Interaction with Rad51 Is Indispensable for Recombination Mediator Function of Rad52

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Homologous recombination in eukaryotic organisms is conserved in mechanism and mediated by a group of genes known as the RAD52 epistasis group. The RAD52 group members were first identified in the baker’s yeast, Saccharomyces cerevisiae, and include RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RAD54/TID1, MRE11, and XR52 (1–4). In S. cerevisiae and in other eukaryotes, homologous recombination is also an important means of eliminating DNA double-stranded breaks induced by ionizing radiation and other lesions that arise during the normal course of DNA replication (4). In mammals, homologous recombination also appears to be indispensable for cell viability and tumor suppression (1, 4).

A DNA double strand break can be repaired by pathways that are based on either end-joining or homologous recombination. In the latter case, the ends of the break are processed by a nuclease to yield 3′ ssDNA tails. These ssDNA1 tails attract recombination proteins, and the resulting nucleoprotein complex conducts a search for a homologous DNA sequence. Next, one of the ssDNA tails invades the homologous DNA target to form a DNA joint where de novo DNA synthesis can take place, eventually leading to an exchange of genetic information between the recombining chromosomes and to restoration of the integrity of the broken chromosome (2, 3).

The enzymatic process responsible for the formation of heteroduplex DNA joints in recombination is called homologous DNA pairing and strand exchange (2). The RAD51 encoded product, the equivalent of the Escherichia coli recombinase RecA, mediates the homologous DNA pairing and strand exchange reaction (5). Electron microscopic analyses have indicated that Rad51, like E. coli RecA protein, forms a highly ordered nucleoprotein filament on DNA (6). Biochemical studies have suggested that pairing and exchange of DNA strands in recombination processes occur within the confines of the Rad51-ssDNA nucleoprotein filament. The reaction phase in which the Rad51-ssDNA nucleoprotein filament is assembled is commonly referred to as the presynaptic phase, and the nucleoprotein filament as the presynaptic filament (2, 6, 7).

Formation of the presynaptic filament requires ATP binding by Rad51 (2). When plasmid length ssDNA substrates are used, presynaptic filament assembly is facilitated by the heterotrimeric single-stranded DNA binding factor, replication protein A (RPA), which functions to remove secondary structure in the ssDNA (5, 8, 9). The beneficial effect of RPA is seen most clearly when it is incorporated after Rad51 has been given an opportunity to nucleate onto the ssDNA template. In contrast, if RPA is added together with Rad51, it interferes with the filament assembly process by competing for binding sites on the ssDNA molecule. However, the inhibitory behavior of RPA can be alleviated by the addition of either of two recombination mediators (10), Rad52 or the Rad55-Rad57 heterodimer (11–14).

We are interested in the molecular basis of the mediator function of Rad52 and the Rad55-Rad57 heterodimer in the above mentioned reaction. Both Rad52 and the Rad55-Rad57 complex bind ssDNA and physically interact with Rad51 (15). In the present study, we have performed a fine mapping of the domain in Rad52 that is responsible for the interaction with...

1 The abbreviations used are: ssDNA, single-stranded DNA; RPA, replication protein A; ScRPA, Saccharomyces cerevisiae RPA; DTT, dithiothreitol; GST, glutathione S-transferase; Oligo, oligonucleotide; aa, amino acid; MOPS, 4-morpholinopropanesulfonic acid.

2 L. Krejci and P. Song, unpublished observation.
Rad51. Furthermore, we have used this information to introduce a small deletion mutation into Rad52 to ascertain the significance of Rad51-interaction in Rad52 mediator function. The combination of genetic and biochemical analyses of the mutant rad52 protein unequivocally demonstrate the requirement for a physical association of Rad52 with Rad51 to effect its mediator function.

**Dissection of Rad52-Rad51 Interaction**

**Materials and Methods**

**Yeast Media and Strains**—Yeast extract-peptone-dextrose (YPD) medium, synthetic complete (SC) medium, and synthetic complete medium without leucine (SC–Leu) and without uracil (SC–Ura) were prepared as described previously (16) except that the synthetic medium contained twice the amount of leucine (60 mg/liter). Yeast extract-peptone-agar (YPEA) contained 10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter potassium acetate. Sporulation medium contained 2.5 g/liter yeast extract and 15 g/liter potassium acetate supplemented with 62.5 mg/ml Leu and 20.6 mg/liter each of adenine, His, Trp, and uracil. All strains are derivatives of Trp-303 (17) except that they are wild type for *RADS* (18, 19). Standard genetic techniques were used to manipulate yeast strains (20). The rad52*409–412* allele was integrated into the yeast genome at the *RADS* locus by a cloning-free PCR-based allele replacement method (21). Specifically, gene-targeting substrates were made by amplifying a region of the *rad52* allele, which comprises the 5′ upstream region of the vector pYES2.4–412 (1) by PCR using the primers and *Pr-52*–C-Adap-B (5′-GATCCGGGGAATTCCGTGCGTCCTTACGGTTCGG-3′) and *Pc*-Adap-A (5′-AAATCCGCTTCAACCAGCATGAAGGATCCCGTTGTAGCTAAG-3′). The underlined sections of the primers correspond to unique tags that match sequences upstream and downstream of *Klyuyveromyces lactis* URA3, respectively. Next, two PCR fragments containing the upstream and the downstream two-thirds of the *K. lactis* URA3 gene were fused individually to the *rad52*409–412* PCR fragment as described in Erdeniz et al. (21).

**Plasmids for Protein Expression**—GST fusion fragments of Rad52 were constructed as follows: GST-Rad52N (aa 1–412, GST-Rad52C (aa 322–504), encoded within the 12% native polyacrylamide gels at 4 °C in TAE buffer. DNA annealing was quantified as the portion of the 32P-labeled Oligo-2 that had been converted into the double-stranded form.

**DNA Substrates**—The 6× 174 viral (+) strand was purchased from New England Biolabs, and the replicative form (about 90% supercoiled DNA) was purchased from Invitrogen. Oligonucleotide 2 is the exact complement of oligonucleotide 1. Oligonucleotide 2. The completed reactions (50 μl) were incubated at 25 °C, and at the indicated times, 9 μl of the annealing reactions was removed and treated with 0.5% SDS, 500 μg/ml protease K, and an excess of unlabelled Oligo-2 (20 μmol) at 35 °C for 5 min in a total volume of 15 μl. The various samples (6 μl) were resolved in 12% native polyacrylamide gels run in TAE buffer. DNA annealing was quantified as the portion of the 32P-labeled Oligo-2 that had been converted into the double-stranded form.

**Protein Purification and Annealing of Proteins**—All of the GST fusion proteins were expressed in *E. coli* strain BL21(DE3), and all of the protein purification steps were carried out at 4 °C. For the purification of the GST fusion proteins, lysate was prepared from *E. coli* cell paste using a French press in buffer G (20 mM NaH₂PO₄, pH 7.4, 0.5 mM EDTA, 1 mM DTT, and 150 mM NaCl) that contained the protease inhibitors aprotinin, leupeptin, and pepstatin A at 5 μg/ml each, as well as 1 mM phenylmethylsulfonyl fluoride). The crude lysate was clarified by centrifugation (100,000 ×g, 90 min), and the supernatant (20 ml) from the centrifugation step was mixed with 1 ml of glutathione-Sepharose 4B (Amersham Biosciences) for 3 h at 4 °C. The beads were washed three times with 20 ml of buffer G containing 150 mM KCl. The bound GST fusion protein was eluted with 5 ml of 10 mM reduced glutathione in buffer G. The eluate was dialyzed against buffer G and concentrated to 10 mg/ml in a Centricron-30 microcentrator.

Plasmids encoding untagged versions of Rad52 protein (pR52.2) and rad52*409–412* mutant protein (pR52.2–409–412) under the control of the GAL-POR promoter were introduced into yeast strain BJ5464-6B. The *Rad51*, *Rad52*, and *rad52* expression was quantified by SDS-PAGE in a 10% gel. The eluate was dialyzed against buffer G and concentrated to 10 ng/ml in a Centricron-30 microcentrator.

Plasmids encoding untagged versions of Rad52 protein (pR52.2) and rad52*409–412* mutant protein (pR52.2–409–412) under the control of the GAL-POR promoter were introduced into yeast strain BJ5464-6B. The expression was quantified by SDS-PAGE in a 10% gel.
Dissection of Rad52-Rad51 Interaction

Fig. 1. The carboxyl terminus of Rad52 is responsible for interaction with Rad51. A, schematic representation of GST fusion proteins consisting of near full-length Rad52 (NMC) as well as the amino-terminal (GST-N), the middle (GST-M), and the carboxyl-terminal (GST-C) portions of Rad52. B, purified GST, GST-NMC, GST-M, and GST-C, 1 μg each and designated by an asterisk, were run in a 10% denaturing polyacrylamide gel and stained with Coomassie Blue. C, GST pull-down assay. Purified GST fusion proteins were incubated individually with Rad51 and then mixed with glutathione-Sepharose beads. The beads were washed twice with buffer containing 300 mM KCl before being treated with SDS to elute bound proteins. The input material (I), the supernatant after mixing with glutathione-Sepharose (S), the KCl wash (W), and the SDS eluate (E) from these binding reactions were subjected to electrophoresis in a 10% denaturing polyacrylamide gel followed by immunoblotting with anti-Rad51 antibodies to determine their Rad51 content.

reaction mixtures (12.5 μl, final volume) containing the indicated amounts of Rad51, Rad52, and RPA were incubated on ice for 45 min in 10.5 μl of buffer R followed by the addition of ssDNA and a 10-min incubation. After the incorporation of linear duplex and spermidine, the completed reactions were incubated and analyzed as described for the standard reaction. For the time course experiments in Fig. 7, the reactions were scaled up four times to 50 μl of buffer R followed by the addition of ssDNA and a 10-min incubation. After the incorporation of linear duplex and spermidine, the completed reactions were incubated and analyzed as described for the standard reaction. For the time course experiments in Fig. 7, the reactions were scaled up four times to 50 μl but were otherwise assembled in the same fashion.

Cellular Sensitivity to γ-Irradiation—Three independent haploid spores from each strain were picked and analyzed for their sensitivity to γ-irradiation, and the average values were reported. Yeast cultures were grown in YPD at 30 °C to the mid-log phase. At this point, the cultures were sonicated using a W-385 device (Heat Systems-Ultrasonics, Farmingdale, NY), and the appropriate number of cells were plated on YPD plates and irradiated in a GammaCell-220 60Co irradiator (Atomic Energy of Canada). In Fig. 8R, cells transformed with pYES10-Rad51 (2μ, RAD51) and with the empty vector pRS426 (25) were grown on selective medium (SC-Ura) containing galactose as the sole carbon source at all stages of the experiment. The yeast cultures were analyzed as described above, except that for each strain, serial 10-fold dilutions were made and 5 μl of the diluted cell suspensions were spotted in duplicate on solid media prior to irradiation.

Determination of Mitotic Recombination Rates, Sporulation Efficiency, and Meiotic Recombination Frequencies—Mitotic rates of interchromosomal heteroallelic recombination were determined as described previously (28). For each strain, nine independent trials were performed. The meiotic interchromosomal heteroallelic recombination frequency, sporulation efficiency, and spore viability were determined as described in Lisby et al. (27) except that strains were grown at 30 °C. Three trials were performed for each strain.

RESULTS

Location of the Rad51 Interaction Domain—Rad52 possesses 504 amino acid residues (28). Results from yeast two-hybrid analyses have suggested that the carboxyl terminus of Rad52 encompassing residues 328 to 504 can interact with Rad51 (29). Exploiting this information, we divided Rad52 into three fragments: Rad52N (aa 34–168), Rad52 M (aa 169–327), and Rad52C (aa 328–504), which were fused individually to glutathione-S-transferase (GST) as depicted in Fig. 1A. These GST fusion proteins were expressed in E. coli and purified using affinity chromatography on glutathione-Sepharose (Fig. 1B). We also expressed and purified a GST fusion protein, termed GST-NMC, which contain the Rad52 protein sequence starting from the third ATG codon (aa 34); the region corresponding to amino acid 1–34 is not required for in vitro Rad52 function.3 To determine which portion of Rad52 contains the Rad51 binding domain, the purified GST fusion proteins were mixed with Rad51 and then immobilized on the glutathione-Sepharose beads. After washing with high salt buffer, the GST fusion proteins and associated Rad51 were eluted from the glutathione-Sepharose beads. After washing with high salt buffer, the GST fusion proteins and associated Rad51 were eluted from the glutathione-Sepharose beads. The results show that Rad51 binds GST-NMC and GST-C, but not GST-N, GST-M, or GST alone. We then asked whether the purified GST fusions could be retained on Affi-gel 15 beads that contained covalently coupled Rad51 protein (24). As expected, the Affi-Rad51 beads were able to bind GST-NMC and GST-C but not GST-M, GST-N, or GST (data not shown). None of the GST-Rad52 fusion proteins was retained on Affi-gel 15 beads containing bovine serum albumin (data not shown). Thus, in agreement with yeast two-hybrid studies (29), the results from our in vitro analyses with purified Rad52 protein fragments revealed that the Rad51 interaction domain is located within the last 177 amino acid residues of Rad52 protein.

Fine Mapping of Rad51 Interaction Domain and Construction of a Rad51 Interaction-defective Mutant—to delimit the region in Rad52 required for interaction with Rad51, additional GST-tagged fragments of Rad52 derived from the carboxyl-terminal residues were generated (Fig 2A). These GST fusions were again purified by affinity chromatography and tested for

3 R. Rothstein and U. H. Mortensen, unpublished observation.
Rad51 interaction by pull-down using glutathione-Sepharose beads as described before (Fig. 1C). The binding of the various GST-Rad52 fusions to Affi-gel-Rad51 beads was also examined. The results from these combined analyses, as summarized in Fig. 2, revealed that amino acids 407–419 of the Rad52 protein are likely involved in binding Rad51.

Overexpression of the Rad52 protein from another yeast, K. lactis, confers a dominant negative phenotype in S. cerevisiae that can be overcome by overexpression of the S. cerevisiae Rad51 (29). The authors of this study (29) suggested that the negative dominance of the K. lactis Rad52 in S. cerevisiae cells is due to the formation of a biologically inactive complex between KIRad52 and ScRad51. Even though the carboxyl terminus of the S. cerevisiae and K. lactis Rad52 counterparts display only a low level of identity (29%), the KIRad52 protein contains a sequence that is highly similar to the one in ScRad52 protein found here to be involved in Rad51 binding (Fig 2B). Consistent with the suggestion that the sequence encoded within amino acid residues 407–419 of Rad52 is critical for Rad51 binding, we found that a small deletion spanning amino acid residues 409–412 within this region completely ablates the ability of Rad52 to interact with Rad51 (Fig. 2A, panel II), as determined by the GST pull-down assay, binding of the GST fusion proteins to Affi-Rad51 beads, and other criteria (see below).

Purification and Biochemical Characterization of a Rad51 Interaction-deficient Rad52 Mutant—The results presented above show that amino acid residues 409–412 are likely to be required for Rad51 binding. To further demonstrate the importance of these four amino acid residues, we introduced the same deletion mutation (Δ409–412) into the untagged Rad52 protein. For biochemical analyses, we overexpressed both the rad52Δ409–412 mutant and the wild-type protein by using the GAL-PGK promoter and galactose induction in the protease-deficient yeast strain BY4746-6B. The level of expression of the wild-type and mutant proteins was very similar (data not shown), and they could be purified to near homogeneity by the same chromatographic procedure (see “Materials and Methods”, Fig. 3A). Approximately 1 mg of each of the wild-type and mutant proteins was obtained from 300 g of starting yeast paste. This represents a 5–10-fold improvement compared with protein yield obtained when the PGK promoter is used for protein expression, as described in our previously published study (12).

Unlike wild-type Rad52 protein, the purified rad52Δ409–412 mutant protein did not bind Affi-Rad51 beads (Fig. 3B), indicating that the four-amino acid deletion indeed eliminates the ability of Rad52 to associate with Rad51. Both Rad51 and Rad52 self-associate to form oligomeric molecules (14, 23, 30). A very large complex of these two proteins can be isolated in a sizing column (23). Accordingly, we subjected the purified rad52Δ409–412 mutant protein to sizing analysis in Sephacryl 400 to obtain independent verification that it does not associate with Rad51 and also to determine whether the Δ409–412 mutation affects self-association. When a mixture of Rad51 and wild-type Rad52 was analyzed, they formed a complex that emerged from the gel filtration column at an earlier position than either Rad51 or Rad52 alone (Fig. 4, compare panels I and II). In contrast, when the rad52Δ409–412 mutant was mixed with Rad51, no apparent shift of the elution profile of either protein was observed (Fig. 4, compare panels V with panels I and III). Importantly, the peak of the rad52Δ409–412 mutant protein migrated at the same position as wild-type Rad52 (Fig. 4, compare panels I and II), strongly suggesting that the mutant rad52 protein has the same oligomeric composition as the wild-type protein. Thus, the results from the gel filtration analyses demonstrated that the rad52Δ409–412 mutant is defective in Rad51 interaction but maintains the ability to self-associate.

As first reported by Mortensen et al. (15) and later confirmed by others (13, 14, 23), Rad52 possesses an ssDNA binding function. We therefore addressed the possibility that the four-amino acid deletion affects the DNA binding activity of the rad52Δ409–412 mutant protein. To do this, increasing amounts of Rad52 and rad52Δ409–412 proteins were incubated with a 32P-labeled 83-mer oligonucleotide. Subsequently, the capacity of these proteins to bind DNA was evaluated by gel mobility shift of the radiolabeled DNA substrate. The results presented in Fig. 5A show that rad52Δ409–412 shifts the DNA fragment just as efficiently as the wild-type protein. We also used 5X ssDNA as substrate to test the DNA binding capacity of the protein species. As in the previous experiment, no differ-
ence in DNA binding activity was observed between wild-type and mutant proteins (data not shown).

In addition to DNA binding, Rad52 also anneals complementary DNA strands (15). Interestingly, the Rad52-mediated DNA annealing reaction occurs efficiently with RPA-coated DNA strands (8, 14), whereas RPA alone slows the spontaneous rate of DNA annealing (8, 14). This annealing reaction is likely to involve a specific interaction between Rad52 and RPA, as the heterologous E. coli SSB and human RPA strongly inhibit the Rad52-ssDNA annealing activity (8). We examined the
rad52\textsuperscript{409–412} mutant protein for its ability to anneal ScRPA-coated complementary single strands. The results from this experiment indicate that the rate and extent of the annealing reaction obtained in the presence of either Rad52 or rad52\textsuperscript{409–412} are essentially identical for both (Fig. 5B and Fig. 5C, panel I). With DNA substrates free of ScRPA, the rad52\textsuperscript{409–412} mutant protein was again as proficient as wild-type Rad52 in its annealing reaction (Fig. 5C, panel II). This observation shows that rad52\textsuperscript{409–412} likely retains the ability to physically interact with RPA.

Taken together, the biochemical analyses documented here allowed us to conclude that rad52\textsuperscript{409–412} mutant has the wild-type level of ssDNA binding and DNA annealing activities and also possesses the same oligomeric state as the wild-type protein. In addition, the ability of the rad52\textsuperscript{409–412} mutant to anneal RPA-coated single strands like the wild-type protein
is consistent with the premise that it retains the ability to interact with RPA.

rad52Δ409–412 Mutant Is Specifically Defective in Mediator Function—The above data have demonstrated that the rad52Δ409–412 mutant protein does not interact with Rad51 but that it otherwise behaves like the wild-type protein in various biochemical attributes. We next tested whether the rad52Δ409–412 mutant protein retains the recombination mediator activity of wild-type Rad52.

We and others have shown that, with plasmid length DNA substrates, the efficiency of the Rad51-mediated DNA strand exchange reaction is greatly enhanced by RPA (8, 12). However, the order of addition of RPA relative to Rad51 is critical for efficient DNA strand exchange. Specifically, if RPA is added after Rad51 has already nucleated onto the ssDNA, a robust strand exchange reaction is observed. In contrast, if RPA is added to the ssDNA at the same time as Rad51, the level of DNA strand exchange diminishes greatly (11–14) (Fig. 6B, panels I and II). As shown before and repeated here (Fig. 6B, panel III), the inhibitory effect of RPA in the latter experiment can be alleviated by adding a substoichiometric amount of Rad52 (10 μM Rad51 and 1.2 μM Rad52). In contrast, when the same experiment was performed with the equivalent amount of rad52Δ409–412 mutant protein, the suppressed level of DNA strand exchange caused by RPA was not relieved. In fact, the presence of the rad52Δ409–412 protein further reduced the already low level of DNA strand exchange caused by RPA co-addition. Because of this result, we also examined lower amounts of rad52Δ409–412 (0.4 to 1.0 μM) for a possible mediator effect but found that it is devoid of such activity at any of these concentrations (Fig. 7, lanes 8–12). On the other hand, the addition of rad52Δ409–412 (0.4–1.2 μM) and RPA to a preformed Rad51-ssDNA complex did not affect the efficiency of DNA strand exchange (data not shown). Taken together, the results establish a direct linkage between the Rad51-interacting activity of Rad52 and its mediator function in the DNA strand exchange reaction.

Repair and Recombination Defects of rad52Δ409–412—The
**Fig. 7. Mediator activity as a function of concentration of Rad52 or rad52Δ409–412.**

A, increasing amounts of either Rad52 (0.4, 0.6, 0.8, 1.0, and 1.2 μM in lanes 3–6, respectively) or rad52Δ409–412 (0.4, 0.6, 0.8, 1.0, and 1.2 μM in lanes 8–12, respectively), Rad51 (10 μM; lanes 2–12), or RPA (2 μM; lanes 2–12) were incubated with ssDNA (30 μM nucleotides) for 10 min followed by the incorporation of the linear duplex (25 μM nucleotides) to complete the reactions; the reaction in lane 2 (Inh) did not contain Rad52 or rad52Δ409–412. In lane 1 (Std), ssDNA (30 μM) was incubated with Rad51 (10 μM) for 5 min followed by the addition of RPA (2 μM) and a 5-min incubation before the duplex substrate (25 μM nucleotides) was incorporated to complete the reaction. Aliquots of the reactions were withdrawn at 30 and 60 min and processed for electrophoresis. The results from the 30-min time point are shown. B, the results from A and from analyzing the gel containing the 60-min time point samples are graphed.

**Fig. 8. γ-Ray sensitivity of the rad52Δ409–412 mutant.**

A, the log fraction of surviving cells (% survival) after exposure to the indicated doses of γ-radiation (krad) of yeast strains with the following genetic backgrounds: wild-type RAD52 (wt), rad52Δ409–412 (rad52), and rad52Δ (null). B, effect of RAD51 overexpression. RAD52 or rad52Δ409–412 cells were spotted in serial 10-fold dilutions and irradiated as indicated (0, 20, 40, and 80 kilorads). The same set of strains was also transformed with a 2μ vector or the same vector containing the RAD51 gene and then analyzed. The rad52Δ409–412 allele is designated rad52.
biochemical experiments described above demonstrated a specific defect in the rad52Δ409–412 mutant protein, namely, that it fails to interact with Rad51 and is devoid of recombination mediator function. To establish the role of the Rad52 mediator activity in vivo, we replaced the chromosomal RAD52 gene with the rad52Δ409–412 allele in the Trp-303 background (see "Material and Methods") and then tested the mutant strain for its γ-ray sensitivity. The rad52Δ409–412 mutant strains show a marked increase in sensitivity to ionizing radiation although not to the same extent as isogenic rad52 deletion strain (Fig. 8A). This result indicates that the interaction with Rad51 is indeed important for the full biological activity of Rad52 protein in double strand break repair in vivo. Interestingly, the γ-ray sensitivity of the rad52Δ409–412 mutant can be complemented fully by the overexpression of the Rad51 protein on a 2μ plasmid (Fig. 8B). Similar complementation by Rad51 overexpression has been observed by Livingston and Kuytor (31) for a rad52 allele (rad52Δ327) that lacks the carboxy-terminal 177 residues.

We also compared the frequencies of meiotic interchromosomal heteroallelic recombination and spore viability of wild-type RAD52 and rad52Δ409–412 strains (data not shown). In both cases, no significant differences were observed, indicating that the Rad51-Rad52 interaction is not essential for successful completion of meiosis. In contrast, the mitotic interchromosomal heteroallelic recombination is reduced 4-fold in the rad52Δ409–412 strain (data not shown). Thus, the overall phenotype of the rad52Δ409–412 strain is very similar to that obtained with the rad52Δ327 strain except that the spore viability of the latter strain is somewhat impaired (32).

**DISCUSSION**

The Rad51 interaction domain was first shown to reside within the carboxy-terminal 177 amino acids residues of Rad52 by a yeast two-hybrid analysis (29). Here we have verified the two-hybrid result by showing biochemically that the carboxy terminus, but not the amino-terminal and middle portions, of Rad52 has the ability to bind Rad51 in the absence of another protein factor or DNA. Next, we finely mapped the Rad51 interaction domain and used this information to conduct a variety of biochemical and genetic experiments to firmly establish the biological and functional significance of the Rad51-Rad52 association.

First we examined a series of deletion fragments derived from the carboxy terminus of Rad52 for complex formation with Rad51. The results strongly suggest that a short sequence encompassing residues 407–419 is involved in mediating interaction with Rad51. This mapping information and a sequence comparison to K. lactis Rad52 prompted us to introduce a short deletion in the region spanning residues 409–412 into the full-length Rad52 protein. We find that the rad52Δ409–412 mutant protein can be stably expressed in yeast cells and that it behaves like wild-type Rad52 during column chromatography, allowing us to use the same procedure to purify both wild-type and mutant proteins to near homogeneity for biochemical experiments. Here we have shown, by several criteria, that the rad52Δ409–412 mutant protein lacks the ability to interact with Rad51. In contrast, its ability to bind DNA and mediate DNA annealing, as well as its oligomerizing properties, are indistinguishable from those of the wild-type protein. These results demonstrated that the short sequence (i.e., aa residues 407–419) in Rad52 identified in our mapping work is likely to be indispensable to and is possibly responsible for Rad51 binding. Furthermore, the biochemical results have verified that the Rad51 interaction-defective rad52Δ409–412 mutant is normal in all other known biochemical attributes of Rad52.

In DNA strand exchange experiments, the rad52Δ409–412 mutant protein over a wide range of concentrations is devoid of the mediator function seen in the Rad52 protein. Consistent with the biochemical result, the rad52Δ409–412 mutation renders cells sensitive to ionizing radiation and confers a 4-fold decrease in mitotic recombination. It has been suggested from yeast two-hybrid and in vitro studies that Rad52 physically interacts with RPA and that this interaction is important for its mediator activity and biological function (8, 13, 14, 33). It could be argued that the lack of mediator function in the rad52Δ409–412 mutant protein is due to an inability to recognize RPA. We have attempted to demonstrate a direct interaction between purified Rad52 and RPA but have thus far been unable to find the conditions to detect such an interaction in the absence of DNA. However, it remains quite possible that the interaction between Rad52 and RPA occurs only when these factors are bound to DNA. Consistent with this premise, it has been demonstrated that Rad52 effectively anneals DNA strands coated with ScRPA (8, 14) but not with heterologous ssDNA-binding proteins (8), implying a direct interaction between Rad52 and RPA as the likely reason for this observed specificity. We have demonstrated that the rad52Δ409–412 mutant is perfectly capable of mediating ssDNA annealing with an ScRPA-coated template. This result strongly suggests that the mutant protein also retains the ability to interact with RPA (14). Given this consideration, our data provide evidence that the lack of recombination mediator function in rad52Δ409–412 is specifically due to its inability to form a complex with Rad51 protein.

The DNA repair deficiency of the rad52Δ409–412 mutant strain can be complemented by Rad51 overproduction. It seems possible that a substantial increase in the intracellular pool of Rad51 may accelerate the assembly of the presynaptic Rad51 filament, thus rendering this process less prone to the competitive effect of RPA even when the Rad52 mediator function has been disabled, as in the case of the rad52Δ409–412 mutant. However, a more attractive explanation is that the elevated Rad51 levels facilitate complex formation between Rad51 and other mediator proteins. This could conceivably lead to more effective loading of Rad51 onto ssDNA via the other recombination mediators. In fact, our observation that Rad51 shows only a weak interaction with the Rad55-Rad57 complex (41) is congruent with the latter view. Mutants of the RAD59 gene show some deficiency in Rad51-dependent recombination events (34, 35). It remains to be seen whether Rad59 also functions as a mediator in Rad51-catalyzed homologous DNA pairing and strand exchange and, if so, whether the mediator activity of Rad59 will require complex formation with Rad51 that is enhanced by increased intracellular Rad51 levels.

The rad52 null mutation renders cells highly defective in recombination, and overexpression of Rad51 does not compensate for the loss of Rad52 (36). Thus, Rad52 must have a function in recombination/repair pathways independent of Rad51 interaction. It is possible that in the cellular setting, Rad52 renders the RPA-coated ssDNA template more accessible to Rad51 even in the absence of a specific interaction with Rad51. Alternatively, or additionally, the Rad51 interaction-defective rad52 variant may enhance the interaction between Rad51 and other recombination mediators without directly contacting Rad51. Also, it seems possible that the severe phenotype associated with RAD52 deletion may be related to the ability of Rad52 to promote the assembly of DNA repair centers as revealed in recent cytological studies (27).

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4 L. Krejci and P. Song, unpublished observation.
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