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Cloning and characterisation of the Schizosaccharomyces pombe rad32 gene: a gene required for repair of double strand breaks and recombination

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ABSTRACT

A new Schizosaccharomyces pombe mutant (rad32) which is sensitive to gamma and UV irradiation is described. Pulsed field gel electrophoresis of DNA from irradiated cells indicates that the rad32 mutant, in comparison to wild type cells, has decreased ability to repair DNA double strand breaks. The mutant also undergoes decreased meiotic recombination and displays reduced stability of minichromosomes. The rad32 gene has been cloned by complementation of the UV sensitive phenotype. The gene, which is not essential for cell viability and is expressed at a moderate level in mitotically dividing cells, has significant homology to the meiotic recombination gene MRE11 of Saccharomyces cerevisiae. Epistasis analysis indicates that rad32 functions in a pathway which includes the rhp51 gene (the S.pombe homologue to S.cerevisiae RAD51) and that cells deleted for the rad32 gene in conjunction with either the rad3 deletion (a G2 checkpoint mutation) or the rad2 deletion (a chromosome stability and potential nucleotide excision repair mutation) are not viable.

INTRODUCTION

DNA repair processes are fundamentally important if cells are to maintain their genetic integrity. Organisms have thus evolved a number of processes by which DNA damage is recognised and repaired, and these include a well characterised nucleotide excision repair pathway and a damage tolerance mechanism involving recombination. Recent studies have begun to yield important data on the mechanisms associated with nucleotide excision repair and the highly conserved nature of the proteins involved (e.g. 1-3). In contrast, somewhat less is known about the molecular mechanisms involved in recombination in eukaryotes.

The fission yeast, S.pombe, has proved to be a good model system for the study of eukaryotic processes, such as the cell cycle (e.g. 4) and more recently nucleotide excision repair (e.g. 5,6). Radiation sensitive mutants in S.pombe were originally assigned to 23 complementation groups (7,8), although this number has been expanded to include several new G2 checkpoint mutants (9). Categorization of the corresponding genes into epistasis groups has been limited and effective only for those genes involved in nucleotide excision repair and a G2 checkpoint pathway. Of the original S.pombe mutants which are sensitive to gamma irradiation, only rad21 and rad22 are now categorized as being defective in recombination (10,11). A more recent search for meiotic recombination mutants has led to the identification of 10 new complementation groups, mutants in three of which are sensitive to DNA damaging agents (12).

Recombination is not only required for the tolerance of DNA damage, but is also central to meiosis. In S.cerevisiae, recombination mutants have been isolated in a number of screens e.g. the rad52 series of mutants which are sensitive to gamma radiation (e.g. 13) as well as several mutants defective in meiotic recombination (e.g. 14). Recent studies have shown that some of the RAD52 series of genes are highly conserved between the yeasts, S.cerevisiae and S.pombe, and higher eukaryotes. e.g. RAD51 (15), RAD52 (16) and RAD54 (17).

We report here the isolation and characterization of a new gamma radiation sensitive S.pombe mutant rad32 and the cloning and sequence analysis of the corresponding gene. The mutant is defective in meiotic recombination, displays spontaneous chromosome instability and is defective in repairing DNA double strand breaks. The rad32 gene has homology to the S.cerevisiae meiotic recombination gene MRE11 and is not essential for cell viability. Epistasis analysis indicates that Rad32 is likely to function in a pathway that includes Rhp51 (the S.pombe homologue to S.cerevisiae Rad51).

MATERIALS AND METHODS

Plasmids, strains and growth conditions

The S.pombe plasmid pUR19 and the genomic library used in this study have been described elsewhere (18). The S.pombe cDNA library was a gift from L. Guarente (19). S.pombe strains used in

* To whom correspondence should be addressed
this study are shown in Table 1, strain GP24 was a gift from G. Smith (Seattle, 12). Plasmids were grown in E. coli strain DH5α, [endA1, hsdR17, (rK-, mK-), supE44, thi-1, recA1, lacIq2-M15 (lacproAB)]. M13 derivatives were grown in DH5αF’ (as DH5α except that it contains an integrated F’). Procedures and media used for the routine growth and maintenance of S. pombe strains were as reported in our previous work (20).

Table 1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spore viability</th>
<th>Percentage</th>
<th>Ade6-704</th>
<th>Leu1-32</th>
<th>Ura4-D18</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP24</td>
<td>ade6-M26</td>
<td>h+</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Irradiation of S. pombe cells

Gamma irradiation was carried out using a 137Cs gamma source, with a dose rate of 12 Gy/min. UV irradiation was carried out directly on freshly plated cells using a Stratagene "Stratalinker".

Pulsed field gel electrophoresis

Preparation of DNA plugs was as described by Noolandi and Turmel (21). Electrophoresis using 0.7% agarose gels (Seakern, FMC) in 0.25 x TBE buffer was carried out using a Rotopher apparatus (Biometra). The following settings were used. 45 V, interval 4000–2000 s log, field angle 105–95°, at 11°C for 90 h. The DNA was stained by immersing the gel in 0.25 x TBE containing 1 μg/ml ethidium bromide for 40 min and destained in water for 5 h.

Genetics and molecular biology methods

S. pombe genetic and molecular biology methods have been described in our previous work (20). Meiotic recombination frequencies were measured using mutant alleles of the ade6 gene, namely ade6-704, ade6-469 and a hotspot mutation, ade6-M26. Crosses were carried out using standard methods and wild type recombinants were scored as white colonies on minimal medium containing 7.5 mg/l adenine (ade6 mutant colonies were pink). The chromosome segregation assay was carried out as described by Niwa et al. (22). General molecular biology protocols were as detailed by Sambrook et al. (23)

RESULTS

Isolation of the rad32 mutant

In order to identify new S. pombe genes required for the repair of DNA double strand breaks, a search was initiated for additional gamma radiation-sensitive mutants; rad32-1 is one of two mutants isolated in such a screen. Wild type S. pombe (972 h+) cells were subjected to a UV dose of 150 J/m², a dose sufficient to give 10% cell survival. Approximately 5 x 10⁶ cells were plated onto YES agar and grown at 29°C for 4 days. Colonies were then replica plated and the replicas exposed to gamma radiation at a dose of 1000 Gy. Colonies failing to grow after irradiation were re-tested for sensitivity to gamma radiation, resulting in two independently isolated mutants. These mutants, named rad31-1 and rad32-1, were outcrossed at least three times with the rad2 strain sp.011 Further characterization of rad31-1 will be described elsewhere (Shayeghi et al. manuscript in preparation).

Phenotype of the rad32 mutant strain

To investigate the radiation sensitivity of the rad32-1 mutant, its survival was analysed after exposure to both gamma and UV radiation (Fig. 1a and b). In addition to being sensitive to gamma radiation, the rad32-1 strain (sp.132) was also found to be moderately sensitive to UV irradiation. rad32-1 is more sensitive to gamma irradiation than a typical nucleotide excision repair mutant and less sensitive to UV radiation than the G2 checkpoint mutants and excision repair mutants (20, 24). Its phenotype thus closely resembles those of the S. pombe mutants previously shown to be required for repair of DNA strand breaks e.g. rad21 (10).

The ability of the rad32 deletion mutant (see below) to repair DNA double strand breaks was investigated using pulsed field gel electrophoresis. Cells were exposed to 125 Gy, a dose sufficient to give 85 and 7% survival of wild type and rad32 mutant cells respectively (Fig. 1b). Analysis of genomic DNA extracted from both wild type and rad32 mutant cells immediately following exposure to ionizing radiation indicated the presence of substantial DNA damage which, after 4.5 h, had been almost completely repaired in the wild type cells (Fig. 2). However the rad32d mutant strain showed only very limited repair of the strand breaks after 4.5 h, confirming that this mutant is defective in double strand break repair.

Table 2. Meiotic recombination at ade6

<table>
<thead>
<tr>
<th>Cross</th>
<th>ade6 recombinants/10⁹ viable spores</th>
</tr>
</thead>
<tbody>
<tr>
<td>M26 x M26</td>
<td>6931 (142.02 x 10⁵)</td>
</tr>
<tr>
<td>rad32d x rad32d</td>
<td>560 (7.11 x 10⁴)</td>
</tr>
</tbody>
</table>

The effect of the rad32 deletion mutation on meiotic recombination frequency was investigated using mutant alleles of the ade6 gene. The alleles used were ade6-607, ade6-469 and ade6-m26, of which the latter displays recombination 'hotspot' activity by stimulating recombination at the ade6 locus 10-15-fold (25). The effect of the rad32d mutation was analysed on both hotspot and non-hotspot recombination (Table 2). The
Figure 1. Survival curves of rad32 mutant strains following UV or gamma-irradiation (a) and (b) survival of rad* strain sp011 (wt), the rad32-1 strain sp132 (rad32), the rad32 deletion strain sp276 (rad32d) and the rad32-1 strain sp132 containing the complementing plasmid pMT2a (rad32 + pMT2a) after UV and gamma irradiation respectively (c) and (d) survival of double mutants of the rad32 deletion in combination with other rad mutations. The rad* strain sp011 (wt), the rad32 deletion (rad32d), the rad13 deletion strain (rad13d), the rad32/rad13 double deletion (rad32d rad13d), the rhp51 deletion strain (rhp51d), the rad32/rhp51 double deletion (rad32d rhp51d)

results indicate that in both cases the rad32 deletion mutation reduces meiotic recombination by at least 15-fold in comparison to wild type.

Table 3. Fidelity of chromosome transmission

<table>
<thead>
<tr>
<th>rad locus</th>
<th>% Loss/generation</th>
<th>Fold increase over wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.005</td>
<td>1</td>
</tr>
<tr>
<td>rad32d</td>
<td>1.52</td>
<td>304</td>
</tr>
<tr>
<td>rad2d</td>
<td>0.63</td>
<td>126</td>
</tr>
<tr>
<td>rad13d</td>
<td>0.1</td>
<td>20</td>
</tr>
<tr>
<td>rad21d</td>
<td>0.01</td>
<td>2</td>
</tr>
</tbody>
</table>

*Data from Murray et al (6)

Chromosome segregation was also measured in the rad32 deletion strain using the minichromosome assay of Niwa et al (22). Table 3 shows that of all the *S. pombe* rad mutants tested to date, rad32 displays the greatest loss of the minichromosome.

Isolation of the rad32 gene

An *S. pombe* genomic library (18) was used to transform the rad32-1 mutant strain sp130 to uracil prototrophy. 30,000 ura* colonies were pooled and subjected to three rounds of UV irradiation at a dose of 100 J/m² for the first round followed by 300 J/m² for the second and third rounds as previously described (24). Individual colonies were tested for co-instability of the rad* and ura* phenotypes. Two plasmids (pMT2a and pMT2d) were isolated from two independently isolated radiation-resistant colonies. These were retransformed into sp.130 (the rad32-1 mutant strain) and found to complement both the UV and gamma radiation sensitive phenotypes of the rad32-1 mutant (Fig. 1a and
Figure 2. Repair of double strand breaks in wild type and rad32 deletion strains. Genomic DNA of wild type cells (sp.011, lanes 1–3) and rad32 deletion cells (sp.276, lanes 4–6) were subjected to pulsed field gel electrophoresis. Lanes 1 and 4, samples from unirradiated cells; lanes 2 and 5, from cells immediately after exposure to 125 Gy; lanes 3 and 6, from cells 4.5 h after irradiation with 125 Gy. Note that DNA from unirradiated rad32d cells shows a low level of DNA strand breaks as witnessed by the slightly reduced size of the chromosomes.

b). The plasmids also complement the slow growth and elongated cell phenotypes of the mutant (data not shown). Restriction mapping and Southern blot analysis (data not shown) indicated that the two plasmids contain overlapping fragments (Fig. 3a).

Mapping of the chromosomal locus of rad32

The 2.2 kb HindIII fragment of the rad32 gene was hybridised to the gridded S.pombe cosmid array of Lehrac and colleagues (26), and its position on the physical map was determined as being on chromosome 1 between rad29 and cdc25 on cosmid 13c5c.

DNA sequence analysis

The sequence of the 3.7 kb fragment of plasmid pMT2a that complemented the rad32 defect was determined in both directions using a series of overlapping clones created using exonuclease III deletion in M13 mp19 and mp18 (27). Sequence analysis revealed an open reading frame of 2154 nucleotides which is interrupted by four small introns of 48, 70, 43 and 46 bp (Fig. 3b). The four introns were confirmed by sequence analysis of a PCR product generated from an S.pombe cDNA library. The predicted rad32 gene product comprises 648 amino acids with a Mr of 73582 Da and isoelectric point of 5.52.

Computer searches revealed that the predicted Rad32 protein has significant homology to the S.cerevisiae meiotic recombination protein, Mre11 (14, GenBank database) (Fig. 4). The sequence similarity between the two proteins is 44% over the first 528 amino acids, with the most highly conserved sequences within the N-terminal 410 amino acids. A search for motifs failed to identify any characteristic functional or structural domains.

Gene deletion

In order to determine whether rad32 is essential for cell viability, the gene was disrupted by replacing the 2.2 kb HindIII fragment by a 1.7 kb fragment containing the ura4 gene (28) (Fig. 3c). A 4.7 kb linear DNA fragment containing the disrupted gene was released from the vector and used to transform a diploid strain (sp.101) to uracil prototrophy. An h90/h+ derivative was then isolated and induced to sporulate. The spores were then subjected to tetrad analysis. Ten tetrads were dissected and the individual spores allowed to form colonies. The ura+ phenotype was always associated with radiation sensitivity, showing that the rad32 gene is not essential for mitotic growth. The radiation sensitivity of the null allele was investigated and is shown in Figure 1a and b. The phenotype is similar to that of the original rad32-l mutant with a slight increase in sensitivity to both UV and gamma irradiation.

Figure 3. (a) Restriction map of the rad32 region. (b) Position of the rad32 open reading frame. The closed boxes represent exons, the open boxes denote position of introns, the N-terminus is on the left hand side of the diagram. (c) Construction of the rad32 deletion (rad32d). The 2.2 kb HindIII fragment was replaced by the ura4 gene, deleting the first three exons and the majority of the fourth exon of the rad32 gene.
indicates that the rad32 gene had been inactivated. Exponentially growing cultures of rad32Δ cells contain high proportions (~75%) of non-viable cells.

During the construction of the null allele, it was noted that the appearance of the rad32 deletion mutation (rad32Δ)-containing spores was infrequent. The viability of rad32Δ spores was low compared to that of wild type spores. The results of the crosses are shown in Table 4. Compared to the products of a cross between two wild type spores where the spore viability is 93%, the viability of spores resulting from a cross between two rad32 deletion strains is dramatically reduced (0.5%). Intermediate values are observed if rad32Δ is crossed with either wild type cells (sp.011) or the rhp51Δ strain (sp.150). These results indicate that Rad32 is likely to play an important role in either meiosis or spore germination or both.

Table 4. Spore viability

<table>
<thead>
<tr>
<th>Cross</th>
<th>Spore viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt × wt</td>
<td>93</td>
</tr>
<tr>
<td>rad32Δ × rad32Δ</td>
<td>0.5</td>
</tr>
<tr>
<td>rad32Δ × wt</td>
<td>58</td>
</tr>
<tr>
<td>rad32Δ × rhp51Δ</td>
<td>52</td>
</tr>
</tbody>
</table>

Epistasis analysis

To determine whether Rad32 functions in a pathway with any other Rad proteins, double mutants were constructed and analysed for their sensitivity to UV or gamma irradiation (Fig. 1c and d). The rad32 deletion mutation in conjunction with the rad13 deletion mutation (defective in excision repair, 5) shows increased sensitivity to radiation when compared to any of the single mutants, suggesting that Rad32 does not function in excision repair. Analysis of a rad32Δ/rhp51Δ double mutant shows no increase in radiation sensitivity over that of the rhp51Δ deletion strain, suggesting that Rad32 functions in a pathway requiring Rhp51. Despite extensive random spore and tetrad analyses no double mutants were obtained between the rad32 deletion mutant and either the rad2 mutant (defective in chromosome segregation, 6) or the rad3 mutant (defective in a G2 checkpoint, 29), suggesting that these combinations may be lethal.

Northern analysis

Northern blot analysis (Fig. 5) detects two transcripts of 2.1 and 2.6 kb, and indicates that the gene is expressed at moderate levels. The 2.1 kb transcript is consistent with the size expected from the Rad32 open reading frame of 648 amino acids. To ensure that both transcripts are encoded by the rad32 gene and to confirm that the rad32 gene had been disrupted, Northern analysis was carried out on RNA from the rad32 deletion strain. This indicated the loss of both transcripts in the rad32 null allele (Fig. 5, lane 2). The existence of two transcripts may be due to the presence of unspliced RNA (the size would be consistent with this possibility) or could be due to the requirement for Rad32 in meiosis, as large 5′ untranslated regions have been identified in several mRNAs required for sexual development in fission yeast (e.g. 30,31).

DISCUSSION

Two new gamma radiation sensitive S.pombe mutants, rad31-l and rad32-1, have been isolated. Using pulsed field gel electrophoresis and measurement of recombination frequencies we have shown that the rad31 mutant is defective in the repair of double strand breaks and in meiotic recombination. Detailed analysis of rad31 will be presented elsewhere (Shayeghi et al., manuscript in preparation). The level of the reduction in meiotic recombination in rad32Δ and the sensitivity to DNA damaging agents suggest that Rad32 is a Class III-type recombination mutant as defined by

Figure 4. Comparison of Rad32 protein with S.cerevisiae Mre11. Conserved amino acids are indicated between the Rad32 and Mre11 sequences, conservative substitutions are indicated . , gaps (−) have been introduced to maximise the alignment.

Figure 5. Northern analysis of S.pombe total RNA hybridised with a rad32 specific probe (1.7 kb of the rad32 cDNA derived by PCR). Lane 1, RNA from a radΔ strain (sp.011); lane 2, RNA from the rad32 deletion strain (sp.276). Two transcripts are observed in the wild type strain, which are both absent from the deletion strain.
DeVaux and co-workers (12) The low spore viability resulting from a S. cerevisiae x S. cerevisiae cross is consistent with the observation that the Rad3 protein is involved in a step subsequent to double strand break formation. Further experiments are currently underway to investigate the role of the Rad3 protein and whether it interacts with any of the other known recombination proteins in S. cerevisiae such as Rad22 (the homologue of Rad52, 11), Rhp51 or Rad21.

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