MOLECULAR AND CELLULAR BIOLOGY, Aug. 2000, p. 5888–5896 Vol. 20, No. 16
0270-7306/00/$04.00+0
Copyright © 2000, American Society for Microbiology. All Rights Reserved.

Rfc5, in Cooperation with Rad24, Controls DNA Damage Checkpoints throughout the Cell Cycle in *Saccharomyces cerevisiae*

TAKAHIRO NAIKI, TOSHIYASU SHIMOMURA,† TAE KONDO, KUNIHIRO MATSUMOTO,* AND KATSUNORI SUGIMOTO

Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-0814, Japan

Received 7 February 2000/Returned for modification 20 March 2000/Accepted 2 May 2000

***RAD24* and *RFC5* are required for DNA damage checkpoint control in the budding yeast *Saccharomyces cerevisiae*. Rad24 is structurally related to replication factor C (RFC) subunits and associates with RFC subunits Rfc2, Rfc3, Rfc4, and Rfc5. *rad24*Δ mutants are defective in all the G_1-, S-, and G_2/M-phase DNA damage checkpoints, whereas the *rfc5-1* mutant is impaired only in the S-phase DNA damage checkpoint. Both the RFC subunits and Rad24 contain a consensus sequence for nucleoside triphosphate (NTP) binding. To determine whether the NTP-binding motif is important for Rad24 function, we mutated the conserved lysine^{115} residue in this motif. The *rad24-K115E* mutation, which changes lysine to glutamate, confers a complete loss-of-function phenotype, while the *rad24-K115R* mutation, which changes lysine to arginine, shows no apparent phenotype. Although neither *rfc5-1* nor *rad24-K115R* single mutants are defective in the G_1- and G_2/M-phase DNA damage checkpoints, *rfc5-1 rad24-K115R* double mutants become defective in these checkpoints. Coimmunoprecipitation experiments revealed that Rad24^{K115R} fails to interact with the RFC proteins in *rfc5-1* mutants. Together, these results indicate that *RFC5*, like *RAD24*, functions in all the G_1-, S- and G_2/M-phase DNA damage checkpoints and suggest that the interaction of Rad24 with the RFC proteins is essential for DNA damage checkpoint control.**

Eukaryotic cells employ a set of surveillance mechanisms to coordinate cell cycle events by permitting the onset of one event only after the completion of the preceding event. The mechanisms that ensure the proper ordering of cell cycle events have been termed checkpoint controls (10). DNA damage triggers the activation of checkpoint pathways that arrest the cell cycle and induce the transcription of genes that facilitate repair. Other checkpoints are activated when DNA replication is blocked. Failure to respond properly to DNA alterations may result in genomic instability, a mutagenic condition that predisposes organisms to cancer (5, 24).

The cell cycle is transiently arrested at different stages depending on the phase at which DNA damage occurs. Three responses have been characterized in the budding yeast *Saccharomyces cerevisiae*, known as the G_1-, S- and G_2/M-phase DNA damage checkpoints (16). Genetic studies have identified genes that are involved in all three checkpoints. These include *RAD9*, *RAD17*, *RAD24*, *MEC3*, *DDC1*, *MEC1(ESR1)*, and *RAD53 (SPK1 or MEC2)* (1, 17, 18, 22, 23, 30–33, 43–45). Several lines of genetic evidence have suggested that *RAD17*, *RAD24*, *MEC3*, and *DDC1* operate in the same checkpoint pathway, while *RAD9* functions separately (17, 18, 20). Indeed, Ddc1, Mec3, and Rad17 physically interact with each other, suggesting that they function as a complex (13). *RAD53* encodes a dual-specificity protein kinase (35), and Mec1 belongs to the ATM protein family (12, 28). Rad53 is phosphorylated in response to DNA damage in a *MEC1*-dependent manner (26, 39). DNA damage-induced Rad53 phosphorylation is also dependent on *RAD9*, *RAD17*, *RAD24*, *MEC3*, and *DDC1* (21, 29, 39, 41).

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* (4). RFC is a structure-specific DNA-binding protein complex that recognizes the primer-template junction. RFC loads PCNA onto the primer terminus, and then DNA polymerases δ and ε bind to the DNA-RFC-PCNA complex to constitute a processive replication complex (2, 15, 42). We have demonstrated that *rfc5-1* mutants are defective in the S-phase DNA damage and DNA replication block checkpoints but not in the G_2/M-phase DNA damage checkpoint (36, 38). *RAD24* encodes a protein structurally related to the RFC subunits (8, 19) and has an essential role in the G_1-, S- and G_2/M-phase DNA damage checkpoints (23, 31, 45). We isolated *RAD24* in a screen for dosage-dependent suppressors of *rfc5-1* and have shown that Rad24 interacts physically with Rfc2 and Rfc5 (29). Consistent with its role in DNA damage checkpoints, *RAD24* overexpression suppresses the sensitivity to DNA-damaging agents and the defect in DNA damage-induced Rad53 phosphorylation in *rfc5-1* mutants. Thus, the RFC proteins and Rad24 appear to form a complex that functions in the DNA damage checkpoint pathway. However, it was not known whether this complex is required for the DNA damage checkpoint only in the S phase or throughout the cell cycle.

Rad24, like the RFC subunits, contains a nucleoside triphosphate (NTP)-binding motif. In order to test if this motif is involved in Rad24 function, we created the substitution mutations *rad24-K115E* and *rad24-K115R* at the conserved lysine residue in the NTP-binding motif. From studies of cells carry-

* Corresponding author. Mailing address: Division of Biological Science, Graduate School of Science, Nagoya University, Chikusa-ku, Nagoya 464-8602, Japan. Phone: 81-52-789-2593. Fax: 81-52-789-2589. E-mail: g44177a@nucc.cc.nagoya-u.ac.jp.

† Present address: Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba 300-2611, Japan.

TABLE 1. Strains used in this study

Strain	Relevant genotype
KSC006	*MAT***a** *ade1 his2 trp1 ura3 leu2*
KSC835	*MAT***a** *rfc5-1*::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC980	*MAT***a** *rad24*Δ::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC1105	*MAT***a** *rfc5-1*::*LEU2 rad24*Δ::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC1133	*MAT***a** *RFC1-HA*::*TRP1 rad24*Δ::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC1151	*MAT***a** *rad24-K115E*::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1152	*MAT***a** *rad24-K115R*::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1160	*MAT***a** *rfc5-1*::*LEU2 rad24-K115E*::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1161	*MAT***a** *rfc5-1*::*LEU2 rad24-K115R*::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1163	*MAT***a** *RFC3-HA*::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC1164	*MAT***a** *RFC4-HA*::*LEU2 ade1 his2 trp1 ura3 leu2*
KSC1168	*MAT***a** *RFC3-HA*::*LEU2 rad24*Δ::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1170	*MAT***a** *RFC3-HA*::*LEU2 rfc5-1*::*LEU2 rad24*Δ::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1172	*MAT***a** *RFC4-HA*::*LEU2 rad24*Δ::*TRP1 ade1 his2 trp1 ura3 leu2*
KSC1174	*MAT***a** *RFC4-HA*::*LEU2 rfc5-1*::*LEU2 rad24*Δ::*TRP1 ade1 his2 trp1 ura3 leu2*

ing the *rad24-K115R* and/or *rfc5-1* mutation, we show that *RFC5*, like *RAD24*, has a role in the DNA damage checkpoints not only in the S phase but also in the G_1 and G_2/M phases. We also show that Rad24 interacts physically with Rfc3 and Rfc4 and that in *rfc5-1* mutants the $Rad24^{K115R}$ protein fails to associate with the RFC proteins. Our results suggest that the interaction of Rad24 with the RFC proteins is essential for DNA damage checkpoint control throughout the cell cycle.

MATERIALS AND METHODS

Strains, media, and general methods. The yeast strains used in this study are isogenic and are listed in Table 1. Standard genetic techniques were used for manipulating yeast strains (9, 11). Synthetic complete (SC) medium containing 0.5% casamino acids and the appropriate supplements was used to maintain selection of *URA3* plasmids.

Plasmids and gene replacement. The *Bam*HI-*Hin*dIII fragment from YCpRAD24 (29) and an *Nde*I-*Bam*HI fragment of the 5′ noncoding sequence of *RAD24* were cloned into *Nde*I-*Hin*dIII-treated YIplac204 (6), resulting in YIpT-RAD24. To construct YIpT-RAD24-K115E and YIpT-RAD24-K115R, the 110-bp *Bam*HI-*Bst*BI fragment of YIpT-RAD24 was replaced by sets of complementary oligonucleotides KE-1 (5′-GATCCTACTACTGTCTGGCCCCAG TGGATGCTCTGAAAGTACGGTCATAA-3′), KE-2 (5′-GAGAGTTCTTTT ATGACCGTACTTTCAGAGCATCCACTGGGGCCAGACAGTAGTAG-3′), ER-1 (5′-AAGAACTCTCAAAAATCTTAGTTCCTAAATACAGACAA AACAGCAACGGAACGTCCTTT-3′), and ER-2 (5′-CGAAAGGACGTTCC GTTGCCTGTTTTGTCTGTATTTAGGAACTAAGATTTTT-3′) or by KR-1 (5′-GATCCTACTACTGTCTGGCCCCAGTGGATGCTCTAGAAGTACGG TCATAA-3′), KR-2 (5′-GAGAGTTCTTTTATGACCGTACTTCTAGAGCA TCCACTGGGGCCAGACAGTAGTAG-3′), ER-1, and ER-2, respectively. The 1.1-kb *Eco*RI-*Sac*I fragment from YIpT-RAD24-K115R was cloned into YCpRAD24-myc (29), generating YCpRAD24-K115R-myc. The substitution at each site was confirmed by sequence analysis. To obtain *rad24*Δ::*ura3* cells, *rad24*Δ::*LEU2* cells were transformed with *Xho*I-digested pLU12 (3) and selected for Ura^+ Leu^-, and the resulting *rad24*Δ::*leu2*::*URA3* cells were plated on medium containing 5-fluoroorotic acid to counterselect against the Ura^+ marker as described before (9). To construct site-specific *rad24* mutations marked with *TRP1*, the plasmids YIpT-RAD24-K115E and YIpT-RAD24-K115R were cleaved with *Cla*I, and the resulting DNA fragments were transformed into *rad24*Δ::*ura3* cells. Correct integration of each mutant gene at the *RAD24* locus was confirmed by PCR. *rad24*Δ cells carrying YCpRAD24-K115R-myc showed no apparent phenotype as observed for *rad24-K115R*::*TRP1* cells with regard to sensitivity to DNA-damaging agents, such as methyl methanesulfonate (MMS) and UV light.

To construct tagged versions of *RFC1*, *RFC3*, and *RFC4*, sequences encoding hemagglutinin (HA) epitope tags were inserted in front of the stop codon. To construct the *RFC1-HA* integration plasmid YIpT-RFC1-HA, a *Bgl*II-*Sal*I fragment from the *RFC1-HA* gene was subcloned into pRS304 (34). To construct the *RFC3-HA* and *RFC4-HA* integration plasmids, an *Msc*I-*Sph*I fragment from the *RFC3-HA* gene and an *Nco*I-*Xho*I fragment from the *RFC4-HA* gene were subcloned into YIplac128 (6), generating YIpL-RFC3-HA and YIpL-RFC4-HA, respectively. YIpT-RFC1-HA, YIpL-RFC3-HA, and YIpL-RFC4-HA were treated with *Sph*I, *Kpn*I, and *Eco*RI, respectively, and transformed into cells. The precise integration, which destroys the endogenous *RFC1*, *RFC3*, or *RFC4* gene, was confirmed by PCR. These integrations did not affect the growth or DNA damage sensitivity of wild-type or *rfc5-1* mutant cells. YCp-RAD53-HA was described previously (36).

UV radiation and MMS sensitivities. The UV radiation sensitivity assay was performed as described previously (37). Cells grown at 30°C were plated on YEPD and then irradiated by UV at 254 nm. After 2 to 3 days of incubation at 30°C, the number of colonies was counted. MMS sensitivity was determined as described (37). Cells were incubated with MMS at 30°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 30°C for 2 to 3 days.

UV and MMS synchrony experiments. To analyze cell cycle delay at the G_2/M transition, log-phase cultures at 30°C were prearrested with 6 μg of α-factor per ml for 120 min, washed with water, and then released into YEPD containing nocodazole (15 μg/ml) for 120 min to synchronize cells in G_2/M. Cells arrested in G_2/M were spread on YEPD plates and irradiated with a 254-nm UV lamp at 75 J/m^2. Cells were then washed to remove nocodazole and released into fresh YEPD containing 1% dimethyl sulfoxide at 30°C. At timed intervals, cells were withdrawn and stained with 4′,6-diamidino-2-phenylindole (DAPI) for microscopic examination. An MMS synchrony experiment to monitor S-phase regulation was carried out as described elsewhere (36). To analyze cell cycle delay at the G_1/S transition, log-phase cultures in YEPD were treated with α-factor (6 μg/ml) for 120 min to synchronize cells in G_1. Cells arrested in G_1 were spread on YEPD plates and irradiated with a 254-nm UV lamp at 75 J/m^2. Cells were then washed to remove α-factor and released into fresh YEPD at 30°C. Cells were withdrawn at different times and subjected to examination as described (32).

Antibody and immunoblotting. 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. For cell cycle arrest, cells were treated with nocodazole (15 μg/ml) or α-factor (6 μg/ml) for 120 min and then irradiated with a 254-nm UV lamp at 150 or 200 J/m^2, respectively. Cells were released into fresh YEPD containing nocodazole or α-factor and incubated for 60 min. Protein extracts for immunoblotting were prepared and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (36). Proteins were transferred to nylon membranes, subjected to immunoblot analysis with the anti-Myc (9E10) or anti-HA (3F10 or 16B12) monoclonal antibody or anti-Rfc2 (a gift from A. Sugino) or anti-Rfc5 antibody and detected by the ECL kit (Amersham). Antibody against Rfc5 was raised by immunizing a rabbit with synthetic peptides corresponding to amino acids 22 to 42 and 119 to 134 of Rfc5 and purified with affinity chromatography. Among the RFC subunits of budding yeast, the amino acid sequences within these regions are specific to Rfc5. This antibody specifically recognized Rfc5 in immunoblots, and the signal was significantly increased when *RFC5* was overexpressed (data not shown).

Immunoprecipitation. Yeast cells were grown in SC medium appropriate to select for *URA3* plasmids. Cells were then diluted in YEPD and allowed to grow for 3 h at 30°C. Cells were next harvested, washed, and resuspended in lysis buffer (36). 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 protein A-Sepharose beads bound with anti-HA (3F10) or anti-Rfc2 antibody. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad). Immunoprecipitates were washed with lysis buffer and subsequently with a wash buffer and boiled immediately in 1× SDS-PAGE sample buffer (36). The proteins were detected after immunoblotting with antibody as described above.

RESULTS

DNA damage sensitivity of cells carrying mutations in the NTP-binding motif of Rad24. *RAD24* encodes a protein structurally related to subunits of the RFC complex (8, 19). One feature of this homology is that both Rad24 and the RFC subunits contain a sequence motif characteristic of NTP-binding and -hydrolyzing proteins. The NTP-binding motif in Rad24, GXXGXXKS, deviates slightly from the classical motif, GXXGXGK(S/T) (14) (Fig. 1). The conserved lysine residue in the NTP-binding motif is involved in electrostatic interactions with the triphosphate tail of NTP, and mutation of this residue reduces NTP-binding and hydrolysis (27). To test whether this motif has a role in Rad24 function, the conserved $lysine^{115}$ was changed into either glutamate or arginine, creating the *rad24-K115E* and *rad24-K115R* mutations, respectively (Fig. 1). Since the *rad24*Δ mutation confers sensitivity to DNA

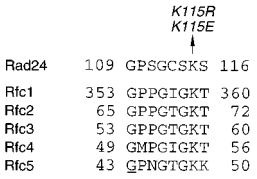

FIG. 1. Mutations of Rad24 at the conserved lysine of the NTP-binding motif. The NTP-binding domain of Rad24 is aligned with those of all RFC subunits from *S. cerevisiae*. The amino acid converted by site-specific mutagenesis in the *RAD24* gene is shown by an arrow with the mutation names. The amino acid underlined is the site in the *rfc5-1* mutation which changes Gly to Glu at amino acid position 43.

damage, we measured the sensitivity of *rad24-K115E* and *rad24-K115R* mutants to MMS treatment and UV irradiation (Fig. 2). *rad24-K115E* mutants showed DNA damage sensitivity very similar to that of *rad24*Δ mutants, while *rad24-K115R* mutant cells were as resistant to DNA damage as wild-type cells.

To further investigate the properties of these *rad24* mutations, we evaluated DNA damage checkpoints in the corresponding mutant cells. It has been shown that *RAD24* is required for the G_1-, S- and G_2/M-phase DNA damage checkpoints (23, 31, 45). We first examined the S-phase checkpoint by monitoring the DNA content of cells experiencing DNA damage after release from a G_1 block (Fig. 3). When released from α-factor arrest and exposed to MMS, wild-type cells exhibited lower rates of DNA synthesis. In contrast, *rad24*Δ mutants showed some delay but progressed through the S phase faster than wild-type cells. The partial defect of *rad24*Δ mutants in the S-phase DNA damage checkpoint was reported previously (23). Under the same conditions, *rad24-K115R* cells proceeded through the S phase as slowly as wild-type cells, whereas *rad24-K115E* mutant cells completed the S phase as fast as *rad24*Δ cells. We next examined the G_2/M-phase DNA damage checkpoint by monitoring mitotic division following UV irradiation (Fig. 4A). When cell cultures were released from nocodazole arrest after UV irradiation, *rad24-K115R* cells delayed nuclear division similar to wild-type cells, while *rad24*Δ and *rad24-K115E* cells went through mitosis much faster than wild-type cells. We further analyzed the G_1-phase DNA damage checkpoint in the *rad24* mutants by monitoring the appearance of budded cells after release from α-factor arrest (Fig. 5A). When cell cultures were released from α-factor arrest after UV irradiation, *rad24-K115R* cells were delayed in bud emergence, similar to the wild-type cells. This delay at the G_1/S progression was equally reduced in *rad24*Δ and *rad24-K115E* cells. Thus, *rad24-K115E* appears to be a complete loss-of-function mutation, whereas *rad24-K115R* appears to be functionally equivalent to the wild-type gene. However, we show below that the *rad24-K115R* mutation confers a DNA damage checkpoint defect when combined with the *rfc5-1* mutation (see below). These results suggest that the NTP-binding motif is important for Rad24 function.

DNA damage checkpoints in *rfc5-1 rad24-K115R* double mutants. We have shown that Rad24 interacts physically with the RFC subunit Rfc5 (29). If these proteins function as a complex, its complex activity should be dependent on the properties of the proteins and abolished by loss of either protein. Consistently, *rfc5-1 rad24*Δ double mutants were defective in DNA damage checkpoints, similar to *rad24*Δ single mutants (Fig. 3, 4B, and 5B). Since the *rad24-K115R* mutation confers no apparent phenotype, we examined the genetic interaction between the *rad24-K115R* and *rfc5-1* mutations in the DNA damage checkpoints. We first evaluated the S-phase DNA damage checkpoint of the *rfc5-1* and *rfc5-1 rad24-K115R* double mutants. As observed previously (36), *rfc5-1* single mutant cells progressed through S phase faster than wild-type cells in the presence of MMS. The defect of *rfc5-1* single mutants in the S-phase checkpoint was similar to that of *rad24*Δ single and *rfc5-1 rad24-K115R* double mutants (Fig. 3). We next analyzed the G_2/M-phase DNA damage checkpoint in *rfc5-1* and *rfc5-1 rad24-K115R* double mutants. Although neither *rfc5-1* nor *rad24-K115R* single mutants were defective in the G_2/M-phase DNA damage checkpoint, *rfc5-1 rad24-K115R* double mutants became defective in this checkpoint; these double mutants failed to delay mitosis, similar to *rad24*Δ mutants (Fig. 4B). We further examined the G_1-phase DNA damage checkpoint in *rfc5-1* and *rfc5-1 rad24* double mutants. Although neither *rfc5-1* nor *rad24-K115R* cells were defective, *rfc5-1 rad24-K115R* double mutants were as defective as *rad24*Δ mutants in the G_1-phase DNA damage checkpoint (Fig. 5B). These results show that *RFC5* is involved in DNA damage checkpoint control not only in the S phase but also in the G_1 and G_2/M phases.

Rad53 phosphorylation in α-factor- or nocodazole-arrested *rfc5-1 rad24-K115R* double mutants. Rad53 is required for DNA damage checkpoint control and is hyperphosphorylated in response to DNA damage. Consistent with the role of Rad24 in the DNA damage checkpoints, *RAD24* is required for DNA damage-induced Rad53 phosphorylation (29, 41). Since *rfc5-1 rad24-K115R* double mutants become as defective as *rad24*Δ cells in the G_1- and G_2/M-phase DNA damage checkpoints, we expected that *rfc5-1 rad24-K115R* cells in the G_1 or G_2/M phase would be defective in DNA damage-induced Rad53 phosphorylation. To test this hypothesis, Rad53 phosphorylation following UV irradiation was examined by immunoblot analysis in cells arrested in the G_1 phase with α-factor or in the G_2/M phase with nocodazole (Fig. 6). Under these conditions, Rad53 hyperphosphorylation occurred in wild-type, *rfc5-1*, and *rad24-K115R* single mutant cells. However, Rad53 phosphorylation in *rfc5-1 rad24-K115R* double mutants was significantly reduced, similar to *rad24*Δ mutants in the G_1 and G_2/M phases. These results are consistent with the finding that *rfc5-1 rad24-K115R* double mutants are defective in the G_1- and G_2/M-phase DNA damage checkpoints and further support the idea that *RFC5* functions in the G_1- and G_2/M-phase checkpoints.

Effect of the *rfc5-1* mutation on the interaction between Rad24^{K115R} and the RFC proteins. We have shown previously that Rad24 associates with Rfc2 and Rfc5 (29). Next, we examined whether Rad24 interacts physically with the other RFC proteins Rfc1, Rfc3, and Rfc4. To detect these RFC proteins,

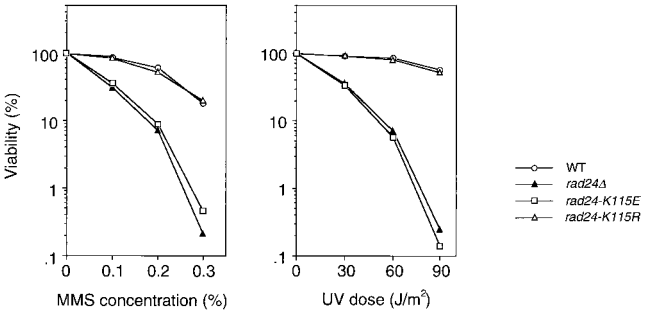

FIG. 2. DNA damage sensitivity in *rad24* mutants. Wild-type (WT) (KSC006), *rad24*Δ (KSC980), *rad24-K115E* (KSC1151), and *rad24-K115R* (KSC1152) cells were grown to log phase at 30°C and treated with MMS or irradiated with UV light. Viability of cells was estimated as described in Materials and Methods.

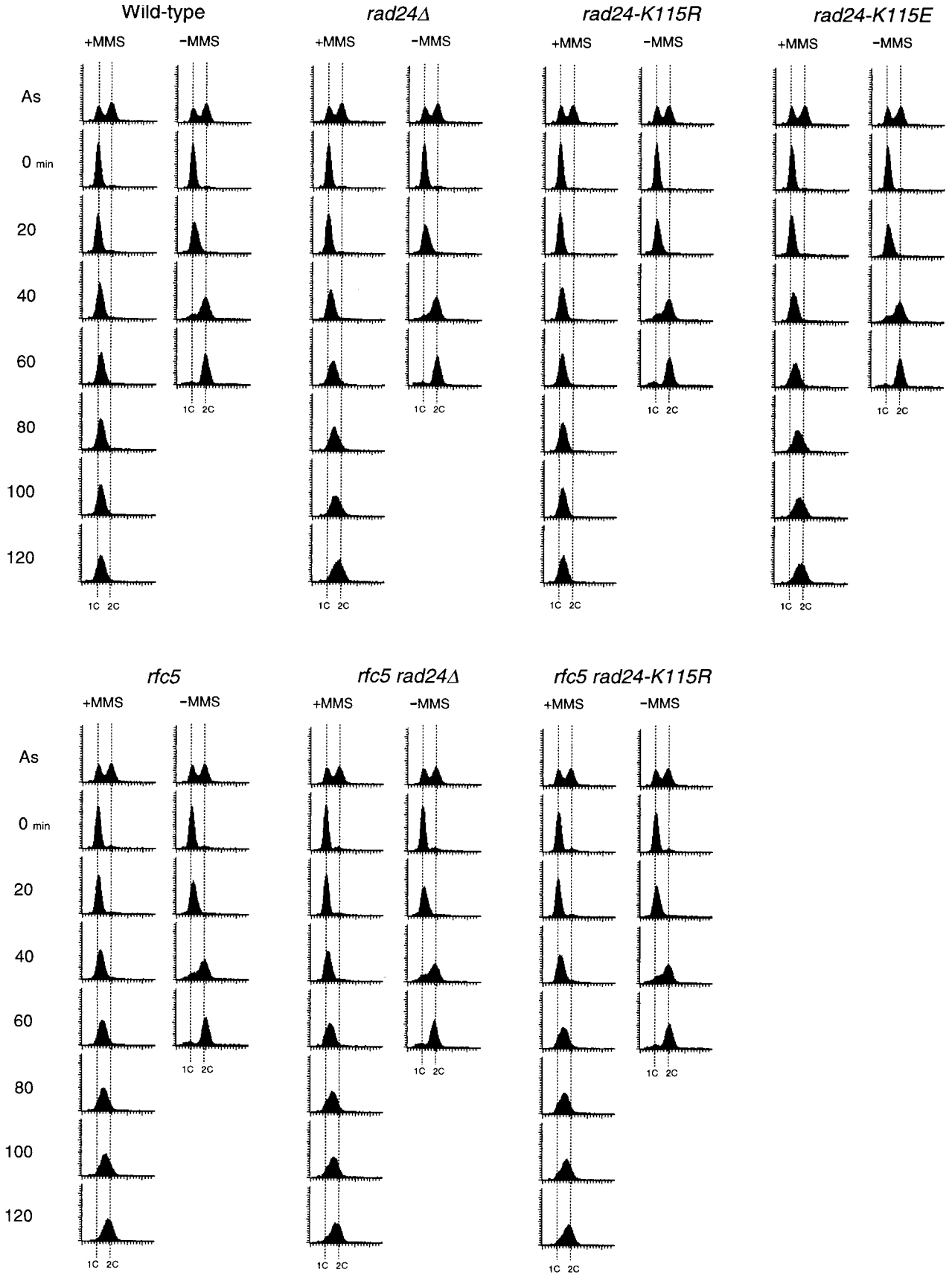

FIG. 3. S-phase DNA damage checkpoint in *rad24* and *rfc5-1 rad24* mutants. Wild-type (KSC006), *rad24*Δ (KSC980), *rad24-K115E* (KSC1151), *rad24-K115R* (KSC1152), *rfc5-1* (KSC835), *rfc5-1 rad24*Δ (KSC1105), and *rfc5-1 rad24-K115R* (KSC1161) cells were synchronized with α-factor in G_1 and released in either the presence (+) or the absence (−) of 0.05% MMS at 30°C as described in Materials and Methods. Aliquots of cells were collected at the indicated times after release from α-factor treatment and examined for DNA content by flow cytometry. Dotted lines indicate the DNA content of 1C and 2C cells. The top panels represent asynchronous (As) cells not treated with MMS at 30°C and are included as a reference.

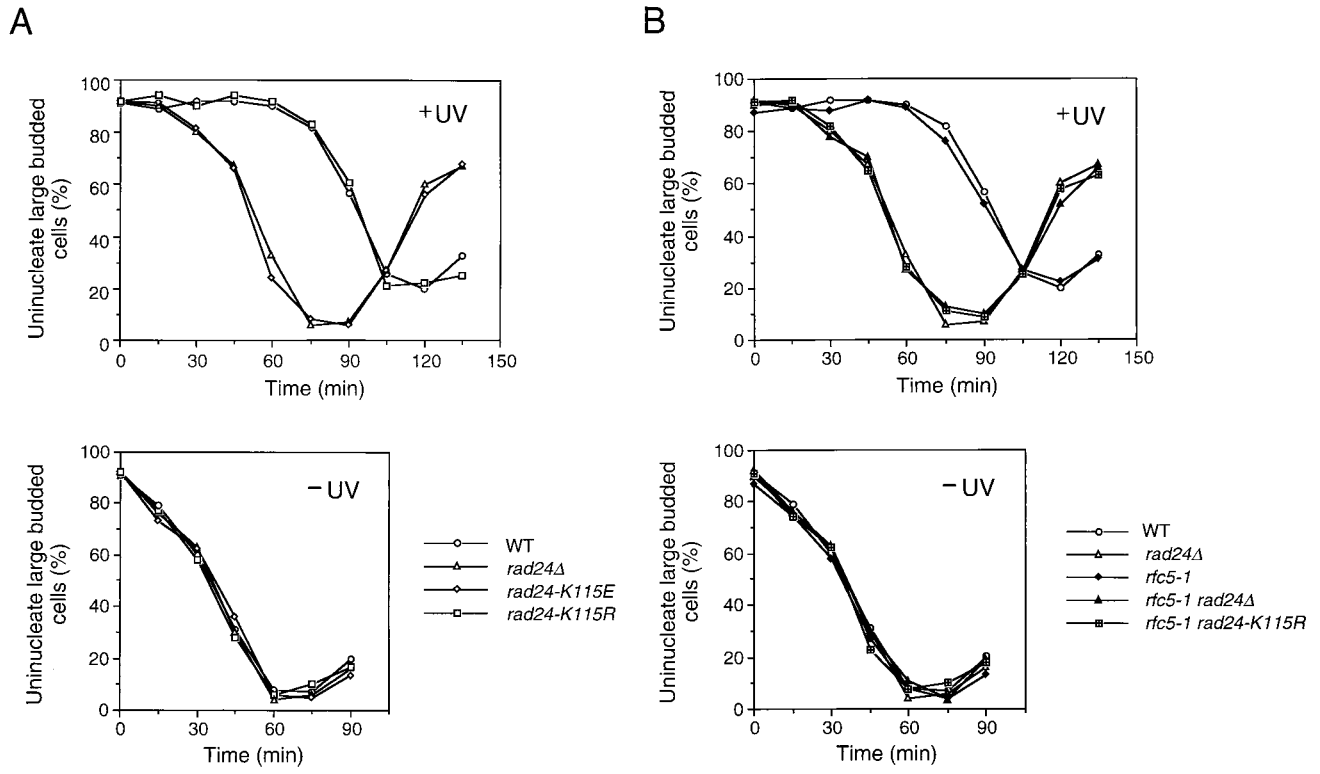

FIG. 4. G_2/M-phase DNA damage checkpoint in *rad24* and *rfc5-1 rad24* mutants. Cells were grown at 30°C, arrested with nocodazole, and irradiated or not irradiated with UV light. At the indicated times after release of UV-irradiated (+UV) and unirradiated (−UV) cultures from nocodazole, the percentage of uninucleate large budded cells was scored by DAPI staining. (A) Wild-type (WT) (KSC006), *rad24*Δ (KSC980), *rad24-K115E* (KSC1151), and *rad24-K115R* (KSC1152) cells; (B) wild-type (KSC006), *rfc5-1* (KSC835), *rad24*Δ (KSC980), *rfc5-1 rad24*Δ (KSC1105), and *rfc5-1 rad24-K115R* (KSC1161) cells.

we constructed cells containing HA-tagged versions of the *RFC1*, *RFC3*, and *RFC4* genes. A low-copy-number plasmid carrying *RAD24-myc* (YCpRad24-myc) or vector alone was transformed into cells containing the HA-tagged *RFC1* (*RFC1-HA*), *RFC3* (*RFC3-HA*), or *RFC4* (*RFC4-HA*) gene. Cell extracts were prepared and subjected to immunoprecipitation using anti-HA antibody. The immunocomplexes were analyzed by immunoblotting with anti-Rfc5 and anti-Myc antibodies. Immunoaffinity-purified anti-Rfc5 antibody recognized Rfc5 in immunocomplexes from *RFC1-HA* cells, but anti-Myc antibody failed to detect Rad24-myc (Fig. 7A). In contrast, Rad24-myc was detected in immunocomplexes from cells expressing Rad24-myc together with Rfc3-HA or Rfc4-HA (Fig. 7B and 7C). These and our previous observations show that Rad24 interacts physically with Rfc2, Rfc3, Rfc4, and Rfc5 but not with Rfc1. During preparation of the manuscript, Green et al. (7) presented similar results.

To understand the phenotype of *rfc5-1 rad24-K115R* double mutants, we next examined the interaction between the $Rad24^{K115R}$ and RFC proteins in wild-type and *rfc5-1* cells. *RFC5 RFC3-HA rad24*Δ and *rfc5-1 RFC3-HA rad24*Δ cells were transformed with YCpRAD24-myc or YCpRAD24-K115R-myc, and extracts prepared from the cells were examined by immunoblotting analysis with anti-Myc and anti-Rfc5 antibodies. The expression levels of the Rfc5 and Rad24 proteins were not significantly altered in either *RFC5* or *rfc5-1* cells (Fig. 8A). Cell extracts were also subjected to immunoprecipitation with anti-HA antibody followed by immunoblotting analysis with anti-Myc and anti-Rfc5 antibodies to detect coprecipitation of the Rad24-myc and Rfc5 proteins (Fig. 8A). In *RFC5* cells, the interaction of Rfc3-HA with $Rad24^{K115R}$-myc was slightly decreased compared to its interaction with Rad24-myc. In *rfc5-1* mutants, coprecipitation of Rad24-myc with Rfc3-HA was reduced compared to wild-type cells and strikingly, coprecipitation of $Rad24^{K115R}$-myc was undetectable. In *rfc5-1* mutants, the interaction between Rfc3-HA and Rfc5 was also decreased, suggesting that interactions among the other RFC proteins may also be affected. To address this possibility, we examined the interaction between Rfc2 and Rfc3 in *rfc5-1* mutants. Cell extracts were prepared from *RFC5 RFC3-HA* and *rfc5-1 RFC3-HA* cells and subjected to immunoprecipitation with anti-Rfc2 antibody. The immunoprecipitates were then analyzed by immunoblotting analysis with anti-Rfc2 and anti-HA antibodies. The interaction of Rfc2 with Rfc3-HA was decreased in *rfc5-1* mutants compared to wild-type cells, although the expression level of Rfc3-HA was not altered (Fig. 8B). Furthermore, in wild-type cells, the interaction of Rfc4-HA with $Rad24^{K115R}$-myc was reduced compared to its interaction with Rad24-myc, while in *rfc5-1* mutants no interaction of Rfc4-HA with $Rad24^{K115R}$-myc was detected (data not shown). These results indicate that the *rfc5-1* mutation causes a defect in the interaction between $Rad24^{K115R}$ and the RFC proteins. Together with the genetic observations provided above, these results suggest that the interaction of Rad24 with the RFC proteins is essential for DNA damage checkpoint control throughout the cell cycle.

DISCUSSION

RAD24 and *RFC5* are required for DNA damage checkpoint control in the budding yeast *S. cerevisiae*. The budding yeast RFC is composed of one large subunit, Rfc1, and four small

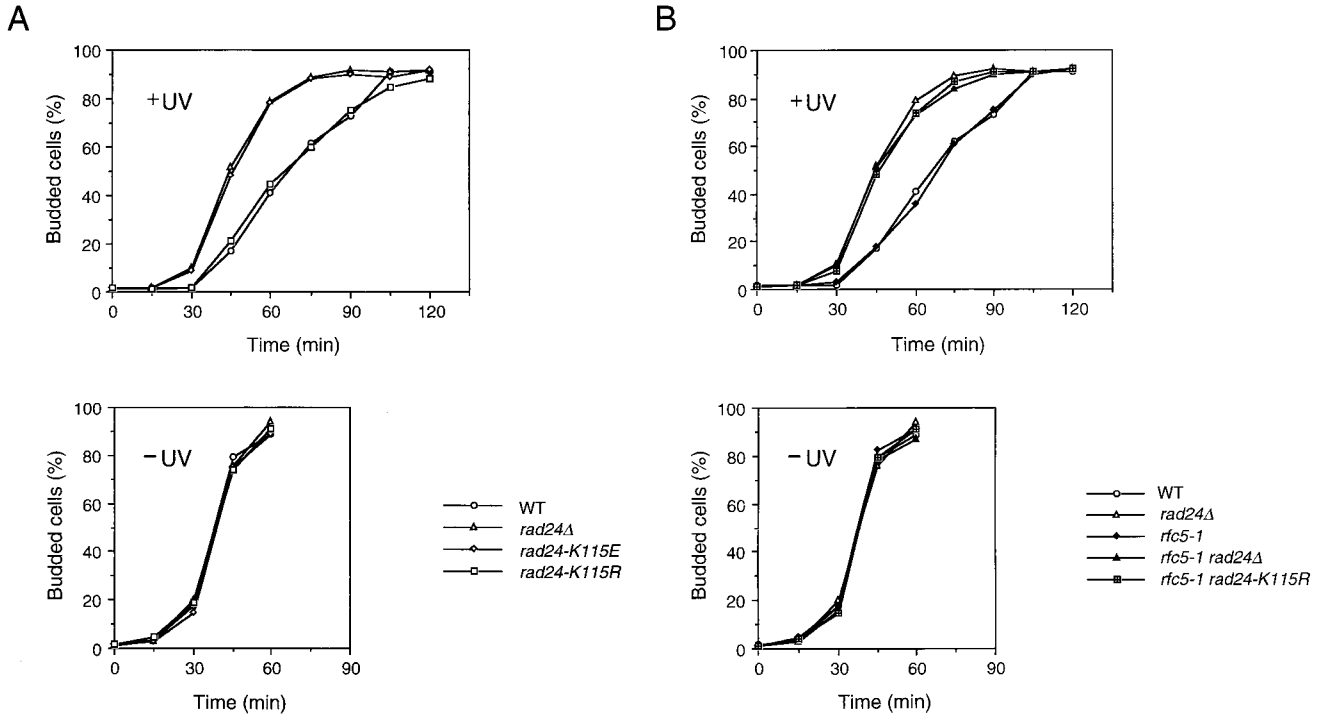

FIG. 5. G_1-phase DNA damage checkpoint in *rad24* and *rfc5-1 rad24* mutants. Cells were grown at 30°C, arrested with α-factor, and irradiated or not irradiated with UV light. The percentage of budded cells was scored at the indicated times after release of UV-irradiated (+UV) and unirradiated (−UV) cultures from α-factor. (A) Wild-type (WT) (KSC006), *rad24*Δ (KSC980), *rad24-K115E* (KSC1151), and *rad24-K115R* (KSC1152) cells; (B) wild-type (KSC006), *rfc5-1* (KSC835), *rad24*Δ (KSC980), *rfc5-1 rad24*Δ (KSC1105), and *rfc5-1 rad24-K115R* (KSC1161) cells.

subunits (Rfc2, Rfc3, Rfc4, and Rfc5). The RFC subunits are related to one another in their primary amino acid sequence. Rad24 and the RFC subunits also have sequence homology, including the presence of an NTP-binding motif. To evaluate the role of the NTP-binding motif in Rad24 function, we created two different mutations in the motif. One mutation, *rad24-K115E*, changes the conserved lysine to glutamic acid, converting a basic residue to an acidic residue. The other mutation, *rad24-K115R*, changes the conserved lysine to arginine, a similar basic amino acid. The phenotype of *rad24-K115E* mutants is identical to that of *rad24* null mutants, suggesting that the NTP-binding motif has an essential role in Rad24 function. It is noted, however, that such an extreme amino acid change may alter the conformation of the entire Rad24 protein. In contrast, *rad24-K115R* mutants show no apparent phenotype with respect to DNA damage sensitivity or DNA damage checkpoint control. The fission yeast Rad17 protein, a structural and functional homolog of Rad24, also contains an NTP-binding motif in the corresponding region. Previously, Griffiths et al. (8) mutated the conserved lysine[118] to arginine or glutamate in the Rad17 protein and obtained results essentially the same as ours; *rad17.K118R* cells showed no phenotype, whereas *rad17.K118E* cells were phenotypically similar to null mutants. These results are consistent with the current view that the function of checkpoint genes is highly conserved in eukaryotes.

RAD24 has an essential role in all the G_1-, S- and G_2/M-phase DNA damage checkpoints. We previously demonstrated that *rfc5-1* mutants are defective for the S-phase DNA damage checkpoint. Rad24 and Rfc5 interact physically and appear to function together in regulating the response to DNA damage. However, there was no evidence to suggest that *RFC5* has a role in the G_1- and/or G_2/M-phase DNA damage checkpoints. To address this possibility, we examined the G_1- and G_2/M-phase checkpoints in *rfc5-1 rad24-K115R* double mutants. Although neither *rfc5-1* nor *rad24-K115R* single mutants are defective in the G_1- and G_2/M-phase DNA damage checkpoints, *rfc5-1 rad24-K115R* double mutants become as defective in these checkpoints as *rad24*Δ mutants. Consistent with the idea that Rad24 and Rfc5 function as a complex that controls DNA damage checkpoints, the *rfc5-1* and *rad24*Δ mutations are genetically nonadditive with respect to the checkpoint defects in the G_1 and G_2/M phases. We also showed that *rfc5-1 rad24-K115R* and *rfc5-1 rad24*Δ double mutants are as defective as *rad24*Δ mutants in the S-phase DNA damage checkpoint. Rad53 is phosphorylated in response to DNA damage, and its phosphorylation correlates with the activation of the checkpoint pathway. Although *rad24-K115R* and *rfc5-1* single mu-

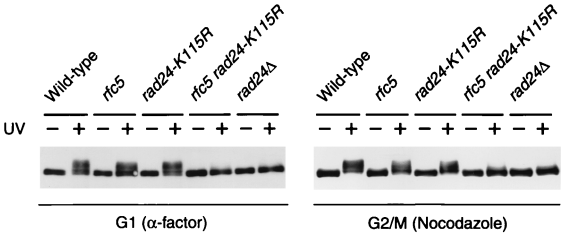

FIG. 6. DNA damage-induced Rad53 modification in G_1- and G_2/M-arrested *rfc5-1 rad24-K115R* mutants. Wild-type (KSC006), *rfc5-1* (KSC835), *rad24-K115R* (KSC1152), *rfc5-1 rad24-K115R* (KSC1161), and *rad24*Δ (KSC980) cells carrying YCpRAD53-HA were grown at 30°C, arrested in G_1 with α-factor or in G_2/M with nocodazole, and unirradiated (−) or irradiated with UV light (+). Cells were then incubated at 30°C, maintaining arrest in medium containing α-factor or nocodazole for 60 min, and subjected to immunoblotting analysis as described in Materials and Methods.

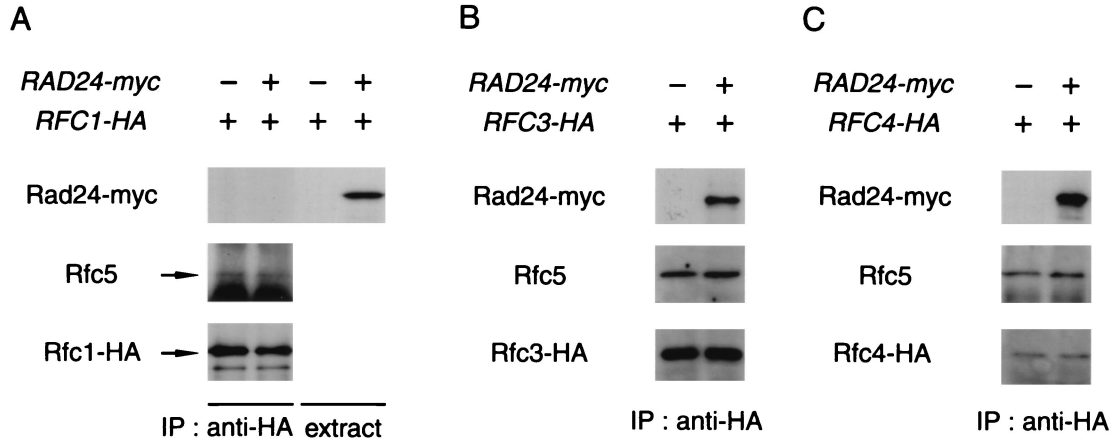

FIG. 7. Physical interaction of Rad24 with RFC proteins. Cells containing *RFC1-HA* (KSC1133) (A), *RFC3-HA* (KSC1163) (B), or *RFC4-HA* (KSC1164) (C) were transformed with YCpRAD24-myc or empty vector. Extracts prepared from the transformants were subjected to immunoprecipitation (IP) with anti-HA antibody. The immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-Myc, anti-Rfc5, or anti-HA antibody. Whole extracts were immunoblotted with anti-Myc antibody.

tants are not defective in DNA damage-induced Rad53 phosphorylation in the G_2/M phase, *rfc5-1 rad24-K115R* double mutants are defective. Thus, the *rfc5-1* mutation in the presence of the wild-type *RAD24* gene is defective only in the S-phase DNA damage checkpoint, while the *rfc5-1 rad24-K115R* double mutation becomes defective in all the G_1-, S- and G_2/M-phase DNA damage checkpoints. These observations suggest that *RFC5*, like *RAD24*, has a role in the DNA damage checkpoints throughout the cell cycle.

To further understand the phenotypes of *rfc5-1* and *rfc5-1 rad24-K115R* double mutants, we examined the physical interaction between the Rad24 and RFC proteins in wild-type and *rfc5-1* mutant cells. Coimmunoprecipitation experiments revealed that the interaction of Rad24^{K115R} with Rfc3 or Rfc4 is decreased in *RFC5* cells, despite the fact that the *rad24-K115R* mutation does not appear to affect the DNA damage responses. In *rfc5-1* mutants, the interaction between Rad24 and the RFC proteins is decreased compared to wild-type cells, and the interactions among the RFC proteins are also impaired. The *rfc5-1* mutation changes glycine to glutamate at amino acid position 43 in the Rfc5 NTP-binding motif (Fig. 1). Involvement of the NTP-binding motif in complex formation may be a common feature of Rad24 and RFC proteins. It was reported that mutation of the NTP-binding motif in the p140, p40, or p36 subunit of the human RFC complex results in decreased complex assembly and/or stability (25). One explanation why *rfc5-1* mutants are defective only in the S-phase checkpoint could be that the interaction between Rad24 and the RFC proteins is decreased specifically in the S phase. However, this possibility is unlikely because the interaction between Rad24 and Rfc3 in *rfc5-1* mutants was constant during the cell cycle (data not shown). It is, rather, possible that the

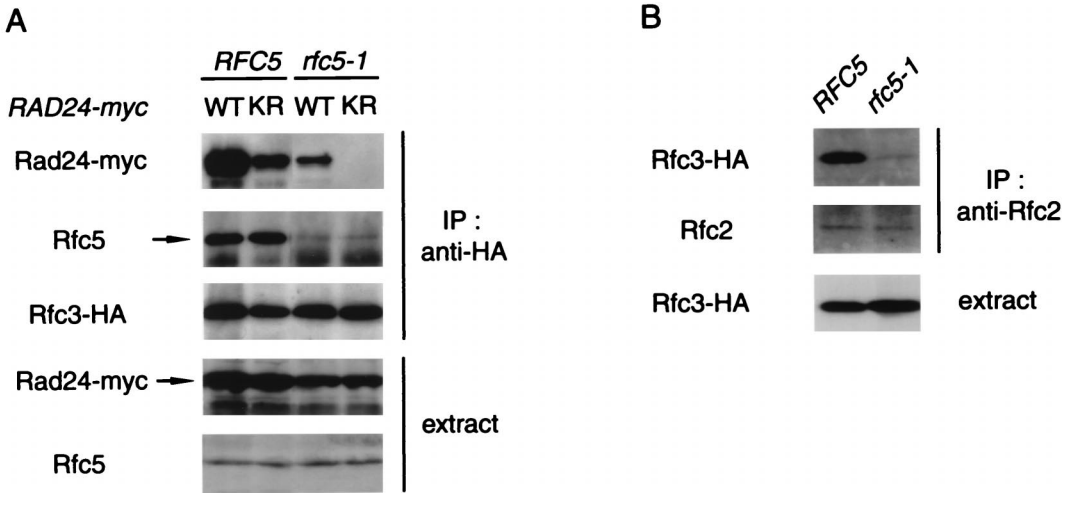

FIG. 8. Effect of the *rfc5-1* mutation on the interaction of Rfc3 with Rad24^{K115R} and Rfc2. (A) Interaction of Rad24^{K115R} with Rfc3 in *RFC5* and *rfc5-1* mutant cells. *RFC5 RFC3-HA rad24*Δ (KSC1168) and *rfc5-1 RFC3-HA rad24*Δ (KSC1170) cells were transformed with YCpRAD24-myc (WT) or YCpRAD24-K115R-myc (KR). Extracts prepared from the transformants were subjected to immunoprecipitation (IP) with anti-HA antibody. The extracts and immunocomplexes were separated by SDS-PAGE and immunoblotted with anti-Myc, anti-Rfc5 and anti-Myc, anti-Rfc5, or anti-HA antibody. (B) Interaction of Rfc3 with Rfc2 in *RFC5* and *rfc5-1* mutant cells. Extracts from *RFC5 RFC3-HA rad24*Δ (KSC1168) and *rfc5-1 RFC3-HA rad24*Δ (KSC1170) cells carrying YCpRAD24-myc were subjected to immunoprecipitation (IP) with anti-Rfc2 antibody. The extracts and immunocomplexes were separated by SDS-PAGE and immunoblotted with the corresponding antibody.

S-phase DNA damage checkpoint is more sensitive to the level of the interaction between Rad24 and the RFC proteins than the G_1- and G_2/M-phase DNA damage checkpoints.

Alternatively, the *rfc5-1* defect may result from alterations in Rfc5 function and/or impaired interactions among the RFC proteins. For example, DNA damage may be processed differently in the S phase and Rfc5 may be more specifically involved in recognition of this damage processing. The significance of the Rad24-RFC protein interaction in the DNA damage checkpoints is demonstrated from studies of *rfc5-1* mutants expressing $Rad24^{K115R}$. When expressed in *rfc5-1* mutants, $Rad24^{K115R}$ shows no detectable association with the RFC proteins. Accordingly, *rfc5-1 rad24-K115R* double mutants are defective in all the G_1-, S- and G_2/M-phase DNA damage checkpoints. Importantly, these *rfc5-1 rad24-K115R* double mutants are as defective as *rad24*Δ mutants in the DNA damage checkpoints. Thus, the interaction between Rad24 and the RFC proteins appears to be critical for the DNA damage checkpoints.

RFC is composed of one large subunit (Rfc1) and four small subunits (Rfc2, Rfc3, Rfc4, and Rfc5). RFC has an established role in recognizing the primer-template junction and loading PCNA onto the primer terminus. We have shown that Rad24 interacts physically with the small RFC subunits Rfc2, Rfc3, Rfc4, and Rfc5 but not with the large RFC subunit Rfc1. Recently, Green et al. (7) purified Rad24 to homogeneity and found that Rfc2 and Rfc3 copurify with Rad24. They also performed coimmunoprecipitation studies with Rad24 and the RFC subunits and obtained results similar to ours; Rad24 associates with Rfc2, Rfc3, Rfc4, and Rfc5 but not with Rfc1. They further showed that Rad24 does not cofractionate with Rfc1. These results suggest that Rad24 forms a complex closely related to but distinct from RFC and that the Rad24 complex functions in the DNA damage checkpoints. Genetic analysis has suggested that *RAD24* is involved in the same checkpoint pathway as *RAD17*, *MEC3*, and *DDC1* (7, 17, 18, 20). Rad17 has been suggested to share structural similarity with PCNA (40) and to function in a complex with Mec3 and Ddc1 (13). Interestingly, overexpression of *DDC1* suppresses the *rad24*Δ mutant phenotype (13). These observations raise the possibility that the Rad24-RFC proteins complex is required for loading the Rad17-Mec3-Ddc1 complex onto specific structures on damaged DNA.

In summary, our results suggest that the Rad24-RFC proteins complex functions in all the G_1-, S- and G_2/M-phase DNA damage checkpoints. It has been proposed that there may be one DNA damage surveillance system that functions throughout the cell cycle, as opposed to multiple distinct mechanisms that operate at different checkpoints (24). Future experiments will be necessary to determine whether the Rad24-RFC protein complex functions as an RFC-related complex, which might recognize DNA damage and recruit other checkpoint proteins.

ACKNOWLEDGMENTS

We thank A. Sugino for materials, M. Lamphier for critical readings of the manuscript, M. Mayer for discussion, and C. Green and N. Lowndes for communicating results prior to publication. K.S. acknowledges H. Mishima, Y. Miyake, H. Takahashi and H. Terasaki for suggestions and encouragement.

T.K. is a recipient of a JSPS predoctoral fellowship. This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas and General Research from the Ministry of Education, Science, Sports and Culture of Japan (K.M. and K.S.).

REFERENCES

1. **Allen, J. B., Z. Zhou, W. Siede, E. C. Friedberg, and S. J. Elledge.** 1994. The *SAD1/RAD53* protein kinase controls multiple checkpoints and DNA damage-induced transcription in yeast. Genes Dev. **8:**2416–2428.
2. **Burgers, P. M. J.** 1991. *Saccharomyces cerevisiae* replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerase δ and ε. J. Biol. Chem. **266:**22698–22706.
3. **Cross, F. R.** 1997. Marker swap plasmids: convenient tools for budding yeast molecular genetics. Yeast **13:**647–653.
4. **Cullmann, G., K. Fien, R. Kobayashi, and B. Stillman.** 1995. Characterization of the five replication factor C genes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**4661–4671.
5. **Elledge, S. J.** 1996. Cell cycle checkpoints: preventing an identity crisis. Science **274:**1664–1672.
6. **Gietz, R. D., and A. Sugino.** 1988. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene **74:**527–534.
7. **Green, C. M., H. Erdjument-Bromage, P. Tempst, and N. F. Lowndes.** 2000. A novel Rad24 checkpoint protein complex closely related to replication factor C. Curr. Biol. **10:**39–42.
8. **Griffiths, D. J. F., N. C. Barbet, S. McCready, A. R. Lehmann, and A. M. Carr.** 1995. Fission yeast *rad17*; a homologue of budding yeast *RAD24* that shares regions of sequence similarity with DNA polymerase accessory proteins. EMBO J. **14:**5812–5823.
9. **Guthrie, C., and G. R. Fink.** 1991. Guide to yeast genetics and molecular biology. Academic Press, New York, N.Y.
10. **Hartwell, L. H., and T. A. Weinert.** 1989. Checkpoints: controls that ensure the order of cell cycle events. Science **246:**229–234.
11. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
12. **Kato, R., and H. Ogawa.** 1994. An essential gene, *ESR1*, is required for mitotic cell growth, DNA repair, and meiotic recombination in *Saccharomyces cerevisiae*. Nucleic Acids Res. **22:**3104–3112.
13. **Kondo, T., K. Matsumoto, and K. Sugimoto.** 1999. Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway. Mol. Cell. Biol. **19:**1136–1143.
14. **Koonin, E. V.** 1993. A superfamily of ATPases with diverse functions containing either classical or deviant ATP-binding motif. J. Mol. Biol. **229:**1165–1174.
15. **Lee, S.-H., A. D. Kwong, Z.-Q. Pan, and J. Hurwitz.** 1991. Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase δ. J. Biol. Chem. **266:**594–602.
16. **Longhese, M. P., M. Foiani, M. Muzi-Falconi, G. Lucchini, and P. Plevani.** 1998. DNA damage checkpoint in budding yeast. EMBO J. **17:**5525–5528.
17. **Longhese, M. P., R. Fraschini, P. Plevani, and G. Lucchini.** 1996. Yeast *pip3/mec3* mutants fail to delay entry into S phase and to slow down DNA replication in response to DNA damage, and they define a functional link between Mec3 and DNA primase. Mol. Cell. Biol. **16:**3235–3244.
18. **Longhese, M. P., V. Paciotti, R. Fraschini, R. Zaccarini, P. Plevani, and G. Lucchini.** 1997. The novel DNA damage checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast. EMBO J. **17:**5216–5226.
19. **Lydall, D., and T. Weinert.** 1997. G2/M checkpoint genes of *Saccharomyces cerevisiae*: further evidence for roles in DNA replication and/or repair. Mol. Gen. Genet. **256:**638–651.
20. **Lydall, D., and T. Weinert.** 1995. Yeast checkpoint genes in DNA damage processing: implications for repair and arrest. Science **270:**1488–1491.
21. **Paciotti, V., G. Lucchini, P. Plevani, and M. P. Longhese.** 1998. Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p. EMBO J. **17:**4199–4209.
22. **Paulovich, A. G., and L. H. Hartwell.** 1995. A checkpoint regulates the rate of progression through S phase in *S. cerevisiae* in response to DNA damage. Cell **82:**841–847.
23. **Paulovich, A. G., R. U. Margulies, B. M. Garvik, and L. H. Hartwell.** 1997. *RAD9*, *RAD17*, and *RAD24* are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. Genetics **145:**45–62.
24. **Paulovich, A. G., D. P. Toczyski, and L. H. Harwell.** 1997. When checkpoints fail. Cell **88:**315–321.
25. **Podust, V. N., N. Tiwari, R. Ott, and E. Fanning.** 1998. Functional interactions among the subunits of replication factor C potentiate and modulate its ATPase activity. J. Biol. Chem. **273:**12935–12942.
26. **Sanchez, Y., B. A. Desany, W. J. Jones, Q. Liu, B. Wang, and S. J. Elledge.** 1996. Regulation of *RAD53* by the *ATM*-like kinase *MEC1* and *TEL1* in yeast cell cycle checkpoint pathways. Science **271:**357–360.
27. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:**430–434.
28. **Savitsky, K., A. Bar-Shira, S. Gilad, G. Rotman, Y. Ziv, L. Vanagaite, D. A. Tagle, S. Smith, T. Uziel, S. Sfez, M. Ashkenazi, I. Pecker, M. Frydman, R. Harnik, S. R. Patanajali, A. Simmons, G. A. Clines, A. Sartiel, R. A. Gatti, L. Chessa, O. Sanal, M. F. Lavin, N. G. J. Jaspers, A. M. R. Taylor, C. F. Arlett, T. Miki, S. M. Weissman, M. Lovett, F. S. Collins, and Y. Shiloh.** 1995. A single ataxia telangiectasia gene with a product similar to PI-3 kinase. Science **268:**1749–1753.
29. **Shimomura, T., S. Ando, K. Matsumoto, and K. Sugimoto.** 1998. Functional

and physical interaction between Rad24 and Rfc5 in the yeast checkpoint pathways. Mol. Cell. Biol. **18:**5485–5491.
30. **Siede, W., J. B. Allen, S. J. Elledge, and E. C. Friedberg.** 1996. The *Saccharomyces cerevisiae MEC1* gene, which encodes a homolog of the human ATM gene product, is required for G_1 arrest following radiation treatment. J. Bacteriol. **178:**5841–5843.
31. **Siede, W., A. S. Friedberg, I. Dianova, and E. C. Friedberg.** 1994. Characterization of G1 checkpoint control in the yeast *Saccharomyces cerevisiae* following exposure to DNA-damaging agent. Genetics **138:**271–281.
32. **Siede, W., A. S. Friedberg, and E. C. Friedberg.** 1993. RAD9-dependent G1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **90:**7985–7989.
33. **Siede, W., G. Nusspaumer, V. Portillo, R. Rodriguez, and E. C. Friedberg.** 1996. Cloning and characterization of *RAD17*, a gene controlling cell cycle responses to DNA damage in *Saccharomyces cerevisiae*. Nucleic Acids Res. **24:**1669–1675.
34. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
35. **Stern, D. F., P. Zheng, D. R. Beidler, and C. Zerillo.** 1991. Spk1, a new kinase from *Saccharomyces cerevisiae*, phosphorylates proteins on serine, threonine, and tyrosine. Mol. Cell. Biol. **13:**3744–3755.
36. **Sugimoto, K., S. Ando, T. Shimomura, and K. Matsumoto.** 1997. Rfc5, replication factor C component, is required for regulation of Rad53 protein kinase in the yeast checkpoint pathway. Mol. Cell. Biol. **17:**5905–5914.
37. **Sugimoto, K., Y. Sakamoto, O. Takahashi, and K. Matsumoto.** 1995. *HYS2*, an essential gene required for DNA replication in *Saccharomyces cerevisiae*. Nucleic Acids Res. **23:**3493–3500.
38. **Sugimoto, K., T. Shimomura, K. Hashimoto, H. Araki, A. Sugino, and K. Matsumoto.** 1996. Rfc5, a small subunit of replication factor C complex, couples DNA replication and mitosis in budding yeast. Proc. Natl. Acad. Sci. USA **93:**7048–7052.
39. **Sun, Z., D. S. Fay, F. Marini, M. Foiani, and D. F. Stern.** 1996. Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. Genes Dev. **10:**395–406.
40. **Thelen, M. P., C. Venclovas, and K. Fidelis.** 1999. A sliding clamp model for the Rad1 family of cell cycle checkpoint proteins. Cell **19:**769–770.
41. **Torre-Ruiz, M.-A., C. M. Green, and N. F. Lowndes.** 1998. *RAD9* and *RAD24* define two additive, interacting branches of the DNA damage checkpoint pathway in budding yeast normally required for Rad53 modification and activation. EMBO J. **17:**2687–2698.
42. **Tsurimoto, T., and B. Stillman.** 1990. Functions of replication factor C and proliferating-cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. Proc. Natl. Acad. Sci. USA **87:**1023–1027.
43. **Weinert, T. A., and L. H. Hartwell.** 1993. Cell cycle arrest of cdc mutants and specificity of the *RAD9* checkpoint. Genetics **134:**63–80.
44. **Weinert, T. A., and L. H. Hartwell.** 1988. The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science **241:**317–322.
45. **Weinert, T. A., G. L. Kiser, and L. H. Hartwell.** 1994. Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. Genes Dev. **8:**652–665.