A novel SMC protein complex in *Schizosaccharomyces pombe* contains the Rad18 DNA repair protein

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In *Schizosaccharomyces pombe*, *rad18* is an essential gene involved in the repair of DNA damage produced by ionizing radiation and in tolerance of UV-induced DNA damage. The *Rad18* protein is a member of the SMC (structural maintenance of chromosomes) superfamily, and we show that, like the other SMC proteins in condensin and cohesin, *Rad18* is a component of a high-molecular-weight complex. This complex contains at least six other proteins, the largest of which is *Spr18*, a novel SMC family member closely related to *Rad18*, and likely to be its heterodimeric partner. SMC proteins have ATP-binding domains at the N- and C-termini, and two extended coiled-coil domains separated by a hinge in the middle. We show that the N-terminal ATP-binding domain of *Rad18* is essential for all functions, and overexpression of an N-terminal mutant has a dominant-negative effect. We have identified an important mutation (S1045A) near the C-terminus of *Rad18* that separates its repair and essential roles. Potential models for the role of the *Rad18–Spr18* complex during DNA repair are discussed.

*Keywords:* ATPase/coiled coils/DNA repair/fission yeast/SMC proteins

**Introduction**

Many genes involved in the response to DNA damage have been identified in the fission yeast *Schizosaccharomyces pombe* (see Lehmann, 1996). Epistasis analysis has enabled these genes to be assigned to different DNA repair pathways. Following treatment with ionizing radiation, repair of DNA double-strand breaks requires genes known to be involved in homologous recombination, suggesting that this mechanism is likely to be the major means of repairing such DNA damage in fission yeast. Photoproducts induced in DNA by UV-irradiation can be repaired both by the classical nucleotide excision repair (NER) system, which is conserved in all eukaryotes, and by a second excision repair process, which is specific to *S.pombe* and a few other organisms (Yonematsu et al., 1997; Yasui and McCready, 1998). In addition to these pathways that repair DNA damage directly, cells are able to tolerate UV damage during DNA replication (Murray et al., 1997) and to utilize cell cycle checkpoint mechanisms to ameliorate the deleterious effects of DNA damage by arresting the cell cycle following damaging treatments (for review, see Caspari and Carr, 1999).

In previous work we used the rad18-X mutant to show that the Rad18 protein was involved in repair of ionizing radiation damage (Lehmann et al., 1995). Double-strand breaks are the principal lesions responsible for killing cells by ionizing radiation. Both Verkade et al. (1999) and we (unpublished observations) have shown that *rad18*-X cells are deficient in repair of radiation-induced double-strand breaks.

Epistasis analysis in response to UV-irradiation suggests that Rad18 is not involved in NER, but that it does play a role in the second excision repair pathway for UV damage. The first step in this pathway is mediated by UV damage-endonuclease (UVDE) (Yonematsu et al., 1997), which nicks UV-irradiated DNA on the 5′ side of UV photoproducts (Avery et al., 1999; Yoon et al., 1999). In a *uvde* background, the *rad18*-X mutation still sensitized the cells to UV-irradiation (Yonematsu et al., 1997), and further epistasis analysis suggested that this was due to a role for *Rad18* in a DNA damage tolerance pathway (Murray et al., 1997). Furthermore, deletion of the *rad18* gene demonstrated that it was essential for cell proliferation, and our data led us to propose tentatively that this essential role was an involvement in DNA replication (Lehmann et al., 1995). Figure 1 summarizes the pathways in which *Rad18* is involved, based on this genetic analysis.

Sequence analysis of *Rad18* (Lehmann et al., 1995) showed that the 131 kDa *Rad18* protein was a member of the SMC (structural maintenance of chromosomes) superfamily. SMC proteins have globular N- and C-terminal domains, which are involved in ATP and Mg<sup>2+</sup> binding, and two extended α-helical coiled-coil domains involved in protein–protein interactions, separated by a hinge region (for review, see Hirano, 1998, 1999; Jessberger et al., 1998). Recent work using *Saccharomyces cerevisiae*, *S.pombe* and *Xenopus laevis* has identified two protein complexes containing heterodimeric pairs of SMC proteins. Smc2 and Smc4 (and their orthologues) form the core of condensin, a five-subunit complex involved in chromosome condensation (Hirano et al., 1997; Sutani et al., 1999). Smc1 and Smc3 (and their orthologues) are the core of a five-subunit cohesin complex, which is required for sister chromatid cohesion (Michaelis et al., 1997; Losada et al., 1998). There is also evidence from bovine cells to suggest that SMC1 and SMC3 proteins form part of a complex with DNA ligase III and DNA polymerase ε, which has DNA strand exchange activity (Jessberger et al., 1996).

We describe in this paper the purification of a high-molecular-weight complex with DNA-dependent ATPase activity, which contains *Rad18* and at least six other polypeptides. The largest of these is a novel SMC protein (*Spr18*), which is likely to be the heterodimeric partner...
of Rad18. We show by site-directed mutagenesis that the N-terminal ATP-binding domain of Rad18 is required for all its functions, and that a C-terminal mutation can separate repair from essential functions.

## Results

**Rad18 is part of a high-molecular-weight complex**

To determine whether Rad18, like other SMC proteins, is a member of a protein complex, we fractionated extracts of *S. pombe* in gel filtration columns. For these experiments we constructed cell strain myc-Rad18, in which the rad18 gene was myc-tagged in the genome at the 5′ end, remaining under the control of its own promoter. When extracts of myc-Rad18 cells were passed down a Superdex 200 column, all the Rad18 protein was found in a high-molecular-weight peak (*M*<sub>r</sub> > 700 kDa). To obtain a more accurate estimate of the size of this complex, the peak fractions from the column were pooled and analysed on an Superose 6 column (Figure 2A). The results show that Rad18 is part of a complex whose apparent *M*<sub>r</sub> is ~1.6 MDa. This complex was stable in the presence of up to 0.7 M NaCl (not shown). Using a polyclonal antibody raised against a Rad18 peptide, we also showed that the gel filtration profile was identical whether or not the Rad18 protein was N-terminally myc-tagged (Figure 2A and B). No change in the gel filtration profile was detected when extracts of γ-irradiated cells were used (data not shown).

As discussed below, the mutation in rad18-X is located in the hinge region, immediately adjacent to the second coiled-coil region (see Figure 5A). This raised the possibility that the mutation might disrupt the coiled-coil structure of the protein and interfere with complex formation. The Rad18 profiles on gel filtration columns using normal and rad18-X extracts were, however, indistinguishable (compare Figure 2C and A), even at salt concentrations up to 0.7 M NaCl. The rad18-X mutation does not, therefore, interfere with complex formation.

### Purification of Rad18

We have developed a purification procedure for the Rad18-containing complex, using extracts from myc-Rad18 cells (Figure 3A). After ammonium sulfate precipitation, the extracts were subjected to gel filtration on Superdex 200. The high-molecular-weight fractions containing Rad18 were pooled and fractionated on a heparin–Sepharose column. The 0.6 M KCl fractions, which contained the Rad18 protein, were incubated with protein G beads covalently cross-linked to anti-myc antibody. After extensive washing, bound proteins were eluted with a peptide corresponding to the c-myc epitope, and analysed both by immunoblotting and colloidal Coomassie staining. Immunoblotting showed that the bound and eluted proteins contained myc-tagged Rad18 (Figure 3B, lane 2). When extracts from untagged control cells (strain 501) were subjected to identical purification procedures, no corresponding protein was bound to the beads (Figure 3B, lane 4). Coomassie staining revealed that, apart from Rad18, there were six specific bands in the eluate from the tagged cells (Figure 3C, lane 3) that were not found in similar experiments using untagged control cells (Figure 3C, lanes 1 and 2). One of these specific bands, p124, had a molecular weight close to that of Rad18 and was of similar intensity.

### ATPase activity

Mutagenesis studies described in detail below implicate ATP-binding activity of Rad18 in all its functions, suggesting that the protein might have ATPase activity. We have therefore measured ATPase activity of the Rad18 complex. We assayed the peptide eluate from the immunosplitinity beads for ATPase activities in the presence and

Fig. 1. Involvement of Rad18 in different cellular responses to DNA damage.
Absence of DNA. Eluate from the myc-Rad18 cell extracts had significant ATPase activity, which was stimulated by single-stranded, and to a greater extent by double-stranded DNA (Figure 3D). In contrast, eluate from untagged control strain 501 containing an equal amount of the non-specific 73 kDa protein (see Figure 3C) had minimal activity, which was unchanged by the addition of DNA. The complex is therefore associated with specific DNA-stimulated ATPase activity.

**Spr18, a heterodimeric partner of Rad18**

In the SMC family, Smc2 and Smc4 form a heterodimeric component of the condensin complex (Hirano *et al.*, 1997), which is involved in chromosome condensation. Smc1 and Smc3 are similarly part of cohesin, involved in sister chromatid cohesion (Michaelis *et al.*, 1997). We therefore anticipated that Rad18 might have a heterodimeric partner, and we considered the possibility that the p124 polypeptide in the complex might be a novel SMC protein partner of Rad18. We searched the *S. pombe* sequence database and found a candidate open reading frame (ORF) in cosmid C14C4 from chromosome 1 (DDBJ/EMBL/GenBank accession No. Z98596) with an orthologue in the *S. cerevisiae* database (ORF SCYOL034W; DDBJ/EMBL/GenBank accession No. Z74776). The predicted 123 kDa protein was related to the SMC family, but was most closely related to Rad18 (see Figure 4A). We designated it Spr18 (SMC partner of rad18). Alignments of Rad18 with Spr18 and its *S. cerevisiae* orthologue are shown in Figure 4B. There is 28% identity between the *S. pombe* and *S. cerevisiae* orthologues, and 20% identity between Spr18, a heterodimeric partner of Rad18. Like rad18, spr18 has a small intron (49 bases) close to the 5' end of the gene (between nt 187 and 188).

In order to determine whether Spr18 interacts with Rad18 in vivo, we carried out immunoprecipitations using the myc-Rad18 strain (Figure 4C). Extracts were immunoprecipitated with antibody directed against a peptide from Spr18 (Figure 4C, lanes 2, 4, 6 and 8) or, as control, with anti-myc antibody (lanes 1, 3, 5 and 7). The immuno-
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### A

- Sp Cut3
- Sc Smc4
- Sc Smc1
- Sp Smc1
- Sp Cut4
- Sc Smc2
- Sp Smc3
- Sp Smc2
- Sp Smc3
- Sp Spr3
- Sp Spr18
- Sc Spr18
- Sc Spr18
- Sc Rho18

### B

<table>
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<td>spr10</td>
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<tr>
<td>acsSpr</td>
<td></td>
</tr>
<tr>
<td>rad10</td>
<td></td>
</tr>
</tbody>
</table>

### C

**IP with Rad18**

- 0.25 M
- 0.5 M
- 0.75 M
- 0.25 M NaCl

### D

- Image 1
- Image 2
- Image 3
- Image 4
- Image 5

### E

- Graph showing % Cells with Normal, Golgi phenotype, Multiple Septa, Enlarged, Other abnormalities.
precipitates were electrophoresed in an SDS–polyacryl-
amide gel and immunoblotted with the anti-myc antibody. Figure 4C shows that myc-tagged Rad18 co-immunopreci-
cipitated with Spr18 in these experiments in the presence of
salt concentrations up to 0.75 M (lanes 1–6). Similar
results were obtained when the blots were probed with
anti-Spr18 (data not shown). The results were not affected
by treatment of the cell extracts with DNase I prior to
immunoprecipitation, demonstrating that the interaction
was not mediated by binding to DNA. When the immuno-
precipitation experiments were carried out with control
untagged strain 501, no bands were detected on the
immunoblot (Figure 4C, lanes 7 and 8). These results
demonstrate that Rad18 and Spr18 do indeed interact,
consistent with the idea that the two proteins form a
heterodimer in S.pombe cells, although we have not
formally eliminated the possibility that the interaction
might be mediated by another protein. In results to be
presented elsewhere, we have shown a similar interaction
between the human orthologues of these proteins
(E.M.Taylor, J.Moghraby and A.R.Lehmann, unpublished
observations).

**Spr18 is the p124**
The peptid elution fractions from the immuno-affinity
column obtained using both the myc-Rad18 and 501
extracts (Figure 3) were analysed by immunoblotting with
the anti-Spr antibody. Spr18, like Rad18, was detected in
the bound and eluted proteins from the myc-Rad18 extract
(Figure 3B, lane 6), but not in the untagged control
extracts (lane 8). The band detected using the anti-Spr18
antibody corresponded with that of the p124 protein in the
Coomassie-stained gel. These results confirm that p124 is the
SMC protein Spr18 and are consistent with the
suggestion that Rad18–Spr18 forms a heterodimeric core
of a large complex similar to the other SMC complexes,
cohesin and condensin. To investigate whether the Rad18–
Spr18 complex contains other known DNA damage response genes, we carried out immunoblotting of the
proteins eluted from the anti-myc affinity column using a
series of antibodies. We were unable to detect the presence
of the DNA damage response proteins Rad9, Rad26,
Cds1, Rqh1 or Rad21 in the Rad18–Spr18 complex (data
not shown).

**Spr18 is an essential gene**
If Spr18 is the partner protein of Rad18, we anticipated
that, like Rad18, it would be an essential gene. We deleted
the entire ORF of the spr18 gene from the genome in a
diploid strain by replacing one copy of the gene with the
ura4+ marker. Following sporulation and plating in the
absence of uracil, no haploid colonies were obtained,
suggesting that the spr18 gene was essential for cell
proliferation. In order to confirm this, tetrads were dis-
sected from the spores and plated on yeast extract plates.

Viability and the *Schizosaccharomyces pombe* Rad18 protein

Viability and the *Ur4+* phenotype segregated in a 2:0
manner, confirming that *spr18*, like *rad18* and the SMC
genes, is essential for cell proliferation. In order to
determine the terminal phenotype of the *spr18* deletion
strain, the spores were germinated in liquid medium with
selection for *Ur4+*, and cell number and morphology
were determined. Germinating spores underwent one or
two divisions and ceased growth with a variety of abnormal
morphologies. Several examples are shown in Figure 4D.
Many cells were elongated, in some cases excessively so
(Figure 4D, examples 1 and 5), often with several septa
(examples 3 and 5), and the nuclear material was barely
detectable, either being present in a single nucleus
(example 4) or spread amorphously through part of the
cell (example 1). A proportion of cells (10–15%) showed
a cut phenotype (Figure 4D, example 2). The distribution
of these different phenotypes is shown in Figure 4E. The
similarity of the terminal phenotype of the *spr18* deletion
mutant, *rad18* deletion (Lehmann et al., 1995) and wild-
type cells overexpressing dominant-negative mutants of
*rad18* (see below), provides further evidence that Rad18
and Spr18 perform similar functions in the cell.

**Mutagenesis of *rad18* defines important structural
domains**
The characteristic SMC structure of Rad18, with globular
head and tail regions and two coiled-coil domains separated
by a hinge, is depicted schematically in Figure 5A. To
gain insight into the roles of the different domains, we
have used site-directed mutagenesis to introduce a series
of mutations into *rad18* cDNA. The cDNA was cloned
under the control of the attenuated thiamine-repressible
**mm1** promoter in the vectors pRep41MH N (Leu+CDS1) o r
pRep42MH N (Ura+) (Maundrell, 1993; Craven et al.,
1998), so that the Rad18 protein was N-terminally myc-
tagged. The effects of the mutations were examined in
different ways, as follows: (i) the ability of the mutant
plasmids to rescue the lethal effect of a *rad18* deletion
was examined by transformation into the *rad18:ura4+/rad18* diploid strain; (ii) the mutated plasmids were
overexpressed in wild-type cells to look for dominant-
negative effects of the mutant gene on cell growth and
viability, as well as on sensitivity to UV- and γ-irradiation;
and (iii) the mutated genes were introduced into *rad18-X*
cells and their impact on the sensitivity of the mutant to
ionizing and UV-irradiation was examined following their
transient overexpression.

The mutations and their effects are summarized in
Figure 5 and Table I. Several of the mutant constructs
had a dominant-negative effect on cell viability
(Figure 5A). The proportion of the population that lost
viability depended on the mutation, but the phenotype of
the dying cells was quite similar in all cases, and resembled
the terminal phenotype of deletion mutants of *rad18*
(Lehmann et al., 1995) or *spr18* (see above). For simplicity

![Fig. 4. Spr18, the SMC partner protein of Rad18. (A) Phylogenetic tree of all the SMC family members in *S.cerevisiae* and *S.pombe*. (B) Sequence alignments of Rad18 with Spr18 and its *S.cerevisiae* orthologue (designated *scspr*). Identical amino acids are highlighted in black, and conserved residues are in grey. (C) Extracts of myc-Rad18 cells were immunoprecipitated with either anti-myc or anti-Spr18 antibody in the presence of the
salt concentrations indicated. The immunoprecipitates were immunoblotted and probed with anti-myc antibody. The right hand lanes (7 and 8) show
results with untagged strain 501. (D) Terminal phenotypes of the *spr18* deletion mutant. Diploid *spr18/*spr18: *ura4* cells were sporulated and
grown in the absence of uracil for 36 h. Cells were fixed and stained with DAPI and calcofluor. (E) Frequency of different phenotypes after 24 h
growth.](1695)
we will refer to this as the Rad18 phenotype, although there is no intended implication that this phenotype is unique to rad18 mutants. This phenotype consisted of cells with a variety of abnormal morphologies in which nuclear segregation was clearly aberrant. Many of them were small, a few septated without prior mitosis, resulting in very long cells either without a nucleus or with only one nucleus often with multiple septa. Examples are shown in Figure 6A (compare with overexpressed wild-type rad18 in Figure 6B).
The N-terminal domain of Rad18 contains the highly conserved Walker Type A box found in many ATPases. We mutated the conserved Lys129 residue in the GXGKS motif to glutamic acid (rad18K129E). When rad18K129E was overexpressed in wild-type cells, it had a dramatic dominant-negative effect on viability, completely abolishing the colony-forming ability of the cells. A similar result has recently been obtained for a different mutation in the ATP-binding site by Verkade et al. (1999). When the rad18K129E was overexpressed in cells growing in liquid culture, cell growth was prevented (Figure 6C) and the cells died with the Rad18 phenotype.

We also generated a milder mutation of Lys129 to glutamine (K129Q). When plasmids containing this construct were overexpressed in wild-type cells, the resulting colonies were very small, and in liquid culture, a significant proportion of the cells (65%) showed the Rad18 phenotype and had reduced viability (Figure 6A). The viable cells were sensitized to both UV- and ionizing radiation (Figure 5B). The mutant plasmid failed to rescue the table:

**Table I. Summary of effects of overexpressing mutant constructs**

<table>
<thead>
<tr>
<th></th>
<th>K129E</th>
<th>K129Q</th>
<th>R706C</th>
<th>R706A</th>
<th>L843P</th>
<th>L843F</th>
<th>S1045A</th>
<th>S1045T</th>
<th>D1072A</th>
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<tbody>
<tr>
<td>WT viabilitya</td>
<td>0</td>
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<td>N</td>
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</table>

aNumbers denote percentage of viable cells. +, >70% viability.
bN, normal; S, more sensitive than normal.
cR, restores wild-type resistance; SS, sensitivity comparable to rad18-X; SSS, sensitivity greater than rad18-X.
dYes, does; no, does not rescue lethality of deletion strain.

The N-terminal domain of Rad18 contains the highly conserved Walker Type A box found in many ATPases.
viability of the deletion mutant and slightly increased the
sensitivity of the rad18-X cells to γ-irradiation (Figure 5A
and C).

The C-terminal globular region contains motifs LSGG
and DE, conserved in all SMC proteins, the latter probably
representing part of the Walker Type B motif involved in
ATP–Mg²⁺ binding. We mutated Ser1045 from the LSGG
sequence to either alanine or threonine (rad18S1045A or
rad18S1045T), and Asp1072 to alanine (rad18D1072A).
Overexpression of these mutated genes had no significant
effect on the viability of wild-type cells. However, the
rad18S1045A mutant plasmid sensitized the cells to both
γ- and UV-irradiation damage, whereas rad18S1045T
had less effect and rad18D1072A had minimal effect
(Figure 5B). The S1045A and S1045T plasmids slightly increased the γ-irradiation sensitivity of the
rad18-X mutant (Figure 5C). Importantly, however, the
rad18S1045A mutant plasmid, but neither of the others,
was able to rescue the lethality of the rad18 deletion
strain (Figure 5A). This significant result shows that
the S1045A mutation is able to separate the repair from
the essential phenotype of the cells. It is possible that the
rad18 mutant plasmid rescued the rad18 deletion because
the mutant gene was overexpressed. However, in results
to be presented in detail elsewhere, we have eliminated
this possibility. We have integrated the S1045A mutation
into the genomic rad18 gene, in which it is expressed at
normal levels. This strain is viable and sensitive to
radiation.

Coiled-coil motifs are amphipathic α-helices character-
ized by heptad repeats with hydrophobic amino acids at
the first and fourth residues of each heptad (Lupas et al.,
1991). We mutated Leu843, in the middle of a long heptad
repeat, either to proline (L843P), which would be expected
to destroy the coiled-coil structure, or to phenylalanine
(L843F), which is hydrophobic like leucine, but has a
completely different structure. The latter mutation behaved
like wild type in all respects (Figure 5B): it restored
viability to the rad18 deletion strain and rescued the
sensitivity to DNA damage of the rad18-X cells (Figure 5C).
In contrast, L843P had a dramatic effect. Only a few
wild-type cells overexpressing the plasmid were able to form colonies, which were very small, and
in liquid culture, most of the cells (80%) had the Rad18
phenotype. Viability of the deletion strain was not restored
with this mutant plasmid.

The mutation in rad18-X, which is sensitive to UV- and
γ-irradiation and elevated temperatures, results in the
change R706C. We considered the possibility that this
change might exert its effects because the mutant protein
contains a cysteine residue, which might form aberrant
disulfide bridges. We therefore mutated Arg706 to alanine
(R706A) and compared its effects with those of R706C.
Overexpression of R706C had no effect on either wild-
type or rad18-X cells and restored the viability of the
deletion strain. The effects of R706A were, however, more
severe, the phenotype being quite similar to that of
rad18K129Q (see Figure 5B and C and Table I). These
findings ruled out the possibility that the phenotype of
rad18-X resulted from aberrant disulfide bridges, and
rather implicated R706 as an important residue for
Rad18 function.

In summary, we have found a gradation in severity of
mutations in rad18. Mutations in the ATP-binding site in
the N-terminus and disruption of the coiled-coil domain
had the most dramatic effects, suggesting that the ATP-
binding site and coiled-coil structures are essential for all
functions. Effects of mutations in the conserved C-terminal
domain were less severe. The S1045A mutation in the
conserved LSGG motif enabled us to separate repair from
essential functions of the protein, since it only affected
the former.

**Overexpression of damage response genes resuces
the dominant-negative phenotype**

We have looked for genetic interactions between rad18
and other genes by examining their ability to suppress the
dominant-negative effects of the K129E mutation in the
ATP-binding site. If the normal and mutant cDNAs were
co-overexpressed from separate plasmids with the same
mnt1 promoter (in pRep41 and 42), the normal plasmid
was able to rescue the growth-inhibitory effects of the
mutant plasmid (Figure 6C). We used this co-expression
system to investigate whether several other genes (rad1,
rad2, rad3, rad17, pcn1 and cdc6) could also rescue the
adverse effects of the mutant plasmid. We found that
when either the cell cycle checkpoint genes rad1 or rad3,
the rad2 gene encoding a structure-specific nuclease or the
cdc6 gene encoding DNA polymerase δ was overexpressed
together with rad18K129E, the growth inhibition was
overcome, whereas pcn1 encoding PCNA and the check-
point gene rad17 had no effect. In order to ensure that
this was not merely the result of the second plasmid
reducing the expression of the mutant gene, we used
Western blotting to determine the levels of expression
after 14 h in the absence of thiamine. Since the genes
were also myc-tagged (in most cases), we were able to
detect both Rad18 and the co-expressing proteins using
an antibody directed against the myc epitope tag. The
expression of the mutant rad18K129E was not affected
by co-expression from the second plasmid (Figure 6D).
Our data therefore provide evidence for a genetic inter-
action between rad18 and rad1, rad2, rad3 and cdc6.
These interactions will be studied in detail in future work.

**Discussion**

**Rad18–Spr18 and other SMC protein complexes**

Although our understanding of many DNA repair pro-
cesses has increased dramatically over the last few years,
the functions of many DNA repair proteins are still poorly
understood. As shown by the scheme in Figure 1, Rad18
plays an important role in several repair processes, but its
exact function is unknown. The other SMC protein family
members are essential for chromosome condensation
(SMC2 and 4) and cohesion (SMC1 and 3) (see Hirano,
1998; Jessberger et al., 1998), although the role that these
proteins play at the molecular level is poorly understood.
We have shown in this paper that Rad18 resembles other
SMC proteins in that it forms a heterodimeric complex
with a closely related partner protein, Spr18, and Rad18–
Spr18 is part of a higher molecular weight complex. This
complex has a molecular weight >1 MDa, is stable in
high salt, contains at least five other proteins and has
DNA-stimulated ATPase activity. Similarly, the condensins
from Xenopus and S.pombe contain multiple subunits.
including, in Xenopus, XCAP-C and XCAP-E (Hirano et al., 1997) and in S. pombe, Cut3 and Cut14 (Tanaka et al., 1999), the orthologues of SMC2 and SMC4. Likewise the cohesins, which include SMC1 and SMC3 (Losada et al., 1998), and the dosage compensation complex from Caenorhabditis elegans, which contains the SMC protein Dpy27 (Chuang et al., 1996), also contain multiple subunits. A complex designated RC-1, which was able to carry out strand exchange reactions, is composed of bovine SMC1 and SMC3 together with DNA polymerase ε and DNA ligase III (Jesberger et al., 1996). Both Rad18 and Spr18 have orthologues in the S. cerevisiae and C. elegans databases and we have isolated human orthologues of both (E.M.Taylor, J.Moghraby and A.R.Lehmann, unpublished results). In view of the similarity in structural composition between the Rad18–Spr18 complex, condensin and cohesin, we consider Rad18–Spr18 to be the third conserved SMC complex in eukaryotes.

Structure–function relationships in Rad18

Structural studies on bacterial SMC-like proteins have been carried out recently by Melby and coworkers (Melby et al., 1998). MukB is the only SMC-like protein in Escherichia coli and has an antiparallel homodimeric structure, with the globular domains attached by rigid coiled-coil domains to the hinge. The coiled-coil regions form the basis for the dimerization. The hinge appeared quite flexible, permitting a scissoring movement. It is probable that in the Rad18 complex, Rad18 and Spr18 form antiparallel heterodimers.

Site-directed mutagenesis studies (Figures 5 and 6) have shown that the globular N-terminal domain and the coiled-coil regions are required for both repair and essential functions of Rad18. This is consistent with the structure suggested above. If we assume that Rad18 forms a similar structure but as a heterodimer with Spr18, we can provide a ready explanation for the dominant-negative effects of overexpressed mutant Rad18 (Figure 6 and Verkade et al., 1999). In the majority of the mutants in which the coiled-coil domains remain intact, the overexpressed mutant Rad18 protein will dimerize and thereby sequester the cellular Spr18, preventing it from dimerizing with the endogenous protein. The L843P mutant probably causes local disruption of the α-helical structure of the more C-terminal coiled coil, but the more N-terminal coiled-coil domain is likely to be unaffected and still capable of interacting with Spr18.

Interestingly, mutations in the conserved C-terminal globular domain had relatively mild effects. Particularly interesting is the S1045A mutation (Figure 5), which destroyed the repair function of the protein, whilst retaining its essential function. This was quite surprising in view of the fact that the motif LSGG, encompassing Ser1045, is conserved in all SMC proteins (with the exception of Spr18, which contains the sequence QSGG). It is also found in a large family of ATP transporter proteins (Ames et al., 1992), in which it has been proposed that the LSGG motif forms an important linker region between different domains. In the latter protein family the serine residue is essential for the transporter function of the S. cerevisiae Ste6p protein involved in transport of the a-factor pheromone (Browne et al., 1996), and it is mutated in the CFTR protein in several cystic fibrosis patients (Kerem et al., 1990; Gregory et al., 1991). This mutation provides an opportunity for further investigation of the repair functions of Rad18 and its homologues.

Function of the Rad18–Spr18 complex

The SMC protein complexes function in chromosome-associated processes, such as condensation, cohesion and dosage compensation. It is thought that the putative scissoring action, which is possible because of the flexibility of the SMC dimer (Melby et al., 1998), enables the complexes to move DNA molecules relative to each other or to other cellular structures. The condensin complex from Xenopus is able to supercoil DNA in the presence of topoisomerase I (Kimura and Hirano, 1997) and to effect topological reconfigurations of DNA molecules (Kimura et al., 1999). The S. cerevisiae SMC1p and SMC2p proteins are able to bind to DNA, and this DNA-binding activity resides in the C-terminus of the molecule (Akmedov et al., 1998). We find that Rad18 binds tightly to DNA-cellulose columns (our unpublished data), suggesting that Rad18 is likely to be a DNA-binding protein, which would be consistent with the DNA-stimulated ATPase activity that we have found associated with the Rad18–Spr18 complex. We previously suggested that Rad18 was involved in recombinational repair processes (Lehmann et al., 1995). Our current work together with published data on the functions of SMC proteins lead us to speculate that the function of the Rad18–Spr18 complex is to bring together and/or hold broken DNA molecules together in the vicinity of double-strand damage to allow repair by recombination to take place (see Figure 7). Such damage could be either a double-strand break produced directly by ionizing radiation, or a UV photoproduct opposite a gap or stalled replication fork generated during replication of UV damage. We envisage that the Rad18–Spr18 complex either holds the ends of the break together or alternatively holds the broken chromosome in register with its sister or homologue to allow the Rhp51 RecA homologue to initiate strand exchange. In this context the Rad18–Spr18 complex presumably plays a complementary role to the cohesins, which are known to hold sister chromatids together, and are also required for a normal response to radiation, since mutants in S. pombe rad21, a
non-SMC component of cohesin, are sensitive to radiation. Recent data suggest that the cohesin complex is present at specific sites along the chromosome arms rather than coating the lengths of the chromosomes (Blat and Kleckner, 1999; Tanaka et al., 1999). In the context of recombinational repair of double-strand DNA damage, it may be that cohesins are necessary to maintain the overall cohesion of the sister chromatids, whereas the Rad18–Spr18 complex has a more specific role at sites of damage. The suggestion for a role for Rad18 in recombination is supported by the 4-fold reduction in intrachromosomal recombination recently reported in a Rad18 mutant of Arabidopsis thaliana (Mengiste et al., 1999). A more complex role for Rad18 in response to DNA damage has been suggested by the work of Verkade et al. (1999), who provided evidence to suggest that under some circumstances Rad18 was required to maintain a checkpoint following DNA damage, although establishment of the checkpoint was clearly normal. Our finding of a genetic interaction between some of the checkpoint genes and rad18 is consistent with these observations. We obtained no evidence, however, for physical association in the Rad18–Spr18 complex of several checkpoint proteins tested. The relationship of Rad18 to the checkpoint machinery is likely, therefore, to be complex and indirect.

Both Rad18 and Spr18 are essential for cell proliferation. Our results in Figure 4D and Figure 6 show that in the absence of functional Spr18, or when dominant-negative mutant rad18 constructs are overexpressed, cells appear to be unable to replicate their DNA, but nevertheless they continue to grow, lay down septa and develop grossly abnormal morphologies. This is indicative of a role both in DNA replication and in mitotic control. Occasional double-strand breaks occur during DNA replication and these are thought to be repaired by recombination. Cells deficient in the recombination-repair genes rhp51 or rhp54 grow poorly (Muris et al., 1993, 1996), presumably because they have difficulty in dealing with damage arising during replication. Double mutants of rhp51 or rhp54 with the DNA damage checkpoint genes are inviable (Muris et al., 1996), showing that checkpoint-mediated mitotic delay is necessary for the cell to deal with this kind of replicative stress. We propose that the inviability of rad18 and spr18 mutants arises from their involvement in linking replication, repair and mitotic control. Further work will be necessary to unravel the origin of these complex phenotypes.

Materials and methods

Antibodies

The 9E10 anti-myc monoclonal antibody used for immunoblotting was obtained from Santa Cruz. For immuno-affinity chromatography we used supernatants from CRL-1729 hybridoma cells obtained from the American Type Culture Collection, and cultured in RPMI with 10% fetal calf serum. The supernatant was bound to protein G–agarose, column (Pharmacia Biotech). In this case 0.5 ml fractions were collected and analysed in vitro.


epistat in ab binding buffer [40 mM HEPES pH 7.8, 2 mM EDTA, 2 mM EGTA, 60 mM β-glycerophosphate, 1 mM NaF, 0.5 mM phenylmethylsulphonyl fluoride (PMSF), 0.2 mM diithiothreitol (DTT), 0.1 mM orthovanadate, 10% glycerol and protease inhibitor cocktail, as described below] in the presence of different concentrations of NaCl. The immune complexes were captured on protein G–agarose beads, washed several times, boiled with gel loading buffer and electroblotted in 10% SDS–PAGE gels. They were blotted onto PVDF membrane filters and probed with the anti-myc antibody.

Purification of the Rad18 complex

For purification, 30 1 of exponentially growing myc-Rad18 cells were harvested, washed and the cell pellet resuspended in an equal volume of lysis buffer A (40 mM HEPES pH 7.8, 0.4 M KCl, 80 mM β-glycerophosphate, 12 mM NaF, 5 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mM orthovanadate, 10% glycerol, and a protease inhibitor cocktail consisting of 5 μg/ml each of trypsin inhibitor, pepstatin, leupeptin, aprotinin, 10 μg/ml bestatin and E-64, and 50 μg/ml chymotrypsin inhibitor). The cells were snap-frozen in liquid nitrogen and lysed using a blender, following addition of copious quantities of dry ice. The lysate was clarified by centrifugation and subjected to ammonium sulfate precipitation. The 15–45% cut containing the Rad18 protein was dissolved in lysis buffer and dialysed overnight against the same buffer. The extract containing ~300 mg of protein was applied in multiple injections to a Superdex 200, 10/30 FPLC column (Pharmacia Biotech). Fractions of 0.25 ml were collected and analysed by Western blotting using the anti-myc monoclonal antibody. The high-molecular-weight fractions containing the Rad18 protein were pooled and rechromatographed on heparin–Sepharose CL-6B (Pharmacia Biotech) equilibrated in buffer B/0.1 M KCl (25 mM HEPES pH 7.8, 2 mM EGTA, 3 mM MgCl2, 0.1 mM DTT, 0.5 mM PMSF, 12% glycerol). After extensive washing, proteins were eluted with buffer B/0.2, 0.4, 0.6 and 1 M KCl. The 0.6 M KCl eluate that contained the Rad18 protein was incubated overnight at 4°C with protein G–agarose beads (Pharmacia Biotech) crosslinked with anti-myc antibody in buffer B/0.3 M KCl containing 0.4 mM EDTA and protease inhibitor cocktail. The beads were washed once with the same buffer, four times in 10 vol. of buffer B/0.5 M KCl, 0.1% NP-40, and twice in buffer B/0.1 M KCl, 0.01% NP-40. Bound proteins were eluted twice at 30°C for 1 h each with peptide corresponding to the myc epitope (AEEQKLISEEDL) in buffer B containing 0.1 M KCl and 15% glycerol to eliminate the peptide. The proteins were separated on 10% SDS–PAGE and stained with colloidal Coomassie Brilliant Blue (Brilliant Blue G, colloidal concentrate; Sigma). Alternatively they were analysed by immunoblotting with antibodies specific for different DNA damage response proteins.

In experiments to estimate the size of the complex, pooled Superdex 200 fractions that contained the high-molecular-weight peak, derived as above, were further subjected to gel filtration on a Superose 6 FPLC column (Pharmacia Biotech). In this case 0.5 ml fractions were collected and analysed by Western blotting. The column was calibrated with HMW Gel Filtration Calibration Markers (Pharmacia Biotech).

ATPase assay

For assaying ATPase activity, elution from the affinity column with the myc peptide was carried out at 30°C for 20 min only, and the eluate was dialysed against buffer B containing 0.05 M KCl. Protein eluates were then incubated for 2 h at 30°C in the presence of 0.1 μCi [γ-32P]ATP (7000 Ci/mmol; ICN) in a 10 μl reaction volume containing 25 mM

Immunoprecipitation

For these experiments we constructed an S.pombe strain myc-tagged in the genome (myc-Rad18). A fragment of DNA containing 950 bp of rad18 upstream sequence and the coding sequence up to the NcoI site at 1760 was cloned into the integrating vector p5Sta18 (Carr et al., 1989), which contains the sup3.5 tRNA tandem (insert, Hottinger, 1982). The S.myc– M.rad18 complex was inserted as an Ndel fragment from pGEMMHi (Craven et al., 1996) into an Ndel site engineered at the start of the S.rad18 ORF. This plasmid was transformed into S.pombe strain 501 (leu1.32 ade6.D18 ade6.704) and selection was applied for growth in the absence of adenine. (The ade6.704 mutation in the 501 strain is supported by the 4-fold reduction in intrachromosomal recombination following DNA damage, although establishment of the checkpoint was clearly normal. The immune complexes were captured on protein G–agarose beads, washed several times, boiled with gel loading buffer and electroblotted in 10% SDS–PAGE gels. They were blotted onto PVDF membrane filters and probed with the anti-myc antibody.

The 9E10 anti-myc monoclonal antibody used for immunoblotting was generously supplied by Drs T.Caspari, J.Murray and A.M.Carr.
Tris·HCl pH 7.5, 4 mM MgCl₂, 20 μM ATP, 1 mM DTT and 50 μg/ml BSA. The reaction was stopped by adding 5 μl of 0.5 M EDTA pH 8.0. One microlitre of each reaction mixture was then spotted on a PEl-cellulose thin layer chromatography (TLC) plate (Sigma) and developed in 0.75 M K₂HPO₄. Radiolabelled ATP and inorganic phosphate were quantitated on a Storm 840 PhosphorImager (Molecular Dynamics). DNA-dependent ATPase activity was assayed in the presence of double- or single-stranded DNA at a final concentration of 15 ng/μl.

Site-directed mutagenesis

A rad18 cDNA clone was constructed by PCR and subcloning from total S.pombe cDNA. The internal NdeI site was destroyed by site-directed mutagenesis, and after removing PCR errors by further mutagenesis, the cDNA was cloned as an NdeI–SstI fragment into the pREP41 MH and pREP42 MH epitope-tagging expression vectors (Crenven et al., 1998). Mutations in the cDNA were introduced at specific sites using the procedure of Kunkel et al. (1987), and all the mutant clones were sequenced completely to ensure that only the desired mutations had been introduced. We regularly found that subcloning of rad18 constructs was unsuccessful when the ligated DNA was transformed into a variety of commonly used E.coli strains. We therefore transformed the ligation mixes directly into S.pombe and successfully obtained the desired products.

Cell survival and complementation of the essential protein

Mutant constructs were introduced either into wild-type or rad18-X cells in the presence of thiamine to maintain the rad18-phenotype (Russel, 1998). Wild-type cells were plated on minimal medium, and rad18-X cells on YEP. For effects on cell viability, wild-type cells were grown for 19 h and the cells were counted, fixed in methanol and stained with DAPI and calcofluor.

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