). We have previously shown that SpPCNA physically interacts with the C terminus of SpMYH (24). In light of the observation that the Hus1/Rad1/Rad9 heterotrimer is structurally related to the Hus1/Rad1/Rad9 heterotrimer (6–8, 38–40), we tested whether SpMYH is able to interact with SpHus1. The physical interaction between SpHus1 and SpMYH was demonstrated using a GST pull-down assay. GST-SpMYH fusion protein bound to glutathione-Sepharose was incubated with extracts derived from fission yeast cells expressing HA-tagged SpHus1. Detection of bound SpHus1 was performed via Western blot analysis using an antibody against the HA peptide. As shown in Fig. 1 A, SpHus1 could be pulled down by GST-SpMYH (lane 4) but not by GST alone (lane 2). To determine a direct interaction between SpHus1 and SpMYH, His-tagged SpHus1, expressed in E. coli, was bound to Ni^{2+}-agarose and incubated with purified SpMYH protein. SpMYH was found to bind to Ni^{2+}-agarose-bound SpHus1 but not to Ni^{2+}-agarose beads associ-
Experimental procedures were similar to those described for their ability to bind the GST-tagged C-terminal domain of SpRad1 and SpRad9 expressed in S. pombe. Protein extracts were prepared from yeast cells expressing HA-tagged SpHus1. SpRad1 and SpRad9 were shown to bind GST-SpMYH but not to GST alone (Fig. 2, B and C). The binding affinities of SpMYH to SpHus1, SpRad1, and SpRad9 are weak; a maximal 5% of input proteins were pulled down (Figs. 1A and 2A). Thus, all three individual PCNA-related checkpoint proteins can directly interact with SpMYH and do so in the absence of the other two partners.

**Oxidative Stress Enhances SpMYH-SpHus1 Interaction and Induces SpHus1 Phosphorylation—**Because SpMYH is involved in repair of oxidative damage, we tested whether the SpHus1-SpMYH interaction was altered after hydrogen peroxide-induced oxidative stress. For this in vivo interaction, we used S. pombe hus1-MYC in which the SpHus1 is C-terminally tagged with 13 MYC epitopes (6). Like wild type cells, hus1-MYC cells arrest progression through cell cycle upon ionizing radiation (6). Cells were grown to log phase, treated with hydrogen peroxide for 1 h, and then cultured in media lacking hydrogen peroxide at various time intervals. Protein extracts from hydrogen peroxide-treated cells were immunoprecipitated using antibodies directed against SpMYH, and the immunoblot was detected with c-Myc antibody. In untreated cells, the SpMYH-SpHus1 interaction could be detected; however, it was weak (Fig. 3A, lane 1). The amount of SpHus1 bound to SpMYH increased slightly immediately after hydrogen peroxide treatment (Fig. 3A, lane 2). When treated cells were allowed to recover in fresh media for 2 h, the amount of SpHus1 precipitated by SpMYH antibody increased dramatically (Fig. 3A, lane 3). The levels of SpHus1 in the immunoprecipitant peaked at 2 h and then decreased after 6 h of recovery. A quantitative analysis of the Western blot showed 16-, 12-, and 6-fold increases of SpMYH-SpHus1 interactions after 2, 4, and 6 h of recovery over untreated cells, respectively. In contrast, the interaction between SpPCNA and SpMYH in the H2O2-treated cells remained almost unchanged, as assayed by co-immunoprecipitation (Fig. 3B). There was a slight decrease of SpMYH-SpPCNA interaction at 6 h after hydrogen peroxide treatment (Fig. 3B, lane 5). Therefore, the SpMYH-SpHus1 interaction is altered, but the SpMYH-SpPCNA interaction remains almost constant in fission yeast cells under oxidative stress.

We investigated whether the increased interaction between SpMYH-SpHus1 is due to differences in protein expression levels. Thus, we determined the total protein levels of SpMYH, SpHus1, and SpPCNA in cell extracts directly through Western blotting. The protein levels of SpMYH decreased slightly, whereas those of SpPCNA did not change in response to hydrogen peroxide treatment (Fig. 3, C and D). As shown in Fig. 3E, SpHus1 protein levels were unchanged, but a minor band with slightly lower mobility was observed in H2O2-treated extracts. This upper band could be observed at 1 h after hydrogen peroxide treatment (Fig. 3E, lane 2), was maximal at 2 h of recovery (lane 2), and remained after 6 h of recovery (lane 5). The upper SpHus1 band is the phosphorylated form, as evidenced by conversion of this band to a lower band after alkaline phosphatase treatment (Fig. 3F, lane 2). SpHus1 has been shown to be phosphorylated by SpRad3 in response to hydroxyurea replication block and bleomycin treatment (9). Our result demonstrates that SpHus1 also becomes phosphorylated in response to oxidative stress. Interestingly, the presence of SpHus1 phosphorylation correlated with the increase in SpHus1-SpMYH interaction after hydrogen peroxide treatment. However, the phosphorylation of SpHus1 was not necessary for SpMYH-SpHus1 interaction, since both phosphorylated and non-phosphorylated SpHus1 could be immunoprecipitated by SpMYH antibodies (Fig. 3A, lane 3).

**Redistribution of SpMYH in H2O2-treated Extracts and in hus1-deleted Cell Extracts—**Previous studies indicate that SpMYH associates with checkpoint proteins Hus1/Rad1/Rad9 in yeast extracts. SpRad1 binds mainly to the C-terminal region of SpMYH. Cell extracts of S. pombe expressing HA-Rad1 were used to bind GST-SpMYH (residues 1–461, G-MYH, lanes 1 and 2), the GST-N-terminal half of SpMYH (residues 1–230, G-N230, lanes 3 and 4), the GST-C-terminal domain of SpMYH (residues 245–461, G-C245, lanes 5 and 6), or GST alone (lanes 7 and 8) immobilized on agarose. Western blot analysis was performed with the antibody against HA. B, GST-SpMYH binds to His-SpRad1 expressed in bacteria. E. coli extracts expressing His-tagged SpRad1 were used to bind the GST-C-terminal domain of SpMYH (residues 245–461, G-C245, lanes 1 and 2) or GST alone (lanes 3 and 4). Western blot analysis was performed with an antibody against the His tag. C, GST-SpMYH binds to His-SpRad9 expressed in bacteria. The experimental procedures were similar to B. S, supernatant; P, pellets.
SpMYH Associates with Checkpoint Proteins Hus1/Rad1/Rad9

SpHus1-MYC (65 kDa), SpRad1(35 kDa), and SpRad9 (50 kDa) form a single complex with a native molecular mass (Mr) of ~450 (6). The size of this complex is larger than the expected trimeric size of ~150 kDa, suggesting the complex may contain other proteins. However, Caspari et al. reported that SpRad1 and SpRad9 did not co-elute with this 450-kDa complex; SpRad9 eluted at peak of 150 kDa, whereas SpRad1 eluted as two peaks of 200 and 70 kDa on a Superdex 200 column (6). To test whether SpMYH and SpHus1 co-elute on gel filtration chromatography, we loaded yeast extracts from the hus1-MYC strain on a Superose 12 column and then performed Western blotting with the respective antibodies. The majority of SpHus1-MYC eluted as two peaks on this column; peak I of larger than 670 kDa (fractions 32–34) and peak II of ~450 kDa (fractions 40–44) (Fig. 4 A and B, and Fig. 5A). Peak I eluted in the void volume, where blue dextran and thyroglobin also co-eluted. The 450-kDa peak II of SpHus1-MYC is similar to that reported by Caspari et al. (6); however, peak I was not observed by the same authors. The elution profiles of SpHus1 were similar for H2O2-treated and untreated cell extracts. This is consistent with the findings of Caspari et al. (6) that the elution profile of SpHus1 does not change after ionizing radiation or hydroxyurea treatment. The phosphorylated SpHus1 form was not readily detected in these fractions from H2O2-treated extracts due to poor gel resolution.

Three peaks of SpMYH in the H2O2-untreated cell extract eluted from the Superose 12 column; peak I consisted of larger than 670-kDa (fractions 30–34 in the void volume), peak II consisted of 150-kDa (fractions 46–50), and peak III consisted of less than 50 kDa (fractions 54–64) (Fig. 4C and Fig. 5B, open circles). The purified SpMYH (calculated Mr of 51) expressed in bacteria eluted from the same column at a position of ~45 kDa (data not shown). Thus, peak III from yeast extract represents the native monomeric SpMYH. This suggests that peaks I and II of SpMYH may be associated with other proteins. The elution profiles of SpHus1 (Fig. 5A) and SpMYH (Fig. 5B) do not coincide, indicating they do not exist as a strong complex. SpMYH in the H2O2-treated cell extract also eluted from the Superose 12 column as three peaks (Fig. 4D and Fig. 5B, filled diamonds). However, the distribution of the three peaks was different from that of SpMYH in the H2O2-untreated cell extract. An increase in peak II accompanied by a decrease of peak III was observed for SpMYH in the H2O2-treated cell extract (Fig. 5B, filled diamonds). There is also an increase of SpMYH at fractions 38–44 that corresponds to molecular mass of 400–500 kDa. These elution profiles were reproducible in two independent experiments. Thus, the portion of SpMYH (Peak II) co-eluting with the SpHus1 increases as a result of H2O2 treatment (compare Fig. 5, A and B, filled diamonds).

To study the association of SpMYH and SpHus1, we then performed coimmunoprecipitation utilizing the SpMYH antibody to precipitate SpHus1 from the Superose 12 fractions. In the H2O2-untreated cell extract, SpHus1 precipitated by the SpMYH antibody mainly distributed in fractions 37–43 (Figs. 4E and 5C, open circles). Fractions 37–43 contain peak II of SpHus1 and correspond to a Mr of ~400–600, which has been previously reported as the native Mr of the 9-1-1 complex (6). However, the precipitable SpHus1 peaking at fraction 39 does not coincide to peak II of SpHus1. Using the H2O2-treated cell extract, the pattern of SpHus1 coimmunoprecipitated by SpMYH antibody differed from that of the untreated cell extract; more SpHus1 was detected in fraction 31 and fractions 45–51 (com-
pare Figs. 4, E, and F, and Fig. 5C, filled diamonds). The distribution pattern of the precipitable SpHus1 (Fig. 5C, filled diamonds) is very similar to the pattern seen in the Western blot of SpHus1 (Fig. 5A, filled diamonds). This suggests that the redistribution of SpMYH in H$_2$O$_2$-treated cells (Fig. 5B) may be due to the increased SpMYH-SpHus1 interaction.

To analyze the influence of SpHus1 on SpMYH association with other proteins, extracts were prepared from hus1-deleted cells and subjected to gel filtration chromatography. As can be seen in Figs. 4, G and H, and 5D, the majority of SpMYH eluted in the void volume from the Superose 12 column (fractions 30–36, M$_r$ larger than 670), and the profiles of SpMYH distribution are very similar for those of H$_2$O$_2$-treated and untreated cell extracts.

SpHus1 Phosphorylation Is Dependent on SpMYH Expression after Hydrogen Peroxide Treatment—Kostrub et al. (9) reported that SpHus1 is phosphorylated by SpRad3 in response to DNA damage. To test a model that SpMYH senses DNA damage and activates cell cycle checkpoint pathways after oxidative stress, we measured the phosphorylation level of SpHus1 in H$_2$O$_2$-treated SpMYH$^+$ cells. We noted a 10-fold reduction of SpHus1 phosphorylation level in SpMYH$^+$/hus1-MYC cells as compared with SpMYH-proficient cells after H$_2$O$_2$-induced oxidative stress and recovery for 2 h (Fig. 6B, lanes 1 and 2, Fig. 6C). To further investigate the correlation of SpMYH expression levels and SpHus1 phosphorylation, an expression vector pSCF172 containing SpMYH cDNA was incorporated into the SpMYH$^+$/hus1-MYC cells. The expression of the SpMYH protein in the transformed cells was detected by Western analysis (Fig. 6A, lanes 3–7). Because the nmt1 promoter controls the expression of SpMYH cDNA in pSCF172, SpMYH protein expression can be regulated by the concentration of thiamine in the minimal medium. As shown in Fig. 6A, lanes 3 and 4, when cells containing plasmid-borne SpMYH cDNA were cultured in medium with low concentrations of thiamine, the expression of SpMYH was fully induced. At 5
μg/ml thiamine, the expression of SpMYH was almost completely suppressed (Fig. 6A, lane 7). As shown in Fig. 6, the percentages of SpHus1 phosphorylation are correlated with the SpMYH expression levels. These results clearly indicate that phosphorylation of SpHus1 in response to oxidative stress is dependent on SpMYH.
DISCUSSION

DNA base lesions induced by oxidative damage are repaired mainly by base excision repair pathways. In light of an absence of OGG1 in *S. pombe*, SpMYH may be the important player in reduction of GO mutagenesis. We have shown that SpMYH-deficient fission yeast has an elevated mutation frequency (23). The most important known biological function of SpMYH is the recognition of A/GO mismatches by removing misincorporated A from the template GO. The high affinity SpMYH for its reaction product apurinic/apyrimidinic/GO may extend to G/GO, T/GO, and C/GO mismatches, similar to that seen for *E. coli* MutY (41). It has been suggested that the base excision repair pathway may involve highly coordinated processes governed by protein-protein and protein-DNA interactions, possibly involving the transient “handing off” of intermediates (42–44). Human MYH has been shown to interact with apurinic/apyrimidinic endonuclease, PCNA, replication protein A, and MSH6 (25, 27). We have shown that SpMYH can interact with SpPCNA and hPCNA (24, 25). The interaction of SpMYH with SpPCNA is important for the ability of SpMYH to repair oxidative DNA damages (24). In this study we show that three fission yeast PCNA-related checkpoint proteins (SpHus1, SpRad1, and SpRad9) can also interact with SpMYH through GST pull-down and co-immunoprecipitation assays and that SpMYH interaction with recombinant SpHus1, SpRad1, and SpRad9 occurs even in the absence of other yeast proteins. In addition, as assayed by co-immunoprecipitation of fractions from a gel filtration column, SpMYH associates with SpHus1 in a complex of ~450 kDa, the reported native molecular mass of the 9-1-1 complex. This is the first demonstration that a DNA base excision protein directly interacts with the PCNA-like 9-1-1 complex.

The PCNA sliding clamp is a homotrimer that interacts with many replication and repair proteins (29, 45–48). So far no one has been able to demonstrate that one PCNA homotrimer can bind three proteins simultaneously. Although Hus1, Rad1, and Rad9 have structures similar to that of PCNA (6–8), it has been shown that more than one subunit of the 9-1-1 complex can associated with the same protein. Bermudez et al. (49) has shown that hRad17 interacts predominately with hRad9 and to a much lesser degree with hRad1 but not with hHus1. Giannattasio et al. (13) demonstrated that ScRad14 interacts strongly with ScDdc1 (SpRad9 homolog) and more weakly with ScMec3 (SpHus1 homolog) but did not test the interaction of ScRad14 with ScRad17 (SpRad1 homolog). It appears that one of the three Hus1/Rad1/Rad9 subunits is involved in major interactions with other proteins. We show here that SpMYH can interact with each subunit of the 9-1-1 complex and with individual subunits in the absence of the other two subunits. Our data indicate that SpMYH may undergo an asymmetrical interaction with the 9-1-1 complex in the decreasing affinity order of SpHus1, SpRad1, SpRad9 (compare Fig. 1, lanes 9 and 10)
after H2O2 treatment can occur during the G1 and G2 phases in
S. cerevisiae (52, 53). Leroy et al. (53) reported that treatment of Saccharomyces cerevisiae cells with H2O2 induces a ScMec1-dependent (human ATR/SpRad3 homolog) phosphorylation of ScRad53 (hChk2/SpOds1 homolog) during S phase but not during G1 and G2 phases. They also showed that the response to DNA damage after H2O2 treatment can occur during the G1 and G2 phases in yeast cells defective in the apurinic/apyrimidinic endonucleases ScApn1 and ScApn2. This may be caused by the accumulation of the intermediates and/or the repair is operated by alternate repair pathways. In human cells both OGG1 and MYH glycosylases are involved in LOH lesions base excision repair, whereas S. cerevisiae and S. pombe do not contain MYH and OGG1, respectively. Therefore, DNA checkpoint activation in response to oxidative stress is likely unique in these two organisms.

We have shown that fission yeast cells deficient in the functions of SpMYH are more sensitive to hydrogen peroxide (23). Here, we show that hydrogen peroxide can induce the phosphorylation of SpHus1 and can enhance the SpMYH-SpHus1 interaction. Moreover, SpHus1 phosphorylation is dependent on SpMYH expression after hydrogen peroxide treatment. Even though the phosphorylation of SpHus1 is not essential for SpHus1-SpMYH interaction, the increase in the in vivo SpHus1-SpMYH interaction correlates with the presence of SpHus1 phosphorylation after hydrogen peroxide treatment. Our result is similar to the finding of Brown et al. (12) who showed that interaction between MSH2 and CHK2 and interaction between MLH1 and ATM are enhanced after ionizing radiation. However, Giannattasio et al. (13) demonstrated that the interaction between ScRad14 and ScDcd1 is not affected by a UV mimetic agent. In contrast, the SpMYH-SpPCNA interaction demonstrated minimal changes after oxidative stress. The data of Fig. 3, A and B, show that when SpMYH-SpHus1 interaction is enhanced, the SpMYH-SpPCNA does not decrease. In addition, SpHus1 and SpPCNA bind to separate regions of SpMYH. Thus, SpMYH-SpHus1 interaction seems independent of SpMYH-SpPCNA interaction. We have shown that the association between MYH and PCNA is important in vivo for MYH function in mutation avoidance (24). It will be interesting to see the phenotype of a functional SpMYH with a defective interaction with the 9-1-1 complex. Our working model is that PCNA acts as the coordinator of base excision repair directing the repair on the newly synthesized DNA strands (24, 25, 27), whereas the MYH-Hus1 interaction occurs to induce the DNA damage response after oxidative stress. It remains to be determined whether MYH is able to bind to PCNA and Hus1 simultaneously.

Several interesting findings emerge from the analyses of SpMYH and SpHus1 by gel filtration chromatography. (i) We observed different elution profiles of SpMYH on a gel filtration column between the H2O2-unintreated and treated cell extracts. Particularly, SpMYH in the H2O2-unintreated extract is shifted to the region (fractions 38–50) with a molecular mass of 150–500 kDa, the same position of second peak of SpHus1. (ii) Although the interaction between SpMYH and SpHus1 increases after H2O2 treatment, as assayed by coimmunoprecipitation (Fig. 3A), both proteins do not co-elute completely on a gel filtration column (Fig. 5, A and B, filled diamonds). Similar findings were reported by Caspari et al. (6), that the majority of SpRad1 and SpRad9 do not co-elute with the 450-kDa native 9-1-1 complex. This suggests that SpMYH may form SpHus1-independent protein complexes. (iii) It is interesting to note that peak II of SpMYH elutes at a Mr of 150–200 (fractions 46–50), which coincides with the expected trimeric size of the 9-1-1 complex and with the reported SpRad9 peak (6). In addition, more SpHus1 was precipitated by SpMYH antibody in this region in the H2O2-treated extract as compared with the untreated extract (fractions 45–51 in Fig. 5C). (iv) Coimmunoprecipitation of SpHus1 with SpMYH antibody demonstrates SpMYH is associated with the 450-kDa native 9-1-1 complex (Fig. 5C). The distribution pattern of the precipitable SpHus1 from the H2O2-treated cell extract is very similar to the pattern of SpHus1 by direct Western blot analysis (compare Figs. 5, A and C, filled diamonds). (v) The redistribution of SpMYH in H2O2-treated cells is dependent on SpHus1, because the profiles of SpMYH distribution are not altered by the H2O2 treatment in hus1-deleted cells. These results suggest that an intact 9-1-1 complex may be critical for the enhanced SpMYH-SpHus1 interaction. Further testing the SpMYH-SpHus1 interaction in SpRad1 and SpRad9 mutants remains to be investigated.

Our findings support a model that MYH is one of the adaptors for checkpoint proteins in recognition of DNA lesions. First, the physically association between SpMYH and the 9-1-1 complex is enhanced after oxidative stress. Second, there is a similar kinetics between SpHus1 phosphorylation and SpHus1-SpMYH interaction after hydrogen peroxide treatment. Third, SpHus1 phosphorylation is dependent on SpMYH expression after hydrogen peroxide treatment. In this model, MYH functions upstream of Rad9/Rad1/Hus1 in the DNA damage signaling pathway. MYH first recognizes the lesions and then recruits Rad9/Rad1/Hus1 to the sites of DNA damage. After binding with SpMYH at the lesion site, SpHus1 is phosphorylated by SpRad3 kinase, signaling the DNA damage response. Several reports also support this model. S. cerevisiae Rad14 and Rad1 as well as human XPA, which are all involved in nucleotide excision repair, are required for the damage response (54, 55). A direct interaction between nucleotide excision repair enzymes and checkpoint proteins has been demonstrated by Giannattasio et al. (13) showing that ScRad14 physically and functionally interacts with the S. cerevisiae 9-1-1 complex. In addition, mismatch repair proteins have been suggested to function as a sensor in signaling apoptosis, based on the findings that mismatch repair deficient cells are more resistant to a variety of chemotherapeutic agents (56–59). Wang and Qin (15) showed that human mismatch repair enzyme MSH2 interacts with the ATR kinase to form a signaling module in response to alkylating agents. Brown et al. (12) showed that MSH2 interacts with CHK2, MLH1 associates with ATM, and the mismatch repair system is required for S-phase checkpoint activation. A series of recent reports pro-

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2 A. Lu, unpublished results.
vides support that the Mre11/Rad50/Nbs1 complex senses double strand breaks in DNA and activates cell cycle checkpoint pathways after exposure to radiation (for review, see Ref. 14). It will be interesting to discover whether other damage-recognition proteins also interact with PCNA-like proteins.

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Interaction of Checkpoint Proteins Hus1/Rad1/Rad9 with DNA Base Excision Repair Enzyme MutY Homolog in Fission Yeast, *Schizosaccharomyces pombe*

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