The extremely conserved amino terminus of RAD6 ubiquitin-conjugating enzyme is essential for amino-end rule-dependent protein degradation

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The *RAD6* gene of *Saccharomyces cerevisiae* encodes a ubiquitin-conjugating enzyme that is required for DNA repair, damage-induced mutagenesis, and sporulation. In addition, RAD6 mediates the multiubiquitination and degradation of amino-end rule protein substrates. The structure and function of RAD6 have been remarkably conserved during eukaryotic evolution. Here, we examine the role of the extremely conserved amino terminus, which has remained almost invariant among RAD6 homologs from yeast to human. We show that RAD6 is concentrated in the nucleus and that the amino-terminal deletion mutation, *rad6*_{Δ1-9}, does not alter the location of the protein. The amino-terminal domain, however, is essential for the multiubiquitination and degradation of amino-end rule substrates. In the *rad6*_{Δ1-9} mutant, β-galactosidase proteins bearing destabilizing amino-terminal residues become long lived, and purified *rad6*_{Δ1-9} protein is ineffective in ubiquitin-protein ligase (E3)-dependent protein degradation in the proteolytic system derived from rabbit reticulocytes. The amino terminus is required for physical interaction of RAD6 with the yeast *UBR1*-encoded E3 enzyme, as the *rad6*_{Δ1-9} protein is defective in this respect. The *rad6*_{Δ1-9} mutant is defective in sporulation, shows reduced efficiency of DNA repair, but is proficient in UV mutagenesis. E3-dependent protein degradation by RAD6 could be essential for sporulation and could affect the efficiency of DNA repair.

[Key Words: *RAD6* gene; *Saccharomyces cerevisiae*; ubiquitin-conjugating enzyme; amino-end rule protein]

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The *RAD6* gene of *Saccharomyces cerevisiae* is required for DNA repair, DNA damage-induced mutagenesis, and sporulation. *rad6* mutants are highly sensitive to killing by a variety of DNA damaging agents, including ultraviolet light (UV), γ-rays, and alkylating agents, and they are defective in postreplication repair of gaps that arise in newly synthesized DNA across from the damage sites in UV-irradiated DNA. In addition, *rad6* mutants are defective in damage-induced mutagenesis and sporulation [for review, see Prakash et al. 1990].

RAD6 encodes a 172-residue, 20-kD protein containing a globular domain comprising approximately the first 149 amino acids and an extended carboxy-terminal tail in which 20 of the 23 residues are acidic (Morrison et al. 1988). RAD6 is a ubiquitin conjugating enzyme, and in vitro it has been shown to multiubiquitinate histones H2A, H2B, and H3 (Jentsch et al. 1987; Sung et al. 1988; Haas et al. 1990). The carboxy-terminal acidic domain is required to target RAD6 to histones (Sung et al. 1988). The *rad6-149* mutant, missing the entire acidic tail domain, is defective in sporulation but proficient in DNA repair and mutagenesis (Morrison et al. 1988). Muta-

tional inactivation of the active site Cys-88 residue results in a Rad6 protein devoid of any ubiquitin-conjugating ability, and these mutants resemble the *rad6* null mutant in defects in DNA repair, mutagenesis, and sporulation, indicating that all of the biological functions of RAD6 are mediated by its ubiquitin-conjugating activity (Sung et al. 1990).

More recently, RAD6 has been shown to function in the protein degradation pathway dependent on the identity of the amino-terminal residue. The amino termini of potential proteolytic substrates are first recognized by a protein variously referred to as ubiquitin-protein ligase, E3, or N-recogin. Both yeast and mammalian E3 proteins contain a binding site for type I substrates and another site for type II substrates. Type I protein substrates have a basic residue, Arg, Lys, or His, at their amino termini, and type II substrates have a bulky hydrophobic residue, Phe, Trp, Tyr, or Leu, at their amino termini (for review, see Finley and Chau 1991; Hershko 1991). In addition, E3 interacts with a region in the body of the proteolytic substrate. Studies from several laboratories have demonstrated a role of RAD6 in E3-dependent ubiq-

uitination and protein degradation in yeast as well as in mammals. Purified yeast RAD6 protein catalyzes multiubiquitination and degradation of protein substrates with destabilizing amino termini in reaction mixtures containing ubiquitin activating enzyme (E1), E3, and the ubiquitin-specific protease from rabbit reticulocytes (Sung et al. 1991). In *S. cerevisiae*, *UBR1* encodes the amino-end-recognizing (E3) enzyme, and the *ubr1* null mutant is unable to degrade *X*- β -galactosidase bearing any of the destabilizing residues (*X*) at the amino terminus. Sporulation is somewhat defective in the *ubr1* mutant, and growth rate is slightly retarded (Bartel et al. 1990). *UBR1*-dependent protein degradation is inactive in the *rad6* null mutant, indicating that RAD6 interacts with *UBR1* in mediating multiubiquitination and degradation of substrates with destabilizing amino termini (Dohmen et al. 1991). In rabbit reticulocytes, only the *E2_{14K}* enzyme has been found to catalyze the E3-dependent multiubiquitination and degradation of amino-end rule substrates (Pickart and Rose 1985; Pickart and Vella 1988). Recently, this protein has been shown to be identical in sequence to the human RAD6 homolog HHR6B (Wing et al. 1992).

The structure and function of RAD6 have been highly conserved during eukaryotic evolution. RAD6 is a single-copy gene in *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Drosophila melanogaster* but is duplicated in human, and the two human homologs share 95% identical amino acid residues (Koken et al. 1991b). The acidic tail is found only in the *S. cerevisiae* protein; otherwise, the various homologs share a high degree of sequence identity. Genetic studies with the RAD6 homolog *rhp6⁺* in *S. pombe* indicate that *rhp6⁺* resembles RAD6 in its roles in DNA repair, mutagenesis, and sporulation. Furthermore, *S. cerevisiae* and *S. pombe* genes can substitute functionally for one another (Reynolds et al. 1990). The *Drosophila* and human genes also complement the DNA repair and mutagenesis defects of the *S. cerevisiae rad6 Δ* mutant (Koken et al. 1991a,b).

Among the RAD6 homologs, the amino-terminal sequence has remained almost invariant. Because this conservation does not extend to other ubiquitin-conjugating enzymes, a role of this sequence in RAD6-specific functions is implicated. To delineate the role of this sequence, we have examined the effects of an amino terminal deletion mutation on various RAD6-dependent functions. We find that this mutation inactivates the amino-end rule-dependent protein degradation pathway and engenders defective sporulation and a reduction in the proficiency of DNA repair.

Results

RAD6 is concentrated in the nucleus

We examined the location of RAD6 in yeast cells by indirect immunofluorescence, using affinity-purified antibodies directed against the rad6-149 protein, which is deleted for the entire acidic tail region of 23 residues. This antibody shows a very high degree of specificity

toward the globular domain of RAD6. We found that RAD6 is concentrated in the nucleus, its location coinciding with the nuclear stain DAPI (Fig. 1A,B). However, some RAD6 also appears to be present in the cytoplasm. In contrast, in the *rad6 Δ* strain, no cross-reactivity was observed over the entire cell (Fig. 1C,D).

Mutagenesis of the highly conserved amino terminus of RAD6 protein

The amino terminus of RAD6 represents the most highly conserved segment in the protein. In this region, the first 15 residues are identical among RAD6 and its homologs, with the exception of a single difference in *S. pombe* (Fig. 2A). This sequence conservation, however, does not extend to other ubiquitin-conjugating enzymes (Fig. 2B), suggesting an involvement of the amino-terminal region in RAD6-specific functions. To examine the role of this sequence, we mutated the translation initiating ATG codon to AAG, so that translation of the mutant protein would initiate from the next methionine codon at position 10. To examine the intracellular expression of the resulting *rad6 Δ_{1-9}* mutant allele, we cloned it into low-copy and multicopy yeast vectors, generating plasmids pR68 and pR64, respectively. In these plasmids, the mutant allele is fused to the normal RAD6 promoter. We also fused the *rad6 Δ_{1-9}* allele to the *S. cerevisiae* highly expressed and constitutive *ADC1*

Figure 1. Nuclear localization of RAD6. Isogenic RAD⁺ (LP3041-6D) and *rad6 Δ* (EMY1) strains were fixed, incubated with affinity-purified anti rad6-149 antibodies, and stained with FITC (Sigma)-conjugated goat anti-rabbit IgG and with DAPI. (A) RAD6 cells visualized for DAPI and (B) for FITC fluorescence. C and D are the same as A and B, respectively, except that *rad6 Δ* cells were used.

Figure 2. The amino-terminal sequence of the RAD6 protein is highly conserved among its homologs from different species, but not among other *S. cerevisiae* ubiquitin-conjugating enzymes. (A) The amino-terminal 50 residues in various RAD6 homologs: *S. cerevisiae* RAD6 (Reynolds et al. 1985); *S. pombe* rhp6+ (Reynolds et al. 1990); *D. melanogaster* DHR6 (Koken et al. 1991a); and human HHR6A and HHR6B (Koken et al. 1991b). (B) Comparison of the first 50 residues of RAD6 protein to other ubiquitin-conjugating enzymes from *S. cerevisiae*: UBC1 (Seufert et al. 1990); UBC4 and UBC5 (Seufert and Jentsch 1990); UBC8 (Qin et al. 1991); and CDC34 (Goebel et al. 1988). Dots indicate identity to the RAD6 protein; dashes indicate gaps introduced in the protein sequence for optimal alignment. The numbers above the RAD6 sequence refer to the amino acid residue of RAD6. In UBC8, the first methionine residue occurs at a position corresponding to residue 13 of RAD6.

promoter in a multicopy vector, yielding plasmid pR661. These *rad6_{Δ1-9}* plasmids were introduced into the *rad6Δ* strain EMY1, and the intracellular levels of mutant proteins were determined by Western immunoblot analysis. The *rad6_{Δ1-9}* allele encodes a shorter protein of approximately the size expected (Fig. 3). The mutant protein was barely detectable in *rad6Δ* cells carrying plasmid pR68, whereas the level of protein in cells carrying the multicopy plasmid pR64 was approximately fourfold lower than wild-type RAD6 levels. An increase of ~50-fold occurred in cells carrying the *ADC1::rad6_{Δ1-9}* plasmid pR661 over the level of protein produced by pR64. Thus, the level of *rad6_{Δ1-9}* protein in *rad6Δ* cells harboring pR661 is >10-fold higher than the level of RAD6 protein in wild-type cells.

The *rad6_{Δ1-9}* protein localizes to the nucleus

The amino terminus of RAD6 contains a high proportion of basic amino acids (see Fig. 2), and the sequence MSTPARRRL fits the common features of nuclear-targeting signals in that it is a short sequence rich in basic residues (Dingwall and Laskey 1991). To determine whether the amino terminus of RAD6 is necessary for nuclear targeting, we examined the cellular location of the *rad6_{Δ1-9}* protein in the *rad6Δ* strain EMY1 harboring plasmid pR64. The results in Figure 4 show that the mutant protein is also located in the nucleus.

Figure 3. Western analysis of cell extracts. Isogenic *RAD6*⁺ strain (LP3041-6D) and *rad6Δ* (EMY1) strain carrying the *RAD6* or the *rad6_{Δ1-9}* plasmid were disrupted by alternate freezing and boiling of the cells. The resulting supernatant following centrifugation was electrophoresed in a 15% polyacrylamide-SDS gel, transferred to nitrocellulose, probed with affinity-purified anti-*rad6*-149 antibodies, and developed by the indirect peroxidase procedure. Equal amounts of cell extract (1×10^7 cells) were loaded for each sample. (Lane 1) *rad6Δ* strain; (lane 2) *RAD6*⁺ strain; (lane 3) *rad6Δ* strain carrying the *RAD6* gene on a low-copy plasmid (pR67); (lanes 4–6) *rad6Δ* strain carrying the *rad6_{Δ1-9}* gene on a low-copy plasmid (pR68), multicopy plasmid (pR64), and multicopy *ADC1* promoter fusion plasmid (pR661), respectively.

The *rad6_{Δ1-9}* mutation confers a defect in sporulation and reduces the efficiency of DNA repair and growth rate

To determine the effects of the *rad6_{Δ1-9}* mutation on various RAD6 functions, we examined UV survival, UV mutagenesis, sporulation, and growth rate of the *rad6Δ* strain harboring the *ADC1::rad6_{Δ1-9}* plasmid pR661. The *rad6Δ* strain is extremely sensitive to UV light; for example, a 10^4 -fold reduction occurs in UV survival at 10 J/m^2 . Introduction of pR661 into the *rad6Δ* strain increased the UV survival by >100-fold, suggesting that the RAD6 amino terminus affects the proficiency of DNA repair (Fig. 5). To determine the effect of the *rad6_{Δ1-9}* mutation on UV mutagenesis, we examined the

Figure 4. Nuclear location of the *rad6_{Δ1-9}* protein. *rad6Δ* strain EMY1 carrying the *rad6_{Δ1-9}* gene on the multicopy plasmid pR64 was examined by indirect immunofluorescence as described in the legend to Fig. 1. DAPI (A) and FITC (B) fluorescence shows the location of the nucleus and the *rad6_{Δ1-9}* mutant protein, respectively.

Figure 5. Survival after UV irradiation of *rad6Δ* strain EMY8 and the isogenic *rad6Δ rad18Δ* strain JWY119 carrying different plasmids. Strains were grown on media for maintaining selection of the plasmids. (■) *RAD*⁺ strain 839; (□) *rad6Δ* + vector pSCW231; (△) *rad6Δ* + pR661 (*ADC1::rad6_{Δ1-9}*); (○) *rad6Δ rad18Δ* + vector pSCW231; (●) *rad6Δ rad18Δ* + pR661 (*ADC1::rad6_{Δ1-9}*).

rate of UV-induced reversion of a *met14* mutation. Whereas no UV mutagenesis occurs in the *rad6Δ* strain, introduction of plasmid pR661 restored UV mutagenesis to a rate 5- to 10-fold higher than in the wild-type strain (Table 1).

The *RAD6* epistasis group of DNA repair genes in *S. cerevisiae* consists of *RAD18*, *REV1*, *REV3*, and other genes (for review, see Prakash et al. 1990). *RAD6* is the only gene in this group that is required both for postreplication repair and damage-induced mutagenesis. The *rad18* mutant resembles *rad6* in the level of UV sensitivity and in deficiency in postreplication repair but differs from *rad6* in being proficient in UV mutagenesis.

Table 1. Effect of the *rad6_{Δ1-9}* mutation on UV-induced reversion of *met14*

<i>met</i> ⁺ revertants ^a per 10 ⁷ survivors in isogenic <i>rad6Δ</i> strain EMY7 or EMY8 carrying different plasmids			
UV dose (Jm ⁻²)	pSCW231 (<i>rad6Δ</i>)	pR67 (<i>RAD6</i>)	pRR661 (<i>rad6_{Δ1-9}</i>)
0	0.0	0.3	0.9
2	0.0	13.0	83.0
4	0.0	48.0	262.0
6	0.0	65.0	618.0

^aValues represent the average of at least three independent experiments.

Although the *rev1* and *rev3* mutants are defective in UV mutagenesis, they are only marginally UV sensitive, suggesting that mutagenic bypass of DNA damage constitutes a minor DNA repair pathway in yeast. Thus, *RAD6* functions with *RAD18* in error-free DNA repair and with *REV1* and *REV3* in mutagenic bypass. The higher level of UV resistance of the *rad6_{Δ1-9}* mutant over that of the *rad6Δ* strain could arise from partial inactivation of the *RAD18*-dependent DNA repair pathway in the *rad6_{Δ1-9}* strain. Alternatively, although the *RAD18* pathway could be completely inactive in the *rad6_{Δ1-9}* mutant, increased UV resistance might result from a more efficient processing of DNA damage by mutagenic bypass. In the latter case, we would expect the UV resistance of *rad6Δ rad18Δ* cells harboring plasmid pR661 to be similar to that of the *rad6Δ* strain carrying pR661. Therefore, to distinguish between these possibilities, we examined the UV sensitivity of a *rad6Δ rad18Δ* mutant strain with or without the plasmid pR661. Because we found no increase in the UV resistance of *rad6Δ rad18Δ* cells upon introduction of plasmid pR661 (Fig. 5), we infer partial inactivation of the *RAD18* pathway rather than a more efficient processing of DNA damage by mutagenic bypass to be the likely reason for the observed level of UV sensitivity in the *rad6_{Δ1-9}* mutant.

The *rad6_{Δ1-9}* mutation renders cells sporulation defective, as no sporulation occurred in the *rad6Δ/rad6Δ* diploid strain carrying plasmid pR661 (Table 2). Western immunoblotting of cell extracts at various times during sporulation indicated that the level of *rad6_{Δ1-9}* protein stays ~10-fold higher than the level of *RAD6* protein in wild-type cells (data not shown). Therefore, the failure of *rad6Δ/rad6Δ* cells harboring pR661 to sporulate is not the result of reduced levels of mutant protein under sporulation conditions. The *rad6_{Δ1-9}* mutation also affects growth rate adversely (Table 3); the growth rate of the mutant was intermediate between that of the wild-type and *rad6* null strains. Growth rate was not affected by the *rad6-149* mutation. This mutation renders cells sporulation defective, but DNA repair and mutagenesis remain unaffected (Morrison et al. 1988).

Defective amino-end-dependent protein degradation in the *rad6_{Δ1-9}* mutant

The *RAD6* gene is specifically required for ubiquitination and subsequent degradation of proteins dependent on the identity of the amino terminal residue (Dohmen

Table 2. Sporulation of *rad6Δ/rad6Δ* strain carrying different plasmids

Strain	Plasmid	Gene	Sporulation (%) ^a
EMY28	pSCW231	<i>rad6Δ</i>	0
EMY26	pR67	<i>RAD6</i>	35
EMY28	pR661	<i>rad6_{Δ1-9}</i>	0

^aBased on a count of >1000 cells for each strain. Strains EMY26 and EMY28 are isogenic.

Table 3. Growth rates of *rad6Δ* strain *EMY1* carrying the *RAD6* or mutant *rad6* gene on a plasmid

Plasmid	Genotype	Doubling time ^a (hr) ± s.d.
pR67	<i>RAD6</i>	1.54 ± 0.15
pSCW231	<i>rad6Δ</i>	2.70 ± 0.07
pR661	<i>rad6_{Δ1-9}</i>	2.12 ± 0.14
pR615	<i>rad6-149</i>	1.51 ± 0.06

^aAverage of at least three independent experiments carried out at 30°C.

et al. 1991; Sung et al. 1991). In this role, RAD6 interacts with the *UBR1*-encoded amino-end-recognizing protein. To examine the effect of the *rad6_{Δ1-9}* mutation on degradation of amino-end rule substrates, as well as that of *rad6-153* and *rad6-149* mutations deleted for the sequence encoding the last 19 or 23 amino acids of the acidic tail, respectively, we used plasmids (KEP268, KEP269, KEP270; Table 4) in which the *Escherichia coli* β-galactosidase gene bearing different amino-terminal codons is fused to the ubiquitin gene placed under the control of the *GAL10* promoter (Bachmair et al. 1986). Expression of Ub-X-β-Gal plasmids in yeast results in the cleavage of ubiquitin, yielding the X-β-Gal protein, where X denotes the amino-terminal residue. Ub-X-β-Gal plasmids encoding Met, a stabilizing residue, Arg, a type I destabilizing residue, or Leu, a type II destabilizing residue at the amino terminus, were introduced into isogenic *RAD⁺* and *rad6Δ* strains. The *rad6Δ* strains, each harboring a different Ub-X-β-Gal plasmid, were then transformed with a plasmid bearing either the *rad6_{Δ1-9}* (pR661), the *rad6-149* (pR615), or the *rad6-153* (pR616) mutant allele, and the stability of X-β-Gal proteins determined by measuring the steady-state levels of β-galactosidase, and by pulse-chase experiments.

In the *RAD⁺* strain, the steady-state level of Met-β-Gal was much higher than that of Arg-β-Gal or Leu-β-Gal, whereas in the *rad6Δ* strain, Arg-β-Gal and Leu-β-Gal increased to levels nearly equal to that of the stable

Table 4. Plasmids used in this study

Plasmid	Gene, vector, and nutritional marker
pR64	<i>rad6_{Δ1-9}</i> gene in 2μ vector (URA3)
pR67	<i>RAD6</i> gene in CEN vector (URA3)
pR68	<i>rad6_{Δ1-9}</i> gene in CEN vector (URA3)
pR615	<i>rad6-149</i> gene in CEN vector (URA3)
pR616	<i>rad6-153</i> gene in CEN vector (URA3)
pR661	<i>ADC1</i> promoter:: <i>rad6_{Δ1-9}</i> gene in 2μ vector (TRP1)
pSCW231	<i>ADC1</i> promoter in 2μ vector (TRP1)
KEP268	<i>GAL10</i> promoter:: <i>Ub-Met</i> -β-Gal in 2μ vector (URA3)
KEP269	<i>GAL10</i> promoter:: <i>Ub-Arg</i> -β-Gal in 2μ vector (URA3)
KEP270	<i>GAL10</i> promoter:: <i>Ub-Leu</i> -β-Gal in 2μ vector (URA3)
pSOB44	<i>ADC1</i> promoter: <i>UBR1/ha</i> in 2μ vector (TRP1)

Met-β-Gal (Fig. 6). The levels of Arg-β-Gal and Leu-β-Gal in the *rad6_{Δ1-9}* mutant resembled those in the *rad6Δ* strain, suggesting that the RAD6-mediated amino-end rule pathway is defective in the *rad6_{Δ1-9}* mutant. In contrast, cells carrying the *rad6-153* gene displayed normal activity, whereas the proficiency of degradation was reduced in the *rad6-149* mutant. These findings are in agreement with our previous in vitro studies, indicating that *rad6-153* protein was as efficient as RAD6 in E3-dependent protein ubiquitination and subsequent degradation, whereas the *rad6-149* protein showed reduced levels of activity (Sung et al. 1991).

Pulse-chase experiments done to verify and extend the results obtained from colorimetric assays of β-gal levels are shown in Figure 7. In *RAD⁺* cells, during the 15-min chase period, Met-β-Gal was stable but Arg-β-Gal and Leu-β-Gal proteins decayed with an approximate half-life of 3 min and 5 min, respectively. Multiubiquitinated species of Arg-β-Gal and Leu-β-Gal were evident, particularly in the 0-min sample, and these proteins also generated a β-Gal cleavage product of ~90 kD. For the stable Met-β-Gal protein, there was no evidence of formation of either the higher molecular weight species representing ubiquitinated β-Gal or of the 90-kD product. In contrast, in the *rad6Δ* and *rad6_{Δ1-9}* strains, Arg-β-Gal and Leu-β-Gal proteins became stabilized like the

Figure 6. *rad6_{Δ1-9}* mutant is defective in the amino-end rule degradation pathway in vivo. Levels of β-Gal activity in *RAD⁺* and various mutant *rad6* strains expressing either Ub-Met-β-Gal, Ub-Arg-β-Gal, or Ub-Leu-β-Gal. Cells were grown to a density of 1×10^7 cells/ml in selection medium containing 2% galactose as the sole carbon source and assayed for β-gal activity as described in Materials and methods. Values shown represent the average of at least three independent experiments. The levels of Arg-β-Gal and Leu-β-Gal in each strain were normalized to the Met-β-Gal activity (set at 100%) in that particular strain. Standard deviation is shown above each bar. (Solid bar) *RAD⁺*; (dark hatched bar) *rad6Δ*; (crosshatched bar) *rad6_{Δ1-9}*; (light hatched bar) *rad6-149*; (open bar) *rad6-153*.

Figure 7. Pulse-chase analysis of X-β-Gal in *RAD*⁺ and various *rad6* mutants. Cultures of exponentially growing cells (0.5×10^7 to 1.0×10^7 cells/ml) expressing either Ub-Met-β-Gal, Ub-Arg-β-Gal, or Ub-Leu-β-Gal were labeled for 5 min with [³⁵S]methionine at 30°C (0 min), followed by a chase at 30°C in the presence of cycloheximide and unlabeled methionine for 15 min (15 min). β-Gal was immunoprecipitated from the cells and subjected to electrophoresis and fluorography as described in Materials and methods. (A–D) The X-β-Gal band is indicated, and the position of the ~90-kD long-lived β-Gal cleavage product is marked by an arrow. Higher molecular weight species representing multiubiquitinated forms of Arg-β-Gal and Leu-β-Gal appear in the *RAD*⁺ strain and, to a lesser extent, in the *rad6*-149 mutant. (E) To show unequivocally the absence of multiubiquitination and degradation of Arg-β-Gal in the *rad6*Δ and *rad6*Δ1-9 strains, the Arg-β-Gal gels in B and C were exposed for a longer period.

Met-β-Gal protein, and we did not observe any multiubiquitinated species or 90-kD cleavage product in either mutant. Degradation of Arg-β-Gal and Leu-β-Gal proteins occurred in the *rad6*-149 mutant but at a slower rate than in the *RAD*⁺ strain; the half-life of Arg-β-Gal was increased to ~12 min, and that of Leu-β-Gal was increased to ~50 min. Thus, our studies indicate that the *rad6*Δ1-9 mutant lacks the ability to function in the amino-end rule protein degradation pathway, whereas the efficiency of this pathway is reduced in the *rad6*-149 mutant.

*The rad6*Δ1-9 protein is defective in E-3-dependent protein degradation in vitro

RAD6-dependent multiubiquitination and degradation of substrates bearing destabilizing amino-terminal residues can be examined in vitro using the proteolytic system derived from rabbit reticulocytes (Sung et al. 1991); the E3 component of this cell-free system is functionally equivalent to the UBR1 gene product of yeast. To assess the ability of the *rad6*Δ1-9 mutant protein to function with E3 in amino-end-dependent protein degradation, we purified the *rad6*Δ1-9 protein to near homogeneity (Fig. 8) from extract of the *rad6*Δ strain EMY8 harboring the plasmid pR661 (*ADC1::rad6*Δ1-9) by a combination of

four-column chromatographic steps (see Materials and methods).

The RAD6 and *rad6*Δ1-9 proteins were tested for their ability to cooperate with E3 in the degradation of [¹²⁵I]-labeled β-lactoglobulin, which has leucine at its amino terminus, a destabilizing amino-terminal residue in both rabbit reticulocytes and yeast. Consistent with our previous findings (Sung et al. 1991), RAD6 promoted the degradation of β-lactoglobulin in the proteolytic system, showing a strict requirement for E3, ubiquitin, and the ubiquitin-specific protease (Table 5). In contrast, the *rad6*Δ1-9 protein, even in an amount fivefold higher than RAD6, was totally ineffective in protein degradation (Table 5). We also examined the formation of ubiquitinated β-lactoglobulin by subjecting reaction mixtures to SDS-PAGE and autoradiography, as described previously (Sung et al. 1991; see Materials and methods). In contrast to the RAD6 protein, which catalyzes the multiubiquitination of β-lactoglobulin (Fig. 9, lane 3), the *rad6*Δ1-9 protein did not function in this reaction (Fig. 9, lanes 4,5).

Conceivably, the failure of *rad6*Δ1-9 protein to mediate multiubiquitination and degradation of β-lactoglobulin could occur upstream of E3 interaction, that is, in thioester formation with ubiquitin, a reaction catalyzed by the ubiquitin-activating enzyme E1. To eliminate this possibility, we examined the formation of ubiquitin

Figure 8. SDS-PAGE of RAD6 and $rad6_{\Delta 1-9}$ proteins. One microgram each of RAD6 (lane 1) and $rad6_{\Delta 1-9}$ (lane 2) proteins, purified as described in Materials and methods, was electrophoresed in a 12.5 % polyacrylamide-SDS gel and stained with Coomassie blue.

thioester (Sung et al. 1988) with the RAD6 and $rad6_{\Delta 1-9}$ proteins, using the same E1 preparation as we used in the protein-degradation experiments. We found that the $rad6_{\Delta 1-9}$ protein readily forms thioester with ubiquitin, indicating that the mutant protein retains the ability to interact with E1 and ubiquitin (Fig. 10).

Defective physical interaction of the $rad6_{\Delta 1-9}$ protein with the UBR1 gene product

The results presented above establish that the $rad6_{\Delta 1-9}$ mutation inactivates the amino-end rule function of RAD6 protein. Using methodology similar to that employed by Dohmen et al. (1991) in demonstrating physical interaction between RAD6 and UBR1 proteins, we examined whether the $rad6_{\Delta 1-9}$ protein interacts with UBR1.

The $rad6\Delta ubr1\Delta$ yeast strain BBY68 was transformed

Table 5. $rad6_{\Delta 1-9}$ protein is ineffective in E3-dependent protein degradation

E2 enzyme	Reaction mix	Degradation (%)	Activity relative to RAD6 (%)
RAD6 ^a	complete	21.5	100
RAD6 ^a	– E3	0.4	2
RAD6 ^a	– ubiquitin	0	0
RAD6 ^a	– protease	0	0
$rad6_{\Delta 1-9}$ ^a	complete	0	0
$rad6_{\Delta 1-9}$ ^b	complete	0	0

RAD6 and $rad6_{\Delta 1-9}$ proteins were examined for the ability to mediate E3 dependent degradation of ¹²⁵I-labeled β -lactoglobulin as described in Materials and methods. The concentration of E2 in the assays was 200 nM^a or 1 μ M^b.

Figure 9. E3-dependent multiubiquitination of β -lactoglobulin occurs with RAD6 but not with $rad6_{\Delta 1-9}$ protein. Reaction mixtures containing RAD6 (200 nM; lanes 1–3) or $rad6_{\Delta 1-9}$ (200 nM in lane 4; 1 μ M in lane 5) were assembled as described in Materials and methods, incubated at 37°C for 20 min, and subjected to electrophoresis in a 12.5% polyacrylamide-SDS gel, followed by autoradiography of the dried gel. (Lane 1) With RAD6 but no ubiquitin; (lane 2) with RAD6 but no E3; (lane 3) with RAD6, E3, and ubiquitin; (lanes 4,5) $rad6_{\Delta 1-9}$ with E3 and ubiquitin. (β Lg) ¹²⁵I-labeled β -lactoglobulin; (Cont.) contaminant in the β -lactoglobulin preparation; (Ub- β Lg) ubiquitin conjugates of β -lactoglobulin in lane 3.

with the plasmid pSOB44 (*ADC1::UBR1/ha*), a high-copy plasmid containing the UBR1 gene carrying a carboxy-terminal 9-residue extension [derived from hemagglutinin (ha) of influenza virus] under the control of the *ADC1* promoter (Dohmen et al. 1991). Cell extract containing the UBR1/ha protein was mixed with the purified RAD6 or $rad6_{\Delta 1-9}$ protein and, after incubation, subjected to immunoprecipitation with protein A-agarose beads bearing affinity-purified anti-RAD6 antibodies. After washing, proteins from immunoprecipitates were

Figure 10. $rad6_{\Delta 1-9}$ protein forms thioester conjugate with ubiquitin. Formation of ubiquitin thioester with RAD6 (lane 1) or $rad6_{\Delta 1-9}$ (lane 2) protein was carried out as described in Materials and methods. The ubiquitin thioester conjugates (uRAD6 and urad6 $_{\Delta 1-9}$) were readily disrupted by treatment with 2-mercaptoethanol (not shown). The minor band below uRAD6 and urad6 $_{\Delta 1-9}$ is a proteolytic product generated by a contaminating protease in the ubiquitin preparation. (Ub) ¹²⁵I-labeled ubiquitin.

eluted by treatment with 2% SDS, and the eluates analyzed by immunoblotting (Fig. 11A,B) with either anti-RAD6 antibodies (Sung et al. 1988) or the monoclonal antibody 12CA5 specific for the ha epitope (Field et al. 1988) in UBR1/ha. In agreement with the result of Dohmen et al. (1991), we found that RAD6 forms a physical complex with the 226-kD UBR1/ha protein, as indicated by coprecipitation of the two proteins (Fig. 11A,B, lanes 2). The *rad6*_{Δ1-9} protein, however, did not coprecipitate the UBR1/ha protein (Fig. 11A,B, lanes 3), indicating a defect in interaction with the UBR1 protein.

Discussion

The amino terminus of RAD6 is extremely conserved, where the first 15 residues are almost invariant in RAD6 and its homologs from yeast to human. Because conservation of this domain is specific to RAD6 and does not extend to the other ubiquitin-conjugating enzymes, we expected this sequence to be required for interactions with specific protein components that mediate RAD6-dependent functions, rather than in interactions with proteins in the ubiquitin-conjugating pathway, such as the ubiquitin-activating enzyme E1 or ubiquitin itself. RAD6 differs from the other ubiquitin-conjugating enzymes in its requirement in DNA repair, mutagenesis, and sporulation and in its capacity to catalyze E3-dependent multiubiquitination of amino-end rule substrates. These functions of RAD6 are also shared by its homologs from higher eukaryotes, as these homologs can complement the DNA repair and mutagenesis defects of the *S.*

cerevisiae rad6Δ mutant (Reynolds et al. 1990; Koken et al. 1991a,b). In rabbit reticulocytes, E2_{14K} is the only ubiquitin-conjugating enzyme that functions in E3-dependent multiubiquitination and degradation of substrates, and this protein is identical in sequence to the human RAD6 homolog HHR6B (Wing et al. 1992). To determine the role of the amino-terminal sequence, we examined the effects of a *rad6*_{Δ1-9} mutation, in which the first 9 residues of the protein are deleted, on RAD6-specific functions. We show that RAD6 is a nuclear protein and that the *rad6*_{Δ1-9} mutation has no adverse effect on nuclear localization. Because of the presence of basic amino acids in residues 10–15, we examined whether these residues were responsible for nuclear entry of the *rad6*_{Δ1-9} protein. We constructed a *rad6*_{Δ1-15} mutant allele by simultaneously changing the ATG codons at positions 1 and 10 to AAG, which results in a protein deleted for the first 15 residues. However, we found that this protein also localizes to the nucleus (data not shown).

The most interesting finding to emerge from our studies is the inability of the *rad6*_{Δ1-9} protein to function in the amino-end-dependent protein degradation pathway in both yeast and rabbit reticulocytes. In the *rad6*_{Δ1-9} mutant, the short-lived β-Gal proteins containing either the arginine- or the leucine-destabilizing residue at the amino terminus become metabolically stabilized, and the severity of the defect in the mutant was identical to that in the *rad6* null mutant. In the proteolytic system derived from rabbit reticulocytes, we show that *rad6*_{Δ1-9} protein has lost the ability to mediate multiubiquitination and degradation of β-lactoglobulin, which bears the destabilizing residue leucine at its amino terminus. Results from the coimmunoprecipitation experiments indicate that the defect of *rad6*_{Δ1-9} protein in the amino-end rule function is the result of a failure to physically interact with the UBR1-encoded E3 enzyme.

The *rad6*_{Δ1-9} mutation has a striking effect on sporulation, as *rad6Δ/rad6Δ* cells carrying this mutant gene do not sporulate. This mutation also lowers the proficiency of DNA repair and reduces growth rate, whereas the rate of UV mutagenesis is elevated a fewfold. Because ubiquitin-conjugating activity is essential for all RAD6 biological functions (Sung et al. 1990), the differential effects of the *rad6*_{Δ1-9} mutation in these cellular processes very likely stem from the inability of the mutant protein to interact with specific protein components that affect these processes. Because the RAD6 amino terminus is essential for interaction with the yeast E3 enzyme encoded by the *UBR1* gene, we suggest that a simultaneous defect in interactions with additional E3 enzymes could underlie the phenotypic defects observed in the *rad6*_{Δ1-9} mutant.

Both in yeast and rabbit reticulocytes, there is evidence for the existence of multiple E3 enzymes that recognize different amino termini. A protein, E3-R, partially purified from *S. cerevisiae* vegetative cells, has been shown to mediate ubiquitination of amino-end rule substrates by RAD6 (Sharon et al. 1991). In conjunction with E3-R, RAD6 also ubiquitinates a substrate with a

Figure 11. *rad6*_{Δ1-9} mutation disrupts physical interaction with the UBR1 protein. Extract from strain BBY68 carrying the plasmid pSOB44 expressing the UBR1/ha protein (lane 1) was incubated with 1 μg of RAD6 (lane 2) or 1 μg of *rad6*_{Δ1-9} (lane 3) protein and then subjected to immunoprecipitation using protein A-agarose beads bearing affinity-purified anti-RAD6 antibodies. Proteins in immunoprecipitates were eluted by SDS treatment, separated in 12.5% (A) and 7% (B) denaturing polyacrylamide gels, transferred onto nitrocellulose, and probed with anti-RAD6 antibodies (A) and with the monoclonal antibody 12CA5 (B) specific for the ha epitope in UBR1/ha. No UBR1/ha protein was coprecipitated even with 3 μg of *rad6*_{Δ1-9} protein (data not shown).

blocked amino terminus. The level of E3-R activity is not affected by the *ubr1* null mutation; therefore, E3-R and UBR1 represent different proteins. In rabbit reticulocytes, in addition to E3 α , which recognizes substrates bearing type I (basic) or type II (bulky hydrophobic) destabilizing amino termini and which specifically interacts with RAD6 (Sung et al. 1991), another protein, E3 β has been purified that recognizes type III substrates that bear Ala, Ser, or Thr at the amino terminus (Heller and Hershko 1990). These residues are destabilizing in reticulocyte lysates but not in yeast, presumably because of the presence of E3 β in reticulocytes. It is not known whether the rabbit RAD6 homolog E2_{14K} interacts with E3 β .

In addition to defective interaction with UBR1, the *rad6* _{Δ 1-9} protein could be defective in interacting with E3-R and also with other E3 proteins that might recognize determinants other than the amino terminus. A common sequence in various E3 proteins might govern their interaction with the RAD6 amino terminus. Our finding that the *rad6* _{Δ 1-9} mutant is defective in both sporulation and UBR1-mediated protein degradation is consistent with the suggestion that E3-dependent ubiquitination of substrates by RAD6 might constitute an essential protein degradation pathway in sporulation. The *rad6-149* mutation also causes sporulation deficiency in *S. cerevisiae* (Morrison et al. 1988), indicating the requirement of the acidic tail in this process. The *rad6-149* protein, however, can function in E3-dependent degradation, albeit at a slower rate. Because the acidic tail is found only in the *S. cerevisiae* RAD6 protein, its indispensability in sporulation would appear to be restricted to this species. An essential role of the RAD6 globular domain in sporulation is indicated from studies with the *S. pombe* *rhp6*⁺ gene. Although *rhp6*⁺ does not contain the acidic tail, it is essential for sporulation in *S. pombe*, as manifested from the inability of the *rhp6* Δ /*rhp6* Δ mutant strain to sporulate. Moreover, the *S. cerevisiae* *rad6-149* gene restores a normal level of sporulation in the *rhp6* Δ /*rhp6* Δ strain (Reynolds et al. 1990). Our studies reported here indicate an involvement of the RAD6 amino terminus in this sporulation specific role of the globular domain. Because the amino-terminal sequence is almost invariant from yeast to human, we expect the sporulation/meiosis-specific role of the RAD6 amino terminus to be conserved among eukaryotes. Furthermore, if the RAD6 amino terminus interacts with related E3 proteins, then we anticipate a parallel evolutionary conservation of these E3 proteins as well.

The reduced efficiency of DNA repair in the *rad6* _{Δ 1-9} mutant could arise as a pleiotropic effect of defective E3 interactions, and decreased growth rate may be a consequence of defective repair of spontaneous DNA lesions. An association between DNA repair and growth rate is suggested from the following observations: (1) the *rad6-149* mutant is defective in sporulation but DNA repair and growth rate remain unaffected; (2) null mutations in the *RAD18* and *RAD5* genes, both members of the *RAD6* epistasis group, have no effect on sporulation, but they diminish both DNA repair and growth rate (Johnson et

al. 1992); and (3), the *SRS2* mutation suppresses both the DNA repair and growth defects in the *rad6* null mutation by channeling DNA lesions into the alternate DNA repair pathway controlled by genes in the *RAD52* epistasis group (Schiestl et al. 1990). RAD6, by its E3-dependent roles, may control the levels of exopeptidases or endopeptidases which, in turn, affect the levels of DNA repair proteins. A decrease in the level of some limiting proteins, resulting from elevated peptidase levels in the *rad6* _{Δ 1-9} mutant, might underlie the DNA repair and growth defects. Another possibility is that the RAD6 amino terminus directly interacts with DNA repair proteins. In that case, the RAD6 amino terminus would be expected to interact with E3 proteins, as well as with DNA repair proteins, by recognition of a common sequence motif. However, we have observed no evidence of any similar sequence shared among UBR1, RAD18, RAD5, and several other DNA repair proteins. We also examined the possibility that amino acids 10–15 provide the *rad6* _{Δ 1-9} protein the residual capacity to interact with DNA repair proteins. However, we found that the UV sensitivity and growth rate, as well as UV mutagenesis, in the *rad6* _{Δ 1-15} mutant were virtually identical to that in the *rad6* _{Δ 1-9} mutant (data not shown).

In summary, we suggest that the RAD6 amino terminus is specifically involved in interactions with various E3 proteins and that E3 dependent ubiquitination of substrates is essential for sporulation and affects the proficiency of DNA repair.

Materials and methods

Strains and plasmids

S. cerevisiae strains used in this study are listed in Table 6. Growth, minimal, and sporulation media were prepared as described (Sherman et al. 1986). A list of plasmids used is given in Table 4.

Yeast transformation and other procedures

Introduction of plasmids into yeast strains was done according to Ito et al. (1983). Determination of UV survival and UV-induced mutagenesis, as well as techniques for Western immunoblotting and affinity purification of antibodies, were as described previously (Morrison et al. 1988). Electrophoresis of proteins on SDS-polyacrylamide gels was performed according to Laemmli (1970).

Construction of *rad6* _{Δ 1-9} plasmids

The initiation codon of the *RAD6* gene was changed from ATG to AAG by site-directed mutagenesis (Zoller and Smith 1984). The mutation was made using the oligonucleotide 5'-TGCTGTGCACTTGACGCTTTA-3', which corresponds to the antisense DNA strand of *RAD6* from positions +12 to -9 (Reynolds et al. 1985). A 613-bp *EcoRI* fragment containing the mutation was used to replace the wild-type *RAD6* sequence in the same fragment from plasmids pTB227, pR67 (Morrison et al. 1988), and pSCW242 (Sung et al. 1990) yielding plasmids pR64, pR68, and pR661, respectively (Table 4). DNA sequence analysis of the mutant gene confirmed that only the desired change had occurred.

Table 6. *S. cerevisiae* strains used

Strain	Genotype	Source
LP3041-6D	<i>MATα leu2-3 leu2-112 trp1Δ ura3-52</i>	Sung et al. (1990)
839	<i>MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3</i>	Morrison et al. (1988)
EMY1	<i>MATα leu2-3 leu2-112 trp1Δ ura3-52 rad6Δ::LEU2⁺</i>	Morrison et al. (1988)
EMY7	<i>MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 rad6Δ::LEU2⁺</i>	Morrison et al. (1988)
EMY8	<i>MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 trp1Δ::URA3⁺ rad6Δ::LEU2⁺</i>	Reynolds et al. (1990)
YPH500	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ63 ura3-52</i>	Sikorski and Hieter (1989)
JWY119	<i>MATα ade5 his7 leu2-3 lys1 met14 pet5 ura3 trp1Δ rad18Δ rad6Δ::LEU2⁺</i>	this study
JWY182	<i>MATα ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 rad6Δ</i>	this study
BBY68	<i>MATα ade2-101 his3-Δ200 lys2-801 trp1-Δ63 ura3-52 rad6Δ::LEU2⁺ ubr1Δ</i>	Dohmen et al. (1991)
EMY26	<i>rad6Δ/rad6Δ (EMY1 $\times$ EMY7)</i>	Sung et al. (1990)
EMY28	<i>rad6Δ/rad6Δ (EMY1 $\times$ EMY8)</i>	Reynolds et al. (1990)

Strains LP3041-6D and EMY1 are isogenic; strains 839, EMY7, EMY8, and JWY119 are isogenic; and strains YPH500 and JWY182 are isogenic.

Construction of JWY119 (*rad6 Δ rad18 Δ*) and JWY182 (*rad6 Δ*) strains

The genomic *RAD18* gene from strain EMY8 and the genomic *RAD6* gene from strain YPH500 were deleted by the method of gene replacement (Rothstein 1983). A *ura3* mutant of EMY8 was obtained by selecting for growth on 5-fluoro-orotic acid (5-FOA) [Boeke et al. 1984]. To delete the *RAD18* gene, the resulting *ura3* strain was transformed with a 6.6-kb *EcoRI* fragment from pJJ239 in which a 3.8-kb *BamHI*–*BglIII* fragment from pNKY51 [Alani et al. 1987] containing a *hisG*–*URA3*–*hisG* construct had been inserted into the *BglIII* site of pJJ193 [Jones et al. 1988]. This deletes the *RAD18* gene from position +130 in the open reading frame to 20 nucleotides beyond the termination codon. *URA*⁺ transformants carrying the *rad18 Δ* mutation, as verified by allelism test, were grown on 5-FOA to select for a *ura3* mutant, yielding the *rad6 Δ rad18 Δ* strain JWY119. The *rad6 Δ* mutation in YPH500 was obtained by transformation with a *HindIII*–*BglIII* DNA fragment of pTB64 [Reynolds et al. 1985] in which the 613-bp *EcoRI* fragment containing the entire *RAD6* open reading frame has been replaced by the 3.8-kb *hisG*–*URA3*–*hisG* DNA fragment from pNKY51. Strain JWY182 was obtained by 5-FOA treatment of *URA*⁺ transformants showing allelism to *rad6*.

Indirect immunofluorescence

Determination of the cellular location of RAD6 and mutant Rad6 proteins was done by indirect immunofluorescence [Pringle et al. 1991]. Affinity-purified antibodies to rad6-149 protein were used as the primary antibody. The secondary antibody used was goat anti-rabbit IgG conjugated to FITC (Sigma). As a control, cells used for immunofluorescence were also incubated with secondary antibody, in the absence of treatment with primary antibody, to make certain there was no cross-reactivity of cellular material to the secondary antibody. Cells were stained with 4',6'-diamidino-2 phenylindole (DAPI) to visualize the nucleus. A Leitz Laborlux D fluorescence microscope equipped with an Olympus PM-10AD photomicrographic system was used to visualize and photograph the fluorescence.

Determination of β -Gal activity

RAD⁺ strain YPH500, *rad6 Δ* strain JWY182, and *rad6 Δ* strain JWY182 carrying plasmids pR661 (*rad6 Δ 1-9*), pR615 (*rad6*–149), or pR616 (*rad6*–153) were each transformed with the Ub–X– β -Gal plasmid KEP268, KEP269, or KEP270. Minimal medium

containing 2% galactose, instead of glucose, was inoculated with cells from an exponentially growing culture in glucose-containing medium. The cells were grown overnight at 30°C and used to inoculate fresh galactose-containing medium. The cells were grown to $\sim 1 \times 10^7$ cells/ml and then assayed for the level of β -Gal as described by Reynolds and Lundblad (1989).

In vivo β -Gal degradation assay

Pulse-chase experiments and immunoprecipitation of β -Gal proteins were carried out by a procedure similar to Bachmair et al. (1986). Briefly, *RAD*⁺ strain YPH500 and *rad6 Δ* strain JWY182 carrying plasmids of interest (see above) were grown at 30°C in medium (for maintaining selection of plasmids) containing 2% galactose (in place of glucose) until the cell density was $\sim 1 \times 10^7$ cells/ml. Cells from 30 ml of suspension were collected by filtration and washed three times at 30°C with 30 ml of selection medium lacking methionine. Cells were then suspended in 0.5 ml of 40 mM potassium phosphate buffer (pH 7.4), 1% galactose, and labeled with 500 μ Ci of [³⁵S]methionine (Amersham; >1000 Ci/mmol) for 5 min at 30°C. After the pulse period, the cells were collected by filtration, washed with 15 ml of selection medium (30°C), and suspended in 0.8 ml of selection medium containing 0.5 mg/ml of cycloheximide and 10 mM methionine. Samples of 0.4 ml were then withdrawn immediately and after a 15-min chase period (0- and 15-min samples, respectively, in Fig. 7), and added to 0.8 ml of ice-cold buffer A [1% Triton X-100, 0.15 M NaCl, 5 mM EDTA, 50 mM HEPES (pH 7.5)] containing protease inhibitors (leupeptin, pepstatin, aprotinin, and chymostatin each at 20 μ g/ml, 5 mM *n*-ethylmaleimide, 1 mM PMSF, 0.5 mM benzamidin), and cells were broken by vortexing vigorously for 3 min at 4°C in the presence of 0.4 ml (~ 0.7 grams) of acid-washed glass beads (0.5 mm). Cellular debris was removed by centrifugation, and β -Gal was immunoprecipitated from 0.95 ml of supernatant by addition of monoclonal antibodies to β -Gal (Promega) and incubation at 4°C for 1 hr with rocking. Protein A-agarose (50 μ l) (Bio-Rad) was then added to the samples, and the incubation was continued for an additional 30 min. The protein A-agarose pellet after a 3-sec centrifugation was washed three times with buffer A containing 0.1% SDS, suspended in SDS-electrophoresis sample loading buffer, boiled for 3 min, and electrophoresed on a 6% SDS-polyacrylamide gel [Laemmli 1970]. After electrophoresis, the gel was subjected to fluorography using EN³HANCE (Du Pont) and following the manufacturer's recommended procedure.

Protein purification

All of the protein purification steps were carried out at 4°C. Buffer A was 50 mM Tris-HCl (pH 7.5) containing 10% sucrose, 200 mM KCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, and 10 µg/ml of each of the following protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A. Buffer B was 20 mM KH₂PO₄ (pH 7.4) containing 10% glycerol, 0.5 mM EDTA, and 0.2 mM DTT. Chromatographic matrices were equilibrated in buffer B containing 150 mM KCl.

The rad6_{Δ1-9} mutant protein was purified from extract of the rad6Δ strain EMY8 harboring pR661 (*ADC1::rad6_{Δ1-9}*). Fifty grams of cells were disrupted through the use of a French press in 100 ml of buffer A, and the crude lysate was clarified by centrifugation (100,000g for 60 min). The supernatant (fraction I) was diluted with 50 ml of 10% glycerol before being applied onto DEAE-Sephacel (1.6 × 12.5 cm); bound proteins were eluted with buffer B containing 400 mM KCl. The protein pool (fraction II; 15 ml) was dialyzed against 1 liter of buffer B to lower the KCl concentration to 100 mM and chromatographed on DEAE-Sephacel (1 × 5 cm) by use of a 120-ml gradient of 150–400 mM KCl in buffer B. The rad6_{Δ1-9} protein elutes from DEAE-Sephacel at ~310 mM KCl, and the protein pool (fraction III; 9 ml) was diluted with 5 ml of 10% glycerol and further fractionated on Mono Q (HR5/5) with a 25-ml of KCl gradient from 150 to 500 mM. The Mono-Q pool (fraction IV; 1 ml with ionic strength of 400 mM KCl) was concentrated to 200 µl in Centricon-10 (Amicon) and subjected to molecular sizing on Sephadex G50 (1 × 25 cm) to give fraction V (1.5 ml), which contained 20 µg of nearly homogeneous rad6_{Δ1-9} protein and was concentrated to 100 µl and stored in small aliquots at –70°C.

Wild-type RAD6 protein was purified as described by Sung et al. (1988).

Immunoprecipitation

Cell lysate was prepared from the *ubr1Δ rad6Δ* strain BBY68 harboring the plasmid pSOB44, in which UBR1 is overexpressed from the *ADC1* promoter. Cells were disrupted with a French press in buffer C [50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, and 0.5 mM DTT, and 10 µg/ml of the protease inhibitors aprotinin, chymostatin, leupeptin, and pepstatin A] at 1 ml/g of cells and clarified by centrifugation (100,000g for 120 min). To 0.3 ml of extract was added 1 µg of purified RAD6 or rad6_{Δ1-9} protein, and after a 12-hr incubation on ice, 30 µl of Affigel protein A (Bio-Rad) bearing 50 µg of anti-RAD6 antibodies (Sung et al. 1988) was added. After mixing by rocking for 2 hr at 4°C, the immunoprecipitates were washed four times with 300 µl of ice-cold buffer C containing 5% glycerol but no protease inhibitors. Proteins were eluted from immunoprecipitates by a 5-min treatment with 50 µl of 2% SDS at 37°C and subjected to SDS-PAGE and immunoblot analysis.

Assay of E3-dependent protein multiubiquitination and degradation

Components of the cell-free proteolytic system—affinity-purified E1 and fractions containing E3 and the ubiquitin-specific protease—were obtained from rabbit reticulocytes as described (Sung et al. 1991). Assay mixtures (25 µl) were assembled in 50 mM Tris-HCl (pH 7.3) containing 5 mM MgCl₂, 9.4 µM ubiquitin, ¹²⁵I-labeled β-lactoglobulin (1.2 × 10⁵ cpm), an ATP-regenerating system (2 mM ATP, 10 mM creatine phosphate, and 0.6 U/ml each of creatine phosphokinase and inorganic pyrophosphatase), 200 nM RAD6 or 200 nM to 1 µM rad6_{Δ1-9} protein,

100 nM E1, E3 fraction (18 µg protein), and the protease fraction (17 µg protein); multiubiquitination and degradation of the ¹²⁵I-labeled protein substrate proceeded at 37°C for 60 min. To process reaction mixtures for quantitation of substrate degradation, 30 µg of BSA was added in 30 µl followed by 30 µl of 40% trichloroacetic acid. After a 10-min incubation on ice, samples were spun at 4°C for 5 min in a microcentrifuge and the radioactivity of the supernatant was determined. To visualize the multiubiquitinated forms of the substrate, 10 µl of reaction mixtures were withdrawn after 20 min of incubation and subjected to electrophoresis in 12.5% SDS–polyacrylamide gels and autoradiography.

Assay of thioester formation

Reaction mixtures (10 µl) containing 6 µM ¹²⁵I-labeled ubiquitin, 200 nM E1 and 500 nM RAD6 or rad6_{Δ1-9} protein in 50 mM Tris-HCl (pH 7.3), with 5 mM MgCl₂, 2 mM ATP, and 0.1 mM DTT were incubated at 30°C for 10 min. After the addition of 10 µl of SDS buffer [4% SDS in 100 mM Tris-HCl (pH 6.8) and 20% glycerol], samples were subjected to electrophoresis in 12.5% SDS–polyacrylamide gels and autoradiography.

Other procedures

Iodination of ubiquitin and β-lactoglobulin with sodium ¹²⁵I (Amersham) using the chloramine T method was done as described (Sung et al. 1988). SDS-PAGE was carried out according to Laemmli (1970). Nitrocellulose blots were probed with 1/300 dilution of the anti-ha monoclonal antibody 12CA5 (2.5 mg/ml; Berkeley Antibody) and 1/1000 dilution of affinity-purified polyclonal anti-RAD6 antibodies (2.5 ml/ml; Sung et al. 1988), and immunoblots were developed with protein A–HRP and 4-chloro-1-naphthol following the instructions of the supplier (Bio-Rad).

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