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Domains Required for Dimerization of Yeast Rad6 Ubiquitin-Conjugating Enzyme and Rad18 DNA Binding Protein

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The *RAD6* gene of *Saccharomyces cerevisiae* encodes a ubiquitin-conjugating enzyme required for postreplicational repair of UV-damaged DNA and for damage-induced mutagenesis. In addition, Rad6 functions in the N end rule pathway of protein degradation. Rad6 mediates its DNA repair role via its association with Rad18, whose DNA binding activity may target the Rad6-Rad18 complex to damaged sites in DNA. In its role in N end-dependent protein degradation, Rad6 interacts with the *UBR1*-encoded ubiquitin protein ligase (E3) enzyme. Previous studies have indicated the involvement of N-terminal and C-terminal regions of Rad6 in interactions with Ubr1. Here, we identify the regions of Rad6 and Rad18 that are involved in the dimerization of these two proteins. We show that a region of 40 amino acids towards the C terminus of Rad18 (residues 371 to 410) is sufficient for interaction with Rad6. This region of Rad18 contains a number of nonpolar residues that have been conserved in helix-loop-helix motifs of other proteins. Our studies indicate the requirement for residues 141 to 149 at the C terminus, and suggest the involvement of residues 10 to 22 at the N terminus of Rad6, in the interaction with Rad18. Each of these regions of Rad6 is indicated to form an amphipathic helix.

Lesions in DNA caused by UV and by various other DNA-damaging agents constitute a block to DNA replication machinery, resulting in a gap in the newly synthesized strand across from the damage site in the template strand. In both prokaryotes and eukaryotes, a variety of error-free and mutagenic postreplicative repair processes have evolved to fill in the gap. Genetic studies of the yeast *Saccharomyces cerevisiae* have been instrumental in the identification of genes that affect postreplicational repair in eukaryotes. The *RAD6* and *RAD18* genes of *S. cerevisiae* are the two most important members of the *RAD6* epistasis group. Mutations in both genes confer a high degree of UV sensitivity, resulting from a defect in postreplicational repair (22). UV-induced mutagenesis is also abolished in the *rad6* and *rad18* mutants (1, 7, 17), and various genetic observations are consistent with the involvement of *RAD6* and *RAD18* in error-free as well as the error-prone modes of postreplicational repair (reference 28 and references therein). Apart from its role in DNA repair, *RAD6* is essential for sporulation, and *rad6* mutations cause an increase in the rate of retrotransposition (18, 21). Mutations in *RAD18* have no effect on sporulation. The effect of *RAD18* on retrotransposition has not been examined.

Rad6 is a ubiquitin-conjugating enzyme of 172 residues (13, 27). The first 149 residues of Rad6 form a globular domain, while the last 23 residues, of which 20 are acidic, constitute a freely extending tail domain (20). The *rad6-153* mutation, which produces a protein missing the last 19 residues of the acidic tail, has no effect on the biological functions of *RAD6* (20). The *rad6-149* mutation, which results in the deletion of all 23 residues of the acidic tail, also has no adverse effect on DNA repair or mutagenesis functions; sporulation is, however, severely impaired by this mutation (20).

Rad6 mediates its role in DNA repair via an association with Rad18 (2). We have purified the Rad6-Rad18 complex from yeast to near homogeneity and shown that it is a heterodimer (3). Unlike Rad6, which shows no affinity for DNA, the Rad6-Rad18 complex binds single-stranded DNA (2). Rad18 contains a C_3HC_4 sequence motif, known as the RING finger motif, and a C_2HC motif (14), either or both of which may be utilized for DNA binding. Rad18 also contains a GKS Walker type A nucleotide binding motif (14). In addition to its role in DNA repair, Rad6 is required for protein degradation dependent upon the identity of the amino-terminal residue in the proteolytic substrate (9, 26). In this role, Rad6 interacts with the *UBR1*-encoded ubiquitin protein ligase, an E3 enzyme, which binds the N end rule protein substrates (9, 29).

The structure and function of *RAD6* have been conserved to a remarkable degree among eukaryotes, such that *RAD6* counterparts from *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and humans complement the DNA repair and mutagenesis defects of the *S. cerevisiae rad6*Δ mutant (15, 16, 24). Consistent with these observations, the Rad6 counterparts of *Schizosaccharomyces pombe* and humans have been shown to physically interact with the *S. cerevisiae* Rad18 protein (2). Furthermore, *S. cerevisiae* Rad6 protein catalyzes efficient multiubiquitination and degradation of N end rule protein substrates in reaction mixtures containing ubiquitin-activating enzyme E1, the ubiquitin protein ligase E3α, and the ubiquitin-specific proteasome derived from rabbit reticulocytes (26). Therefore, the domains of Rad6 that direct interactions with the E1 and E3α enzymes have also been conserved.

Rad6 forms a binary complex either with Ubr1 or with Rad18 (2). Our previous studies have indicated an involvement of the highly conserved Rad6 amino terminus in an interaction with the Ubr1 protein (29). The $rad6_{\Delta 1-9}$ mutation, which produces a protein lacking the first nine amino acid residues, confers a total defect in the degradation of N end rule protein substrates, and the $Rad6_{\Delta 1-9}$ protein lacks the ability to interact with the Ubr1 protein (29). The $Rad6_{\Delta 1-9}$ protein, however, interacts normally with Rad18 (2). In contrast to the lack of any perceptible effects of the *rad6-149* mutation on DNA repair, the $rad6_{\Delta 1-9}$ mutation reduces the efficiency of N end-dependent protein degradation (19, 29). These observations

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TABLE 1. Oligonucleotides used to generate Rad6 and Rad18 peptides by PCR

Oligonucleotide stock no.	Oligonucleotide sequence[a]
RAD18	
1791	GCG GAT CCT AAA AAG ACA GTT GTT AAG CTG GG
1868	GCG AAT TCC GAT GAA CCT GTT TTT ATT TTC ATT TTT GC
2127	GCG AAT TCA TTA ATT GTT ACC GGG TGG GTC
2128	GCG GAT CCG ACA GTT CAG GTT CAG TGG GAC
3818	GCG GAT CCA ACA AGG GTG GAA TTT C
3819	GCG GAT CCA AAT TTA ATA GGA AAG GAT GG
3820	GCG AAT TCT TTG AAC CTG TTT TTA TTT TC
3821	GCG AAT TCT TAT CGT TTT TGA AAT TTT CAA
A25178	GCG GAT CCA GGA AAT TAC TTG AAA ATT TC
A25179	GCG AAT TCT TCC TGA TAA GCC TAG CAA AAT C
RAD6	
1581	AAA GAT AAA GCG CCA TGG CCA CAC CAG CTA GA
1582	TTT GAT TGA ACA AAA AGG TAA
N054	GCG AAT TCT TCA TAT CAT CCT CCC AAG ATT TC
N055	GCG GAT CCG GTG TAT CTG CTT CAC CAT TAC
88.148	GAG TTT GAT TCA GAA TAT CCC AAT

[a] Restriction sites for *Bam*HI (GGATCC), *Eco*RI (GAATTC), and *Nco*I (CCATGG) are underlined.

have suggested that both the amino-terminal and carboxyl-terminal regions of Rad6 affect its interaction with Ubr1.

In this work, we identify the domains of Rad6 and Rad18 necessary for dimerization. In particular, we show that a region of 40 amino acids (residues 371 to 410) in Rad18 is sufficient for interaction with Rad6. This Rad18 segment may represent a helix-loop-helix motif. For Rad6, we provide evidence for the involvement of the carboxyl-terminal region, between residues 141 and 149, in interaction with Rad18. Our studies also suggest the involvement of residues 10 to 22 in the amino-terminal region of Rad6 in complex formation with Rad18.

MATERIALS AND METHODS

PCR. DNA amplifications were carried out with *Taq* DNA polymerase (Perkin-Elmer) at 2 mM $MgCl_2$ in the buffer recommended by the manufacturer.

Plasmids for protein expression. The *rad18-5* mutant was obtained serendipitously during our attempt to place the *RAD18* gene under the control of the *tac* promoter by cloning it into a derivative of pKK233-2 (Pharmacia). Sequence analysis of the *rad18-5* mutation indicated it to be a 10-bp deletion from nucleotides +1083 to +1092 of the 1,461-nucleotide-long *RAD18* open reading frame (ORF), resulting in a mutant protein lacking the 126 C-terminal amino acids and containing an additional 39 amino acids resulting from the frameshift.

The pGEX glutathione *S*-transferase (GST) gene fusion vectors (Pharmacia) were used to generate plasmids in which various Rad18 or Rad6 polypeptides were overexpressed. For GST::Rad18 fusions, a 500-bp *Rsa*I-*Stu*I fragment from pJJ136, a 2μm *URA3* plasmid containing the entire *RAD18* gene (14), was cloned into the *Sma*I site of pGEX-3X, generating plasmid pR18.31. Plasmid pR18.31 thus contains the DNA encoding the C-terminal 188 amino acids, from residue 300 to residue 487, of Rad18 protein fused to the *GST* gene in pGEX-3X. To generate a shorter Rad18 polypeptide, pR18.31 was digested with *Bam*HI and *Xba*I and the ends were filled in and religated, yielding pR18.40, which contains the GST::$Rad18_{323–487}$ fusion. The other GST::Rad18 fusions were constructed by PCR to obtain DNA fragments encoding peptides of different lengths with pJJ136 as the DNA template and the oligonucleotides listed in Table 1. The PCR products were digested with *Bam*HI and *Eco*RI and ligated into pGEX-2T (or pGEX-3X for pR18.44), yielding the following: GST::$Rad18_{331–411}$ (pR18.44), GST::$Rad18_{412–487}$ (pR18.46), GST::$Rad18_{351–380}$ (pR18.49), GST::$Rad18_{381–410}$ (pR18.50), GST::$Rad18_{351–410}$ (pR18.51), GST::$Rad18_{371–400}$ (pR18.53), and GST::$Rad18_{371–410}$ (pR18.67) (Table 2).

TABLE 2. Plasmids for overexpression of Rad18 and Rad6 peptides

Plasmid	Vector (gene)	Peptide residues	Oligonucleotides[a]
Rad18			
pR18.31	pGEX-3X (GST)	300–487	
pR18.40	pR18.31 (GST)	323–487	
pR18.44	pGEX-3X (GST)	331–411	1791 and 1868
pR18.46	pGEX-2T (GST)	412–487	2127 and 2128
pR18.49	pGEX-2T (GST)	351–380	3818 and 3821
pR18.50	pGEX-2T (GST)	381–410	3819 and 3820
pR18.51	pGEX-2T (GST)	351–410	3818 and 3820
pR18.53	pGEX-2T (GST)	371–400	A25178 and A25179
pR18.67	pGEX-2T (GST)	371–410	A25178 and 3820
Rad6			
pR6.99	pSE380 (trc)	1–172	1581 and 1582
pR6.102	pGEX-1T (GST)	1–172	
pR6.108	pGEX-2T (GST)	23–153	N055 and N054
pR6.109	pGEX-2T (GST)	1–153	1581 and N054
pR6.113	pGEX-3X (GST)	100–153	88.148 and N054

[a] Oligonucleotide numbers refer to those given in Table 1.

The *RAD6* gene, containing an *Nco*I site introduced at the translation-initiating ATG codon by PCR with oligonucleotides 1581 and 1582 (Table 1), was cloned as an *Nco*I-*Eco*RI fragment in the *trc* promoter expression vector pSE380 (Invitrogen Corp.), yielding plasmid pR6.99. To obtain a GST::$Rad6_{1–172}$ fusion, the *Nco*I-*Eco*RI fragment from pR6.99 with the *Nco*I site filled in was ligated into pGEX-1T, yielding plasmid pR6.102. Different portions of the *RAD6* gene were amplified by PCR with pTB227, a 2μm *URA3* plasmid containing the entire *RAD6* gene (20), with oligonucleotides listed in Table 1. After digestion with the appropriate restriction enzymes, the PCR fragments were cloned into pGEX vectors, yielding the following constructs: GST::$Rad6_{23–153}$ (pR6.108), GST::$Rad6_{1–153}$ (pR6.109), and GST::$Rad6_{100–153}$ (pR6.113) (Table 2).

The *rad6-164*, *rad6-153*, and *rad16-149* mutant alleles, which lack 8, 19, and 23 C-terminal residues, respectively, have been described previously (20). The *rad6-1* mutant allele contains a TAG nonsense codon at nucleotide +424 and results in a truncated protein containing 141 rather than 172 amino acids (25). *Eco*RI fragments containing either the wild-type *RAD6* or these *rad6* mutant alleles were cloned into a 2μm *URA3* vector, generating plasmids pTB227, pR6.33, pR6.32, pR6.31, and pTB248, which contain the wild-type *RAD6* gene or the *rad6-164*, *rad6-153*, *rad6-149*, and *rad6-1* mutant alleles, respectively (Table 3).

For overexpression of Rad18, the *RAD18* gene was placed under the control of the *ADC1* promoter to generate the 2μm *TRP1* plasmid pR18.22. We also constructed plasmid pR18.36, in which the *RAD18* and *RAD6* genes are expressed from the inducible *GAL1* promoter and the constitutive *ADC1* promoter, respectively. This plasmid was used for purification of the Rad6-Rad18 complex from yeast. We also used this plasmid to construct C-terminal deletions

TABLE 3. Plasmids used in immunoprecipitation and UV survival experiments

Plasmid	Relevant characteristics
pR18.22	2μm, *TRP1 ADC1*::*RAD18*
pTB236	2μm, *URA3*
pTB227	2μm, *URA3 RAD6*
pR6.33	2μm, *URA3 rad6-164*
pR6.32	2μm, *URA3 rad6-153*
pR6.31	2μm, *URA3 rad6-149*
pTB248	2μm, *URA3 rad6-1*[a]
pTB386	2μm, *URA3 GAL1*
pR18.36	2μm, *URA3 GAL1*::*RAD18 ADC1*::*RAD6*
pR18.78	2μm, *URA3 GAL1*::$rad18_{1–380}$ *ADC1*::*RAD6*
pR18.79	2μm, *URA3 GAL1*::$rad18_{1–410}$ *ADC1*::*RAD6*

[a] The *rad6-1* allele encodes the Rad6-141 protein (25).

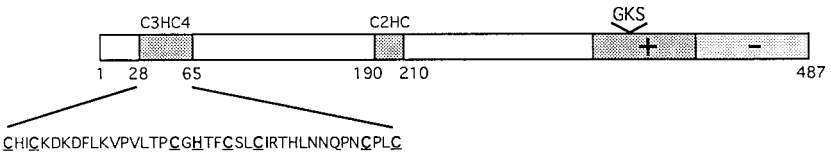

FIG. 1. Schematic representation of the Rad18 sequence features. The positions of the C_3HC_4 RING finger domain and the C_2HC motif ($CX_2CX_{12}HX_3C$) in the Rad18 protein are indicated; the cysteine and histidine residues shown to participate in zinc coordination in RING finger motifs (4, 6, 11) are in boldface type and underlined. The basic and acidic carboxyl-terminal domains are specified by the + and − symbols, respectively. The GKS sequence of the ATP binding-hydrolysis motif is indicated.

of *RAD18*. Plasmids pR18.78 and pR18.79, derived from pR18.36 (Table 3), encode $Rad18_{1–380}$ and $Rad18_{1–410}$ proteins, which lack 107 and 77 C-terminal amino acids, respectively.

Proteins. The Rad6-Rad18 complex was purified from yeast cells harboring plasmid pR18.36, which coexpresses both Rad18 and Rad6 proteins, by a combination of ammonium sulfate precipitation and column chromatography on Q-Sepharose, phenyl-Sepharose, Mono S, and Mono Q (3). The Rad18-5 mutant protein was expressed in *Escherichia coli* from plasmid pJJ309; it was solubilized with sodium dodecyl sulfate (SDS) from the insoluble fraction of lysed cells and purified by preparative polyacrylamide gel electrophoresis and electroelution as described previously (2). GST-Rad6 and GST-Rad18 fusion proteins were prepared from *E. coli* BL21 and bound by affinity chromatography on glutathione-Sepharose as described by the manufacturer (Pharmacia). Elution from the beads was done with 10 mM glutathione or by boiling in SDS loading buffer.

Rad6 was purified from yeast strain CMY135 carrying pSCW242 (2μm, *TRP ADC1-RAD6*) as described previously (27).

To obtain radiolabeled GST–^{32}P-$Rad18_{371–410}$, the *RAD18* fragment was cloned into pGEX-2KT (Pharmacia), in which a sequence motif for phosphorylation has been inserted between the GST sequence and the cloning site. The protein was expressed in *E. coli* BL21, bound to the glutathione-Sepharose beads, radiolabeled with bovine heart protein kinase (Sigma), and eluted with glutathione as directed by the manufacturer (Pharmacia).

Yeast extracts and immunoprecipitations. YR6-15 (*MAT***a** *ade5 his7 leu2-3 met14 pet5 trp1*Δ *rad6*Δ::*ura3*), a strain harboring a genomic deletion mutation of the *RAD6* gene, was transformed with two multicopy plasmids—pR18.22, which carries the *RAD18* gene under the control of the *ADC1* promoter, and either pTB227, pR6.33, pR6.32, pR6.31, or pTB248, in which the *RAD6* gene or the *rad6* mutant alleles are under the control of the natural promoter of the *RAD6* gene—or with the control vector pTB236 with no *RAD6* insert (Table 2). The strains were grown in synthetic complete medium lacking tryptophan and uracil until a density of 1×10^7 to 2×10^7 cells per ml was reached. Extracts were obtained as described previously (2) with a French press. Immunoprecipitations were carried out with 25 μl of protein A-agarose beads bearing anti-Rad6 or anti-Rad18 antibodies (3 mg/ml) and 1.2 ml of extract (2). Proteins were eluted in 25 μl of SDS loading buffer, and aliquots of 5 μl were run on a 5 to 20%-gradient polyacrylamide denaturing gel.

Antibodies. Polyclonal anti-Rad6 and anti-Rad18 antibodies were raised in rabbits and affinity purified on antigen-Sepharose columns as previously described (2).

Western blots and far-Western blots. Western blotting and far-Western blotting were performed as described previously (2). Briefly, proteins were run on denaturing (SDS) polyacrylamide gels and electrotransferred onto nitrocellulose membranes. The membranes were blocked by rocking for 1 h in phosphate-buffered saline containing 0.05% Tween 20 and 1.5% bovine serum albumin (BSA) (PBT). For far-Western blot analyses, the membrane was reacted with the putative interacting protein (3 h in PBT at room temperature) and then treated as in a regular Western blot: it was probed with anti-Rad6 or anti-Rad18 antibodies and then with protein A-horseradish peroxidase. The colorimetric reaction was done with 4-chloro-1-naphthol as the substrate.

To detect interaction between the different GST-Rad6 and Rad18 proteins (see Fig. 6B), the nitrocellulose blot was probed directly with GST–^{32}P-$Rad18_{371–410}$ in phosphate-buffered saline containing 0.2% Tween 20 and 1.5% BSA. After three 30-min washes with the same buffer, the blot was wrapped in plastic film and subjected to autoradiography.

Survival after UV irradiation. Cells of the *rad18*Δ yeast strain YR18.6 (*MAT*α *leu2-3,112 gal1 reg1-501 pep4-3 prb1-1122 trp1*Δ *ura3-52 rad18*Δ::*URA3*) carrying the plasmid pTB386 (2μm *GAL1* vector), pR18.36 (2μm *GAL1*::*RAD18 ADC1*::*RAD6*), pR18.78 (2μm *GAL1*::$rad18_{1–380}$ *ADC1*::*RAD6*), or pR18.79 (2μm *GAL1*::$rad18_{1–410}$ *ADC1*::*RAD6*) were spread onto synthetic complete medium plates containing 0.01% galactose. Plates were kept at 30°C for 6 h before being exposed to UV irradiation and then incubated at 30°C in the dark for 5 days.

RESULTS

Identification of the Rad18 domain for interaction with Rad6. Rad18 protein possesses a C_3HC_4 zinc finger motif in its amino terminus. Within the carboxyl-terminal one-third of the protein, Rad18 has sequence motifs that are expected to be involved in the binding and hydrolysis of ATP and regions with clustered basic and acidic residues (Fig. 1). We showed previously that the majority of cellular Rad18 protein exists as a highly stable binary complex with Rad6 protein (2). The interaction between Rad6 and Rad18 proteins can be revealed conveniently in a far-Western blot assay in which Rad18 and its mutant derivatives are first run in a denaturing polyacrylamide gel and then transferred onto a nitrocellulose membrane. After extensive washing with buffer to renature proteins, the nitrocellulose membrane is incubated with purified Rad6 protein to allow complex formation to occur. Binding of Rad6 to Rad18 and its various derivatives (Fig. 2A) is then visualized by probing with affinity-purified anti-Rad6 antibodies (Fig. 2B). Although Rad18 protein bound the added Rad6 protein efficiently (Fig. 2B, lane 1), complex formation did not occur with the Rad18-5 mutant protein that is missing the carboxyl-terminal 126 amino acid residues of Rad18 protein (Fig. 2B, lane 2). Thus, it appeared that the carboxyl terminus of Rad18 is required for the interaction with Rad6 protein. In fact, the results presented below indicate that a domain consisting of 40 amino acid residues situated in the carboxyl terminus of Rad18 confers high affinity for Rad6 protein.

For defining the domain within the carboxyl terminus of Rad18 protein that mediates the interaction with Rad6, different segments of the Rad18 coding sequence encompassing various portions of the carboxyl terminus were fused to the coding sequence of GST and the resulting hybrid GST-Rad18 fusion proteins were expressed in *E. coli* and purified by affinity chromatography on glutathione-Sepharose (Fig. 2A). These purified GST-Rad18 fusion proteins were then subjected to far-Western analysis (Fig. 2B), as described above. As shown in Fig. 2B, since GST-$Rad18_{323–487}$ interacted with Rad6 protein (lane 4) as well as did full-size Rad18 (lane 1) and GST-$Rad18_{300–487}$ (lane 3), it is clear that the Rad6 interaction domain lies within residues 323 to 487 of Rad18 protein. The acidic carboxyl-terminal portion beyond residue 411 is dispensable for interaction with Rad6, as GST-$Rad18_{331–441}$ also interacted strongly with Rad6 protein (lane 5). The Rad6 interaction domain was further localized to a 60-amino-acid segment from residues 351 to 410, as GST-$Rad18_{351–410}$ interacts proficiently with Rad6 (lane 7); GST-$Rad18_{381–410}$, however, interacts only weakly with Rad6 (lane 9), indicating that this region is involved in interaction but most likely lacks a portion of the interaction domain, while GST-$Rad18_{351–380}$ and GST-$Rad18_{371–400}$ do not interact with Rad6 (lanes 8 and 10) at all, indicating that they lack an essential part of the interaction domain.

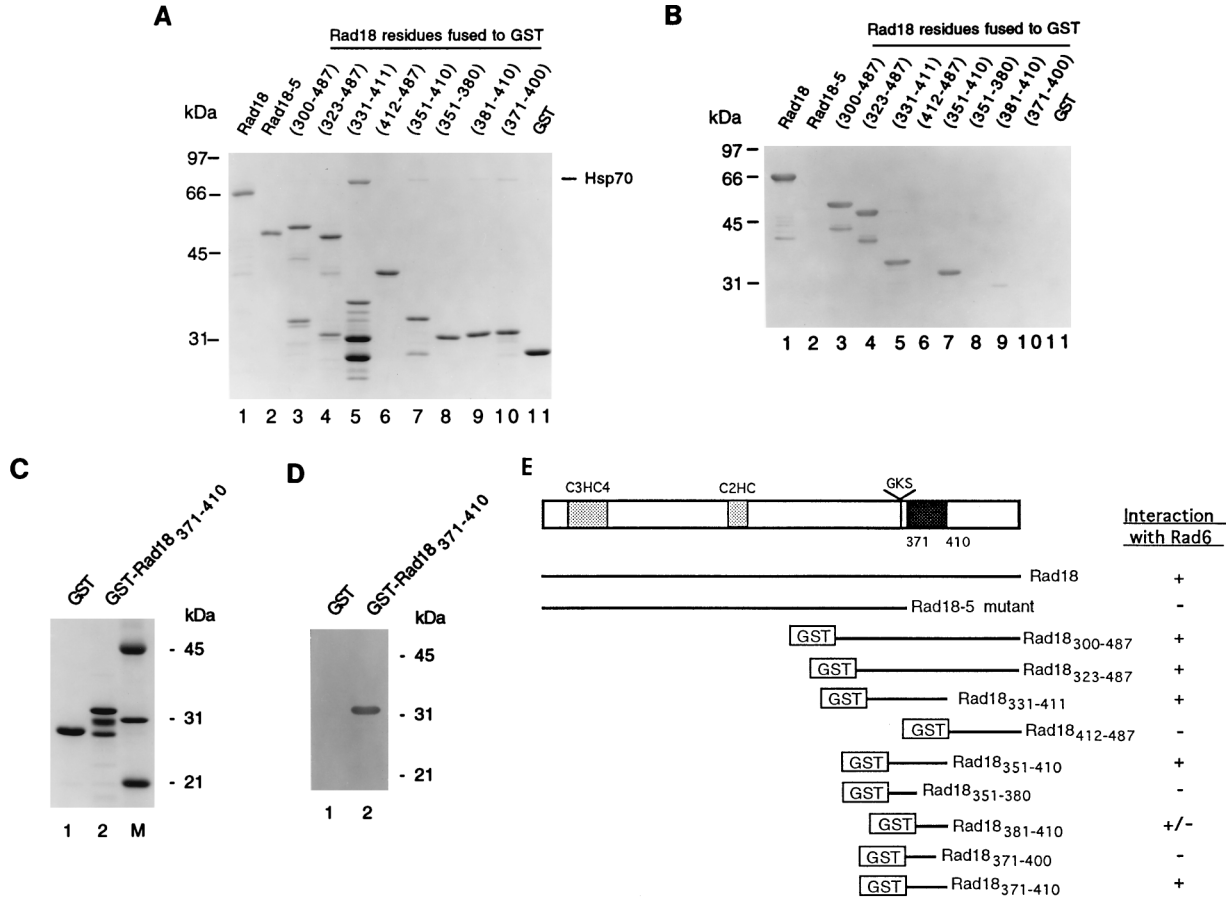

FIG. 2. The domain of Rad18 sufficient for interaction with Rad6 lies close to the carboxyl terminus between residues 371 and 410. (A) Polyacrylamide gel stained with Coomassie blue. The smaller-size bands are proteolytic fragments. (B) Far-Western analysis in which a nitrocellulose blot was incubated first with Rad6 protein to allow for complex formation with the immobilized Rad18 protein and Rad18-derived polypeptides and then with anti-Rad6 antibodies to reveal the complex. The proteins analyzed in panels A and B were Rad18 (lane 1), Rad18-5 (lane 2), GST-Rad18$_{300–487}$ (lane 3), GST-Rad18$_{323–487}$ (lane 4), GST-Rad18$_{331–411}$ (lane 5), GST-Rad18$_{412–487}$ (lane 6), GST-Rad18$_{351–410}$ (lane 7), GST-Rad18$_{351–380}$ (lane 8), GST-Rad18$_{381–410}$ (lane 9), GST-Rad18$_{371–400}$ (lane 10), and GST (lane 11). (C) Polyacrylamide gel stained with Coomassie blue. (D) Far-Western blot. The proteins analyzed in panels C and D were GST (lane 1) and GST-Rad18$_{371–410}$ (lane 2). M, molecular size standard. (E) Schematic representation of the Rad18 ORF, the proteins made, and the result of their interaction with Rad6. In the representation of the Rad18 ORF, the RING domain (C_3HC_4), the C_2HC domain, and the Rad6 binding domain (residues 371 to 410) are denoted by shaded boxes; the position of the GKS sequence of the ATP binding motif is indicated. Under the Rad18 ORF, Rad18 protein and the portions of it are represented by horizontal bars. The GST portion of the fusion proteins is represented by a box.

Since the 60-amino-acid-long Rad6 interaction domain of Rad18 (residues 351 to 410) encompasses the Walker type A nucleotide binding motif GKS, which corresponds to residues 365 to 367, we determined if this site was essential for interaction with Rad6. To do this, we examined the interaction of GST-Rad18$_{371–410}$ protein, which is missing the nucleotide binding motif, with Rad6. As shown in Fig. 2C and D, the GST-Rad18$_{371–410}$ fusion protein interacts strongly with Rad6, indicating that the type A nucleotide binding motif is dispensable for interaction. Taken together, these results indicate that the Rad18 segment of 40 amino acids encompassing residues 371 to 410 is sufficient for interaction with Rad6. Figure 2E summarizes the data for the various Rad18 constructs used in these experiments.

Functional significance of Rad18 interaction with Rad6. The delineation of the interaction domain in Rad18 to amino acids 371 to 410 has allowed us to examine the extent to which interaction with Rad6 is important for Rad18 function in DNA repair. For this purpose, we constructed yeast plasmids that carried the *RAD6* gene expressed from the *ADC1* promoter and the wild-type or mutant *rad18* gene expressed from the galactose-inducible *GAL1* promoter (Table 3). Cells of the *rad18*Δ strain carrying the wild-type *RAD18* gene or the C-terminally deleted *rad18* gene were spread onto plates containing synthetic complete medium with 0.01% galactose and incubated for 6 h at 30°C prior to UV irradiation. Expression of these proteins in galactose-grown cells was verified by immunoblotting. As shown in Fig. 3, the *rad18*Δ strain is highly UV sensitive. Introduction into *rad18*Δ of plasmid pR18.78, which produces the Rad18$_{1–380}$ protein that lacks 30 of the 40 residues of the Rad6 interaction domain, conferred only a small increase in UV resistance. By contrast, expression of Rad18$_{1–410}$ protein, which contains all of the Rad6 interaction domain, restored wild-type levels of UV resistance to *rad18*Δ cells. These results indicate that interaction with Rad6 is indispensable for the DNA repair function of Rad18; they also reveal that the carboxy-terminal 77 amino acids are not essential for Rad18 function in DNA repair.

The acidic domain of Rad6 is dispensable for interaction with Rad18. The carboxyl terminus of Rad6 is highly acidic, such that 20 of the last 23 residues are acidic (Fig. 4). We have previously generated Rad6 mutant proteins missing portions of

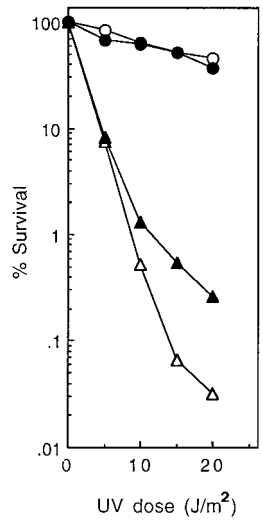

FIG. 3. Effect of deletion of the Rad6 interaction domain from Rad18 on UV survival. Survival after UV irradiation of the *rad18*Δ strain YR18.6 harboring plasmids containing the wild-type *RAD18* gene or genes encoding the C-terminally truncated Rad18 proteins was determined as described in Materials and Methods. Symbols indicate *rad18*Δ-harboring plasmids as follows: ○, pR18.36 (*GAL1*::*RAD18*); ●, pR18.79 (*GAL1*::*rad18*$_{1–410}$); ▲, pR18.78 (*GAL1*::*rad18*$_{1–380}$); △, pTB386 (*GAL1* vector).

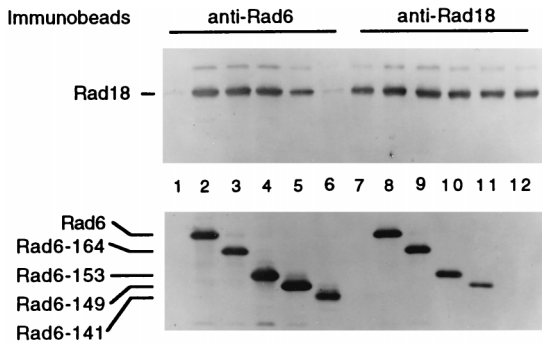

FIG. 5. The helix proximal to the acidic tail in Rad6 is required for interaction with Rad18. Immunoprecipitates were run on a gradient (5 to 20%) polyacrylamide gel, and the proteins were transferred onto a nitrocellulose membrane. Rad6 and Rad18 bands were revealed by probing with affinity-purified antibodies. Anti-Rad6 immunoprecipitates (lanes 1 to 6) and anti-Rad18 immunoprecipitates (lanes 7 to 12) from the YR6-15 (*rad6*Δ) strain carrying plasmid pR18.22 (*ADC1*::*RAD18*) and either pTB236 (2μm control plasmid) (lanes 1 and 7), pTB227 (2μm, *RAD6*) (lanes 2 and 8), pR6.33 (2μm, *rad6-164*) (lanes 3 and 9), pR6.32 (2μm, *rad6-153*) (lanes 4 and 10), pR6.31 (2μm, *rad6-149*) (lanes 5 and 11), or pTB248 (2μm, *rad6-141*) (lanes 6 and 12).

the acidic domain (Fig. 4), and here, we used immunoprecipitation to examine whether these mutant proteins—Rad6-164, Rad6-153, and Rad6-149—retain the ability to interact with Rad18. Immunoprecipitation experiments with both anti-Rad6 and anti-Rad18 immunobeads were carried out with extracts from yeast strains that expressed Rad18 and either wild-type Rad6 or one of the aforementioned mutant Rad6 proteins. After being washed, the beads were resuspended in SDS loading buffer and the eluted proteins were run in a gradient (5 to 20%) denaturing polyacrylamide gel, followed by immunoblot analysis to reveal coprecipitation of Rad18 protein with the wild-type or mutant Rad6 proteins. The results in Fig. 5 show that Rad18 interacts with the Rad6-164, Rad6-153, and Rad6-149 proteins, indicating that the acidic carboxyl-terminal domain of Rad6 is in fact dispensable for interacting with Rad18 protein.

Protein encoded by the *rad6-1* allele is defective in interaction with Rad18. *rad6-1* is a nonsense mutation at nucleotide position +424 in the *RAD6* ORF which changes the lysine AAG codon to the amber TAG codon (25). This results in a deletion of the carboxyl terminus after residue 141. We examined whether Rad6-141 mutant protein is capable of interacting with Rad18 protein. As shown in Fig. 5, the Rad6-141 mutant protein failed to coprecipitate with the Rad18 protein, regardless of whether anti-Rad6 or anti-Rad18 immunobeads were used for the immunoprecipitation. Since Rad6-149 protein interacts with Rad18 fairly efficiently, we conclude that the region of Rad6 protein in the vicinity of residues 141 to 149 is required for interaction with Rad18 protein.

The lack of interaction of Rad6-141 protein with Rad18 is unlikely to be due to improper folding of the C-terminally deleted Rad6 protein. As shown in Fig. 5, the Rad6-141 protein produced by the *rad6-1* allele is expressed in yeast at a level comparable to that of wild-type Rad6 protein. Furthermore, purified Rad6-142 protein retains normal ubiquitin acceptor activity as determined by ubiquitin thiolester formation (10), indicating that this protein interacts normally with ubiquitin-activating enzyme and ubiquitin.

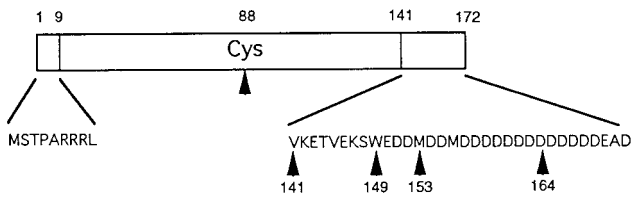

FIG. 4. Schematic representation of the Rad6 protein. The sequences of the first 9 and the last 31 amino acid residues are shown. The position of the unique cysteine (Cys) residue essential for thioester formation with ubiquitin is denoted. The last amino acid residues of the various carboxyl terminal deletion mutants are indicated by arrowheads.

Role of the Rad6 carboxyl terminus in DNA repair. The Rad6-164, Rad6-153, and Rad6-149 proteins, in which portions or all of the acidic carboxyl terminus is deleted, interact normally with Rad18 (Fig. 5), and these mutations have no adverse effect on the DNA repair or UV mutagenesis functions of *RAD6* (20). By contrast, the Rad6-141 protein lacks the ability to interact with Rad18 (Fig. 5) and the *rad6-1* allele which encodes this protein is inactive in DNA repair and damage-induced mutagenesis. The *rad6-1* mutation renders cells highly UV sensitive and defective in postreplicative bypass of UV-damaged DNA templates (22). The formation of mutations in response to treatments with UV and other DNA-damaging agents is also severely impaired in the *rad6-1* mutant (17, 23). These results imply that interaction with Rad18 is important for Rad6 function in DNA repair and damage-induced mutagenesis.

Possible involvement of the Rad6 amino terminus in interaction with Rad18. The amino-terminal region of Rad6 protein is highly conserved among eukaryotes. In fact, the first 15 residues are almost invariant among *S. cerevisiae* Rad6 and its counterparts from *Schizosaccharomyces pombe*, *D. melanogaster*, and humans (29). In addition to forming a binary complex with Rad18 (2), Rad6 also complexes with the *UBR1*-encoded ubiquitin protein ligase E3, essential for multiubiquitination and degradation of N end rule protein substrates (9, 26, 29). We have shown previously that the *rad6*$_{\Delta 1–9}$ mutation, which produces a protein lacking the first

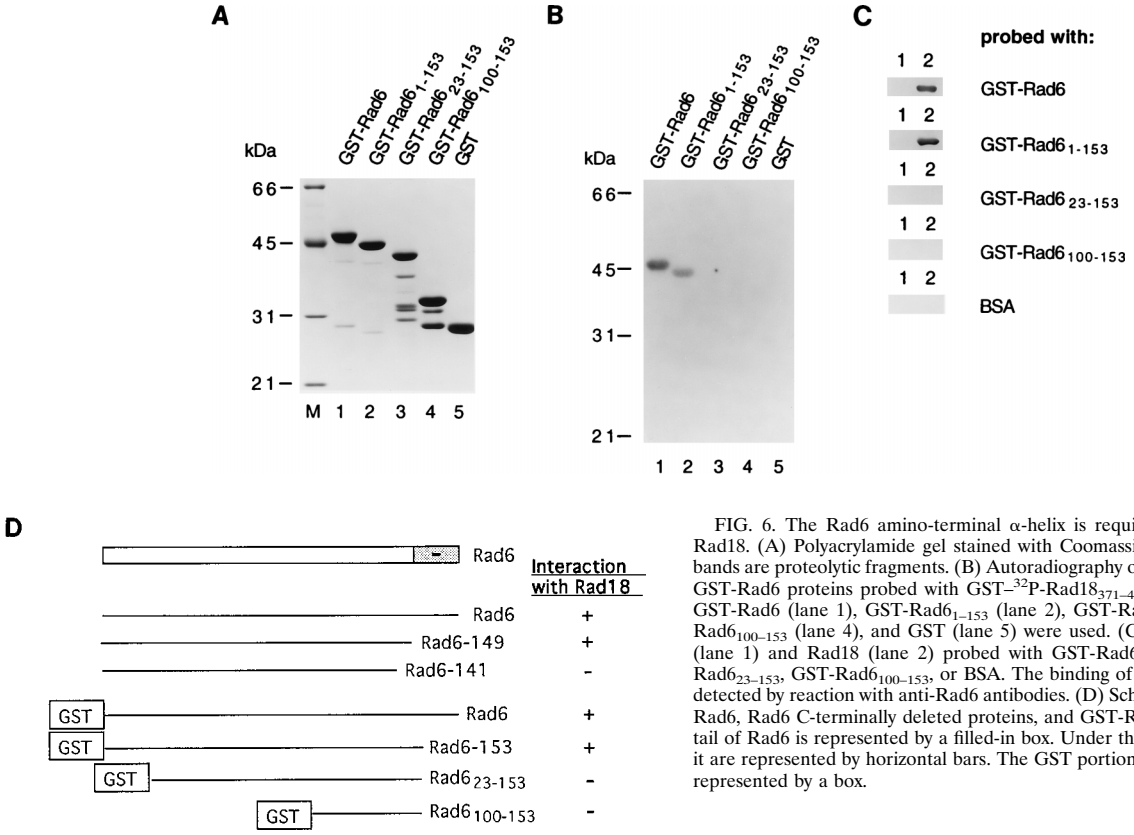

FIG. 6. The Rad6 amino-terminal α-helix is required for interaction with Rad18. (A) Polyacrylamide gel stained with Coomassie blue. The smaller-size bands are proteolytic fragments. (B) Autoradiography of a far-Western blot with GST-Rad6 proteins probed with GST–^{32}P-Rad18$_{371–410}$. For panels A and B, GST-Rad6 (lane 1), GST-Rad6$_{1–153}$ (lane 2), GST-Rad6$_{23–153}$ (lane 3), GST-Rad6$_{100–153}$ (lane 4), and GST (lane 5) were used. (C) Western blots of BSA (lane 1) and Rad18 (lane 2) probed with GST-Rad6, GST-Rad6$_{1–153}$, GST-Rad6$_{23–153}$, GST-Rad6$_{100–153}$, or BSA. The binding of Rad6 or GST-Rad6 was detected by reaction with anti-Rad6 antibodies. (D) Schematic representation of Rad6, Rad6 C-terminally deleted proteins, and GST-Rad6 proteins. The acidic tail of Rad6 is represented by a filled-in box. Under that, Rad6 and portions of it are represented by horizontal bars. The GST portion of the fusion proteins is represented by a box.

nine residues, is defective in multiubiquitination and degradation of N end rule substrates and that the Rad6$_{Δ1–9}$ mutant protein does not interact with the Ubr1 enzyme (29). These results have indicated the involvement of the first nine Rad6 residues in complex formation with Ubr1. Interaction with Rad18, however, is not affected by the *rad6*$_{Δ1–9}$ mutation (2).

To investigate whether the Rad6 amino terminus is involved in the interaction with Rad18, we employed various GST-Rad6 fusion proteins (Fig. 6A) in the far-Western blot assay (Fig. 6B). The nitrocellulose blot containing GST-Rad6 (lane 1), GST-Rad6$_{1–153}$ (lane 2), GST-Rad6$_{100–153}$ (lane 4), and GST-Rad6$_{23–153}$ (lane 3) was probed with ^{32}P-labeled GST-Rad18$_{371–410}$, which we have shown in prior experiments to contain the Rad6 interaction domain (Fig. 2), and then subjected to autoradiography to detect complex formation. As shown in Fig. 6B, the radiolabeled GST-Rad18$_{371–410}$ probe bound GST-Rad6 (lane 1) and GST-Rad6$_{1–153}$ (lane 2) but failed to interact with GST-Rad6$_{100–153}$ (lane 4) or with GST-Rad6$_{23–153}$ (lane 3). To exclude the possibility that lack of interaction is due to improper configuration of amino-terminally deleted Rad6 proteins when they are bound to nitrocellulose, we carried out another experiment in which Rad18 protein was run on a polyacrylamide gel, transferred to nitrocellulose, and probed with various GST-Rad6 fusion proteins and complex formation was revealed by probing with anti-Rad6 antibodies (Fig. 6C). Again, we observed the interaction of Rad18 protein with GST-Rad6 and GST-Rad6$_{1–153}$ but not with GST-Rad6$_{23–153}$ and GST-Rad6$_{100–153}$ (Fig. 6C). Taken together, these results suggest that the amino-terminal 23 residues in Rad6 protein affect the interaction with the Rad18 protein. However, in the absence of other biochemical data for Rad6$_{23–153}$, the possibility of an altered conformation affecting interaction cannot be excluded.

DISCUSSION

Rad6 contains 172 residues, of which the first 149 residues constitute a globular domain and the last 23 predominantly acidic residues form a freely extending tail domain (20). The ubiquitin-conjugating activity of Rad6 is required for N end rule-dependent protein degradation and for postreplicative DNA repair and damage-induced mutagenesis. Rad6 mediates its role in N end rule protein degradation via interaction with the *UBR1*-encoded E3 enzyme and in DNA repair via its association with Rad18. Previous studies have indicated the involvement of Rad6 amino-terminal and carboxyl-terminal regions in interactions with Ubr1. In particular, the Rad6$_{Δ1–9}$ protein, which lacks the first 9 residues, does not interact with Ubr1, and consequently the *rad6*$_{Δ1–9}$ mutation does not degrade proteins with destabilizing amino-terminal residues. The Rad6-149 protein, which lacks the last 23 residues, interacts with Ubr1 but with a reduced efficiency. Neither the *rad6*$_{Δ1–9}$ nor the *rad6-149* mutation affects interaction with Rad18.

Here, we identify regions in Rad6 that affect complex formation with Rad18. Although the Rad6 proteins with increasing numbers of C-terminal residues deleted (up to residue 149) can interact with Rad18, a deletion of eight additional residues, as in the Rad6-141 protein, results in a complete loss of Rad18 binding activity, suggesting the involvement of residues 141 to 149 in Rad18 binding (Fig. 6D). The *rad6-1* mutant allele, which encodes the Rad6-141 protein, resembles the *rad6*Δ mutation in its defects in DNA repair and damage-induced mutagenesis. Thus, interaction with Rad18 is crucial for Rad6 action in DNA repair. Our studies also suggest the involvement of the Rad6 amino terminus in the interaction with Rad18, as the Rad6$_{23–153}$ protein, which lacks the first 22 residues, is unable to interact with Rad18 (Fig. 6D). Since the Rad6$_{Δ1–9}$ protein binds Rad18 proficiently, these results sug-

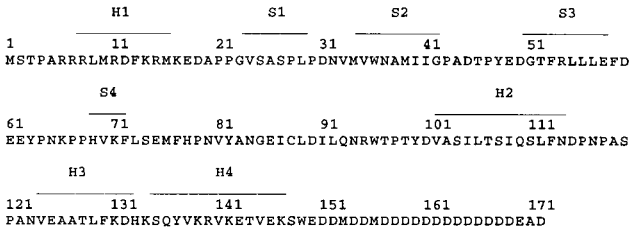

FIG. 7. Predicted sequence and secondary structure of Rad6. The positions of α-helices (H1 to H4) and β-strands (S1 to S4), which are overlined and marked, are inferred from the X-ray crystal structure of Ubc1 protein of *A. thaliana* (8). The X-ray crystal structure determined for Rad6 has confirmed these predictions (12a).

gest the involvement of Rad6 region in the vicinity of amino acids 10 to 22 in the interaction with Rad18.

The *UBC1*-encoded E2 enzyme from *Arabidopsis thaliana* has a high degree of sequence similarity with the *S. cerevisiae* Rad6 protein (8). The Ubc1 protein contains 152 amino acids, but it lacks the acidic C-terminal tail extension. The X-ray crystal structure of Ubc1 enzyme shows it to be an α-β protein, with four α-helices and a four-stranded antiparallel β-sheet (8). Figure 7 shows the positions of α-helices and β-sheets in Rad6 corresponding to those in Ubc1. At the amino terminus, residues 8 to 16 form an amphipathic helix, and at the carboxyl terminus, residues 135 to 147 form another amphipathic α-helix.

The amino terminus of Rad6 is highly conserved, such that the first 15 residues of Rad6 are almost identical among yeast and other eukaryotes. Importantly, this sequence conservation does not extend to the other ubiquitin-conjugating enzymes that do not belong to the Rad6 group (16). The involvement of residues 1 to 9 in Ubr1 binding and of residues 10 to 22 in Rad18 binding supports the idea that the highly conserved amino terminus is critical for Rad6-specific functions. Our mapping studies have suggested a role of the α-helix formed by residues 8 to 16 in Rad6 in interaction with Rad18. Since a deletion of residues 141 to 149 also inactivates Rad6 binding to Rad18, the α-helix formed by residues 135 to 147 may also be involved in Rad18 binding. The region of Rad6 between amino acids 137 to 149 contains several very highly conserved residues (Y137, K139, R140, V141, V145, E146, S148, and W149) which are mostly identical in all the Rad6 homologs but are not conserved in the other E2 enzymes (16). The high degree of conservation of these N-terminal and C-terminal Rad6 regions may explain the ability of Rad6 counterparts from *Schizosaccharomyces pombe*, *D. melanogaster*, and humans to complement the DNA repair and mutagenesis defects of the *S. cerevisiae rad6*Δ mutant and the fact that the protein products of *RAD6* counterparts of *Schizosaccharomyces pombe* and humans physically interact with *S. cerevisiae* Rad18.

The region of Rad18 required for stable association with Rad6 was delimited to 40 amino acids, from residues 371 to 410. Since a GST-Rad18 peptide comprised of these 40 amino acids interacted as efficiently with Rad6 as the complete Rad18 protein, this region of Rad18 is sufficient for binding Rad6. Deletion of this region from Rad18 confers a high level of UV sensitivity, indicating that interaction with Rad6 is critical for Rad18 function. The Rad6 interaction domain in Rad18 does not include the C_3HC_4 or the C_2HC motifs, which may be involved in zinc binding. Secondary-structure predictions of this region have suggested the presence of two α-helices separated by a loop region. The helix-loop-helix motif of MyoD directs its interaction with Jun (5). The Rad6-interacting region of Rad18 bears resemblance to helix-loop-helix motifs present in MyoD and in various other proteins (Fig. 8). In particular, a number of nonpolar residues have been conserved in Rad18 regions corresponding to helix 1 and helix 2 of helix-loop-helix motifs. These nonpolar residues may associate with the two amphipathic α-helices H1 and H4 in Rad6 (Fig. 7) via hydrophobic interactions, which would explain the stability of the Rad6-Rad18 complex in 1 M NaCl. The loop region of MyoD is dispensable for interaction with Jun (5), and this region shows little conservation among various proteins (Fig. 8).

The X-ray crystal structure of Rad6 indicates a high degree of similarity to the structure of *A. thaliana* Ubc1 protein. The globular core of Rad6 displays the same four α-helices and four anti-parallel β-sheets, and the C-terminal acidic domain constitutes an unstructured domain (12a). With the identification of the Rad6 binding domain in Rad18, it may now be feasible to determine the three-dimensional structure of Rad6 bound to the $Rad18_{371–410}$ peptide and to define the manner of this interaction.

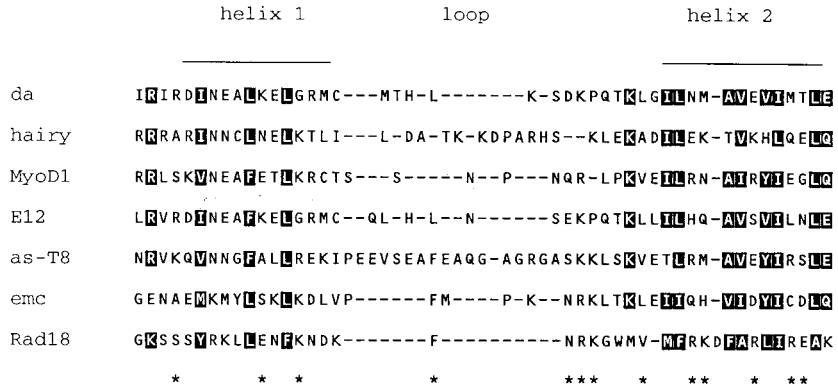

FIG. 8. Comparison of the Rad18 sequence involved in the interaction with Rad6 with the sequences of helix-loop-helix motifs in other proteins. The amino acids of Rad18 (residues 365 to 403) are compared to amino acids from MyoD1 (120 to 161), E12 (346 to 389), and *Drosophila daughterless* (da; 565 to 608), *hairy* (48 to 95), *achaete-scute* T8 (as-T8; 105 to 109), and *extramacrochaetae* (emc; 34 to 76) proteins. The amphipathic helices are indicated by the solid lines. The Rad18 sequence is from reference 14. All the other sequences are from reference 12. White letters in black boxes represent conserved residues; asterisks indicate identical amino acids in Rad18 and in at least one of the other sequences at the same position.

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