RETRACTED ARTICLE: Molecular Dissection of Interactions between Rad51 and Members of the Recombination-Repair Group

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Recombination is important for the repair of DNA damage and for chromosome segregation during meiosis; it has also been shown to participate in the regulation of cell proliferation. In the yeast Saccharomyces cerevisiae, recombination requires products of the RAD52 epistasis group. The Rad51 protein associates with the Rad51, Rad52, Rad54, and Rad55 proteins to form a dynamic complex. We describe a new strategy to screen for mutations which cause specific disruption of the interaction between certain proteins in the complex, leaving other interactions intact. This approach defines distinct protein interaction domains and protein relationships within the Rad51 complex. Alignment of the mutations onto the constructed three-dimensional model of the Rad51 protein reveals possible partially overlapping interfaces for the Rad51-Rad52 and the Rad51-Rad54 interactions. Rad51-Rad55 and Rad51-Rad51 interactions are affected by the same spectrum of mutations, indicating similarity between the two modes of binding. Finally, the detection of a subset of mutations within Rad51 which disrupt the interaction with mutant Rad52 protein but activate the interaction with Rad54 suggests that dynamic changes within the Rad51 protein may contribute to an ordered reaction process.

In the yeast Saccharomyces cerevisiae, genes of the RAD52 epistasis group are required for both homologous recombination and the repair of double-strand-breaks (DSBs) (10). Mutations in these genes result in severe cellular sensitivity to ionizing radiation and alkylating agents (e.g., methyl methanesulfonate [MMS]), reduced spontaneous and DNA damage-induced mitotic recombination, and the production of inviable spores in meiotic recombination (36).

Biochemical data suggest that some products of the RAD52 epistasis group (Rad51, Rad52, Rad54, Rad55, and replication protein A [RPA]) assemble disassemble on DNA. The Rad51 protein is a key component of this complex. It has significant sequence and functional similarity to Escherichia coli RecA protein, the crystal structure of which has been determined (47). The two proteins share a region of 30% identity, comprising amino acid residues 154 to 374 of Rad51 and 35 to 240 of RecA, corresponding to a large middle domain essential for recombination. Indeed, Rad51 protein also possesses some of the RecA functional activities, e.g., binding of single-stranded DNA (ssDNA) and double-stranded DNA, ATP hydrolysis, formation of nucleoprotein filaments, and formation of heteroduplex DNA (51, 54).

Rad51 interacts with itself, with Rad52 (9, 43), with Rad54 (7, 17), and with Rad55, which in turn associates with Rad57 (15, 18). In accordance with the biochemical and two-hybrid data obtained for these interactions, there are also many genetic data supporting their cellular relevance (7, 11, 41). The importance of the N-terminal part of Rad51 has been demonstrated in Rad51 self-association and in the interaction with Rad52 (31). The details of these two interactions have not been explored further.

Recently, much attention has been paid to the biochemical function of Rad51 and its associated proteins, Rad51, Rad52, Rad54, and the Rad55-Rad57 heterodimer. Rad52 shows annealing activities (32, 50) and promotes the exchange of RPA for Rad51 protein on ssDNA (28, 52), and human Rad52 binds double-strand breaks (56). Rad54 belongs to a SWI2/SNF2 protein family, whose members modulate chromatin structure (57). Biochemical studies show that Rad54 forms a dimer or oligomer on DNA and promotes Rad51-dependent homologous DNA pairing through changes in DNA double-helix conformation (37). Both RAD55 and RAD57 are sequence homologs of RAD51, and they form heterodimers that assist Rad51 in interacting with the ssDNA. The heterodimer may be involved in overcoming an inhibition of strand exchange by RPA (52).

The sequence of the RAD genes is conserved in a wide variety of eukaryotic organisms, suggesting their importance to eukaryotic cellular function in general. An interesting feature of Rad51p is its crucial role in the mouse, where the rad51 mutant displays early embryonic lethality (24) but also impairs spontaneous and DSB-induced conservative recombination without affecting cell viability (22). The physical interaction of HsRad51 with several tumor suppressor genes, namely, p53, BRCA1, and BRCA2, implies its possible role(s) in tumorigenesis (26, 48).

Here we describe a new approach to dissect protein interactions within the multiprotein complex and the application of this technique to the yeast recombination-repair complex. By
| Primers used in this study | Oligonucleotide | Sequence (5’ to 3’) |
|---------------------------|-----------------|--------------------|
| scRAD51-FOR               | CCGGATTCTGTAAGTCTCAAGTTCAAGAACAA |
| scRAD51-REV               | ACGCGTCGACTTACCTCAAGTTCAAGTTCAAGAACAA |
| scRAD52-FOR               | GGAGAATTCATTCGCGAAGATCGCATGCTTGAAGTTCAAGAACAA |
| scRAD52-REV               | ACGCGTCGACTTACCTCAAGTTCAAGTTCAAGAACAA |
| scRAD54-FOR               | GGAGAATTCATTCGCGAAGATCGCATGCTTGAAGTTCAAGAACAA |
| scRAD54-REV               | ACGCGTCGACTTACCTCAAGTTCAAGTTCAAGAACAA |
| scRAD51-crev              | GAATTCATTCGCGAAGATCGCATGCTTGAAGTTCAAGAACAA |
| scRAD51-39SD              | GTTTCGGATCATAAGATAAGTTCAAGAACAA |
| scRAD51-39SS              | GTTTCGGATCATAAGATAAGTTCAAGAACAA |
| scRAD51-R2               | GTTTCGGATCATAAGATAAGTTCAAGAACAA |
| disrup51-FOR              | AGCGACAAAGAGCAGACGTAGTTATTTTTTAAAGGCCTACTAATTTGT |

**TABLE 1. Primers used in this study**

**MATERIALS AND METHODS**

**Media and plasmids.** Yeast and bacterial media, as well as all the standard yeast genetic methods, were used as described previously (2). 5-Fluoroorotic acid medium was prepared by the method of Booke et al. (5). The vectors pGBT9 and pGAD10 have been described elsewhere (6). Coding sequences of RAD51, RAD52, and RAD54 were amplified from genomic clones by PCR using the primers scRAD51-FOR plus scRAD51-REV, scRAD52-FOR plus scRAD52-REV, and scRAD54-FOR plus scRAD54-REV, respectively (Table 1). The PCR products were digested with BamHI-Sall, EcoRI-Sall, and EcoRI-Pol, respectively, and cloned into the same site within GBT9 to generate pGBT9-RAD51, pGBT9-RAD52, and pGBT9-RAD54, respectively. pGBT9-RAD55 and pGAD10-RAD51 were kindly provided by R. Rothstein (University of California, Plasmids pRS413-rad51x, carrying different rad51 mutations, were constructed by inserting the BritEI-Bsu36I fragment from pGAD-rad51x into the BritEI-Bsu36I site of pRS413-RAD51 (kindly provided by L. Simmington, Columbia University). Plasmid pKD413-rad51A27V was constructed by ligating the StuI-Bsu36I fragment produced by PCR using disrupt51-FOR and scRAD51-R2 primers, with pGAD10-rad51A27V as template. pKD413-G393S and pKD413-G393D were produced by site-directed mutagenesis using primers scRAD51-393S and scRAD51-393D as forward primers and scRAD51-crev as a reverse primer. The resulting fragments were digested with NruI and cloned into the pRS413-RAD51 vector. All PCR products were isolated from agarose gels by Gene Clean II (Bio 101, Inc.), and the final constructs were verified by DNA sequencing.

**Yeast strains.** Yeast strains used to study two-hybrid interactions were CBY14.1a and CBY14.1a (ade2 his3 leu2 trp1 URA3:UASGalI-HIS3 gal4A gal80A LYS::UASGALI-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (4) and P669-a and P669-a (trp1 leu2 ura3 his3 gal4A gal80A LYS::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ) (16). The LMI strain used for rad51 complementation studies is a derivative of W303 (rad51::URA3 ade2 can1 his3 leu2 trp1 ura3) (5). The RAD51 gene was replaced with the URA3 gene by transformation of yeast cells with the PCR fragment of the URA3 gene with 60 bp of the RAD51 sequence at the 5’ and 3’ ends of the fragment, compatible with the sequence outside the operon reading frame. Yeast strains were transformed by the method of Gietz et al. (12).

**Detection of two-hybrid interactions.** Individual interactions were examined using isogenic CBY14.1a plus CBY14.1a and P669-a yeast two-hybrid strains. The diploids were selected on synthetic complete medium lacking Trp and Leu (SC-Trp-Leu) plates and replica plated to SC-Trp-Leu-His plates supplemented with 30 m M 3-amino-1,2,4-triazole. The cells were incubated at 30°C for 3 to 4 days and then subjected to a β-galactosidase assay (2). The β-galactosidase activity was quantified by the method of Guarente (14). Additional selection on SC-Trp-Leu-His was performed with the P669-a (4a) diploid strain since it carries the ADE2 reporter gene (16). To investigate the temperature sensitivity of the protein interactions, the same studies were done at two additional temperatures, 25 and 34°C.

**Mutational screen.** Mutations in the RAD51 gene were introduced by propagating the two-hybrid plasmid pGAD10-RAD51 through an Escherichia coli mutD5 mutant strain, GM4708 (35). Mutagenesis was induced by cultivation for different periods (16, 19, 21, and 24 h) in Luria-Bertani (LB) medium containing ampicillin (75 μg/ml). The mutation rate followed the Gaussian distribution, with an optimum at 20 h of incubation, and was determined by loss of function of the LEU2 plasmid to complement the leuB mutation in E. coli strain HB101. The mutated plasmid (pGAD10-rad51x) was used to transform the yeast strain CBY14.1a. Around 10,000 transformants were then manually patched on selective media so that each colony would be easily identified by its position, with its row and column numbers in a chess-like pattern. Eighty plates (140 mm) containing 131 individual colonies, were then replica plated onto four lawns with the CBY14.1a strain containing pGBT9-RAD51, pGBT9-RAD52, pGBT9-RAD54 or pGBT9-RAD55 fusion plasmids. Diploids were recovered on SC-Trp-Leu plates and afterwards replica plated on SC-Trp-Leu-His triple-dropout plates supplemented with 30 mM 3-amino-1,2,4-triazole and placed at 30°C. The cells were then subjected to a β-galactosidase filter assay, and pGAD10-rad51x plasmids were isolated from candidate colonies and subsequently sequenced. Due to the organized pattern of the colonies, each transformant was compared for its ability to interact with the RAD51, Rad52, Rad54, or RAD55 proteins, respectively. Thus, colonies where specific interactions were disrupted while others remained intact could be isolated.

**MMS sensitivity.** Transformants of each clone were grown to stationary phase in yeast extract-peptone-dextrose medium (YPD) and subjected to titer determination in four 10-fold serial dilutions with sterile water. Aliquots of each dilution were plated in duplicate on SC-His plates in the presence and absence of various concentrations of MMS. After preparation, the plates were immediately wrapped in foil to prevent evaporation, and they were used within 12 h. The cells were incubated in the dark at 30°C, and colonies were counted daily for 6 days.
RESULTS

Delineation of the two-hybrid interactions between members of the RAD52 epistasis group. Since the yeast two-hybrid system was capable of detecting at least some Rad51 interactions, we examined systematically which regions of Rad51 protein are required for these protein interactions. First, full-length versions of Rad51, Rad52, Rad54, or Rad55 protein (each fused to the binding domain of Gal4p) were transformed into a haploid strain of one mating type. Full-length Rad51 (fused to the activation part of Gal4p) was transformed into a haploid strain of the opposite mating type. Thereafter, the two haploid strains were mated and the diploids were examined for Rad51 interaction with each of the other constructs (Fig. 1B, lanes wt). In addition, several N- and C-terminal deletion mutants of Rad51 were assayed, and it was found that N-terminal deletion of 93 amino acids from Rad51 (residues 93 to 400) did not affect interaction with its partners (data not shown) with the exception of Rad54, whose interaction was substantially reduced (data not shown). In contrast, Rad51 constructs with larger N-terminal (residues 151 to 400) or C-terminal (residues 1 to 285) deletions failed to interact with any of its full-length partners.

Screen for interaction mutants. Since the truncation studies did not provide satisfactory detailed information about the interactive regions of the Rad51 protein, we modified the basic protocol of the two-hybrid screen. In this revised protocol, random mutations are introduced into the Rad51 protein, and each mutant is simultaneously tested for specific interaction with the Rad51-associated proteins. The Rad51 construct was mutagenized by passage through a mutD strain (35) and screened for mutations that disrupt specific interaction (see Materials and Methods). Thirty-seven diploid colonies showed detectable loss of Rad51 interaction with one or more partner proteins. To avoid the interference of mutations elsewhere in the plasmid, mutants were recloned into a new pGAD10 vector and the interaction assay was repeated; this left 35 mutants for further characterization. Sequence analysis of these clones identified 19 different mutants containing 22 mutational changes, 19 of which were transitions and 3 of which were transversions. Sixteen mutants represent multiple isolation of the same mutations, probably reflecting the clonal origin. No frameshift mutations were found, confirming the results from truncation studies (see above), which showed that even a small C-terminal deletion disrupted individual Rad51 interactions.

Two or more mutations in a single mutant were separately cloned into the pGAD10 vector to find which mutation caused the selected phenotype (data not shown). Finally, Western blots revealed no significant difference in the level of expression from two-hybrid vectors among any of the 19 rad51 mutants in the final set, with the exception of the G211S and A27V mutants, which are expressed at low levels or are proteolytically unstable (data not shown).

Individual mutants were grouped into seven classes, based on their ability to interact with wild-type Rad51 partner proteins or with the Rad52-K353E mutant protein (Fig. 1A). The K353E allele of the Rad52 gene was independently isolated in a screen for mutants with reduced affinity for wild-type Rad51 protein and can be suppressed by co-overexpression of RAD51 (data not shown). Three classes of mutations identified affect interactions of Rad51 with Rad52 and/or Rad54 (classes I to III). Mutants with class I and II mutations disrupt interactions with Rad52 (class I) and Rad54 (class II), respectively. Mutants with Class III mutations show disruption of the interaction with both Rad52 and Rad54 proteins. A fourth class of mutants disrupts the interaction of Rad51 with both Rad55 and Rad54. Class V mutants include those which activate the Rad51-Rad54 interaction. The last two classes include separately isolated mutants that either increase (class VI) or decrease (class VII) the interaction of Rad51 with the Rad52-K353E protein.

MMS sensitivity of rad51 mutants. To determine the effect of disruption of a specific protein-protein interaction on survival after DNA damage in vivo, we examined the sensitivity of all mutants to MMS. A single-copy vector containing different rad51 mutants (pRS413-rad51x) or the scRAD51 gene (pRS413-RAD51) was transformed into the rad51Δ strain, LM1. Cells transformed with scRAD51 were fully complemented, since an equal number of colonies appeared in the presence and absence of MMS (Fig. 2). Two of the three mutations (G210C and A248T) that disrupt the interaction of the Rad51 protein with both Rad52 and Rad54 (class III) conferred the highest sensitivity to MMS, indistinguishable from that of the rad51Δ strain (Fig. 2). The third mutant with the same phenotype (class III, G211S) showed higher resistance to MMS, probably reflecting an incomplete interaction deficiency, since some residual interaction is still observed (Fig. 1B, lane G211S). rad51 cells expressing the rest of the mutants conferred various degrees of sensitivity to MMS but did not complement the loss of Rad51. None of the mutants could fully complement the rad51Δ strain, suggesting the importance of both the Rad51-Rad52 and Rad51-Rad54 interactions for the DNA repair process.

Temperature sensitivity of Rad51 interactions. The interaction assays described above were all carried out at 30°C. We also examined wild-type Rad51 interactions and all interactions of mutant proteins with wild-type partner proteins at lower and higher temperatures, 23 and 34°C, respectively (Fig. 1A). None of the wild-type Rad51 interactions were as robust at 34°C as at the lower temperature, but the Rad51 self-association and the interaction of Rad51 with Rad55 were especially compromised. The mutants of class IV (L68P, L99P, and L104P), which abolish Rad51-Rad55 interaction, also disrupt Rad51 self-association at higher temperatures (Fig. 1A). Interestingly, all three mutations involve a change from leucine to proline, in close proximity to each other, suggesting severe structural changes within the Rad51 protein. This could also explain the isolation of the interaction-disrupting mutant (L68P) despite the inability to detect such a disruption when 93 residues from the N-terminus of Rad51 are deleted.
FIG. 1. (A) Classification of rad51 mutants based on their two-hybrid interaction with Rad51-associated partners. The plus signs indicate the two-hybrid interaction of rad51 mutant indistinguishable from wild-type Rad51. Black and red arrows depict disruption or activation of the corresponding interaction. Numbers in parentheses represent the average β-galactosidase activity (in Miller units) of three independent experiments. The ts superscript indicates the temperature-sensitive phenotype, where individual interaction is disrupted at 34°C. Rad52-K corresponds to the Rad52-K353E mutant protein. The colors representing individual classes are also used in other figures for easier identification. (B) The two-hybrid assay between individual Rad51 mutant proteins and wild-type Rad51, Rad52, Rad54, and Rad55 proteins. The mutants are grouped into classes according to disruption of the appropriate interaction as follows: class I, Rad52 disruption; class II, Rad54 disruption; class III, Rad52 and Rad54 disruption; class IV, Rad54 and Rad55 disruption; and class V, mutants that activate the interaction to Rad54. The cells were grown on selective medium (SC-Trp-Leu-Ade); two other reporter genes (HIS3 and lacZ) were used as additional controls of the appropriate interactions. wt, wild type.
Molecular modeling of the Rad51 protein. The positions of the 19 Rad51 mutations exhibit no obvious clustering along the linear sequence of the protein that might point to an interaction domain, with two exceptions (Fig. 3A). First, all three mutations that disrupt the Rad51-Rad55 interaction (class IV) are located within 20 residues in the N-terminal part of the protein, revealing a possible interaction domain or somewhat improper folding, since all three mutations involve conversions to proline residues. Second, all mutations that disrupt the interaction of Rad51 with Rad52 (class I and class II) are located in the C-terminal half of the protein.

A three-dimensional (3D) model of the Rad51 protein was constructed to reveal additional information about the possible interaction surfaces of the protein. Since Rad51 is 30% identical to RecA, we chose a structural model of the RecA protein as a template for our predictions. To find how well conserved the individual residues are in the RecA protein, all 64 members of the RecA family were aligned; this revealed that only the core of the RecA protein is conserved throughout the family (data not shown). Furthermore, homologous residues from the Rad51-RecA alignment also match the core region of the RecA protein (data not shown), allowing us to use the RecA structure as a template. The 3D model of the core of the Rad51 protein was generated by homology modeling (Fig. 4).

Comparison of the template structure with the homology model in terms of charge distribution and location of hydrophilic and hydrophobic residues revealed a very similar pattern (data not shown). There are N-terminal and C-terminal regions where no structure could be predicted, and there are a few insertions where the predicted geometry has low reliability. These regions were excluded from the analysis.

Rad52 binding domain. The identification of rad51 mutants that specifically disrupt Rad51-Rad52 and Rad51-Rad54 protein interactions, while other Rad51 interactions remain intact, indicates possible separate domains for these interactions. To address this theory, individual mutations were localized onto a 3D model of the Rad51 protein. Four of the mutations that disrupt the interaction with Rad52 (G210C, G211S, A248T, and A320V) can be aligned into this model, and they localize in a single region at the top of the model (Fig. 4A). Magnification of this domain (Fig. 4B) reveals the accessibility of this region for protein interaction, with the substitutions G210C and G211S possibly localized on a binding epitope. Three other class I mutations, affecting Rad52 specifically, lie at the C-terminal part of the Rad51 protein, whose structure could not be predicted in our model. We suggest that the C-terminal part might fold close to the predicted domain or, alternatively, that there might be two regions for interaction with Rad52p.

Rad54 binding domain. Of the 13 rad51 mutations which disrupt interaction with Rad54, 6 can be localized to a possible interacting region with an orientation similar to that of the Rad52 interaction domain (Fig. 4A and C). Among the seven mutations that disrupt interaction with Rad54 alone (Fig. 1, class II) and the three mutations that disrupt interaction with Rad54 and Rad52 (class III), five residues constitute a domain (G210C, G211S, S231P, A248T, and A320V). Residue L310S is possibly buried in the structure, and so its effect on the interaction might be rather indirect. For instance, a serine residue has quite a low propensity for inclusion in an α-helix.
FIG. 3. (A) Linear representation of mutations within Rad51 protein that affect interactions with the Rad51, Rad52, Rad54, and Rad55 proteins. Horizontal lines illustrate the amino acid sequence of the Rad51 protein. Vertical bars represent the mutations defective in the interactions with individual Rad51 partners. The blue and red bars indicate the mutations that disrupt the interaction with only Rad54 or Rad52, respectively. The black bars correspond to the mutations disrupting interaction with both Rad52 and Rad54 proteins. Mutations affecting Rad51-Rad54 as well as Rad51-Rad55 association are depicted by green bars. The yellow bars indicate the mutations that significantly increase the interaction with Rad51. The same Rad51 mutated residues were used to examine the interaction between the Rad51 and Rad52 mutant protein (Rad52-K353E). This mutant protein has lower overall affinity to the Rad51 protein and shows additional phenotypes with the rad51 mutants compared to wild-type Rad52. (B) Amino acid sequence of S. cerevisiae Rad51 protein (residues 1 to 400) aligned with the corresponding homologous part of the E. coli RecA protein (residues 33 to 239). Mutations affecting protein-protein interactions are shown above the Rad51 sequence with the same color coding as in panel A. Bold letters and stars indicate identical and similar amino acids, respectively.
and could significantly change the structure of the protein. The other seven mutations affecting the interaction to Rad54 cannot be localized in the Rad51 model. Six of these mutations are located at the N terminus, thus perhaps creating an additional binding site for this protein (Fig. 3A). Alternatively, the mutations could influence the conformation of this domain since they introduce proline residues into the Rad51 sequence (Fig. 1, class IV). Furthermore, the T146A mutation is located at a

FIG. 4. Model of the Rad51 protein with the proposed Rad52 interaction domain. (A) This model represents the core of Rad51 protein (residues 154 to 375), with localized mutations affecting the Rad51-Rad52 interaction shown in red. Mutations that could be localized within this model constitute the surface region accessible for the Rad52 protein. (B) The same Rad52 interaction domain viewed from the bottom. For this view, the Rad51 protein model in panel A was rotated 90° backward with respect to the viewer. (C) Location of the Rad54p interaction domain within the structural model of Rad51 protein. The Rad51 monomers with localized mutations that disrupt the interaction with Rad54 are indicated in purple. Residues in yellow indicate two mutations that increase the efficiency of Rad51-Rad54 interaction. (D) Model of one turn of a Rad51 filament, showing a highlighted monomer with the suggested Rad54 interaction domain. Note the opposite orientation of mutations disrupting and activating this interaction.
possible linker between the core and the N-terminal domain and A27V maps to the N-terminal domain, which does not have any effect on Rad51 interaction when deleted.

**Activation of the Rad51-Rad54 interaction.** Class V represents two mutants that activate the interaction with the Rad54 protein (Fig. 1B). Interestingly, both mutations cause a dramatic charge change, since residue E186 mutates to K and residue K342 mutates to E. Mutated residues in Rad51 are located very close to each other (distance, 12 Å), at a site close to the polymer axis, contrasting with the suggested Rad54 interaction domain (Fig. 4D). This axis region has been proposed to be involved in the binding of DNA (47) and ATP (40), suggesting that their binding could have similar effects.

Since electron microscopy also suggests an overall structural similarity of Rad51 and RecA protein filaments (34), a 3D model of the Rad51 polymer was constructed (Fig. 4D). Since RecA packs in the P61 crystals to form a polymer, the individual monomers of Rad51 protein were packed to form a continuous spiral of protein. Within the proposed polymer structure, the putative Rad54 binding domain is located at the outer accessible site of the polymer, opposite the mutations that activate the Rad54 interaction and the proposed DNA binding regions corresponding to the L1 and L2 disorder loops of RecA protein.

**Rad52 mutant protein affects interaction with Rad51.** A mutant of the Rad52 protein, with reduced affinity to wild-type Rad51 (Rad52-K353E), has also been combined with the above-described rad51 mutants (Fig. 1A and 3A). All mutations disrupting the interaction with wild-type Rad52 also affect the interaction with Rad52-K353E. In addition, four rad51 mutants that interact with wild-type Rad52 are defective in their interaction with this mutant protein (Fig. 1A, classes II and V). Other Rad51 mutations, A264T and I208T, isolated after the initial screen, also exhibit this property (Fig. 1A, class VII). The mutations all colocalize near the predicted Rad52 interaction domain, in the core of Rad51 protein, and probably further sensitize the already weakened Rad51–Rad52-K353E interaction.

A total of six Rad51 mutations exhibit enhanced interaction with Rad52-K353E (Fig. 1A, classes II and IV). Five of these mutations (L86P, L99P, G103E, L104P, and T146A) map to the N-terminal region, while one (C377Y) is located at the C-terminal end of the Rad51 protein (Fig. 3A). A second screen carried out to identify additional mutations of this type yielded two other alleles, V61M and C366T (class VI). Both mutations specifically activated Rad52 interaction, while other Rad51 interactions were not affected. The data suggest two possible interaction-enhancing and -stabilizing regions, one at the N terminus (spanning residues 81 to 146) and the other at the C terminus (spanning residues 366 to 377) (Fig. 3A). Interestingly, all mutations activating the Rad52-K353E interaction also inhibit Rad51 interaction with Rad54 and vice versa, suggesting opposite binding modes toward Rad51. These regions might stabilize Rad52 or Rad54 interactions and perhaps block the binding of the other protein.

**DISCUSSION**

The modified strategy of the two-hybrid system described above has allowed us to isolate rad51 mutations which specifically disrupt interactions between Rad51 and its associated proteins, including Rad51 itself. All of the Rad51 mutant proteins identified in this screen exhibit a reduced ability for DNA repair. In addition, most of the mutations are positioned close to or in the regions where the above proteins differ by deletion or insertion of residues. These regions, in turn, correspond to the turns and loops predicted in the structural model of Rad51p. This further indicates that the mutations probably do not disturb the whole structure but, rather, affect domains with some flexibility that are important for protein-protein interactions.

The MMS sensitivity is often partial compared to that of the rad51Δ strain, suggesting that some interaction remains. An alternative explanation may be that loss of the interaction can be compensated by the activity of another protein; the yeast Rad52 and Rad54 homologs could play such a role (3, 19, 45). In addition, the two alleles conferring the most severe MMS sensitivity disrupt the interaction with both Rad52 and Rad54, indicating that in these cases, cell survival is as low as in the rad51Δ strain. This clearly illustrates the importance of the identified protein-protein interactions in vivo, although some mutants may not have a direct effect on DSB repair, recombination, meiosis, etc.

Comparison of different Rad51 proteins from higher eukaryotes suggests that the same interaction mechanism might have been conserved throughout evolution. Indeed, there are reports describing some of these interactions in higher eukaryotes (13, 42). Intriguingly, the A320V mutation, resulting in disrupted interaction with Rad52, as well as a defect in DSB repair, is located only 3 amino acids away from the aligned human Rad51 F259V mutation, which was recently reported to impair binding with human Rad52 and to decrease homologous pairing (21). This comparison further supports the evolutionary conservation of Rad51 protein-protein interactions.

In contrast, all the mutated residues in Rad51 are only poorly conserved within the yeast Rad51-homologous proteins, Rad55 and Rad57. The absence of these residues may reveal a basic difference between the Rad51, Rad55, and Rad57 proteins, indicating that Rad55 and Rad57 have retained only part of the biological function of Rad51. Both are required in the presynaptic formation of the Rad51 nucleoprotein filament (53) and to gain access to otherwise inaccessible regions of chromatin (49). Furthermore, the Rad55 interaction was more temperature sensitive, being lost at 30°C, while the Rad51 interaction required higher temperatures (34°C) before disruption was apparent (Fig. 1A, class IV). This suggests a higher binding affinity of the Rad51 protein to itself than to Rad55. The fact that we were not able to isolate mutations that specifically disrupt only the process of self-association indicates the presence of a quite flexible binding surface, which might allow Rad51 to compensate for mutated residues. Intriguingly, at high temperatures, disruption of the Rad51–Rad55 interaction was always associated with disruption of the Rad51–Rad55 interaction, demonstrating their close relationship and suggesting that these interactions might be integral to the RecA-like filament of Rad51. We conclude that the Rad51 and Rad55 proteins share a similar but quite complex Rad51 interaction domain consisting of several flexible regions. The N-terminal part of Rad51 seems to be important for these interactions but
could reflect interfilament rather than direct subunit-subunit contacts.

To reveal individual interaction domains within the Rad51 protein, we constructed a 3D model based on structural data from the RecA protein. Mutations localized in this model indicate a possible core binding domain for both Rad52 and Rad54 proteins, situated on the outer face of the filament at a site accessible for protein interactions. The proteins may compete for this binding site, since the two domains partially overlap (compare Fig. 4A and C) on each side of a potential interaction “epitope” containing residues G210 and G211, while N- and C-terminal regions play a role in stabilization of individual interactions. We cannot confirm the localization of the Rad52 interaction domain at the N-terminal part of the protein, suggested by Donovan et al. (9), since not a single mutation disrupting the interaction with the Rad52 protein was found in this region. This is in agreement with recent data from Kurumizaka et al. (21), showing that the N-terminal part of human Rad51 protein also does not exhibit binding to the human Rad52 protein.

Apart from the suggested binding domains, there are also other regions that might affect these interactions. In the Rad52 and Rad54 interactions with Rad51, there are other mutations in the C- and N-terminal regions, respectively, that might be directly involved in the binding process or stabilize these interactions. In particular, the N terminus constitutes a separate domain (1), and we suggest that mutations here alter conformation, resulting in blocked access of the Rad54 protein to the binding site. Surprising results were found for the Rad51-Rad54 interaction, where two interaction-activating mutations were isolated (E186K and K342E). Both activators reverse the charge of the wild-type residues and localize to the inner part of the filament. Interestingly, the E186K mutation corresponds to residue P67 in the P-loop of RecA (Fig. 3B), a region responsible for the binding of ATP (40), and specific substitution of this residue results in separation of RecA functions (20). In addition, the K342E mutation corresponds to residue T210 of RecA located between the L2-loop, which is proposed to be a DNA binding domain (47), and α-helix G, which is proposed to be involved in conformational change following ATP hydrolysis (38). Therefore, the effects of mutated residues on the efficiency of the interaction might mimic the transition between alternative conformations as a part of a chain of events, in this case represented by the binding of ATP (29, 30), affecting the binding efficiency of the Rad54 protein. Determining the conformation of interacting amino acids may be a common strategy by which distal elements influence specific affinity.

In the Rad51 interaction with Rad52-K353E, compensatory mutations were found. These suppressor mutations, which increase the efficiency of interaction with Rad52-K353E are located within residues 86 to 104 and 366 to 377 at the N- and C-terminal regions of Rad51, respectively. Interestingly, the mutations activating the Rad51 interaction with either Rad52-K353E or Rad54 proteins are often antagonistic; e.g., mutations increasing the affinity to Rad54 disrupt the association of Rad51 with Rad52-K353E, and Rad51 activators of the Rad52-K353E interaction often disrupt the Rad54 interaction. The presence of these activating mutations strongly argues for competition between Rad52 and Rad54 for their overlapping binding sites, suggested by the 3D model superposition.

In summary, the data indicate that the assembly of the whole complex and/or cycle of Rad51 reaction might be sequential with competitive binding sites. Binding of a substrate, ATP, or the protein components of the recombination-repair complex might alternate conformations within the Rad51 protein that could play a role in stabilization of the complex and/or in the accessibility of Rad51 to other proteins. Rad51 is much less active than RecA protein in its reactions (43, 54), indicating that an additional cofactor(s) alters Rad51 activity. Indeed, Rad52 probably stimulates the action of Rad51 by displacing the RPA from ssDNA during the formation of the Rad51 nucleoprotein filament (33, 44), the Rad55-Rad57 heterodimer promotes strand exchange activity (53), and Rad54 promotes Rad51-mediated homologous DNA pairing (37). Therefore, protein-protein interaction seems to be essential for nucleation as well as for the proper functioning of Rad51. The basis for a model of Rad51 assembly might be as follows: (i) RPA binds to the resected ssDNA tails; (ii) Rad52 nucleates Rad51 to this site, together with the Rad55-Rad57 heterodimer; (iii) the Rad51 filament is assembled in a cooperative manner, displacing the RPA from binding sites on ssDNA; (iv) after assembly, the Rad51 nucleoprotein filament permits Rad54 to bind, which stimulates Rad54-mediated ATP hydrolysis and DNA strand separation (27); and (v) consequently, Rad54 stimulates Rad51 to overcome kinetic impediments limiting homologous pairing. This highlights the fact that to comprehend the mechanism of homologous recombination, one needs to study both the single reactions performed by individual proteins and the multistage reactions catalyzed by a set of collaborating proteins. Finally, the above-described mutants could facilitate dissection of the individual steps in the assembly of recombination-repair proteins and permit detection of intermediate stages in the process of homologous recombination. In particular, they will permit assessment of the function or temporal order of specific interactions. It is clear that other biochemical and/or genetic studies will confirm or specify the role of individual Rad51-mediated protein interactions and result in additional insight into these reactions.

This novel strategy of selecting the mutants based on interaction phenotype rather than loss of function provides an important tool for the dissection of other complex multiprotein structures. The strategy allows a determination of the interaction regions and an identification of the effects of individual components on the biochemistry of the process. Furthermore, the activation of the interaction between Rad52 and Rad54 with Rad51 suggests the possibility of isolation of compensatory mutations within these two proteins. This work can be further developed, and preliminary results indicate that the interactions could be switched off and on as desired.

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