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Yeast Rad52 and Rad51 Recombination Proteins Define a Second Pathway of DNA Damage Assessment in Response to a Single Double-Strand Break

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Saccharomyces **cells with a single unrepaired double-strand break adapt after checkpoint-mediated G_2/M arrest. We have found that both Rad51 and Rad52 recombination proteins play key roles in adaptation. Cells lacking Rad51p fail to adapt, but deleting *RAD52* suppresses *rad51*Δ. *rad52*Δ also suppresses adaptation defects of *srs2*Δ mutants but not those of *yku70*Δ or *tid1*Δ mutants. Neither *rad54*Δ nor *rad55*Δ affects adaptation. A Rad51 mutant that fails to interact with Rad52p is adaptation defective; conversely, a C-terminal truncation mutant of Rad52p, impaired in interaction with Rad51p, is also adaptation defective. In contrast, *rad51*-K191A, a mutation that abolishes recombination and results in a protein that does not bind to single-stranded DNA (ssDNA), supports adaptation, as do Rad51 mutants impaired in interaction with Rad54p or Rad55p. An *rfa1-t11* mutation in the ssDNA binding complex RPA partially restores adaptation in *rad51*Δ mutants and fully restores adaptation in *yku70*Δ and *tid1*Δ mutants. Surprisingly, although neither *rfa1-t11* nor *rad52*Δ mutants are adaptation defective, the *rad52*Δ *rfa1-t11* double mutant fails to adapt and exhibits the persistent hyperphosphorylation of the DNA damage checkpoint protein Rad53 after HO induction. We suggest that monitoring of the extent of DNA damage depends on independent binding of RPA and Rad52p to ssDNA, with Rad52p's activity modulated by Rad51p whereas RPA's action depends on Tid1p.**

Saccharomyces cerevisiae cells with a single unrepaired double-strand break (DSB) arrest prior to mitosis, but after eight or more hours they adapt and resume cell cycle progression even though they still harbor a broken chromosome (24, 25, 40, 49). Arrest in these cells depends on a network of checkpoint proteins that sense DNA damage and trigger a cascade of protein kinases (reviewed in references 7, 18, 27, 36, and 55). These checkpoint proteins include Rad17p, Rad24p, Mec3p, and Ddc1p that appear to act as DNA damage sensors (19, 20, 28), as well as Rad9p, which may be both a sensor and a modulator of protein kinases (5, 12). These proteins activate the ATM/ATR homologue, Mec1p, which phosphorylates both Rad53p and Chk1p, two protein kinases that control different aspects of the DNA damage response (38, 39). Both the Ddc1p-Rad17p-Mec3p and Mec1p-Ddc2p complexes have been shown to bind directly to a HO endonuclease-induced DSB (20, 28, 37). Rad53p is required for the phosphorylation of both Dun1p, a protein kinase that controls several DNA damage-inducible genes (1, 4), and Cdc5p, a polo-like kinase that has been implicated in the regulation of several steps in mitosis (for review, see reference 32). Both Rad53p and Chk1p must be functional to ensure mitotic arrest of DNA-damaged cells (38). An observation relevant to the work described below is that *RAD51* mRNA transcripts are up-regulated in response to DNA damage and that this induction is dependent both on the intact homologous recombination machinery (i.e., *RAD52*) and on the DNA damage signal transduction cascade enforced by Rad53 checkpoint kinase (3).

A recent study has examined the phosphorylation and protein kinase activity of Rad53p after induction of a single unrepaired DSB (34). Following the induction of the DSB, Rad53p kinase activity increases an hour or more after the DNA damage is inflicted, suggesting that cells respond to the damage only after the DNA has been degraded to form 3′-ended single-stranded DNA (ssDNA) tails. Kinase activity remains elevated for 8 to 12 h and decreases at the time cells adapt. In a similar fashion, Chk1p becomes hyperphosphorylated in parallel with Rad53p and these activated forms of Chk1p disappear at the time of adaptation (34). This suggests that adaptation requires the dephosphorylation and/or turnover of the activated kinases. This hypothesis is strengthened by the fact that overexpressing the phosphatase Ptc2 suppresses prolonged G_2/M arrest of DNA-damaged cells and suppresses all adaptation-defective mutations tested (26).

Several adaptation-defective mutations, which cause cells to remain permanently arrested when cells have a single, unrepaired DSB, have been identified (24, 25, 49). This prolonged arrest depends on the continued activity of both the Mec1p and Rad53p checkpoint kinases (25, 34). These adaptation-defective mutations appear to affect the process in different ways. Deletion of the genes encoding yKu70p and yKu80p enhances the DNA damage signal by causing a twofold in-

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crease in the rate of 5′-to-3′ exonuclease resection, thus apparently increasing the extent of ssDNA and prolonging G_2/M arrest (24). Consistent with this idea, cells also become permanently arrested when there are two DSBs, each resected at a normal rate (24). In both cases, a single amino acid substitution mutation, *rfa1-t11* (Rfa1-L45E), in the largest subunit of the ssDNA binding complex, RPA, suppresses permanent arrest (24, 50). These results suggest that the cell monitors the extent of DNA damage by measuring the extent of ssDNA (10). This idea has been strongly supported by results of recent experiments by Zou and Elledge (58) showing that the recruitment of Mec1-Ddc2 to sites of DNA damage is defective in an *rfa1-t11* mutant.

In contrast, the adaptation-defective *cdc5-ad* mutation does not alter 5′-to-3′ resection of DNA and is not suppressed by *rfa1-t11* (34). Nevertheless, permanent arrest in *cdc5-ad* cells depends on the continued activity of Rad53p and Mec1p (34). Cdc5p appears therefore to be both a downstream target of Rad53p kinase (directly or indirectly) and its feedback regulator. Deletion of the casein kinase II proteins also causes permanent cell cycle arrest in the face of DNA damage, but these less severe mutations have not been analyzed in much detail (49).

Four other adaptation-defective mutations have recently been described. The recombination protein Tid1p, a homologue of Rad54p, interacts with the Rad51p strand exchange protein (6). In mitotic cells, a *tid1* deletion has a relatively minor role in gene conversion repair of a DSB but does play an important role in a *RAD51*- and *RAD54*-independent pathway of DNA repair known as break-induced replication (42). Nearly 100% of G_2/M-arrested *tid1*Δ cells permanently arrest, as do *yku70*Δ cells, in the presence of a single unrepaired DSB (25). Unlike *yku* mutations, *tid1*Δ does not affect the rates of resection of DSB ends. Like *yku70*Δ, *tid1*Δ is suppressed by *rfa1-t11*. The three other adaptation-defective mutations are distinct in that they also prevent cells from resuming cell cycle progression when DNA damage has been repaired. A deletion of the Srs2 helicase (53) prevents both adaptation and recovery. Recent evidence has suggested that Srs2p acts to displace Rad51p from ssDNA (23, 54), although in the checkpoint studies we carried out, it appears that Srs2p's checkpoint role is at a later step, when DNA has been repaired in a *RAD51*-independent process. Interestingly, however, *rad51*Δ suppressed the recovery defect. The remaining two adaptation- and recovery-defective mutations, *ptc2*Δ and *ptc3*Δ, together abolish PP2C phosphatase activity that is apparently required to turn off the checkpoint kinase cascade beginning with Mec1p (26).

Here we report that another protein involved in recombinational repair of DSBs is also adaptation defective. Rad51p is an evolutionarily conserved strand exchange protein that binds to ssDNA in vitro to form filaments that might compete with RPA in the assessment of how much ssDNA has been generated at DSB ends (11, 13, 14, 31, 41, 47). The adaptation defect of *rad51*Δ is partially suppressed by *rfa1-t11*, which encodes a mutant protein that has recently been shown to be inefficiently replaced by Rad51p in vitro (17). Quite surprisingly, a *rad52*Δ *rfa1-t11* double mutant is also adaptation defective. Based on these results, we suggest that Rad52p, which is modulated by Rad51p, and RPA, which is modulated by Tid1p, independently monitor the extent of DNA damage and determine whether cells will adapt in the face of a single unrepaired DSB.

MATERIALS AND METHODS

Strains. All strains are derivatives of JKM179 (*ho*Δ *MAT*α *hml*Δ::*ADE1 hmr*Δ::*ADE1 ade1-100 leu2-3,112 lys5 trp1*::*hisG' ura3-52 ade3*::*GAL*::*HO*). Construction of the *yku70*Δ::*URA3* strain (JKM181), the *rad52*Δ::*TRP1* strain (JKM168), the *rad9*Δ::*KAN* (YSL60) strain, and the *rfa1-t11* strain (YSL40) was previously described (24). The *tid1*Δ::*URA3* strain (YSL300) is a segregant from a cross between HK764 (a gift from H. Klein) and JKM179. The *rad51*Δ::*URA3* strain (YSL302) was constructed by transformation using a *Hin*dIII fragment of pJH724. The *rad54*Δ::*LEU2* (YSL303), *rad55*Δ::*LEU2* (YSL304), and *sgs1*Δ::*URA3* (YSL305) strains were obtained by transformation with a *Bgl*II fragment of pXRAD::*LEU2* (a gift from L. Symington), a *Hin*dIII fragment of pSTL11 (a gift from S. Lovett), and a *Xho*I fragment of pJH1340, respectively. The *rad59*Δ::*KAN* strain (YSL306) was constructed by crossing the JKM179 derivative CSHYKO (a gift from C. Greider) with the isogenic *MAT***a** strain, JKM139. XW652 has been previously described (57). Additional *tid1*Δ derivatives of the JKM179 background were constructed by transformation with PCR-amplified *KAN*::*MX* cassettes from the Research Genetics collection of *Saccharomyces* strains harboring deletions of different yeast genes. These isogenic *tid1*Δ strains showed adaptation behavior nearly identical to that of the backcrossed strain.

Plasmids pR51.3, pR51.4, and pR51.5 (kindly provided by P. Sung) express wild-type *RAD51*, *rad51*-K191A, and *rad51*-K191R, respectively, under the *PGK* promoter (48). pRS413 plasmids carrying Rad51-Y388H, Rad51-L99P, and Rad51-T146A (21) were generously provided by L. Krejci. The promoter and open reading frame of each *rad51* mutant was cloned into the *URA3*-containing centromeric plasmid pRS316.

Analysis of G_2/M checkpoint adaptation. Cells were grown in preinduction medium (yeast extract-peptone [YEP]-lactate) at 30°C overnight and were spread onto agar plates containing COM-galactose medium. G_1 (unbudded) cells were micromanipulated onto a grid, and cells were then monitored for growth and division.

Measurement of DNA degradation. A DSB was induced in YEP-lactate-grown cells by adding 2% galactose. Genomic DNA was isolated at intervals and subjected to slot blot hybridization by using a strand-specific RNA probe as previously described (24). Alternatively, the extent of resection and stability of 3′ ends was examined on Southern blots of DNA separated on denaturing gels, where higher-molecular-weight bands hybridizing to a short probe homologous to sequences near the HO cut site represent partial digestion products caused as restriction sites become single stranded (24, 56).

Fluorescence-activated cell sorter (FACS) analysis. Flow cytometry analysis was performed with a Becton Dickinson fluorescence-activated cell analyzer, as described by Paulovich and Hartwell (33), by using either propidium iodide or sytox green (Molecular Probes Co.). Each sample was also visualized with a fluorescence microscope to analyze cell morphology and the location of the nuclei in the cells.

Western blotting and in situ autophosphorylation assay. Crude-extract preparation, the Western blotting procedure, and the in situ autophosphorylation assay have been described by Pellicioli et al. (34).

Chromatin immunoprecipitation. Cultures were induced with 2% galactose, harvested, and then analyzed by chromatin immunoprecipitation as described previously (8, 45). Polyclonal antibodies directed against Rad51p were kindly provided by P. Sung and D. Bishop. Immunoprecipitated DNA and input DNA were analyzed by PCR using the oligonucleotides 5′ CTTGCTCTTGTTCCCA ATGTTTG 3′ and 5′ CCGCATGGGCAGTTTACCT 3′ located distally to *MAT*. PCR conditions were chosen so that a linear relationship existed between the amount of PCR product and the amount of template DNA, and this relationship was verified with a calibration curve prepared for each set of PCRs. PCR products were visualized on 2% agarose gels stained with ethidium bromide, and images provided by either a Bio-Rad Gel Doc 1000 or Alpha Innotech AlphaImager 1220 were analyzed using Quantity One software (Bio-Rad).

RESULTS

Cells lacking Rad51p fail to adapt. A *MAT*α strain, JKM179, carrying a galactose-inducible *HO* gene (*ade3*::*GAL*::*HO*) and deletions of the recombination donor cassettes *HML* and *HMR* was grown in YEP-glycerol-succinate and

TABLE 1. Effect of recombination-defective mutations on adaptation

Strain[a]	% Adaptation ± SD 24 h post-HO induction
Wild type	93.5 ± 4.0
*rad51*Δ	27.6 ± 4.9
*rad52*Δ	84.0 ± 0.8
*rad54*Δ	74.8 ± 16.0
*rad55*Δ at 23°C	84.0 ± 8.5
*rad55*Δ at 30°C	89.0 ± 4.8
*rad59*Δ	88.4 ± 3.2
*rad51*Δ *rad59*Δ	34.0 ± 6.3
*tid1*Δ	1.4 ± 0.1
*srs2*Δ	24.8 ± 4.4
*sgs1*Δ	70.1 ± 13.8
*rad52*Δ409-420	63.4 ± 10.5

[a] All strains are derivatives of JKM179, containing *MAT*α but lacking both *HML* and *HMR*. An irreparable DSB was induced from a *GAL*::*HO* gene integrated at the *ade3* locus.

placed onto a synthetic complete-galactose plate to induce HO-mediated cleavage of the *MAT* locus (24). Individual unbudded G_1 cells were micromanipulated onto a grid so that their cell cycle progression could be monitored microscopically. By 8 h, nearly all cells had grown into the dumbbell shape characteristic of G_2/M-arrested cells. By 24 h, more than 90% of wild-type cells had resumed growth and produced microcolonies with three or more cells and buds, as expected for cells able to adapt to the presence of a single DSB (Table 1 and Fig. 1A).

We examined the effects of deleting a series of genes in the *RAD52* epistasis group, important in homologous recombination. Neither *rad52*Δ, *rad54*Δ, *rad55*Δ, *rad57*Δ, nor *rad59*Δ mutants exhibited any significant defect in arrest or adaptation; however, a *rad51*Δ deletion mutant had a clear defect in adaptation. Approximately 75% of *rad51*Δ cells remained arrested at the first cell division (Fig. 1A). Another 20% of the cells completed the first division but became permanently arrested at the next G_2/M boundary, even at 48 h. This differs

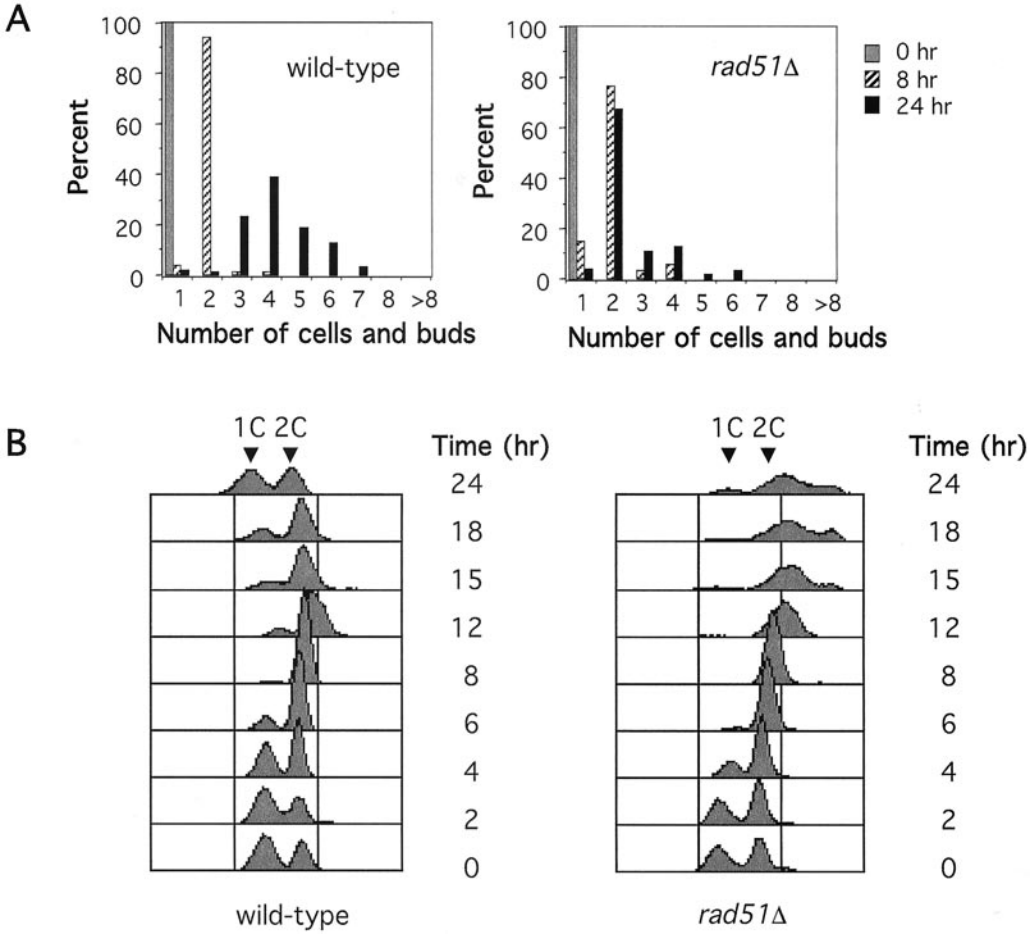

FIG. 1. Arrest of cell cycle progression by a single unrepaired DSB in wild-type and mutant cells. (A) Arrest and adaptation of cells experiencing an irreparable DSB in G_1. At least 300 G_1 unbudded cells were initially plated (gray bar). The numbers of cells and buds at 8 h (hatched bar) and 24 h (black) are shown. After 24 h, wild-type cells initially plated as G_1 unbudded cells onto galactose-containing medium to induce HO endonuclease have mostly adapted and resumed cell division, whereas adaptation-defective *tid1*Δ and *rad51*Δ strains remain arrested prior to anaphase. (B) Results of FACS analysis of wild-type and mutant cells induced for HO expression at 0 h. The apparent increase in DNA content of checkpoint-arrested cells above the 2C level is caused by light scattering of G_2/M-arrested cells because of their greatly enlarged size and is not an indication of continued DNA replication (53).

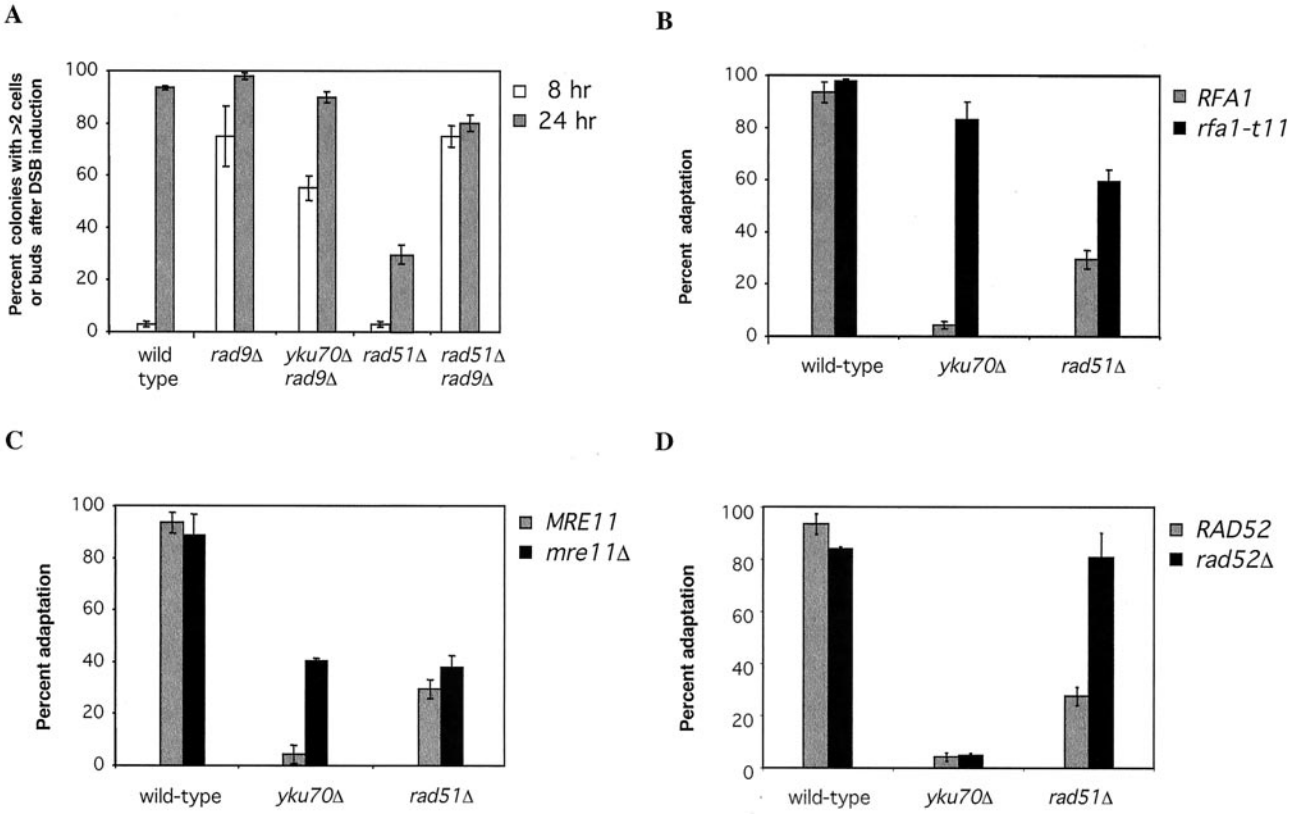

FIG. 2. Adaptation of wild-type and mutant cells. Cells progressing beyond the two-cell-plus-bud stage at 24 h after HO induction were scored as having adapted. (A) Effect of *rad9*Δ on the arrest and adaptation of wild-type and mutant cells at 8 and 24 h. Also shown are the effects of *rfa1-t11* (B), *mre11*Δ (C), and *rad52*Δ (D) on the adaptation of wild-type and mutant cells at 24 h.

from the more severely adaptation-defective phenotype of *yku70*Δ or *tid1*Δ mutant cells, nearly all of which arrest at or prior to the first cell division. As with *yku70*Δ and *tid1*Δ cells, permanently arrested, DNA-damaged *rad51*Δ cells exhibit a terminal morphology with an especially enlarged mother cell (25 and data not shown). As expected, FACS analysis of *rad51*Δ cells with a single, unrepaired DSB shows that they become arrested with the fully replicated (2C) DNA content expected for cells that had replicated their DNA but failed to progress through the cell cycle (Fig. 1B). We note that *rad51*Δ has no effect on the establishment of DNA damage-induced arrest but affects the cell's exit from arrest.

Permanent arrest of *rad51*Δ cells is bypassed by the *rad9*Δ checkpoint mutation and partially suppressed by *rfa1-t11*. G_2/M arrest of *rad51*Δ cells depends on a functional checkpoint system. Hence, a *rad9*Δ *rad51*Δ double mutant did not show significant cell cycle arrest after induction of HO cleavage (Fig. 2A). Again, this result is similar to what was previously observed with *yku70*Δ (24) and *tid1*Δ (25). Thus, the permanent arrest phenotype of this new adaptation-defective mutation is dependent on a functional DNA damage arrest checkpoint system.

A key finding concerning both *yku70*Δ and *tid1*Δ was that the *rfa1-t11* mutation restores adaptation in a strain with a single unrepaired DSB (24, 25). *cdc5-ad* is not suppressed by *rfa1-t11* (34). Here we found that *rad51*Δ is only partially suppressed by *rfa1-t11* (Fig. 2B). We also found that *rfa1-t11* partially suppresses *rad51*Δ *tid1*Δ, but the double mutation is suppressed to a lesser degree than is either single mutation, suggesting that the defects caused by *rad51*Δ and *tid1*Δ mutations may not be in the same pathway.

Previously it was shown that the permanent arrest of *yku70*Δ cells at the G_2/M checkpoint could be partially suppressed by the absence of functional Mre11p, although *tid1*Δ cells were not affected (24, 25). The absence of Mre11p did not significantly affect the failure of adaptation of *rad51*Δ cells (Fig. 2C). Thus, the arrest phenotype of *rad51*Δ cells is checkpoint dependent but different from those of *yku70*Δ, *tid1*Δ, and *cdc5-ad* cells.

***rad51*Δ does not alter the rate of formation of ssDNA by 5′-to-3′ exonucleases.** The failure of *yku70*Δ strains to adapt was explained by the observation that they exhibited a twofold higher rate of 5′-to-3′ resection of DNA ends, thus creating a larger amount of ssDNA (24). The defect in *rad51*Δ cells must affect adaptation in a different way, as it does not affect the rate of 5-to-3′ resection as measured by slot blot hybridization (Fig. 3). In addition, *rad51*Δ does not significantly alter the stability of the 3′-ended ssDNA (data not shown). These results are similar to those found for *tid1*Δ (25).

Epistatic relationships among adaptation-defective mutants. We also tested double adaptation-defective mutants to understand their epistatic relationships. *tid1*Δ *rad51*Δ cells arrest and fail to adapt, similar to *tid1*Δ cells, but unlike *tid1*Δ alone, the double mutation cannot be suppressed by *rfa1-t11*

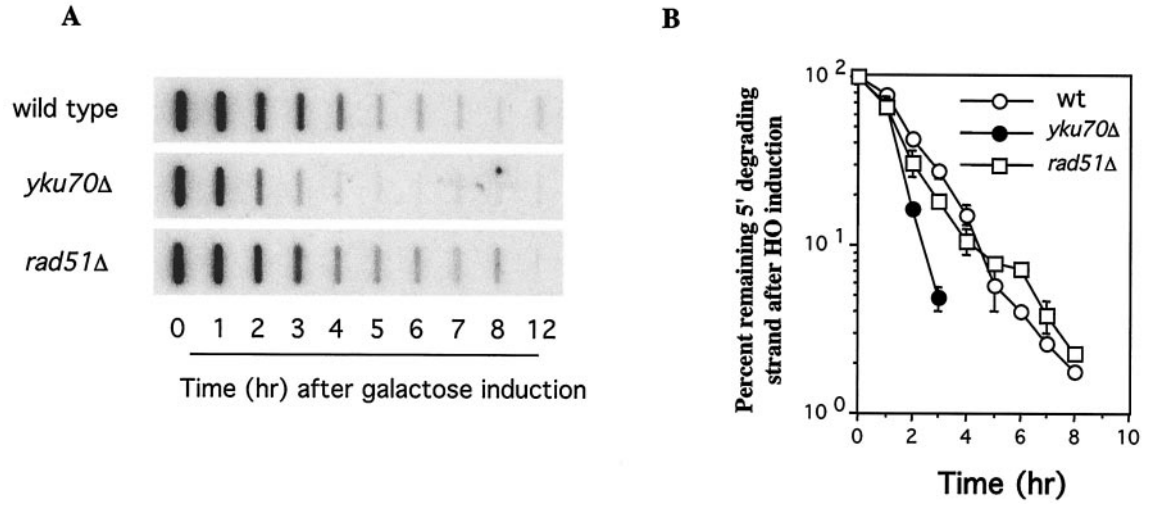

FIG. 3. 5′-to-3′ resection of an HO-induced DSB in adaptation-defective mutants. (A) Slot blot hybridization was performed with a probe specific for the strand ending 5′ distal to the HO-cleaved *MAT* locus. (B) Percentages of the sequences remaining at different times. wt, wild type.

(Table 2). The more severe arrest phenotype of *yku70*Δ cells is also epistatic to *rad51*Δ (that is, the double mutant resembles the *yku70*Δ strain rather than the *rad51*Δ strain). As expected, the double mutant *yku70*Δ *tid1*Δ is indistinguishable from either single mutant (data not shown).

A *rad52*Δ mutation suppresses *rad51*Δ. Although a *rad52*Δ mutation had no discernible effect on adaptation, we were surprised to discover that the absence of *RAD52* suppressed the adaptation defect of the *rad51*Δ strain (Fig. 2D). This is especially intriguing because our recent studies have shown that the absence of Rad52p abolishes Rad51p binding to HO-cleaved DNA, as detected by chromatin immunoprecipitation (45); hence we might have expected *rad52*Δ and *rad51*Δ strains to have identical phenotypes. Instead, *rad52*Δ suppresses *rad51*Δ; however, *rad52*Δ had no effect on either *yku70*Δ or *tid1*Δ (24, 25) or on *cdc5-ad* (49). This result raised the question of whether some aspect of checkpoint monitoring of DNA damage was integrated with one or more processes of *RAD52*-dependent but *RAD51*-independent recombination. Although the DSB we study is created in a haploid strain with no extensive homology elsewhere in the genome (i.e., *HML* and *HMR* have been deleted), it is possible that, as the ends are resected for many kilobases, recombination is attempted between dispersed repeated DNA sequences such as Ty elements. Alternatively, recombinational interactions may occur between the two unequally resected broken sister chromatid strands after replication of the broken DNA molecules.

TABLE 2. Suppression of adaptation defects caused by *rad51*Δ and other mutations

Strain description	% Adaptation ± SD at 24 h after galactose induction
Strains with epistatic relationships	
*rad51*Δ	27.6 ± 4.9
*rad51*Δ *srs2*Δ	29.5 ± 3.5
*rad51*Δ *tid1*Δ	0.3 ± 0.1
*srs2*Δ *tid1*Δ	0.0 ± 0.0
*srs2*Δ *rad52*Δ	85.3 ± 3.6
rfa1-t11	95.1 ± 3.5
*tid1*Δ *rfa1-t11*	86.0 ± 8.4
*rad51*Δ *rfa1-t11*	64.0 ± 15.2
*srs2*Δ *rfa1-t11*	18.8 ± 7.6
*rad51*Δ *tid1*Δ *rfa1-t11*	28.0 ± 4.1
*rad52*Δ *tid1*Δ *rfa1-t11*	17.8 ± 9.9
*rad51*Δ *rad52*Δ *tid1*Δ	14.3 ± 12.1
*rad52*Δ *rfa1-t11*	25.0 ± 2.8
Strains with suppression by Rad51 mutants	
*rad51*Δ pPGK::*RAD51*, 2μm	68.3 ± 8.3
*rad51*Δ pPGK::*rad51*-K191A, 2μm	71.9 ± 3.7
*rad51*Δ pPGK (empty vector) *CEN ARS*	25.7
*rad51*Δ pPGK::*rad51*-K191A *CEN ARS*	71.7
*srs2*Δ pPGK (empty vector) *CEN ARS*	20.8
*srs2*Δ pPGK::*rad51*-K191A *CEN ARS*	33.0
*rad51*Δ pPGK::*rad51*-K191R, 2μm	82.3 ± 2.1
*rad51*Δ pADH::*rad51*-L99P, 2μm	77.3 ± 10.3
*rad51*Δ pADH::*rad51*-S146A, 2μm	62.8 ± 6.1
*rad51*Δ pADH::*rad51*-Y388H, 2μm	27.9 ± 7.2

Recent studies have revealed a *RAD52*-dependent, *RAD51*-independent process that requires the *RAD59* gene, so that recombination in a *rad51*Δ *rad59*Δ double mutant is reduced almost to the very low level seen in a *rad52*Δ strain (2, 15, 42). We therefore examined whether the permanent checkpoint arrest of *rad51*Δ cells could be suppressed by *rad59*Δ, but an isogenic *rad59*Δ *rad51*Δ strain was not different from the *rad51*Δ mutant (Table 1). This indicates that the role of *RAD52* in the checkpoint response is revealed only in the absence of Rad51p.

To examine how *rad52*Δ may suppress *rad51*Δ, we created a *rad51*Δ *rad52*Δ *tid1*Δ strain. This triple mutant adapted more than the *rad51*Δ *tid1*Δ strain but less than the *rad51*Δ strain (Table 2). This result indicates again that Rad52p affects adaptation by a pathway different from that used by Tid1p or that Tid1p acts upstream of these other proteins.

The adaptation-defective phenotypes of *rad51*Δ mutants are in many respects similar to those of *srs2*Δ mutants (53). About 20% of *srs2*Δ cells also arrest in the second cell cycle; moreover, *srs2*Δ is also suppressed by *rad52*Δ. A *rad51*Δ *srs2*Δ dou-

ble mutant has the same adaptation defect as either single mutant (Table 2). However, the *srs2*Δ strain also has a defect in recovery when a DSB is repaired after a long checkpoint-mediated delay. *rad51*Δ cells are slow to recover, but do so, and *rad51*Δ suppresses the recovery defect of the *srs2*Δ mutant (53).

Separation-of-function mutations in Rad51p implicate its interaction with Rad52p during adaptation. Further support for the importance of the Rad51p-Rad52p interaction in the checkpoint response has come from analysis of three DNA repair-defective mutants of Rad51p. The Rad51-Y388H mutant protein is defective in its interaction with Rad52p, as shown by two-hybrid tests (21). We transformed a *rad51*Δ strain with a centromeric plasmid expressing Rad51-Y388H transcribed from its own promoter and found that the cells remained adaptation defective (Table 2). In contrast, when *rad51*Δ was complemented with plasmids expressing Rad51-L99P and Rad51-T146A, which are defective in interactions with Rad54p and/or Rad55p (21), adaptation was restored (Table 2).

The repair-defective Rad51p-K191A protein that fails to bind ssDNA is still active in regulating adaptation. A K191A mutation in Rad51p prevents in vitro DNA binding and strand exchange activity and renders cells radiation sensitive (29, 52). We transformed cells with both a high-copy-number plasmid, pR51.4, and a centromeric plasmid derivative, each expressing Rad51-K191A, and found that these cells could still adapt. These results suggest that it is not the normal recombination activity of Rad51p—such as DNA binding and strand exchange—that regulates adaptation but rather its interaction with some other proteins; this interpretation is again consistent with the idea that adaptation is dependent on an interaction between Rad51p and Rad52p.

To confirm that Rad51-K191A protein is indeed defective in binding to ssDNA, we used chromatin immunoprecipitation techniques to examine how Rad51-K191A protein binds to DNA adjacent to an unrepaired DSB (45). The *rad51*Δ strain YSL306 (*GAL*::*HO MAT*α *hml*Δ *hmr*Δ) was transformed with centromeric plasmids expressing either wild-type Rad51p or Rad51-K191A. DNA and proteins were cross-linked with formaldehyde at intervals after the creation of an HO-induced DSB at *MAT*. The results of this experiment showed that Rad51-K191A binds to ssDNA ends with 50- to 100-fold less efficiency than the wild type during the first 2 h following HO cleavage (Fig. 4). The very weak binding seen in a strain expressing Rad51-K191A (from plasmid pR51.4) may be a reflection of the fact that the protein is overexpressed from a high-copy vector.

As Western blotting confirmed that the level of Rad51-K191A expressed from the centromeric plasmid is about 10-fold higher than that of the wild type (data not shown), we also entertained the hypothesis that when the mutant protein is expressed at this level, Rad51-K191A may act by sequestering Rad52p, making the cells phenotypically Rad52$^-$ as *rad52*Δ suppresses *rad51*Δ (Table 2). However, this is unlikely to be the explanation. Whereas overexpressing Rad51-K191A suppresses *rad51*Δ alone, it does not suppress the adaptation defect of an *srs2*Δ mutant, although *rad52*Δ does suppress the adaptation defect of an *srs2*Δ mutant (Table 2). Hence, overexpressing Rad51-K191A does not create a phenocopy of *rad52*Δ.

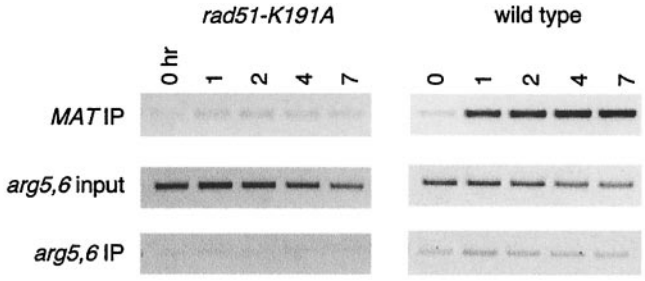

FIG. 4. Lack of binding of Rad51-K191A protein to ssDNA in vivo. Rad51p binding to ssDNA adjacent to a HO endonuclease-induced DSB was monitored by chromatin immunoprecipitation (IP) in a strain that cannot repair the break by homologous recombination. The amount of input DNA was monitored by PCR amplification of the *arg5,6* gene, and the same primer pairs were used to show the low background of *arg5,6* DNA in the samples immunoprecipitated by antibody against Rad51.

Changes in the phosphorylation and kinase activity of Rad53p in different genetic backgrounds. The presence of damaged DNA stimulates the phosphorylation of Rad53p by the Mec1 protein kinase, activating Rad53p as a protein kinase (39). This activated state is evident in Western blots as slower-migrating phosphorylated forms of the protein. It is also possible to measure Rad53 kinase activity by autophosphorylation by using an in-gel assay (34). When wild-type cells carry an unrepaired DSB, Rad53p phosphorylation and kinase activity increase dramatically approximately 1 h after HO-mediated DNA cleavage and peak at 6 to 8 h after HO induction (Fig. 5) at the time that all cells in the population are arrested. Rad53p activation persists until 8 to 15 h, when most cells adapt and resume cell cycle progression. Thus, cells turn off the Rad53p checkpoint kinase, even though DNA damage continues to be present.

In adaptation-defective cells, however, Rad53p is maintained in a kinase-activated state even after 24 h. As shown in Fig. 5, Rad53p is hyperphosphorylated and activated in *rad51*Δ cells at 24 h (Fig. 5A) (25). We then examined Rad53p phosphorylation and kinase activity in strains carrying *rfa1-t11* (Fig. 5B). *rfa1-t11* suppresses the activated state of Rad53p in *rad51*Δ cells (Fig. 5B), similar to what is seen in *rfa1-t11 tid1*Δ cells (data not shown). The effect of *rfa1-t11* on kinase activity in *rad51*Δ cells appears to be more complete than the partial suppression of the cells' permanent cell cycle arrest.

Overexpression of Rad51p does not alter the DNA damage checkpoint response. In a recent study of Rad51p binding to HO-cleaved DNA in these same strains, it was found that the abundance of Rad51p is quite limited. As continued 5′-to-3′ resection of DNA continues, Rad51p fails to "fill up" the newly generated ssDNA after about 1 to 2 h (i.e., beyond a total of about 5 kb on either side of the DSB); however, the ssDNA binding protein RPA does occupy all the ssDNA (45). However, overexpression of Rad51p by about 10-fold allowed Rad51p to bind to newly generated ssDNA over at least 12 kb on one side of the DSB that was monitored (45). These observations raise the question of whether the activation of the checkpoint signal or its intensity may be influenced by the

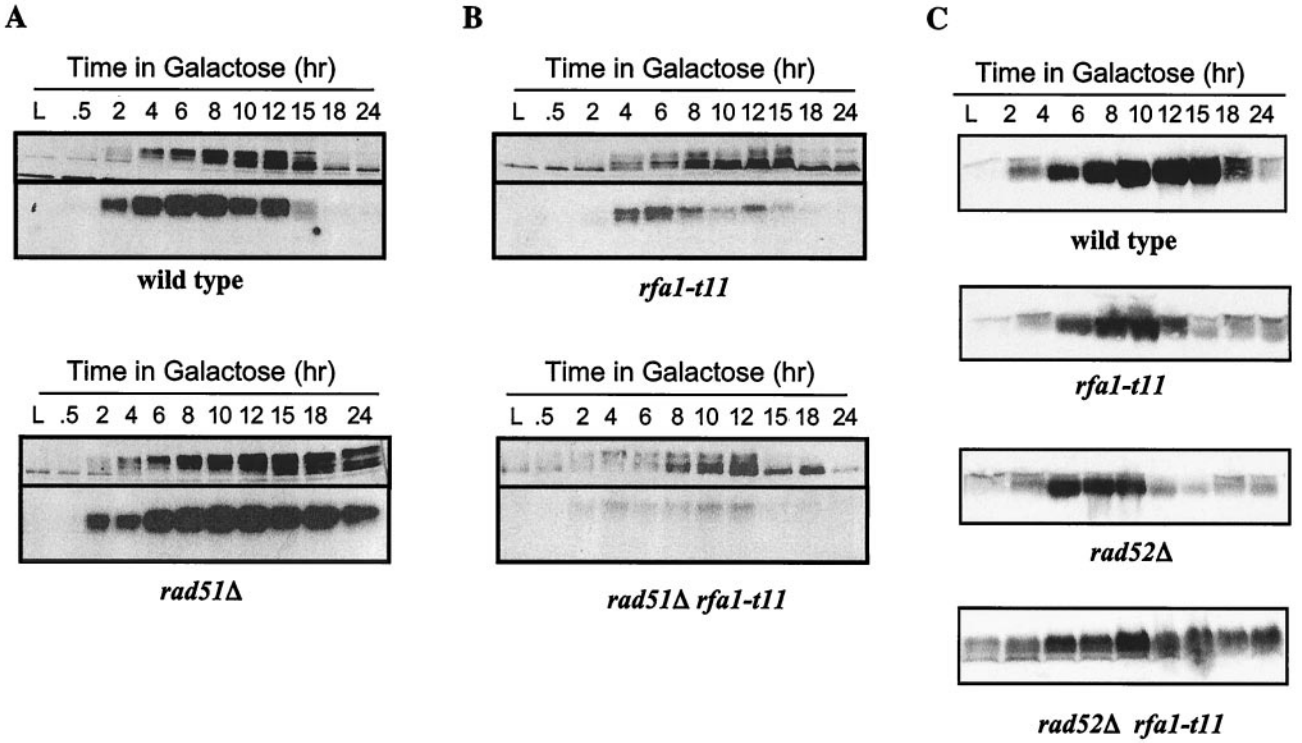

FIG. 5. Phosphorylation and kinase activity of Rad53p. The activation of Rad53p kinase is shown both by Western blot analysis (top of each set), in which phosphorylated forms of Rad53p exhibit slower migration, and by an in-gel activity autophosphorylation assay. (A) Activation of Rad53p by a single HO-induced DSB in logarithmically growing wild-type and adaptation-defective mutant cells. L represents cells in logarithmic growth prior to HO induction. (B) Suppression of the adaptation defect of *rad51*Δ cells by the *rfa1-t11* mutation. (C) Persistent hyperphosphorylation of Rad53p in *rad52*Δ *rfa1-t11* cells after induction of an irreparable DSB. This is not seen for either *rad52*Δ or *rfa1-t11* alone. No autophosphorylation assay was performed.

amount of ssDNA bound by RPA but not by Rad51p. However, this does not seem to be the case, as an isogenic strain overexpressing *RAD51* showed a similar extent and duration of G_2/M arrest after HO induction of a single DSB (data not shown).

A *rad52*Δ *rfa1-t11* double mutant fails to adapt. Both *rfa1-t11* and *rad52*Δ cells are adaptation proficient; in fact, *rfa1-t11* cells adapt somewhat faster than wild-type cells (34). However, a double mutant *rfa1-t11 rad52*Δ fails to adapt, exhibiting an arrest pattern similar to that seen for *rad51*Δ cells (Table 2). This arrest appears to be checkpoint mediated because *rad9*Δ *rad52*Δ *rfa1-t11* cells do not permanently arrest. Moreover, Rad53p remains hyperphosphorylated at 24 h in *rad52*Δ *rfa1-t11* cells (Fig. 5C). This persistent activation of the checkpoint is notably different from what occurs in cells with *rad52*Δ or *rfa1-t11* alone. We note that *rad52*Δ and especially *rad52*Δ *rfa1-t11* cause persistent, but low-level, activation of Rad53p kinase even before HO expression, but the response after induction of the unrepaired DSB is evident.

These results indicate that the ability to adapt depends on the presence of either Rad52p or wild-type RPA. Moreover, whereas an *rfa1-t11 tid1*Δ strain adapts, a *rfa1-t11 tid1*Δ *rad52*Δ strain does not. A *rad52*Δ *rad51*Δ strain adapts, but a *rad52*Δ *rad51*Δ *rfa1-t11* strain does not (Table 2). A *rad52*Δ *rad51*Δ *tid1*Δ strain also does not adapt.

If the interaction between Rad51p and Rad52p is essential for regulation of adaptation, then a *rad52* mutant that is impaired in interaction with Rad51p may also be adaptation defective. We examined the C-terminal deletion mutant *rad52*Δ409-420, which lacks Rad51p binding and is recombination defective (22). Cells with the *rad52*Δ409-420 mutation alone are slightly impaired in adaptation (Table 1), but those with the mutation in combination with *rfa1-t11* are about as adaptation defective as the *rad52*Δ *rfa1-t11* double mutant (Fig. 6).

DISCUSSION

Previous work has suggested that both the establishment of a checkpoint response and its maintenance depends on the extent of ssDNA created by 5′-to-3′ resection at the ends of DSBs (10, 24, 58). Our finding that two additional ssDNA binding proteins, Rad51p and Rad52p, play a role in adaptation from the G_2/M checkpoint further emphasizes the role of ssDNA in checkpoint control. Our results also indicate that Rad52p and RPA function in parallel to regulate adaptation; whereas neither *rad52*Δ nor *rfa1-t11* strains are adaptation defective, the double mutant is unable to adapt after DNA damage. Rad51p and Rad52p play different roles in adaptation. From results for the various mutation combinations summarized in Table 2, we propose that the regulation of adaptation consists of two separate monitors that determine the nature and extent of DSB damage, one represented by Rad52p and the other by RPA. RPA and Rad52p, independently and

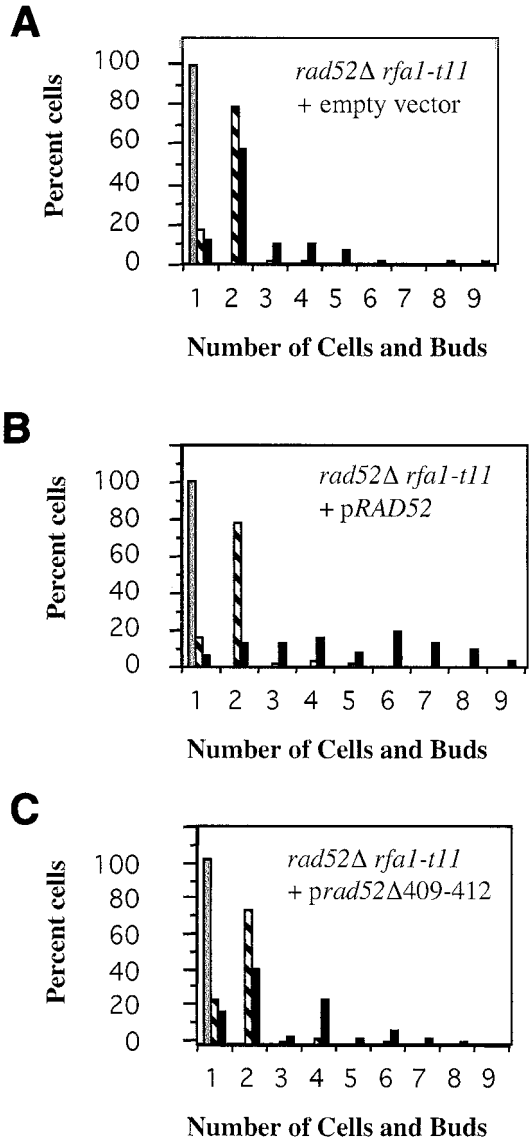

FIG. 6. A C-terminal truncation of Rad52 (Δ409-420) is adaptation defective. A *rad52Δ rfa1-t11* strain was transformed with a centromeric plasmid carrying either *RAD52* (B) or *rad52*Δ409-420 (C) or with an empty vector (A). G_1 cells were plated onto YEP-galactose as described above. Cells were scored at 8 h (hatched bars) and 24 h (black bars) after induction of the DSB.

possibly also through their mutual interactions (11, 14, 41), serve as monitors of the presence of unrepaired ssDNA and thus control the cell's decision to adapt. It is also important to emphasize that none of the proteins discussed here prevent the imposition of the checkpoint, which must be initially sensed in a Rad51-, Rad52-, Tid1-, and Rfa1-t11-independent fashion; rather, these recombination proteins affect the termination of the checkpoint.

Several checkpoint proteins have been shown to bind to HO-induced DSB ends (20, 28, 37). The observed increase in the intensity of Ddc1-green fluorescent protein (GFP) and Ddc2-GFP foci over time (28) suggests that the level of Ddc1p and Ddc2p binding to ssDNA is proportional to the extent of 5′-to-3′ resection of DNA adjacent to the DSB. When cells adapt, however, the intensity of Ddc2-GFP foci decreases (28), suggesting either that the checkpoint protein is displaced from ssDNA or that the ssDNA substrate has changed. It is possible that both Rad52p and RPA complexes may interact with one or both of the checkpoint complexes (Mec1p-Ddc2p and Rad17p-Mec3p-Ddc1p) to modulate the signals during checkpoint-mediated G_2/M arrest. So long as one of these DNA damage sensors remains bound to DNA, cells will remain checkpoint arrested.

There appear to be two pathways to remove or inactivate the sensor protein(s), one involving RPA and the other involving Rad52p. These proteins themselves may not displace or inactivate the sensors, but each may facilitate the binding of other proteins that can remove or inactivate them. Given that *rad52*Δ prevents Rad51p filament formation but that the mutant is adaptation proficient, it is unlikely that Rad51p filament formation itself regulates checkpoint signaling. Moreover, overexpression of Rad51p did not modulate adaptation, even though normally Rad51p is unable to bind to more than about 10 kb of ssDNA. By the time cells adapt, as much as 50 kb or more has apparently been resected, although over time the 3′ ends of ssDNA do also become degraded. Rad52p itself may form multiple rings on ssDNA (51), and this activity may be modulated by the interaction of Rad52p with Rad51p. Similarly, once RPA is bound, it will facilitate the binding of some other proteins, a process that apparently involves Tid1p. Either when Rad52p is absent or when RPA carries the t11(L45E) mutation, the regulation of adaptation is normal, but when either of these two ssDNA binding pathways is blocked at a downstream step (*rad51*Δ affecting Rad52p or *tid1*Δ affecting RPA), the checkpoint is maintained. This suggests that there are several steps in creating protein complexes that result in inactivation of the checkpoint.

Recently, Zou and Elledge (58) reported that the *rfa1-t11* mutation reduces the binding of Mec1p at a HO nuclease-induced DSB. We note, however, that there is significant Mec1p-dependent hyperphosphorylation of Rad53p in an *rfa1-t11* strain after induction of a DSB, albeit the persistence of this state is shorter (34). Even more striking, in a *rad52 rfa1-t11* mutant the checkpoint remains permanently activated, suggesting that Rfa1-t11 mutant protein is entirely competent in recruiting sufficient DNA damage checkpoint proteins to impose permanent cell cycle arrest. Thus, it is possible that the establishment of the DNA damage checkpoint does not involve an interaction between Mec1p and RPA but that the maintenance of the checkpoint depends on this interaction.

Link between homologous recombination and G_2/M checkpoint control. Rad52p plays a central role in recombination. It binds to 3′-ended ssDNA created at DSBs, both at the end and along the strand (51). In vitro, Rad52p plays an important role in the formation of the Rad51p filament and in strand exchange (22, 30, 31, 41, 46, 47). Rad52 has been shown genetically and physically to be necessary for *RAD51*-dependent recombination. In fact, in vivo, the absence of Rad52p prevents the association of Rad51p with ssDNA, as assessed by chromatin immunoprecipitation (45). Hence, one may have expected that *rad52*Δ and *rad51*Δ strains should have similar adaptation phenotypes, but this is not the case. Here we show that Rad52p plays a role in DNA damage sensing in which

Rad51p apparently needs to make contact with Rad52p but does not necessarily form a filament. We imagine that the Rad52p complex, modulated by Rad51p, monitors whether the DSBs ends have engaged in homologous recombination.

Currently, it is unclear how Rad52p performs its signaling role during adaptation to the DNA damage checkpoint. One can imagine that Rad52p, through its ability to bind to ssDNA and to promote homologous recombination, is used to assess the progress of the DNA repair and to convey this information to the checkpoint complex that may also reside at the site of a DSB (20, 28, 37). However, the role of Rad51p is clearly different from its normal function in recombination, because the Rad51-K191A mutant that exhibits markedly reduced ssDNA binding and is devoid of recombination activity is nevertheless competent for adaptation. It is possible, however, that the mutant protein makes transient contacts with DNA that are sufficient for some role in checkpoint response but not for recombination. Moreover, a partially active Rad51-Y388H mutant, defective in its interaction with Rad52p (21), is adaptation defective, whereas two other Rad51p mutants that are similarly sensitive to methylmethane sulfonate but are defective in interactions with Rad54p and/or Rad55p are adaptation competent. We supported this conclusion by showing that a Rad52Δ409-429 protein, defective in its interaction with Rad51p, is also adaptation defective, especially as measured in combination with *rfa1-t11*.

We have considered whether *rad52*Δ may suppress *rad51*Δ by altering the extent of ssDNA, for example by allowing degradation of long 3′-ended molecules or decreasing the rate of resection. This seems unlikely for several reasons. First, *rad52*Δ does not suppress *yku70*Δ even though a reduction in the extent of resection by *mre11*Δ does so (24), and second, cells with the *rad52*Δ mutation alone are not defective in adaptation. We examined directly the extent of resection and the stability of 3′ ends on denaturing gels, where higher-molecular-weight bands represent partial digestion products caused when restriction sites become single stranded (24, 44, 56). If 3′ ends were extensively degraded, these bands would disappear, as the probe is within 500 bp of the HO cleavage site; conversely, if 5′-to-3′ resection was faster, higher-molecular-weight fragments would appear more rapidly. In fact, DNA from *rad52*Δ *rad51*Δ cells at intervals after HO induction was indistinguishable from that from *rad51*Δ or *rad52*Δ single mutants (data not shown).

One of the most interesting aspects of our results is that they reveal different functional groupings of recombination proteins than would be expected from the roles of these proteins in homologous recombination. Whereas Rad54p and Rad55p/Rad57p play important roles in DSB-mediated homologous recombination (42), they do not influence adaptation. There is also a *RAD52*-dependent, *RAD51*-independent homologous recombination pathway in mitotic cells that depends on *RAD50*, *RAD59*, and *TID1* (2, 42), but that pathway also does not seem to be involved in regulating adaptation, as *rad59*Δ did not affect this process. However, *tid1*Δ strains are profoundly adaptation defective (25). Tid1p exhibits only very weak two-hybrid interaction with Rad51p (6), but there is evidence that Tid1p is capable of improving Rad51p's in vitro strand exchange activity (35). Yet in checkpoint responses, Tid1p and Rad51p are not epistatic (e.g., the *rad51*Δ *rad52*Δ strain adapts but the *tid1*Δ *rad52*Δ and *rad51*Δ *tid1*Δ *rad52*Δ strains do not; similarly, the *tid1*Δ *rfa1-t11* strain adapts but the *tid1*Δ *rad51*Δ *rfa1-t11* strain does not).

One may also ask whether *rad51*Δ and *rad52*Δ may play a role in the related, but distinct, S phase checkpoint that is seen when cells are exposed to agents such as hydroxyurea (HU) that stall DNA replication. A previous study found that the *rad51*Δ strain is HU sensitive (1), and the *rad51*Δ mutation may therefore represent an adaptation defect rather than a problem in DNA repair. We think that this is unlikely, because *rad51*Δ, *rad52*Δ, and *mre11*Δ deletion mutants all have similar HU sensitivities and are similar in their abilities to recover after removal from HU exposure (K. Patterson and J. E. Haber, unpublished data). That three mutants with very different checkpoint phenotypes have similar HU sensitivities is most consistent with the presence of a defect in recombinational repair of lesions generated during HU arrest and subsequent growth in the absence of the inhibitor.

We suggest that adaptation depends on two interacting conditions, the extent of ssDNA and the state of homologous recombination. The amount of ssDNA is monitored through RPA, while the homologous recombinational repair depends on Rad52p. However, these two parts of the checkpoint system likely interact, given that *RAD52* physically and genetically interacts with RPA (9, 17, 43) and given the results of the genetic studies we discussed here. We further suggest that RPA and Rad52 proteins each interact with one or more of the checkpoint proteins that continue signaling in adaptation-defective mutants. Once RPA is engaged with the checkpoint, it requires Tid1p to turn off that part of the checkpoint that interacts with RPA. This may involve the ATP-dependent chromatin-remodeling activity assigned to Tid1p, Rad54p, and other members of the Swi2/Snf2 class of proteins (16), but it may also be carried out through allosteric interactions. In any case, in a *tid1*Δ mutant, the RPA-associated checkpoint proteins apparently continue to signal. The checkpoint system also interacts with Rad52p. Once Rad52p is engaged with the checkpoint, it requires Rad51p to disengage. In *rad51*Δ cells, the Rad52p-associated checkpoint continues to signal. However, the way in which Rad51p interacts with Rad52p appears to be distinct from the normal recombinational role of Rad51p, as Rad51-K191A that cannot bind DNA is still adaptation proficient but Rad51-Y338P that does not interact with Rad51p is defective. Presumably Rad51-K191A can still interact with Rad52p.

In summary, we have found that a number of proteins involved in homologous recombination play important roles in cellular responses to DNA damage. We suggest that RPA primarily signals the presence of the damaged DNA itself whereas Rad52p signals whether the damage has been successfully taken up into recombination structures. This would explain why cells that are capable of carrying out normal recombination—even though it is a slow process in which there is extensive ssDNA created—are not signaled to arrest (34). In the special case in which a DSB cannot be repaired—the case we study here—both Rad52p and RPA act to assess the extent of the damage itself.

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