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A Ubiquitin Mutant with Specific Defects in DNA Repair and Multiubiquitination

JEAN SPENCE,[1] SETH SADIS,[1] ARTHUR L. HAAS,[2] AND DANIEL FINLEY[1]*

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115,[1] and Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 52336[2]

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The degradation of many proteins involves the sequential ligation of ubiquitin molecules to the substrate to form a multiubiquitin chain linked through Lys-48 of ubiquitin. To test for the existence of alternate forms of multiubiquitin chains, we examined the effects of individually substituting each of six other Lys residues in ubiquitin with Arg. Substitution of Lys-63 resulted in the disappearance of a family of abundant multiubiquitin-protein conjugates. The *UbK63R* mutants were not generally impaired in ubiquitination, because they grew at a wild-type rate, were fully proficient in the turnover of a variety of short-lived proteins, and exhibited normal levels of many ubiquitin-protein conjugates. The *UbK63R* mutation also conferred sensitivity to the DNA-damaging agents methyl methanesulfonate and UV as well as a deficiency in DNA damage-induced mutagenesis. Induced mutagenesis is mediated by a repair pathway that requires Rad6 (Ubc2), a ubiquitin-conjugating enzyme. Thus, the *UbK63R* mutant appears to be deficient in the Rad6 pathway of DNA repair. However, the *UbK63R* mutation behaves as a partial suppressor of a *rad6* deletion mutation, indicating that an effect of *UbK63R* on repair can be manifest in the absence of the Rad6 gene product. The *UbK63R* mutation may therefore define a new role of ubiquitin in DNA repair. The results of this study suggest that Lys-63 is used as a linkage site in the formation of novel multiubiquitin chain structures that play an important role in DNA repair.

Eukaryotic cells employ a protein known as ubiquitin in processes such as cell cycle control, DNA repair, the stress response, and organelle biogenesis (19, 28, 32, 58). The covalent linkage of ubiquitin to substrate proteins serves to signal their degradation by a multisubunit, 26S protease (44). The pathway of ubiquitin conjugation involves an initial, ATP-dependent step of ubiquitin C-terminal activation, catalyzed by the Uba1 enzyme. Ubiquitin is then transferred to any of a large number of ubiquitin-conjugating (Ubc) enzymes (32). These enzymes share the ability to accept ubiquitin in a thiolester form from Uba1 and are required for the donation of ubiquitin to protein substrates. Substrate selection can be controlled by specificity factors such as Ubr1 (58), E3α (28), E3β (28), and E6-associated protein (48).

Analysis of the *UBC* gene family has allowed the resolution of diverse functions of ubiquitination (32). For example, Ubc3 (Cdc34) is required for cell cycle progression from the G_1 to S phase (24), and Ubc10 (Pas2), a peroxisome-associated protein, is required for peroxisome biogenesis (61). Other *ubc* mutations confer more complex phenotypes. In the case of the Ubc4/Ubc5 family, this may reflect a broad diversity of substrates, as mutations in these genes result in defective degradation of the bulk of short-lived proteins (9, 52, 53).

The most pleiotropic *ubc* phenotype to be described is that of *ubc2* (*rad6*) mutants (33, 43). Despite being proficient in bulk protein turnover, *rad6* mutants are acutely sensitive to UV light, methyl methanesulfonate (MMS), X rays, and other mutagens as well as defective in DNA damage-induced mutagenesis. Complex formation between the Rad6 and Rad18 proteins may target Rad6 to DNA damage sites (5). Rad6 is also required for degradation of proteins through the N-end rule pathway, whose substrates include Gpa1, the α subunit of a heterotrimeric G protein involved in pheromone-dependent signal transduction (38). The participation of Rad6 in N-end rule turnover is mediated by complex formation with the Ubr1 protein (15, 56), which recognizes proteins bearing specific N-terminal amino acids (6). This complex is active in ubiquitination, forming Lys-48-linked multiubiquitin chains on target proteins (8, 15, 56).

Efficient degradation of a variety of short-lived proteins requires that they be ligated to a chain of ubiquitin molecules joined through Lys-48 of ubiquitin (8, 21, 29). An arginine substitution at this position prevents breakdown of model substrates in vitro (8) and is lethal in *Saccharomyces cerevisiae* (21). With some model substrates, unidentified residues other than Lys-48 can serve as ubiquitin acceptor sites, although no functional consequences have been associated with these linkages (26, 34). To assay the functional effects of ubiquitin mutations, yeast strains in which the four natural ubiquitin genes are replaced with a synthetic, mutated ubiquitin gene have been engineered (21). Here we describe the effects of substituting lysine residues of ubiquitin, particularly Lys-63.

UbK63R mutants both grow and degrade proteins at normal rates but are highly sensitive to UV irradiation and the alkylating agent MMS. These phenotypic defects appear to result from dramatic deficiencies in the formation of specific ubiquitin-protein conjugates. In particular, Lys-63 is required to form the most abundant family of multiubiquitin-protein conjugates in *S. cerevisiae*. Other ubiquitin conjugates are present at normal levels in the mutant. Thus, the effects of the *UbK63R* mutation on ubiquitination are, like those on DNA repair, highly specific. To account for these observations, we propose that Lys-63 can be used as a chain linkage site and that its usage, while restricted to a minority of substrates, is in at least some cases obligatory or highly preferred over Lys-48.

* Corresponding author. Mailing address: Department of Cell Biology, Harvard Medical School, 25 Shattuck St., Boston, MA 02115. Phone: (617) 432-3492. Fax: (617) 432-1144.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques. Yeast culture media were prepared essentially as described by Rose et al. (46). Except where otherwise noted, yeast strains were grown at 30°C in YPD medium, and a previously described strain (21), SUB280 (*MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-*Δ*200 trp1-1[am] ubi1-*Δ*1*::*TRP1 ubi2-*Δ*2*::*ura3 ubi3-*Δ*ub-2 ubi4-*Δ*2*::*LEU2* [pUB39] [pUB100]), was used as the relevant wild-type strain. pUb39 is a *LYS2*-marked plasmid that expresses ubiquitin from a *CUP1* promoter (18). pUB100 expresses the Ubi1 tail (18). As previously described (18), the *ubi2-*Δ*2*::*ura3* allele was generated from *ubi2-*Δ*1*::*URA3* by selecting for resistance to 5-fluoro-orotic acid (7). SUB280 behaves essentially indistinguishably from the wild type (SUB62; *MAT***a** *lys2-801 leu2-3*, *112 ura3-52 his3-*Δ*200 trp1-1[am]*) (20) under favorable growth conditions, as a result of the expression of ubiquitin from pUB39 at a level close to that of wild-type cells. Sporulation of SUB280 derivatives requires the presence of an additional plasmid expressing ubiquitin from the sporulation-inducible *UBI4* promoter. SUB328 is identical to SUB280 except that it contains plasmid pUB146 instead of pUB39. pUB146 (a gift of E.-C. Park) is a *URA3*-marked plasmid expressing wild-type ubiquitin (fused to a short peptide derived from Ard1 [42]) from a galactose-regulated promoter. SUB413 was identical to SUB280 except for the *UBK63R* mutation. SUB493 and SUB492 were identical to SUB413 and SUB280, respectively, except that the ubiquitin-expressing plasmids were marked with *URA3* rather than *LYS2*.

The *rad6* mutation used in this study is a complete deletion and was constructed by using the *URA3* allele of pNKY51 (2). After transformation, the Ura$^-$ marker of the recipient strain (either SUB62 or SUB280) was regenerated by selecting for resistance to 5-fluoro-orotic acid. The *rad52* mutation is a complete deletion of the *RAD52* coding sequence which removes no more than 20 bp of flanking sequence. It was prepared by inserting the above-described fragment from pNKY51 into the *Bam*HI site of plasmid pDR52, a gift of E. Perkins. The resulting plasmid, pNU44, was cut with *Sal*I and transformed into SUB280. The Ura$^-$ marker was then regenerated as described above. Standard techniques were used for manipulation and sequencing of DNA (47).

Construction of ubiquitin gene derivatives. Lys-to-Arg mutations in the ubiquitin gene were introduced into plasmid Yep96 (21) following PCR-mediated, site-directed mutagenesis. All such constructions involved digestion of PCR fragments spanning the ubiquitin-coding element of Yep96 with *Bgl*II and *Kpn*I and cloning them into the corresponding sites in Yep96. A 5.5-kb *Cla*I fragment containing the *LYS2* gene was then cloned into the unique *Cla*I site of each mutant plasmid, yielding plasmids comparable to pUB39 (18), which served as wild-type control.

Assays of induced mutagenesis and sensitivity to DNA-damaging agents. UV (and MMS) sensitivity was tested in cells grown to exponential phase in YPD. Cultures were exposed to 254-nm UV light after being spread onto YPD plates. Assays of induced mutagenesis were similar except that the liquid cultures were prepared in SD medium supplemented with amino acids and uracil (46), and plates contained the same medium with or without the relevant nutritional component (either uracil or lysine). The yield of revertants obtained after UV irradiation was normalized to that of unselected cells irradiated for the same time period. For assay of MMS sensitivity, liquid cultures were washed and resuspended at 0.1 M potassium phosphate (pH 7.0). MMS was added, and the sample was incubated at 30°C for 30 min with agitation. Freshly prepared $Na_2S_2O_6$ was then added to a final concentration of 5%, and after 5 min at room temperature, the cells were washed and spread onto YPD plates.

Assays of protein degradation. The turnover of bulk short-lived proteins was measured in cultures growing exponentially in SD medium supplemented with amino acids as described previously (46), but with the omission of methionine and arginine. ^{35}S-amino acids (Tran^{35}S-label; ICN) were added to 0.2 ml of culture, and after 5 min, cells were washed twice in chase medium (growth medium supplemented with 0.2 mg of methionine and 0.5 mg of cycloheximide per ml) and resuspended in 0.2 ml of chase medium. Duplicate 10-μl aliquots were withdrawn at each time point and spotted onto discs of 3MM filter paper (Whatman) that had been previously spotted with 25 μl of 50% trichloroacetic acid. Filters were then washed as described previously (11), and radioactivity was determined by liquid scintillation counting. Degradation is measured as time-dependent loss of trichloroacetic acid-insoluble counts from the filter. To measure turnover of canavanyl proteins, the same protocol was followed except that canavanine was added to 0.6 mg/ml 7 min prior to labeling, and labeling was carried out for 2 min. Turnover of Lys–β-Galactosidase was measured essentially as described previously (21). Plating efficiency assays for canavanine sensitivity were as described previously (20).

Immunoblot analysis. Exponentially growing cells were washed, resuspended in distilled H_2O, and UV irradiated for 40 s at 0.5 J/m^2/s with shaking. An equal volume of 2× YPD was then added, and cells were incubated for 10 min at 23°C in the dark. The cells were harvested and lysed in 9.5 M urea–2% Nonidet P-40–5% β-mercaptoethanol, using glass beads. Clarified lysate was added to an equal volume of 20% glycerol–10% β-mercaptoethanol–4.6% sodium dodecyl sulfate (SDS)–0.125 M Tris-HCl (pH 6.8) before loading onto SDS-polyacrylamide gels. After electrophoresis as described previously (21), proteins were transferred to polyvinylidene difluoride membranes in 25 mM Tris base–19 mM glycine–0.01% SDS–20% methanol. For two-dimensional isoelectric focusing/SDS-polyacrylamide gel electrophoresis (PAGE) analyses, ampholines of pH range 3 to 10 were added to the clarified lysate, and electrophoresis was performed as described previously (41). Antibodies to ubiquitin-protein conjugates were prepared and affinity purified as described elsewhere (25). The detection method involved either ^{125}I-protein A (Amersham) or enhanced chemiluminescence, using horseradish peroxidase-conjugated donkey anti-rabbit antibodies (Amersham).

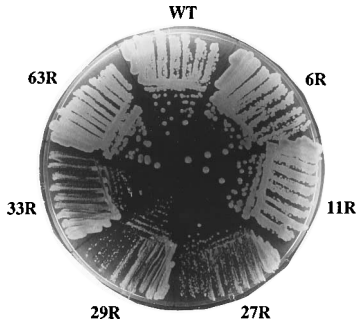

FIG. 1. Effects of Lys-to-Arg mutations in ubiquitin on growth of yeast cells. Colony sizes of ubiquitin mutants streaked onto YPD plates after 3 days of growth at 30°C are shown. WT, wild-type control strain SUB280. Remaining strains express the mutant ubiquitin indicated and lack wild-type ubiquitin.

RESULTS

Lysine substitution mutations in ubiquitin. To test for the functionality of multiubiquitin chains linked through residues other than Lys-48, we constructed arginine substitutions for each of the six other lysines of ubiquitin (amino acids 6, 11, 27, 29, 33, and 63). The mutated genes, expressed from the *CUP1* promoter (16), were transformed into a yeast strain (SUB328) in which the genes encoding ubiquitin-ribosomal protein fusions (*UBI1* to *UBI3*) and polyubiquitin (*UBI4*) have been deleted (21). In this strain, the ribosomal protein-encoding portions of *UBI1* to *UBI3* are expressed free of ubiquitin (21). The viability of this strain is dependent on the presence of a *URA3*-marked plasmid (pUB146) expressing wild-type ubiquitin. By using a negative selection against its *URA3* marker (7), pUB146 could be eliminated from the strain once any one of the mutated ubiquitin genes had been introduced, indicating that these mutations are nonlethal. However, substitution of either Lys-27, Lys-29, or Lys-33 resulted in a moderate growth defect (Fig. 1).

Loss of specific ubiquitin-protein conjugates in the *UbK63R* mutant. A mutation affecting an alternate multiubiquitination site would be expected to cause the disappearance of a specific subset of ubiquitin-protein conjugates. In contrast, a mutation that caused a general reduction in the efficiency of ubiquitin conjugation would be expected to cause comparable decreases in the levels of many or all conjugates. Whole-cell extracts from each lysine substitution mutant were analyzed on SDS-polyacrylamide gels, transferred to a filter, and visualized with an antibody that detects ubiquitin and ubiquitin-protein conjugates (Fig. 2A). Toward the top of the gel, we observed diffuse staining that presumably represents heterogeneous ubiquitin-protein conjugates, because it disappeared in mutants lacking the major Ubc enzymes Ubc4 and Ubc5 (Fig. 2C). However, in the low-molecular-weight region of the gel, many discrete bands were evident. Strikingly, at least four of these bands were missing in the *UbK63R* mutant (Fig. 2A). These bands are labeled A, B, C, and X. The intensities of bands A and B were weak in untreated cells (Fig. 2B), but upon UV treatment, they appeared as major bands (Fig. 2A).

Each of the remaining mutants showed a conjugate profile identical to that of the wild type, albeit with minor differences

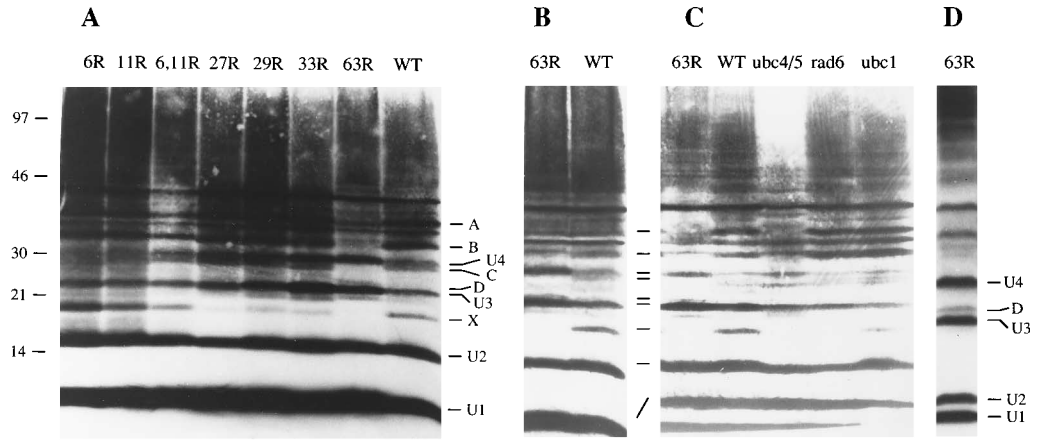

FIG. 2. Immunoblot detection of ubiquitin-protein conjugates in ubiquitin and Ubc enzyme mutants. (A) Immunoreactive bands in UV-treated Lys-to-Arg mutants of ubiquitin. Whole-cell extracts were analyzed on SDS–15% polyacrylamide gels and electroblotted. Filters were incubated with an affinity-purified antibody to ubiquitin. Designations at the left indicate the molecular masses (in kilodaltons) of standards. The position of free ubiquitin (U_1) was determined by using a purified standard. Bands U_n are apparently free multiubiquitin chains (see text and Fig. 4). The numerical designation is based on comigration with in vitro-polymerized free chains (10). WT, wild type. See text for other designations. (B) As panel A, except that UV light treatment was omitted. (C) Immunoreactive bands in Ubc enzyme mutants exposed to UV light. Note that in the *ubc4 ubc5* lane, the band under U_4 is not C but U_4', as discussed in the legend to Fig. 4. The strains *ubc1* (54), *rad6*, and *ubc4 ubc5* (52) are congenic to SUB62 (20). (D) Analysis of a *UbK63R* extract, prepared as for panel A, on an SDS–13% polyacrylamide gel, showing improved resolution of bands D and U_3.

in band intensity. The lack of effect of the Lys-6 and Lys-11 substitutions is not due to redundancy of these sites, since a *UbK6R,K11R* double mutant was also indistinguishable from the wild type both in its conjugate profile (Fig. 2A) and in its growth rate (data not shown). There was a reduction in the intensity of band X among the growth-deficient *UbK27R*, *UbK29R*, and *UbK33R* mutants. However, overexposures of the blot shown in Fig. 2A show that these reductions are partial (Fig. 3). In contrast, band X is undetectable in *UbK63R* mutants (Fig. 3 and 4).

Interestingly, one band (band D) was present at significantly higher levels in *UbK63R* mutants (Fig. 2A). Band D is resolved most clearly from a neighboring band in Fig. 4D. Bands A, B, C, and D are distributed periodically, with apparent molecular mass increments of approximately 6 kDa. These bands could therefore represent progressively ubiquitinated forms of a single ubiquitin acceptor protein. In this interpretation, band D would represent the monoubiquitinated species and would accumulate in the mutant because the *UbK63R* mutation acts as a terminator of chain elongation. This model was tested by two-dimensional isoelectric focusing/SDS-PAGE analysis.

Two-dimensional analysis of ubiquitin conjugates from *UbK63R* mutants. In two-dimensional gels loaded with whole-cell extracts from *S. cerevisiae*, most ubiquitin-immunoreactive material is found within two series of discrete spots, one vertical and one diagonal. Remarkably, the diagonal strip is missing in *UbK63R* extracts (Fig. 4B). This diagonal of spots extends through the upper right-hand (basic) quadrant of the gel upward and to the left of conjugate A (Fig. 4A and C). The diagonal pattern presumably results from progressive ubiquitination of the same substrate, with more highly ubiquitinated forms asymptotically approaching the isoelectric point of ubiquitin (6.7) as they increase in molecular weight. Conjugate D falls onto this diagonal as well, as shown in Fig. 5, but is often lost during isoelectric focusing because of its basicity. Thus, conjugates A, B, C, and D appear to represent tetra-, tri-, di-, and monoubiquitinated forms of the same protein. Certain components of the diagonal, including conjugates A and B, are visualized more clearly upon UV induction (Fig. 4C), as previously noted in one-dimensional analyses. The defect in the formation of multiubiquitinated derivatives of a specific protein in the *UbK63R* mutant supports the hypothesis that Lys-63 is used as a multiubiquitination site.

We note that the existence of more than one family of highly basic, Lys-63-dependent conjugates is suggested by the splitting of spot C into C_1 and C_2 (Fig. 4C). One may extrapolate from the diagonal to estimate that the acceptor protein(s) is highly basic and slightly less than 20 kDa in apparent molecular mass. In higher eukaryotes, the most abundant ubiquitin-protein conjugates are formed from small, basic proteins as well, histones H2A and H2B. However, bands A to C do not appear to represent ubiquitin-histone conjugates, as shown by immunoblotting analysis of partial deletion mutants of the four histone types (data not shown) (reference 39 and references therein).

In addition to the diagonal of spots described above, a second series of spots focuses directly above ubiquitin (Fig. 4) and is distributed in a periodic pattern that includes ubiquitin itself. These spots are not derived from processing of polyubiquitin, because the polyubiquitin gene is deleted in these strains. The pattern extends to the top of the gel, indicative of very long adducts. The spots labeled U_2 to U_5 comigrate electrophoretically with Lys48-linked free ubiquitin chains synthesized in vitro by the Ubc enzyme E2-25K (data not shown) (10). From these data, and the recent biochemical analysis of U_2 by van Nocker et al. (57), it can be concluded that the species U_2 to U_5 represent free multiubiquitin chains. The existence of such free chains in yeast cells is consistent with previous evidence of a mammalian enzyme that can both synthesize free Lys-48 chains and use them as conjugative substrates (10). The apparent free chains are present at comparable levels in all Lys-

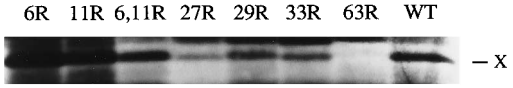

FIG. 3. Band X levels in Lys-to-Arg mutants of ubiquitin. A fluorographic overexposure of a portion of Fig. 2A is shown. WT, wild type.

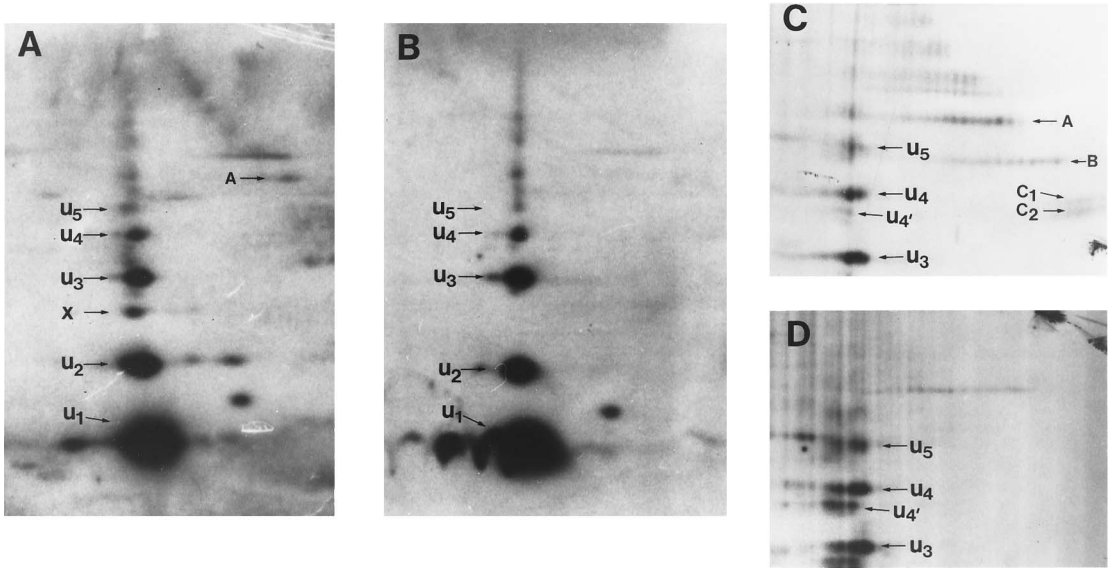

FIG. 4. Two-dimensional analysis of ubiquitin-protein conjugates in wild-type and mutant yeast strains. (A) Wild type (SUB280); (B) *UbK63R* (SUB413); (C) wild type (SUB280), UV treated; (D) *ubc4 ubc5*, UV treated. Proteins were separated in the first (horizontal) dimension by isoelectric focusing and in the second dimension on either SDS–18% (A and B) or 15% (C and D) polyacrylamide gels. Designations are described in the text and in the legend to Fig. 2. Horizontal spreading of spots A and B in panel C is a result not of isoelectric variants but of gel overloading, as required to provide detectable amounts of certain conjugates. Note that in panel D, both spots C_1 and C_2 disappear in the *ubc4 ubc5* sample, whereas in Fig. 2C, the lower member of the C_1/C_2 doublet appears to be present in the *ubc4 ubc5* lane. This band is shown by in panel D to represent not C_2 but U_4', an apparent free chain variant which is enhanced in extracts from *ubc4 ubc5* mutants. (An enhanced spot corresponding to U_3' can also be seen in panel D.) Thus, *ubc4 ubc5* mutants are (like *UbK63R*) defective for the synthesis of both C_1 and C_2. Immunoblots from UV-irradiated *UbK63R* mutants resemble that shown in panel B. In particular, spots A to C are undetectable.

to-Arg mutants, suggesting that they are formed predominantly, if not exclusively, through Lys-48 linkages (Fig. 2A).

The isoelectric point of band X differs from those of the other, highly basic conjugates that disappear in the *UbK63R* mutants and is similar to that of free multiubiquitin chains (Fig. 4A). Thus, band X may be a free ubiquitin chain whose electrophoretic mobility is shifted as a result of a variant Lys-63 chain linkage site. Alternatively, band X may a ubiquitinated or diubiquitinated form of a small protein whose isoelectric point is very close to that of ubiquitin.

Ubc enzymes involved in Lys-63-dependent ubiquitin conjugation. Ubc4 and Ubc5 mediate turnover of the bulk of short-lived proteins and appear to be functionally interchangeable (52). Figure 4D shows that these enzymes are required for synthesis of ubiquitin conjugates A to C. Mutants lacking a related enzyme, Ubc1 (53), had wild-type levels of bands A to C (Fig. 2C). Deletion of the *RAD6* (*UBC2*) gene also had no effect on the levels of bands A and B (Fig. 2C). This result was of interest because bands A and B are induced by UV light, and Rad6 plays a crucial role in the resistance of yeast cells to UV light (see below). The level of band X was also reduced in *ubc4 ubc5* double mutants. However, this effect was incomplete, and partial reductions in band X levels were also observed in *ubc1* and *rad6* (*ubc2*) mutants (Fig. 2C and data not shown).

A DNA repair defect in *UbK63R* mutants. As shown above, the levels of bands A and B increase upon UV irradiation in wild-type cells. When *UbK63R* mutants were subjected to UV irradiation, the frequency of survivors was far below that of the wild type (Fig. 6A). No sensitivity to UV light was found for the other Lys-to-Arg mutants, including those with growth deficiencies (Fig. 6A). The *UbK63R* mutant was also sensitive to the DNA-alkylating agent MMS (Fig. 6B). When exposed to 0.6% MMS, the *UbK63R* mutants exhibited an approximately 1,000-fold-greater loss of colony-forming ability than wild-type cells and required a day more than wild-type cells to form colonies after MMS treatment. Several lines of evidence indicate that the sensitivity of *UbK63R* mutants to DNA-damaging agents is specific and does not result from a general ubiquitin conjugation deficiency. First, *UbK63R* mutants were quantitatively indistinguishable from wild-type cells in their ability to degrade substrates of the ubiquitin pathway such as the bulk of short-lived proteins and abnormal, canavanine-containing pro-

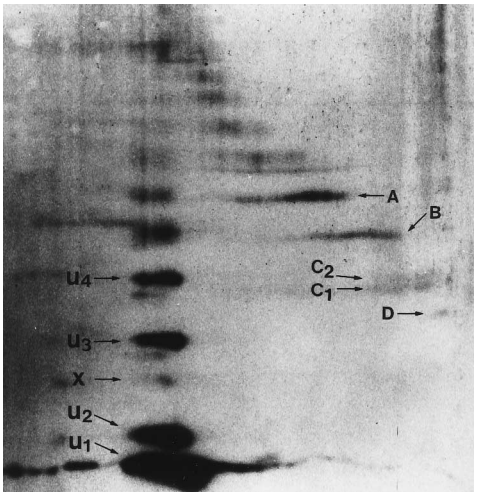

FIG. 5. Conjugate D is highly basic. Two-dimensional analysis of ubiquitin-protein conjugates in UV-treated *ubc1* mutants (first dimension, isoelectric focusing; second dimension, SDS–15% polyacrylamide) is shown. This gel shows that spot D falls onto the diagonal of spots that includes A, B, and C, consistent with the proposal that spot D represents the monoubiquitinated form of this conjugate family. See text for additional details. Similar results have been obtained for *rad6* (*ubc2*) mutants and wild-type (SUB280) cells.

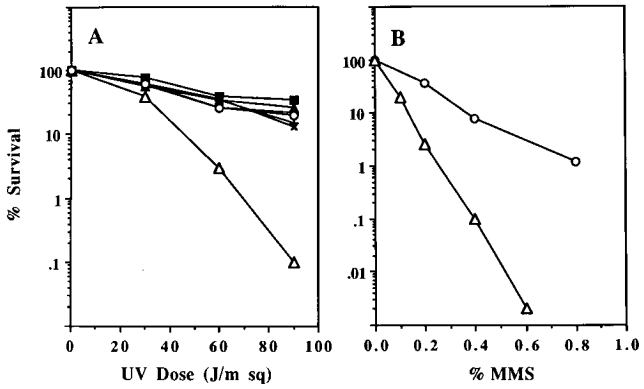

FIG. 6. Sensitivity of *UbK63R* mutants to DNA-damaging agents. (A) Viability of yeast strains following exposure to graded doses of 254-nm UV light. Circles, wild type (SUB280); open triangles, *UbK63R* (SUB413); crosses, *UbK6R*; diagonal crosses, *UbK11R*; squares, *UbK27R*; filled triangles, *UbK33R*. Datum points for *UbK29R* closely overlap those for *UbK33R* and are not shown. (B) Viability of wild-type (circles) and *UbK63R* (triangles) strains following exposure to MMS.

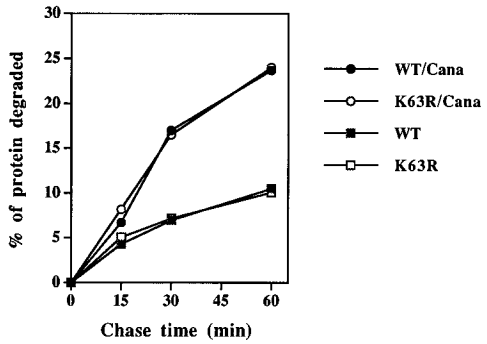

FIG. 7. Turnover of pulse-labeled proteins in wild-type cells (WT; SUB280) and *UbK63R* mutants (SUB413). Proteins were labeled in the presence of the amino acid analog canavanine (Cana) to measure degradation of abnormal proteins or in the absence of canavanine to measure degradation of normal short-lived proteins, as indicated. We note that despite being proficient in turnover of the bulk of canavanyl proteins, *UbK63R* mutants exhibit low plating efficiencies in the presence of canavanine.

teins (Fig. 7) (11, 21, 52). Second, immunoblotting experiments showed that the UV sensitivity of *UbK63R* mutants could not be attributed to attenuated ubiquitin levels, either before or after irradiation (Fig. 2 and 4). Third, the wild-type growth rate of the mutants at 30°C (Fig. 1) indicates that UbK63R is functional, since ubiquitin is essential for growth (21, 32).

The results presented above suggest that Lys-63 has a specific role in DNA repair. If this were attributable to the formation of a Lys-63-linked multiubiquitin chain, different amino acid substitutions at position 63 should result in quantitatively equivalent repair defects, because no residue other than lysine appears to act as a ubiquitin acceptor. To test this prediction, a nonconservative substitution of cysteine at position 63 was constructed. This mutant proved to be similar to the *UbK63R* mutant in its sensitivity to UV across the dose-response curve (data not shown). *UbK63R* mutants are slightly temperature sensitive for growth, but this phenotype appears to be unrelated to their specific multiubiquitination and DNA repair defects described above, which are strongly expressed at 30°C. This is suggested by the observation that the nonconservative *UbK63C* substitution results in a substantially enhanced degree of temperature sensitivity (data not shown). The temperature-sensitive phenotype is not specific to K63 but is typical of substitutions in this surface of ubiquitin, such as F4Y, T12A, E64K, and T66S (50).

The DNA repair defect of *UbK63R* mutants lies in the Rad6 pathway. There are three major epistasis groups, or pathways, for the repair of UV-induced DNA damage in *S. cerevisiae*. One pathway involves the Ubc enzyme Rad6 (Ubc2). There is a qualitative resemblance between *UbK63R* and *rad6* mutants in that they are both sensitive to UV light and MMS. This finding suggested that the functions of Rad6 in DNA repair might be executed in part through Lys-63-dependent ubiquitination. To test this possibility, we assayed DNA damage-induced mutagenesis, which is mediated exclusively by the Rad6 pathway (37, 43).

Yeast strains carrying a point mutation in the *URA3* gene were spread onto plates lacking uracil and UV irradiated. Both strains generated spontaneous revertants at a low frequency (Table 1; Fig. 8). However, irradiation resulted in a dramatic increase in Ura^+ colonies in the wild-type strains. In *UbK63R* mutants, the frequency of UV-induced reversion was substantially lower (Fig. 8A). UV-induced reversion of the *lys2-801* allele was also reduced in the *UbK63R* mutants (Fig. 8B). These results indicated that *UbK63R* mutants are defective in Rad6 pathway function. The mutagenesis assay also showed, unlike the survival assay, that the *UbK63R* mutation affects DNA repair rather than some other process that may influence the survival of UV-irradiated cells, such as degradation of UV-damaged or oxidized proteins.

The Rad6 pathway of DNA repair involves many genes, mutations in which generally result in a sensitivity to DNA-damaging agents which is less severe than that of *rad6* itself. To test whether the functions of Rad6 in DNA repair are generally dependent on Lys-63 of ubiquitin, the effect of the *UbK63R* mutation on additional Rad6-dependent repair processes was determined. The *UbK63R* mutation had no effect on either spontaneous mutation rates (Table 1) or sensitivity to γ irradiation (Fig. 9). Thus, Lys-63 is involved in a specific subset of Rad6-mediated repair events. *UbK63R* mutants are also indistinguishable from the wild type in many *RAD6*-dependent processes that do not obviously involve DNA repair, such as sporulation and the degradation of proteins through the N-end

TABLE 1. Frequencies of spontaneous reversion of the *ubi2-Δ2::ura3* allele to Ura^+ in *UbK63R* mutants

Strain[a]	Frequency of Ura^+ revertants/10^8 cells in:						Mean	Median
	Culture 1	Culture 2	Culture 3	Culture 4	Culture 5	Culture 6		
Wild type	4.0	2.1	2.4	2.0	2.6	2.1	2.5	2.2
UbK63R	1.8	1.7	2.0	1.4	1.7	3.0	1.9	1.8

[a] Wild type, strain SUB280; *UbK63R*, strain SUB413. Each strain was streaked onto YPD plates, and after 2 days of growth, whole colonies were inoculated into 3 ml of YPD medium and grown for 2 days. Cultures were then diluted appropriately in H_2O and spread onto synthetic minimal medium plates supplemented with amino acids, in the presence or absence of uracil. Colonies were counted after 3 days of growth. The total yield of revertants counted ranged from 98 to 287 in different cultures.

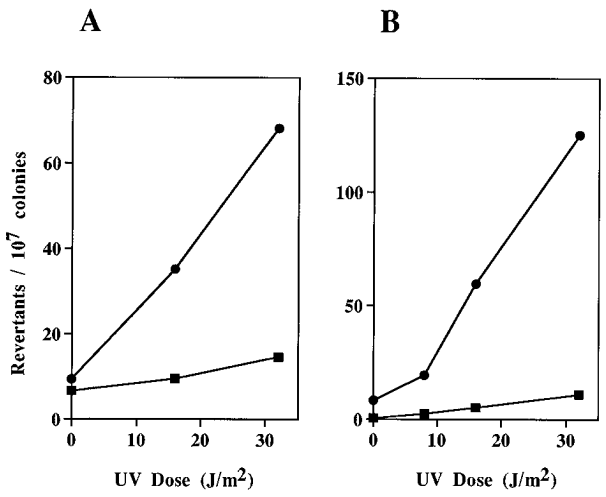

FIG. 8. *UbK63R* mutants are defective in UV-induced mutagenesis. (A) UV-induced reversion of *ubi2*-Δ*2*::*ura3* in *UbK63R* mutants (squares; SUB413) and wild-type cells (circles; SUB280). (B) UV-induced reversion of *lys2-801* in *UbK63R* mutants (squares; SUB493) and wild-type cells (circles; SUB492). See Materials and Methods for details.

rule pathway (Table 2). *UbK63R* mutants also exhibited no sensitivity to the antifolate compound trimethoprim, which is highly toxic to *rad6* mutants (Table 2) (27).

Epistatic interactions between *UbK63R* and other DNA repair mutations. The nature of the repair pathway involving Lys-63 was further addressed through epistasis analysis. If a double mutant exhibits greater sensitivity to UV than either of the corresponding single mutants, the repair pathways affected by the mutations are suggested to be independent. For example, the strong UV sensitivity of the *rad52 UbK63R* double mutant suggests that Lys-63 does not participate in the *RAD52*-requiring recombinational repair pathway (Fig. 10A). In contrast, a *rad6 UbK63R* double mutant is no more sensitive to UV than is the *rad6* mutant itself (Fig. 10B). This result suggests

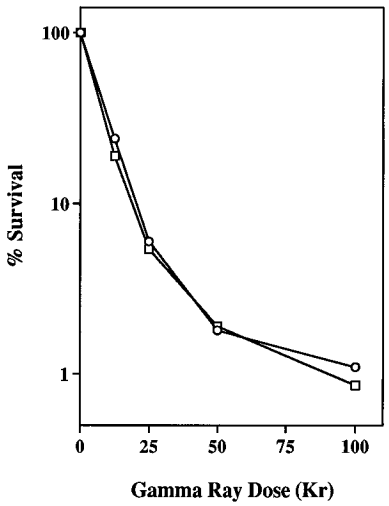

FIG. 9. Survival of *UbK63R* mutants (squares; SUB413) and wild-type cells (circles; SUB280) following γ irradiation. Cultures were grown to stationary phase in YPD, irradiated with a cobalt-60 source, diluted, and spread onto YPD plates. Colonies were counted after 3 days of growth. In similar experiments carried out with exponentially growing cultures, the sensitivity of *UbK63R* mutants to γ irradiation was similarly indistinguishable from wild-type sensitivity.

TABLE 2. Comparison of *UbK63R* and *rad6* mutant phenotypes[a]

Genotype	Resistance to:			Mutagenesis		Trimethoprim resistance	Spore formation	Turnover of Lys–β-Gal
	UV	MMS	γ rays	Induced	Spontaneous			
Wild type	+	+	+	+	+	+	+	+
rad6	−	−	−	−	++	−	−	−
UbK63R	−	−	+	−	+	+	+	+

[a] Note that those phenotypes shared by *rad6* and *UbK63R* are more severe in the *rad6* mutant. Resistance to trimethoprim was determined by spreading cells onto YPD plates containing the drug at 0.2%. Plating efficiency of *rad6* mutants was less than 10^{-3}, whereas wild-type and *UbK63R* cells formed colonies at control efficiencies. Spore formation was assayed microscopically in cells expressing UbK63R from both *CUP1* and *UBI4* promoters. Lys–β-Galactosidase (Lys–β-Gal) is a substrate of the N-end rule pathway of protein degradation (4).

that the *UbK63R* mutation affects the Rad6 pathway, consistent with the induced mutagenesis data of Fig. 8. Assuming that Lys-63 chains participate in the Rad6 pathway, a simple model would be that Rad6 mediates formation of these chains. The UV sensitivity of a *rad6* deletion strain would in this case be predicted to be indistinguishable from that of a *rad6 UbK63R* strain. Surprisingly, this was not observed. Rather, *UbK63R* behaves as a partial suppressor of *rad6* (Fig. 10B). This effect has been consistently observed in four independently constructed strains. The data suggest that in *rad6* mutants, Lys-63-dependent ubiquitination persists and continues to affect the efficiency of DNA repair mechanisms. The relative level of UV resistance conferred by *UbK63R* in the absence of Rad6 indicates that Rad6-independent repair pathways function more efficiently in the absence of Lys-63.

A third process contributing to the survival of UV-irradiated cells is excision repair. Among genes of the excision repair group is *rad4* (23). The sensitivity of *rad4 UbK63R* mutants to UV light is no greater than that of the *rad4* mutant itself (Fig. 10C). Thus, epistasis analysis suggests that Lys-63 functions within either the Rad6-mediated or excision repair pathway but does not distinguish between the two. Because defects in induced mutagenesis have been observed only in Rad6 pathway mutants, and because elevated rates of induced mutagenesis are characteristic of excision repair defective strains, it is unlikely that Lys-63 functions within the excision repair pathway.

The difficulty of observing additive effects between *rad4* and *UbK63R* mutants may be related to their disparate degrees of UV sensitivity. An alternative possibility is suggested by the observation that in excision repair-deficient strains, induced mutations arise after DNA replication, whereas in excision repair-proficient strains, they arise before DNA replication (31, 35). It has been proposed that the former process reflects the filling of daughter strand gaps and that the latter involves filling of excision repair-generated gaps in which the template strand contains a lesion (37). Thus, certain aspects of Rad6 pathway function may be perturbed in excision repair-deficient strains. Additional epistasis experiments may further clarify the nature of the repair pathway in which Lys-63 participates.

DISCUSSION

A ubiquitination defect in *UbK63R* mutants. In the *UbK63R* mutant of ubiquitin, a number of otherwise highly abundant ubiquitin conjugates are reduced to undetectable levels. This effect is highly selective, as the levels of all other detectable conjugates are unaffected. Therefore, this effect cannot be ascribed to a general defect in the capacity of UbK63R to

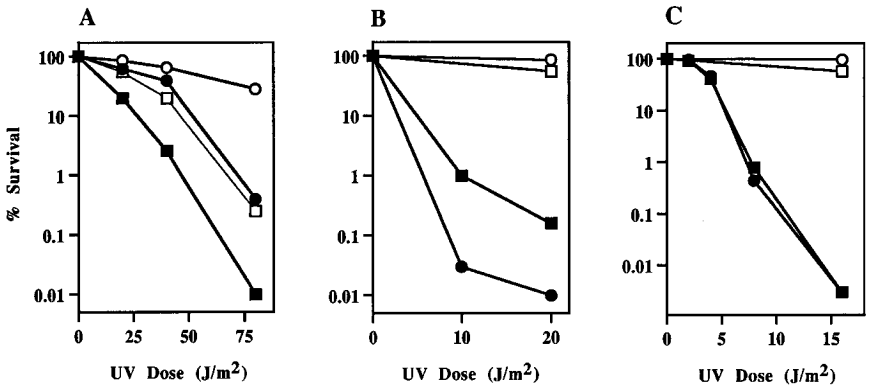

FIG. 10. Epistasis tests. In all panels, open circles represent the wild type (SUB280) and open squares represent *UbK63R* mutants. (A) *UBK63R* and *rad52* are nonepistatic. Closed circles, *rad52*; closed squares, *UbK63R rad52*. (B) Partial suppression of the UV-sensitive phenotype of a *rad6* mutant by *UbK63R*. Closed circles, *rad6*; closed squares, *UbK63R rad6*. (C) The sensitivities of *rad4* mutants and *rad4 UbK63R* double mutants to UV light are similar. Closed circles, *rad4*; closed squares, *UbK63R rad4*. All strains used in these assays are congenic to SUB280.

function in conjugation. Moreover, the inability of *UbK63R* mutants to form conjugates A to C cannot be ascribed to a defect in the interaction of UbK63R with the Ubc enzymes required for their formation, Ubc4 and Ubc5, because other Ubc4/5-dependent processes, such as bulk protein degradation, have been shown to be quantitatively equivalent in mutant and wild-type cells (Fig. 7). Similarly, Rad6 (Ubc2) functions normally in the degradation of N-end rule substrates in the presence of UbK63R (Table 2). Taken together, these results strongly suggest that Lys-63 functions as a major multiubiquitination site in *S. cerevisiae*. Consistent with this view, the crystal structure of ubiquitin shows Lys-63 to be a surface residue (59). Lys-63 is also highly susceptible to chemical acetylation (30).

That residues other than Lys-48 can be modified by ubiquitination has been previously reported (26, 34). However, the experimental systems used in these studies have artificial aspects, and no evidence has been obtained for the functional significance of these linkages. In this study, we have focused on ubiquitin conjugates that are observed when ubiquitin is expressed at normal levels and which contain natural substrate proteins in vivo. However, the constraints of this experimental system have not yet allowed us to show directly that Lys-63 functions as a multiubiquitination site, which requires peptide mapping of ubiquitin-ubiquitin linkages.

We found no evidence by one-dimensional immunoblotting analysis or by general phenotypic characterization that other lysine residues in ubiquitin function as multiubiquitination sites for natural substrate proteins. Further characterization of the strains that we have constructed, particularly two-dimensional analysis of their ubiquitin conjugate patterns, should be of value in clarifying this issue.

Alternate forms of multiubiquitin chains. Alternative chain structures would provide a simple basis by which different classes of ubiquitin conjugates could potentially be distinguished by components of the ubiquitin pathway. Recent studies suggest that the arrangement of ubiquitin molecules into Lys-48 chains may be critical for efficient recognition by the 26S protease (14). Within a Lys-48 chain, ubiquitin molecules that are covalently linked may interact noncovalently as well (12, 13). The crystal structures of diubiquitin and tetraubiquitin conjugates reveal multiple contacts among ubiquitin groups (12, 13). In the putative Lys-63 chains, these contacts could not be formed, because of steric hindrance resulting from displacement of the chain linkage site. Thus, the solution structures of Lys-48 and Lys-63 chains may differ in their packing interactions as well as ubiquitin-ubiquitin linkage sites. Structural differences between Lys-48 and Lys-63 chains could affect their relative capacities to target proteins for degradation or the resistance of the chains to disassembly by deubiquitinating enzymes. The spots A to C seen in Fig. 4 are apparently the most abundant multiubiquitin-protein conjugates in the cell and are also unique among detectable ubiquitin-protein conjugates in the multiplicity of bound ubiquitin groups. Ubiquitin-protein conjugates that are intermediates in degradation are expected to be present at low levels, because of their rapid degradation. Therefore, the distinct abundance of these conjugates suggests that they may be relatively poor substrates for degradation.

Ubiquitination and DNA repair. *UbK63R* mutants exhibit a specific sensitivity to DNA-damaging agents such as UV and MMS. Their defect in induced mutagenesis suggests that Lys-63 functions within the repair pathway that requires the Rad6 (Ubc2) Ubc enzyme. The Rad6 pathway of repair of *S. cerevisiae* involves at least 24 gene products and mediates resistance to a wide variety of DNA-damaging agents (22, 37, 43). The evolutionary conservation of *RAD6* genes from *S. cerevisiae* to humans (36, 60) suggests that this repair pathway is universal among eukaryotes. Mutagenesis by this pathway is believed to result from translesion DNA synthesis by the Rev3 gene product (40). Rad6-mediated ubiquitination might serve either to regulate the activity of Rev3 and other repair enzymes or to provide a more accessible template for repair, presumably by catalyzing degradation of chromatin proteins near the lesion.

Although *UbK63R* and *rad6* mutants were sensitive to both UV light and MMS, *UbK63R* mutants did not exhibit elevated levels of spontaneous mutagenesis or sensitivity to γ irradiation. Thus, *UbK63R* mutants exhibited a subset of the DNA repair phenotypes of *rad6* mutants. These observations, and the suppressive effect of the *UbK63R* mutation on *rad6*, suggest that the role of ubiquitination in DNA repair may be more complex than previously expected. Since this effect of *UbK63R* on DNA repair is manifest in the absence of Rad6 function, other Ubc enzymes can be predicted to participate in DNA repair. Although in a *rad6* genetic background, the inability to perform Lys-63-dependent ubiquitination events leads to resistance rather than sensitivity to UV, the ubiquitination events responsible for UV resistance in wild-type cells may be identical to those imparting sensitivity in the absence of Rad6

enzyme; when Rad6 pathway function is mutationally inactivated, remaining pathway components may nonetheless engage damaged sites nonproductively and thus inhibit repair by other pathways. Mutations affecting Srs2, a helicase (45) that is known to function in the Rad6 pathway, have also been found to partially suppress *rad6* deletion mutations (1, 49). Similar suppressive effects have been observed between excision repair mutations (27).

These indications of a novel Ubc enzyme involved in DNA repair are supported by the recent characterization of the *hus5* gene of *Schizosaccharomyces pombe*. Partial loss of function mutations in *hus5* result in UV sensitivity in *Schizosaccharomyces pombe* (17). The *hus5* gene encodes a Ubc enzyme (3) which is homologous to Ubc9 of *S. cerevisiae* (51). *ubc9* mutants, which are defective in the degradation of Clb2 and Clb5 cyclins (51), are also UV sensitive, like *hus5* mutants (54, 55). It will be interesting to test for mechanistic interactions among Ubc9, Rad6, and Lys-63 chains in the UV response and to identify the signaling mechanisms that control the ubiquitination events involved in DNA repair.

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