Physical Interaction between Recombinational Proteins Rhp51 and Rad22 in Schizosaccharomyces pombe*

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In eukaryotes, Rad51 and Rad52 are two key components of homologous recombination and recombinational repair. These two proteins interact with each other. Here we investigated the role of interaction between Rhp51 and Rad22, the fission yeast homologs of Rad51 and Rad52, respectively, on the function of each protein. We identified a direct association between the two proteins and their self-interactions both in vivo and in vitro. We also determined the binding domains of each protein that mediate these interactions. To characterize the role of Rhp51-Rad22 interaction, we used random mutagenesis to identify the mutants Rhp51 and Rad22, which cannot interact each other. Interestingly, we found that mutant Rhp51 protein, which cannot interact with either Rad22 or Rti1 (G292D), lost its DNA repair ability. In contrast, mutant Rad22 proteins, which cannot specifically bind to Rhp51 (S379L and P381L), maintained their DNA repair ability. These results suggest that the interaction between Rhp51 and Rad22 is crucial for the recombinational repair function of Rhp51. However, the significance of this interaction on the function of Rad22 remains to be characterized further.

Double strand breaks (DSBs) in chromosome are very harmful, and the failure in repair of DSB results in severe genomic instabilities that can lead to cell death or, in higher eukaryotes, to cancer (1). In eukaryotes, two major pathways have been known to deal with DSBs (2). The nonhomologous end-joining (NHEJ) pathway takes advantage of the undamaged homologous DNA strands, resulting in accurate repair of DSBs.

In Saccharomyces cerevisiae, RAD52 epistasis group genes are involved in HR pathway (3). These genes, including RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RPA, MRE11, and XR52, are well conserved throughout eukaryotes. In addition to these genes, there are several species-specific genes involved in HR, such as RAD59 (4) and RHD54/TID1 (5) in S. cerevisiae, rti11°/rad22B° (6) in Schizosaccharomyces pombe, and BRCA1 (7), BRCA2 (8), Rad51B-D, Xrec2, and Xrec3 (9) in mammals. RAD52 is the only gene among the RAD52 epistasis group that is required for virtually all homologous recombination events (3). Purified S. cerevisiae and human Rad52 proteins (ScRad52 and HsRad52, respectively) have an annealing activity of two complementary single-stranded DNAs (10), and they promote the strand exchange activity of Rad51 in the presence of RPA (11). In addition, HsRad52 and S. pombe Rad52 (Rad22) appear to bind to DSBs (12, 13). Rad51 is an eukaryotic structural and functional counterpart of Escherichia coli RecA (14). Purified ScRad51 and HsRad51 have the homologous pairing strand exchange activity that is the core catalytic activity of HR (15). Rti1/Rad22B (hereafter referred to simply as Rti1) is another Rad52 homolog found in S. pombe (6, 16). Deletion of both rad22° and rti1° leads to more severe defects compared with each single mutant, suggesting that the role of ScRad52 would be diverged to Rad22 and Rti1.

It has been reported that there are many protein-protein interactions in which ScRad51 and ScRad52 are involved. In accordance with its enzymatic role, ScRad51 is the center of the interactions between HR proteins in that it interacts with itself, ScRad52, ScRad54, ScRad55, and the large subunit of RPA (17–21). ScRad52 also interacts with itself and with all three subunits of RPA (22).

In this report we explored the interactions between Rad51 and Rad52, the two key molecules of HR, using their fission yeast homologs Rhp51 and Rad22, respectively (23, 24). We determined their binding domains and investigated the effect of Rhp51-Rad22 interaction on the DNA repair function of each protein.

EXPERIMENTAL PROCEDURES

Strains and Media—S. pombe haploid strains JY746 (h+ ade6-M16 leu1-32 ura4-D18) and ED668 (h+ ade6-M16 leu1-32 ura4-D18) were used as wild type cells. rhp51 deletion strain JAC151Δ (h+ ade6-704 leu1-32 ura4-D18 rhp51::ura4+) and rad22 deletion strain H6863 (h+ ade6-M16 leu1-32 ura4-D18 rad22::ura4+) (24) were used as hosts for complementation by Rhp51 and Rad22, respectively. S. cerevisiae strain Y190 (MATa ura3–52 his3–200 lys2–801 ade2–101 trpl–1 his3–201 ura3–52 GAL4 ura3–52 GAL1–ura3–52 GAL1–ura3–52 HIS3 URA3–GAL1–ura3–52) (25) was used as a host for yeast two-hybrid assay. S. pombe cells were grown and maintained in standard rich media (YES) or in minimal media (EMM) supplemented with appropriate nutrients as described in Alfa et al. (26).

Plasmids and Enzymes—For yeast two-hybrid analysis, derivatives of pGBT9 (Clontech), named pGBT9–1 and pGBT9–2, which have different reading frames, were generated by inserting or deleting nucleotides into the EcoRI site. Various restriction or PCR fragments of the rhp51+ or rad22+ genes were fused in-frame with GAL4 DNA-binding domain (GAL4BD; pGBT9 derivatives) or GAL4 activation domain (GAL4AD; pDP4, -7, -12) (26), respectively. rhp54° (27), rad55° (28), and rad22° (29) strains were used as wild type cells. RPA (30) was purified from E. coli.

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GAL4 DNA-binding domain; Sc, Saccharomyces cerevisiae; MMS, methylmethane sulfonate; GST, glutathione-S-transferase; GAL4AD, GAL4 trans-activation domain; GAL4BD, GAL4 DNA-binding domain; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; RPA, replication protein A.

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† The abbreviations used are: DSB, double strand break; HR, homologous recombination; MMS, methylmethane sulfonate; GST, glutathione-S-transferase; GAL4AD, GAL4 trans-activation domain; GAL4BD, GAL4 DNA-binding domain; Sc, Saccharomyces cerevisiae; Hs, Homo sapiens; RPA, replication protein A.
(28), rad57'' (29), rti1'' (6), ssb1'', and ssb2'' (30) genes were amplified from their respective cDNA or genomic DNA by PCR and cloned into the BamHI site of pGBT9 derivatives or pJF plasmids. For the complementation assay, SpeI-PstI fragment of pSL551 (31) was replaced by the cognate fragment from mutagenized rhp51, resulting in the full-length rhp51 genes containing each mutation. The BamHI-SmaI fragments of rad22 open reading frame containing each mutation were cloned into the equivalent sites of pREP81 (32) for the complementation assay. Restriction enzymes and modifying enzymes were purchased from New England Biolabs, Boehringer Mannheim, Promega, or Takara.

**Role of Rhp51-Rad22 Interaction**

Random Mutagenesis and Screening of Binding Mutants—The hydrazine mutagenesis method was employed (34, 35) to introduce random point mutations into rhp51'' or rad22'' genes. Plasmid DNA pBD51-(102–365) and pAD22-(310–469) harboring Rhp51 residues 102–365 in pGBT9-2 and Rad22 residues 310–469 in pDP12, respectively, were subjected to mutagenesis. Each plasmid was incubated in 0.5 ml of reaction buffer (50 mM sodium pyrophosphate (pH 7.0), 1 M NaCl, 2 mM EDTA, and 0.5 M hydroxylamine) at 75 °C for 30 min and purified by spin-column. After amplification through E. coli, the mutagenized pBD51-(102–365) or pAD22-(310–469) plasmids were transformed into S. cerevisiae Y190 harboring pDP22 or pBD51, respectively, for yeast two-hybrid screening. Each transformant was replica plated onto solid media lacking histidine. The colonies showing a His'' phenotype were subjected to a β-galactosidase color assay. Plasmid DNAs were recovered from the white colonies and recloned into the same intact plasmid to eliminate mutations in plasmid backbone. The changes of nucleotide sequence in insert DNAs were determined using Sequenase version 2.0 kit (Amersham Pharmacia Biotech). In case multiple mutations were found in a single clone, they were separated into each mutation by restriction enzyme digestion or site-directed mutagenesis.

**Survival Test**—Complementation of methylmethane sulfonate (MMS) and UV sensitivity of rhp51Δ and rad22Δ strains by mutant rhp51 and rad22 genes has been described previously (31).

**RESULTS**

**Homo- and Heterotypic Interactions of Rhp51 and Rad22**—We examined the association of Rhp51 and Rad22 in the wild type cell by co-immunoprecipitation. Fig. 1A shows that Rad22 protein can be co-precipitated with Rhp51, indicating that these two proteins can form a protein complex not only when they are overproduced (31) but also at normal levels. We also confirmed their interaction by GST pull-down assay. Recombinant Rad22 protein was co-precipitated with GST-Rhp51 fusion protein but not with GST protein (Fig. 1B, upper panel, lane 4), indicating that these two proteins can associate directly without the help of any other proteins in S. pombe. The interaction between Rhp51 and Rad22 was also visualized by yeast two-hybrid β-galactosidase plate assay (Fig. 1C). As expected, cells harboring both reciprocal sets of Rhp51 and Rad22 proteins fused with GAL4AD and GAL4BD turned blue. In addition, both proteins were self-interacting. Taken together, these results indicate that Rhp51 and Rad22 proteins directly associate both in vivo and in vitro and that each of them also self-associates.

**Mapping Domain of Rhp51 Confers Interaction with Rad22 or with Itself**—To determine the region of Rhp51 required for...
respectively. RecA homologous region, and the conserved ATP-binding motifs, indicate the homologous region among eukaryotic Rad51 homologs, the conserved ATP-binding motifs, and the conserved ATP-binding motifs respectively.

We determined the causal mutations by nucleotide sequence analysis. For self-interaction with Rad22, the carboxy-terminal two-thirds of RsRp51 (residues 102–365, fragment 2) was required, which encompasses the central RecA homology region and the carboxy-terminal region. On the other hand, for self-association of RsRp51, a shorter fragment (residues 102–312, fragment 12) was sufficient. Therefore the carboxy-terminal 53 amino acids appeared to have the residues critical for Rad22 binding. However, the amino-terminal region (residues 1–102), which corresponds to the region of ScRad51 responsible for the interaction with ScRad52 or with itself, was not required for the Rad22 binding to RsRp51.

Mapping Domains of Rad22 Conferring Interaction with Itself or with RsRp51—Yeast two-hybrid analysis was also applied to verify the region of Rad22 responsible for the interaction with RsRp51 or with itself (Fig. 3). Fusion proteins of GAL4AD with truncations of Rad22 and GAL4BD with RsRp51 or with Rad22 were subjected to a β-galactosidase plate assay. As shown in Fig. 3, the regions for RsRp51 and self-association were completely separated in the Rad22 protein. For self-interaction, the amino-terminal region (residues 1–162, fragment 6) was sufficient, whereas the carboxy-terminal region (residues 310–469, fragment 11) dealt with the interaction with RsRp51. However, as the fragment became shorter, the signals for RsRp51 binding progressively decreased, suggesting that the entire structure may also be required for the interaction with RsRp51 or for stabilization of the protein expression.

Screening of Point Mutations of RsRp51 or Rad22 That Interrupt Protein-Protein Interactions of Both Proteins—To identify the significance of RsRp51-Rad22 interaction in recombinational repair, we designed a screening method to identify mutations in RsRp51 that disrupt the interaction with Rad22 and vice versa. Based on the binding domain mapping, we introduced random point mutations into plasmids pBD51-(102–365) and pAD22-(310–469) by the hydroxyamine mutagenesis method. In yeast two-hybrid screening using these mutant libraries as prey and pAD22 or pBD51 as bait, we isolated five RsRp51 and four Rad22 mutant clones that did not show a blue color. We determined the causal mutations by nucleotide sequence analysis, separated multiple mutations in a single clone into single mutations by site-directed mutagenesis, and thereby acquired five RsRp51 and four rad22 mutant genes carrying single point mutation.

All five mutations of the rsrp51 gene were localized in the central RecA-homologous core region (Fig. 4A). None of the mutations was found in the conserved ATP-binding motifs (36) or the corresponding residues of putative RecA DNA binding sites (37). Except for one mutation that resulted in the stop codon (Q228NS), all four mutations caused single amino acid substitution. As shown in Fig. 4A, G177S, C179F, Q228NS, and G282D completely impaired the Rad22 binding of RsRp51, whereas the L274F mutation reduced its binding ability. Western blot analysis revealed that all mutant proteins except Q228NS were expressed well (data not shown), indicating that the failure in interaction was not caused by the lack of protein expression.

Each of the rad22 Mutations Caused a Single Amino Acid Substitution—Interestingly, all four mutations (S377F, S379L, P381S, and P381L) were found in very close proximity, and two were different substitutions of the same amino acid (Fig. 4B), suggesting that this region could be a binding epitope for RsRp51.

Interaction between RsRp51 Mutants and Recombination Factors—We performed two-hybrid analysis employing RsRp51, Rad22, RhsRp54, RhsRp55, RhsRp57, Rti1, and the large and middle subunits of RPA. These results revealed that RsRp51 interacts with itself, Rad22, RhsRp54, RhsRp57, and Rti1, whereas Rad22 interacts with itself, RsRp51, and Rti1. However, we could not find the interactions between Rad22 and the large or middle subunit of RPA that have been reported in S. cerevisiae (data not shown). Our results were similar to those of Tsutsumi et al. (38), except that the self-interactions of RsRp51 and RhsRp57 and the interaction between RsRp51 and Rti1 were newly found. Based upon these results, we examined whether the mutant RsRp51 could interact with its binding partners other than Rad22. All five mutations had different effects on the interaction of RsRp51 with its binding partners (Fig. 5A). The RsRp51 C179F could not bind with any of the proteins examined, indicating that this cysteine residue could be crucial for the entire interaction. Interactions between full-length RsRp51 or Rad22 fused with GAL4AD and various truncations of RsRp51 fused with GAL4BD were analyzed by β-galactosidase plate assay. The hatched boxes in Rad22 indicate homologous regions among Rad52 homologs.
structure of Rhp51, for example, by disulfide bridge formation. Rhp51 G177S and G282D, which had failed to interact with Rad22, were also unable to interact with Rad22. However, Rad22 and Rti1 may bind a similar epitope in Rhp51. Rhp51 G177S also did not interact with Rhp54. Rhp51 Q228NS, the nonsense mutation, did not interact with itself or Rti1 and showed reduced interaction with Rad22 and Rhp54. Rhp51 L274P interacted with Rhp57, Rad22, and Rti1 but not with Rhp51 or Rhp54. These results showed that none of the five mutants was restricted to a single interaction. However, given that Rad22 and Rti1 are homologs, Rhp51 G282D could be considered a Rad22-specific mutant.

Interaction between Rad22 Mutants and Recombination Factors—In our two-hybrid analysis, Rad22 interacted not only with Rhp51 but also with itself and Rti1, a Rad22 homolog (data not shown). We also investigated the interactions of mutant Rad22 with Rad22 or Rti1 (Fig. 5B). Rad22 S379L and P381S did not interact with Rad22 and Rti1. On the other hand, Rad22 S377F and P381S did interact with Rad22 and Rti1. These results suggested that S379L and P381S specifically disrupted the interaction of Rad22 with Rhp51, leaving other interactions intact. Immunoblot experiment revealed that Rad22 S379L, S377F, and P381S were not detected with our antibody (data not shown). However, because anti-Rad22 antibody recognizes the carboxyl-terminal region of Rad22, it is unclear whether the immunoblot results reflect the lack of protein expression. Rather, it is likely that the structural change of these three proteins may cause the failure in detection of protein by immunoblot.

Effects of Protein-Protein Interaction on DNA Repair Function of Rhp51 and Rad22—To investigate the significance of protein-protein interactions on the function of Rhp51, we examined whether the mutant rhp51 genes could rescue the DNA damage sensitivity of the rhp51 deletion mutant (Fig. 6A). All five mutant rhp51 genes barely rescued MMS and UV sensitivity of the rhp51 deletion mutant. The complementation efficiency of these mutant rhp51 genes was slightly better than an empty plasmid, suggesting that these mutations severely impaired the DNA repair ability of Rhp51. Interestingly, Rhp51 G282D, which is specifically unable to interact with Rad22 homologs, was biologically inactive, indicating that the interaction with Rad22 homologs would be indispensable for the DNA repair function of Rhp51. However, we could not evaluate the significance of the individual interaction between Rhp51 and other binding partners because of the lack of specific mutation that disrupted each individual interaction.

We also examined whether mutant rad22 genes could rescue DNA damage sensitivity of rad22 deletion mutants. As expected, Rad22 S377F and P381S were unsuccessful in complementing MMS and UV sensitivity. However, Rad22 S379L and P381L, which were specific to the interaction with Rhp51, were biologically active in their DNA repair function (Fig. 6B). These results suggested that the interaction with Rhp51 might not be essential for the DNA repair ability of Rad22.

DISCUSSION

Domains of Rhp51 for Protein-Protein Interactions—The amino-terminal region of ScRad51, where sequence homology exists between eukaryotic Rad51 homologs but is missing in RecA, is known as a domain of self-interaction and for ScRad52 binding (18). However, recent studies of ScRad51 and HsRad51 argued that the amino-terminal domain is not required for the interaction with either Rad52 or Rad51 but is responsible for the DNA binding (39–41). In our mapping, we also found the binding region of Rhp51 for the Rad22 or itself located in the broad regions spanning the central RecA homology region and the carboxyl terminus but not in the amino terminus. In addition, point mutations that affect these interactions were dis-
tributed throughout the central RecA homology region of Rhp51. Therefore it is likely that binding domains would be constituted by the cooperation of several domains or that distantly located residues might shape a single domain.

A comparison of multiple interactions between mutant Rhp51 and its binding partners revealed that a single mutation could affect multiple interactions. Among the mutations, at least three mutations appeared to have a severe influence on the global structure of protein by substitution of a cysteine residue (C179F), by introduction of proline (L274P), or by the truncation of polypeptide (Q228NS). In contrast, G177S and G282D seem to be involved specifically in the interactions of Rhp51 with Rad22 and Rti1. Amino acid sequence alignment of Rhp51, ScRad51, and HsRad51 demonstrated that both Gly177 and Gly282 are well conserved in Rad51 homologs (Fig. 7A, Table I). Interestingly, the neighboring residues of Gly177 and Gly282 were found to be involved in the Rad51-Rad52 interaction in ScRad51 and HsRad51 demonstrated that both Gly177 and Gly282 are well conserved in Rad51 homologs (Fig. 7A, Table I). Interestingly, the neighboring residues of Gly177 and Gly282 were found to be involved in the Rad51-Rad52 interaction in ScRad51 and HsRad51. Mutation in Phe259 of HsRad51, which corresponds to Phe281 of Rhp51, disrupted the interaction with HsRad51 in GST pull-down assay (41).

Three-dimensional modeling by similar mutational study in ScRad51 revealed that Gly210, Gly211, and Ala284 of Rhp51 constituted a single Rad52-binding domain (40). Therefore, it is highly likely that residues around Gly177 and Gly282 of Rhp51 could also comprise a single domain that is responsible for the Rad22 binding.

Domains of Rad22 for Protein-Protein Interactions—Unlike broad mapping of Rhp51 domains, those of Rad22 for Rhp51 binding and self-association were separately located in the carboxyl and the amino terminus, respectively. Yeast two-hybrid assays demonstrated that the Rad51-binding domains of ScRad52 and HsRad52 also exist in their C-terminal regions (17, 42). In addition, the existence of mutations that specifically disrupt binding with Rhp51 (S379L, P381S) also supports the possibility that the carboxyl-terminal region indeed forms Rhp51-binding domain. Because the carboxyl-terminal region lacks sequence homology between Rad52 homologs, Rad51-Rad52 interaction, although conserved from yeast to human, is likely to be species-specific. Interestingly, sequence alignment of Rad22 and Rti1 demonstrates that two short regions in the carboxyl terminus of Rad22 (residues 334–346 and 365–384) have a relatively higher sequence similarity than other regions in the carboxyl terminus (Fig. 7B). Moreover, Ser279 and Pro281 residues are conserved between Rad22 and Rti1. Because Rti1 also binds with Rhp51, these two regions may serve as an Rhp51-binding domain of Rad22 and Rti1. The lack of Rhp51 binding in the short fragments of residues 310–380 or 366–469 (Fig. 3) suggests that both of these regions are required and that they might comprise a single Rhp51-binding domain.

Significance of Rhp51-Rad22 Interaction in Recombinational Repair—All five mutant Rhp51 proteins were unable to interact with at least two binding partners and were nonfunctional in DNA repair. Therefore, although the significance of each individual interaction is uncertain, some or all of such interactions are likely to be required for the proper functioning of Rhp51.

On the other hand, four Rad22 mutants were divided into two groups. Rad22 S379L and P381L were specifically defective in interaction with Rhp51 and biologically active, whereas Rad22 S377F and P381S had neither protein binding ability nor DNA repair activity. The latter phenotypes could be the result of a lack of protein expression because Rad22 S377F and P381S were not observed in immunoblot. However, because Rad22 S379L, which is functional, was not detected either by immunoblot and our anti-Rad22 antibody recognized the carboxyl terminus region of Rad22, these phenotypes are presumed to be caused by a severe change in protein structure rather than by lack of expression.

In our experiments, the significance of Rhp51-Rad22 inter-
action appears to be different for each protein. Impairment of this interaction significantly affected Rhp51 function (Rhp51 G282D) but not that of Rad22 (Rad22 S379L and P381L). We can postulate at least two possibilities for this discrepancy. First, Rad22 S379L and P381L may retain enough binding activity to complement in vivo, although their Rhp51 binding is undetectable by yeast two-hybrid analysis; this is probably because we employed a multicopy plasmid with an attenuated promoter for the complementation assay. Second, Rad22 impairing Rhp51 function (Rhp51 G282D) but not that of Rad22 (Rad22 S379L and P381L).

Our observation that interaction between Rhp51 and Rad22 is essential to the DNA repair function of Rhp51 suggests that there would be an essential but unknown role of Rad22 other than as a co-factor of strand exchange reaction. One possible role of Rad22 in terms of interaction with Rhp51 is that of a mediator to direct Rhp51 into the site of action, as there are a few reports that Rad52 homologs bind to the end of duplex DNA. However, because there is a controversy on this property of Rad52, this possibility will require extensive verification.

**Fig. 7. Amino acid sequence alignments of each Rhp51 and Rad22 protein.** A, the positions of mutations that disrupted the protein-protein interactions of Rhp51, ScRad51, and HsRad51 are compared. Each mutation is **bolded** and indicated by an **arrowhead**. B, Rhp51 binding regions of Rad22 are aligned with Rti1. **Bolded letters** indicate Rad22 residues in which the mutation affects interaction with Rhp51; **boxes** indicate a close similarity between Rad22 and Rti1 regions.

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