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Mutations in yeast Rad51 that partially bypass the requirement for Rad55 and Rad57 in DNA repair by increasing the stability of Rad51–DNA complexes

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Yeast Rad51 promotes homologous pairing and strand exchange *in vitro*, but this activity is inefficient in the absence of the accessory proteins, RPA, Rad52, Rad54 and the Rad55–Rad57 heterodimer. A class of *rad51* alleles was isolated that suppresses the requirement for *RAD55* and *RAD57* in DNA repair, but not the other accessory factors. Five of the six mutations isolated map to the region of Rad51 that by modeling with RecA corresponds to one of the DNA-binding sites. The other mutation is in the N-terminus of Rad51 in a domain implicated in protein–protein interactions and DNA binding. The Rad51-I345T mutant protein shows increased binding to single- and double-stranded DNA, and is proficient in displacement of replication protein A (RPA) from single-stranded DNA, suggesting that the normal function of Rad55–Rad57 is promotion and stabilization of Rad51–ssDNA complexes.

Keywords: DNA repair/Rad51/Rad55/Rad57/recombination

Introduction

The repair of DNA double-strand breaks (DSBs) is essential to maintain genome integrity and for the accurate segregation of homologous chromosomes during meiosis. Recombinases of the RecA family play a central role in homology-dependent DSB repair (Gasior *et al*., 2001). These proteins assemble into filaments on single-stranded DNA (ssDNA) that is formed at stalled replication forks or by nucleolytic processing of DSBs (Flory *et al*., 1984; Egelman and Stasiak, 1986; Ogawa *et al*., 1993; Sung and Robberson, 1995). The nucleoprotein filament is the active form of the recombinase and is capable of searching for homology within intact duplex DNA to initiate synapsis and strand exchange (Kowalczykowski *et al*., 1994; Sung and Robberson, 1995).

Yeast and human encode two RecA homologs, Rad51 and Dmc1, as well as Rad51-related proteins, referred to as Rad51 paralogs (Gasior *et al*., 2001; Thompson and Schild, 2001). Yeast *RAD51* is expressed in mitotic and meiotic cells and is required for resistance to ionizing radiation, spontaneous and induced mitotic recombination and for meiotic recombination (Aboussekhra *et al*., 1992; Basile *et al*., 1992; Shinohara *et al*., 1992). Deletion of *RAD51* in vertebrates results in cell inviability and early embryonic lethality in mice (Lim and Hasty, 1996; Tsuzuki *et al*., 1996). Depletion of Rad51 using a conditional chicken DT40 cell line results in a G_2/M phase arrest, the accumulation of cytologically visible chromosomal breaks and eventual cell death (Sonoda *et al*., 1998). These data suggest that the essential role of *RAD51* in vertebrates is to repair breaks generated during DNA replication. *DMC1* is expressed only during meiosis and, although it has some redundant functions with *RAD51*, it also has unique functions and acts specifically in the inter-homolog recombination pathway (Bishop *et al*., 1992; Schwacha and Kleckner, 1997; Shinohara *et al*., 1997). The Rad51 paralogs of *Saccharomyces cerevisiae* are encoded by the *RAD55* and *RAD57* genes and are determined by genetic studies to function in the same pathway as *RAD51* (Kans and Mortimer, 1991; Lovett, 1994; Rattray and Symington, 1995). The vertebrate Rad51 paralogs are encoded by the *RAD51B*, *RAD51C*, *RAD51D*, *XRCC2* and *XRCC3* genes (Thompson and Schild, 2001). Mutation of any of these genes in the chicken DT40 cell lines does not cause inviability, but the cells show high sensitivity to DNA cross-linking agents and increased frequencies of spontaneous chromosome aberrations (Takata *et al*., 2001).

Purified yeast Rad51 forms right-handed helical filaments on ssDNA and double-stranded DNA (dsDNA) with structural similarity to those formed by RecA (Ogawa *et al*., 1993; Sung and Robberson, 1995). Formation of filaments on ssDNA is stimulated in the presence of the heterotrimeric DNA-binding protein, replication protein A (RPA) (Sung and Robberson, 1995; Sugiyama *et al*., 1997). The addition of RPA to the Rad51 reaction is thought to allow the formation of continuous filaments by removal of secondary structures from ssDNA (Sugiyama *et al*., 1997). Once assembled, the Rad51 nucleoprotein filament is capable of interacting with a second DNA molecule to initiate strand exchange. The *in vitro* strand exchange assay is used frequently as a metric for pre-synapsis, synapsis and strand exchange steps of the reaction. Rad51 by itself has very weak strand exchange activity and can be stimulated by the addition of RPA, but only if Rad51 is allowed to nucleate on the ssDNA prior to the addition of RPA (Sung, 1994). If RPA is added before, or simultaneously with Rad51, then RPA inhibits the reaction (Sung, 1997b). The inhibition by RPA is presumed to be a consequence of the higher affinity of RPA for ssDNA and faster binding kinetics compared with Rad51.

The inhibitory effect of RPA can be overcome by the inclusion in the reaction of accessory proteins, also known as mediators. Rad52 is the best characterized of the mediators of Rad51 filament assembly (Sung, 1997a; Benson *et al*., 1998; New *et al*., 1998; Shinohara and Ogawa, 1998). Rad52 interacts with both RPA and Rad51,

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and the interaction with Rad51 is necessary for overcoming the RPA inhibitory effect (Shinohara and Ogawa, 1998). Rad52 is thought to replace RPA bound to ssDNA with Rad51, or provide a seeding site within the RPA-bound ssDNA for subsequent cooperative binding by Rad51 (Song and Sung, 2000). The Rad55 and Rad57 proteins, which form a stable heterodimer, can also overcome the inhibition to Rad51-promoted strand exchange imposed by RPA (Sung, 1997b), but the mechanism of mediation is unknown. Consistent with their *in vitro* roles in Rad51-promoted strand exchange, *rad52*, *rad55* and *rad57* mutants fail to assemble Rad51 foci during meiotic recombination (Gasior *et al*., 1998). However, in mitotic cells, there appears to be some redundancy between Rad52 and Rad55–Rad57 for the assembly of radiation-induced Rad51 foci (Gasior *et al*., 2001). The role of Rad55 and Rad57 as accessory proteins for Rad51 is also supported by the observation that *RAD51* expressed from a high copy number plasmid partially suppresses the radiation sensitivity of *rad55*, *rad57* and *rad55 rad57* mutants (Hays *et al*., 1995; Johnson and Symington, 1995). The cold sensitivity for DNA repair conferred by *rad55* and *rad57* deletion mutants is suggestive of a role for these proteins in stabilizing a protein complex (Game and Mortimer, 1974; Lovett and Mortimer, 1987; Hays *et al*., 1995; Johnson and Symington, 1995).

Mediators for recombinase functions are also found in bacteriophage T4 and bacteria. The strand exchange activity of T4 UvsX is stimulated by UvsY, which interacts directly with UvsX and gene 32 protein (ssDNA-binding protein) to replace gene 32 protein bound to ssDNA with UvsX (Bleuit *et al*., 2001). In *Escherichia coli*, the RecBCD nuclease–helicase complex facilitates loading of RecA onto 3′ ssDNA generated at Chi sites (Anderson and Kowalczykowski, 1997), and the RecF, RecO and RecR proteins overcome the inhibition to RecA-mediated strand exchange by ssDNA-binding protein (SSB) (Umezu *et al*., 1993). The UV sensitivity of *recF*, *recO* and *recR* mutants can be suppressed by high copy expression of *recA*, consistent with their roles as accessory proteins. Furthermore, several extragenic suppressors of the UV sensitivity of *recF* mutants (*srf* mutants) map to *recA* (Volkert and Hartke, 1984). The *recA srf* alleles also suppress the UV sensitivity of *recO* and *recR* mutants, suggesting that RecFOR functions at the same step in recombination (Wang *et al*., 1993). Alleles of *recA* that are constitutive for the SOS response (*tif* alleles) also confer the *srf* phenotype (Wang *et al*., 1993). Most of the *srf* and *tif* alleles map to regions of RecA involved in monomer–monomer or polymer–polymer interactions, and the mutant proteins are better at displacing SSB from ssDNA (Kowalczykowski *et al*., 1994).

The similarity between Rad55, Rad57 and RecFOR in their roles as mediators of the strand exchange activity of their cognate recombinases suggested the possibility that alleles of *RAD51* with altered function could be isolated as suppressors of the DNA repair defect conferred by *rad55* and *rad57* mutations. Here, we describe six alleles of *RAD51* that partially suppress the radiation sensitivity of *rad55* and *rad57* mutants, but not *rad52* or *rad54* mutants. Biochemical characterization of one of the mutant Rad51 proteins revealed higher affinity and more stable binding to DNA, suggesting that one function of Rad55 and Rad57 is promotion and stabilization of the Rad51 nucleoprotein filament.

Results

Identification of alleles of RAD51 that partially suppress the ionizing radiation sensitivity of a rad57 strain

Based on the observation that *RAD51* present on a high copy number plasmid partially suppresses the γ-ray sensitivity of a *rad55 rad57* strain, we sought to identify alleles of *RAD51* that when expressed at low copy (from a *CEN* plasmid) could also suppress the *rad55 rad57* mutant phenotype. Because Rad55 and Rad57 act as an obligate heterodimer and the phenotype of the double mutant is no more severe than that of the single mutants, the screen was conducted in a *rad51 rad57* yeast strain. The strain utilized contained a deletion of the *RAD51* gene in case the mutations were recessive. A *CEN/ARS/HIS3* plasmid carrying the *RAD51* open reading frame (ORF) and promoter elements was mutagenized randomly and the pool of mutant plasmids transformed into the double mutant strain. Transformants were selected and individual isolates were assayed at 23°C for their resistance to γ-irradiation. Suppression by mutant *rad51* plasmids was expected to be more obvious at 23°C due to the cold sensitivity for DNA repair of *rad57* mutants. Those transformants that survived irradiation at frequencies equal to or higher than that seen with the double mutant strain harboring wild-type *RAD51* expressed from a high copy number (2μ) plasmid were analyzed further.

To establish that the phenotype was the result of the plasmid and not some other change in the strain, the plasmid containing the putative *rad51* mutation was isolated from yeast, amplified in *E.coli* and then retested in the *rad51 rad57* strain. Rad51 protein levels from each suppressing *CEN* plasmid were assayed by western blot analysis and shown to be equal to wild-type *RAD51* expressed from the same *CEN* plasmid (data not shown).

Of ~3000 transformants screened, six plasmids were obtained capable of conferring increased γ-ray resistance without overexpressing Rad51. From the DNA sequence, it was determined that each contained a single point mutation within the *RAD51* ORF. The residues altered and the putative function of these residues based on alignment with RecA are listed in Table I. Two alleles were identified that contained different point mutations in codon 345; one resulted in alteration of Ile345 to a threonine while the other change was to serine. Figure 1 is a partial alignment of the amino acid sequence of *S.cerevisiae* Rad51 compared with *E.coli* RecA and human Rad51. Each of the amino acid changes is indicated. Val328 and Ile345 are both conserved in human Rad51, and the corresponding region of RecA is implicated in DNA binding and/or ATP-induced conformational changes (Story *et al*., 1992, 1993). Leu119 corresponds to Ile61 in the human protein. The N-terminal domain of human Rad51, which is highly conserved among Rad51 proteins, has DNA-binding activity (Aihara *et al*., 1999). The N-terminal region of yeast Rad51 is implicated in monomer–monomer interactions and interaction with Rad52 and Rad54 (Krejci *et al*., 2001). Although none of the altered residues is

conserved with *E.coli* RecA, Ile345 immediately precedes two invariant glycine residues that are thought to be involved in DNA binding or ATP-induced conformational changes in RecA (Story and Steitz, 1992). These alleles of *rad51* will be referred to as *srp* (suppressor of rad51 paralogs) alleles.

The rad51 srp alleles suppress the γ-ray sensitivity of rad55 and rad57 mutants, but not rad52 or rad54 mutants

Each of the plasmids containing the *srp* alleles was able to complement the radiation sensitivity of a *rad51* strain (data not shown), indicating that they have retained *RAD51* function. Rad57 acts as an obligate heterodimer with Rad55; therefore, the *srp* alleles were expected to suppress the radiation sensitivity conferred by a *rad55* mutation as well as a *rad57* mutation. The *CEN* plasmids expressing the *srp* alleles, wild-type *RAD51* expressed from the same *CEN* plasmid or *RAD51* expressed from a 2μ plasmid were used to transform *rad51 rad55* and *rad51 rad57* strains. Serial dilutions of the strains containing each plasmid were grown at 23°C after 50 krad γ-irradiation and compared with an unirradiated control (Figure 2). When *RAD51* was expressed at low copy number from a *CEN* plasmid, only a few colonies were formed from undiluted *rad51 rad55* or *rad51 rad57* cells after irradiation. When *RAD51* was expressed from a 2μ plasmid, a 10- to 50-fold suppression of the γ-ray sensitivity was observed in both strain backgrounds. Each of the *rad51 srp* plasmids conferred partial suppression of both the *rad55* and the *rad57* mutant phenotypes when expressed from a *CEN* plasmid, with ~10- to 50-fold increased plating efficiency after γ-irradiation when compared with wild-type *RAD51*.

Table I. *rad51* alleles recovered from the screen

Mutated residue	Putative function[a]
Rad51-L119P	Rad51 self-interaction, Rad52–Rad54 interaction, DNA interaction[b]
Rad51-V328I	DNA interaction (L2 loop)
Rad51-P339S	DNA interaction (L2 loop)
Rad51-I345T	DNA interaction and/or ATP-induced conformational change
Rad51-I345S	DNA interaction and/or ATP-induced conformational change
Rad51-G359S	β-strand 6

[a]Putative function based on alignment with RecA (Kowalczykowski *et al*., 1994).
[b]Putative function for Leu119 based on studies of human and yeast Rad51 (Aihara *et al*., 1999; Krejci *et al*., 2001).

According to the *in vitro* strand exchange data, both the Rad55–Rad57 heterodimer and Rad52 play some role in enabling Rad51 to overcome the inhibition imposed by RPA (Sung, 1997a,b; Benson *et al*., 1998; New *et al*., 1998; Shinohara and Ogawa, 1998). To determine whether the Rad52 and Rad55–Rad57 mediators play redundant roles in the Rad51-promoted reaction, we tested the ability of the *rad51 srp* alleles to suppress a *rad52* phenotype. In *S.cerevisiae*, *rad52* mutants show the most severe recombination and repair phenotypes of all the *rad52* group mutants. Consequently, Rad52 protein is thought to have other roles in recombination in addition to its mediator function. *rad52Δ327* is a C-terminal truncated allele of *RAD52* encoding amino acids 1–327. The truncated protein was shown to have partial DSB repair activity *in vivo*, but to be defective in its interaction with Rad51 and unable to function as a mediator *in vitro* (Boundy-Mills and Livingston, 1993; Milne and Weaver, 1993). As in the case of *rad55* and *rad57* mutants, overexpression of *RAD51* partially suppresses the methyl methanesulfonate (MMS) sensitivity of a *rad52Δ327* strain (Boundy-Mills and Livingston, 1993; Milne and Weaver, 1993). To assess whether any of the *rad51 srp* alleles also suppressed the Rad52 mediator defect, each was transformed into a *rad51 rad52Δ327* strain. As expected, *RAD51* expressed from a 2μ plasmid suppressed the radiation sensitivity of the *rad51 rad52Δ327* strain, but none of the *rad51 srp* alleles did (Figure 2).

Rad54 protein stimulates the Rad51-promoted pairing reaction primarily during the synapsis and strand exchange phases, thus the *rad51 srp* alleles were not expected to suppress the requirement for *RAD54* in DNA repair (Petukhova *et al*., 1998; Mazin *et al*., 2000; Van Komen *et al*., 2000; Solinger *et al*., 2001). As anticipated, no suppression of the DNA repair defect was observed when each of the *rad51 srp* alleles was expressed in a *rad51 rad54* strain (data not shown).

To determine whether there is synergism between the *rad51-L119P* mutation and alleles in the C-terminal region of *RAD51*, a *rad51-L119P*, *I345T* double mutant was constructed. However, the level of suppression of the *rad57* DNA repair defect was the same as observed for

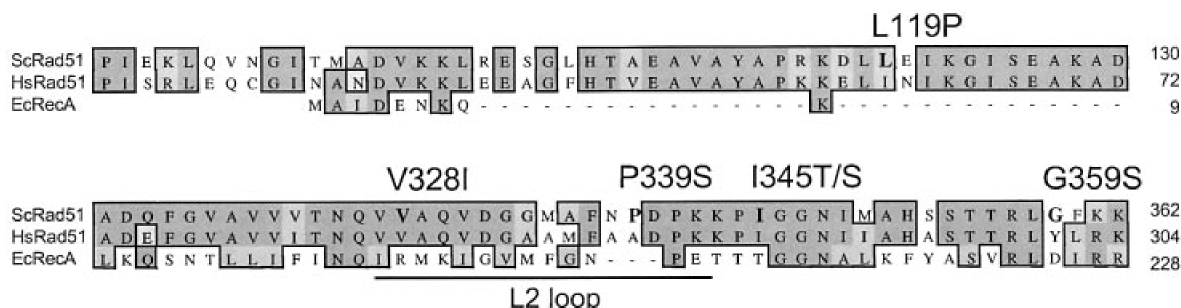


Fig. 1. Partial alignment of ScRad51 with HsRad51 and RecA. The *srp* mutations are indicated above each mutated amino acid (in bold). The L2 loop of RecA, corresponding to one of the DNA-binding sites, is indicated.

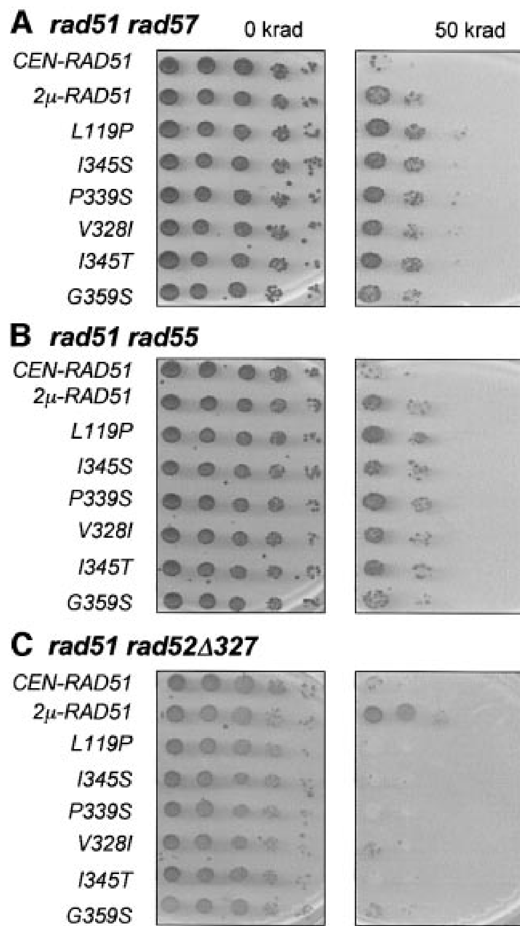


Fig. 2. Suppression of the γ-ray sensitivity of *rad55* and *rad57* mutants by the plasmid-borne *rad51 srp* alleles. (**A**) Serial dilutions of *rad51 rad57* strains containing each of the *rad51* mutant plasmids, wild-type *RAD51* expressed from the same *CEN/HIS/ARS* plasmid (*CEN-RAD51*) and wild-type *RAD51* expressed from a 2μ plasmid (2μ-*RAD51*) were spotted onto YPD plates and left unirradiated or irradiated at 50 krad. (**B**) Serial dilutions of *rad51 rad55* strains containing each of the plasmids described above were spotted onto YPD plates and treated with 0 or 50 krad. (**C**) Serial dilutions of *rad51 rad52Δ327* plasmid-containing strains irradiated with 0 or 50 krad. For strains tested in (A–C), all survival was assessed following incubation at 23°C.

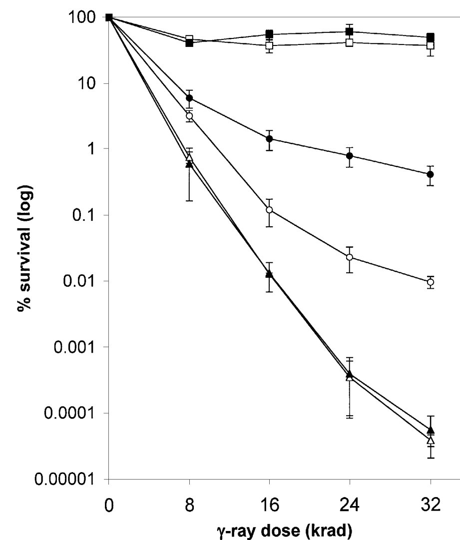


Fig. 3. Partial suppression of the γ-ray sensitivity conferred by the *rad57* mutation by *rad51-I345T* expressed from the chromosome. Survival was assessed following incubation at 30°C. The log percentage survival was plotted as a function of dose for each strain (filled squares, *rad51-I345T*; open squares, wild-type; filled circles, *rad51-I345T rad57::LEU2*; open circles, *rad57::LEU2*; filled triangles, *rad51::LEU2*; open triangles, *rad51::LEU2 rad57::LEU2*).

the two single mutations (data not shown). The *rad51 srp* alleles showed the same level of suppression of the *rad55* and *rad57* DNA repair defects whether expressed from a high copy number plasmid or from a *CEN* plasmid (data not shown), suggesting that *RAD55* and *RAD57* have additional roles in DNA repair, or that other factors are limiting in their absence.

Suppression of the rad57 DNA repair defect by rad51-I345T expressed in single copy

Although *CEN* plasmids are maintained at low copy number, some mutations confer a slightly different phenotype when present on a *CEN* plasmid compared with the native chromosomal locus (U.Mortensen and R.Rothstein, personal communication). To test whether suppression of the *rad57* phenotype could occur when the *srp* alleles were expressed from the chromosomal *RAD51* locus, a representative *srp* allele was used to replace the native *RAD51* locus. As the mutations in most of the *srp* alleles map to the region of Rad51 implicated in DNA binding, the mechanism of suppression is probably the same. Therefore, the *rad51-I345T* allele was chosen as representative because two independent mutations were identified affecting this residue, which is also conserved in the human Rad51 protein. The strain containing the *rad51-I345T* allele is as resistant as the wild-type strain at both 30 (Figure 3) and 23°C (data not shown), suggesting that this allele retains *RAD51* function. At 30°C, strains containing *rad51::LEU2* and *rad51::LEU2 rad57::LEU2* are both equally sensitive, while the *rad57::LEU2* strain is moderately more resistant. The *rad51-I345T rad57::LEU2* strain, when compared with *rad57::LEU2*, shows an increase in survival of 1–2 orders after 32 krad γ-irradiation, similar to the level of suppression seen when this allele is expressed from a *CEN* plasmid (Figure 3). At 23°C, the *rad57::LEU2* strain is as sensitive as the *rad51::LEU2* and *rad51::LEU2 rad57::LEU2* strains due to the cold sensitivity conferred by the *rad57* mutation. The *rad51-I345T rad57::LEU2* strain, when compared with the *rad57::LEU2* strain, shows a 50-fold increase in resistance after 32 krad of γ-irradiation at 23°C (data not shown). Thus the *rad51-I345T* mutation shows similar suppression of the DNA repair defect of the *rad57::LEU2* strain at both temperatures. However, it should be noted that the *rad51-I345T rad57::LEU2* strain, like the *rad57::LEU2* strain, is more sensitive to γ-irradiation at 23°C than at 30°C.

The *rad51-I345T rad57* strain was crossed to a *RAD51 rad57* strain to test for dominance. The resulting diploid was ~2-fold less resistant to γ-irradiation than the *rad51-I345T rad57* homozygous diploid, but >20-fold more resistant than the *RAD51 rad57* diploid (data not shown). Thus, the *rad51-I345T* mutation is dominant.

Partial suppression of the rad57 mating-type switching defect by the rad51-I345T allele

Ionizing radiation produces a variety of DNA lesions in addition to DSBs. To determine the effect of the *rad51-I345T* allele on the repair of a single DSB, a mating-type switching assay was performed. The repair of an HO endonuclease-induced DSB was monitored at the DNA level after induction of HO endonuclease for 1 h. To measure the formation of switched products, the DNA samples were digested with *Sty*I, which cuts within *MAT* Y**a** but not Yα sequences. The appearance of a 0.9 kb *Sty*I fragment is indicative of repair of the DSB from the *HMR***a** locus (Figure 4A). In the wild-type and *rad51-I345T* strains, switching was efficient and completed 3 h after induction of HO. In the *rad57* mutant, the 0.7 kb cut fragment persisted for several hours, but disappearance of the cut DNA was not concomitant with conversion to the *MAT***a** product. In the *rad51-I345T rad57* double mutant, a low level of switched product was detected 3 h after induction of HO, indicating partial suppression of the *rad57* mating-type switching defect.

To quantitate mating-type switching, HO endonuclease was induced in cultures of each strain for 3 h. Cells were then plated on YPD to repress expression of HO and survivors were tested for their mating type. Approximately 50% of wild-type and 60% of *rad51-I345T* cells switched from *MAT*α to *MAT***a** following HO induction whether grown at 23 (data not shown) or 30°C (Figure 4B). The strain containing the *rad51-I345T* mutation consistently showed slightly increased switching compared with the wild-type strain. At 30°C, the *rad51-I345T* mutation suppressed the mating-type switching defect of the *rad57* strain by ~4-fold (Figure 4B). At 23°C, suppression of the *rad57* defect by *rad51-I345T* was weaker, with a 2-fold increase in the number of switched products compared with the *rad57* strain (data not shown). Low levels of switched products (2–6%) were detected in the *rad57* mutant, consistent with a previous study suggesting that *RAD55* and *RAD57* are not essential for mating-type switching (Johnson and Symington, 1995).

Rad51-I345T overcomes the inhibition imposed by RPA

The mediator role of the Rad55–Rad57 heterodimer was discovered using an *in vitro* strand exchange assay (Sung, 1997b). The addition of the Rad55–Rad57 heterodimer enables Rad51 to overcome the inhibition imposed by RPA, presumably by mediating Rad51 filament formation. Consistent with this model, the formation of meiosis-specific Rad51 foci requires Rad55 and Rad57 (Gasior *et al*., 1998). Because the *rad51 srp* alleles partially suppress the γ-ray sensitivity of *rad57* mutants, one possibility is that the Rad51 srp mutant proteins are capable of overcoming the inhibition imposed by RPA in the absence of Rad55 and Rad57. This notion was tested biochemically using the purified Rad51-I345T protein (Figure 5A).

The ability of wild-type and mutant proteins to overcome the RPA inhibition of pre-synaptic filament formation was examined by measuring the ssDNA-dependent ATPase activity of Rad51. If ssDNA is pre-coated with RPA, the ATPase activity of Rad51 is inhibited because Rad51 is unable to compete with RPA for binding to the

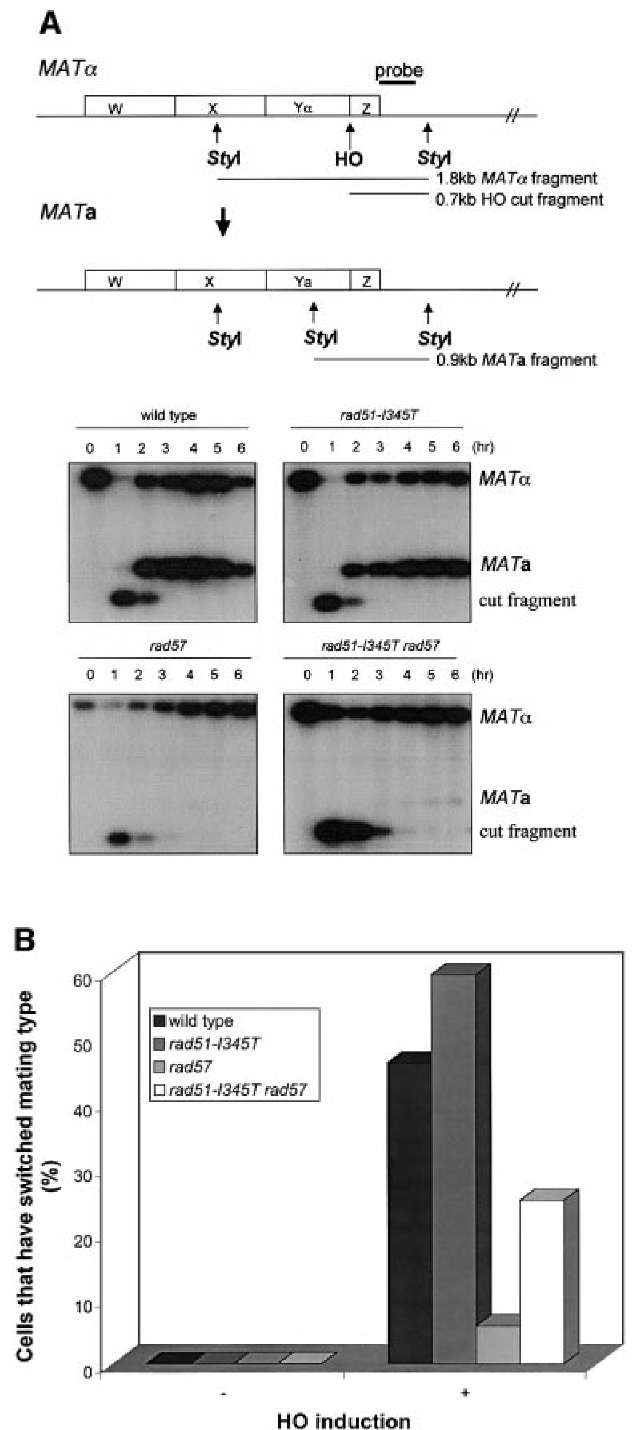
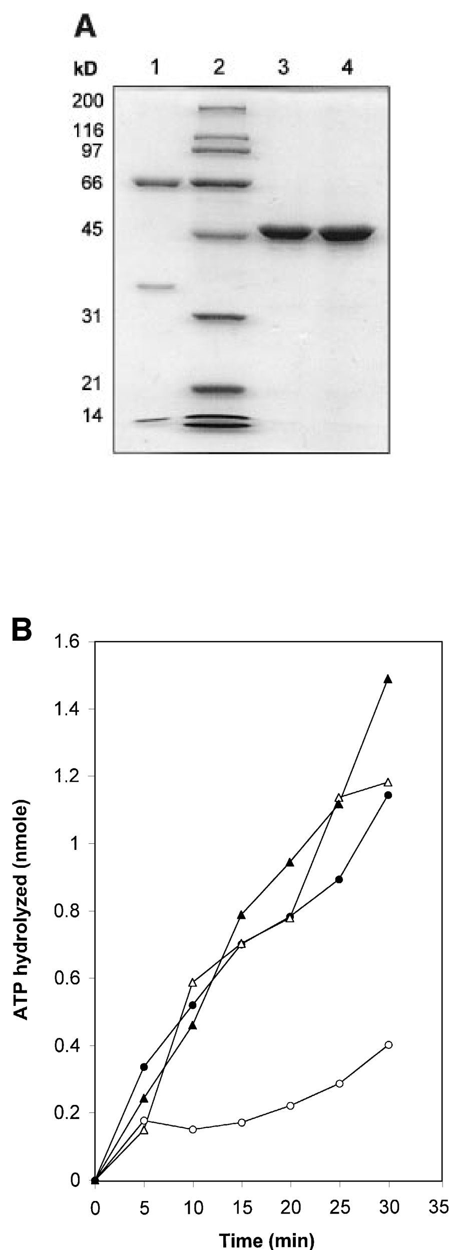


Fig. 4. (**A**) The *rad51-I345T* mutation partially suppresses the mating-type switching defect of a *rad57* strain. Schematic representation of the *MAT*α and *MAT***a** loci indicating the locations of *Sty*I sites and the hybridization probe is shown at the top of the figure. HO endonuclease produces a 0.7 kb fragment from the 1.8 kb *MAT*α *Sty*I fragment. A 0.9 kb *Sty*I fragment is produced when the mating-type switches from *MAT*α to *MAT***a**. DNA was isolated from cultures grown at 30°C prior to galactose induction (0 h time point) and at 1 h intervals after HO induction. The strains tested are indicated above each autoradiogram. (**B**) Suppression of the *rad57* mating-type switching defect quantified genetically. HO endonuclease was induced in each strain for 3 h. Cells plated on YPD and incubated at 30°C were assessed for their mating type. Shown is a representative experiment displaying the reproducible 4-fold suppression of *rad57* by *rad51-I345T*. Black bars, wild-type; dark gray bars, *rad51-I345T*; light gray bars, *rad57*; white bars, *rad51-I345T rad57*.



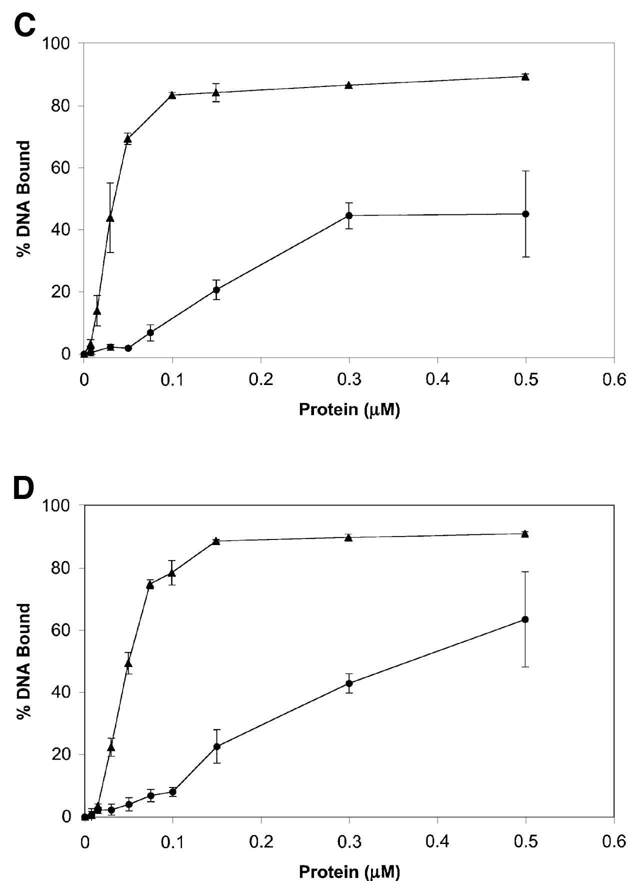


Fig. 5. Rad51-I345T overcomes the inhibition to ssDNA binding imposed by RPA and exhibits higher affinity for single- and double-stranded DNA. (**A**) Purified proteins used in the *in vitro* assays. Lane 1, RPA (3.75 μg); lane 2, molecular weight size standards; lane 3, Rad51 (7.5 μg); lane 4, Rad51-I345T (9 μg). (**B**) ssDNA-dependent ATPase was compared for wild-type Rad51 versus Rad51-I345T using poly(dT) as the ssDNA substrate. Rad51 (filled circles), Rad51-I345T (filled triangles), Rad51 with RPA-coated DNA (open circles), Rad51-I345T with RPA-coated DNA (open triangles). (**C**) Increasing concentrations of either wild-type Rad51 (filled circles) or Rad51-I345T (filled triangles) were incubated with a constant amount of ssDNA and passed through alkali-treated nitrocellulose and DEAE filters. The percentage bound represents the amount of DNA retained on nitrocellulose compared with the total DNA. (**D**) As (C) except using dsDNA as the substrate.

ssDNA. ATP hydrolysis with RPA-coated ssDNA as a cofactor is then a measure of the ability of Rad51 to overcome the inhibition imposed by RPA. The wild-type and Rad51-I345T proteins showed similar rates of ATP hydrolysis using as a DNA cofactor poly(dT), which has little or no secondary structure (Figure 5B). As expected, ATP hydrolysis by Rad51 was inhibited when the DNA was coated with RPA. However, in the case of Rad51-I345T, ATP hydrolysis was the same with either naked or RPA-coated ssDNA (Figure 5B). Thus, RPA does not prevent Rad51-I345T from gaining access to DNA.

Rad51-I345T shows increased affinity for DNA

Most of the *rad51 srp* mutations map to the region of Rad51 that by alignment with RecA corresponds to the L2 DNA-binding domain. This observation suggests that the mutant proteins might have an altered affinity for DNA. Consistent with this hypothesis, generation of random mutations within the region corresponding to the L2 loop of the human Rad51 protein resulted in proteins with increased ssDNA-binding activity *in vitro* (Kurumizaka *et al.*, 1999). Binding to ssDNA and dsDNA by Rad51-I345T was tested using a nitrocellulose filter-binding assay. When increasing concentrations of protein were incubated with a constant amount of either ssDNA or dsDNA, 6- to 8-fold less of the Rad51-I345T protein was required to achieve half-maximal binding compared with wild-type Rad51 (Figure 5C and D). This increased binding to DNA was evident for both ssDNA and dsDNA. The sigmoidal curves for both wild-type Rad51 and Rad51-I345T suggest cooperative binding to both substrates. The shift of the curve for the Rad51-I345T protein indicates a higher affinity for both ssDNA and

dsDNA. The increased level of complex formation may reflect a more stable interaction between the Rad51-I345T protein and DNA compared with wild-type Rad51.

Discussion

The nucleoprotein filament formed by interaction between Rad51 and ssDNA is essential for homologous pairing and strand exchange. Formation of the nucleoprotein filament is impeded by RPA, which has higher affinity for ssDNA and is more abundant than Rad51 (Sugiyama *et al*., 1997). Consequently, additional factors, known as mediators, are required to promote assembly of the Rad51 nucleoprotein filament in the presence of RPA. Rad52 and Rad55–Rad57 have both been identified as mediators based on their ability to stimulate Rad51-promoted strand exchange in the presence of RPA (Sung, 1997a,b; New *et al*., 1998; Shinohara and Ogawa, 1998), but these factors are clearly non-redundant *in vivo* (Rattray and Symington, 1995). Efforts to understand the mechanism of mediation by Rad55–Rad57 have been hampered by the difficulty in purifying the heterodimer. To shed light on this problem, we isolated gain-of-function alleles of *RAD51* that partially suppress the requirement for *RAD55* and *RAD57* in DNA repair, but not the need for *RAD52*. Thus, the mechanism by which the two mediators stimulate Rad51 can be separated genetically.

Rad51-I345T protein, a representative of the gain-of-function mutants, was compared with wild-type Rad51 protein for its ability to overcome the inhibition imposed by RPA. As Rad51 is an ssDNA-dependent ATPase, pre-incubating the DNA cofactor with RPA inhibits Rad51 ATPase activity by competing with Rad51 for binding sites on the ssDNA (Sugiyama *et al*., 1997). Pre-coating ssDNA with RPA fails to inhibit the ATPase activity of the Rad51-I345T protein (Figure 5B). Thus, Rad51-I345T is able to compete with RPA for binding to ssDNA and displace it. The RecA-803 protein, identified genetically as a suppressor of the UV sensitivity conferred by *recF* mutations, also shows increased ability to compete with SSB for binding to ssDNA (Lavery and Kowalczykowski, 1992; Madiraju *et al*., 1992). These data suggest that the primary function of the mediator proteins in prokaryotes and eukaryotes is to assist loading, or stabilization, of the recombinase to ssDNA in the presence of SSBs. The purified RecO and RecR proteins facilitate loading of RecA onto ssDNA in the presence of SSB by a direct interaction between RecO and SSB (Umezu and Kolodner, 1994). The RecF, RecO and RecR proteins also play additional roles in stabilization of the RecA filament and preventing RecA polymerization onto dsDNA from adjacent ssDNA (Shan *et al*., 1997; Webb *et al*., 1997).

Formation of joint molecules in reactions employing ssDNA circles and linear duplex molecules as substrates was the assay originally used to define the Rad55–Rad57 heterodimer as a mediator in enabling Rad51 to overcome the inhibition imposed by RPA. When Rad51-I345T was used in the strand exchange assay, even under stimulatory conditions, joint molecules were not resolved to nicked circles (data not shown). Instead, large aggregates of DNA were observed. This could result from an inability to complete strand exchange due to unproductive binding of Rad51 to dsDNA, or perhaps the products of reinvasion by the displaced strand (Chow *et al*., 1988). RecA mutant proteins that show increased binding to DNA also promote formation of aggregates when tested in the *in vitro* strand exchange assay (Lavery and Kowalczykowski, 1990). DNA aggregates are likely to represent the products of invasion of the displaced strand from the linear duplex into another duplex molecule (Chow *et al*., 1988). The continuing cycle of re-invasion is thought to result in the large DNA networks seen on agarose gels as smeared species with slower migration than the predicted product bands. As mentioned earlier, based on sequence alignment with RecA, Ile345 maps to a region of Rad51 implicated in DNA binding. Studies with the human Rad51 protein have shown that the I287S mutation, corresponding to Ile345 of yeast Rad51, results in a protein with higher affinity for single-stranded DNA (Kurumizaka *et al*., 1999). Thus, it seemed plausible that the *rad51-I345T* allele may act to suppress the defects caused by the *rad57* mutation by more stable binding of the mutant protein to DNA.

Rad51 binding to both ssDNA and dsDNA exhibits a sigmoidal dependence on protein concentration indicative of cooperative binding (Shinohara *et al*., 1992). Rad51-I345T also appears to bind ssDNA and dsDNA cooperatively, but the concentration of protein required to exhibit half-maximal binding to the DNA is 6- to 8-fold less than wild-type protein (Figure 5). This observation suggests that Rad51-I345T has a higher affinity for DNA. Also different between wild-type and mutant proteins was the maximum amount of DNA bound. This property could be explained in terms of the dynamic interaction between Rad51 and DNA. With wild-type protein, the equilibrium between bound and unbound protein would appear to be such that the maximum amount of DNA trapped in complexes was never more than 60%. An intrinsic decrease in the K_{eq} of Rad51-I345T could result in formation of a higher level of trappable complex. The RecA mutant proteins encoded by the suppressors of *recF*, *recO* and *recR* mutants (*recA srf* alleles) have been determined to have higher affinity for ssDNA (Lavery and Kowalczykowski, 1992). However, none of the mutations in the *srf* alleles maps to residues implicated in DNA binding by RecA. Instead, the mutations are located in the regions thought to be involved in filament–filament interactions (Story *et al*., 1992). In this case, the mechanism of suppression appears to operate through decreasing the formation of large RecA protein aggregates and promoting productive association with ssDNA (Kowalczykowski *et al*., 1994).

The cold sensitivity of *rad55* and *rad57* mutants has suggested that the Rad55–Rad57 heterodimer is involved in stabilizing a protein or protein–DNA complex (Lovett and Mortimer, 1987; Hays *et al*., 1995; Johnson and Symington, 1995). A critical early step in recombinational repair is the formation of the Rad51 filament on ssDNA. Therefore, it is quite plausible that the Rad55–Rad57 heterodimer serves to stabilize the interaction of Rad51 with ssDNA. Consistent with this idea, the requirement for Rad55–Rad57 is partially alleviated by the Rad51-I345T protein, which exhibits more stable binding to DNA than wild-type Rad51.

The current model for Rad52 in Rad51-mediated strand exchange is that Rad52 is required to target Rad51 to the ssDNA and displace RPA (Song and Sung, 2000).

Table II. Yeast strains

Strain	Genotype[a]	Reference or source
W1588-4C	*MAT***a**	R.Rothstein
W1588-4A	*MAT*α	R.Rothstein
HKY595-1C	*MAT*α *rad51::LEU2*	H.Klein
HKY595-3B	*MAT***a** *rad51::LEU2*	H.Klein
HKY597-2B	*MAT***a** *rad55::LEU2*	H.Klein
HKY597-2C	*MAT*α *rad55::LEU2*	H.Klein
HKY587-2C	*MAT***a** *rad57::LEU2*	H.Klein
HKY598-8B	*MAT*α *rad57::LEU2*	H.Klein
J883	*MAT***a** *rad52Δ327*	R.Rothstein
YAR179-2C	*MAT***a** *rad51::LEU2 rad54::LEU2 ade2::hisG-URA3-hisG*	Rattray and Symington (1995)
LSY411	*MAT*α *rad51::URA3 rad5-535*	Rattray and Symington (1994)
LSY516-1C	*MAT*α *rad51::URA3 rad57::LEU2*	This study
LSY516-2D	*MAT***a** *rad51::URA3 rad57::LEU2*	This study
LSY989-1	*MAT***a** *rad51::LEU2 rad57::LEU2*	This study
LSY989-2	*MAT*α *rad51::LEU2 rad57::LEU2*	This study
LSY990	*MAT***a** *rad51::LEU2 rad55::LEU2*	This study
LSY991	*MAT*α *rad51-I345T rad5-535*	This study
LSY992-1	*MAT***a** *rad51-I345T rad57::LEU2*	This study
LSY992-2	*MAT*α *rad51-I345T rad57::LEU2*	This study
LSY1007-1	*MAT***a** *rad51::LEU2 rad52Δ327*	This study
LSY1007-2	*MAT*α *rad51::LEU2 rad52Δ327*	This study
LSY1009	*MAT*α *ade3::GAL10-HO*	This study
LSY1022-2	*MAT*α *rad57::LEU2 ade3::GAL10-HO*	This study
LSY1023-1	*MAT*α *rad51-I345T rad57::LEU2 ade3::GAL10-HO*	This study
LSY1024-2	*MAT*α *rad51-I345T ade3::GAL10-HO*	This study
RDKY2275	*MAT***a** *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1D1.6R can1 GAL* pRDK273, pRDK274, pRDK275	Nakagawa *et al*. (2001)
MCY14	*MAT***a** *suc2-437 lys2-801*	S.Jinks-Robertson
SJR13	*MAT*α *lys2-802*	S.Jinks-Robertson

[a]All strains are in the *RAD5* corrected W303 background (*his3-11, 15 leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 RAD5*) except RDKY2275, MCY14 and SJR13; only differences from this genotype are noted.

A Rad51 mutant that can compete with RPA for binding to ssDNA should bypass the need for Rad52 in forming the pre-synaptic filament. One possible explanation for why Rad51-I345T still requires Rad52 *in vivo* is that the mutant protein shows higher affinity and more stable binding to both ssDNA and dsDNA. Therefore, Rad52 is still required to target Rad51-I345T to ssDNA and prevent unproductive association of Rad51-I345T with duplex DNA. However, once targeted to the ssDNA, Rad51-I345T is bound more stably and no longer requires stabilization by the Rad55–Rad57 heterodimer. Based on the properties of the Rad51-I345T mutant protein, we suggest that the Rad52 and Rad55–Rad57 mediators have distinct functions in the formation of the Rad51 nucleoprotein filament. ssDNA formed by resection of double-stranded ends is immediately bound by RPA. Rad52 associates with RPA and Rad51, and targets Rad51 to ssDNA. We propose that Rad55 and Rad57 stabilize the initial Rad51–ssDNA complex to facilitate cooperative binding by additional Rad51 monomers, resulting in filament formation.

The role of the vertebrate Rad51 paralogs in recombinational repair is currently unknown. Like *rad55* and *rad57* mutants, *rad51b*, *rad51c*, *rad51d*, *xrcc2* and *xrcc3* cell lines show less severe defects in recombinational repair than *rad51* mutants, and overexpression of *RAD51* suppresses the sensitivity of the paralog mutant cell lines to DNA-damaging agents (Takata *et al*., 2001). Given these similarities, it seems likely that mutations in human *RAD51* corresponding to the yeast *rad51 srp* alleles might suppress the requirement for the vertebrate Rad51 paralogs in DNA repair and genome stability.

Materials and methods

Media, growth conditions and genetic methods

Standard genetic methods were followed. Rich medium (YPD) and synthetic complete (SC) medium lacking the appropriate amino acid or nucleic acid base were prepared as described previously (Sherman *et al*., 1986). Selection for Ura^- cells was performed on SC medium containing 5-fluoro-orotic acid (5-FOA) at 1 mg/ml (Boeke *et al*., 1987). Yeast mating, sporulation and tetrad dissection were performed as previously described (Sherman *et al*., 1986). The DNA repair and recombination defects of *rad55* and *rad57* strains are greater at lower temperatures, and most of the assays using these mutants were performed at room temperature (23°), unless otherwise indicated. For most purposes, yeast cells were grown at 30°C. Transformations were performed by the lithium acetate method (Ito *et al*., 1983).

Yeast strains and plasmids

Saccharomyces cerevisiae strains used in this study are listed in Table II. All strains except RDKY2275 are derivatives of strains W303-1A or W303-1B containing the corrected *RAD5* allele (W1588-4C and W1588-4A) except where noted. Double mutants were identified by phenotype among haploid progeny generated by crossing the appropriate haploid parents. To construct strain LSY991, LSY411 was co-transformed with a *Bam*HI fragment from plasmid pRS413::*rad51-I345T* and a replicating vector containing the *HIS3* gene, and selecting for His^+ transformants. Recombination of the fragment with the homologous chromosomal locus results in loss of the *URA3* marker and 5-FOA resistance. His^+ transformants were screened for 5-FOA resistance, and positive clones were verified further by their resistance to 50 krad γ-irradiation (Gammacell-220 ^{60}Co irradiator, Atomic Energy of Canada). The *rad51* ORF of 5-FOA-resistant, γ-ray-resistant colonies was amplified by PCR and directly sequenced to confirm the presence of the *rad51-I345T* allele. Segregation of *rad51-I345T* in crosses could be

distinguished from *RAD51* by the increased resistance to γ-irradiation in the presence of a *rad57* mutation. Alternatively, LSY991 was crossed to strains containing a marked *rad51* allele that would segregate from the *rad51-I345T* allele during genetic crosses. To construct strain LSY1007, HKY595-1C was crossed to J883. Haploid progeny were irradiated with 50 krad, and tetrads showing 2:2 segregation for γ-ray sensitivity were scored as *rad51::LEU2 rad52Δ327* double mutants. To construct strain LSY1009, LSY679 (*ade2-1*) was transformed to Ura^+ with *Bst*EII-linearized plasmid YIP*ade3::HO* (Sandell and Zakian, 1993). Ura^+ transformants were grown on non-selective medium and then streaked onto medium containing 5-FOA to select for pop-out recombination events. White colonies contain the *ade3::HO* disruption, whereas red colonies contain the undisrupted *ADE3* allele. The presence of the *GAL10*-regulated *HO* gene was confirmed by the poor growth of white colonies on medium containing galactose.

Plasmid pRS413:*RAD51* was generated by cloning the 3.7 kb *Bam*HI fragment from pRS423::*RAD51* into the *Bam*HI site of pRS413. The *rad51-I345T* point mutation was reconstituted in plasmid pEZ5139 (Zaitseva *et al*., 1999) using the Gene Editor *in vitro* Site-Directed Mutagenesis System (Promega, Madison, WI) and mutagenic oligonucleotide 5′-CCAGATCCAAAGCCTACCGGTGGTAATATT-3′.

Genetic screen for rad57 suppressors

Plasmid pRS413::*RAD51* was mutagenized randomly by growth for ~30 generations in *E.coli* strain XL1-Red [*endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac mutD5 mutS mutT* Tn*10* (Tet^r) Stratagene]. Two different pools were screened. Each pool of mutagenized plasmids was used to transform yeast strain LSY516-2D to His^+. Cultures (1 ml) of individual His^+ transformants were grown to saturation, harvested and resuspended in 1 ml of water. A 5 μl aliquot of each suspension was spotted onto selective plates, irradiated at 50 krad and incubated at 23°C for 5 days. Plasmids were recovered and re-transformed into LSY516-2D. Rad51 protein levels were measured for each transformant capable of growth after 50 krad. Cell extracts were prepared (Strahl-Bolsinger *et al*., 1997) and analyzed by western blotting using affinity-purified rabbit anti-Rad51 (kindly provided by P.Sung).

γ-irradiation survival assays

Cells were grown in liquid medium to mid-log phase. The cultures were serially diluted and aliquots of each dilution were plated on solid medium. The plates were irradiated in a Gammacell-220 containing ^{60}Co for the designated dose. The dose rate of the Gammacell-220 was 53 rad/s. The plates were incubated for 3 days at 30°C or 5 days at 23°C, before survivors were counted. Each strain was assayed three separate times and the mean values are presented (Figure 3). For the spot assays, strains were grown as described above, serial dilutions were spotted onto YPD plates, left unirradiated or irradiated at 50 krad, and were incubated at 23°C for 5 days.

Analysis of mating-type switching

The physical analysis of mating-type switching was performed as described previously with strains LSY1009-1, LSY1022-2, LYS1023-1 and LSY1024-2 grown at 30°C (White and Haber, 1990). Mating-type switching was assayed genetically by inducing HO endonuclease in the above strains for 3 h at 23 or 30°C and then plating diluted cultures onto YPD. Following incubation at either 23 or 30°C, survivors were assayed for mating type by the ability to mate with tester strains MCY14 (*MAT***a**) or SJR13 (*MAT*α). Approximately 100 colonies were assayed for each strain at each temperature, and the experiment was repeated twice.

Protein purification

RPA was purified from strain RDKY2275 as described previously (Nakagawa *et al*., 2001). Rad51 was expressed in *E.coli* strain BL21 (DE3)/pLysS (Novagen Inc., Madison, WI) using plasmid pEZ3951 and purified as described (Zaitseva *et al*., 1999). The Rad51-I345T protein was purified by the same procedure as Rad51.

DNA binding assay

DNA binding was measured by retention of protein–DNA complexes on alkali-treated nitrocellulose filters as described previously (Wong and Lohman, 1993). Linear DNA labeled at the 3′ ends was obtained by digesting pUC19 with *Eco*RI and filling-in the recessed 3′ ends with DNA polymerase I (Klenow fragment) in the presence of [α-^{32}P]dATP. Labeled DNA was made single stranded by heat denaturation and then quenched on ice prior to use in ssDNA-binding assays. Increasing amounts of protein (0.0075, 0.015, 0.03, 0.05, 0.075, 0.1, 0.15, 0.3 and 0.5 μM) were incubated with a fixed amount (0.6 μM nucleotides) of either ssDNA or dsDNA in buffer A [20 mM Tris acetate pH 7.9, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 7.5 μg/ml bovine serum albumin (BSA), 5 mM adenosine 5′-[γ-thio]triphosphate (ATP[γ-S])]. Reactions were incubated at 37°C for 15 min and then passed through the nitrocellulose and DEAE membranes in a vacuum manifold apparatus. DNA binding was monitored as the signal retained on the alkali-treated nitrocellulose filter over the total amount of DNA retained on both filters. Data were quantified with a Molecular Dynamics Storm 445 SI phosphoimager and IMAGE-QUANT software. Numbers presented are the mean of two trials.

ATPase assay

Reactions contained 25 mM Tris acetate pH 7.9, 10 mM magnesium acetate, 1 mM ATP, 1 mM DTT, 10 μCi/ml [γ-^{32}P]ATP, 0.6 μM poly(dT) DNA and 0.2 μM Rad51 or rad51-I345T. Poly(dT) was either free or pre-incubated with 0.06 μM RPA for 2 min at 37°C. Reactions were incubated at 37°C for the time indicated and then quenched with 10 mM EDTA. A 2 μl aliquot of each reaction was spotted onto polyethyleneimine cellulose thin-layer chromatography plastic sheets. The plates were developed in 0.8 M acetic acid, 0.8 M LiCl, dried and the amounts of ^{32}Pi and [γ-^{32}P]ATP quantitated using a Molecular Dynamics Storm 445 SI phosphoimager and IMAGE-QUANT software.

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