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Functional and Physical Interaction between Rad24 and Rfc5 in the Yeast Checkpoint Pathways

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The *RFC5* gene encodes a small subunit of replication factor C (RFC) complex in *Saccharomyces cerevisiae* and has been shown to be required for the checkpoints which respond to replication block and DNA damage. Here we describe the isolation of *RAD24*, known to play a role in the DNA damage checkpoint, as a dosage-dependent suppressor of *rfc5-1*. *RAD24* overexpression suppresses the sensitivity of *rfc5-1* cells to DNA-damaging agents and the defect in DNA damage-induced Rad53 phosphorylation. Rad24, like Rfc5, is required for the regulation of Rad53 phosphorylation in response to DNA damage. The Rad24 protein, which is structurally related to the RFC subunits, interacts physically with RFC subunits Rfc2 and Rfc5 and cosediments with Rfc5. Although the *rad24*Δ mutation alone does not cause a defect in the replication block checkpoint, it does enhance the defect in *rfc5-1* mutants. Furthermore, overexpression of *RAD24* suppresses the *rfc5-1* defect in the replication block checkpoint. Taken together, our results demonstrate a physical and functional interaction between Rad24 and Rfc5 in the checkpoint pathways.

The survival of eucaryotes depends on the accurate transmission of genetic information from one generation to the next. Successful mitotic division requires the events of the cell cycle to be ordered such that the initiation of late cycle events is dependent on the completion of early events. The mechanisms that ensure the proper ordering of cell cycle events have been termed checkpoint controls (7). When DNA replication is delayed and DNA damage occurs, checkpoint controls activate cell cycle arrest enough to complete DNA replication and repair DNA damage (4, 18).

In the budding yeast *Saccharomyces cerevisiae*, checkpoint pathways induce cell cycle arrest in G_1 or G_2/M and retard S-phase progression in response to DNA damage. Other checkpoints prevent cells with incompletely replicated DNA from exiting the S phase. A number of genes that are involved in the DNA damage checkpoint and/or the replication block checkpoint have been identified elsewhere (4, 18). These include *RAD9*, *RAD17*, *RAD24*, *POL2*, *MEC1*/*ESR1*, *RAD53*/*SPK1*/*MEC2*/*SAD1*, *RFC5*, *MEC3*, and *DDC1*. Among these genes, *RAD9*, *RAD17*, *RAD24*, *MEC3*, and *DDC1* are involved not only in the G_2/M-phase but also in the G_1- and S-phase DNA damage checkpoints (11, 12, 17, 22–24, 31–33). *POL2*, encoding a large subunit of DNA polymerase (Pol) ε, is required for the checkpoints responding to replication block and DNA damage in S phase (15, 16). *MEC1* and *RAD53* are necessary for the checkpoints operating in response to both DNA damage and incomplete DNA replication (1, 33). *RAD53* encodes a dual-specificity protein kinase (25), and Mec1 belongs to the phosphatidylinositol kinase family that includes human ataxia-telangiectasia-mutated (ATM) proteins (9, 21). Rad53 is phosphorylated in response to DNA damage and DNA replication block in a *MEC1*-dependent manner (20, 29).

Replication factor C (RFC) is required for DNA replication and repair and consists of one large and four small subunits. In *S. cerevisiae*, the large subunit of RFC is encoded by *RFC1*/*CDC44* and the four small subunits are encoded by *RFC2*, *RFC3*, *RFC4*, and *RFC5* (3). RFC is a structure-specific DNA-binding protein complex that recognizes the primer-template junction. RFC loads proliferating cell nuclear antigen (PCNA) onto the primer terminus and then Pols δ and ε bind to the DNA-RFC-PCNA complex to constitute a processive replication complex (2, 10, 30). A temperature-sensitive mutant of *RFC5* whose lethality can be suppressed by overexpression of the Rad53 kinase has been identified (28). We have demonstrated that *RFC5* is required for the checkpoints operating in response to DNA replication block and DNA damage in S phase (26, 28). Phosphorylation of Rad53 is reduced in *rfc5-1* mutants in response to DNA damage during the S phase, suggesting that *RFC5* functions upstream of *RAD53*. However, it is not yet known how Rfc5 regulates the checkpoint pathway.

To identify genes that interact with *RFC5* in the checkpoint pathway, we isolated dosage-dependent suppressors of *rfc5-1* mutants. One of the suppressor genes was found to be *RAD24*, a gene which has been shown to play a role in the DNA damage checkpoint. Overexpression of *RAD24* suppresses the DNA damage sensitivity and Rad53 phosphorylation defect in *rfc5-1* mutants. *RAD24* encodes a protein structurally related to subunits of the RFC complex, and the Rad24 protein associates with the components of RFC. Although *rad24*Δ alone does not cause a defect in the replication block checkpoint, its introduction does exacerbate the defect in *rfc5* mutants. Thus, Rad24 and Rfc5 interact physically and functionally in the checkpoints responding to DNA damage and replication block.

MATERIALS AND METHODS

Strains, media, and general methods. Yeast strains used in this study are listed in Table 1. DNA was manipulated by standard procedures (19). Standard genetic techniques were used for manipulating yeast strains (8). Media used to maintain selection of *TRP1* and *URA3* plasmids are synthetic complete media containing 0.5% Casamino Acids and the appropriate supplements.

Screening of dosage-dependent suppressors of *rfc5-1*. To isolate dosage-dependent suppressors of *rfc5-1*, *rfc5-1* (KSC766) mutants were transformed with an *S. cerevisiae* YEp13 genomic library. Approximately 40,000 transformants grown at 25°C were replica plated to yeast extract-peptone-dextrose (YEPD) containing 1 mg of hydroxyurea (HU) per ml at 37°C. The plasmids were recov-

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TABLE 1. List of strains used in this study

Strain[a]	Genotype
KSC766	*MAT***a** *rfc5-1 ade2 his2 trp1 ura3 leu2 lys2*
TSY401	*MAT***a** *ade1 his2 trp1 ura3 leu2*
TSY418	*MAT***a** *rad24Δ::LEU2 ade1 his2 trp1 ura3 leu2*
TSY437	*MAT***a** *rad24Δ::TRP1 ade1 his2 trp1 ura3 leu2*
TSY535	*MAT***a** *RFC5-HA::LEU2 rad24Δ::TRP1 ade1 his2 trp1 ura3 leu2*
TSY601	*MAT***a** *rfc5-1::LEU2 ade1 his2 trp1 ura3 leu2*
TSY602	*MAT***a** *rfc5-1::LEU2 rad24Δ::LEU2 ade1 his2 trp1 ura3 leu2*
TSY612	*MAT***a** *RFC5-Myc::LEU2 rad24Δ::TRP1 ade1 his2 trp1 ura3 leu2*

[a] All strains except KSC766 are isogenic. KSC766 is congenic to other strains.

ered and retransformed into KSC766 cells. Plasmids that conferred the suppression were subjected to restriction and partial sequence analysis. After elimination of plasmids containing the *RFC5* gene, four plasmids were further tested to see whether they suppressed the HU sensitivity of the *rad53* mutant (*spk1-101* [28]). Two of the plasmids did not suppress the *spk1-101* phenotype and were found to contain an overlapping region on chromosome V. Subcloning analysis along with a search of DNA databases localized the suppressor gene to *RAD24*.

Plasmids. A 4-kb *Kpn*I-*Spe*I fragment from pRS416 carrying the *RAD24* gene (obtained from T. Weinert) was cloned into *Kpn*I-*Xba*I-digested YEplac112 and YEplac195 (5), creating YEpT-RAD24 and YEpRAD24, respectively. To create the *rad24* disruption construct, the N-terminal and C-terminal region of the *RAD24* gene was amplified by PCR with the primers R24N-5′(GGGCTCGAG AGATCATCACAATGCG) and R24N-3′(GCATCTAAAGCTTCTTGTAC) or R24C-5′(CCCGCATGCGGAAAGGGACAGAAGGCT) and R24C-3′(GGGC TCGAGGTAATGTGCATAGATTTGTG). The *rad24* disruption plasmid was constructed by a three-part ligation of the *Xho*I-*Hin*dIII-treated PCR-amplified N-terminal fragment and the *Sph*I-*Xho*I-treated PCR-amplified C-terminal fragment with *Sph*I-*Hin*dIII-linearized YIplac128 (5). A null allele for *RAD24* (*rad24Δ::LEU2*) selecting for leucine prototrophy was obtained after sporulation of diploid cells transformed with *Xho*I-digested *rad24* disruption plasmid. Disruption for *RAD24* was confirmed by Southern blotting. The *rad24Δ::TRP1* strains were obtained by replacing *LEU2* with *TRP1* in the *rad24Δ::LEU2* strains with pLW1 (a gift from M. Shirayama). To construct the *rfc5-1* integration plasmid pIR-1, the *Hin*dIII-*Sma*I fragment from the recovered *rfc5-1* mutation (28) was cloned into YIplac128 digested with *Hin*dIII and *Sma*I. The *rfc5-1::LEU2* strains were obtained by transforming pIR-1 into TSY401 cells after treatment with *Mlu*I. The *Nco*I-*Sal*I fragment from R5HC (28), whose *Nco*I site was blunted with Klenow fragment, was cloned into YIplac128 cleaved with *Sma*I and *Sal*I, creating the *RFC5-HA* integration plasmid pTS-I5H. The *Eco*RI-*Bam*HI fragment from pTS-I5H and the *Bam*HI-*Hin*dIII fragment containing DNA sequences of four Myc epitopes were cloned into YIplac128 cleaved with *Eco*RI and *Hin*dIII, creating the *RFC5-Myc* integration plasmid pTS-I5M. The *RFC5-HA* and *RFC5-Myc* integration strains were obtained by transformation with *Bgl*II-digested pTS-I5H and pTS-I5M, respectively. Precise integration was confirmed by PCR. The *RFC5-HA*- and *RFC5-Myc*-integrated cells, in which the endogenous *RFC5* gene is disrupted, grow as well as do wild-type cells. To create the *RAD24-HA* and *RAD24-Myc* plasmids (YCpRAD24-HA and YCpRAD24-Myc), the *RAD24* sequence was amplified by PCR with the 5′ primer CTCGA ATTCTTTCAGGAATATAACTCT and the 3′ primer CTCGGATCCCGAGT ATTTCCAGATCTGAAT, creating a *Bam*HI restriction site at the C-terminal end. The *Nco*I-*Bam*HI-digested PCR fragment, together with the *Kpn*I-*Nco*I fragment from the *RAD24* gene and the *Bam*HI-*Sal*I fragment containing DNA sequences of two hemagglutinin (HA) epitopes, were subcloned into YCplac33 (5), creating YCpRAD24-HA. The *Nco*I-*Bam*HI-digested PCR fragment, together with the *Kpn*I-*Nco*I fragment from the *RAD24* gene and the *Bam*HI-*Hin*dIII fragments containing DNA sequences of two HA and four Myc epitopes, were subcloned into YCplac33 (5), creating YCpRAD24-Myc. The tagged *RAD24* genes complement the sensitivities of the *rad24*Δ mutant to methyl methanesulfonate (MMS) and UV, indicating that the tagged versions are fully functional. YCpT-RFC5 was constructed by inserting the *Hin*dIII-*Sal*I fragment of the *RFC5* gene into YCplac22 (5). YCpRFC5, YEpPOL30, YEpT-POL30 and YCp-RAD53-HA were described previously (26, 28).

Immunoblotting. Immunoblotting analysis was performed as previously described (26). Yeast cells were grown in synthetic complete medium selectable for *TRP1* and/or *URA3* plasmids. Cells were then diluted in YEPD and allowed to grow for 3 h before cells were treated with MMS. Cells were pelleted, washed with chilled water, and resuspended in sodium dodecyl sulfate (SDS) sample buffer U. An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were clarified by 15 min of centrifugation, and 2-mercaptoethanol was added to a final concentration of 1%. The samples were boiled for 5 min and fractionated on SDS-polyacrylamide gels. Proteins were then transferred to nylon membranes; subjected to immunoblot analysis with the mouse monoclonal anti-HA (BAbCO or Boehringer Mannheim), rat monoclonal anti-HA (Boehringer Mannheim), rabbit polyclonal anti-HA (MBL), rabbit polyclonal anti-Myc (MBL), or rabbit anti-Rfc2 serum (a gift from A. Sugino) antibodies, and were detected with the ECL kit (Amersham).

Immunoprecipitation. Yeast cells were grown in synthetic complete medium selectable for *URA3* plasmids. Cells were then diluted in YEPD and allowed to grow for 3 h. Cells (20 U of optical density at 600 nm) were pelleted, washed, and resuspended in 150 μl of lysis buffer (26). An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were clarified by 15 min of centrifugation at 4°C. The supernatant was diluted with lysis buffer and incubated at 4°C for 2 h with 30 μl of protein A-Sepharose beads bound with anti-Rfc2, anti-HA, or anti-Myc antibodies. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad). Immunoprecipitates were washed four times with lysis buffer, twice with another buffer (20 mM HEPES-Na [pH 7.5], 10 mM $MgCl_2$, 4 mM $MnCl_2$), and boiled immediately in 1× SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (26). The proteins were detected after immunoblotting with antibodies described above.

Sucrose density gradient centrifugation. Cells were pelleted, washed, and resuspended in lysis buffer. An equal volume of glass beads was added, and the cells were lysed by vortexing. Extracts were clarified by 15 min of centrifugation at 4°C, and 200 μl of the supernatant was separated by sucrose density gradient sedimentation (4 ml of 10 to 40% sucrose gradient in lysis buffer centrifuged in an SW60 rotor at 40,000 rpm for 12 h at 4°C). The gradients were fractionated from the top (200 μl/fraction) and subjected to immunoblotting with antibodies described above.

UV radiation and drug sensitivities. The UV radiation sensitivity assay was performed as described previously (27). Cells grown at 37°C were plated on YEPD and then irradiated by UV light at 254 nm. After 2 to 3 days of incubation at 37°C, the number of colonies was counted. MMS sensitivity was determined as described elsewhere (27). Cells were incubated with 0 to 0.06% MMS at 37°C for 30 min. Incubation was terminated by addition of sodium thiosulfate to a final concentration of 5%. Aliquots were plated on YEPD, and the number of colonies was counted after incubation at 37°C for 2 to 3 days. The HU sensitivity assay was performed as described previously (28).

Immunofluorescence microscopic analysis. Yeast cells were grown in YEPD medium at 30°C. To examine spindle elongation at 37°C, the culture was synchronized in the G_1 phase by addition of 6 μg of α-factor per ml at 30°C for 1 h. After 1 h, α-factor (6 μg/ml) was subsequently added, and the culture was shifted to 37°C for 1 h. HU was added to a final concentration of 10 mg/ml to the culture during the last 30 min of incubation with α-factor. Cells were then washed to remove α-factor and released into YEPD containing 10 mg of HU per ml at 37°C. To examine spindle elongation at 30°C, the culture was synchronized in the G_1 phase by addition of 6 μg of α-factor per ml at 30°C for 2 h. HU was added at 10 mg/ml to the culture during the last 30 min of incubation with α-factor. Cells were then washed to remove α-factor and released into YEPD containing 10 mg of HU per ml at 30°C. Aliquots of cells were removed and processed for DNA flow cytometry analysis, viability assessment, and indirect immunofluorescence microscopy as described previously (26). For analysis of suppression of the *rfc5-1* checkpoint defect by *RAD24* overexpression, yeast cells were grown in synthetic complete medium selectable for *URA3* plasmids, diluted in YEPD, and allowed to grow at 30°C for 3 h. The culture was synchronized in G_1 with α-factor and released into YEPD containing 10 mg of HU per ml at 37°C, and aliquots of cells were removed and processed as described above.

RESULTS

Isolation of the *RAD24* gene as a dosage-dependent suppressor of *rfc5-1*. The *rfc5-1* mutation is defective for the checkpoints responding to DNA damage and replication block (26, 28). We have shown that the *rfc5-1* mutation confers a growth defect and HU sensitivity at the restrictive temperature. Overexpression of *POL30*, which encodes PCNA, suppresses the growth defect but not the HU sensitivity in *rfc5-1* mutants (28) (see Fig. 1). On the other hand, both defects are suppressed by a high dosage of the checkpoint control gene *RAD53* (28). To identify genes involved in the checkpoint control, we isolated genes which suppress the HU sensitivity of the *rfc5-1* mutant in a dosage-dependent manner. *rfc5-1* mutants were transformed with an *S. cerevisiae* YEp13 genomic library, and transformants grown at 25°C were replica plated to YEPD containing 1 mg of HU per ml at 37°C. In addition to plasmids containing *RFC5*, four plasmids which suppressed the *rfc5-1* growth defect on YEPD containing HU at 37°C were recovered. These plasmids were further tested for whether they suppressed the HU sensitivity of *rad53* mutants. *RAD53* is considered to function downstream of *RFC5* in the checkpoint pathway (26, 28). Two plasmids suppressed the HU sensitivity of *rad53* mutants, while the other two did not (data not shown). The suppressor genes on the latter plasmids were chosen for more-detailed analysis, since their function is presumably more closely related to that

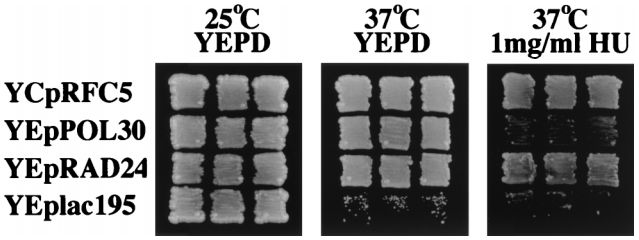
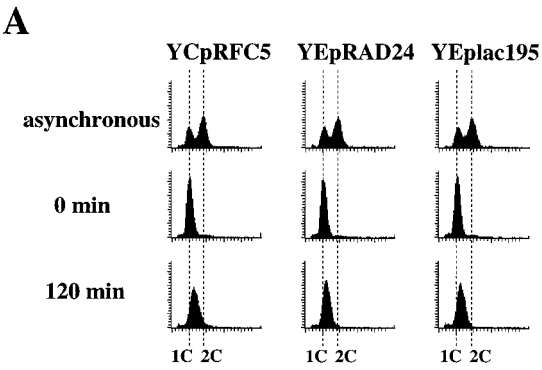


FIG. 1. *RAD24* overexpression suppresses the *rfc5* growth defect. *rfc5-1* mutant (KSC766) cells transformed with the YCpRFC5, YEpPOL30, YEpRAD24, or YEp vector (YEplac195) were streaked and incubated on YEPD medium at 25°C or on YEPD medium in the presence or absence of 1 mg of HU per ml at 37°C.

of *RFC5*. Restriction and DNA sequence analysis indicated that the plasmids contain an overlapping region on chromosome V. Subcloning analysis and a DNA database search identified the suppressor gene as *RAD24* (6, 14). As shown in Fig. 1, a high-copy-number plasmid carrying *RAD24* (YEpRAD24) suppressed both the growth defect and HU sensitivity in *rfc5-1* mutants.

Effect of *RAD24* overexpression on the *rfc5-1* defect in the replication block checkpoint. We have shown that *rfc5-1* mutants are partially defective for the checkpoint responding to DNA replication block (26). Since overexpression of *RAD24* suppressed the growth defect of *rfc5-1* mutants on HU-containing medium (Fig. 1), we examined the effect of *RAD24* overexpression on the *rfc5-1* defect in the replication block checkpoint. *rfc5-1* cells carrying the YCpRFC5, YEpRAD24, or YEp vector were synchronized with α-factor and released into medium containing HU at 37°C. Flow cytometric analysis showed that DNA replication was efficiently blocked in those cells until 120 min after release into HU (Fig. 2A). Most (99%) of the *RFC5* cells were arrested as large budded cells with short spindles, and 31% of *rfc5-1* mutant cells exhibited elongated spindles at 120 min after release into HU. Overexpression of *RAD24* decreased the population of *rfc5-1* mutants with elongated spindles; 19% of *rfc5-1* mutants carrying YEpRAD24 showed elongated spindles at 120 min after release (Fig. 2B). Furthermore, cell survival following HU treatment was higher in *rfc5-1* mutants carrying YEpRAD24 than in those carrying the YEp vector (Fig. 2B). Thus, high levels of Rad24 can partially suppress the *rfc5-1* checkpoint defect responding to replication block.

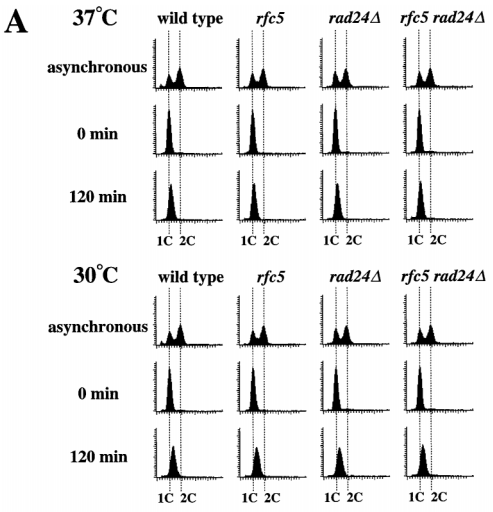
***rfc5 rad24* double mutants are more defective for the checkpoint responding to replication block than are single *rfc5* mutants.** Although the *rad24*Δ mutant shows no apparent defect in the replication block checkpoint (14, 33), *RAD24* overexpression suppresses the checkpoint defect in *rfc5-1* mutants. To determine whether *RAD24* is involved in the replication block checkpoint, we examined the effect of the *rad24*Δ mutation on the checkpoint defect in *rfc5-1* mutants. DNA content and spindle elongation were analyzed in *rfc5-1* and *rfc5-1 rad24*Δ mutants following the release of α-factor-arrested cells into medium containing HU at 37°C (Fig. 3). If cells are defective for the replication block checkpoint, HU-treated cells should enter into mitosis, as evidenced by spindle elongation prior to completion of DNA replication. Flow cytometric analysis showed that DNA replication was efficiently blocked in wild-type and *rfc5-1*, *rad24*Δ, and *rfc5-1 rad24*Δ mutant cells until 120 min after release into HU (Fig. 3A). Most (98%) of the wild-type and *rad24*Δ cells were arrested as large budded cells with short spindles, while 30% of *rfc5-1* mutant cells exhibited elongated spindles at 120 min after release into HU. However, 56% of *rfc5-1 rad24*Δ mutants showed elongated spindles at 120 min after release (Fig. 3B and C). Cell survival following HU treatment was lower in *rfc5-1 rad24*Δ double mutants than in either single mutant (Fig. 3B). Thus, *rfc5-1 rad24*Δ double mutants show a more pronounced defect in the replication block checkpoint than do single *rfc5-1* mutants. Since *rfc5-1* mutants are defective for DNA replication at 37°C (28), this exacerbated defect might be a secondary consequence of more perturbed DNA replication by the *rad24*Δ mutation. To exclude this possibility, we next examined DNA content and spindle elongation in *rfc5-1 rad24*Δ mutants following the release of α-factor-arrested cells into HU at 30°C (Fig. 3). *rfc5-1 rad24*Δ mutant cells exhibited no apparent replication defect at 30°C, since they grew as well as did wild-type cells and did not accumulate in the S phase at 30°C. Flow cytometric analysis showed that DNA replication was efficiently blocked in cells until 120 min after release into HU (Fig. 3A). Most of the wild-type, *rfc5-1*, and *rad24*Δ cells were arrested as large budded cells with short spindles, while 21% of *rfc5-1 rad24*Δ mutant cells exhibited elongated spindles at 120 min after release into HU. *rfc5-1 rad24*Δ mutants became more sensitive to HU



B

plasmid	short spindle	elongated spindle	viability
YCpRFC5	99±0%	1±0%	95±1%
YEpRAD24	81±1%	19±1%	30±3%
YEplac195	69±1%	31±1%	14±3%

FIG. 2. Suppression of spindle elongation and sensitivity of HU-treated *rfc5* mutants by *RAD24* overexpression. (A) Flow cytometric analysis of the DNA content of G_1-synchronized cells released into medium containing HU. *rfc5-1* mutants (TSY601) carrying YCpRFC5, YEpRAD24, or YEplac195 were synchronized in G_1 by α-factor treatment and released into YEPD containing 10 mg of HU per ml at 37°C as described in Materials and Methods. Aliquots of cells were collected at 0 and 120 min after release from α-factor and examined for DNA content by flow cytometry. Dotted lines indicate the DNA content of 1C and 2C cells. The top panels represent asynchronous cells not treated with HU at 30°C and are included as a reference. Typical data from at least two independent experiments is presented. (B) Spindle elongation and sensitivity of HU-treated *rfc5* mutants. *rfc5-1* mutants (TSY601) carrying YCpRFC5, YEpRAD24, or YEplac195 were synchronized in G_1 and released into YEPD containing 10 mg of HU per ml as described above. Cells were collected and fixed in formaldehyde at 120 min after release into HU. Nuclear and microtubular structures were visualized with DAPI (4′,6-diamidino-2-phenylindole) and antitubulin antibodies, respectively. At least 200 cells were examined. Viabilities were determined at 120 min after release in HU. Results are means plus or minus standard errors of at least two independent cultures per strain.



B

strain	short spindle	elongated spindle	viability
37°C			
wild type	98±1%	2±1%	98±1%
rfc5	70±2%	30±2%	18±2%
rad24Δ	98±0%	2±0%	96±1%
rfc5 rad24Δ	44±2%	56±2%	5±1%
30°C			
wild type	98±1%	2±1%	98±1%
rfc5	96±0%	4±0%	96±1%
rad24Δ	98±0%	2±0%	96±2%
rfc5 rad24Δ	79±1%	21±1%	72±2%

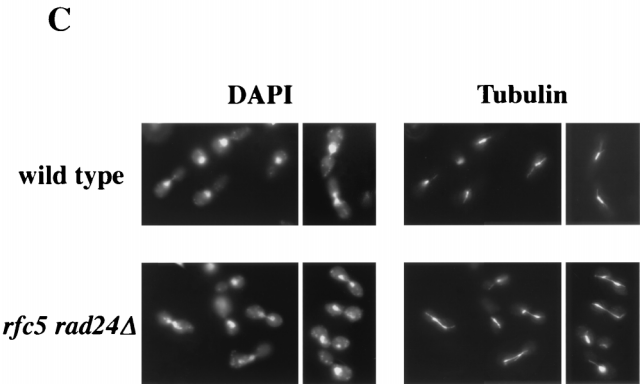


FIG. 3. Effects of the *rad24*Δ mutation on the replication block checkpoint in *rfc5-1* mutants. (A) Flow cytometric analysis of the DNA content of G_1-synchronized cells released into medium containing HU. Wild-type (TSY401) and *rfc5-1* (TSY601), *rad24*Δ (TSY418), and *rfc5-1 rad24*Δ (TSY602) mutant cells were synchronized in G_1 and released into YEPD containing 10 mg of HU per ml at 30 or 37°C as described in Materials and Methods. Aliquots of cells were collected at 0 and 120 min after release into HU and examined for DNA content by flow cytometry. Dotted lines indicate the DNA content of 1C and 2C cells. The top panels represent asynchronous cells not treated with HU at 30°C and are included as a reference. Typical data from at least two independent experiments is presented. (B) Spindle elongation and viability of cells in the presence of HU. Wild-type (TSY401) and *rfc5-1* (TSY601), *rad24*Δ (TSY418), and *rfc5-1 rad24*Δ (TSY602) mutant cells were synchronized in G_1 and released into YEPD containing 10 mg of HU per ml at 30 or 37°C as described in Materials and Methods. Cells were collected and fixed in formaldehyde. Nuclear and microtubular structures at 120 min after release into medium with HU were visualized with DAPI (4′,6-diamidino-2-phenylindole) and antitubulin antibodies, respectively. At least 200 cells were examined. Viabilities were determined at 120 min after release in HU. Results are means plus or minus standard errors of at least two independent cultures per strain. (C) Photomicrographs of wild-type and *rfc5-1 rad24*Δ mutant cells at 120 min after release from the G_1 block into medium containing HU. Wild-type (TSY401) and *rfc5-1 rad24*Δ (TSY602) mutant cells were synchronized in G_1 and released into YEPD containing 10 mg of HU per ml at 37°C. Cells were collected at 120 min after release and fixed in formaldehyde. Nuclear and microtubular structures were visualized with DAPI and antitubulin antibodies, respectively.

treatment even at 30°C (Fig. 3B). These results confirm that the *rad24*Δ mutation enhanced the checkpoint defect in *rfc5-1* mutants.

Effects of *RAD24* overexpression on the response to DNA damage in *rfc5-1* mutants. Several lines of evidence have implicated *RAD24* in the DNA damage checkpoint (17, 22, 32, 33). The *rfc5-1* mutation is also defective for the DNA damage checkpoint and is sensitive to DNA damage (26). To investigate the relationship between *RFC5* and *RAD24*, we examined the effects of *RAD24* overexpression on the DNA damage sensitivity of *rfc5-1* mutants. Overexpression of *POL30* suppresses the growth defect but not the sensitivity to DNA damage in *rfc5-1* mutants (26, 28). We therefore examined whether *RAD24* overexpression would suppress the DNA damage sensitivity of *rfc5-1* mutants carrying YEpT-POL30. *RAD24* overexpression restored the ability of *rfc5-1* cells carrying YEpT-POL30 to survive exposure to MMS and UV irradiation (Fig. 4).

Rad53 is an essential protein kinase that plays a role in the DNA damage checkpoint pathway (1). Exposure of cells to MMS leads to the phosphorylation of Rad53, resulting in the accumulation of a lower-mobility form of Rad53 (20, 29). We have shown that the phosphorylation of Rad53 is reduced in response to MMS treatment in *rfc5-1* mutants, providing evidence that Rfc5 is required for the DNA damage-induced phosphorylation of Rad53 (26). Since overexpression of *RAD24* can suppress the sensitivity to MMS of *rfc5-1* mutants, we expected that its overexpression would also suppress the defect in the MMS-induced Rad53 phosphorylation of *rfc5-1* mutants. To test this hypothesis, the phosphorylation state of Rad53 was examined in vivo by immunoblot analysis in cells expressing the Rad53-HA protein. When treated with MMS at 37°C, Rad53-HA in wild-type cells became highly phosphorylated as indicated by the appearance of isoforms with a lower electrophoretic mobility. In contrast, the phosphorylation of Rad53-HA in *rfc5-1* mutants was greatly reduced (Fig. 5). However, the DNA damage-induced phosphorylation of Rad53 was partially restored in *rfc5-1* mutants by the introduction of YEpT-RAD24, as evidenced by the appearance of smeared, shifted bands corresponding to Rad53 (Fig. 5). Thus, suppression of the DNA damage sensitivity of *rfc5-1* mutants by *RAD24* overexpression was correlated with the modification of Rad53. These observations suggest that overexpression of *RAD24* suppresses the DNA damage sensitivity of the *rfc5-1* mutation by activating Rad53. This is consistent with our earlier observation that *RAD53* overexpression can suppress the DNA damage sensitivity of *rfc5-1* mutants (26).

Since overexpression of *RAD24* restored the DNA damage-induced phosphorylation of Rad53 in *rfc5-1* mutants, it is possible that Rad24 is also required for activation of Rad53 kinase. To examine this possibility, we tested the phosphorylation state of Rad53 in *rad24*Δ mutants that suffered from DNA damage. When wild-type cells were treated with MMS, Rad53-HA underwent modification. In contrast, the MMS-induced modification of Rad53-HA was reduced in *rad24*Δ mutants (Fig. 6). This result indicates that Rad24, like Rfc5, is required for the DNA damage-induced phosphorylation of the Rad53 kinase.

Rad24 proteins associate with subunits of the RFC complex. The coding sequence of the *RAD24* gene is 1,977 bp in length,

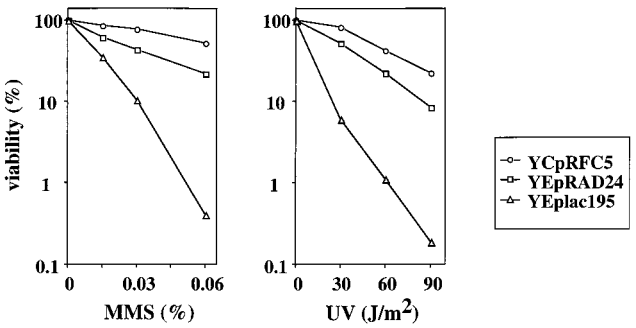


FIG. 4. Effects of *RAD24* overexpression on DNA damage sensitivity in *rfc5* mutants. *rfc5-1* mutant (KSC766) cells carrying YEpT-POL30 were transformed with YCpRFC5, YEpRAD24, or YEplac195. The transformants in log-phase culture grown at 37°C were treated with the indicated concentrations of MMS for 30 min or irradiated at the indicated doses with UV light. Viability of cells was estimated as described in Materials and Methods.

and the predicted protein consists of 659 amino acids, corresponding to a molecular mass of 76 kDa, and contains a nucleoside triphosphate binding motif (6, 14). Rad24 is most homologous to the fission yeast Rad17, and they show a well-conserved structural organization. Rad24 is also structurally related to components of the RFC complex (6, 14). RFC subunits contain eight domains termed the RFC boxes (3). Rad24 contains homology to RFC boxes II, III (nucleotide binding motif), and VIII but lacks sequences corresponding to RFC boxes I (the DNA ligase homology domain), IV, V (DEAD box), VI, and VII.

The genetic interaction presented above and sequence similarities between the RFC subunits and Rad24 raised the possibility that Rad24 associates with the RFC complex. To examine the physical interaction between Rad24 and the RFC complex, we tagged the *RAD24* gene with the HA or Myc epitope and analyzed its association with the RFC subunits Rfc2 and Rfc5. When extracts from cells harboring a low-copy-number-tagged *RAD24* plasmid (YCpRAD24-HA or YCpRAD24-Myc) were subjected to immunoblot analysis, we detected an appropriately sized protein immunoreactive with the anti-HA or anti-Myc antibody (data not shown). Isogenic *rad24*Δ cells with or without an integrated copy of *RFC5-HA* (*RFC5-HA rad24*Δ or *RFC5 rad24*Δ cells, respectively) were transformed with the YCpRAD24-Myc or YCp vector. Extracts were prepared from the transformed cells and subjected to immunoprecipitation with an antibody to the HA epitope. The immunoprecipitates were then analyzed by immunoblotting with antibodies to the HA epitope, the Myc epitope, and Rfc2. When immunoblotted with the anti-HA antibody, bands migrating at about 40 kDa were detected in the *RFC5-HA* cells, while no band was detected by the anti-HA antibody in the *RFC5* cells (Fig. 7A). When immunoblotted with the anti-Myc antibody, bands corresponding to Rad24-Myc were observed in the immunocomplex from the cells coexpressing Rfc5-HA and Rad24-Myc, while Rad24-Myc proteins were absent in the immunocomplex from the cells expressing only Rad24-Myc or Rfc5-HA (Fig. 7A). Consistent with the previous finding that Rfc2 and Rfc5 are subunits of the RFC complex (3, 28), immunoblotting with the anti-Rfc2 antibody revealed that Rfc5-HA coprecipitated with Rfc2 (Fig. 7A). Extracts were also prepared from *rad24*Δ mutants carrying YCpRAD24-Myc or the YCp vector and subjected to immunoprecipitation with anti-Rfc2 or control serum. The immunoprecipitates were then analyzed by immunoblotting with antibodies against the Myc epitope and Rfc2. A signal corresponding to the Rad24-Myc proteins was observed in extracts from cells carrying YCpRAD24-Myc after immunoprecipitation with the anti-Rfc2 antibody (Fig. 7B). We next examined coimmunoprecipitation of Rfc2 and Rfc5 with Rad24 in the reciprocal experiment. *RFC5-Myc rad24*Δ or *RFC5 rad24*Δ cells were transformed with the YCpRAD24-HA or YCp vector. Extracts were prepared from the transformed cells and subjected to immunoprecipitation with an antibody to the HA epitope. The immunoprecipitates were then analyzed by immunoblotting with antibodies against the HA epitope, the Myc epitope, and Rfc2. Rfc5-Myc was observed in the immunoprecipitates from cells coexpressing Rfc5-Myc and Rad24-HA. Rfc2 was found to coprecipitate with Rad24-HA in a tagged-Rad24-specific manner (Fig. 7C). These results show that the Rad24 protein interacts physically with components of the RFC complex.

We investigated whether Rad24 associates with the RFC complex or with RFC proteins in smaller complexes. Extracts from cells coexpressing Rfc5-HA and Rad24-Myc were fractionated by sucrose density gradient centrifugation and subjected to immunoblotting with the anti-HA and anti-Myc antibodies. As shown in Fig. 8, Rad24-Myc cosedimented with Rfc5-HA as a 10S particle. It has been shown that the purified yeast RFC complex sediments as an 8.7S particle (34). Altogether, these results strongly suggest that Rad24 proteins associate with the RFC complex.

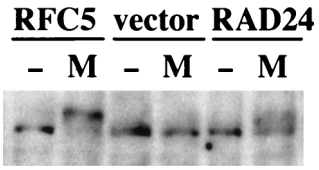


FIG. 5. Effects of *RAD24* overexpression on modification of Rad53 in *rfc5* mutants. *rfc5-1* mutant (KSC766) cells were transformed with YCpRAD53-HA and YCpT-RFC5 (RFC5), YEpT-RAD24 (RAD24), or YEplac112 (vector). The transformants grown at 25°C were shifted to 37°C for 1 h and then incubated with YEPD (−) or YEPD containing 0.1% MMS (M) at 37°C for 2 h. The cells were subjected to immunoblotting analysis as described in Materials and Methods.

DISCUSSION

In this paper, we provide evidence demonstrating that the interaction between *RFC5* and *RAD24* is linked with the checkpoint control in the budding yeast. First, *RAD24* overexpression suppressed the *rfc5-1* defect in the replication block checkpoint. Second, *rfc5-1 rad24*Δ mutants showed a more pronounced defect in the replication block checkpoint than did single *rfc5-1* mutants. Third, *RAD24* overexpression suppressed the DNA damage sensitivity and restored the DNA damage-induced phosphorylation of Rad53 in *rfc5-1* mutants. Fourth, Rad24, like Rfc5, was required for MMS-induced Rad53 phosphorylation. Finally, Rad24 proteins were found to interact physically with components of the RFC complex, Rfc2 and Rfc5, and to cosediment with Rfc5. Taken together, these findings

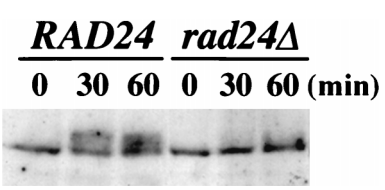


FIG. 6. Modification of Rad53 in *rad24*Δ mutants. *RAD24* (TSY401) and *rad24*Δ (TSY418) mutant cells carrying YCp-RAD53-HA were grown at 30°C. The cells were incubated with 0.04% MMS for the indicated time and subjected to immunoblotting analysis as described in Materials and Methods.

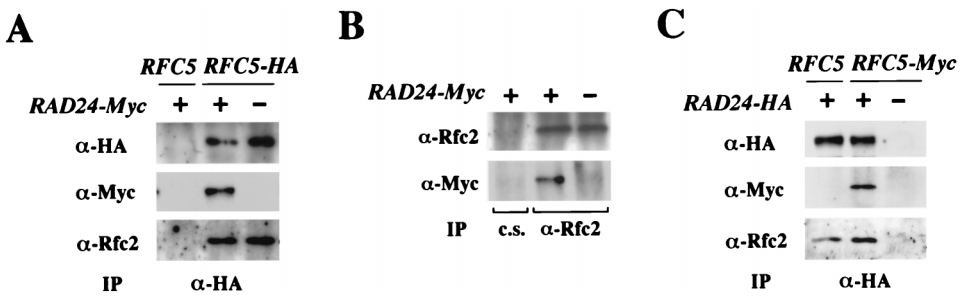


FIG. 7. Association of Rad24 with subunits of RFC. (A) Coimmunoprecipitation of Rad24 and Rfc2 with Rfc5. Cell extracts prepared from *RFC5 rad24*Δ (TSY418) and *RFC5-HA rad24*Δ (TSY535) cells carrying YCpRAD24-Myc (+) or YCplac33 (−) were immunoprecipitated (IP) with anti-HA antibody. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-HA antibody (top), anti-Myc antibody (middle), or anti-Rfc2 serum (bottom). (B) Coimmunoprecipitation of Rad24 with Rfc2. Cell extracts prepared from *rad24*Δ (TSY418) cells carrying YCpRAD24-Myc (+) or YCplac33 (−) were immunoprecipitated (IP) with preimmune control serum (c.s.) or anti-Rfc2 serum. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-Rfc2 serum (top) or anti-Myc antibody (bottom). (C) Coimmunoprecipitation of Rfc2 and Rfc5 with Rad24. Cell extracts prepared from *RFC5 rad24*Δ (TSY437) and *RFC5-Myc rad24*Δ (TSY612) cells carrying YCpRAD24-HA (+) or YCplac33 (−) were immunoprecipitated (IP) with anti-HA antibody. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-HA antibody (top), anti-Myc antibody (middle), or anti-Rfc2 serum (bottom).

strongly support a model in which Rfc5 and Rad24 interact physically and functionally in the checkpoint pathways.

The budding yeast RFC has been purified to homogeneity by assaying replication activity in vitro. The purified RFC complex is composed of five different subunits, each of which is encoded by an essential gene (3). The amino acid sequence of Rad24 has similarities with those of the five subunits of RFC in three of the eight domains termed the RFC boxes. The *RAD24* gene encodes a predicted protein of 659 amino acids with a molecular mass of 76 kDa. Although the peptide corresponding to Rad24 is not detected in highly purified fractions of yeast RFC (3), we demonstrated the association of Rad24 with Rfc2 and Rfc5 by immunoblot analysis following immunoprecipitation and the cosedimentation of Rad24 with Rfc5 in sucrose density gradient centrifugation. One likely explanation for these results is that Rad24 proteins may bind unstably or indirectly to the RFC complex and therefore dissociate during the purification steps. Thus, Rad24 appears to associate with the RFC complex but not with RFC proteins in smaller complexes. The physical interaction between Rfc5 and Rad24 was not affected by treatment with MMS or arrest with nocodazole in M phase (data not shown). Therefore, the checkpoint or DNA replication status does not appear to regulate the interaction but rather the other properties, for example, the activity of the Rad24-RFC complex. The RFC complex possesses a structure-specific DNA binding activity, displaying a preference for DNA molecules mimicking DNA replication substrate, and an ATPase activity that is stimulated by DNA (10, 30). The fact that Rad24 contains a nucleotide binding motif raises the possibility that Rad24, like RFC proteins, may possess ATP binding activity. It will be interesting to see whether ATPase activity of the RFC-Rad24 complex can be stimulated by recognizing the primer terminus or aberrant structures resulting from DNA damage and replication delay. *RAD24* overexpression appears to suppress the *rfc5-1* mutation through the physical interaction between Rad24 and Rfc5, although we cannot exclude the other possibilities, for instance, that high levels of Rad24 could activate checkpoint pathways independently of *RFC5*.

RAD24 has been suggested to have a role in DNA replication and/or repair, because overexpression of *RAD24* strongly reduces the growth rate of mutants that are defective in the DNA replication-repair proteins Rfc1, Pol α, and Pol δ (14). Although it remains possible that an increased dosage of *RAD24* could rescue the *rfc5-1* defect in DNA replication or DNA repair, the strongest evidence for a functional interaction between *RFC5* and *RAD24* in the checkpoint comes from the analysis of double mutants. *rfc5-1 rad24*Δ mutants were more defective for the replication block checkpoint than were single *rfc5-1* mutants. Of particular note, at a temperature that does not affect DNA replication, neither single mutant exhibited the checkpoint defect, yet the double mutant was defective for the checkpoint. It is therefore less possible that the observed checkpoint defect results from a general disturbance of the whole DNA replication apparatus.

One plausible explanation of the *RFC5-RAD24* interaction is that these two genes function redundantly in the same checkpoint pathways but that the function of *RAD24* is modest relative to that of *RFC5* in the replication block checkpoint. The additive defect in the replication block checkpoint in the double mutants suggests that *rfc5-1* mutants may still have some residual checkpoint activity at the restrictive temperature due to the leakiness of the conditional mutation. Another explanation of the *RFC5-RAD24* interaction is that *RAD24* and *RFC5* function in different but overlapping checkpoint pathways and that an increased dosage of *RAD24* can compensate for loss of function of *RFC5*. For example, the signal that induces the *RFC5*-mediated checkpoint pathway differs from the signal that induces the *RAD24*-mediated checkpoint pathway; *RFC5* may be involved primarily in recognizing the primer terminus and monitoring stalled DNA replication, whereas *RAD24* might be required for recognizing the aberrant DNA structures resulting from DNA replication delay.

RAD24 has been shown to play a role in all known DNA damage checkpoint controls in the G_1, S, and G_2/M phases. It

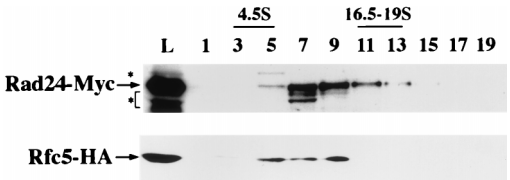


FIG. 8. Cosedimentation of Rfc5 and Rad24. A cell extract prepared from *RFC5-HA rad24*Δ (TSY535) cells carrying YCpRAD24-Myc was separated in a 10 to 40% sucrose gradient, and the load on the gradient (L) and fractions (removed from the top of the gradient) were analyzed by immunoblotting with anti-HA (upper panel) and anti-Myc (lower panel) antibodies. Bovine serum albumin (4.5S) and thyroglobulin (16.5-19S) were separated simultaneously in an independent gradient as markers. The upper band marked with an asterisk is a protein other than Rad24-Myc, which is recognized by the anti-Myc antibody. The lower bands marked with an asterisk are likely proteolytic products of Rad24-Myc.

has been demonstrated that the Rad53 protein kinase is phosphorylated in response to DNA damage, and thus, this biochemical modification correlates with the activation of the checkpoint pathway. We have shown that *rfc5-1* mutants are sensitive to DNA damage and defective for the phosphorylation of Rad53 in response to DNA damage. Similar to *rfc5-1*, *rad24*Δ was defective for the phosphorylation of Rad53 in response to DNA damage. Overexpression of *RAD24* partially suppressed the DNA damage sensitivity and restored the phosphorylation of Rad53 in *rfc5-1* mutants. These observations are consistent with our finding that Rfc5 and Rad24 interact physically and regulate the DNA damage checkpoint pathway. Lydall and Weinert (13) showed that the functions of *RAD17*, *RAD24*, and *MEC3* in response to DNA damage are genetically indistinguishable and proposed that these genes play similar roles in DNA damage processing directly linked to the checkpoint control in *S. cerevisiae*. We are now examining the interaction of *RFC5* with *RAD17* and *MEC3* in the checkpoint control.

The observations presented here provide evidence indicating that the interaction between *RFC5* and *RAD24* is linked with the checkpoint pathway in the budding yeast. However, it remains to be precisely determined how *RFC5* and *RAD24* are involved in the checkpoint signal transduction. Further experiments will be aimed at elucidating the biochemical properties of the RFC-Rad24 complex and its interaction with the other components in the checkpoint pathway.

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