In vivo assembly and disassembly of Rad51 and Rad52 complexes during double-strand break repair

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Assembly and disassembly of Rad51 and Rad52 complexes were monitored by immunofluorescence during homologous recombination initiated by an HO endonuclease-induced double-strand break (DSB) at the MAT locus. DSB-induced Rad51 and Rad52 foci colocalize with a TetR–GFP focus at tetO sequences adjacent to MAT. In strains in which HO cleaves three sites on chromosome III, we observe three distinct foci that colocalize with adjacent GFP chromosome marks. We compared the kinetics of focus formation with recombination intermediates and products when HO-cleaved MAT α recombines with the donor, MAT α . Rad51 assembly occurs 1 h after HO cleavage. Rad51 disassembly occurs at the same time that new DNA synthesis is initiated after single-stranded (ss) MAT DNA invades MAT α . We present evidence for three distinct roles for Rad52 in recombination: a presynaptic role necessary for Rad51 assembly, a synaptic role with Rad51 filaments, and a postsynaptic role after Rad51 dissociates. Additional biochemical studies suggest the presence of an ssDNA complex containing both Rad51 and Rad52.

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Introduction

Homologous recombination repairs double-strand breaks (DSBs) both in mitotic and meiotic cells. Our knowledge of the mechanism of recombination has been obtained from biochemical and cytological analyses of the proteins involved in recombination, as well as physical analysis of recombination intermediates (Pâques and Haber, 1999; Symington,

2002). In eucaryotes, homologous recombination requires genes of the RAD52 epistasis group: RAD50, -51, -52, -54, -55, -57, -59, MRE11, XRS2, and TID1. These genes have originally been identified in the budding yeast *Saccharomyces cerevisiae*, and are evolutionarily conserved from yeast to humans.

Biochemical analysis has established the importance of the bacterial RecA homolog, Rad51, to carry out the search for homologous sequences and strand exchange (Shinohara *et al*, 1992). Rad51 forms helical filaments on single-stranded DNA (ssDNA) (Ogawa *et al*, 1993; Sung and Robberson, 1995). *In vitro*, formation of the Rad51 filament is promoted by ssDNA-binding protein, RPA (Sung and Robberson, 1995; Sugiyama *et al*, 1997). RPA removes secondary structure from ssDNA, which inhibits Rad51 function. However, Rad51 by itself cannot bind to RPA-coated ssDNA; assembly requires the so-called mediator proteins Rad52 and the Rad55–Rad57 complex. Rad52 forms a heptameric ring on the ssDNA (Shinohara *et al*, 1998; Passy *et al*, 1999; Kagawa *et al*, 2002; Singleton *et al*, 2002) and binds to both RPA and Rad51 (Shinohara *et al*, 1992, 1998). Rad52 apparently recruits Rad51 on ssDNA covered with RPA and promotes formation of the Rad51 filament (Sung, 1997a; Benson *et al*, 1998; New *et al*, 1998; Shinohara and Ogawa, 1998). The Rad55–57 heterodimer also facilitates formation of the functional Rad51 complex on RPA-coated ssDNA (Sung, 1997b). *In vivo*, the requirement for Rad55 and Rad57 in the repair of DSBs is less stringent than Rad52 (Pâques and Haber, 1999; Symington, 2002).

In addition to presynaptic roles in recombination, Rad52 protein exhibits a distinct activity: the pairing of two complementary DNA strands (Mortensen *et al*, 1996; Shinohara *et al*, 1998; Sugiyama *et al*, 1998). This strand-annealing activity is stimulated by an interaction with RPA (Shinohara *et al*, 1998; Sugiyama *et al*, 1998). Sugiyama and Kowalczykowski (2002) proposed a role for Rad52's annealing activity in the postsynaptic phase of recombination, allowing the second end of a DSB to anneal to a D-loop created by strand invasion of the other DSB end.

Immunostaining is one method that is used to detect the presence of specific recombination proteins bound to chromosomes. Proteins such as Rad51 and Rad52 form foci (punctate staining) on chromosomes and in the nuclei of both mitotic cells suffering from DNA damage and meiotic cells (Bishop, 1994; Gasior *et al*, 1998, 2001). Genetic analysis of these foci suggests that they may represent sites of ongoing recombination. Rad52 is required for Rad51 focus formation both in mitosis and meiosis. Rad52 foci form in the absence of Rad51 function, consistent with the mediator function of Rad52 in the assembly of Rad51 onto ssDNA. Recent analysis using a GFP–Rad52 fusion protein raises an intriguing possibility concerning the nature of these foci (Lisby *et al*, 2001, 2003). When more than 20 DSBs per cell are introduced by

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ionizing radiation, only one or two Rad52–GFP foci were observed, suggesting the presence of a recombination center, where multiple recombination reactions occur.

One of the best-analyzed homologous recombination reactions is mating-type (*MAT*) switching in *S. cerevisiae* (Haber, 1998; Pâques and Haber, 1999). *MAT* switching is a site-specific recombination event in which mating-type information is transferred to the *MAT* locus by gene conversion using as templates the heterochromatic and untranscribed donor sequences *HMLα* or *HMRA*. The event is initiated by the introduction of a DSB at the *MAT* locus by HO endonuclease. DSB formation is followed by 5'-to-3' resection of DSB ends, forming ssDNA with a 3'-OH (White and Haber, 1990). Rad51 facilitates a search for homology and invasion of ssDNA of the *MAT* distal end into the donor duplex (*HMLα* or *HMRA*). Invasion of the 3'-OH end into the duplex triggers initiation of DNA synthesis. The mechanism of events following DNA synthesis is still largely unknown.

Using a strain in which the *MAT* locus is tagged with a GFP fusion protein bound to nearby sequences, we analyzed the formation of Rad51 and Rad52 foci in response to a single HO-induced DSB. Rad51 and Rad52 foci colocalize with the GFP-tagged DSB site. In strains bearing multiple HO cut sites, we find that the number of foci is proportional to the number of DSBs. This argues that each focus represents a site of recombination. Kinetic analysis of focus formation relative to the appearance of recombination intermediates and products reveals a new view on the assembly of recombination complexes. We propose that Rad52 carries out three distinct functions during *MAT* switching.

Results

Localization of recombination proteins to a single DSB site

In order to study the assembly and disassembly of Rad51 (and Rad52) foci at the site of a DSB, we used a strain in which the *MAT* locus is marked with tetracycline repressor protein (TetR) fused to GFP (Michaelis *et al*, 1997). An array of 112 tetracycline operators (*tetO*) was introduced 2 kb distal to the HO cleavage site at *MAT* (Figure 1A). The TetR–GFP fusion protein is expressed from a constitutive promoter and HO endonuclease from an inducible *GAL10* promoter. Induction of HO creates a single DSB at the *MAT* locus. Exponentially growing yeast cells exposed to galactose were collected and chromosomes were surface spread. The spreads were stained simultaneously with anti-Rad51 (or anti-Rad52) and anti-GFP antibodies, followed by staining with chromophore-conjugated secondary antibodies, and were examined under an epifluorescent microscope.

GFP foci on nuclear spreads

In cells subjected to surface spreading, nearly 90% of the nuclei contained one or two GFP spots (Figure 2). In cells from asynchronous cultures, approximately 60% of nuclei showed a single focus of GFP (Figure 2A), whereas the remainder had two closely positioned spots (Figure 2B). Cells with two GFP spots were likely to be in G₂, since most cells show two closely positioned spots when arrested by nocodazole (data not shown). Thus, to simplify our analysis described below, we restricted our observations to cells containing only one GFP spot, which presumably corre-

Figure 1 Experimental design. (A) Schematic diagram of the *MAT* locus visualized by GFP. A *tetO* array, able to bind TetR–GFP, is located 2 kb distal to the *MAT* locus. (B) Mating-type switching from *MATα* to *MATa*. The location of *StyI* sites used to monitor DSB formation and products by Southern analysis. (C) PCR analysis of the SIE recombination intermediate.

spond to cells in the G₁ and S phases. Irrespective of cell cycle, a DSB is formed and repaired efficiently in asynchronous cultures (Supplementary Figure 1).

Rad51 and Rad52 foci colocalize with the site of a DSB

Nuclear Rad51 foci form specifically in response to the DSB created by the galactose-induced HO endonuclease, as fewer than 3% of uninduced cells exhibit such foci. The spontaneously induced focus is located randomly in respect of the GFP focus (Figure 2C). Upon continuous exposure of wild-type (wt) cells to galactose, nuclei containing a Rad51 focus appear at *t* = 1 h (Figure 2D), with the number of Rad51 focus-positive nuclei increasing up to 50% during a 3-h incubation (Figure 3A). The number of nuclei with a single GFP spot gradually starts to decrease after 2 h. The loss of the GFP spot most likely reflects the 5'-to-3' degradation of DSB ends (Lee *et al*, 1998; Vaze *et al*, 2002). The number of Rad51 focus-positive nuclei, if one includes GFP-negative nuclei, increases up to 80% at 5 h (data not shown). Importantly, in 90% of cases where there is a GFP spot, the Rad51 focus partly overlaps with the GFP focus, whereas the remainder show a side-by-side configuration (Figure 2D). This may reflect the degree of spreading between the two sites that are 2 kb apart. The average number of foci per Rad51

Figure 2 Rad51 and Rad52 focus formation at the *MAT* locus. (A–J) wt (YDB057) and *sir3* mutants (YDB058 and YDB236) were incubated with and without galactose. YDB057 (A–E) and YDB058 (F, G) contain a GFP-binding site near the *MAT* locus. YDB236 (H–J) contains three GFP-binding sites on chromosome *III*. Chromosome spreads were stained with anti-Rad51 (red; A–D, F, H–J) or anti-Rad52 (red; E, G) and anti-GFP (green) antibodies and examined under an epifluorescence microscope. DNA was stained with 4', 6'-diamidino-2-phenylindole (DAPI; blue). Bars indicate 2 μ m. (K, L) Colocalization of Rad51 and Rad52 with the GFP spot. Chromosome spreads from wt (K; YDB057) and the *rad54* mutant (L; YTM132) were stained simultaneously with anti-GFP (green), anti-Rad51 (pink), anti-Rad52 (red), and DAPI (blue). Bars indicate 2 μ m.

focus-positive nucleus is 1.1 at 3 h following DSB induction, consistent with the fact that a small proportion of cells have a focus that is independent of an HO-induced DSB.

When chromosome spreads were stained with anti-Rad52 antibodies, there was a pattern of focus formation similar to Rad51 (Figures 2E and 3B). Rad52 foci are induced specifically in response to a DSB and colocalize with the GFP spot. The average number of Rad52 foci per focus-positive nucleus

at $t = 3$ h of incubation is 1.2. These results indicate that a focus containing either Rad51 or Rad52 marks the site of the DSB.

The number of Rad51 foci is proportional to that of DSBs

We then determined if there is a one-to-one correspondence between the numbers of Rad51 foci and DSBs. We analyzed DSB formation in a *sir3* derivative, which is defective in the silencing of the *HML* and *HMR* donor cassettes; consequently, HO can cleave *HML* and *HMR* as well as *MAT*. In this strain, there are no intact templates to complete recombination. This strain also contains a *tetO* array near the *MAT* locus as described above.

Following HO induction, nuclear spreads were prepared and stained with anti-Rad51 and anti-GFP antibodies. Rad51 foci are induced after 1 h, reach a plateau after 3 h (Figure 3C). At later time points, the Rad51 foci become much brighter. This could be due to longer ssDNA regions that would form longer Rad51 filaments. In nuclei with three Rad51 spots, the three foci often occupy a small portion of the spread chromatin, with one of them colocalized with the *MAT* adjacent GFP spot (Figure 2F). The mean number of Rad51 foci per focus-positive nucleus in the *sir3* mutant increases up to 2.6 at $t = 3$ h (Figure 3E). These data indicate a near one-to-one correspondence of the number of Rad51 foci with the number of DSBs, suggesting that each focus reflects a single recombination site. Similar results were obtained for Rad52 focus formation in *sir3* cells (Figures 2G and 3D). The average number of Rad52 foci in *sir3* mutant strains is 2.7 after a 3-h exposure to galactose (Figure 3E). The above experiments demonstrate that the nuclear foci containing either Rad51 or Rad52 each correspond to the site of a single DSB.

To support our conclusion that, in most cases, three DSBs produce three distinct foci, we constructed a *sir3* mutant with *lac* operator (*lacO*)-binding sites (Robinett *et al*, 1996; Straight *et al*, 1996) near both *HML* and *HMR* as well as the *tetO*-binding site near *MAT*. This strain expresses not only TetR-GFP but also LacI-GFP (see Materials and methods). Nuclei often show three GFP spots on spreads. After the induction of DSBs, chromosome spreads were stained with anti-Rad51 and anti-GFP antibodies (Figures 2I and J). We found that 52% of chromatin spreads contained three Rad51 foci after a 3-h incubation with galactose, each of which overlaps or is closely adjacent to a single GFP spot.

We also see some early spreads containing two Rad51 foci in the *sir3* mutant, both of which are present in near vicinity to GFP spots (Figure 2H). These spreads are likely to come from cells with two DSBs, arguing against that TetR-GFP does not have an ability to bind Rad51 after HO induction.

To address the concern that the chromosome-spreading technique disrupts recombination centers, we examined intact *sir3* mutant cells tagged with GFP-bound operator arrays after HO induction (Figure 4A). Under these conditions, efficient cutting by HO endonuclease is observed at *MAT* as well as at the unsilenced *HML* and *HMR* loci (Supplementary Figure 1). Consistent with the results obtained from spread nuclei, we found that 57 % of intact cells contained three GFP foci following a 2-h induction of *HO* (Figure 4C). The average number of GFP foci in the *sir3* mutant was 2.5 after a 2-h exposure to galactose, similar to that found in spread nuclei.

Figure 3 Kinetic analysis of Rad51 and Rad52 foci. (A–D) wt (YDB057; A, B) and the *sir3* (YDB058; C, D) cells were exposed to galactose at $t = 0$. At indicated times, chromosome spreads were prepared and stained with either anti-Rad51 (A, C) or anti-Rad52 (B, D) together with anti-GFP. Nuclei containing Rad51 focus (closed circles) or Rad52 focus (closed squares) were counted and percentages per focus-positive nuclei were plotted. Results for one experiment are shown. GFP-positive nuclei (closed rectangles) were also counted and plotted against total nuclei. At least 100 nuclei were analyzed at each time point. (E) The numbers of Rad51 (open bars) and Rad52 (closed bars) foci per focus-positive nuclei at a 3-h incubation were counted and the average numbers of each focus per focus-positive nuclei are shown.

These data support the conclusion that nuclear foci containing Rad51 or Rad52 represent individual DSB sites.

We noted that there was also an increase in the number of cells containing only one or two GFP foci following a 2-h *HO* induction in the *sir3* strain. To determine if the increase in nuclei containing fewer than three foci reflects the formation of recombination centers (in which several DSBs are brought to one nuclear location), we replaced the TetR–GFP construct in the *sir3* mutant strain above with a TetR–CFP fusion. This enabled us to distinguish between loss of foci due either to colocalization of the tagged DSB sites or to resection through

the repressor binding arrays adjacent to the DSB sites. Consistent with the results described above, we found that 56% of intact cells contained three foci (Figure 4B; two GFP and one CFP) following a 2-h induction of *HO*, with an average of 2.5 foci per focus-positive nucleus (data not shown). We observed colocalization of the CFP and GFP foci in only 13% of focus-positive nuclei following a 2-h *HO* induction (5% contained a single GFP⁺CFP⁺ focus and 8% contained two distinct GFP foci, only one of which was colocalized with the CFP focus). Moreover, 22% of the focus-positive nuclei containing fewer than three foci did not

Figure 4 Intact cell analysis of DSB sites marked with GFP and CFP. (A, B) GFP foci (A; YDB236) and GFP and CFP foci (B; YDB244) were observed in intact *sir3* cells by 3D fluorescence microscopy. Cells were fixed following a 2-h incubation with galactose. Each image shown is a projection of a series of 16 sections through the cell spaced 0.2 μ m apart. Bars indicate 2 μ m. (C) The number of GFP foci per focus-positive cell was scored in *sir3* cells (YDB236) at indicated times following exposure to galactose. At least 100 focus-positive cells were scored at each time point.

contain a CFP focus. The loss of the CFP spot is likely the result of resection through the *tetO* arrays inserted adjacent to the DSB site at *MAT*. These results strongly suggest that the decrease in the number of nuclei containing three foci and the corresponding increase in the number of nuclei containing two foci following *HO* induction is due to hyper-resection of the DSB in the absence of a donor for repair, and not to colocalization of these foci into a recombination center.

Finally, we carried out immunostaining of chromosome spreads from wt and Rad52-YFP cells after induction of DNA damage (Supplementary Figure 3; T Miyazaki and A Shinohara, unpublished results). wt cells show several foci of Rad51 or Rad52 on spreads after the treatment with Zeocin, which causes DSBs. This is consistent with the previous observation that γ -rays induce Rad51 foci in a dose-dependent manner (Gasior *et al*, 1998, 2001). On the other hand, Rad52-YFP cells frequently formed only one Rad52 (or YFP) focus on spreads. Furthermore, Rad51 also shows aggregation, which is colocalized with the Rad52-YFP focus. These results suggest that Rad52-YFP forms one focus after the induction of exogenous DNA damage, strongly suggesting that the fusion of Rad52 to YFP has an ability to induce the aggregation of recombination proteins.

Kinetic analysis of Rad51 and Rad52 focus formation during repair of a single DSB

To study the kinetics of assembly and disassembly of Rad51 and Rad52 at the *MAT* locus, we carried out experiments when the DSB is induced transiently. After a 1-h exposure to galactose, the cells were washed and resuspended in medium containing glucose to turn off *HO* expression. Chromosome spreads were prepared from cells taken at time intervals following induction, and stained with anti-Rad51 or anti-Rad52 antibodies. Nuclei containing a single Rad51 (or Rad52) focus near the GFP focus were scored and plotted versus time (Figure 5). As above, we confined our analysis to nuclear spreads with only one GFP spot. In these nuclei, Rad51 focus formation begins at $t = 0$ (the time when the cells were shifted to glucose-containing medium), peaks at $t = 30$ min and then disappears (Figure 5A). Rad52 foci begin to appear at $t = 0$. However, in contrast to Rad51, Rad52 focus formation peaks at $t = 60$ min, 30 min later

Figure 5 Cumulative analysis of Rad51 and Rad52 foci. (A) wt cells (YDB057) were transiently exposed to galactose for 1 h. At indicated times, chromosome spreads were prepared and stained for Rad51 or Rad52 and GFP. Time 0 is the time when the medium was exchanged. The nuclei containing Rad51 (closed circles) or Rad52 (closed squares) focus per single GFP-positive nuclei were counted. At least 100 nuclei were analyzed at each time point. (B) The kinetics of the foci are compared with that of DSBs and products. Results of experiment #6 are shown: DSBs (closed triangles) and products (closed diamonds). (C) Noncumulative curves of focus positive nuclei were converted into cumulative curves as described in Materials and methods. Rad51 assembly, closed circles; Rad51 disassembly, open circles; Rad52 assembly, closed squares; Rad52 disassembly, open squares. (D) Cumulative curves for Rad51 foci are from five independent experiments. (E) Lifespans of Rad51 and Rad52 foci were calculated from five independent noncumulative curves and the average time of each lifespan was obtained.

than Rad51, and then disappears during further incubation. Thus, the kinetics of Rad52 focus dissociation is delayed relative to Rad51. These results were reproduced in three independent time-course analyses (see below).

Comparison of the kinetics of focus formation with the appearance of recombination intermediates

To study the relationship between the assembly/disassembly of the recombination machinery and DNA transactions during DSB repair, we compared the kinetics of Rad51 and Rad52 focus formation with those of recombination intermediates and products. We analyzed the formation of a single DSB and the appearance of the recombination product (switching from *MAT α* to *MAT β*) by Southern blotting (Figure 1B). In addition, we measured the kinetics of joint molecule formation between the donor and recipient molecules indirectly by PCR (White and Haber, 1990). The sequence differences between the *HMR α* donor and the *MAT α* recipient locus enable us to detect an intermediate molecule in which *MAT* distal ssDNA has invaded the donor sequence and has initiated synthesis of

a short patch of DNA (30 bp) using the 3'-OH end of the invading strand as a primer (Figure 1C). We refer to this intermediate as a strand invasion-extension (SIE) structure. The kinetics of the formation of DSBs and products as well as SIE (Supplementary Figure 1) was similar to that reported previously (White and Haber, 1990; Sugawara *et al*, 1995).

We converted the kinetic data of Rad51 and Rad52 focus formation and of DSBs into cumulative curves as described (Padmore *et al*, 1991). In brief, we calculated the lifespan of a particular stage, for example, focus-positive nuclei. The average lifespan of Rad51 and Rad52 foci is 30 and 44 min, respectively (Figure 5E; the difference in lifespan between the proteins is statistically significant; Student's *t*-test, $P=0.014$), whereas that of the DSB is 110 min. Rad51 and Rad52 foci are shorter-lived intermediates than DSBs (Figure 5B). By this approach, the kinetics of entry into and exit out of the particular stage are obtained as a cumulative curve (Figure 5C). Cumulative curves of Rad51 focus formation data from five independent experiments were highly consistent, as shown in Figure 5D.

Using the cumulative curves, we compared cytological events with DNA events. Two typical results are presented

in Figure 6. To simplify the results, the data are also presented in a schematic view (Figure 6G). There is about a 1-h lag between the introduction of the DSB and the formation of a detectable Rad51 focus. This gap may reflect the time that cells spend assembling a detectable amount of Rad51 and Rad52 on the ssDNA. In each of our experiments, the Rad51 focus appears at the same time or slightly earlier (10 min) than the Rad52 focus (Figures 6C and F). However, the Rad51 focus disappears 20 min earlier than Rad52. Thus, the Rad52 focus has a longer lifespan than Rad51, indicating that Rad52 associates with chromosomes after the disassembly of a detectable Rad51 focus. The timing of Rad51 disassembly correlates with the formation of the SIE intermediate at the *MAT* distal end (Figures 6A and D). In contrast, Rad52 disappears concomitantly with the formation of the product as well as disappearance of the DSB (Figures 6B and E). It is important to note that Rad51 and Rad52 are both present for a short time prior to the strand invasion/primer extension step.

Colocalization of Rad51 and Rad52 in wt *MAT* switching

The above kinetic analysis of the foci clearly indicates coexistence of Rad51 and Rad52 on chromosomes. We checked

Figure 6 Comparison of focus formation with DNA events. Results from two independent time-course analyses (#6, A–C; #7, D–F) are presented. Assembly and disassembly of Rad51 foci are compared with the formation of SIE intermediates (A, D). Assembly and disassembly of Rad52 foci are compared with the formation of product and DSB appearance/disappearance (B, E). All curves of the foci and DNA events are indicated (C, F). The times when half of the cells enter into a particular stage are calculated. A schematic summary for #6 experiment is presented in (G). Rad51 assembly, closed circles; Rad51 disassembly, open circles (black); Rad52 assembly, closed squares; Rad52 disassembly, open squares (red); DSB entry, closed triangles; DSB exit, open triangles (green); SIE formation, open diamonds (purple); products, closed diamonds (blue).

colocalization of the two proteins by quadruple staining with GFP and DAPI using rabbit anti-Rad51 and rat anti-Rad52 antibodies. As shown in Figure 2K, Rad51 and Rad52, which also coexist with the GFP spot, often colocalize on wt chromosomes after the introduction of the DSB. The frequency of nuclei with colocalized foci is low: around 10% at 30 min. This is not surprising given the short lifespan of the complex containing both proteins, compared to what is seen when the DSB cannot be repaired, as in a *rad54* mutant (see below). This colocalization is not due to crossreaction of primary and secondary antibodies (data not shown).

Mutant analysis

Our results suggest that Rad51 assembles onto ssDNA simultaneously or just prior to the assembly of Rad52. This is unexpected, given that *in vitro* studies had suggested that Rad52 facilitates Rad51 assembly onto ssDNA and might bind prior to Rad51. We analyzed the role of Rad52 in Rad51–ssDNA assembly using *rad51* and *rad52* mutant strains as well as the role of Rad55 and Rad54 in assembly and disassembly of Rad51 and Rad52 foci. Cells from *rad51*, *rad52*, *rad54*, and *rad55* mutant strains accumulate unusual recombination intermediates (a longer ssDNA, which is shown by the presence of larger restriction fragments detected by Southern hybridization; see Sugawara *et al*, 1995), which suggests that the substrate for Rad51 and Rad52 assembly is somewhat different between wt and the mutants. Therefore, we focused our analysis on early time points following DSB induction (Figure 7 and see also Supplementary Figure 2). At least three independent time-course analyses were carried out for each mutant strain, with reproducible results. One of each experiment is shown here.

rad51. No Rad51 focus is observed in the *rad51* mutant, as expected (Figure 7A) and the SIE intermediate is not detected (data not shown). Interestingly, at 0 h, about 25% of cells show a Rad52 focus, which is not localized with GFP, probably a spontaneously induced focus. The *rad51* strain does form an HO-induced Rad52 focus (Figure 7B), localized with GFP, indicating that Rad52 binds to the ssDNA in the absence of Rad51 (Gasior *et al*, 1998). However, the Rad52 focus does not disassemble in the mutant. Thus, assembly of a Rad52 focus does not require *RAD51* function, whereas its disassembly is *RAD51*-dependent. The kinetics of Rad52 focus formation in the *rad51* mutant are slightly slower than the cumulative curve of Rad52 assembly in wt cells. This does not simply mean that Rad52 assembles on chromosomes slower than in wt, since the cumulative curve of Rad52 in wt probably constitutes several assembly reactions. Moreover, the amount of Rad52 needed in the initial phase of recombination may be too low to be detected by immunocytology (see Discussion).

rad52. As expected from various studies, a *rad52* mutant did not show any detectable HO-induced Rad51 foci (Figure 7C), indicating that Rad52 is required for detectable Rad51 focus formation. Neither Rad52 foci (Figure 7D) nor the SIE intermediates formed in the *rad52* mutant strain following DSB induction.

rad55. Previous biochemical analyses have shown that the Rad55–Rad57 heterodimer has a mediator function that helps

Figure 7 Rad51 and Rad52 focus formation in various mutants. The *rad51* (A, B; YTM134), *rad52* (C, D; YTM171), *rad55* (E, F; YTM167), and *rad54* (G, H; YTM132) mutants were analyzed for HO-induced Rad51 and Rad52 focus formation. The DSB was induced by incubating cells in medium containing galactose for 1 h. The percentages of Rad51 (closed circles in A, C, E, G) and Rad52 (closed squares in B, D, F, H) focus-positive nuclei were plotted against total nuclei. In each figure, noncumulative (closed circles or squares) and cumulative curves (open circles or squares) of the focus in wt are indicated as dotted lines.

the assembly of a Rad51 filament onto RPA-coated ssDNA (Sung, 1997b). Although there are reports that the *rad55* mutant is partially defective in mating-type switching (Schmuckli-Maurer and Heyer, 1999; Fortin and Symington, 2002), the defect of the *rad55* mutant in the formation of SIE or products is comparable to those in the *rad51* and *rad52*

mutants (data not shown), as shown previously (Sugawara *et al*, 2003). Although the maximum percentage of Rad51 focus-positive *rad55* cells is comparable to that in wt strains, *rad55* mutant cells exhibit a 90-min delay in Rad51 focus formation relative to wt (Figure 7E). This indicates that *RAD55* is required for timely assembly of Rad51 on chromosomes. The *rad55* mutant forms Rad52 foci, which accumulate to higher levels than in wt during further incubation (Figure 7F). Surprisingly, in contrast to the accumulation of foci observed in other mutants tested, the *rad55* mutant exhibits disassembly of both Rad51 and Rad52 foci, even without repairing the DSB. In addition, the foci formed in *rad55* cells are fainter than those in other mutants, particularly at late time points following DSB induction (compare with *rad54* below; see Supplementary Figure 2). These suggest that Rad55 (and likely Rad57) plays a role in stabilizing both the Rad51 and Rad52 complexes formed on ssDNA, consistent with a previous report (Fortin and Symington, 2002).

rad54. Rad54 protein functions in a synaptic or postsynaptic phase of recombination (Petukhova *et al*, 1998; Mazin *et al*, 2000; Solinger and Heyer, 2001). A *rad54* mutant forms neither SIE intermediates nor the product of DSB repair (data not shown). As expected, both Rad51 and Rad52 foci appear, but fail to disappear in the *rad54* mutant (Figures 7G and H). This indicates that Rad54 is required for the disassembly of Rad51 and Rad52. In the *rad54* mutant, at $t = 2$ h, 73% of Rad51 foci are colocalized with Rad52 (Figure 2L). Thus, Rad51 and Rad52 seem to bind to the same recombination intermediates prior to strand invasion.

***In vitro* formation of an ssDNA complex containing both Rad51 and Rad52**

To confirm formation of a DNA complex with both Rad51 and Rad52, we carried out *in vitro* experiments using a biotinylated-dT₆₀ ssDNA (Figure 8A). This DNA was incubated first with a saturating amount of Rad52 and then various amounts of Rad51, and then purified using magnetic beads coated with streptavidin. Purified ssDNA complexes were analyzed for the presence of Rad51 or Rad52. Under these conditions, Rad51 cannot efficiently form a complex with dT₆₀ (Figure 8B, lanes 1–4). However, the presence of Rad52 promotes the binding of Rad51 to the Rad52–ssDNA complex (Figure 8B, lanes 5–8), indicating that Rad52 promotes the formation of a complex containing both Rad51 and Rad52. Interestingly, the amounts of Rad52 in the complex increased by the addition of Rad51 (Figures 8B and E), indicating a cooperative effect between the two proteins. The ratio of Rad52 to Rad51 is around 1.1 (Figure 8E). Increased amounts of Rad52 promote the recruitment of Rad51 onto the ssDNA complex (Figure 8C). The presence of nucleotides does not affect the formation of complex (data not shown). These indicate that Rad51 and Rad52 bind to the same single ssDNA molecule simultaneously *in vitro*.

Although we used saturating amounts of the proteins in the above experiments, we cannot deny a possibility that a portion of the DNA is occupied by Rad51 and the remaining portion is by Rad52. We next used a dT₃₀ ssDNA as a substrate. Rad52 forms a heptameric complex bound to ssDNA. Chemical footprint analysis suggests a periodicity of every four nucleotides upon Rad52 binding (Parsons *et al*,

Figure 8 *In vitro* complex formation of Rad51–Rad52–ssDNA. (A) Schematic diagram of the assay. (B) Rad52 protein (1 μM) was incubated with a reaction mixture containing 3 μM (nucleotide) biotinylated-dT₆₀ for 5 min, followed by the addition of various concentrations of Rad51 protein. After a 10-min incubation, the ssDNA complexes were purified and further processed as described in Materials and methods. One-tenth of the eluates were analyzed with Western blotting. Upper panel, Coomassie staining; lower panel, Western blotting using anti-Rad51 antibody. Lanes 1–4, in the absence of Rad52; lanes 5–8, in the presence of 1 μM Rad52. Lanes 1 and 5, no Rad51; lanes 2 and 6, 0.25 μM Rad51; lanes 3 and 7, 0.5 μM Rad51; lanes 4 and 8, 1.0 μM Rad51. (C) Various concentrations of Rad52 were incubated with 3 μM biotinylated-dT₆₀ for 5 min, followed by the addition of 1 μM Rad51. After a 10-min incubation, the mixtures were analyzed as described in Materials and methods. Upper panel, Coomassie staining; lower panel, Western blotting. Lanes 1–4, in the presence of 1 μM Rad51; lanes 5–8, in the absence of Rad51. Lanes 1 and 5, no Rad52; lanes 2 and 6, 0.25 μM Rad52; lanes 3 and 7, 0.5 μM Rad52; lanes 4 and 8, 1.0 μM Rad52. (D) Same as for (B) except 3 μM biotinylated-dT₃₀ was substituted for biotinylated-dT₆₀. Upper panel, Coomassie staining; lower panel, Western blotting. (E) The relative molar amounts of proteins bound to 3 μM biotinylated-dT₆₀ were quantified (B). Rad51 (1 μM) and/or Rad52 (1 μM) were used.

2000), and X-ray crystallographic analysis supports this site size (Kagawa *et al*, 2002; Singleton *et al*, 2002). Thus, the footprint of a Rad52 heptameric ring should be 28 nucleotides. A single Rad52 ring would cover a dT₃₀ oligonucleotide, possibly without a binding site for any other protein. Rad51 was also bound to the Rad52–dT₃₀ complex (Figure 8D). Thus, it is likely that Rad51 and Rad52 can form a complex with ssDNA at least under the conditions described here.

Discussion

Recombination protein foci mark the site of DSB repair

We visualized the site of recombination by tagging an HO endonuclease cut site with GFP and showed that Rad51 and Rad52 foci colocalize with the GFP tag in response to an HO-induced DSB, demonstrating that the foci mark the site of recombination. Furthermore, the analysis of chromosome spreads described here shows that the number of foci per nucleus corresponds to the number of DSBs induced. On the other hand, recent analysis of a Rad52–YFP fusion protein in living yeast cells showed that the number of Rad52 foci did not correspond to the number of ionizing radiation-induced DSBs. Based on their results, Lisby *et al* (2001, 2003) proposed that a ‘recombination center’ carries out multiple recombination events simultaneously, and that DSBs are recruited to this site. Recombination centers might be too fragile to survive during chromosome spreads. However, this is unlikely since we did not observe colocalization of DSB sites even in intact cells (Figure 4A). Although the types of DNA damage used in the current study are different from that by Lisby *et al* (2001, 2003), we believe that the difference could be due to the fusion of Rad52 to YFP. We showed that DNA damage-induced Rad51 or Rad52 foci do not form a center in wt, while Rad52–YFP does form a center even on chromosome spreads (Supplementary Figure 3; T Miyazaki and A Shinohara, unpublished results). Consistent with this, Gasior *et al* (2001) showed that γ -rays induce multiple Rad51 foci on spreads. These imply a potential danger in the use of GFP fusion protein in recombination proteins without a proper control: whole-cell immunostaining for a given protein.

Model of MAT switching: three functions of Rad52 during recombination

Based on the results described here, we propose a model of protein assembly during MAT switching. We could identify three distinct complexes of Rad52 at each stage of MAT switching: presynaptic, synaptic and postsynaptic roles. In the presynaptic stage, Rad52 promotes the assembly of a Rad51 nucleoprotein filament on ssDNA. This activity has already been inferred by various biochemical and cytological analyses. Purified Rad52 protein facilitates the formation of Rad51 complex on the RPA-coated ssDNA *in vitro* (Sung, 1997a; New *et al*, 1998; Shinohara and Ogawa, 1998). Furthermore, meiotic Rad51 focus formation is compromised in *rad52* cells (Gasior *et al*, 1998). Chromatin immunoprecipitation (ChIP) analysis supports this finding as well (Sugawara *et al*, 2003; Wolner *et al*, 2003). Previous studies indicate a step-by-step assembly of Rad51 filaments (Gasior *et al*, 2001). First, RPA binds to the ssDNA and then recruits Rad52 to form a stoichiometric Rad52–RPA–ssDNA complex. Finally, Rad51 is recruited to the site through interaction with

Rad52. Together with the Rad55–Rad57 complex, Rad52 helps in the formation of the Rad51 presynaptic filament. However, our analysis gives new insight into this process. Although we did not observe Rad52 foci prior to Rad51 foci, Rad51 focus formation is dependent on Rad52. One interpretation is that substoichiometric levels of Rad52, below our threshold of detection, are sufficient to promote the assembly of Rad51 on ssDNA. Alternatively, a Rad52 complex, which can assist in Rad51 binding to RPA-coated ssDNA, may be too unstable to detect by the current methods. Furthermore, it is possible that Rad52 and Rad51 assemble on chromosomes simultaneously as a complex, consistent with our biochemical results described here. Sung and his colleagues have identified a free stable complex of Rad51 and Rad52 *in vivo* by immunoprecipitation (Sung, 1997a; Song and Sung, 2000).

Once Rad51 assembles on the ssDNA, Rad52 binds to the Rad51 filament to form a complex containing both Rad51 and Rad52. We detected foci with both Rad51 and Rad52 prior to the strand invasion and initiation of DNA synthesis in wt cells, indicating that Rad51 and Rad52 bind to similar recombination intermediates. We note further that we could detect Rad51–Rad52 colocalized foci even in *rad54* cells, which are completely deficient in strand invasion. These results argue that a complex containing Rad51 and Rad52 is an active species for homology search and strand invasion. This is in sharp contrast to the current models in which the Rad51–ssDNA complex is the active machinery for this process. The Rad51–Rad52 complex may carry out strand invasion more efficiently than the Rad51 filament. Consistent with this, we could detect a stable ssDNA complex containing both Rad51 and Rad52 *in vitro*. Previous biochemical analysis indicates that Rad52 alone promotes Rad51-mediated strand exchange even in the absence of RPA (Shinohara and Ogawa, 1998; Baumann and West, 1999). This cannot be simply explained by the mediator function of Rad52, but rather indicates an active role for Rad52 in Rad51-mediated recombination. A synaptic role of Rad52 was proposed previously (New and Kowalczykowski, 2002).

After strand invasion followed by DNA synthesis, which is facilitated by Rad54, Rad51 dissociates from the recombination intermediate. In contrast, Rad52 foci remain visible on chromosomes following the disappearance of Rad51, indicating a postsynaptic function for Rad52. Rad52 might bind to the displaced strand in a D-loop. Alternatively, Rad52 is likely to associate with the ssDNA on the other end of the DSB, which is not engaged in the initial strand invasion event. In either case, Rad52 could play a role in the completion of the recombinational repair event by promoting the strand annealing.

In late times, we could only detect Rad52 foci that did not have Rad51. This indicates that Rad51 cannot bind to a Rad52–ssDNA complex that forms at a later stage in the recombination, suggesting a functional difference of Rad52 in the presynaptic and postsynaptic phases. The Rad52 complex at the late stage may involve a structure or form of Rad52 that does not bind to Rad51. Biochemical analysis has revealed that excess Rad52 protein may be inhibitory to Rad51-mediated strand exchange in the presence of RPA, and possibly the binding of Rad51 (Sung, 1997a; Baumann and West, 1999). Alternatively, structural hindrance might inhibit the binding of Rad51. We entertain the possibility that the nature of the Rad52 complex might give specificity for Rad51

binding. If Rad52 is located on the displaced strand, Rad51 might not form a stable complex with the DNA since it lacks a free end. During the initial assembly of Rad51 filament on DSB ends, the end might be critical for promotion of Rad51 assembly. For example, the Rad55–Rad57 complex might interact with the ends and facilitate the assembly of Rad51 with Rad52. The displaced strand might lack a binding site for the Rad55–Rad57 complex, and thus Rad51 cannot assemble on this strand. More provocatively, formation of a Rad52 complex on the displaced ssDNA might be coupled with the initial strand invasion event. This coupling of micromolecule assembly would ensure the possible transfer of Rad52 from the Rad51–Rad52-invading complex to the displaced ends and the dissociation of Rad51 from it.

Functions of Rad55/Rad57 and Rad54

Consistent with the results from the ChIP analysis (Sugawara *et al*, 2003), we found that Rad55, and possibly its partner Rad57, is required for the efficient focus formation of Rad51. Rad51 could form foci with ssDNA, but this complex is apparently nonfunctional, indicating that the complex formed in the absence of Rad55 is different from that formed in its presence. In addition, we found that Rad51 complex formed in the absence of Rad55 is very unstable. Therefore, Rad55 has two functions: to assist in the assembly of Rad51 and to stabilize the Rad51 filament.

Rad54 is required for the disassembly of Rad51 and Rad52 foci, consistent with biochemical and ChIP analyses, which demonstrate a postsynaptic role for Rad54 in recombination (Sugawara *et al*, 2003).

Both the *rad55* and *rad54* mutants accumulate hyper-resected ssDNA intermediates (Sugawara *et al*, 1995), but they differ in the nature of their protein assembly defects. By comparing these mutants, we can uncover the role of the other proteins in Rad52 assembly. The Rad52 focus in the *rad55* mutant strain is fainter than that in the *rad54* mutant, particularly at later time points following DSB induction. In addition, the kinetics of Rad52 assembly in the *rad55* strain are slower than in *rad54* cells. This may suggest that Rad55 or Rad51 assists or stabilizes the Rad52 complex.

Our results described here are consistent with one recent report using the ChIP analysis (Sugawara *et al*, 2003), but inconsistent with the other (Wolner *et al*, 2003). Particularly, the behavior of Rad52 is quite different between our and the latter study (Wolner *et al*, 2003). We found that Rad52 focus is formed in both the *rad51* and *rad54* mutants. On the other hand, Wolner *et al* showed that Rad52 binding to *MAT* is completely deficient in the *rad51* and *rad54* mutants. There

are several differences to point out. First, we used a strain that can repair a DSB at the *MAT* locus and transiently expressed the DSBs. Second, we mainly examined early time points, for example, less than 2 h after the induction of the DSB, whereas Wolner *et al* mainly used a strain unable to repair the break and drew their conclusion based on the results at later time points. Furthermore, our cytological analysis could detect all protein complexes. However, the ChIP analysis could only analyze the local association of the proteins depending on the primers used in the experiment. Some recombination protein might be unevenly distributed on the recombination intermediates: near or far from DSB ends. Further studies are necessary to clarify these discrepancies.

Materials and methods

Strains

The tetracycline operator (*tetO*) array and Tet repressor–GFP fusion protein (TetR–GFP) have been described (Michaelis *et al*, 1997). YDB057 *ho HML::HMRa*-(*URA3*) *MATα matdist::tetO(112)*-(*NAT1*) *HMRade3::GAL10-HO ade1-110 trp1 leu2::TetR-GFP*-(*LEU2*) and a *sir3::TRP1* derivative, YDB058, were obtained by insertion of a *tetO* array 2 kb distal to *MAT* and a TetR–GFP fusion construct at the *LEU2* locus. The *lac* operator (*lacO*) array and Lac repressor–GFP fusion protein (LacI–GFP) have been described (Robinett *et al*, 1996; Straight *et al*, 1996). YDB236 *ho HMLα hmlprox::lacO(256)*-(*LEU2*) *MATα matdist::tetO(112)*-(*NAT1*) *HMRahmrdis::lacO(100)*-(*ADE1*) *ura3-52 ade3::GAL10-HO ade1-110 trp1 leu2::TetR-GFP*-(*LEU2*) *HIS3::URA3pro::lacI-GFP*-(*KanMX*) *trp1 sir3::TRP1*, a derivative of YDB057, was obtained by insertion of *lacO* arrays 1.5 kb proximal to *HML* and 2 kb distal to *HMR*, and a *URA3::LACI-GFP* fusion construct at the *HIS3* locus. YDB244 *ho HMLα hmlprox::lacO(256)*-(*LEU2*) *MATα matdist::tetO(112)*-(*NAT1*) *HMRahmrdis::lacO(100)*-(*ADE1*) *ura3-52 ade3::GAL10-HO ade1-110 trp1 ade1::TetR-3X(CFP)*-(*HPH1*) *HIS3::URA3pro::lacI-GFP*-(*KanMX*) *trp1 sir3::URA3* was derived by insertion of a TetR–3X(CFP) fusion construct at the *ADE1* locus. Details of the construction and integration of *lacO*/LacI and *tetO*/TetR constructs will be described elsewhere (Bressan *et al*, 2004).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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