Fission yeast Rad50 stimulates sister chromatid recombination and links cohesion with repair

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To study the role of Rad50 in the DNA damage response, we cloned and deleted the *Schizosaccharomyces pombe* RAD50 homologue. The deletion is sensitive to a range of DNA-damaging agents and shows dynamic epistatic interactions with other recombination–repair genes. We show that Rad50 is necessary for recombinational repair of the DNA lesion at the mating-type locus and that *rad50Δ* shows slow DNA replication. We also find that Rad50 is not required for slowing down S phase in response to hydroxyurea or methyl methanesulfonate (MMS) treatment. Interestingly, in *rad50Δ* cells, the recombination frequency between two homologous chromosomes is increased at the expense of sister chromatid recombination. We propose that Rad50, an SMC-like protein, promotes the use of the sister chromatid as the template for homologous recombinational repair. In support of this, we found that Rad50 functions in the same pathway for the repair of MMS-induced damage as Rad21, the homologue of the *Saccharomyces cerevisiae* Scc1 cohesin protein. We speculate that Rad50 interacts with the cohesin complex during S phase to assist repair and possibly re-initiation of replication after replication fork collapse.

Keywords: DNA replication/Rad21/Rad50/
recombinational DNA repair/sister chromatid cohesion

Introduction

During the cell cycle, DNA is likely to be damaged as a result of failures in DNA replication or by exposure to damage-inducing agents. In mammalian cells, different repair pathways have evolved to repair different types of DNA damage, and DNA repair defects can predispose organisms to cancer. One of the most genotoxic lesions is a DNA double strand break (DSB). A DSB can be repaired by homologous recombination (HR) or by DNA end joining. In the process of HR, a DSB is repaired by copying the missing information from the sister chromatid or homologous chromosome, resulting in the exact restoration of the DNA (for a review see Pâques and Haber, 1999). In the non-homologous DNA end joining (NHEJ) pathway, where the two DNA ends are connected without the need for longer stretches of

homology, repair of a DSB is error prone and frequently leads to small deletions (for a review see Lewis and Resnick, 2000).

The RAD52 epistasis group of *Saccharomyces cerevisiae* consists of several genes which are involved in recombinational DSB repair. Mutants of these genes are sensitive to ionizing radiation and several other DNA-damaging agents, and have a role in mitotic and meiotic recombination. Based on the phenotypes of the mutated genes, two subgroups can be distinguished within the RAD52 epistasis group. The first group consists of the RAD51, -52, -54, -55 and -57 genes. They are involved in both mitotic and meiotic recombination; mutants of these genes are deficient in DSB repair and mating-type switching. They probably act in a protein complex and are thought to be responsible for DNA strand transfer during HR. Homologues of these genes have been found throughout the eukaryotes, suggesting a conserved function of these proteins (for a review see Petrini *et al.*, 1997). The second group consists of *S.cerevisiae* RAD50, XRS2 and MRE11. When these genes are mutated, cells are deficient in DSB repair and meiotic recombination, but proficient in mitotic recombination. The three proteins are thought to act in a protein complex (reviewed in Petrini, 1999). In *S.cerevisiae*, Rad50, Mre11 and Xrs2 also have a role in NHEJ, together with Yku70, Yku80, Lig4 and others (reviewed in Lewis and Resnick, 2000). However, in *Schizosaccharomyces pombe*, Rad50 is not required for NHEJ (Manolis *et al.*, 2001). In *S.cerevisiae*, Rad50 and interacting proteins have also been reported to function in telomere length maintenance (Boulton and Jackson, 1998), mating-type switching (Ivanov *et al.*, 1994), replication or post-replicative repair (Merrill and Holm, 1998), chromatin configuration at meiotic DSB sites (Ohta *et al.*, 1998) and the S-phase checkpoint response (Kironmai and Muniyappa, 1997).

Homologues of Rad50 and its interacting proteins have been found in higher eukaryotes, suggesting a conserved function of the protein complex. In human, the RAD50 gene is located in a region containing an acute myeloid leukaemia tumour suppressor gene, and hRad50 interacts with hMre11 (Dolganov *et al.*, 1996). It was shown that a mutation in p95/Nbs1, which interacts with the human Mre11–Rad50 complex and is probably an Xrs2 homologue, is responsible for the Nijmegen breakage syndrome, which results in increased cancer incidence in human and failure to induce some DNA damage checkpoints (Carney *et al.*, 1998; Varon *et al.*, 1998; Petrini, 2000). Mutations in MRE11 have also been implicated in cancer predisposition (reviewed in Petrini, 2000). These findings significantly increase the importance of understanding the role of the Rad50–Mre11–Nbs1 protein complex. Disruptions of *Mre11* (Xiao and Weaver, 1997) and *Rad50* (Luo *et al.*, 1999) in mouse are not viable,

complicating the analysis of these genes in higher eukaryotes. At the moment, it is unknown what the precise role of Rad50 and its interacting proteins is in different processes which are essential for the stability, recombination and reliable replication of DNA. It is unknown how DSBs are detected, how it is decided which DSB repair pathway is utilized for repair and how the repair is coordinated with the checkpoint-dependent cell cycle delay.

To study the role of the Rad50 protein in the repair of DNA damage in *S.pombe*, we cloned and deleted the *S.pombe* RAD50 homologue. Our data suggest that Rad50 is involved in mating-type switching and DNA replication and has a role in choosing the sister chromatids for recombinational repair. Based on our genetic data, we speculate that, during S phase, the Rad50 complex interacts with the Rad21 cohesin complex to assist repair and possibly the re-initiation of collapsed replication forks.

Results

Sequence of the *S.pombe* RAD50 homologue

Based on sequence information available in the database of *S.cerevisiae*, *Caenorhabditis elegans* and human RAD50 homologues, we designed degenerate primers and were able to clone and sequence the *S.pombe* RAD50 homologue which we designated *rad50⁺* (the gene has also been sequenced recently in the *S.pombe* genome sequencing project, and is located on cosmids SPAC1556.01C and SPAP4C9.01C). Sequence analysis of the *rad50⁺* gene revealed an open reading frame (ORF) encoding 1291 amino acids. This ORF is interrupted by three putative introns (49, 43 and 37 bp, respectively), all containing 5' and 3' splice sites as well as the branch site as published by Prabhala *et al.* (1992). The putative introns are all located in regions with high amino acid sequence conservation (see Figure 1A) and would cause a frameshift if not excised, making it very likely that these introns are spliced as predicted. An alignment of the amino acid sequences of *rad50⁺* and the RAD50 homologues of human, mouse, *S.cerevisiae* and *C.elegans* shows a very strong conservation at the N- and C-termini, where the ATP/GTP nucleotide-binding motif (Walker A; P-loop nucleotide-binding motif) and the DA box (Walker B, second half motif believed to be associated with the P-loop) are located (Walker *et al.*, 1982; Figure 1A). In the middle section, the degree of conservation is low, and long stretches with a high probability of coiled-coil formation are found (Figure 1C). In the middle of the protein, a putative 'half zinc finger' is found which is remarkably conserved throughout the different homologues (Figure 1A). It was postulated that this site could coordinate binding of a zinc atom mediated by dimerization of the protein (Sharples and Leach, 1995). Phylogenetically, Rad50 is closest to its *S.cerevisiae* counterpart (Figure 1B), showing an overall identity of 35.0% (as determined with BestFit). Surprisingly, the conservation between the *C.elegans* Rad50 homologue and the mouse homologue (28.7% identity) is lower than between the *S.pombe* and mouse homologue (30.2% identity).

Sensitivity of *rad50Δ* to DNA-damaging agents and epistasis analysis

We deleted the complete ORF of *rad50⁺*, replacing it with the kanMX6 cassette. We found that the deletion was sensitive to γ -irradiation, methyl methanesulfonate (MMS) and UV light (data not shown). In *S.cerevisiae*, RAD50, MRE11, RAD51, RAD55 and RAD52 have all been assigned to the same epistasis group (called the RAD52 epistasis group) and are thought to be involved in the recombinational repair of DNA DSBs (reviewed in Game, 1993). We studied the epistatic interactions between *rad50Δ* and selected *S.pombe* homologues of the *S.cerevisiae* RAD52 epistasis group genes in early stationary cells (see Table I for genotypes of the strains).

As can be seen in Figure 2A, for the repair of γ -ray-induced damage, *rad32Δ* (MRE11 homologue; Tavassoli *et al.*, 1995), *rhp51Δ* (RAD51 homologue; Muris *et al.*, 1993) and *rad22Δ* (RAD52 homologue; Ostermann *et al.*, 1993) are epistatic with *rad50Δ*; the double mutants are not more sensitive than one or both of the single mutants. The *rhp55* deletion (RAD55 homologue; Khasanov *et al.*, 1999) is not epistatic with *rad50*; the double mutant is more sensitive than either single mutant.

We also performed an epistasis analysis with the same mutants for MMS-induced DNA damage. As can be seen in Figure 2B, *rad50* is epistatic with *rad32* and *rhp51*. As for γ -irradiation, *rad50Δ* is not epistatic with *rhp55Δ*. Surprisingly, the *rad22Δ rad50Δ* double mutant is not only much more sensitive than either of the single mutants, but also much more sensitive than any of the other mutants or double mutant combinations. Thus *rad22* and *rad50* are not epistatic for the repair of MMS-induced DNA damage, whereas they are epistatic for the repair of γ -ray-induced damaged.

Slow growth of a *rad50* deletion is reduced in *smt0* cells

Mating-type switching in *S.pombe* is initiated by a DNA lesion, probably a single strand break (SSB) or some other DNA modification, in the *mat1* cassette (Arcangioli, 1998; Dalgaard and Klar, 1999). It has been proposed that mating-type switching is caused by a replication-stimulated recombination pathway, in which the ongoing replication fork is interrupted at the DNA lesion, resulting in the formation of a DSB which is repaired by a recombinational mechanism (Arcangioli, 1998; Arcangioli and de Lahondès, 2000).

We observed a strong slow growth phenotype in an *h⁻ rad50Δ* strain (data not shown) and speculated that non- or mis-repair of the mating type lesion might be responsible for this phenotype. In an *smt0* strain, no DNA lesion is present in the mating-type region (Engelke *et al.*, 1987; Styrkársdóttir *et al.*, 1993; Arcangioli and de Lahondès, 2000). To study if the absence of this lesion improved the growth rate of a *rad50Δ* strain, we determined the total number of cells and the fraction of dead and living cells at different time points of logarithmically growing liquid cultures of *rad50Δ* and *rad50⁺* strains in *h⁻* and *smt0* backgrounds. As can be seen in Table II, the doubling time and percentage of dead cells of *smt0 rad50Δ* indeed significantly decreased compared with *h⁻ rad50Δ*, although they were not restored to wild-type levels. This suggested that the inability to repair the DNA lesion in the

Fig. 1. (A) Alignment of the human (hs), mouse (mm), *S.cerevisiae* (sc), *S.pombe* (sp) and *C.elegans* (ce) *RAD50* proteins. Putative sequence motifs are indicated in the figure. The inverted triangle above the alignment marks the position of a putative intron (see also Results). The dots above the alignment mark the positions of the degenerated primers used for PCR. P-loop = P-loop nucleotide-binding motif (ProfileScan); C-C = half zinc finger sequence; it was postulated that this site could co-ordinate protein dimerization (Sharples and Leach, 1995); DA BOX = second half motif for nucleotide binding, associated with the P-loop. (B) Dendrogram showing the relationship between the different *RAD50* homologues. (C) Graph showing the probability of coiled-coil formation for the amino acid sequence of Rad50 (Paircoil, see Materials and methods).

mating type region partially accounts for the slow growth phenotype in a *h⁻ rad50Δ* strain and that the *rad50⁺* gene is involved in the proper repair of this lesion.

Rad50 is involved in DNA replication

The initiation of the mating-type switching process in *S.pombe* (Arcangioli, 1998; Arcangioli and de Lahondès, 2000) is very similar to the postulated HR-dependent repair of a replication fork that is stalled due to the presence of damaged DNA in bacteria (for a review see Kowalczykowski, 2000). Therefore, we speculated that the

residual slow growth phenotype in *smt0 rad50Δ* could be the result of problems during DNA replication, probably due to the inability to replicate spontaneously damaged DNA or to reinstate the replication fork after collapse.

We tested the sensitivity of *rad50⁺* and *rad50Δ* strains (EH68 and EH65; Table I) to hydroxyurea (HU), which is an inhibitor of ribonucleotide reductase and stalls the replication fork by depletion of nucleotides. As can be seen in Figure 3A, the *rad50Δ* strain is significantly more sensitive to HU than the wild-type, suggesting a role for Rad50 in S phase.

Table I. Strains used in this study

Strain	Genotype	Source and/or reference
EH6	<i>h⁻ rad50::kanMX6 ura4-D18</i>	this study
EH65	<i>smt0 rad50::kanMX6 ura4-D18</i>	this study
EH68	<i>smt0 ura4-D18</i>	this study; Engelke <i>et al.</i> (1987)
5-163	<i>h⁻ ura4-D18</i>	strain collection Bern
EH62	<i>smt0 rad32::ura4⁺ ura4-D18</i>	this study; Tavassoli <i>et al.</i> (1995)
EH61	<i>smt0 rad50::kanMX6 rad32::ura4⁺ ura4-D18</i>	this study
EH69	<i>smt0 rhp51::his3⁺ his3-D1</i>	this study; R.Krähenbühl (Bern)
EH57	<i>smt0 rad50::kanMX6 rhp51::his3⁺ his3-D1</i>	this study
EH66	<i>smt0 rhp55::ura4⁺ ura4-D18</i>	this study; Khasanov <i>et al.</i> (1999)
EH63	<i>smt0 rad50::kanMX6 rhp55::ura4⁺ ura4-D18</i>	this study
EH60	<i>smt0 rad22::ura4⁺ ura4-D18</i>	this study; Ostermann <i>et al.</i> (1993)
89-3531	<i>h⁺ rad2::ura4 ura4-D18</i>	strain collection Bern; Murray <i>et al.</i> (1994)
EH59	<i>smt0 rad22::ura4⁺ rad50::kanMX6 ura4-D18</i>	this study
EH44/EH45	<i>smt0/smt0 ade6-M210/ade6-M216ura4-D18/ura4⁺</i>	this study
EH42/EH46	<i>smt0/smt0 ade6-M210/ade6-M216 ura4-D18/ura4⁺ rad50::kanMX6/rad50::kanMX6</i>	this study
EH118	<i>smt0 ura4-D18 ade6-M375 int::pUC8/ura4⁺/ade6-469</i>	this study; Schuchert and Kohli (1988)
EH119	<i>smt0 ura4-D18 ade6-M375 int::pUC8/ura4⁺/ade6-469 rad50::kanMX6</i>	this study
EH85	<i>smt0 rad21-45</i>	this study; Birkenbihl and Subramani (1992)
EH80	<i>smt0 rad50::kanMX6 rad21-45</i>	this study

Using fluorescence-activated cell sorting (FACS) analysis, we also looked at the kinetics of DNA replication in synchronized *rad50⁺* and *rad50Δ* strains (EH45 and EH46, Table I). As can be seen in Figure 3B, in *rad50Δ* the progression through S phase is delayed compared with the wild-type and a significant fraction of the cells remains in G₁ in the *rad50* deletion. Using a method described by Cha *et al.* (2000), we estimated that the duration of S phase in the *rad50⁺* strain is 33 min, whereas it is increased to 53 min in the *rad50Δ* strain. We also looked at the kinetics of DNA replication in the presence of MMS and HU. As can be seen in Figure 3B, replication in wild-type as well as in *rad50Δ* is significantly slowed down in the presence of 0.01% MMS. In the presence of 20 mM HU, replication is virtually blocked in the wild-type as well as in *rad50Δ*.

The *S.pombe rad2⁺* gene is a homologue of the *FEN1* gene (Murray *et al.*, 1994), and is involved in Okazaki fragment processing during lagging strand DNA synthesis (Allewa and Doetsch, 1998). It has been reported previously that deletions of the *S.pombe RAD51* and *RAD54* homologues are synthetically lethal with *rad2Δ* (Muris *et al.*, 1996). We crossed *rad50Δ* with *rad2Δ*, pulled 20 tetrads and found that *rad50Δ* is also synthetically lethal with *rad2Δ* (see Figure 3C), suggesting that Rad50 is necessary to retain cell viability when Okazaki fragment processing is defective.

Rad50 is involved in telomere length maintenance

In *S.cerevisiae*, Rad50 is involved in telomere length maintenance (Boulton and Jackson, 1998; Kironmai and Muniyappa, 1997). To check whether *S.pombe* Rad50 has a role in telomere length maintenance, we cultured wild-type and *rad50Δ* strains for at least 100 generations in liquid media. Genomic DNA was isolated, digested, separated on a gel, blotted and hybridized with a telomere-specific probe. As can be seen in Figure 4, the telomeres in *rad50Δ* are shorter than in *rad50⁺* parental strains, suggesting that Rad50 plays a role in telomere length maintenance.

Chromosome loss frequency is strongly increased in *rad50Δ*

It has been reported previously that deletions of the *S.pombe RAD51* and *RAD54* homologues show an increased frequency of minichromosome loss (Muris *et al.*, 1996). We developed a new chromosome loss/inter-homologue recombination assay, which is explained in Figure 5 (for explanation see figure legend). We determined the chromosome loss frequency in a fluctuation test (see Table III) and found that the frequency of chromosome loss is strongly increased in *rad50Δ*: the 705-fold increase in the average number of haploids per 1×10^6 plated cells is statistically significant (Mann–Whitney U-test) and translates to a 490-fold increase of chromosome loss per cell division (as determined with the Lea–Coulson method of the median).

Recombination between homologous chromosomes is increased in *rad50Δ*

With the assay described in Figure 5, we were also able to determine the frequency of inter-homologue crossover recombination between the centromere and the *ura4* locus. A crossover event between the centromere and the *ura4* locus leads to fluoro-orotic acid (FOA)-resistant diploids (*ura4-D18/ura4-D18*) that are white on low adenine media. FOA-resistant colonies were not the result of a mutation in the *ura4⁺* gene, as haploid strains EH45 and EH46 (Table I) did not give any FOA-resistant colonies using the same protocol plating the same numbers of cells (data not shown). White FOA-resistant colonies that were detected in the assay were all diploid, as confirmed with Coulter counter size analysis (data not shown). Therefore, they were not the result of a chromosome loss event (with concurrent gene conversion between the *ade6* alleles to give an *ade6⁺* gene) but of a mitotic crossover between the homologous chromosomes between *ura4⁺* and the centromere. As can be seen in Table IV, the frequency of this inter-homologue recombination event per 1×10^6 plated cells is increased 31-fold in *rad50Δ*. This increase is

Fig. 2. Epistasis analysis for γ -irradiation (A) and MMS (B) sensitivity between *rad50* Δ and deletions of other *S.pombe* homologues of the *RAD52* epistasis group. The survival (%) (y-axis) of the different single mutants and double mutant combinations is plotted against the γ -ray dose (Gy; x-axis) and the MMS concentration (%) (x-axis), respectively. For genotypes, see Table I.

statistically significant (Mann–Whitney U-test) and translates to a 28-fold increased recombination rate per cell division as determined with the Lea–Coulson method of the median.

Frequency of sister chromatid recombination is decreased in *rad50* Δ

We wanted to know if the increase in recombination in *rad50* Δ was due to a general increase in recombination, or

if it was specific for recombination between homologous chromosomes. Therefore, we used an assay to determine the sister chromatid recombination frequency as described by Schuchert *et al.* (1988). In the haploid strain used for this assay, a *ura*⁺ marker is flanked by the *ade6-469* and *ade6-M375* alleles. Homologous recombination between the *ade*⁻ alleles of one sister chromatid (intra-sister recombination) or between alleles on different sister chromatids (inter-sister recombination) leads to a

functional *ade⁺* gene. As shown in Table V, we found that the frequency of sister chromatid recombination per 1×10^6 plated cells was decreased 11-fold. This decrease is statistically significant (Mann–Whitney U-test) and translates to a 15-fold decrease per cell division as determined with the Lea–Coulson method of the median.

***rad50* is epistatic with the *rad21* cohesin gene for the repair of MMS-induced DNA damage**

We found that in *rad50Δ*, recombination between homologous chromosomes is increased whereas recombination between sister chromatids is reduced. Thus, Rad50 probably stimulates the use of the sister chromatid as a template for HR. The overall structure of Rad50 is reminiscent of that of SMC proteins, which are involved in sister chromatid cohesion. This suggested to us that Rad50 might provide a link between sister chromatid cohesion and repair. Interestingly, the *rad21-45* allele of

the sister chromatid cohesion gene *rad21⁺*, the *S.pombe* *SCC1* homologue, is radiation sensitive and repair deficient (Birkenbihl and Subramani, 1992). To determine if *rad50⁺* and *rad21⁺* function in the same pathway for the repair of damaged DNA, we carried out an epistasis analysis with *rad50Δ* and *rad21-45*. As can be seen in Figure 6A, the *rad21-45 rad50Δ* double mutant is no more sensitive than *rad50Δ*, whereas *rad21-45* is more sensitive than the wild-type for MMS-induced DNA damage. Three independent repetitions of this experiment also showed that the *rad50Δ rad21-45* double mutant was no more sensitive than *rad50Δ*. Therefore, we conclude that *rad21* and *rad50* are epistatic and function in the same pathway for the repair of MMS-induced DNA damage. Surprisingly, as can be seen in Figure 6B, *rad21* and *rad50* are not epistatic and function in different pathways for the repair of γ -ray-induced DNA damage.

Discussion

Sensitivity of *rad50Δ* to DNA-damaging agents and epistasis analysis

Different DNA-damaging agents cause different DNA lesions which might be repaired using different mechanisms. The main genotoxic lesions caused by γ -irradiation are DNA DSBs, which in *S.pombe* are repaired predominantly by HR in G₂. MMS alkylates the DNA; alkylated DNA can block DNA synthesis and stall the replication fork. These alkylated sites can be excised enzymatically, leading to formation of SSBs (Laval,

Fig. 3. Analysis of replication problems in *rad50Δ*. (A) Sensitivity of *rad50Δ* to different HU concentrations was determined in a spot test. Numbers above the figure indicate approximate number of cells spotted. (B) Tetrad analysis of a cross between *rad50Δ* and *rad2Δ*. The plus signs and triangles below the colonies show the presence of wild-type and deletion alleles of *rad2* and *rad50*, respectively. Genotypes from non-growing colonies were deduced assuming a 2:2 segregation. (C) Analysis of replication progression using FACS analysis of cells released from G₁ arrest (see Materials and methods). Progression through S phase is reflected by a transition from the G₁ peak to the G₂ peak.

1977). When *S.cerevisiae* is exposed to low (0.05%) MMS concentrations, SSBs can be detected in the DNA (Chlebowicz and Jachymczyk, 1979). The presence of SSBs is not necessarily an immediate threat to cell viability, but is likely to cause problems during DNA replication. Indeed, DNA synthesis in mammalian cells is inhibited when cells are exposed to low (1 mM ~0.01%) concentrations of MMS (Dahle *et al.*, 1978). In *S.cerevisiae*, DSBs are only detectable when high (0.5%) MMS concentrations are used (Chlebowicz and Jachymczyk, 1979). In this study, we showed that also in *S.pombe*, DNA replication is slowed down in the presence

Fig. 4. Southern blot analysis of telomere length in wild-type and *rad50Δ* strains cultured for at least 100 generations in liquid media. Telomeres of the *rad50Δ* strain are shorter than those of the wild-type strain, showing that Rad50 is involved in telomere length maintenance.

of 0.01% MMS, suggesting that the DNA damage caused by MMS causes problems during replication and is not repaired until S phase.

Whereas *rad22⁺* and *rad50⁺* function in the same pathway for repair of γ -ray-induced damage, they function in different pathways for the repair of MMS-induced damage. At first sight, this observation might be surprising because no differences have been found for epistatic interactions between *rad22* and *rhp55* for MMS and γ -irradiation (Khasanov *et al.*, 1999). Our epistasis analysis probably reflects that the different fission yeast homologues of the *S.cerevisiae* 'RAD52 epistasis group' function in different inter-related pathways and that the epistatic interactions depend on the kind of DNA damage and phase of the cell cycle in which the damage is repaired. The synergistic contributions of *rad50⁺* and *rad22⁺* to the repair of MMS-induced damage therefore might reflect different requirements for these genes in S-phase repair. In our hands, the *rad22Δ* mutant is more sensitive to γ -irradiation than was shown by Muris *et al.* (1997). The sensitivities of other mutants used in this study are within the previously reported range, suggesting that the differences are not due to experimental differences. A possible explanation is that the *rad22Δ* strain used by Muris *et al.* (1997) might have contained a suppressor mutation. Strains deleted for *rad22* have a strong slow growth phenotype and, therefore, suppressor mutations that improve the growth rate might occur and take over frequently. As we crossed the *rad22* deletion into the *smt0* background, we might have lost the suppressor.

Fig. 5. Diagram explaining the chromosome loss/inter-homologue recombination assay. A non-sporulating diploid (EH44/EH45 and EH42/EH46; see Table I for genotype) is grown under non-selective conditions in liquid medium until stationary. Cells are plated in appropriate dilutions on YEA medium containing FOA (selective for *ura4⁻* cells) with limiting amounts of adenine (*ade6⁻* cells turn red). The original diploid does not grow on this medium because it is *ura4⁺*. Red FOA-resistant colonies are the result of concomitant loss of the *ura4⁺* gene (making them FOA resistant) and the *ade6-M216* allele (making them red), due to loss of chromosome III. White diploid FOA-resistant colonies are the result of a crossover event between the centromere and *ura4*. Depending on the chromosome segregation pattern after this recombination event, two *ura4-D18* alleles can be pulled to the same spindle pole, forming an FOA-resistant diploid cell which is still *ade⁺* due to the interallelic complementation of the *ade6-M216* and *ade6-M210* alleles.

Table III. Frequency of chromosome III loss in wild-type and *rad50Δ* diploids

Average No. of haploids per 1×10^6 plated cells ^a		Haploidization frequency per division ^b	
Wild-type	<i>rad50Δ</i>	Wild-type	<i>rad50Δ</i>
1.7×10^2	1.2×10^5 (705× ^c ; $P < 0.05^d$)	1.36×10^{-5}	6.66×10^{-3} (490×) ^c

^aBased on 10 independent cultures.^bCalculated with the Lea–Coulson method of the median (Lea and Coulson, 1949).^cFold increase compared with wild-type.^dProbability as determined with the Mann–Whitney U-test.**Table IV.** Inter-homologue crossover frequency between the centromere and the *ura4* locus on chromosome III in wild-type and *rad50Δ* diploids

Average No. of recombinants per 1×10^6 plated cells ^a		Inter-homologue recombination frequency per division ^b	
Wild-type	<i>rad50Δ</i>	Wild-type	<i>rad50Δ</i>
1.8×10^3	5.5×10^4 (31× ^c ; $P < 0.05^d$)	1.40×10^{-4}	3.85×10^{-3} (28×) ^c

^aBased on 10 independent cultures.^bCalculated with the Lea–Coulson method of the median (Lea and Coulson, 1949).^cFold increase compared with wild-type.^dProbability as determined with the Mann–Whitney U-test.**Table V.** Sister chromatid recombination frequency in wild-type and *rad50Δ*

Median No. of <i>ade+</i> recombinants per 1×10^6 plated cells ^a		Inter/intra-sister recombination frequency per division ^b	
Wild-type	<i>rad50Δ</i>	Wild-type	<i>rad50Δ</i>
5.0×10^4	4.7×10^3 (11× ^c ; $P < 0.05^d$)	2.11×10^{-3}	1.44×10^{-4} (15×) ^c

^aBased on 10 independent cultures.^bCalculated with the Lea–Coulson method of the median (Lea and Coulson, 1949).^cFold increase compared with wild-type.^dProbability as determined with the Mann–Whitney U-test.

What is the primary function of Rad50?

Because *rad50* mutations in different organisms result in many pleiotropic phenotypes, it is challenging to find out what the primary function is of the Rad50 protein. In *S.cerevisiae*, Rad50 has been implicated in NHEJ (reviewed in Lewis and Resnick, 2000), S-phase checkpoint response (Kironmai and Muniyappa, 1997; D'Amours and Jackson, 2001; Usui *et al.*, 2001) and HR (Bressan *et al.*, 1999; for a review see Pâques and Haber, 1999). In *S.pombe*, however, Rad50 is not required for NHEJ (Manolis *et al.*, 2001), suggesting that the protein might play a regulatory rather than a catalytic role in NHEJ in *S.cerevisiae*. In this study, we reported that *S.pombe rad50Δ* cells delay S-phase progression in response to MMS and HU treatment to a similar extent

Fig. 6. Epistasis analysis between *rad50Δ* and *rad21-45* for MMS (A) and γ-ray (B) survival. The genes are epistatic for MMS-induced DNA damage, the *rad50Δ rad21-45* double mutant is not more sensitive than the *rad50Δ* single mutant. However, the genes are not epistatic for γ-ray-induced damage, the double mutant being more sensitive than either of the single mutants.

as wild-type cells. This suggests that in these circumstances, *S.pombe* Rad50 is not involved in establishing the intra S-phase checkpoint. Because of these differences, *S.pombe* is a useful organism to study the primary role of the Rad50 protein.

We showed that in *rad50Δ*, the spontaneous recombination frequency between homologous chromosomes is increased, whereas sister chromatid recombination is reduced. This suggested to us that the Rad50 protein might establish an interaction with the sister chromatid during repair, and thus promotes the use of the sister chromatid as a template for HR. Also in *S.cerevisiae*, the spontaneous inter-homologue recombination frequency is increased in *rad50Δ* strains (Malone *et al.*, 1990). To our knowledge, sister chromatid recombination has not been measured in *S.cerevisiae rad50Δ*. Bressan *et al.* (1999) showed that in *S.cerevisiae mre11Δ*, the spontaneous sister chromatid recombination frequency is not decreased, whereas ionizing-radiation induced sister chromatid recombination is slightly decreased compared with the wild-type. However, *S.cerevisiae* spends more of its time in G₁ than *S.pombe*, which is predominantly a G₂ organism, and a defect in sister chromatid recombination might therefore be masked. The fact that haploid *rad50Δ* cells in G₂ are much more sensitive than diploid G₁ *rad50Δ* cells in *S.cerevisiae* has been interpreted to suggest that Rad50 and interacting proteins are involved in sister chromatid interactions (Pâques and Haber, 1999; and references cited therein).

The overall structure of the Rad50 protein is reminiscent of the SMC family of proteins. Members of this family have a role in chromatin condensation, sister chromatid cohesion and chromosome stability (for a review see Strunnikov and Jessberger, 1999). Also the results of a similarity search using Propsearch, which is based on the comparison of several protein properties other than amino acid sequence, showed a high similarity to SMC proteins (data not shown). Besides Rad18 (Lehmann *et al.*, 1995), Rad50 is the second *S.pombe* protein which is involved in DNA repair and shows similarity to SMC proteins. Rad50 is proposed to be an antiparallel homodimer, with DNA-binding catalytic domains on both ends, therefore capable of connecting two DNA strands (Hopfner *et al.*, 2000). Thus the protein would be ideally suited to make a connection between sister chromatids or homologous chromosomes to check the presence of a repair template and aid HR. HR is thought to be initiated with the 5' to 3' resection of the DNA strands, possibly by endo/exo nuclease activity of the Mre11 protein (Haber 1998). This resection can lead to loss of genetic information and is a potentially deleterious event if no sister chromatid is available for recombinational repair. Therefore, it is likely that the availability of a sister chromatid or homologous chromosome influences which repair pathway is chosen. In mammalian cells, Ku70, Rad50 and Mre11 physically interact with each other, and it has been suggested that this interaction is necessary to make a choice between HR and NHEJ (Goedecke *et al.*, 1999). We propose that the primary role of *S.pombe* Rad50 is to check the presence of a sister chromatid and, if present, to promote HR using the sister as a template. We speculate that Rad50 regulates the nuclease activity of Rad32 (Mre11).

Role of Rad50 in mating-type switching, DNA replication and telomere maintenance

During the process of mating-type switching in *S.pombe*, the replication fork runs into a DNA lesion in the mating-type region which is then repaired by HR with one of the donor cassettes (Arcangioli and de Lahondès, 2000). This process is very similar to the postulated HR-dependent repair of a replication fork that is stalled due to the presence of damaged DNA in bacteria (for a review see Kowalczykowski, 2000). We showed that Rad50 plays a role in the recombinational repair of the lesion at the mating-type locus and that non- or mis-repair of this lesion in *rad50Δ* leads to cell death.

Muris *et al.* (1996) proposed that the *S.pombe* homologues of Rad51 and Rad54 play a role during DNA replication (see also Lehmann *et al.*, 1995). Merrill and Holm (1998) found that in *S.cerevisiae*, several RAD52 group mutants, including *rad50*, are synthetically lethal with the *pol30-104* mutation (Pol30 is the *S.cerevisiae* PCNA homologue), suggesting a function of these genes in DNA replication. We tested if the residual slow growth phenotype in *smt0 rad50Δ* could be the result of problems during DNA replication and found that replication in *rad50Δ* is delayed compared with wild-type. The *rad50Δ* strain is sensitive to HU, consistent with the idea that Rad50 is required for the recombination-dependent restart of collapsed replication forks. Rad2 is the *S.pombe* Fen1 homologue and is required for the removal of the lagging strand RNA primer (Murray *et al.*, 1994; Alleva and

Doetsch, 1998). In the absence of Fen1/Rad2, the last remaining ribonucleotide of the primer cannot be removed, and the Okazaki fragments cannot be ligated, leading to nicked DNA (Bambara *et al.*, 1997). We found that *rad50Δ* is synthetically lethal with *rad2Δ*, suggesting that Rad50 functions in an alternative, presumably recombinational, pathway to remove the remaining RNA primer leftovers.

In *rad50Δ*, the telomeres are shortened. *Saccharomyces cerevisiae* Rad50 has also been shown to be involved in telomere length maintenance (Boulton and Jackson, 1998; Kironmai and Muniyappa, 1997). Nugent *et al.* (1998) found that *S.cerevisiae rad50* is epistatic with the telomerase, and it was proposed that the exonuclease activity of Mre11 prepares the single strand substrate for the telomerase. In mammalian cells, telomeres end in a large duplex loop that is formed by invasion of the 3' telomeric overhang into the duplex telomeric repeat array. Formation of this loop is catalysed by the Trf2 protein (Griffith *et al.*, 1999). Zhu *et al.* (2000) showed that the Rad50–Mre11–Nbs1 complex is associated with the Trf2 protein and they proposed that the Rad50 protein complex is involved in formation and stabilization of this loop structure. Whereas Rad50 and Mre11 are found at the telomere throughout the cell cycle, Nbs1 localizes to the telomeres only in S phase, suggesting a role for Nbs1 in telomere replication. Possibly, Rad50 is involved in this process by signalling the presence of a sister chromatid (indicative of completed DNA replication) and subsequent initiation of telomere addition by the telomerase (see also Pâques and Haber, 1999). Recent data might suggest that components of the Rad50 protein complex provide accessory functions and recruit the telomerase to the telomeres (Ranganathan *et al.*, 2001; Tsukamoto *et al.*, 2001). In the absence of the telomerase, telomeres can be elongated in a recombination-dependent manner, which is dependent on RAD52 group genes in *S.cerevisiae* (reviewed in Kass-Eisler and Greider, 2000). In *S.pombe* telomerase-deficient mutants, survivors are formed that have circularized all their chromosomes, whereas a smaller population maintains their telomeres, presumably by a recombinational mechanism (Nakamura *et al.*, 1998).

Mating-type switching, recombination-dependent replication and alternative recombination-dependent telomere lengthening require recombination with the sister chromatid. We therefore believe that defects in these processes in *rad50Δ* might well be explained by a role for Rad50 in initiating recombination using the sister chromatid as a template.

Rad50 functions in the same pathway as Rad21 for the repair of MMS-induced DNA damage

The *S.pombe rad21⁺* gene, the homologue of the *S.cerevisiae SCC1* sister chromatid cohesion gene, was characterized initially as a radiation-sensitive mutant, suggesting that sister chromatid cohesion plays a role in the repair of DSBs. Several lines of evidence suggest that in different organisms a link exists between sister chromatid cohesion and recombinational repair during meiosis (reviewed in Heemst and Heyting, 2000). We showed that for the repair of MMS-induced DNA damage (which leads to problems in S phase), *rad50⁺* functions in the same pathway as *rad21⁺*, the *S.pombe SCC1*

homologue. Interestingly, *rad50* and *rad21* are not epistatic for the repair of γ -ray-induced damage. Because γ -ray-induced DNA damage in asynchronous *S.pombe* cells is repaired predominantly during G₂, whereas MMS leads to problems in S phase (see beginning of Discussion), we think it is likely that the genetic interaction between *rad21* and *rad50* for MMS-induced damage is S phase specific. At the moment, we can only speculate why a genetic interaction between *rad21* and *rad50* would be S phase specific. We proposed that Rad50 plays a role in preventing the initiation of homologous recombination when no sister chromatid is available for repair. Possibly, during G₂, the cell cycle machinery is able to signal the presence of a sister chromatid to the repair machinery. However, during S phase, the DNA is only partially replicated, and an additional mechanism is needed to signal the availability of a sister. The *S.cerevisiae* cohesin complex, consisting of the Scc1, Scc3, Smc1 and Smc3 proteins, is loaded on the DNA after the passage of the replication fork (Uhlmann and Nasmyth, 1998) and the presence of the complex indicates that the DNA has been replicated. Possibly Rad21 supports Rad50 in the task of determining the presence of a sister chromatid during S phase, explaining the epistatic interaction between the two mutant genes for the repair of MMS damage. We can speculate that, during S phase, the Rad50 protein complex interacts with the cohesin complex to assist recombinational repair in regions that have already replicated. This interaction may be important for re-initiation of replication after replication fork collapse. The precise nature of this interaction remains unknown. Because Rad50 is an SMC-like protein, the interaction between Rad21 and Rad50 might be reminiscent of the interaction between Scc1 and the Smc1/3 proteins in the cohesin complex. We have not been able to co-immunoprecipitate Rad21 with Rad50, and vice versa, or see any significant Rad50-dependent change in Rad21 phosphorylation (Birkenbihl and Subramani, 1995). However, any possible Rad50–Rad21 complex is likely to be chromatin bound and would thus be insoluble and not detectable with our assays.

Materials and methods

Strains and media

For strain construction and propagation, standard genetic methods and media were used (Gutz *et al.*, 1974). Strains used and constructed in this study are shown in Table I.

PCR, cloning, DNA sequencing and gene disruption of the *S.pombe* RAD50 homologue

Based on sequence information from the *S.cerevisiae*, human and *C.elegans* RAD50 homologues, conserved regions were chosen for primer synthesis. Degenerated primers derived from the amino acid sequences MRGRC [ATG(A/C)G(A/C/G/T)GG(A/C/G/T)(A/C)G(A/C/G/T)TG] and VITHDE [TC(A/G)TC(A/G)TG(A/C/G/T)GT(A/G/T)AT(A/C/G/T)AC] were used for PCR (1.5 μ g of *S.pombe* genomic DNA as template, annealing temperature 39°C, 35 cycles). A fragment of the expected length was amplified weakly. PCR on the eluted fragment using internal forward and reverse primers derived from the amino acid sequence DEPTTN [forward: GA(C/T)GA(A/G)CC(A/C/G/T)AC(A/C/G/T)AC(A/C/G/T)AA] gave a product of the expected length in combination with both external primers, confirming that we indeed amplified a fragment of the *S.pombe* RAD50 homologue; we designated the gene *rad50*⁺. The PCR fragment was cloned into a T-vector (Promega). Using this fragment to hybridize the *S.pombe* cosmid library (Hoheisel *et al.*, 1993), cosmid clone ICRFc60D1232D was identified. After restriction mapping and Southern analysis, a 5.6 kb *EcoRV*

fragment containing the *rad50*⁺ fragment was identified, subcloned and partially sequenced by automated fluorescence sequencing (Microsynth GmbH, Balgach, Switzerland). As this fragment did not contain the 5' part of the *rad50*⁺ gene, a partially overlapping 4.1 kb *XhoI* fragment containing the 5' end was also subcloned and partially sequenced. For construction of a deletion, we precisely deleted the complete ORF of *rad50*⁺, and replaced it with the kanMX6 cassette as described by Bähler *et al.* (1998).

Protein sequence analysis

Sequence alignments and analysis were done using the Wisconsin sequence analysis package (GCG) and the ISREC BOXSHADE server (http://www.isrec.isb-sib.ch/software/BOX_form.html). Non-sequence-dependent protein similarity was detected using Propsearch (Hobohm and Sander, 1995; <http://www.embl-heidelberg.de/prs.html>).

Sensitivity to DNA-damaging agents

For γ -ray sensitivity, cells were irradiated in suspension in supplemented liquid yeast extract (YEL) with a ¹³⁷Cs source (12.5 Gy/min) and immediately plated in duplicate on supplemented yeast extract (YEA). Cells were grown for 5 days at 30°C and colonies were counted. Relative survival was calculated by dividing the absolute survival by the survival of cells of the same strain not exposed to DNA-damaging agents. For MMS survival, cells were plated in duplicate directly on MMS-containing plates made up the previous day. For HU sensitivity, a spot test was performed: cells were spotted in different dilutions on plates containing different concentrations of HU and grown for 3 days at 30°C. Experiments were repeated at least three times and gave similar results.

Determination of doubling time and percentage of dead cells in liquid cultures

For studying the growth kinetics of different *S.pombe* strains (5-163, EH6, EH65 and EH68; see Table I for genotype), single cells were grown to colonies and pre-cultured in YEL. After inoculating the appropriate amount of cells, cultures of the different strains were grown overnight until they simultaneously had reached a cell density of $\sim 0.5 \times 10^6$ /ml the next morning. Samples for counting and viability determination were sonicated to disrupt cell clumps. Cell density was determined in triplicate for each culture with a Coulter counter. Appropriate dilutions of each culture were plated in triplicate on supplemented YEA plates. Viability of the cells was determined in triplicate in a microscope the next morning by counting the number of microcolonies (resulting from a living cell) and single cells (which were considered to be dead). For each single determination, at least 500 cells and microcolonies were counted. Determination of cell density and viability as described above was repeated after ~ 7 h of logarithmic growth, taking care that cell density was below 5×10^6 /ml. From the determination of total cell density and viability, the doubling time and percentage of dead cells was calculated for the different strains. A repetition of the experiment gave highly similar results.

Tetrad analysis

Strains 89-3531 and EH65 were crossed and tetrad analysis was performed using standard procedures. Plates were allowed to grow for 5 days and were replica plated on minimal media without uracil (to test for the presence of the *rad2::ura4* deletion) and on YEA + G418 (to test for the presence of the *rad50::kanMX6* deletion).

Synchronization and FACS analysis

To study DNA replication, *rad50*⁺ (EH45) and *rad50* Δ (EH46) strains were nitrogen starved for 20 h in EMM2 without nitrogen at 25°C. To get rid of the high percentage of dead cells in the *rad50* Δ culture, which remain elongated after nitrogen starvation, both cultures were centrifuged on a 7–30% lactose gradient, and the small cells on top of the gradient were harvested. The resulting cultures were virtually free of elongated (dead) cells. FACS analysis was performed according to Alfa *et al.* (1993). A repetition of the experiment gave similar results.

Telomere length

Telomeres were detected using the 1.9 kb *ApaI* fragment of plasmid pEN42 (Nimmo *et al.*, 1994). Genomic DNA from *rad50*⁺ and *rad50* Δ strains (EH68 and EH65, respectively; see Table I) was isolated as described in Moreno *et al.* (1991), digested with *ApaI* and separated on a 1.2% agarose gel. DNA was blotted on Bio-Rad Zeta Probe membranes and hybridized with the standard protocol as supplied by the manufacturer. Several repetitions of this experiment showed a similar reduction of telomere length in *rad50* Δ .

Chromosome loss/inter-homologue recombination assay and sister chromatid recombination assay

Non-sporulating diploids of strains EH44/EH45 and EH42/EH46 (see Table I) were made freshly by protoplast fusion (Alfa *et al.*, 1993) and re-streaked on MMA plates supplemented with uracil. For each diploid, a minimum of 10 independent colonies were inoculated in supplemented YEL medium and grown until stationary phase. Appropriate dilutions were plated on supplemented YEA plates to determine the viable cell number and on YEA + FOA + uracil (without adenine) to select for colonies that lost the *ura4⁺* gene. Haploid FOA-resistant colonies that lose *ura4⁺* due to chromosome loss turn red on this medium, whereas diploid colonies that lose the *ura4⁺* marker because of recombination between the centromere and *ura4* remain white due to intragenic complementation of *ade6-M210* and *ade6-M216* (explained in Figure 5). White and red colonies were counted after 5 days at 30°C and the frequencies of chromosome loss and inter-homologue recombination per division were determined with the method of the median (Lea and Coulson, 1949).

For the sister chromatid recombination assay, a minimum of 10 independent single colonies from strains EH118 and EH119 (see Table I for genotype; constructed from PS3; Schuchert and Kohli, 1988) were inoculated in 10 ml of YEL + supplements and grown to stationary phase. Appropriate dilutions were plated on supplemented YEA for determination of viable cell numbers. To select for *ade⁺* recombinants, appropriate dilutions were plated on MMA + uracil and colonies were counted after 5 days at 30°C. The frequency of sister chromatid recombination events per division was determined with the method of the median (Lea and Coulson, 1949).

Statistically significant differences in the chromosome loss and recombination assays described above were detected using the non-parametric Mann-Whitney U-test. Repetitions of these experiments gave similar results.

Acknowledgements

Special thanks go to Oliver Fleck for help during the initial stages of the project, Peter Munz for help with the genetics and viability assays, and Thomas Caspari and Alan Lehmann for reading the manuscript and helpful discussions. This work was supported in part by grants from the Swiss National Science Foundation (J.K.) and the Human Frontiers Science Programme (A.M.C.). E.H. was supported by fellowships from the Swiss National Science Foundation and a Marie Curie Fellowship from the European Union (contract No. MCF1-1999-01425).

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Received April 11, 2001; revised and accepted October 4, 2001