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Biochemistry

Mutation of cysteine-88 in the *Saccharomyces cerevisiae* RAD6 protein abolishes its ubiquitin-conjugating activity and its various biological functions

(DNA repair/mutagenesis/sporulation/histone ubiquitination)

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ABSTRACT **The *RAD6* gene of *Saccharomyces cerevisiae* is required for DNA repair, DNA damage-induced mutagenesis, and sporulation. RAD6 protein is a ubiquitin-conjugating enzyme (E2) that has been shown to attach multiple molecules of ubiquitin to histones H2A and H2B. We have now examined whether the E2 activity of RAD6 is involved in its various biological functions. Since the formation of a thioester adduct between E2 and ubiquitin is necessary for E2 activity, the single cysteine residue (Cys-88) present in RAD6 was changed to alanine or valine. The mutant proteins were overproduced in yeast cells and purified to near homogeneity. We show that the rad6 Ala-88 and rad6 Val-88 mutant proteins lack the capacity for thioester formation with ubiquitin and, as a consequence, are totally devoid of any E2 activity. The *rad6 Ala-88* and *rad6 Val-88* mutations confer a defect in DNA repair, mutagenesis, and sporulation equivalent to that in the *rad6* null allele. We suggest that the biological functions of RAD6 require its E2 activity.**

The *RAD6* gene of *Saccharomyces cerevisiae* is required for a variety of cellular processes. *rad6* mutants are extremely sensitive to DNA-damaging agents such as UV light, ionizing radiation, and alkylating agents (1–3), and they lack the ability to convert DNA lesions into mutations (3–5). In addition, *rad6* mutants are defective in meiotic recombination and do not undergo sporulation (6, 7).

The *RAD6* gene encodes a 172-residue, 20-kDa protein whose carboxyl terminus is striking in that 20 of the last 23 residues are acidic (8). The 23-residue polyacidic sequence adopts a disordered, linear configuration and is appended as a freely extending tail to a globular domain comprising the first 149 residues (9).

RAD6 is among at least five ubiquitin-conjugating (E2) enzymes that can be isolated from cell extracts by affinity chromatography on ubiquitin-Sepharose (10). Ubiquitin, so named because of its ubiquitous presence in eukaryotes, is a 76-residue polypeptide that exists in the cell either in its free state or covalently conjugated to certain proteins. Ubiquitin conjugation serves to mark cellular proteins for degradation via an ATP-dependent proteolytic system or to modulate the biological activity of the proteins in a nonproteolytic fashion (reviewed in ref. 11). The sequence of biochemical events leading to the attachment of ubiquitin to target proteins has been clarified by using pertinent enzymatic components isolated from rabbit reticulocytes (refs. 12 and 13; reviewed in ref. 11). The carboxyl-terminal glycine of ubiquitin is joined covalently, in an ATP-dependent reaction, first to the ubiquitin-activating enzyme (E1) and then to ubiquitin-conjugating enzymes (E2), through thioester linkages. The E2 enzymes then catalyze the formation of an isopeptide bond between ubiquitin and the ε-amino groups of lysines in the protein substrates, a step that may or may not require an additional protein factor designated E3.

The E2 activity of RAD6 (10), in the absence of E3, mediates the ligation of as many as seven molecules of ubiquitin onto histones H2A and H2B (14). Since a multiply ubiquitinated configuration appears to be a requisite feature of protein substrates destined for degradation, RAD6 ubiquitin ligase may mark histones for degradation *in vivo* (14). Alternatively, polyubiquitination of histones may facilitate the opening of chromatin by perturbing nucleosome structure. The histone-ubiquitinating activity of RAD6 is strongly dependent on the carboxyl-terminal polyacidic tail, as the protein product of *rad6-149*, missing the entire acidic tail, is minimally active in histone ubiquitination (14). Yeast strains carrying the *rad6-149* mutation are proficient in DNA repair and in DNA damage-induced mutagenesis but are defective in sporulation (9).

The aforementioned observations with the *rad6-149* mutation suggested that RAD6 may modulate its DNA repair and mutagenesis functions by means other than its E2 activity. Several examples occur, among DNA repair proteins, where different biological functions of a protein are mediated by distinct biochemical activities. For example, the *Escherichia coli* RecA protein, required for genetic recombination, DNA repair, and DNA damage-induced mutagenesis, possesses different biochemical activities that allow it to promote homologous pairing and to inactivate the LexA repressor (15, 16). The RecA protein binds to single-stranded DNA in the presence of ATP and promotes pairing of a single strand with duplex DNA, resulting in the formation of long heteroduplex regions (17, 18). ATP hydrolysis by RecA is essential for strand exchange to occur. In cells that have been exposed to DNA-damaging agents, RecA also functions as a protease, promoting the cleavage of the LexA repressor, resulting in elevated transcription of ≈20 SOS-inducible genes (15, 16). Mutations that differentially inactivate one but not the other biochemical activity of RecA affect only the relevant biological process. Thus, *recA* mutants defective in the protease activity are proficient in recombination but devoid of the ability to induce SOS genes or to undergo UV mutagenesis (15), whereas a deficiency in ATPase activity affects recombination but not SOS induction (19). The *RAD3* gene of *S. cerevisiae* is also multifunctional; it is required for excision repair of UV-damaged DNA and is also essential for cell viability. The RAD3 protein possesses a single-stranded DNA-dependent ATPase activity and a DNA helicase activity (20, 21). *rad3* mutants lacking the ATPase and DNA

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Abbreviations: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme.

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helicase activities are defective in excision repair but viable (22), indicating that the viability function of RAD3 is not dependent on these activities.

To examine the significance of the E2 activity of RAD6 in its various biological roles, we have changed Cys-88 in the RAD6 protein to alanine and valine. We show that the resulting rad6 mutant proteins lack E2 activity and that these mutations confer a defect in DNA repair, mutagenesis, and sporulation. Hence, our observations provide evidence that these biological functions of RAD6 are dependent upon its E2 activity.

MATERIALS AND METHODS

Yeast Strains. The *S. cerevisiae* strains used are listed in Table 1.

Construction of *rad6* Mutant Alleles. Mutations of Cys-88 in the RAD6 protein were obtained by *in vitro* site-directed mutagenesis using a *dut ung E. coli* strain to enrich for the mutant allele (23). Two alleles, *rad6 Ala-88* and *rad6 Val-88*, were generated by changing the TGT (Cys) codon to GCT (Ala) and GTT (Val), respectively. The nature of the change in each mutant was verified by DNA sequencing.

Plasmids. Table 2 lists the plasmids used. pR611 is a low-copy centromeric (*CEN*) plasmid derived from YCp50. pR611 contains 5′ and 3′ flanking DNA regions of *RAD6* but lacks the 0.6-kilobase *Eco*RI fragment containing the protein-coding region of *RAD6* (8). An *Eco*RI fragment containing either the *rad6 Ala-88* or the *rad6 Val-88* mutant allele was inserted into the unique *Eco*RI site of plasmid pR611 to generate pR647 and pR654, respectively. Overproduction of RAD6 protein was achieved by fusing the *RAD6* protein-coding region, contained within the 0.6-kilobase *Eco*RI fragment, to the promoter of the highly expressed constitutive alcohol dehydrogenase I (*ADC1*) gene of *S. cerevisiae*, yielding the plasmid pSCW242 (9). The mutant proteins were overproduced by replacing the *RAD6*-containing *Eco*RI fragment of pSCW242 with the *Eco*RI fragment containing the mutant allele *rad6 Ala-88* or *rad6 Val-88*, generating plasmids pR648 and pR655, respectively.

Protein Purification. The wild-type (RAD6) and mutant (rad6) proteins were purified by a procedure that does not entail affinity chromatography on ubiquitin-Sepharose (14), as the two rad6 mutant proteins do not form a thioester adduct with ubiquitin (see *Results*). Strain EMY1 (*rad6Δ*) was used as the host for plasmids pSCW242, pR648, and pR655, corresponding to fusions of the *RAD6*, *rad6 Ala-88*, and *rad6 Val-88* genes, respectively, to the *ADC1* promoter. The cell breakage buffer was 50 mM Tris/HCl, pH 7.5/10% sucrose/10 mM EDTA/200 mM KCl/10 mM 2-mercaptoethanol, and the column buffer (buffer A) was 15 mM KH_2PO_4, pH 7.4/10% (vol/vol) glycerol/0.5 mM EDTA/0.5 mM dithiothreitol. The column equilibration buffer (buffer B) was buffer A with 180 mM KCl. The following protease inhibitors were used up to the penultimate chromatographic step: benzamidine hydrochloride and phenylmethylsulfonyl fluoride at 1 mM, and aprotinin, chymostatin, leupeptin, and pepstatin A at 5 μg/ml. Cells were cultured in synthetic medium lacking tryptophan, and 50 g was disrupted by passage through a French press at 137.8 MPa (20,000 psi) in 75 ml of cell breakage buffer. Cell breakage and subsequent steps were carried out at 0–4°C. The clear supernatant obtained after high-speed centrifugation (100,000 × *g*, 90 min) was layered onto a column of DEAE-Sephacel (2.6 × 9.5 cm; 50 ml total). The column was washed with 150 ml of buffer B and bound material was eluted with 400 mM KCl in buffer A. Fractions corresponding to the protein peak were identified using the Coomassie dye binding assay of Bradford (24), pooled (20 ml), and diluted with 10% glycerol until the conductivity was equivalent to that of 170 mM KCl in water. The diluted pool was applied onto another column of DEAE-Sephacel (1.6 × 5 cm; 10 ml total), which was washed with 40 ml of buffer B and developed with a 150-ml gradient of 180–410 mM KCl in buffer A. Fractions containing RAD6 or rad6 protein (eluted at 300–330 mM KCl) were identified by $NaDodSO_4$/PAGE, pooled (10 ml), and diluted with 0.5 volume of water. Further enrichment of the RAD6 and rad6 proteins was achieved using an FPLC Mono Q column (HR 5/5; Pharmacia), which, after sample loading, was washed with 5 ml of buffer B and developed with a 25-ml gradient of 200–550 mM KCl in buffer A. The RAD6 and rad6 proteins (eluted at 420–430 mM KCl; 5 mg in 1.5 ml) were judged by $NaDodSO_4$/PAGE to be nearly homogeneous and were concentrated to 10 mg/ml with a Centricon-10 concentrator (Amicon) before being stored in 100-μl aliquots at −70°C.

E1 was purified by a combination of affinity chromatography on ubiquitin-Sepharose and FPLC on a Mono Q column essentially as described (14).

Assays. Reaction mixtures were assembled in buffer R [50 mM Tris/HCl, pH 7.5/6 mM $MgCl_2$/2 mM ATP/0.1 mM dithiothreitol with bovine serum albumin at 100 μg/ml, containing approximately 50 mM KCl and 3 mM KH_2PO_4 (pH 7.4) due to the enzyme additions] and contained 200 ng of RAD6 or rad6 protein, 400 ng of E1, and 300 ng of ^{125}I-labeled ubiquitin, with or without 1 μg of bovine histone H2B. After incubation at 30°C, the assay mixtures were combined with 10 μl of stop buffer (200 mM Tris/HCl, pH 6.8/6% $NaDodSO_4$/30% glycerol/0.06% bromophenol blue/15% 2-mercaptoethanol), boiled for 3 min, and electrophoresed in denaturing 13% gels at 1 mA/cm for 4 hr. The gels were dried, and the radioactive bands were revealed by autoradiography.

Table 2. Plasmids used

Plasmid	Vector	Allele
pR67	*CEN*	*RAD6*$^+$
pR611	*CEN*	*rad6Δ*
pR647	*CEN*	*rad6 Ala-88*
pR654	*CEN*	*rad6 Val-88*
pSCW242	2-μ *ADC1*	*RAD6*$^+$
pR648	2-μ *ADC1*	*rad6 Ala-88*
pR655	2-μ *ADC1*	*rad6 Val-88*

The *CEN* plasmids are derivatives of YCp50; the 2-μ plasmids are derivatives of the yeast 2-μm plasmid.

Table 1. Strains used

Strain	Genotype	Source
LP3041-6D	*MATa leu2-3 leu2-112 trp1Δ ura3-52*	This study
EMY1	*MATa leu2-3 leu2-112 trp1Δ ura3-52 rad6Δ::LEU2*$^+$	This study
839	*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3*	F. Sherman*
EMY7	*MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 rad6Δ::LEU2*$^+$	This study
RS33-2C/6Δ	*MATa arg4-17 his4-17 leu2-3 leu2-112 trp1-289 ura3-52 rad6Δ::LEU2*$^+$	This study
EMY26	EMY1 × EMY7	This study

The *rad6Δ* strain EMY1 was derived from LP3041-6D and the *rad6Δ* strain EMY7 was derived from strain 839 by gene replacement.

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To detect the formation of thioesters between ubiquitin and RAD6 or rad6 protein, 2-mercaptoethanol was omitted from the stop buffer and the boiling step was eliminated.

Other Procedures. Yeast cells were transformed with plasmid DNA by the method of Ito *et al.* (25). $NaDodSO_4$/PAGE was carried out according to Laemmli (26), and proteins in gels were revealed by staining with 0.1% Coomassie blue R. ^{125}I-labeling of bovine ubiquitin with chloramine T and Western analysis with affinity-purified anti-RAD6 antibodies were carried out as described (14).

RESULTS

Mutagenesis of Cys-88 in the RAD6 Protein. In the E2-catalyzed ligation of ubiquitin to cellular target proteins, E2 itself forms a transient covalent intermediate with ubiquitin in an ATP-dependent reaction mediated by E1 (12, 13). The chemical linkage between E2 and ubiquitin is readily cleaved by treatment with sulfhydryl-containing reagents, indicating that it is thioester in nature (12). The *RAD6*-encoded protein (8) possesses a sole cysteine, corresponding to the 88th residue. To inactivate the E2 function of RAD6, we altered Cys-88 to alanine and valine, as these changes are least likely to disrupt the conformation of the protein (27).

The *rad6 Ala-88* and *rad6 Val-88* mutant alleles were cloned into the yeast low-copy centromeric vector pR611 to yield plasmids pR647 and pR654, respectively. To verify that the mutant *rad6* alleles are expressed in yeast, the *rad6Δ* strain EMY1 was transformed with plasmids pR647 and pR654, and the intracellular level of the mutant rad6 proteins was determined by Western immunoblot analysis. The amount of rad6 Ala-88 and rad6 Val-88 mutant proteins in strain EMY1 was comparable to that produced by the wild-type *RAD6* allele carried on the centromeric plasmid pR67. Furthermore, the mutant proteins displayed the same electrophoretic mobility as the wild-type protein (Fig. 1). These observations indicated that the rad6 Ala-88 and rad6 Val-88 mutant proteins have similar stability *in vivo* as the wild-type RAD6 protein.

Purification of Wild-Type RAD6 and rad6 Ala-88 and rad6 Val-88 Mutant Proteins. To overproduce RAD6, rad6 Ala-88, and rad6 Val-88 proteins in yeast for purification and subsequent biochemical analyses, the coding frames for the proteins were placed under the control of the *ADC1* promoter. Cells of the *rad6Δ* strain EMY1 harboring the relevant plasmids were disrupted in a French press, and extracts containing the RAD6 and rad6 proteins were fractionated by anion-exchange chromatography on DEAE-Sephacel and on an FPLC Mono Q column. No contaminating proteins were observed when 2-μg samples of the purified RAD6 and rad6 proteins were analyzed in a denaturing 13% polyacrylamide gel stained with Coomassie blue (Fig. 2). During purification, the mutant rad6 proteins did not display any anomalous chromatographic properties, nor were they degraded more rapidly than the wild-type RAD6 protein by proteases present in the cell extracts.

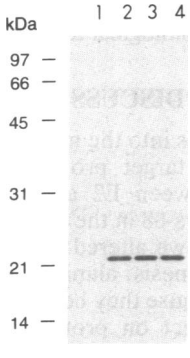

FIG. 1. Western analysis of cell extracts containing RAD6 and rad6 proteins. The nitrocellulose blot of a $NaDodSO_4$/13% polyacrylamide gel was probed with affinity-purified anti-RAD6 antibodies and developed by the indirect peroxidase procedure (21). Lanes 1–4 correspond to extracts from 5×10^6 cells of the *rad6Δ* strain EMY1 harboring pR611 (vector, no insert), pR67 (*RAD6*), pR647 (*rad6 Ala-88*), and pR654 (*rad6 Val-88*), respectively. To prepare extracts, cells were suspended in 2× electrophoresis sample buffer (125 mM Tris/HCl, pH 6.8/4% $NaDodSO_4$/20% glycerol/5% 2-mercaptoethanol/0.04% bromophenol blue) at 10^6 cells per μl, frozen in liquid nitrogen, and then boiled for 3 min; this freezing–boiling cycle was repeated twice more. The samples were clarified by centrifugation before being analyzed.

rad6 Ala-88 and rad6 Val-88 Mutant Proteins Lack E2 Activity. Since E2 enzymes mediate the attachment of ubiquitin to target proteins via their ability to form a thioester conjugate with ubiquitin (12), we first examined whether the purified rad6 Ala-88 and Val-88 proteins could accept activated ubiquitin from E1. The mutant proteins were incubated, alongside wild-type RAD6, with ^{125}I-labeled ubiquitin, E1, and ATP, and a prolonged reaction time of 1.2 hr was chosen to enhance the limit of detection. Two major radioactive species were observed with the RAD6 protein, corresponding to the RAD6–ubiquitin (uRAD6, 30 kDa) and E1-ubiquitin (uE1, >130 kDa) thioesters (Fig. 3*A*, lane 1). In contrast, with the rad6 Ala-88 and rad6 Val-88 proteins, no rad6–ubiquitin conjugate was observed (lanes 2 and 3), indicating that Cys-88 is indeed the site of ubiquitin attachment in RAD6.

We have previously shown that RAD6 catalyzes the ligation of multiple molecules of ubiquitin onto histones H2A and H2B (14). To confirm that the inability of rad6 Ala-88 and rad6 Val-88 proteins to form a thioester linkage with ubiquitin is associated with loss of histone-ubiquitinating activity, the mutant proteins were used in ubiquitin-conjugation assays containing H2A and H2B as substrates. While incubation of histone H2B with wild-type RAD6 protein resulted in the attachment of multiple ubiquitin molecules to H2B (Fig. 3*B*, lane 1), as reported previously (14), this histone-modifying activity was not detected with rad6 Ala-88 and rad6 Val-88 proteins (lanes 3 and 4). Similar results were obtained with histone H2A (results not shown). Thus, rad6 Ala-88 and rad6 Val-88 proteins lack the ability to form a thioester conjugate with ubiquitin and, as a consequence, cannot catalyze ubiquitination of histones.

***rad6 Ala-88* and *rad6 Val-88* Mutants Are Defective in DNA Repair, Damage-Induced Mutagenesis, and Sporulation.** The effects of the Cys-88 → Ala and Cys-88 → Val mutations on the DNA-repair function of RAD6 were examined in the *rad6Δ* strain EMY7. Fig. 4 shows the UV survival of EMY7

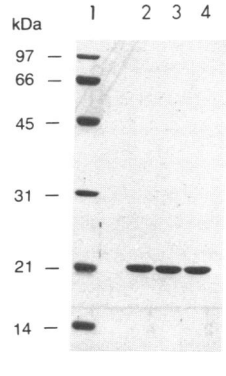

FIG. 2. Purity of RAD6 and rad6 proteins as determined by $NaDodSO_4$/13% PAGE (1 mA/cm, 4 hr) followed by staining with Coomassie blue R. Lane 1, molecular mass markers (1 μg each); lanes 2–4, RAD6, rad6 Ala-88, and rad6 Val-88 proteins (2 μg each), respectively.

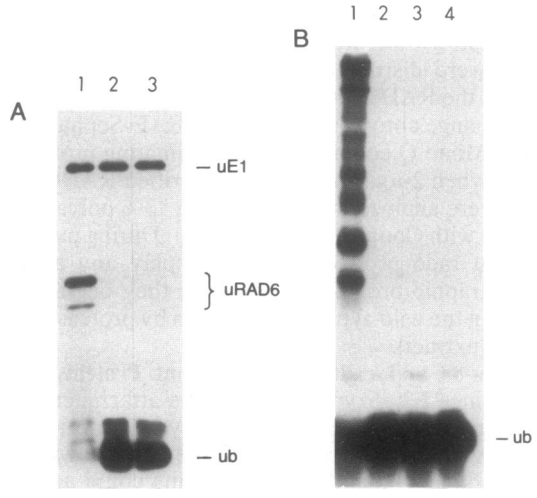

FIG. 3. Mutation of Cys-88 to alanine or valine inactivates the RAD6 E2 activity. (*A*) RAD6 (lane 1), rad6 Ala-88 (lane 2), and rad6 Val-88 (lane 3) proteins were incubated with E1, ^{125}I-labeled ubiquitin, and MgATP for 1.2 hr at 30°C, and thioesters formed between ubiquitin and other components of the reaction mixtures were visualized by omitting 2-mercaptoethanol from the sample buffer. ub, Ubiquitin; uE1, ubiquitinated E1; uRAD6, ubiquitinated RAD6. The smaller uRAD6 band corresponds to the thioester adduct between ubiquitin and a degradation product of RAD6, generated as a result of a contaminating protease in the ubiquitin preparation (data not shown). (*B*) To examine whether the mutant proteins were devoid of the RAD6 histone-polyubiquitinating activity, histone H2B was added to the standard assay mixture containing RAD6 (lane 1), rad6 Ala-88 (lane 3), or rad6 Val-88 (lane 4) protein. Incubation time was 30 min and the reaction mixtures were boiled in stop buffer containing 5% 2-mercaptoethanol prior to electrophoresis. To confirm that the ladder of radioactive bands in lane 1 were ubiquitinated species of H2B, a no-histone control (lane 2) was included for RAD6.

harboring centromeric plasmids carrying the *RAD6* or mutant *rad6* alleles. Up to 10 J/m^2, survival was not affected in the EMY7 strain carrying pR67 (*RAD6*), whereas at 8 J/m^2, a

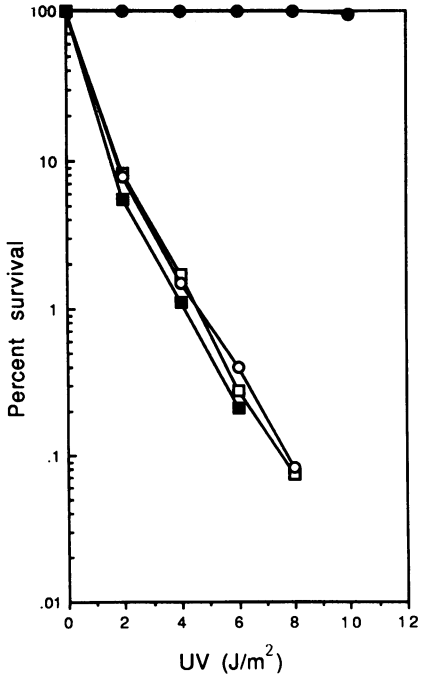

FIG. 4. Survival after UV irradiation of EMY7 (*rad6Δ*) carrying *RAD6* and *rad6* genes on low-copy centromeric plasmids. ●, pR67 (*RAD6*); ○, pR611 (*rad6Δ*); ■, pR647 (*rad6 Ala-88*); □, pR654 (*rad6 Val-88*).

Table 3. Effect of *rad6 Ala-88* and *rad6 Val-88* mutations on UV-induced reversion of *arg4-17*

UV dose, J/m^2	Frequency of *ARG4*$^+$ revertants per 10^6 viable cells in the *rad6Δ* strain RS33-2C/6Δ carrying various plasmids		
	pR67 (*RAD6*$^+$)	pR647 (*rad6 Ala-88*)	pR654 (*rad6 Val-88*)
2	143	0	0
4	252	0	0

Spontaneously arising *ARG4*$^+$ revertants have been subtracted.

1000-fold decrease in survival occurred in EMY7 carrying pR611, which lacks any *RAD6* or *rad6* insert. EMY7 cells harboring pR647 (*rad6 Ala-88*) or pR654 (*rad6 Val-88*) displayed the same extreme sensitivity to UV light as EMY7-(pR611). Similar results were obtained when another *rad6Δ* strain, EMY1, was used as the host strain for these plasmids (data not shown). To test the ability of the *rad6 Ala-88* and *rad6 Val-88* mutants to mediate DNA damage-induced mutagenesis, we examined UV-induced reversion of the ochre allele *arg4-17*. No UV-induced *ARG4*$^+$ revertants were formed in the *rad6Δ* strain RS33-2C/6Δ carrying the *rad6 Ala-88* or *rad6 Val-88* mutant gene on pR647 or pR654 (Table 3). Thus, the *rad6 Ala-88* and *rad6 Val-88* mutants are defective in both DNA repair and mutagenesis.

The role of Cys-88 in the sporulation function of RAD6 was investigated in the *rad6Δ/rad6Δ* diploid strain EMY26, obtained from a cross between the EMY1 and EMY7 haploid strains. As expected, no sporulation occurred in EMY26, whereas introduction of pR67 (*RAD6*) resulted in 50% sporulation (Table 4). The *rad6 Ala-88* or *rad6 Val-88* mutant allele, however, did not restore any sporulation ability to EMY26.

We also examined whether the rad6 null phenotypes associated with the two Cys-88 mutations could be at least partially overcome by increased expression of the mutant alleles. Experiments similar to those described above were carried out with pR648 and pR655 (Table 2), in which the coding regions of *rad6 Ala-88* and *rad6 Val-88*, respectively, have been fused to the *ADC1* promoter, which enhances the expression of these mutant alleles ≈50-fold. We observed no restoration of any of the *RAD6* functions upon the introduction of pR648 and pR655 into *rad6Δ* strains (data not shown), indicating that *rad6 Ala-88* and *rad6 Val-88* are completely devoid of any *RAD6* biological activity.

DISCUSSION

Previous investigations into the mechanistic aspects of ubiquitin conjugation to target proteins revealed a transient thioester linkage between E2 and ubiquitin (12, 13). To determine whether Cys-88 in the RAD6 protein is the acceptor site for ubiquitin, we altered this residue by oligonucleotide-directed mutagenesis; alanine and valine were chosen to replace Cys-88 because they bear the most resemblance to cysteine in their effect on protein conformation (27). In immunoblotting experiments and during protein purification, we found that both *rad6 Ala-88* and *rad6 Val-88* produce a stable protein with an electrophoretic mobility and chromato-

Table 4. Sporulation of the *rad6Δ/rad6Δ* strain EMY26 carrying the *rad6 Ala-88* and *rad6 Val-88* alleles

Plasmid	Vector:*rad6* allele	Sporulation,* %
pR67	*CEN:RAD6*$^+$	51
pR611	*CEN:rad6Δ*	0
pR647	*CEN:rad6 Ala-88*	0
pR654	*CEN:rad6 Val-88*	0

*From counts of at least 1000 cells for each determination.

graphic properties indistinguishable from those of wild-type RAD6 protein, suggesting that the change of Cys-88 to alanine or valine does not result in any protein misfolding. We found that the two rad6 mutant proteins are inactive in thioester formation with ubiquitin and consequently lack any histone-polyubiquitinating activity. These observations demonstrate that Cys-88 is required for the formation of the RAD6–ubiquitin thioester and that this intermediate is essential for the ubiquitination of histones.

Formerly, we reported studies with the *rad6-149* allele, which codes for a protein lacking the entire carboxyl-terminal polyacidic tail of wild-type RAD6 (9, 14). The rad6-149 protein is normal in its capacity to form a thioester conjugate with ubiquitin but has much lower histone-ubiquitinating activity than RAD6, indicating a pivotal role of the polyacidic tail in histone recognition (14). Yeast strains bearing the *rad6-149* mutation are proficient in DNA repair and UV mutagenesis but are defective in sporulation (9). One possible interpretation of these observations was that the E2 activity of RAD6 is required only for its sporulation function, whereas its DNA repair and mutagenesis functions are mediated by additional biochemical activities present in RAD6. Many DNA repair and replication proteins, such as RecA, RAD3, and the large tumor antigen of simian virus 40, are multifunctional and possess multiple biochemical activities (15, 20–22, 28). To determine the role of the E2 activity of RAD6 in its various biological functions, we examined the *rad6 Ala-88* and *rad6 Val-88* mutants for sensitivity to UV light, ability to effect UV-induced mutagenesis, and competency to undergo sporulation. The *rad6 Ala-88* and *rad6 Val-88* mutants displayed a degree of UV sensitivity equivalent to that conferred by the *rad6*Δ mutation, and like the *rad6*Δ mutants, they lacked the ability to convert DNA lesions into genetic mutations. The two mutant alleles also could not overcome the sporulation incompetence of the *rad6*Δ/*rad6*Δ strain. In addition, the slow-growth phenotype and reduced cell viability observed in *rad6*Δ strains were equally evident in the *rad6 Ala-88* and *rad6 Val-88* mutants. Moreover, the phenotypic characteristics of the *rad6 Ala-88* and *rad6 Val-88* alleles were not altered by their enhanced expression achieved by the use of the *ADC1* promoter, indicating that the biological efficacy of RAD6 is not merely weakened but totally abolished by the two Cys-88 mutations. Taken together, the findings reported in this paper implicate the RAD6 ubiquitin ligase activity in the execution of *RAD6*-mediated biological functions.

The proficiency in DNA repair and mutagenesis observed in *rad6-149* mutants can be explained by a targeting mechanism whose functional integrity is not contingent upon the presence of the RAD6 polyacidic tail, which specifically directs RAD6 to sites of DNA damage, where it modulates chromatin structure by the ubiquitination of histones and/or nonhistone proteins. Alternatively, or in addition, the RAD6 ubiquitin ligase may activate DNA-repair proteins that do not require the polyacidic tail for their modification. Repair proteins that may be involved in directing RAD6 to damage sites or the biological substrates of RAD6 could include those encoded by genes in the *RAD6* epistasis group, such as *RAD18, REV1, REV2,* and *REV3*.

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