Vital roles of the second DNA binding site of Rad52 in yeast homologous recombination

Naoto Arai 1*, Wataru Kagawa 2, Kengo Saito 2, Yoshinori Shingu 3,4,
Tsutomu Mikawa 3,4,5, Hitoshi Kurumizaka 5 and Takehiko Shibata 3,4*

1 Department of Applied Biological Science, Nihon University College of Bioresource Sciences, Fujisawa-shi, Kanagawa 252-0880, Japan.
2 Laboratory of Structural Biology, Graduate School of Advanced Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan.
3 Cellular and Molecular Biology Laboratory, RIKEN Advanced Science Institute, Wako-shi, Saitama, 351-0198, Japan.
4 Department of Supramolecular Biology, Graduate School of Nanobioscience, Yokohama City University, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan.
5 RIKEN SPring-8 Center, 1-1-1 Kouto, Sayo, Sayo, Hyogo 679-5148, Japan.

Running head: DsDNA-binding to Rad52 in Rad51-dependent homologous recombination

* Address correspondence to: Naoto Arai, Department of Applied Biological Science, Nihon University College of Bioresource Sciences, 1866 Kameino, Fujisawa-shi, Kanagawa 252-0880, Japan. E-mail: narai@brs.nihon-u.ac.jp; Takehiko Shibata, Cellular & Molecular Biology Laboratory, RIKEN Advanced Science Institute, Hiroswawa 2-1, Wako-shi, Saitama 351-0198, Japan, Fax. 81-48-462-4676; E-mail: tshibata@postman.riken.go.jp

RecA/Rad51 proteins are essential in homologous DNA recombination, and catalyze the ATP-dependent formation of D-loops from a single-stranded DNA and an internal homologous sequence in a double-stranded DNA. RecA and Rad51 require a “recombination mediator” to overcome the interference imposed by the prior binding of SSB/RPA to the single-stranded DNA. Rad52 is the prototype of recombination mediators, and the human Rad52 protein has two distinct DNA binding sites: the first site binds to single-stranded DNA and the second site binds to either double- or single-stranded DNA. We previously showed that yeast Rad52 extensively stimulates Rad51-catalyzed D-loop formation even in the absence of RPA, by forming a 2:1 stoichiometric complex with Rad51. However, the precise roles of Rad52 and Rad51 within the complex are unknown. In the present study, we constructed yeast Rad52 mutants in which the amino acid residues corresponding to the second DNA binding site of the human Rad52 protein were replaced with either alanine or aspartic acid. We found that the second DNA binding site is important for the yeast Rad52 function in vivo. Rad51-Rad52 complexes consisting of these Rad52 mutants were defective in promoting the formation of D-loops, and the ability of the complex to associate with double-stranded DNA was specifically impaired. Our studies suggest that Rad52 within the complex associate with double-stranded DNA to assist Rad51-mediated homologous pairing.

Homologous DNA recombination plays a critical role in the repair of DNA double-strand breaks in mitotic cells (1-4). D-loops that include heteroduplex joints are general intermediates of homologous recombination. D-loop formation is the base pairing of an invading single-stranded DNA (ssDNA) tail, derived from one end of the DNA double-strand break, with its complementary sequence in an internal region of a homologous double-stranded DNA (dsDNA), which serves as the template to repair the break. RecA of Escherichia coli is the prototype of homologous pairing proteins that catalyze ATP-dependent D-loop formation (5,6), and Rad51 is an orthologue of RecA in eukaryotes (7-9). Following the D-loop formation, RecA/Rad51 promotes the extension of a core heteroduplex joint, by displacing a strand of the parental dsDNA with the invading ssDNA (branch migration) in an ATP hydrolysis-dependent reaction (5,10,11). D-loop formation is observed in all domains of life. RecA catalyzes this reaction in prokaryotes (5,6), RadA catalyzes the reaction in Archaea (12), and Rad51 and Dmc1 catalyze the reaction in eukaryotes (8,13-18).

When an ssDNA region is formed after double-stranded breakage, the ssDNA is covered by a single-strand binding protein, SSB (single strand binding protein) in bacteria and RPA (replication protein A) in eukaryotes. The binding of SSB or RPA to ssDNA stimulates, or is
required for D-loop formation by RecA or Rad51 in order to unfold the secondary structures of the ssDNA, especially when the ssDNA is long enough to fold tightly (8,19,20). However, the prior binding of SSB or RPA to ssDNA inhibits the initial binding of RecA or Rad51. Cells have a family of proteins called recombination mediators, which load RecA or Rad51 onto ssDNA by overcoming the inhibitory effects of SSB or RPA (21,22). Rad52, which is essential for homologous recombination in Saccharomyces cerevisiae, is the prototype of the mediators (21), and is ubiquitous in various eukaryotes, except for plants and fly. The yeast Rad52 physically interacts with RPA and Rad51, and facilitates the displacement of RPA by Rad51 (23,24). In addition to its mediator function, Rad52 also possesses potent ssDNA annealing activity in vitro (25). This activity is believed to be important for capturing the second end of the double-strand break. Precisely, after the 3' ssDNA tail derived from the first end of the double-strand break has been incorporated into a D-loop and repair DNA synthesis initiated at the 3' termini enlarges the D-loop, the second end is annealed with the displaced strand of the D-loop. Thus, Rad52 is considered to primarily function on ssDNA.

Although Rad52 has been shown to bind to dsDNA, the functional relevance of this activity is unknown. A crystallographic study combined with mutational analyses of human Rad52 revealed two distinct DNA binding sites (26,27). The first site, located inside the groove that runs around the ring structure, is a binding site for ssDNA. The second site is located at the entrance of the groove, and is a binding site for dsDNA or ssDNA. In the first site, arginine-55 (R55) is the key residue for ssDNA binding. Mutating this residue greatly impairs the ssDNA binding activity of human Rad52. The corresponding residue in yeast Rad52 is R70, and in vivo studies revealed homologous recombination deficiencies in yeast when this residue was mutated (28,29). Subsequent studies suggested that R70 is important for the annealing activity of Rad52, which is required for the second end capture of the double-strand break (30-33). On the other hand, the second DNA binding site of human Rad52 is composed of lysine-102 (K102) and lysine-133 (K133), and in vitro studies revealed that these residues are important for the ssDNA annealing activity of Rad52. However, no clear in vivo defects have been demonstrated yet for this DNA binding site.

We previously reported that the yeast Rad51 and Rad52 proteins form a stoichiometric 2:1 complex, and that the complex promotes D-loop formation efficiently (34). The D-loop formation promoted by the complex does not appear to involve the mediator and ssDNA annealing activities of Rad52. This observation led us to investigate the possible role of the putative second DNA binding site of yeast Rad52 in the D-loop formation activity of the complex. In the current study, we found that yeast Rad52 also harbors the second DNA binding site identified in human Rad52. The DNA binding site was important for homologous recombination in yeast cells, and was indispensable for D-loop formation by the Rad51-Rad52 complex. Based on these results, we discuss the possible roles of the second DNA binding site of Rad52 in yeast.

Materials and Methods

Enzymes and reagents - Taq DNA polymerase, T4 polynucleotide kinase, calf intestine alkaline phosphatase, and all restriction endonucleases were purchased from Takara Shuzo Co., Ltd. Pfu polymerase was obtained from Promega Co. Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories. The micro Spin S-400 HR column, the Probe Quant G-50 Micro Column, and the Q-Sepharose FF, Mono Q HR5/5, and Sephacryl S-300 HR matrices were obtained from GE Healthcare Biosciences.

Yeast strains - Saccharomyces cerevisiae XS560-1C-1D2 (a, rad52-8::TRP1, leu2-3,112, trp1-Δ, ura3-52, his3-Δ, can1) was provided by Dr. Munenori Furuse (RIKEN); YPH499 (a, ura3-52, lys2-801 amber, ade2-101 ochre, trp1-Δ63, his3-Δ200, leu2-Δ1) was obtained from Stratagene.

Standard reaction buffer - The standard reaction buffer consisted of 30 mM MOPS-KOH (pH 7.1), 12 mM MgCl₂, 50 mM KCl, 1 mM dithiothreitol, 50-100 µg/ml bovine serum albumin, and 0.02% (v/v) IGEPAL CA630 (SIGMA), supplemented with 2 mM ATP, unless otherwise stated.

DNA - DNAs used in these experiments were prepared as described previously (34). The DNA concentrations are expressed as the concentrations of nucleotide residues in the DNA. The followings are the details of each DNA species.

Negatively supercoiled dsDNA (Form I DNA) - We prepared pUC119 and pNS11 form I DNA
by the previously described methods (35), which
included the disruption of cells with lysozyme-
and Sarcosyl-treatments, phenol-extraction, and
purification by sucrose-density gradient
centrifugation. Note that, to avoid the induction
of non-B form structures in negatively
supercoiled DNA and to minimize contamination
and damage, the purification procedure for the
form I DNA was devoid of denaturation, gel
electrophoresis and contact with intercalators,
such as ethidium bromide. The pNS11 plasmid is
a pUC119 derivative containing ARG4 and part of
the DED81 gene of S. cerevisiae (see 34).

\[ pUC119 \text{ circular ssDNA} \]

We prepared pUC119 circular DNA by the previously
described methods (35).

\[ \text{Cy5- or biotin labeled pNS11 linear ssDNA} \]

\[ \text{fragment} \]

The pNS11 ssDNA\text{259 mer} that contained a downstream 20-base region of a
meiotic double-strand break site in ARG4 in S.
cerevisiae (36), was prepared as described
previously (34). Cy5-labeled pNS11 ssDNA\text{259 mer}
was prepared by amplification using a
5'-Cy5-labeled AG4sfD primer (prepared by
SIGMA-GENOSYS), as previously described
(34). The 5'-biotinylated pNS11 ssDNA\text{120 mer}
was purified by polyacrylamide gel electrophoresis
(SIGMA-GENOSYS).

\[ \text{Preparation of rad52K117A/R148A and}\]

\[ \text{rad52K117D/R148D ORF} \]

Both the lysine-117 and arginine-148 residues (numbering from the
first ATG) of the wild type Rad52 on pNS145 were replaced by alanine (K117A/R148A) or
aspartic acid (K117D/R148D), using a
QuikChange Site-Directed Mutagenesis Kit
(Stratagene). The replacement of both the AAG
(K117) and AGA (R148) codons with the GCG
(alanine) or GAC (aspartic acid) codon was
confirmed by DNA sequence analyses.

\[ \text{Construction of the plasmid for Rad52}\]

\[ \text{expression in yeast cells} \]

The expression vector for the mutant and wild type Rad52 in S.
cerevisiae, containing the ADH1 promoter and terminator, ARSH4-CEN6 (a yeast centromere
sequence, CEN, and autonomously replicating sequence, ARS: 37) and the LEU2 marker
(named pNS31), was constructed by inserting the
BamHI fragment containing the ADH1 promoter and terminator of pAURI23 (Takara Shuzo Co.,
Ltd.) into the PvuII site of pRS415 (Stratagene).

The BamHI fragment was blunt-ended by a treatment with Klenow fragment before insertion.

The open reading frame of wild type RAD52
(pNS145), rad52K117A/R148A (pNS178) or
rad52K117D/R148D (pNS179) on pET3a for expression in E. coli (see below) was removed by
EcoT114I-digestion and partial digestion with
NdeI, and then blunt-ended by a treatment with
Klenow fragment. The fragment containing the
open reading frame of rad52 was inserted in the
Smal site of pNS31. The cells of the rad52Δ strain (XS560-1C-1D2) were transformed with
the plasmids.

\[ \text{Spot test for methyl methanesulfonate (MMS)}\]

\[ \text{sensitivity of rad52Δ transformants expressing}\]

\[ \text{mutant rad52} \]

The SD liquid medium (2% glucose, and 0.67% yeast nitrogen base without
amino acid (Difco)) for the spot test was supplemented with 2 µg/ml uracil and 2 µg/ml histidine. Cells from an overnight culture (10 ml)
were concentrated to 1 ml by centrifugation at 200xg for 3 min. The number of cells was counted under a microscope using an Improved
Neubauer hemocytometer, and was adjusted to
approximately 10^5 cells/µl. Aliquots (10 µl) of a
10-fold dilution series of each transformant were
spotted onto SD plates containing 2 µg/ml uracil
and 2 µg/ml histidine with 0.25, 0.59 or 1.18 mM
MMS. The plates were sealed with Parafilm and
incubated at 30°C for 5 days.

\[ \text{Quantitative tests for MMS sensitivity of}\]

\[ \text{rad52Δ transformants expressing mutant rad52} \]

The SD liquid medium for quantitative analysis
was supplemented with 4 µg/ml adenine sulfate,
2 µg/ml uracil, 2 µg/ml histidine, 4 µg/ml lysine,
and 4 µg/ml tryptophan (and 6 µg/ml leucine for
YPH499 strain without the plasmid). Cells from
an overnight culture (10 ml) were concentrated to
1 ml by centrifugation at 200xg for 3 min. After
the dilution of the cell cultures, the cells were
spread on freshly prepared SD plates containing
2 µg/ml uracil and 2 µg/ml histidine with 0.25, 0.59 or 1.18 mM
MMS. The plates were sealed with Parafilm and
incubated at 30°C for 7 days.

\[ \text{Purification of mutant and wild type Rad52}\]

The wild type Rad52 and the mutant proteins,
rad52K191A/R148A and rad52K191D/R148D, were expressed from the third ATG codon of the cloned RAD52 on pET3a (pNS145) in E. coli
BLR (DE3). Rad52 was purified as described
previously (34); i.e., disruption by lysozyme-treatment of the expressing cells,
determined from the extinction coefficient, the molar concentration of Rad52 was rad52K191A/R148A; 101 µM rad52K191D/R148D). The concentration of RPA was determined based on the extinction coefficient (8.8x10^4 M^-1 cm^-1 at 280 nm) reported by Sugiyama et al. (39). It is noted that the purified RPA is the authentic yeast RPA produced in yeast cells.

The natural form of RPA was purified from S. cerevisiae YPH501 (α/α, ursa-5,2, lys2-801 amber, ade2-101 ochre, trp1-Δ63, his3-Δ200, leu2-Δ1) as described by Alani et al. (40), except the DEAE 650M step was replaced by a Mono Q step. The purified Rad52 preparation was dialyzed against storage buffer, and then 20 µl-aliquots were frozen by dipping into liquid nitrogen, and stored at –80°C. The molar concentration of Rad51 was determined from the extinction coefficient (2.42x10^4 M^-1 cm^-1 at 280 nm) reported by New et al. (38). The mutant and wild type Rad52 does not have tags of any kinds.

**Purification of Rad51** - The wild type Rad51 (cloned in pET3a) proteins were prepared by a procedure consisting of expression in E. coli BLR (DE3), cell disruption by lysozyme- and Brij58-treatments, polymin P-precipitation and ammonium sulfate-fractionation, and a series of column chromatography steps on Q-Sepharose FF, Hydroxyapatite, Sephacryl S300HR, and Mono Q, as described previously (34). The purified Rad52 preparation was dialyzed against storage buffer, and then 20 µl-aliquots (final concentration 170 µM) were stored at –80°C. The molar concentration of the Rad51 was determined from the extinction coefficient (1.29x10^4 M^-1 cm^-1 at 280 nm) reported by Sugiyama et al. (39). The Rad51 does not have tags of any kinds.

**Purification of RPA (replication protein A)** - The natural form of RPA was purified from S. cerevisiae YPH501 (α/α, ursa-5,2, lys2-801 amber, ade2-101 ochre, trp1-Δ63, his3-Δ200, leu2-Δ1) as described by Alani et al. (40), except that the DEAE 650M step was replaced by a Mono Q step. The purified RPA was dialyzed against storage buffer containing 0.02% IGEPAC CA630, and ammonium sulfate-fractionation, and a series of column chromatography steps, using SP-Sepharose FF, Hydroxyapatite, Sephacryl S300HR, and Mono Q, as described previously (34). The purified Rad52 preparation was dialyzed against storage buffer, and then 20 µl-aliquots were frozen by dipping into liquid nitrogen, and stored at –80°C.

The concentration of RPA was determined based on the extinction coefficient (8.8x10^4 M^-1 cm^-1 at 280 nm) reported by Sugiyama et al. (39). We confirmed that the purified RPA stimulated Rad51-promoted strand-exchange between circular ssDNA and linear dsDNA (data not shown). It is noted that the purified RPA is the authentic yeast RPA produced in yeast cells.

**DNA binding assay** - DNA (0.8 µM Cy5-labeled pNS11 ssDNA259_mer or 15 µM pUC119 form I DNA) was incubated with Rad52 in the standard reaction buffer (10 µl), except that it contained 20 mM KCl and lacked both MgCl₂ and ATP, at 37°C for 10 min. The free and bound DNA were separated by 1% SeaKem GTG agarose (LONZA Group Ltd.) gel electrophoresis with TAE buffer, and analyzed by Typhoon 9410 image analyzer for the Cy5-labeled pNS11 ssDNA259_mer or a Gel Doc XR system (Bio-Rad Laboratories) for the form I DNA, after ethidium bromide staining.

**ATPase assay** - [α-³²P]ATP (0.1 mM) was incubated with 0.88 µM RPA and 5.0 µM pUC119 circular ssDNA in the standard reaction buffer (10 µl), but containing 7 mM MgCl₂, at 37°C for 10 min. After the incubation, 1.0 µM Rad51 and the indicated concentrations of Rad52 were added to the reaction mixture, which was further incubated for 30 min. The reaction was terminated by adding 10 µl of stop solution (25 mM EDTA, 3 mM ATP, 3 mM ADP, 3 mM AMP). Aliquots (15 µl) of the sample were spotted on polyethylenimine plastic film (POLYGRAM CELL 300 PEI for TLC, MACHERY-NAGEL GmbH & Co.), and developed in a mixture of 0.5 M lithium chloride and 1 M formic acid (41). After the plastic film was exposed to a Phosphor Screen (GE Healthcare Bioscience) for approximately 1 hr, the radioactivity was analyzed by a Typhoon 9410 Variable Image Analyzer.

**Immunoprecipitation with an anti-Rad51 antibody** - The purified anti-Rad51 antibody (90 µg: 34) was mixed with 330 µl of Protein A agarose bead suspension (CALBIOCHEM) in PBS-0.05% IGEPAL CA630 (total 1.8 ml), and gently shaken at 4°C for 16 hr. The beads were washed twice with 1 ml of 200 mM triethanolamine (pH 8.2), and were suspended in 500 µl of the same buffer containing 20 mM dimethyl pimelimidate (DMP, purchased from PIERCE Biotechnology, Inc.), followed by an incubation at room temperature for 30 min to form a covalent bond between the antibody and the protein A. To stop the reaction, the beads were collected by centrifugation at 3,000 rpm for 1 min at room temperature with a microcentrifuge, resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, and incubated at room temperature for 15 min. After washing 3 times with 1 ml TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% IGEPAL CA630), the beads were resuspended in 1 ml of TBS.

Rad52 (62.5 pmol) and Rad51 (25 pmol) were mixed in the storage buffer (total 5 µl) using a Protein LoBind Eppendorf-tube (Eppendorf AG), and then incubated on ice for 30 min for...
complex formation. After 100 µl of TBS and 100 µl of the anti-Rad51 antibody-protein A agarose beads were added to the Rad52 and Rad51 mixture, the mixture was incubated at room temperature for 1 hr with mixing. The beads were gently washed twice with 200 µl of TBS, and the proteins captured by the anti-Rad51 antibody were eluted by suspending the beads in 12 µl of sample buffer (3% SDS, 100 mM DTT, 10% glycerol, 0.02% BPB and 65 mM Tris-HCl (pH 6.8)) and heating for 1 min at 90-95°C. Aliquots (10 µl) were fractionated by polyacrylamide gel electrophoresis in the presence of SDS, and the proteins were stained with Coomassie Brilliant Blue. The optical densities of the protein-bands were measured by the Gel Doc XR system.

Circular dichroism (CD) spectra – CD spectra of wild-type and mutant Rad52 were measured at 25 °C in the storage buffer and 10 µM wild type or mutant Rad52, by use of the Jasco J-720 spectropolarimeter. The light path length was 1 mm. [9] is the observed molar ellipticity in mdeg.

D-loop assay - D-loops were analyzed by detecting the radioactive signals from ssDNA that was paired with homologous dsDNA after agarose gel-electrophoresis, as described previously (34). Unless otherwise stated, the reaction mixture for D-loop formation (final 20 µl) contained 0.4 µM [32P]pNS11 ssDNA259 mer, 60 µM pNS11 form I DNA, 0.4 µM Rad51, and 1.0 µM Rad52 in the standard reaction buffer. The complex of Rad52 and Rad51 was allowed to form by mixing Rad52 and Rad51 in the storage buffer and incubating them for at least 30 min on ice. Under the standard conditions for D-loop formation, the standard reaction mixture (19 µl), containing Rad52, Rad51 and ssDNA, was incubated at 37°C for 10 min without the form I DNA. Then, D-loop formation was initiated by the addition of 1 µl of 1,200 µM pNS11 form I DNA, and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 1.7 µl of 0.5 M EDTA and 1.3 µl of 10% SDS, and deproteinized by incubating the mixture at 37°C for 15 min after the addition of 2 µl of 0.25% bromphenol blue-50% (v/v) glycerol were added to the reaction products, which were separated by electrophoresis for 30 min at 5 V/cm on a 1% agarose gel with TAE buffer (40 mM Tris-acetate (pH 8.0), 1 mM EDTA). The gel was dried on Gel Bond film (BMA Inc.), and was exposed to a Phosphor Screen (GE Healthcare Biosciences) for approximately 16 hr. The distribution of radioactivity was analyzed with a Typhoon 9410 Variable Image Analyzer (GE Healthcare Bioscience). The amounts of D-loops formed were determined by the shift of the radioactivity derived from the [32P]pNS11 ssDNA259 mer to the position of pNS11 form I DNA.

Recovery of protein-ssDNA complexes with magnet-beads - pNS11 ssDNA120 mer bound to Dynabeads was prepared by the incubation of 5'-biotinylated pNS11 ssDNA120 mer (0.4 µM) and 2 µl of a Dynabead M-280 streptavidin suspension (streptavidin-associated magnetic beads, Dynal), in 100 µl of the standard reaction buffer containing 0.05% IGEPA CA630, for 20 min at room temperature. Previously mixed Rad52 (1.0 µM) and Rad51 (0.4 µM) were incubated with the pNS11 ssDNA120 mer bound to the Dynabeads in the standard reaction buffer (final 100 µl) at 37°C for 10 min, in a Protein LoBind Eppendorf-tube. The pNS11 ssDNA120 mer ssDNA bound to the Dynabeads was recovered from the mixture by the use of a magnet. Sample buffer (10 µl) was added to the recovered Dynabeads and heated for 1 min at 90-95°C. After applying the magnet again, the proteins in the supernatant were fractionated by polyacrylamide (10%) gel-electrophoresis in the presence of SDS, and stained with Coomassie Brilliant Blue. The optical densities of the protein-bands were measured by the Gel Doc XR system.

Analyses of form I DNA and proteins in the Rad52-Rad51-ssDNA-form I DNA complex (ternary complex) - pNS11 ssDNA120 mer-Dynabeads were prepared by the method described above, with the following modifications. A magnet was applied to 140 µl of a Dynabead M-280 streptavidin suspension (Dynal), and the beads were recovered. To the beads, a solution (280 µl) consisting of 2 µM 5'-biotinylated pNS11 ssDNA120 mer, 10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 50 mM KCl, and 0.1% IGEPA CA630 was added, and the mixture was incubated at room temperature for at least 20 min. The pNS11 ssDNA120 mer-Dynabead suspension (20 µl) was added to 75 µl of the standard reaction buffer, containing 0.1% IGEPA CA-630 and previously mixed Rad52 (final 1.0 µM) and Rad51 (final 0.4 µM), in a Protein LoBind Eppendorf-tube and incubated at 37°C for 10 min. After 5 µl of 220 µM pUC119 form I DNA was added to the mixture, the incubation was continued for 10 min. After the incubation, the Dynabeads were immediately
recovered by applying a magnet. The Dynabeads were suspended in 15 μl of stop solution (1.5% SDS/30 mM EDTA) and incubated at room temperature for at least 5 min. After applying the magnet again, aliquots of the supernatant (3 μl and 10 μl) were used for the analysis of the form I DNA and the proteins (Rad52 and Rad51), respectively. For the analysis of the form I DNA, 6 μl of 200 μg/ml proteinase K were added to 3 μl of the supernatants and incubated at 37°C for 15 min. After the addition of 1 μl orange G-dye (0.5% orange G and 50% glycerol), the supernatant was loaded onto a 1% SeaKem GTG agarose gel and the DNA molecules were separated by electrophoresis.

The amounts of the form I DNA and form II DNA, which were contained in the form I DNA preparation, were measured by the Typhoon 9410 imager after staining for 10 min with 1.0 μg/ml ethidium bromide and destaining with distilled water for 1-2 h.

For the analysis of the Rad52 and Rad51 proteins, 2 μl of a dye-DTT solution (0.1% bromophenol blue/25% glycerol/100 mM dithiothreitol) were added to 10 μl of the supernatants and heated for 2 min at 90-95°C, and 10 μl of the supernatants were analyzed by SDS-PAGE (10% acrylamide). After staining by Coomassie Brilliant Blue R250, the optical densities of the protein bands were measured by the Gel Doc XR system.

**Results**

Amino acid replacements, K117A/R148A and K117D/R148D, in yeast Rad52 cause deficiencies in homologous recombination repair in vivo - The conserved, N-terminal domain of human Rad52 has two distinct DNA binding sites along the outside of the ring. The first site is located at the bottom of the groove formed around the stem of the ring (26,27), and appears to be specific for ssDNA. The second DNA binding site accommodates either dsDNA or ssDNA, and is located at the opening of the groove. Lysine-102 (K102) and lysine-133 (K133) are key residues located within the second DNA binding site, and have been shown to directly interact with dsDNA (27). The K102 and K133 residues of human Rad52 correspond to the lysine-117 (K117) and arginine-148 (R148) residues, respectively, of *S. cerevisiae* Rad52 (Fig. 1A: 26). These basic amino acid residues are located within the stem region of the β-β-β-α fold made up by a highly conserved amino acid sequence among Rad52 of human, yeast and other eukaryotes and yeast Rad59 (Fig. 1A: 26). Note that the amino acids for yeast Rad52 are counted from the first ATG codon closest to the native *RAD52* promoter of *S. cerevisiae* (29), and that the third ATG codon is the translation-start site of the native Rad52 (42,43). In order to understand the roles of these basic residues in *in vivo* functions of Rad52, we introduced mutations in the yeast *RAD52* that replaced both K117 and R148 with either alanine or aspartic acid (K117A/R148A or K117D/R148D, respectively). We separately expressed the two mutant and wild-type Rad52 proteins in rad52-defective yeast cells, using a vector containing a yeast centromere sequence (*CEN6*) and an autonomously replicating sequence (ARS). While the expression of the wild-type Rad52 fully restored the resistance of the rad52 null cells to the methyl methanesulfonate (MMS), the expression of either rad52 mutant only slightly restored the MMS resistance (Fig. 1, B and C). Thus, the cells expressing the rad52 mutants were much more sensitive to MMS, as compared to the cells expressing the wild-type Rad52. The K117D/R148D replacement caused a more severe phenotype than the K117A/R148A replacement (Fig. 1, B and C). These results suggest that the region in yeast Rad52 corresponding to the second DNA binding site of human Rad52 is important for homologous recombination repair in yeast.

K117 and R148 in yeast Rad52 are important for dsDNA binding - We next purified the two mutant rad52 proteins containing the dual amino acid replacements (K117A/R148A and K117D/R148D) that were overexpressed in *Escherichia coli* cells (Fig. 2A). We note that wild type and mutant Rad52, as well as Rad51, were expressed without purification tags, and were purified to near homogeneity. Gel-mobility shift assays revealed that the K117A/R148A and K117D/R148D mutants were slightly defective in ssDNA binding (Fig. 2C), and significantly defective in dsDNA binding (Fig. 2D). The gel profiles of ssDNA and dsDNA binding by the mutant rad52 proteins did not significantly change at KCl concentrations between 20 and 100 mM (data not shown).

Previously, human Rad52 was shown to form ternary complexes with ssDNA and dsDNA (26,27). In the complex, the dsDNA was shown to bind to the second DNA binding site. We therefore investigated whether the corresponding
location in yeast Rad52 also exhibited DNA binding properties similar to those of human Rad52. To do so, the rad52 mutants were first incubated with biotinylated pNS11 ssDNA120-mer. Afterwards, dsDNA (plasmid DNA) lacking sequence homology with the ssDNA was added to the Rad52-ssDNA complex. The biotinylated ssDNA was captured with streptavidin-conjugated, magnetic beads (Dynabeads), and the presence of dsDNA in the captured fraction was confirmed by agarose gel electrophoresis (Fig. 2E). The rad52 mutants were significantly defective in dsDNA binding, as compared with the wild-type protein (Fig. 2, F and G). By contrast, the amounts of Rad52 bound to the biotinylated ssDNA were nearly the same between the wild-type and mutant proteins (See Fig. 5B, lanes 3-5), suggesting that K117 and R148 are not essential for ssDNA binding. This is consistent with the studies of human Rad52 (27).

Mutations in K117 and R148 do not affect protein folding of Rad52 – To examine whether the defects in DNA binding displayed by the two Rad52 mutants are not the result of misfolded proteins, we measured CD spectra of wild type and the two mutant Rad52 (Fig. 3A). We confirmed that there were no significant changes in the spectra of K117A/R148A and K117D/R148D mutants. Thus, the defects in dsDNA binding of rad52 K117A/R148A and rad52K117D/R148D proteins are likely to be direct effects of the amino acid replacements, rather than the secondary effects of the global change in the protein folding.

K117 and R148 are not important for either the recombination mediator function or the stoichiometric complex formation with Rad51 - We next tested whether K117 and R148 of Rad52 participate in the mediator function, which is the loading of Rad51 onto RPA-coated ssDNA (Fig. 3B). When Rad51 binds to ssDNA and ATP, it displays ATPase activity. By measuring the ATPase activity of Rad51 in the presence of RPA-bound ssDNA and Rad52, it is possible to indirectly monitor the displacement of RPA by Rad51. The ATP hydrolysis activity of Rad51 was observed with substoichiometric amounts of the wild-type Rad52 (Fig. 3C), which is consistent with the previous report (44). The rad52 mutants were nearly proficient in this assay (Fig. 3C). These results indicate that the ability of Rad52 to load Rad51 onto RPA-coated ssDNA is independent of K117 and R148.

We previously reported the formation of a stoichiometric complex between yeast Rad51 and Rad52, and the capability of this complex to efficiently promote the formation of D-loops under reaction conditions that are optimal for the bacterial RecA protein (34). We examined whether K117 and R148 affect the stoichiometric complex formation between Rad52 and Rad51. Immunoprecipitation experiments using an anti-Rad51 antibody in the absence of DNA showed that the mutations of K117 and R148 did not affect the complex formation (Fig. 3, D and E). Thus, the stoichiometric complex formation by Rad51 and Rad52 does not require K117 and R148.

K117 and R148 of Rad52 are important for the D-loop formation catalyzed by the Rad51-Rad52 complex - To further investigate the roles of K117 and R148, we next examined whether the mutations in K117 and R148 affect the functions of the Rad51-Rad52 complex. As shown in our previous study, the complex promoted the formation of D-loops under reaction conditions optimal for RecA (34). The reaction was free of Ca²⁺ ions and contained 12 mM Mg²⁺ ions (standard conditions for D-loop formation by RecA: 45,46), which are non-optimal conditions for either Rad51 or Rad52 to promote the formation of D-loops alone. Rad51 alone required Ca²⁺ ions to efficiently promote the reaction, and the D-loop formation activity of Rad52 alone was sensitive to high concentrations of Mg²⁺ ions (Supplementary Fig. S1: 47). Furthermore, RPA was absent from the reaction. Thus in the present reaction conditions, Rad52 does not function as a recombination mediator or independently catalyze the formation of D-loops.

To measure the D-loop formation activity of the Rad51-Rad52 complex, we used ³²P-labeled ssDNA259 mer and negatively (right-handed) supercoiled, closed circular dsDNA (Form I DNA) containing a homologous sequence to the ssDNA (Fig. 4A). Rad52 was clearly required for the formation of D-loops (34: Fig. 4B, lanes C1 to C3 and C6) and mutant Rad52 did not form D-loops by themselves (Fig. 4B, lanes C4 and C5). When the wild-type Rad52 was substituted with either of the rad52 mutants, the amount of D-loop formation significantly decreased, even after a prolonged incubation of the reaction mixture (Fig. 4, B, C, D and E). These results suggest that the second DNA binding site in yeast...
Rad52 is important for the Rad51-Rad52 complex to promote D-loop formation.

Mutations in K117 and R148 do not affect the ssDNA binding activity of the stoichiometric Rad51-Rad52 complex - We previously showed that, for the Rad51-Rad52 complex to promote D-loop formation efficiently, it must bind to the ssDNA first, before binding to dsDNA (34). We examined the ability of the stoichiometric complex containing the rad52 mutants to bind to biotinylated pNS11 ssDNA120 mer. The ssDNA binding activity of the complex was judged by detecting the amount of protein bound to the ssDNA, which was captured with streptavidin beads (Fig. 5A). The experiment was performed using the same buffer conditions as for the D-loop formation assay. We found that the stoichiometric complexes containing rad52 mutants bound to ssDNA with efficiencies similar to that of the wild-type Rad52 (Fig. 5, B and C, lanes 6-8). The amounts of Rad51 in the complex did not significantly change (Fig. 5, B and D). In addition, the stoichiometry of Rad51 and Rad52 was near a 2:1 ratio, and was independent of the mutations in Rad52 (Fig. 5E). Thus, we concluded that the mutations affect neither the binding of the complex to ssDNA nor the formation of the stoichiometric Rad51-Rad52 complex on ssDNA.

K117 and R148 are important for the dsDNA binding activity of the Rad51-Rad52 complex - We next addressed the question of whether the second DNA binding site of Rad52 is important for the Rad51-Rad52 complex to bind to dsDNA, after binding to ssDNA. In the D-loop formation reaction catalyzed by the prototypical bacterial RecA, dsDNA binds to the recombinase after the complex formation between RecA and ssDNA. The resulting ternary complex is important for aligning the homologous sequences between the two DNA molecules (48). To examine the effects of the mutations in the second DNA binding site on the ternary complex formation by the Rad51-Rad52 complex, ssDNA, and dsDNA, we reconstituted the complex using a biotinylated pNS11 ssDNA120 mer Rad51, Rad52, and a heterologous dsDNA (Fig. 6A). The complex was then captured with the streptavidin beads, and the bound proteins and DNA were fractionated through polyacrylamide and agarose gels, respectively. The efficiency of the complex formation was judged from the amounts of Rad51, Rad52, and dsDNA detected. In the absence of Rad52, the amount of Rad51 that was captured decreased significantly (Fig. 6, B and D), probably due to the competition between ssDNA and dsDNA for Rad51 binding. In the presence of both Rad51 and Rad52, the amounts of these proteins that were captured were nearly the same between the wild type and mutant Rad52 (Fig. 6, B and C). By contrast, relatively little dsDNA was captured when either the K117A/R148A or K117D/R148D mutant was complexed with Rad51 (Fig. 6, F and G). These results indicate that the second DNA binding site of Rad52 is required for the dsDNA binding activity of the Rad51-Rad52 complex.

Discussion

In the present study, we demonstrated that the second DNA binding site, previously identified in human Rad52, is also conserved in yeast Rad52, and mutations in the site severely affect homologous recombination in yeast cells. In contrary to the general consensus that the primary role of Rad52 is the mediator function, we found that the second DNA binding site is not essential for the mediator activity (Fig. 3, B and C). This result suggests that Rad52 has other important roles in yeast. Previous studies by others have shown that Rad52 plays an important role in capturing the second end of the double-strand break. This step involves annealing between the second end of the double-strand break and the displaced strand in the D-loop. Studies of the second DNA binding site in human Rad52 have revealed that this site is important for ssDNA annealing (27). Thus, one possible role for the second DNA binding site in yeast Rad52 could be to capture the second end of the double-strand break.

We examined the possible role of the second DNA binding site of Rad52 in the D-loop formation promoted by the Rad51-Rad52 complex. Previously, we showed that Rad52 and Rad51 form a stoichiometric 2:1 complex, in both the absence and presence of ssDNA (34). This complex is capable of promoting the formation of D-loops under in vitro conditions that are unfavorable for Rad51 to function alone. Furthermore, a previous study showed that in the presence of ATP, Rad51 prevents Rad52-mediated annealing of ssDNA (49). This suggests that the ssDNA annealing activity of Rad52 is also absent in the D-loop formation reaction promoted by the Rad51-Rad52 complex. The current study shows that the second DNA binding site of Rad52 is indispensable for the
complex to promote D-loop formation (Fig. 4). Therefore, our present study reveals another possible role of the second DNA binding site of Rad52 in homologous recombination in yeast.

We previously suggested that the homologous alignment of ssDNA and dsDNA is achieved within a stoichiometric Rad51-Rad52 complex (34), as in the case of the D-loop formation catalyzed by bacterial RecA (48,50,51). For the Rad51-Rad52 complex to promote D-loop formation efficiently, the complex must bind to ssDNA first, prior to interacting with dsDNA (34). In the present study, we found that the second DNA binding site of Rad52 was not important for the ssDNA binding activity of the stoichiometric complex (Fig. 5). On the other hand, the second DNA binding site was important for the dsDNA binding and D-loop formation activities of the complex bound to ssDNA (Figs. 4 and 6). These and previous observations (34) on the D-loop formation activity of the stoichiometric Rad51-Rad52 complex suggest that (i) Rad51 is the primary catalyst in the complex, because of the ATP-dependence of the formation, (ii) like bacterial RecA, the complex forms a ternary complex with ssDNA and dsDNA, and (iii) the association with dsDNA is dependent on the second DNA binding site of Rad52. Thus, we propose that in the D-loop formation reaction promoted by the Rad51-Rad52 complex, Rad52 functions as a dsDNA chaperone for Rad51. This mechanism is clearly different from the mediator activity of Rad52, which requires only sub-stoichiometric amounts of Rad52 relative to Rad51 (44) but is independent of the second DNA binding site of Rad52 (Fig. 3C), and from the second end capture, which involves ssDNA annealing.

This study revealed that the binding of dsDNA to the second DNA binding site of Rad52 is important for D-loop formation by the Rad51-Rad52 complex, and this function may be important in homologous recombination in vivo, as discussed above. It would be interesting to determine why two types of D-loop forming proteins, one ATP-independent (Rad52, *Ustilago maydis* BRCA2 (Brh2: 52) and RecO (53)) and the other ATP-dependent (Rad51 and RecA), are both required for the various homologous recombination systems in eukaryotes and prokaryotes.

References

Acknowledgements

We thank Mr. Hideki Nabekawa and Mr. Yoshitomo Yanagida, of Nihon University, for technical support. This research was supported in part by a Grant-in-Aid for Scientific Research on Innovative Areas 21113004, Scientific Research (C) 19570168, from the Ministry of Education, Culture, Science, Sports and Technology, Japan, and by Nihon University Individual Research Grant 08-125. We thank Prof. Yoshifumi Nishimura (Yokohama City University) for his generous support in measuring CD using the Jasco J-720 spectropolarimeter.
Figure legends

Figure 1. Effects of the amino acid replacements in the second DNA binding site of Rad52 on the repair of methyl methanesulfonate (MMS)-induced DNA damage in vivo. A. Alignment of human Rad52 and S. cerevisiae Rad52. The amino acid sequences of the N-terminal conserved domain of human and yeast Rad52 are aligned with the secondary structure identified by an X-ray crystallographic analysis of human Rad52 (26). Identical and similar amino acid residues are enclosed by orange and yellow boxes, respectively. B and C. Wild type and mutant Rad52 proteins (rad52K117A/R148A and rad52K117D/R148D) were expressed under the ADH1 (alcohol dehydrogenase 1) promoter on a single-copy plasmid in rad52Δ haploid transformants. Spot tests for MMS sensitivity of cells grown on solid medium containing MMS are shown in B. WT, wild type Rad52; K117A/R148A, rad52K117A/R148A; K117D/R148D, rad52K117D/R148D; vector, a control transformant containing a vector without the Rad52 clone. Quantitative tests of the recovery from MMS-induced damage are shown in C. We repeated these experiments at least 3 times for the quantitative representation. ● (closed circles), wild type Rad52; ▲ (closed triangles), rad52K117A/R148A; ■ (closed squares), rad52K117D/R148D, ○ (open circles), control transformants without cloned Rad52; ◊ (open diamonds), wild type RAD52 cells (YPH499) containing empty vector; x (crosses), wild type RAD52 cells without any plasmid.

Figure 2. DNA binding deficiencies of rad52K117A/R148A and rad52K117D/R148D. A and B. Preparations of Rad51, Rad52, and the mutants used in this study. Purified proteins (1 µg) were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. A. Rad52: Lane 1, molecular weight markers (Bio Rad precision plus standard); lane 2, wild type Rad52; lane 3, rad52K117A/R148A; lane 4, rad52K117D/R148D. B. Rad51: Lane 1, molecular weight markers; lane 2, Rad51. C and D. ssDNA and dsDNA binding activities of rad52K117A/R148A and rad52K117D/R148D. The indicated amounts of mutants or wild type Rad52 were incubated with Cy5-labeled pNS11 ssDNA 259 mer (in dsDNA binding activities of rad52K117A/R148A and rad52K117D/R148D) at 37°C, and analyzed by agarose gel electrophoresis. Lanes 2-5, wild type Rad52; lanes 7-10, rad52K117A/R148A; lanes 12-15, rad52K117D/R148D. Lanes 1, 6, 11, storage buffer. ori, origin of the gel electrophoresis; ss, free Cy5-pNS11 ssDNA259 mer; form I, free pUC119 form I DNA. The lane numbers coincide with the lane numbers in C. ○ (open squares), K117D/R148D.

Figure 3. The effects of the K117A/R148A and K117D/R148D replacements on Rad52 structure, mediator activity, and on the stoichiometric complex formation with Rad51. A. Circular dichroism spectra of mutant and wild type Rad52. ○ (open circles), wild type; △ (open triangles), K117A/R148A; ◊ (open squares), K117D/R148D. B and C. The recombination mediator functions of the mutant rad52 were assessed by the ability of Rad51 to gain access to RPA-coated ssDNA, and hydrolyze ATP in the presence of the mutant rad52 (B). ATPase activity of Rad51 is shown in C. ● (closed circles), the complete system containing wild type Rad52; ▲ (closed triangles), the complete system containing rad52K117A/R148A; ■ (squares), the complete system containing rad52K117D/R148D; ○ (open circles), Rad51 alone (without RPA and Rad52); ◊ (open diamonds) RPA alone; ◆ (closed diamonds) wild type Rad52 alone; x (cross marks) storage buffer without RPA, Rad51, and Rad52. D and E. The ability to form complexes of Rad52 and Rad51 was examined by immuno-precipitation using anti-Rad51 antibody-protein A agarose. The precipitated proteins were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining (D). W, wild type Rad52; A, rad52K117A/R148A; D, rad52K117D/R148D. Lane 1, mixture of wild type Rad51 (5 pmol) and wild type Rad52 (5 pmol) directly loaded on gel. The ratio of Rad52 to Rad51 was calculated and shown in E. The lane numbers coincide with the lane numbers in D. We repeated these experiments 3 times for the quantitative representation.

Figure 4. Effects of K117A/R148A and K117D/R148D replacements in Rad52 on D-loop formation by the Rad51-Rad52 complex. A. Reaction scheme. pNS11 form I DNA and [32P]pNS11 ssDNA259 mer.
were used as DNA substrates for the D-loop formation assay. Products were deproteinized and analyzed by agarose gel-electrophoresis. B. Effects of titrating Rad52 concentration on the D-loop formation. C. Graphical representation of the results from B. D. Time courses of D-loop formation reactions. E. Graphical representation of the results from D. C1-C6, Controls; W, wild type; A, rad52K117A/R148A; D, rad52K117D/R148D; ss, position of the free [32P]pNS11 ssDNA259 mer. The amounts of D-loops formed were calculated by the signals derived from [32P]pNS11 ssDNA259 mer and shown in C and E. We repeated these experiments at least twice for the quantitative representation. • (closed circles), wild type Rad52 and Rad51; ▲ (closed triangles), rad52K117A/R148A and Rad51; ■ (closed squares), rad52K117D/R148D and Rad51.

Figure 5. ssDNA binding of the Rad51-Rad52 complex containing the rad52 mutants. A. Reaction scheme. Rad52-Rad51 complex were incubated at 37˚C for 10 min with biotinylated pNS11 ssDNA120 mer connected to streptavidin-attached magnetic beads. The beads were collected by using a magnet. The co-recovered proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining (B). Lanes 2-8, proteins were incubated with biotinylated pNS11 ssDNA120 mer; lanes 9-11, proteins were incubated with pNS11 ssDNA120 mer without biotin; lane 1, a mixture of Rad51 (5 pmol) and wild type Rad52 (5 pmol) was directly loaded. W, wild type Rad52; A, rad52K117A/R148A; D, rad52K117D/R148D. The amounts of Rad52 (C) and Rad51 (D) bound to ssDNA are represented as the relative values to the amount (indicated as 1.0) of each protein in the complex of wild type Rad52 and wild type Rad51 (lane 6 in B). We repeated these experiments at least twice for the quantitative representation. Ratios of Rad52 to Rad51 were calculated (E). The column numbers coincide with the lane numbers in B.

Figure 6. Ternary complex formation by the Rad51-Rad52 complex. A. Reaction scheme. Previously mixed Rad51 and Rad52 were incubated with biotinylated pNS11 ssDNA120 mer bound to streptavidin-conjugated magnetic beads at 37˚C for 10 min. Heterologous form I DNA (pUC119) was then added to the reaction mixture (final 100 µl), and incubated for 10 min. After the magnetic beads were recovered by the magnet, the co-recovered proteins (Rad52 and Rad51) and form I DNA were analyzed by SDS-PAGE (B) and agarose gel electrophoresis (F), respectively. W, wild type Rad52; A, rad52K117A/R148A; D, rad52K117D/R148D; M, a mixture of wild type Rad51 (10 pmol) and wild type Rad52 (10 pmol). The amounts of Rad52 (C) and Rad51 (D) recovered with the precipitated ssDNA are represented as the relative values to the amount (indicated as 1.0) of each protein in the complex of wild type Rad52 and wild type Rad51 (lane 3 in B). Ratios of Rad52 to Rad51 were calculated (E). The amounts of the recovered dsDNA are represented (G) as relative values to the amount of input pUC119 form I DNA (lane 11 in F). The column numbers coincide with the lane numbers in gel profiles. We repeated these each series of experiments 3 times for the quantitative representation.
Figure 1
Figure 3

A

B

circular ssDNA
(5.0 μM pUC119)
↓
+0.88 μM RPA
↓
37°C, 10 min
↓
+(Rad52+1.0 μM Rad51)
↓
37°C, 30 min

C

Produced ADP, (pmol)

Rad52, (μM)

D

Rad52  Rad51
- W A D W A D

Rad52 →
Rad51 →
1 2 3 4 5 6 7 8

E

Rad52/Rad51 ratio

Lane number

3 4 5
Figure 4
Figure 5
Figure 6
Vital roles of the second DNA binding site of Rad52 in yeast homologous recombination
Naoto Arai, Wataru Kagawa, Kengo Saito, Yoshinori Shingu, Tsutomu Mikawa, Hitoshi Kurumizaka and Takehiko Shibata

J. Biol. Chem. published online March 28, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M110.216739

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/03/28/M110.216739.DC1

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2011/03/28/jbc.M110.216739.full.html#ref-list-1