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The *Saccharomyces cerevisiae* DNA Repair Gene *RAD23* Encodes a Nuclear Protein Containing a Ubiquitin-Like Domain Required for Biological Function

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In eukaryotes, the posttranslational conjugation of ubiquitin to various cellular proteins marks them for degradation. Interestingly, several proteins have been reported to contain ubiquitin-like (ub-like) domains that are in fact specified by the DNA coding sequences of the proteins. The biological role of the ub-like domain in these proteins is not known; however, it has been proposed that this domain functions as a degradation signal rendering the proteins unstable. Here, we report that the product of the *Saccharomyces cerevisiae RAD23* gene, which is involved in excision repair of UV-damaged DNA, bears a ub-like domain at its amino terminus. This finding has presented an opportunity to define the functional significance of this domain. We show that deletion of the ub-like domain impairs the DNA repair function of *RAD23* and that this domain can be functionally substituted by the authentic ubiquitin sequence. Surprisingly, RAD23 is highly stable, and the studies reported herein indicate that its ub-like domain does not mediate protein degradation. Thus, in RAD23 at least, the ub-like domain affects protein function in a nonproteolytic manner.

Excision repair of UV-damaged DNA in eukaryotes is a complex process involving a large number of genes. In humans, xeroderma pigmentosum (XP) results from a defect in excision repair of UV-damaged DNA. XP patients exhibit extreme sensitivity to sunlight and suffer from a high incidence of skin cancers. Cell fusion studies have identified seven complementation groups among XP cells, XPA to XPG. In addition, UV-sensitive excision-deficient cell lines corresponding to 10 complementation groups have been identified in rodents. Excision repair genes have been remarkably conserved among eukaryotes from yeasts to humans (see reference 22 for references).

A total of 11 genes are known to function in excision repair in the yeast *Saccharomyces cerevisiae*. Mutations in six of these genes, *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD14*, render cells highly UV sensitive via impairment of the incision step of excision repair (4, 23). Genetic studies with the *RAD25* gene of *S. cerevisiae* and its human homolog *XPB* suggest a requirement for this gene in the incision step as well (6, 22). The products of these seven genes are likely to mediate the recognition of damaged DNA and endonucleolytic scission of the damaged DNA strand. Mutations in the remaining four genes, *RAD7*, *RAD16*, *RAD23*, and *MMS19*, cause a moderate degree of UV sensitivity and a reduced proficiency in excision repair (23). Excision repair is less efficient in nucleosomal regions (28). Some of these gene products may have a role in the displacement of histones from DNA during excision repair, increasing the accessibility of damaged DNA in chromatin to the excision repair complex (3).

With the aim of analyzing the structure and function of the *RAD23* gene and its encoded protein, we determined the sequence of *RAD23*. Interestingly, we found that RAD23 bears a sequence at its amino terminus that shows remarkable homology to ubiquitin. The finding of a ubiquitin-like (ub-like) domain in a yeast protein with known biological function has permitted us to analyze the role of this domain.

Conjugation of ubiquitin, a 76-residue protein found in all eukaryotes, to intracellular proteins marks the proteins for degradation via an ATP-dependent proteolytic system. Ligation of ubiquitin to an acceptor protein involves the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and the ε-amino group of a lysine residue in the protein. Subsequent attachment of additional ubiquitin moieties to the Lys-48 residue in the preexisting ubiquitin molecules results in the formation of a multiubiquitin chain. The multiubiquitinated protein substrates are then degraded by the ATP-dependent proteosome (for reviews, see references 10 and 14).

In *S. cerevisiae*, ubiquitin is generated from processing of precursor proteins encoded by the *UBI1* to *UBI4* genes. In the *UBI1*, *UBI2*, and *UBI3* genes, the ubiquitin sequence is fused to ribosomal proteins (9), whereas in *UBI4*, ubiquitin is fused at its C terminus to itself, forming a ubiquitin polymer (21). Transient fusion of ubiquitin to ribosomal proteins, as in UBI1, UBI2, and UBI3, enhances the efficiency with which these proteins are incorporated into ribosomes (9). Several proteins containing ub-like sequences have been identified in eukaryotes (2, 8, 30). Unlike the *UBI1* to *UBI4* gene products, however, in which free ubiquitin is generated by the deubiquitination activity of a ubiquitin-specific processing protease, the ubiquitin portion of these proteins is not cleaved and hence constitutes an integral component of the protein. On the basis of studies in *S. cerevisiae* with the engineered hybrid ubiquitin–proline–β-galactosidase (ub–P–β-Gal) protein, it has been suggested that the ub-like moiety in these proteins functions as an autonomous degradation signal by providing the site of attachment of additional ubiquitin molecules (16). In this study, we have analyzed the functional significance of the ub-like sequence in RAD23. Our results indicate a requirement of the ub-like sequence in RAD23 function, but they provide no support in favor of this domain acting as a degradation signal.

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MATERIALS AND METHODS

Yeast strains. The *S. cerevisiae RAD*$^+$ strain used in this study was LP3041-6D (*MATa leu2-3 leu2-112 trp1Δ ura3-52*). The isogenic *rad23Δ* strain JWY50 was constructed by transformation of the *RAD*$^+$ strain LP3041-6D with the *Eco*RI fragment of the *RAD23* gene (Fig. 1) in which the internal *Bgl*II fragment (+48 to +1113) had been replaced by the yeast *URA3* gene flanked by the *hisG* gene of *Salmonella typhimurium* (1). Transformants which were Ura$^+$ and carried the *rad23Δ* mutation as verified by genetic analyses were treated with 5-fluoro-orotic acid to select for Ura$^-$, resulting in strain JWY50.

Yeast genetic methods were performed as described previously (32).

Construction of plasmids. Plasmids in which the ub-like domain was modified were constructed as follows. A 1.75-kb *Spe*I-*Nae*I fragment from positions −184 to +1568 containing the entire *RAD23* open reading frame and 5′ and 3′ flanking sequences was cloned into *Xba*I-*Sma*I-digested M13mp18, generating clone 1823.200. The nucleotides immediately 5′ of the start codon were modified by site-directed mutagenesis to introduce an *Nco*I restriction site at position −1 (AAATGG was changed to CCATGG). Replicative-form DNA from the resulting phage (1823.204) was digested with *Sal*I-*Eco*RI, and the 1.75-kb fragment containing the *RAD23* gene was cloned into *Sal*I-*Eco*RI-digested pUC18, generating plasmid pJW123. The *Sal*I-*Eco*RI fragment from pJW123 was subsequently cloned into *Sal*I-*Eco*RI-digested centromeric (*CEN*) (YCplac33) and 2μm (YEplac195) vectors (11) to yield plasmids pJW129 and pJW128, respectively. Deletion of codons for amino acids 2 to 60 of RAD23 was achieved by site-directed mutagenesis of phage 1823.204 to give phage 1823.90319. The *Eco*RI-*Sal*I fragment carrying the mutant *rad23* gene was cloned into *Eco*RI-*Sal*I-digested YCplac33 and YEplac195 vectors, resulting in plasmids pJW160 and pJW161, respectively. The *rad23Δ48-60* construction was made by digestion of pJW123 with *Bsm*I and *Ava*I, blunt ended with T4 polymerase in the presence of deoxynucleoside triphosphates (dNTPs), and ligated to generate pJW133. The 1.75-kb *Eco*RI-*Sal*I fragment of pJW133 containing the mutant *rad23* gene was cloned into *Eco*RI-*Sal*I sites of YCplac33 and YEplac195, yielding plasmids pJW136 and pJW134, respectively. An *S. cerevisiae* ubiquitin gene was used to replace the N-terminal 60 codons of *RAD23*. A 204-bp *Eco*RI-*Sal*I fragment from Yep46 (7) containing most of the ubiquitin gene (−8 to +196) was blunt ended with T4 polymerase and dNTPs and cloned into S1 blunt-ended *Nco*I-*Bsm*I-digested pJW123 to generate pJW137. The 1.75-kb *Eco*RI-*Sal*I fragment of pJW137 containing the *ub-rad23* fusion gene was cloned into the *CEN* yeast expression vector YCplac33 to give pJW138. The *ub-rad23* gene codes for the first 65 amino acids of *S. cerevisiae* ubiquitin fused to the *RAD23* gene beginning at codon 61, which was changed from Gly to Arg in the fusion protein.

Plasmids which had selected lysine codons in the ub-like domain changed to arginine by site-directed mutagenesis (34) of phage 1823.204 were constructed as follows. Codons for residues 49, 55, and 63 were changed from Lys (AAA) to Arg (AGA), using the respective oligonucleotides 5′-TAC TCGGGTAGAGTGCTACAA-3′, 5′-CAAGATTCCAGAAC CGTATCTG-3′, and 5′-GCGGGCTAAGAGATGGGGAC3′, which correspond to the sense DNA strand of *RAD23* from positions +136 to +156, +154 to +174, and +179 to +198. A 1.75-kb *Eco*RI-*Sal*I fragment containing the mutation(s) was cloned into *Eco*RI-*Sal*I-digested YCplac33 and YEplac195 vectors, resulting in *rad23*–Arg-49 plasmids pJW140 and pJW141, *rad23*–Arg-49 Arg-55 plasmids pJW196 and pJW197, and *rad23*–Arg-49 Arg-55 Arg-63 plasmids pJW206 and pJW207, respectively.

The DNA sequences of all mutant constructions were

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-412          GAATTCATATAGTGTACATATATACCGATAATAATATCAAACCTCTATTCTT
-360  TGGTCTTTAACTTTTCTTTGAGTGAATTACAATACCATTGTTATATCAATCACTATACAC
-300  GGCTCGGTGGCGAAATTGAAATTTTTTTATTTTCTTCTCTTTCATTATGGCTAGGCAAGA
-240  AATAGCGACAGCAAAACTTAAGAAAAAATGATAGAACAAGGTCCCATCCAGTATTGACTA
-180  GTAGCTAAAGCTAGAAGGTACACTTAGGTTTTTCTCCCTGTTTTTAAGTTTGAACCATTG
-120  ATAAGGCCGTTCGCAGACAAGCTTGCTACCATCCAATTTTCCTTCCTCTAAAGACTTAAT
 -60  AAAGGCATCTAACTCCACCTTTAAATCACAGATCACACAAAGACAACATACAATAGAAAA
  +1  ATGGTTAGCTTAACCTTTAAAAATTTCAAGAAGGAAAAAGTTCCTTTAGATCTGGAACCT
   1   M  V  S  L  T  F  K  N  F  K  K  E  K  V  P  L  D  L  E  P

 +61  TCAAACACAATTTTAGAGACCAAGACCAAGCTTGCTCAATCCATTTCTTGTGAAGAATCT
  21   S  N  T  I  L  E  T  K  T  K  L  A  Q  S  I  S  C  E  E  S

+121  CAAATAAAACTGATCTACTCGGGTAAAGTGCTACAAGATTCCAAAACCGTATCGGAATGC
  41   Q  I  K  L  I  Y  S  G  K  V  L  Q  D  S  K  T  V  S  E  C

+181  GGGCTAAAAGATGGGGACCAAGTTGTCTTCATGGTTTCTCAAAAAAAGTCCACGAAGACC
  61   G  L  K  D  G  D  Q  V  V  F  M  V  S  Q  K  K  S  T  K  T

+241  AAAGTAACAGAACCTCCAATTGCTCCTGAGAGCGCCACTACCCCCGGAAGAGAAAATTCT
  81   K  V  T  E  P  P  I  A  P  E  S  A  T  T  P  G  R  E  N  S

+301  ACAGAAGCATCCCCCAGTACGGATGCTTCTGCAGCTCCTGCAGCCACTGCTCCCGAAGGC
 101   T  E  A  S  P  S  T  D  A  S  A  A  P  A  A  T  A  P  E  G

+361  TCACAACCGCAAGAAGAACAAACCGCCACTACAGAACGTACTGAATCTGCCTCTACACCG
 121   S  Q  P  Q  E  E  Q  T  A  T  T  E  R  T  E  S  A  S  T  P

+421  GGATTCGTGGTGGGAACCGAGAGGAACGAGACCATCGAGAGAATCATGGAAATGGGCTAC
 141   G  F  V  V  G  T  E  R  N  E  T  I  E  R  I  M  E  M  G  Y

+481  CAAAGAGAGGAAGTCGAACGAGCCTTGAGAGCAGCCTTTAATAATCCAGATAGAGCGGTG
 161   Q  R  E  E  V  E  R  A  L  R  A  A  F  N  N  P  D  R  A  V

+541  GAATATCTACTGATGGGTATTCCAGAAAATCTGCGTCAACCGGAACCACAGCAACAAACA
 181   E  Y  L  L  M  G  I  P  E  N  L  R  Q  P  E  P  Q  Q  Q  T

+601  GCCGCCGCAGCGGAACAACCATCGACAGCCGCCACCACTGCGGAACAACCGGCTGAAGAC
 201   A  A  A  A  E  Q  P  S  T  A  A  T  T  A  E  Q  P  A  E  D

+661  GACTTATTTGCACAAGCTGCCCAAGGCGGTAATGCTTCATCCGGTGCGCTTGGCACAACT
 221   D  L  F  A  Q  A  A  Q  G  G  N  A  S  S  G  A  L  G  T  T

+721  GGAGGAGCTACAGATGCTGCGCAAGGTGGACCTCCAGGTTCCATTGGCCTCACTGTAGAA
 241   G  G  A  T  D  A  A  Q  G  G  P  P  G  S  I  G  L  T  V  E

+781  GATTTACTATCGTTGAGACAGGTCGTTTCAGGTAACCCAGAAGCTTTAGCCCCATTGTTG
 261   D  L  L  S  L  R  Q  V  V  S  G  N  P  E  A  L  A  P  L  L

+841  GAAAACATAAGTGCTAGATATCCTCAATTACGTGAACATATCATGGCAAACCCAGAAGTG
 281   E  N  I  S  A  R  Y  P  Q  L  R  E  H  I  M  A  N  P  E  V

+901  TTTGTGTCCATGTTGCTAGAAGCCGTGGGTGACAATATGCAAGATGTTATGGAAGGTGCG
 301   F  V  S  M  L  L  E  A  V  G  D  N  M  Q  D  V  M  E  G  A

+961  GATGATATGGTGGAAGGAGAGGATATAGAAGTTACAGGAGAGGCTGCTGCTGCAGGACTG
 321   D  D  M  V  E  G  E  D  I  E  V  T  G  E  A  A  A  A  G  L

+1021 GGACAAGGTGAAGGTGAAGGTTCTTTCCAAGTTGACTATACCCCCGAAGACGATCAAGCT
 341   G  Q  G  E  G  E  G  S  F  Q  V  D  Y  T  P  E  D  D  Q  A

+1081 ATTTCGCGCCTCTGTGAATTGGGCTTTGAAAGAGATCTTGTTATCCAGGTGTATTTTGCG
 361   I  S  R  L  C  E  L  G  F  E  R  D  L  V  I  Q  V  Y  F  A

+1141 TGCGATAAAAACGAAGAAGCTGCAGCAAATATTCTATTCAGCGATCATGCCGACTGAGAT
 381   C  D  K  N  E  E  A  A  A  N  I  L  F  S  D  H  A  D

+1201 TGTAGTAATGTTATGGCTTGAAGTATCTTCACTTATTCTCGACAATGTTGCATGATTTTT
+1261 GAGCCAAGGGAAAAAAAAAGCTTACGTAGAAATCAATAACGTATGTGTATTTATATATGC
+1321 TAGAAGGGAAAGAAAAGAAAACAGAGAGATGGTAATATTAATCTCAATAATAATAATAAT
+1381 AATAATAATAACAATAATAATAATAATAACAACAACAAATAATGATTATACTGCTTTTAG
+1441 CGCATATATACAGTTTTGTTAAAGAGTTTCAAAATTCTTGTATACCTGGTACTTTTGCCA
+1501 TCGGGGACAGGTGCATTACCAGGTCGGGAAGCTAACCCAGTAGCTATTCCAACGCCGGCC
+1561 GCTGCGTCCAATTCCAATGGATTATCGCTCAAGAACAGTACCTCGCTAGCCTTCGCACCG
+1621 ATATCCCGCAAGATGTTGGCGTAAGACTGTGTTTCGGTTTTCTTTCCAGAAGTGTTGATG
+1681 TCGAAATAACCATCTATATAAGAATTC
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FIG. 1. Nucleotide sequence and predicted amino acid sequence of the 2,119-bp *Eco*RI DNA fragment containing the *RAD23* gene. Numbers with either a + or − sign correspond to the DNA sequence; the other numbers correspond to the protein sequence. DNA fragments obtained by digestion with various restriction enzymes were cloned into M13 derivative phages. The nucleotide sequence of both strands of the *RAD23* gene was determined by the dideoxynucleotide triphosphate chain termination method (27).

confirmed by the dideoxynucleoside triphosphate chain termination method (27).

Antibodies. For the production of rabbit polyclonal antiserum, RAD23 protein was expressed in *Escherichia coli* JM105 by use of the *lac* promoter in plasmid pR23.1 (*lac-RAD23*) and isopropylthiogalactopyranoside (IPTG) induction. Polyclonal antibodies specific for ubiquitin were raised in rabbits by the method of Haas and Bright (12), in which bovine ubiquitin is cross-linked to bovine γ-globulin and the resulting conjugate is denatured by boiling in sodium dodecyl sulfate (SDS) to increase the antigenicity of ubiquitin. To prepare affinity matrices for purification of antibodies from sera, RAD23 expressed in and purified from *E. coli* was cross-linked to cyanogen bromide-Sepharose 4B (Pharmacia) and bovine ubiquitin was cross-linked to activated CH-Sepharose 4B (Pharmacia) at a ligand concentration of 5 mg per ml of matrix. Antibodies were immobilized on the affinity columns in phosphate-buffered saline (PBS; 10 mM NaH_2PO_4 [pH 7.2] containing 150 mM NaCl) and eluted with 100 mM diethylamine (pH 11.5), and the eluate was neutralized immediately with Tris-HCl (pH 7.0) (13). Purified antibodies were concentrated to an optical density at 280 nm (OD_{280}) of ≥3 in a Centricon-30 (Amicon) and dialyzed against PBS.

SDS-PAGE and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli (17); 10% gels were used for RAD23 protein, and 8% gels were used in the analysis of ubiquitinated RAD23 species. In immunoblotting, proteins in gels were transferred electrophoretically onto nitrocellulose membrane (Schleicher & Schuell) and probed with antibodies as described by Towbin et al. (31).

Immunoprecipitation. Affinity-purified anti-RAD23 antibodies were cross-linked to protein A-agarose (Bio-Rad) by using dimethylpimelimidate in 0.1 M sodium borate (pH 9.0) (13); 10 OD_{280} units of antibodies was used per milliliter of matrix. To prepare extract for immunoprecipitation, yeast cells were suspended in buffer (1% SDS–15 mM dithiothreitol in 25 mM Tris-HCl [pH 7.5]) at 2 ml/g, frozen in liquid nitrogen, and boiled for 5 min. After this freezing-boiling cycle was repeated, the cell lysate was diluted with 9 volumes of buffer A (50 mM Tris-HCl [pH 7.5] containing 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and the protease inhibitors benzamidine [2 mM], phenylmethylsulfonyl fluoride [1 mM], and aprotinin, chymostatin, leupeptin, and pepstatin A [20 μg/ml each]) and clarified by centrifugation (100,000 × *g* for 60 min). Nine milliliters of the clarified extract was mixed with 100 μl of anti-RAD23 agarose beads at 4°C for 12 h. The beads were collected by centrifugation, washed at 0°C three times with 500 μl of ice-cold buffer A and once with 600 μl of buffer B (30 mM Tris-HCl [pH 7.5] containing 50 mM NaCl, 0.2 mM EDTA, and 0.1% SDS), and treated at 37°C for 5 min with 80 μl of 2% SDS in 20 mM Tris-HCl (pH 7.0) to elute bound yeast proteins in the immunoprecipitate.

Indirect immunofluorescence. The method of Pringle et al. (24) was used to determine the subcellular location of RAD23 protein. Briefly, affinity-purified antibodies to RAD23 protein were used as the primary antibody, and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibodies (Sigma) were used as the secondary antibody. Cells used for immunofluorescence were also treated with secondary antibody, in the absence of treatment with primary antibody, as a control for cross-reactivity of secondary antibody to cellular material. The nuclei were stained by addition of the fluorescent dye 4′,6′-diamidino-2-phenylindole (DAPI) to the mounting medium. A Leitz Laborlux D fluorescence microscope equipped with an Olympus PM-10AD photomicrographic system was used to visualize and photograph the fluorescence.

Pulse-chase analysis. *S. cerevisiae* LP3041-6D (*RAD*$^+$) and JWY50 (*rad23*Δ) were grown in complete synthetic medium at 30°C until the cell density was ~10^7 cells per ml. Cells from 30 ml of suspension were collected by filtration and washed three times with 30 ml of synthetic medium lacking methionine. The filtered, washed cells were suspended in 0.5 ml of 40 mM potassium phosphate (pH 7.4)–2% glucose and labeled with 500 μCi of [^{35}S]methionine (>1,000 Ci/mmol; Amersham) for 10 min. Following the pulse period, cells were collected by filtration, washed with 15 ml of complete medium, and suspended in 0.8 ml of medium containing 0.5 mg of cycloheximide per ml and 10 mM methionine. The protein labeling was terminated immediately (no chase) or at various times afterward by addition of 0.4 ml of the suspension to 0.8 ml of ice-cold buffer A and vortexing vigorously for 3 min at 4°C in the presence of 0.4 ml (~0.7 g) of acid-washed glass beads (0.5 mm). Cellular debris was removed by centrifugation, and RAD23 was immunoprecipitated quantitatively by addition of 50 μl of anti-RAD23 immunobeads. The immunoprecipitate was washed twice with 200 μl of buffer A containing 0.1% SDS and 1 M NaCl, twice with 200 μl of Tris-HCl (pH 7.5), and twice with 200 μl of buffer A containing 0.1% SDS. RAD23 was eluted from the immunoprecipitate by boiling for 3 min in 60 μl of SDS-electrophoresis sample loading buffer, and 20 μl of the eluate was electrophoresed in an SDS–10% polyacrylamide gel. The gel was subjected to fluorography using En^{3}Hance (Du Pont) as recommended by the manufacturer. All buffers, medium, and incubations during the pulse and chase periods were at 30°C.

Nucleotide sequence accession number. The GenBank accession number of the *RAD23* gene is L25428.

RESULTS

The *RAD23* gene. *RAD23* encodes an acidic protein (pI = 4.07) of 398 amino acids with a predicted molecular weight of 42,366 (Fig. 1). The N-terminal 81 amino acids of RAD23 comprise a basic region (pI = 9.71), whereas the remainder of the protein is highly acidic (pI = 3.77). The acidic region contains 74 of the 78 small amino acids Ala and Gly present in the protein. This acidic region of RAD23 may form a compact globular domain with affinity for basic target proteins. RAD23 does not contain any DNA binding or nucleotide binding sequence motifs.

***RAD23*-encoded protein contains a ub-like sequence at the amino terminus.** The N-terminal region of RAD23, from residues 2 to 77, bears striking homology to ubiquitin. The alignment of this region of RAD23 with *S. cerevisiae* ubiquitin is colinear, without any gaps, and the two proteins share 22% identical and 43% similar (identical plus conserved changes) residues (Fig. 2A). The observed homology is highly significant, as indicated by the adjusted score of 13 (11a). When the variability of the ubiquitin sequence among eukaryotes is considered, the identity between the N terminus of RAD23 and ubiquitin increases to 29% (Fig. 2B).

Ubiquitin is synthesized as a precursor protein, where it is joined at its C terminus either to itself, forming a linear polyubiquitin protein (21), or to ribosomal proteins (9), and mature ubiquitin is generated from these precursors by proteolytic cleavage. Like RAD23, BAT3 (2) and GdX (8, 30) proteins also contain a ub-like domain at the N terminus

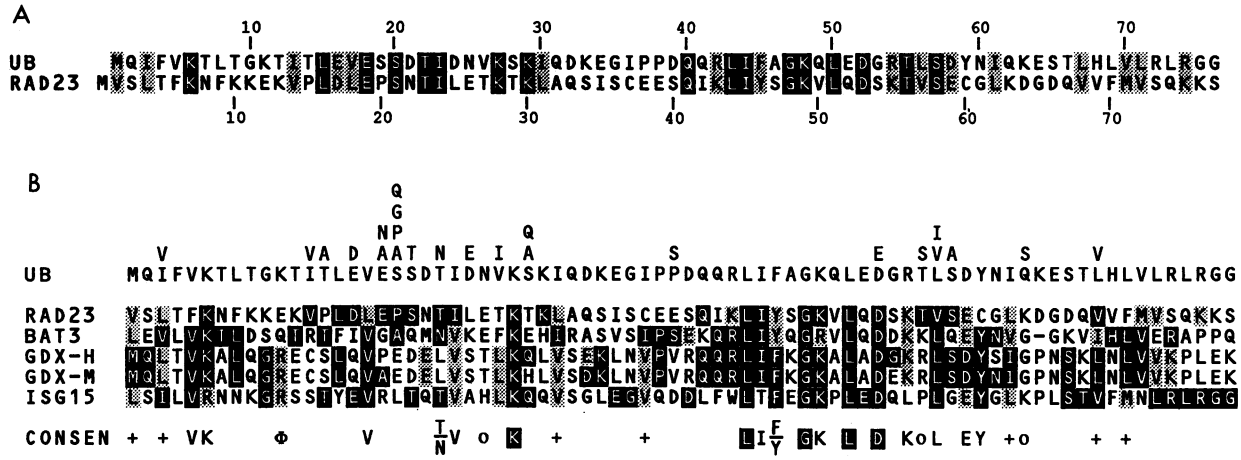

FIG. 2. Homology of RAD23 amino-terminal sequence with ubiquitin. (A) Protein sequence alignment between RAD23 and *S. cerevisiae* ubiquitin (21). The Bestfit program was used for optimal alignment between the two proteins (25). Shown is the entire ubiquitin (UB) sequence (residues 1 to 76) and the N-terminal region of RAD23 (residues 1 to 77). Identical residues between the two sequences are in reverse lettering, while similar residues (V = I = L = M; K = R; D = E; and F = Y) are shaded. Numbers indicate the amino acid residue of ubiquitin (top) and RAD23 (bottom). (B) Protein sequence comparison between ubiquitin from various species and ub-like domains of RAD23 (residues 2 to 77), BAT3 (2) (residues 17 to 92), GdX (8, 30) (residues 1 to 76), and ISG15 (26) (residues 82 to 157) proteins. Optimal alignment to ubiquitin was done as described for panel A. UB, *S. cerevisiae* ubiquitin sequence. The residues in ubiquitin from other organisms (18–20, 29, 33) which differ from those in *S. cerevisiae* ubiquitin are indicated above the corresponding residue in the UB line. A dash indicates a gap introduced in the sequence for optimal alignment to ubiquitin. The consensus sequence for the ub-like domains is given in the CONSEN line. In deriving the consensus sequence, mouse GdX-M and human GdX-H sequences were treated as one, except at those residues where they differed. For assignment to the consensus sequence, a residue in at least three of four sequences (RAD23, BAT3, GdX, and ISG15) had to be identical or similar, and that residue had to share identity or similarity with the residue in ubiquitin. Reverse-lettered amino acids indicate identity among all ubiquitin and ub-like sequences. Symbols: +, hydrophobic; o, hydrophilic; Φ, extremely hydrophilic.

(Fig. 2B). Unlike ubiquitin, RAD23, BAT3, and GdX lack the C-terminal glycine residue at the end of the ub-like moiety, necessary for the proteolytic cleavage of ubiquitin from the precursor molecule (10). *ISG15*, an interferon-induced gene, encodes a 15-kDa protein that contains two ub-like domains (26). The carboxyl half of ISG15 shows greater homology to ubiquitin, including the presence of the C-terminal glycine (Fig. 2B) eight amino acids from the C terminus. The functional significance of the ub-like domains in these proteins is not known.

The ub-like domain is required for RAD23 function. To examine the role of the ub-like domain in RAD23 function, we deleted amino acids 2 to 60 from the RAD23 protein. This mutation, designated *rad23 Δ2-60* (Fig. 3A), removes most of the region of RAD23 protein that shares homology with ubiquitin (Fig. 2A). The low-copy-number *CEN* or the multicopy 2μm plasmid carrying the *rad23Δ2-60* gene was introduced into the *rad23Δ* yeast strain JWY50, whose genomic *RAD23* gene had been deleted by gene replacement. Immunoblot analysis with anti-RAD23 antibodies revealed that the *CEN rad23Δ2-60* plasmid produces substantially less protein (Fig. 3B, lane 4) than does the *CEN* plasmid carrying the wild-type *RAD23* gene (Fig. 3B, lane 3). The *rad23Δ2-60* gene carried on the multicopy 2μm plasmid (Fig. 3B, lane 5) produced protein at levels comparable to that of RAD23 produced from the *CEN RAD23* plasmid (Fig. 3B, lane 3). UV survival of the *rad23Δ* strain carrying the *rad23 Δ2-60* gene on the *CEN* plasmid or the 2μm plasmid was intermediate between that of the *RAD*$^+$ and *rad23Δ* strains (Fig. 3C), indicating a requirement of the ub-like domain in RAD23 function.

We also constructed an internal deletion mutation in *RAD23* that removes residues 48 to 60 (*rad23Δ48-60*); Fig. 3A), which are located in a highly conserved region of the ub-like domain (Fig. 2A). The level of protein in the *rad23Δ* strain carrying the *rad23Δ48-60* gene on the 2μm plasmid was substantially more abundant (Fig. 3B, lane 7) than that of RAD23 produced from the *CEN* plasmid (Fig. 3B, lane 8). The *rad23Δ48-60* mutation lowered the UV survival to the same level as did the *rad23Δ2-60* mutation (Fig. 3C); thus, the region of residues 48 to 60 is essential for the function of the ub-like domain.

We next examined whether the RAD23 ub-like domain can be functionally substituted for by the authentic ubiquitin sequence, by replacing amino acids 1 to 60 of RAD23 with amino acids 1 to 65 of ubiquitin from *S. cerevisiae* (Fig. 3A). The resulting fusion, ub-rad23, carried on a *CEN* plasmid, was introduced into the *rad23Δ* strain. The intracellular level of ub-rad23 protein (Fig. 3B, lane 6) was comparable to that of RAD23 produced from the *CEN* plasmid (Fig. 3B, lane 8). Interestingly, the *ub-rad23* gene restored the UV survival of the *rad23Δ* strain to *RAD*$^+$ levels (Fig. 3C). Thus, ubiquitin can functionally substitute for the ub-like domain of RAD23.

RAD23 is concentrated in the nucleus. We determined the cellular location of RAD23 by indirect immunofluorescence using affinity-purified anti-RAD23 antibodies. We found that RAD23 is concentrated in the nucleus (Fig. 4), whereas in the *rad23Δ* strain, we saw no evidence of cross-reactivity to anti-RAD23 antibodies (Fig. 4). Since the UV sensitivity of the *rad23Δ2-60* mutant could conceivably arise from the failure of the mutant protein to localize in the nucleus, we also examined the cellular location of this protein. However, the rad23Δ2-60 mutant protein is also localized in the nucleus (data not shown), indicating that the ub-like domain in RAD23 is not required for nuclear transport of the protein.

RAD23 protein is multiply ubiquitinated in vivo. The availability of high-specificity anti-RAD23 antibodies has allowed us to use immunoprecipitation to investigate whether ubiq-

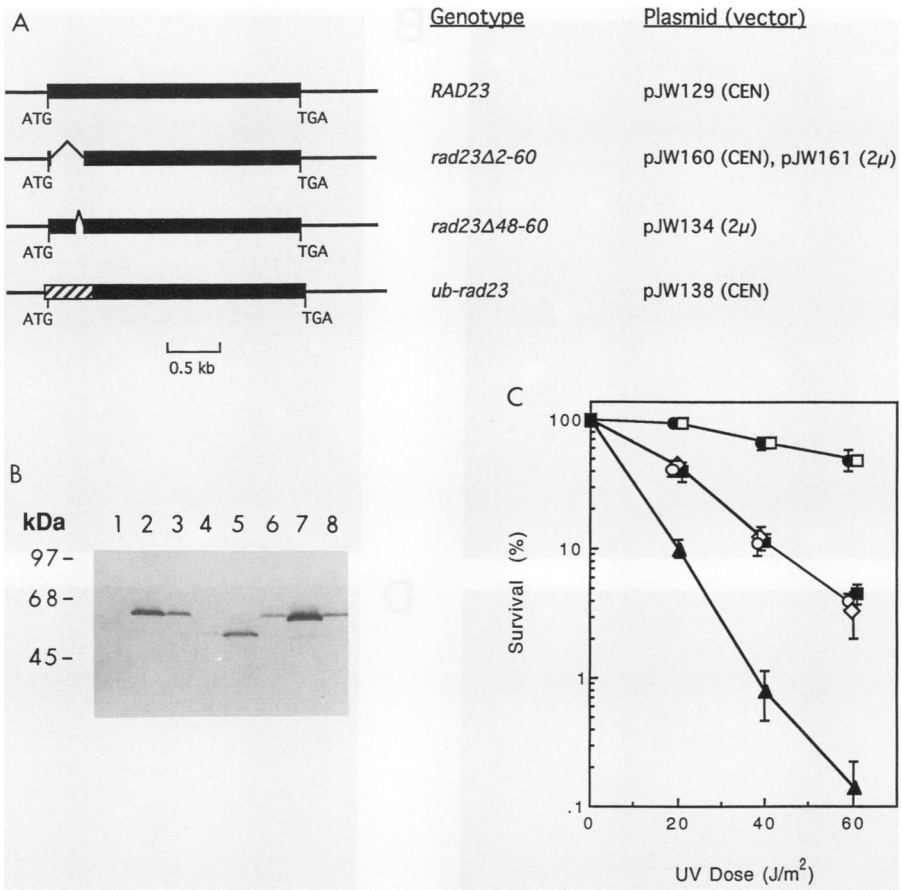

FIG. 3. Requirement of the ub-like domain in RAD23 function. (A) Plasmids carrying mutations of the ub-like domain of RAD23. ■, *RAD23* open reading frame; ⌒, deletion of residues 2 to 60 of RAD23; ⌒, deletion of residues 48 to 60 of RAD23; ▨, replacement of the RAD23 ub-like domain by the *S. cerevisiae* ubiquitin sequence; ——, 5′ and 3′ flanking regions of the *RAD23* open reading frame. (B) Immunoblot analysis of cell extracts from rad23 mutants. Extracts from the *RAD*$^+$ strain LP3041-6D and the isogenic *rad23*Δ strain JWY50 carrying various plasmids were analyzed by immunoblot analysis. In SDS-PAGE, RAD23 migrates at ~57 kDa. Lane 1, JWY50 (*rad23*Δ); lane 2, LP3041-6D (*RAD*$^+$); lanes 3 to 8, JWY50 (*rad23*Δ) carrying plasmids pJW129 (*CEN RAD23*) (lanes 3 and 8), pJW160 (*CEN rad23Δ2-60*) (lane 4), pJW161 (2μm *rad23Δ2-60*) (lane 5), pJW138 (*CEN ub-rad23*) (lane 6), and pJW134(2μm *rad23Δ48-60*) (lane 7). The nitrocellulose blot was probed with a 1/1,000 dilution of affinity-purified anti-RAD23 antibodies (OD_{280} = 3). The equivalent of 2 × 10^6 cells was analyzed in all lanes. (C) Survival after UV irradiation of the *rad23*Δ strain JWY50 carrying different plasmids. Cells were grown in synthetic complete medium lacking uracil for maintaining selection for the plasmids. Shown is the average of at least three independent experiments; bars represent 1 standard deviation of the data from the mean value. Symbols: ●, pJW129 (*CEN RAD23*); □, pJW138 (*CEN ub-rad23*); ○, pJW160 (*CEN rad23Δ2-60*); ■, pJW161 (2μm *rad23Δ2-60*); ◇, pJW134 (2μm *rad23Δ48-60*); ▲, YCplac33 (*CEN* vector).

uitinated forms of RAD23 protein exist in vivo. Extracts from *rad23*Δ cells, *RAD*$^+$ cells, and *RAD*$^+$ cells harboring the 2μm multicopy *RAD23* plasmid pJW128 were subjected to immunoprecipitation with protein-A agarose beads bearing anti-RAD23 antibodies. Bound yeast proteins were eluted from immunoprecipitates by SDS treatment and analyzed by immunoblotting with anti-RAD23 antibodies for RAD23-related species larger than the 57-kDa unmodified form. In this analysis, over a wide range of loadings, we could not detect any larger species of RAD23. However, it remained possible that ubiquitinated forms of RAD23 are present in such low amounts as to preclude detection by direct probing with anti-RAD23 antibodies. For this reason, we probed nitrocellulose blots with affinity-purified antibodies directed against ubiquitin. Since these antibodies do not cross-react with RAD23 protein, we could use much longer reaction times to optimize the detection of minor ubiquitinated RAD23 species that may be present. RAD23 protein precipitated from *RAD*$^+$ cells harboring pJW128 contains a ladder of larger species immunoreactive with the antiubiquitin antibodies (Fig. 5, lane 2). These bands are derived from RAD23 because they are absent in extract of *rad23*Δ cells (Fig. 5, lane 1) and are present in a much lower level in extract of *RAD*$^+$ cells without pJW128 (data not shown). To establish that these larger RAD23 forms contain ubiquitin, we overproduced ubiquitin or Myc-ubiquitin by use of the *CUP1* promoter in *RAD*$^+$ cells harboring pJW128. Myc-ubiquitin consists of a 13-residue segment of the human c-Myc protein fused to the amino terminus of ubiquitin; it is ≈1.5 kDa larger than authentic ubiquitin and is conjugated to acceptor proteins with high efficiency (15). As shown in Fig. 5, overproduction of ubiquitin increased the level of the ubiquitinated RAD23 species (compare lane 3 with lane 2), and expression of Myc-ubiquitin resulted in the appearance of novel species (lane 4) slightly larger than the immunoreactive forms seen in lanes 2 and 3. These observations indicate that RAD23 is indeed ubiquitinated in vivo. We estimate that considerably less than 0.1% of cellular RAD23

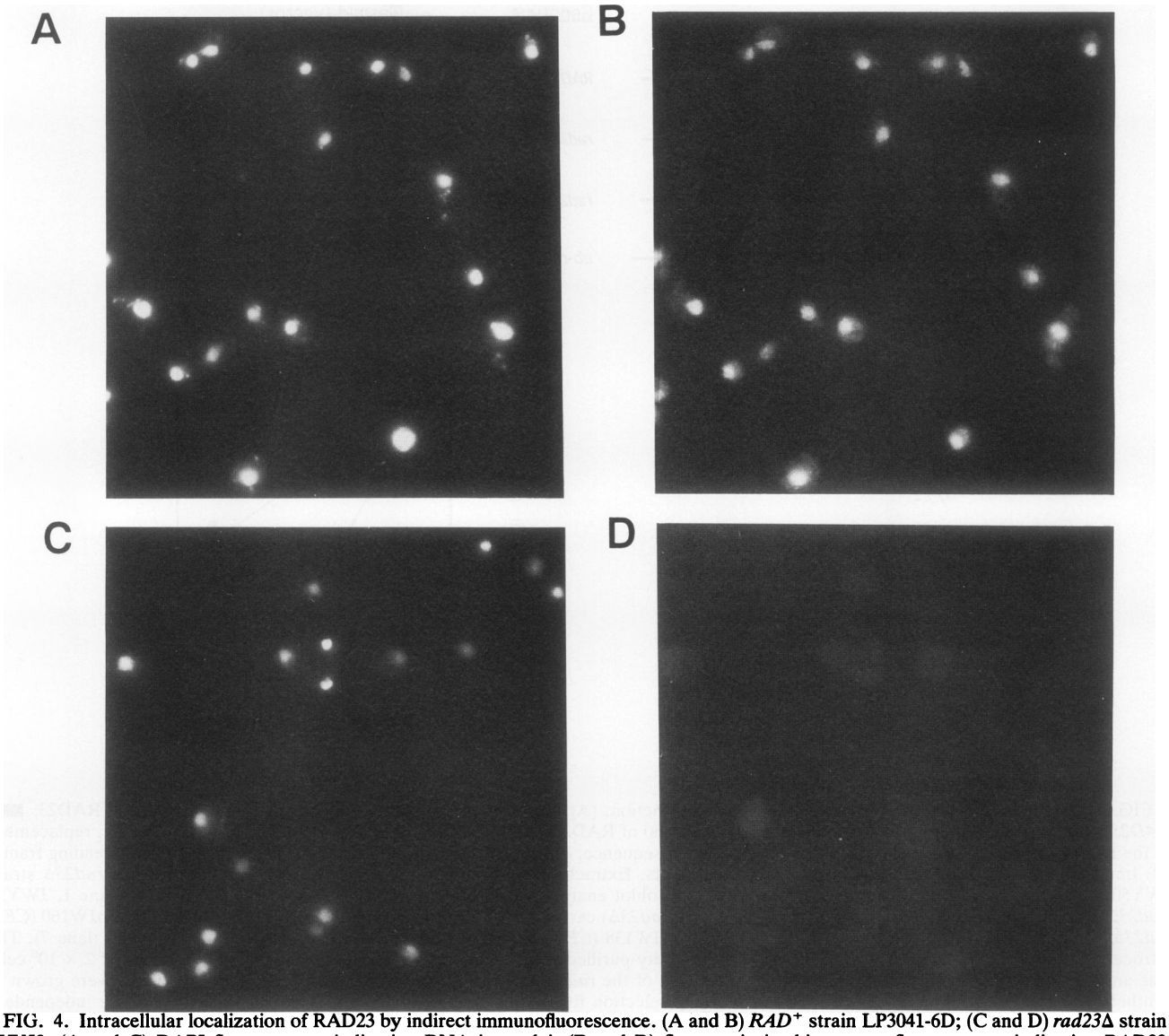

FIG. 4. Intracellular localization of RAD23 by indirect immunofluorescence. (A and B) *RAD*$^+$ strain LP3041-6D; (C and D) *rad23*Δ strain JWY50; (A and C) DAPI fluorescence, indicating DNA in nuclei; (B and D) fluorescein isothiocyanate fluorescence, indicating RAD23 protein. For panel D, the exposure time was five times longer than for panel B.

is conjugated to ubiquitin, however. Consistent with this low level of ubiquitination, we find that RAD23 is a very stable protein in vivo. Pulse-chase analysis revealed no detectable reduction in RAD23 levels during a 6-h chase period (Fig. 6), and we estimate RAD23 to be present at ~10,000 copies per cell.

Mutation of lysine 49 has no effect on RAD23 ubiquitination. To test whether ubiquitin attachment to RAD23 protein occurs at lysine 49, which is equivalent to lysine 48 in authentic ubiquitin where attachment of a multiubiquitin chain occurs, or at the neighboring lysine residues at positions 55 and 63, we constructed the *rad23*–Arg-49, *rad23*-Arg-49 Arg-55, and *rad23*-Arg-49 Arg-55 Arg-63 alleles by site-directed mutagenesis. Immunoblot analysis (Fig. 7) of extracts from *rad23*Δ cells transformed with low-copy-number *RAD23* and *rad23* mutant plasmids revealed that rad23–Arg-49, rad23–Arg-49 Arg-55, and rad23–Arg-49 Arg-55 Arg-63 proteins are expressed at the same level as wild-type RAD23, whereas the rad23Δ48–60 protein is present at ≈10% of the level of the others. The same conclusions concerning the relative intracellular levels of RAD23 and the various rad23 proteins were reached with cells harboring 2μm multicopy plasmids that express these proteins (data not shown). To examine whether the rad23 mutant proteins are also ubiquitinated, they were isolated by immunoprecipitation and probed for the presence of ubiquitinated species. Because the level of rad23Δ48–60 protein is much lower than levels of the other proteins, it was necessary to use five times more extract and anti-RAD23 agarose beads to obtain a sufficient quantity of this mutant protein. Rather surprisingly, we found (i) the same pattern and amount of ubiquitinated species in the rad23–Arg-49 as well as the other two mutants carrying lysine-to-arginine changes as in wild-type RAD23 (Fig. 8, lanes 2 to 5) and (ii) that the level of ubiquitinated intermediates in the rad23Δ48–60 protein is in fact much higher than in RAD23 or any of the rad23-Arg

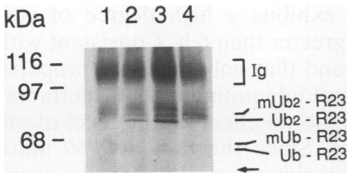

FIG. 5. Multiubiquitinated forms of RAD23. Extracts from various yeast strains were subjected to immunoprecipitation with anti-RAD23 protein A-agarose beads as described in Materials and Methods. The SDS eluates (20 μl containing ≈ 10 μg of RAD23) of immunoprecipitates were electrophoresed in an 8% denaturing polyacrylamide gel. Proteins in the gel were transferred onto nitrocellulose, which was probed with a 1/300 dilution of affinity-purified antiubiquitin antibodies (OD_{280} = 3). Lanes 1 to 4 contain samples from *rad23*Δ strain JWY50, *RAD*$^+$ strain LP3041-6D harboring pJW128 (2μm *RAD23*), LP3041-6D harboring pJW128 and pPM39 (2μm *CUP1-ub*), and LP3041-6D harboring pJW128 and pPM40 (2μm *CUP1-myc ub*), respectively. Yeast cells were grown to a titer of 10^8 cells per ml in YPD medium containing 100 μM copper sulfate to induce the *CUP1* promoter in plasmids pPM39 and pPM40. Ub-R23 and Ub_2-R23, mono- and diubiquitinated species of RAD23, respectively; mUb-R23 and mUb_2-R23, RAD23 containing one and two molecules of Myc-ubiquitin, respectively. RAD23 species with more than two molecules of ubiquitin can also be discerned but are not marked. Ig, immunoglobulin that was eluted during SDS treatment of immunoprecipitates. The arrow marks the position of unmodified RAD23.

mutants included in this analysis (Fig. 8; compare lane 6 with lanes 2 to 5). These observations clearly indicate that lysine 49 as well as lysines 55 and 63 in the ub-like domain of RAD23 are not required for ubiquitin attachment. The UV sensitivity of the rad23–Arg-49, rad23–Arg-49 Arg-55, and rad23–Arg-49 Arg-55 Arg-63 mutants was the same as that of the wild-type strain (Fig. 9). Thus, the UV sensitivity associated with the *rad23*Δ*48–60* mutation cannot be explained by a lack of ubiquitination of its encoded protein. The increased susceptibility of the rad23Δ48–60 protein to ubiquitination provides a plausible explanation as to the lower intracellular level of the protein (Fig. 7, lanes 6 and 7).

DISCUSSION

The N terminus of RAD23 (residues 2 to 77) shows striking homology to ubiquitin. The protein products of several other

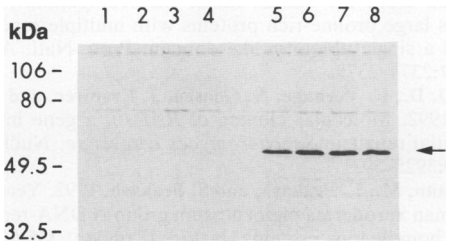

FIG. 6. The RAD23 protein is very stable in vivo. The stability of RAD23 in vivo was determined by labeling the cells for 10 min with [^{35}S]methionine (lanes 1 and 5) followed by a chase period in the presence of cycloheximide and unlabeled methionine for 2 h (lanes 2 and 6), 4 h (lanes 3 and 7), and 6 h (lanes 4 and 8). Immunoprecipitations in lanes 1 to 4 are from the *rad23*Δ strain JWY50, and those in lanes 5 to 8 are from the isogenic *RAD*$^+$ strain LP3041-6D. The arrow indicates the RAD23 protein (lanes 5 to 8). Numbers at the left indicate the positions of prestained molecular weight markers.

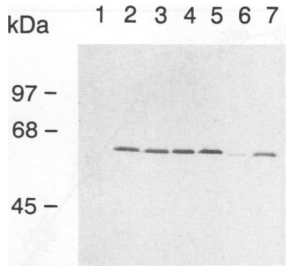

FIG. 7. Intracellular levels of rad23 mutant proteins. Extracts of the *rad23*Δ strain JWY50 (lane 1) and JWY50 harboring the low-copy-number *CEN* plasmids pJW129 (*RAD23*; lane 2), pJW140 (*rad23*–Arg-49; lane 3), pJW196 (*rad23*–Arg-49 Arg-55; lane 4), pJW206 (*rad23*–Arg-49 Arg-55 Arg-63; lane 5), and pJW136 (*rad23*Δ*48–60*; lanes 6 and 7) were subjected to immunoblot analysis with anti-RAD23 antibodies as described in the legend to Fig. 2. The equivalent of 2 × 10^6 cells was loaded in lanes 1 to 6; the equivalent of 1.2 × 10^7 cells was loaded in lane 7.

genes, *BAT3*, *GdX*, and *ISG15*, also contain such ub-like sequences. The ub-like domain in RAD23, BAT3, and GdX is not cleaved and is therefore an integral component of these proteins. The identification of the ub-like domain in RAD23 represents the first finding of such a sequence in *S. cerevisiae*. The presence of this sequence in RAD23 has offered us the opportunity to investigate the biological significance of this domain. Our studies demonstrate the requirement of the ub-like domain in RAD23 function. Yeast cells carrying the *rad23*Δ*2-60* mutant allele missing the sequence encoding residues 2 to 60 are UV sensitive. Replacement of the ub-like domain in RAD23 by the authentic ubiquitin sequence results in normal levels of UV resistance,

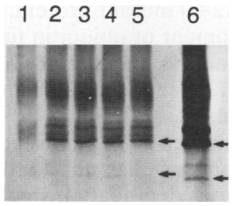

FIG. 8. Mutant rad23 proteins with Lys-to-Arg alterations in the ub-like domain are also multiubiquitinated. Extracts from strain JWY50 (*rad23*Δ) harboring pPM39 (2μm *CUP1-ub*; lane 1) and JWY50 harboring pPM39 plus one of the multicopy 2μm plasmids pJW128 (*RAD23*; lane 2), pJW141 (*rad23*–Arg-49; lane 3), pJW197 (*rad23*–Arg-49 Arg-55; lane 4), pJW207 (*rad23*–Arg-49 Arg-55 Arg-63; lane 5), and pJW134 (*rad23*Δ*48–60*; lane 6) were subjected to immunoprecipitation with anti-RAD23 immunobeads as described in Materials and Methods. Cells were cultured in YPD medium containing 100 μM copper sulfate to induce the *CUP1* promoter in plasmid pPM39. Because of the much lower level of rad23Δ48–60 in extract, it was necessary to scale up the immunoprecipitation fivefold to obtain enough of the protein for probing. The SDS eluate of the immunoprecipitate that contained rad23Δ48–60 protein was concentrated from 400 to 80 μl in a Centricon 30; 20 μl of this concentrate (≈5 μg of rad23Δ48–60; lane 6) and 20 μl of the unconcentrated SDS eluates (≈10 μg of RAD23 or rad23 protein; lanes 2 to 5) from the other immunoprecipitations were analyzed for the presence of ubiquitinated species as described in the legend to Fig. 5. The arrows mark the mono- and diubiquitinated forms of the RAD23 and rad23 proteins. Notice the downward shift in the molecular weight of the ubiquitinated species in the case of rad23Δ48–60 (compare lane 6 with lanes 2 to 5).

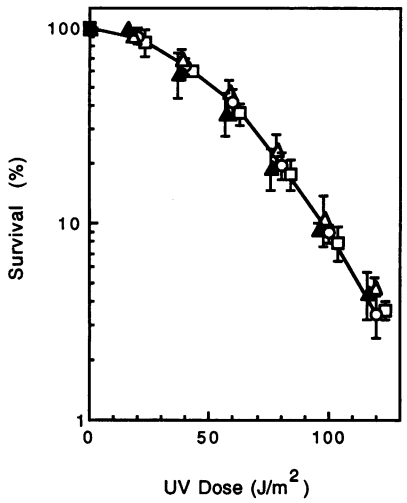

FIG. 9. The *rad23*-Arg mutants have the same UV sensitivity as wild-type cells. Strain JWY50 (*rad23*Δ) carrying *CEN* plasmids containing the *RAD23* gene (pJW129) or *rad23*-Arg mutants (pJW140 [Arg-49], pJW196 [Arg-49 Arg-55], and pJW206 [Arg-49 Arg-55 Arg-63]) were grown in complete synthetic medium lacking uracil. Shown is the average of three independent experiments; error bars represent 1 standard deviation from the arithmetic mean. Symbols: ○, *RAD23*; △, *rad23*–Arg-49; ▲, *rad23*–Arg-49 Arg-55; □, *rad23*–Arg-49 Arg-55 Arg-63.

indicating a structural and functional similarity of this domain with ubiquitin.

Since RAD23 is a nuclear protein, and the ub-like sequence is the only basic region in the protein, we considered the possibility that this portion of the protein controls the entry of RAD23 into the nucleus. The inability of the mutant protein to gain nuclear entry would explain the DNA repair defect of the rad23Δ2-60 mutant protein. In fact, it has been suggested that attachment of ubiquitin to proteins facilitates their translocation across membranes (10). However, we found that like RAD23, the rad23Δ2-60 mutant protein is also located in the nucleus.

On the basis of studies with the fusion protein ub–P–β-Gal, it has been proposed that ub-like domains in proteins function as autonomous degradation signals. ub–P–β-Gal, an engineered ubiquitin gene fusion, has a very short half-life of ~5 min in *S. cerevisiae* because the ubiquitin moiety in the fusion protein acts as a primary degradation signal via the formation of a multiubiquitin chain linked to the Lys-48 residue in the ubiquitin domain. Lys-48 in ubiquitin is the site where attachment of the multiubiquitin chain occurs in proteolytic substrates. A mutation of this site to Arg inactivates the formation of the multiubiquitin chain and subsequent proteolysis of substrates (5). To determine whether the region of the RAD23 protein flanking Lys-49 that is equivalent to Lys-48 in ubiquitin is required for function, we constructed a *rad23Δ48-60* mutation in which residues 48 to 60 have been deleted. This mutation causes the same degree of UV sensitivity as the *rad23Δ2-60* mutation does, indicating a requirement of this portion of the ub-like domain in RAD23 function.

To test the idea that the ub-like domain in RAD23 functions as a primary degradation signal, we examined the stability of RAD23 protein in pulse-chase experiments and investigated the multiubiquitination of RAD23 and various mutant rad23 proteins. We found that RAD23 is fairly abundant and exhibits a high degree of stability, with a half-life much greater than 6 h. Consistent with these observations, we found that only a minute proportion of RAD23 (<<0.1%) is multiubiquitinated. Furthermore, a change of Lys-49 to Arg had no effect on the level of multiubiquitination of RAD23. Since the *rad23Δ48-60* mutation also removes the Lys residue at position 55, we examined whether Lys-55 was involved in the multiubiquitin chain formation. However, the level of multiubiquitination was not affected in the Arg-49 Arg-55 double mutant. Normal levels of multiubiquitination also occur in the Arg-49 Arg-55 Arg-63 triple mutant. As determined by immunoblotting, the intracellular levels of Arg-49, Arg-49 Arg-55, and Arg-49 Arg-55 Arg-63 mutant proteins were the same as that of the wild-type protein. Thus, none of these Lys residues are utilized in RAD23 multiubiquitination. UV survival was also not affected by the Arg-49, Arg-55, and Arg-63 mutations. We also examined the multiubiquitination of the rad23Δ48-60 mutant protein. Unexpectedly, we found a much higher extent of multiubiquitination for this mutant protein than for RAD23 protein. Correspondingly, the intracellular level of rad23Δ48-60 mutant protein was significantly lower than that of the wild-type protein.

Our observations strongly suggest that the ub-like domain affects RAD23 function in a nonproteolytic manner. This domain in RAD23 could influence DNA repair in several plausible ways. For example, during posttranslational folding, this domain could confer upon RAD23 protein a proper conformation necessary for its subsequent interaction with the other components of the excision repair machinery assembled at the site of DNA damage. Alternatively, the ub-like domain may participate directly in physical interactions with the other excision repair proteins facilitating the assembly of the repair complex.

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