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Inhibition of Proteolysis and Cell Cycle Progression in a Multiubiquitination-Deficient Yeast Mutant

DANIEL FINLEY,[1]* SETH SADIS,[1] BRETT P. MONIA,[2] PAUL BOUCHER,[3] DAVID J. ECKER,[2] STANLEY T. CROOKE,[2] AND VINCENT CHAU[3]

Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115[1]; Department of Pharmacology, Wayne State University School of Medicine, Detroit, Michigan 48201[3]; and Isis Pharmaceuticals, Carlsbad, California 92008[2]

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The degradation of many proteins requires their prior attachment to ubiquitin. Proteolytic substrates are characteristically multiubiquitinated through the formation of ubiquitin-ubiquitin linkages. Lys-48 of ubiquitin can serve as a linkage site in the formation of such chains and is required for the degradation of some substrates of this pathway in vitro. We have characterized the recessive and dominant effects of a Lys-48-to-Arg mutant of ubiquitin (UbK48R) in *Saccharomyces cerevisiae*. Although UbK48R is expected to terminate the growth of Lys-48 multiubiquitin chains and thus to exert a dominant negative effect on protein turnover, overproduction of UbK48R in wild-type cells results in only a weak inhibition of protein turnover, apparently because the mutant ubiquitin can be removed from multiubiquitin chains. Surprisingly, expression of UbK48R complements several phenotypes of polyubiquitin gene (*UBI4*) deletion mutants. However, UbK48R cannot serve as a sole source of ubiquitin in *S. cerevisiae*, as evidenced by its inability to rescue the growth of *ubi1 ubi2 ubi3 ubi4* quadruple mutants. When provided solely with UbK48R, cells undergo cell cycle arrest with a terminal phenotype characterized by replicated DNA, mitotic spindles, and two-lobed nuclei. Under these conditions, degradation of amino acid analog-containing proteins is severely inhibited. Thus, multiubiquitin chains containing Lys-48 linkages play a critical role in protein degradation in vivo.

The degradation of short-lived proteins is a complex and highly regulated process (21, 29). In eukaryotes, the primary mechanism of selective degradation involves ligation of the C terminus of ubiquitin to ε-amino groups of lysine residues within the proteolytic substrate. Coupling of ubiquitin to proteolytic substrates is mediated by a family of ubiquitin-conjugating enzymes, which has at least 12 distinct members in *Saccharomyces cerevisiae* (35, 49). These enzymes are required for a wide range of cellular functions, including cell cycle control (35), DNA repair (35), peroxisome biogenesis (57), and heavy metal resistance (37). In the case of higher eukaryotes, ubiquitination has been implicated in the degradation of mitotic cyclins (25), p53 (50), c-mos, c-fos, and other regulators of growth and the cell cycle (9, 41).

Ubiquitination targets proteins for degradation by the 26S protease. This particle contains over 20 distinct subunits, including multiple peptidase and ATPase activities and a deubiquitinating activity (19, 24, 45). The mechanisms by which these activities are coordinated and controlled by substrate ubiquitination are unknown. However, in vitro experiments have indicated that the efficacy of ubiquitination in signaling degradation is a function of the multiplicity and arrangement of ubiquitin groups bound to the substrate (8, 21, 26). Substrates of the N-end rule pathway, such as Arg–β-galactosidase (β-Gal), are modified in rabbit reticulocyte extracts by a multiubiquitin chain with ubiquitin-ubiquitin linkages at Lys-48 (8, 54). Both multiubiquitination and degradation of Arg–β-Gal were blocked when UbK48R was substituted for wild-type ubiquitin, suggesting that degradation requires multiubiquitin chain formation. However, there may be substrate-to-substrate differences in the degree to which degradation is inhibited by preventing Lys-48 chain synthesis (19, 28, 31).

One possible basis for this variation is the existence of alternative forms of multiubiquitin chains. Lys-63 of ubiquitin can apparently be used as a multiubiquitination site on endogenous substrates in vivo (52). In the present work, a failure to form Lys-48 chains was seen to inhibit various processes despite the continued presence of Lys-63 as a potential compensatory attachment site. The phenotypic effects of preventing Lys-48 chain synthesis are more dramatic than those seen with Lys-63, consistent with the view that obligatory Lys-48 chain formation is a dominant mode of degradative signaling in the ubiquitin pathway.

MATERIALS AND METHODS

Yeast strains, media, and genetic techniques. Standard techniques were used for strain construction, transformation, and tetrad dissection (48). Cultures were grown at 30°C except where indicated. Synthetic media consisted of 0.7% Difco yeast nitrogen base supplemented with amino acids and uracil as described previously (48) and either 2% glucose (synthetic glucose medium) or 2% galactose, 2% glycerol, 2% ethanol, and 40 μg of aspartate per ml (synthetic galactose medium). Uracil and specific amino acids were omitted where necessary for plasmid selections, while for pulse-labeling experiments methionine was omitted and arginine was omitted in experiments involving canavanine. Ubiquitin overproduction from the *CUP1* promoter was induced by the addition of $CuSO_4$ to 100 μM.

Strains SUB62 (*MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) and SUB60 (*MAT***a** *ubi4-Δ2::LEU2 lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1*) have been described elsewhere (22). SUB280 (*MAT***a** *lys2-801 leu2-3,112 ura3-52 his3-Δ200 trp1-1 ubi1::TRP1 ubi2-Δ2::ura3 ubi3-Δub2 ubi4-Δ2::LEU2* [pUB39]

* Corresponding author. Phone: (617) 432-3492. Fax: (617) 432-1144.

[pUB100]) was constructed from previously described (20, 22) yeast strains. The pUB39 plasmid is similar to Yep96 but marked with the *LYS2* gene (20). pUB100 carries a Ubi1 tail expression cassette (20) inserted into the *Sma*I site of pUN90 (17). The *ubi2*-Δ2::*ura3* allele has been described elsewhere (52).

Strain SUB312, in which wild-type ubiquitin is expressed from a galactose-inducible promoter, was constructed by transforming SUB280 with the plasmid pUB23 and selecting against the *LYS2*-marked pUB39 plasmid with α-amino adipic acid (6). pUB23 is a previously described plasmid that expresses ubiquitin in the form of a fusion protein with *Escherichia coli* β-Gal (Met–β-Gal [2]). SUB313 to -315 were constructed from SUB312 by transformation with plasmids pUB39, pUB70, and pUB115, respectively.

Canavanine sensitivity tests were carried out as described previously (22). To measure starvation sensitivity, cultures growing exponentially in yeast nitrogen base supplemented with glucose, lysine, histidine, and uracil were washed with distilled H_2O and then resuspended in the same medium but without auxotrophic nutrients and ammonium sulfate. The concentration of viable cells was determined at time zero and on day 8 of starvation by spreading appropriately diluted samples onto YPD (48) plates.

Plasmid constructions. All ubiquitin gene mutations were carried in plasmids derived from Yep96, which expresses a synthetic yeast ubiquitin gene off the *CUP1* promoter. Yep96 is identical to Yep46 (16) except that the 1.7-kb *Pvu*II-*Sma*I fragment of Yep46 is deleted. Previously described mutated ubiquitin genes (14, 15, 39) were cloned into the *Bgl*II and *Kpn*I sites of Yep96. pUB70 was constructed from pUB39 by digestion with *Bgl*II and *Xho*I, treatment with the Klenow fragment of DNA polymerase I (38), and recircularization. This results in deletion of codons 4 through 50 of ubiquitin and out-of-frame expression of the remainder of the gene. pUB115 is identical to pUB39 except for the Lys-to-Arg mutation at codon 48. The experiments whose results are shown in Fig. 5 employed derivatives of human ubiquitin. Human ubiquitin differs from yeast ubiquitin at only three residues, has a virtually superimposable three-dimensional structure (56), supports growth of yeast cells indistinguishably from yeast ubiquitin (data not shown), and is kinetically indistinguishable from yeast ubiquitin in conjugation and degradation assays carried out with extracts from mammalian cells (58).

Quantitation of ubiquitinated forms of Lys–β-Gal. Cultures were grown to exponential phase in synthetic galactose-$CuSO_4$ medium, pulse-labeled with tran^{35}S-label (ICN) for 5 min, harvested by centrifugation, and resuspended in growth medium supplemented with 0.5 mg of methionine per ml, 0.5 mg of cycloheximide per ml, and 1 mM trichodermin. Samples were withdrawn at given time points, pelleted, and resuspended in buffer A (0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM Tris-HCl [pH 7.5]) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, and a cocktail (2) of proteinase inhibitors. Samples were lysed with glass beads (2) and centrifuged at 12,000 × *g* for 10 min at 4°C. Equivalent trichloroacetic acid-precipitable counts per minute from each supernatant were incubated with anti-β-Gal monoclonal antibody (Promega) for 60 min at 4°C. Protein A-Sephacryl (Pierce) was added, and the incubation was continued for 30 min at 4°C. Immunoprecipitates were harvested by centrifugation and washed at 4°C with buffer A containing 0.1% sodium dodecyl sulfate (SDS). The final pellet was resuspended in SDS-containing electrophoretic sample buffer (22) and incubated at 95°C for 4 min. Sephacryl beads were pelleted by centrifugation, and the supernatants were loaded onto a 6% polyacrylamide–SDS gel. After electrophoresis, the gel was fixed, processed for fluorography with En^{3}Hance (NEN), dried, and exposed to a PhosphorImager cassette. The image was visualized with a Molecular Dynamics PhosphorImager. Band intensities of the unmodified and ubiquitinated forms of Lys–β-Gal were determined by area integration of each lane by using the ImageQuant software package. Peak recognition parameters were smoothing of 2 to 3, slope sensitivity of 0.003 to 1.0, and upward count and downward count of 1 to 2. The baseline was determined with the best-fit option. Because of peak broadening, decreased signal, and increased background, ubiquitinated species containing more than four ubiquitin groups could not be reliably quantitated.

Immunofluorescence staining, DAPI staining, and flow cytometry. Cultures were grown in synthetic galactose medium to an optical density at 600 nm of 0.3, washed, resuspended in synthetic glucose-$CuSO_4$ medium for an additional 6 h, and harvested. Flow cytometry was carried out with cells stained with propidium iodide as described previously (23). Staining was quantitated with a system 2150 Ortho Diagnostics cytofluorograph, with excitation by an argon laser at 488 nm, and emission was read at 630 nm. The data were processed with an Applescanner (Macintosh). For 4′,6-diamidino-2-phenylindole (DAPI) staining and immunofluorescence, cells were fixed in 4% formaldehyde and treated with Zymolyase. Microtubules were then stained with the YOL-34 monoclonal antibody to a tubulin and a second antibody of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (23). DNA was stained with DAPI as described previously (23).

Degradation assays. Cultures grown for 6 h in synthetic glucose-$CuSO_4$ medium were harvested by centrifugation and resuspended in the same medium with or without a supplement of 300 μg of canavanine per ml. After 1 h, cells were labeled with [^{35}S]methionine for 10 min. Methionine and cycloheximide were then added to final concentrations of 20 mM and 0.5 mg/ml, respectively, and an aliquot of the cell suspension was withdrawn and placed in SDS sample buffer. The remaining cells were pelleted by centrifugation, washed, and resuspended in synthetic glucose-$CuSO_4$ medium containing methionine and cycloheximide as described above. Aliquots were transferred to SDS sample buffer at given time points. Samples were subjected to electrophoresis in a 15% polyacrylamide–SDS gel. Radioactivity in the dried gel (excluding the solvent front) was quantitated directly, using an AMBIS radioimaging system.

RESULTS

Dominant effects of UbK48R overexpression. The elongation of a Lys-48 multiubiquitin chain occurs through the addition of a new ubiquitin group to the Lys-48 residue of the last ubiquitin added. UbK48R is fully competent as a conjugative donor but when added to such a chain fails to regenerate a free Lys-48 for chain elongation, and thus it can act as a terminator of chain growth. This mechanism suggests that UbK48R should be a dominant negative inhibitor of chain formation and degradation. To assess this possibility, ubiquitin derivatives were overexpressed in wild-type cells from the *CUP1* promoter. In the absence of the inducing agent copper, ubiquitin is expressed from this promoter at a level essentially equivalent to that of total endogenous ubiquitin from the genes *UBI1* to *UBI4* (16). Maximal induction by copper results in an approximately 50-fold induction and thus a substantial excess of *CUP1* promoter-driven ubiquitin (16). Under inducing conditions, a dominant effect of UbK48R overexpression on the degradation of Lys–β-Gal, a short-lived protein that is

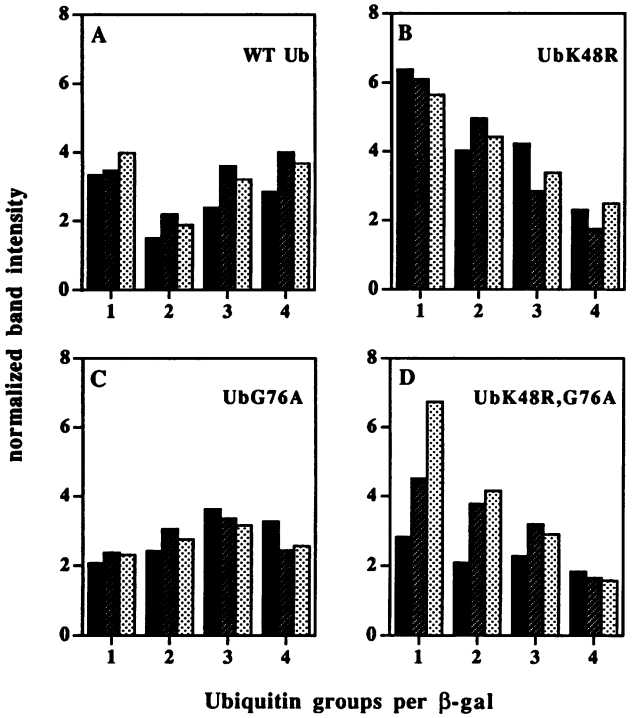

FIG. 1. Dominant effects of overexpressing ubiquitin variants on the length of multiubiquitin chains bound to Lys–β-Gal. Band intensities from the experiment whose results are shown in Fig. 2 were quantitated with a PhosphorImager. The intensity was determined for each Ub_n–Lys–β-Gal conjugate band shown and then normalized to the intensity of unmodified Lys–β-Gal in the same sample. Normalized values are expressed as percentages. WT, wild type. ■, 0 min; ▩, 10 min; ▨, 30 min.

degraded through the N-end rule pathway (2, 54), was observed. The half-life of Lys–β-Gal was approximately 15 min when wild-type ubiquitin was overproduced and 25 min when UbK48R was overproduced. A comparable degree of inhibition by UbK48R has been observed with the transcriptional repressor α2 (32) and a Cln3 cyclin–β-Gal hybrid protein (49). Because of the weak nature of these effects, these data do not prove that K48 chains play a major or obligatory role in degradation of these proteins. Northern (RNA) hybridization studies indicate that the weakness of these effects cannot be explained by a compensatory induction of endogenous ubiquitin genes as a consequence of UbK48R expression (data not shown).

Figure 1 shows that the weak nature of the UbK48R dominant effect on degradation is attributable at least in part to the limited impact of UbK48R overexpression on the process of multiubiquitination itself. Multiubiquitin chain dynamics were assessed with the substrate Lys–β-Gal. A significant fraction of Lys–β-Gal is ubiquitinated, and quantitation of the levels of monoubiquitinated, diubiquitinated, triubiquitinated, and tetraubiquitinated derivatives can be achieved with a PhosphorImager. When wild-type ubiquitin was overexpressed by inducing the *CUP1* promoter, comparable levels of the four conjugates were observed, with a slight underrepresentation of the diubiquitinated form (Fig. 1A). Each conjugate was present at a level approximately 2 to 4% of that of unmodified β-Gal. A number of more highly ubiquitinated forms of Lys–β-Gal could also be detected, but their levels could not be reproducibly quantitated because of their lower abundance. During the chase incubation, Lys–β-Gal was rapidly degraded (Fig. 2), and its ubiquitinated forms disappeared at a rate equivalent to that of Lys–β-Gal itself. This is reflected in Fig. 1A as relatively time-independent normalized conjugate levels over the two-half-life duration of the chase. These results indicate that steady-state levels of modification are established rapidly with respect to the time of the pulse-labeling (5 min) and are consistent with the view that the ubiquitinated forms of Lys–β-Gal are intermediates in degradation.

When UbK48R was overexpressed, a progressive decrease in the intensities of more highly ubiquitinated species was seen (Fig. 1B). However, the activity of UbK48R in capping of growing chains is not pronounced, as the intensity of band n+1 was approximately 70% of that of band n. Although the turnover rate of Lys–β-Gal under these conditions was decreased by approximately twofold, the level of the ubiquitin conjugates relative to unmodified Lys–β-Gal was once again maintained over time, indicating that these ubiquitinated forms are also at steady state. Thus, UbK48R overexpression leads to shorter multiubiquitin chains on Lys–β-Gal but does not generate a time-dependent accumulation of the monoubiquitinated Lys–β-Gal species. One explanation for this result is that UbK48R can cap multiubiquitin chains as proposed but that the capped chains are not stable.

Capping could be bypassed either through multiubiquitination at residues other than Lys-48 or by removal of Lys-48 from the growing chain followed by the addition of wild-type ubiquitin. To distinguish between these possibilities, we constructed a double mutant of ubiquitin, UbK48R,G76A. The Gly-to-Ala mutation at position 76, which forms the C termi-

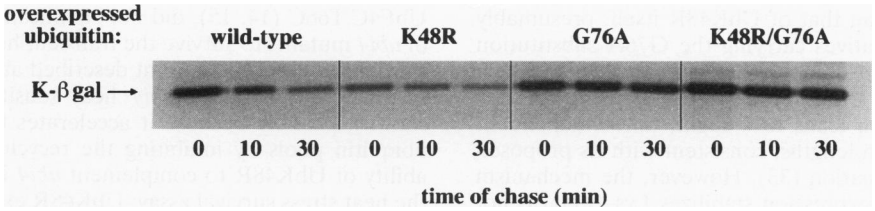

FIG. 2. Dominant effects of overexpressing ubiquitin variants on degradation of Lys–β-Gal. Lys–β-Gal was coexpressed with ubiquitin derivatives, as indicated, in wild-type *S. cerevisiae* (strain SUB62) and was pulse-labeled. Chase incubations were carried out for 0, 10, and 30 min. Cells were then lysed, and Lys–β-Gal was immunoprecipitated, analyzed by SDS-polyacrylamide gel electrophoresis, and detected by fluorography. The half-lives of Lys–β-Gal under these conditions are approximately 15 min when wild-type ubiquitin is overexpressed, 25 min when UbK48R is overexpressed, and 60 min when either UbG76A or UbR48K,G76A is overexpressed. Substrates such as Cln3–β-Gal show considerably greater stabilization by UbK48R,G76A than by UbG76A or UbK48R (49).

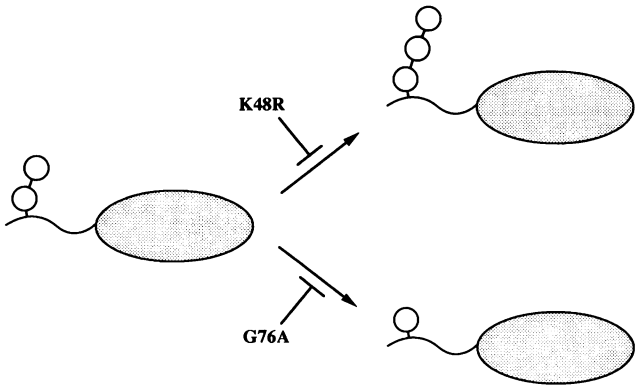

FIG. 3. Model of the effect of UbK48R,G76A on the dynamics of multiubiquitin chains. Lys–β-Gal is represented by ovals, and wild-type ubiquitin is represented by circles. The multiubiquitin chain is bound to Lys–β-Gal through Lys-15 and Lys-17 of the previously described artificial extension of the β-Gal N terminus (54), which is derived from LacI (wavy line). The model shows possible fates of a diubiquitinated form of Lys–β-Gal. In principle, the kinetic pathways available include extension and shortening of the chain, which are inhibited by the *K48R* and *G76A* mutations, respectively. Consequently, chains terminated with UbK48R,G76A may have relatively static lengths and therefore accumulate as reflected in the time dependence of chain length observed in Fig. 1D. The corresponding single mutations influence chain length but do not result in static chains, because each mutation blocks only one of two pathways of chain modification. A third potential fate of diubiquitinated Lys–β-Gal is degradation of Lys–β-Gal. However, it is unlikely that β-Gal derivatives containing one, two, or three ubiquitin moieties are efficient substrates for degradation (7, 36, 49). For example, overexpression of UbK48R,G76A leads to marked stabilization of Cln3–β-Gal, despite a dramatic accumulation of Cln3–β-Gal derivatives attached to one to four ubiquitin molecules (49).

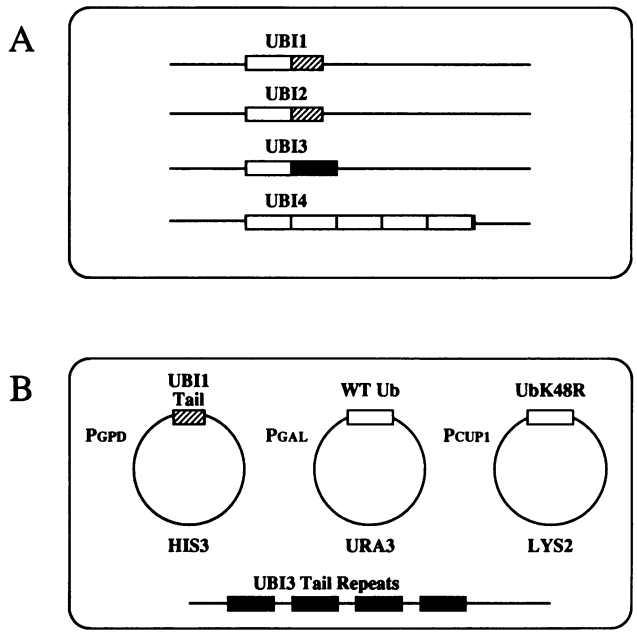

FIG. 4. Arrangement of genes for ubiquitin and ubiquitin tails in wild-type *S. cerevisiae* (A) and UbK48R tester strain SUB315 (B). The indicated plasmids are, from left to right, pUB100, pUB23, and pUB115. See Materials and Methods for details.

nus of ubiquitin, inhibits deubiquitination (33) and is recessive lethal in *S. cerevisiae* (data not shown). As shown in Fig. 1D, overproduction of UbK48R,G76A leads to a striking, time-dependent bias toward short chain lengths. Because no time-dependent alteration of chain length results from overexpression of either UbK48R or UbG76A, the time-dependent chain lengthening in the double mutant is a synthetic effect of combining the two mutations (Fig. 3). The existence of this synthetic phenotype indicates that removal of UbK48R from growing chains is responsible for the weakness of its effect on chain length. These results also indicate that the chains bound to β-Gal are dynamic in nature (18) and that the major pathway for degradation-independent disassembly of multiubiquitin chains is through release of the terminal ubiquitin moiety. The effect of UbK48R,G76A on chain length is realized more slowly than that of UbK48R itself, presumably because ubiquitin derivatives carrying the G76A substitution are poor substrates for ubiquitin-activating enzyme, which catalyzes the first step of the conjugation pathway (39, 44). The single G76A substitution leads to a slight, time-independent bias toward longer chain lengths, consistent with its proposed inhibition of deubiquitination (33). However, the mechanism by which UbG76A overexpression stabilizes Lys–β-Gal is not clear (Fig. 2).

Complementation of *ubi4* by expression of UbK48R. The results described above suggested that the functions of Lys-48 multiubiquitination in vivo would best be studied through analysis of recessive rather than dominant effects of the *UbK48R* mutation. In *S. cerevisiae*, the ubiquitin gene family includes four members (Fig. 4A). *UBI1*, *UBI2*, and *UBI3* encode fusion proteins composed of an N-terminal ubiquitin moiety and a C-terminal tail polypeptide, which is cleaved from ubiquitin and incorporated into the ribosome (20, 42). These genes supply the bulk of ubiquitin under favorable growth conditions and are not expressed under conditions unfavorable for growth, often called stress conditions (42). These include high temperature, starvation, and exposure to certain chemicals such as amino acid analogs. Under such conditions, the *UBI4* gene is induced, and its presence is then required for survival (22). *UBI4* encodes polyubiquitin, a tandem array of ubiquitin precursors that is rapidly processed proteolytically to monomers (42).

Surprisingly, expression of UbK48R from the *CUP1* promoter in *ubi4* mutants results in virtually complete complementation of its heat-sensitive phenotype (Fig. 5). This result was not significantly dependent on copper induction. Other ubiquitin mutants were found to be severely deficient with respect to the wild type when tested in this assay. For example, the expression of ubiquitin derivatives that support protein degradation in vitro at 30% of the wild-type rate, UbH68K and UbF4C,T66C (14, 15), did not significantly restore the ability of *ubi4* mutants to survive the transient heat exposure. Expression of the UbG76A mutant described above actually resulted in an accentuation of *ubi4* heat sensitivity (33) (data not shown), possibly because it accelerates the depletion of free ubiquitin pools by inhibiting the recycling of ubiquitin. The ability of UbK48R to complement *ubi4* is not a peculiarity of the heat stress survival assay; UbK48R expression also leads to virtually complete complementation of the *ubi4* defect in plating efficiency in the presence of canavanine and a substantial rescue of the starvation sensitivity of *ubi4* mutants (Table 1). In each case, complementation of *ubi4* was observed in both the presence and absence of the inducing agent copper.

The simplest interpretation of the *ubi4* complementation

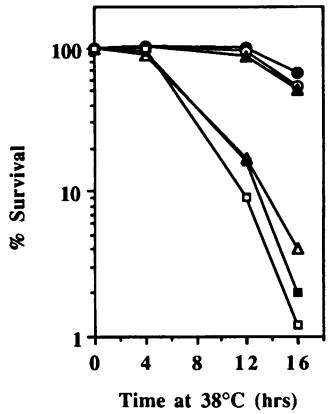

FIG. 5. Complementation of the heat sensitivity of *ubi4* by UbK48R expression. Cultures growing exponentially at 30°C in synthetic glucose medium were diluted, spread onto plates, and shifted to 38°C. After the times indicated, the plates were returned to 23°C to allow colony formation. Colonies were counted after 4 days of growth. Open circles, wild-type strain (SUB62). The remaining samples were *ubi4* strains (SUB60) transformed with plasmids expressing either wild-type ubiquitin (solid circles), UbK48R (solid triangles), UbF4C, T66C (open triangles), UbH68K (solid squares), and *ubi4* itself (open squares).

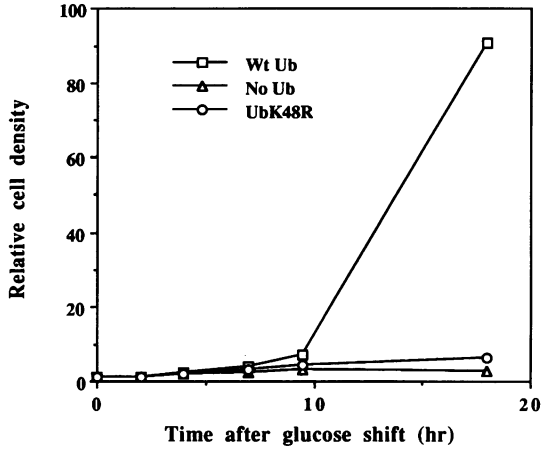

FIG. 6. Recessive lethality of the *UbK48R* mutation. Cells growing exponentially in synthetic galactose medium were centrifuged, washed three times in distilled H_2O, and resuspended in synthetic glucose medium. At the indicated times, the optical density at 600 nm was determined for each culture. Optical density values were normalized to a time zero value of 1. Squares, SUB313. Triangles, SUB314. Circles, SUB315. The SUB313 culture was diluted 10-fold after 13 h to prevent overgrowth.

data is that Lys-48 chains are not required for survival of stress conditions. However, it is also possible that UbK48R expression complements *ubi4* through a mechanism involving an increase in the level of free wild-type ubiquitin (see Discussion). We therefore developed a more stringent assay for recessive effects of the *UbK48R* mutation.

UbK48R does not support vegetative growth of *S. cerevisiae*. To assess the role of Lys-48 chains in vegetative growth, strains lacking all chromosomal ubiquitin genes were constructed from previously described ubiquitin gene deletion mutants (Fig. 4B). The *Δubi1-4* quadruple deletion mutants express the ribosomal tails of Ubi1 to Ubi3 independently of ubiquitin, which requires that they be overproduced, because fusion to ubiquitin serves directly or indirectly to promote incorporation of the tail proteins into ribosomes (20). Overproduction of the Ubi3 tail results from tandem integration at the *Ubi3* locus of a derivative of *Ubi3* in which the ubiquitin coding element is deleted. The Ubi1 tail is expressed from a plasmid off the strong promoter of the *GPD* gene.

When ubiquitin is expressed from the *CUP1* promoter in the context of these deletions and artificial constructs, the resulting strain, SUB280, exhibits essentially wild-type ubiquitin levels, growth rates, and protein turnover rates. Plasmid shuffling in SUB280 and its derivatives is straightforward because the markers available for plasmid transformation, *lys2* and *ura3*, are each amenable to both positive and negative selection (4, 6). By plasmid shuffling, a derivative of SUB280 in which ubiquitin was expressed from a galactose-dependent promoter was produced. This strain (SUB312) ceases to grow when withdrawn from galactose, indicating that ubiquitin is essential for growth of *S. cerevisiae*. Cessation of growth is rapid, in part because the induced *GAL* promoter does not express ubiquitin at wild-type levels, resulting in a mild constitutive growth deficiency. By expressing UbK48R in this strain (Fig. 4B) during a shift from galactose to glucose, the ability of UbK48R to complement the ubiquitin-deficient phenotype of the *Δubi1-4* mutant was tested. When expressed from the *CUP1* promoter under either inducing or noninducing conditions, UbK48R proved completely defective in supporting the growth of yeast cells (Fig. 6), suggesting that formation of Lys-48-linked multiubiquitin chains is an essential process in *S. cerevisiae*. The lethality of the *UbK48R* mutation was confirmed by the mutated gene's inability to sustain growth of *S. cerevisiae* when the galactose-induced wild-type ubiquitin gene was lost (as opposed to glucose repressed). Loss of the wild-type gene was checked by monitoring the linked *URA3* marker of the plasmid (Fig. 4B). In contrast to results with UbK48R, expression of wild-type ubiquitin from an uninduced *CUP1* promoter allowed Ura^- segregants to be readily selected (data not shown).

TABLE 1. Complementation of *ubi4* by UbK48R expression

Plasmid product[a]	Transformant	% Canavanine resistant	% Starvation resistant[b]
None	a	<0.1	0.0005
	b	<0.1	<0.0005
Ub	a	40	20
	b	40	8
UbK48R	a	70	0.05
	b	10	0.01

[a] Ubiquitin was expressed from Yep46 (16) and related plasmids in strain SUB60.

[b] The complementation effect in starving cells might be incomplete partly as a result of insufficient expression of UbK48R off the *CUP1* promoter during prolonged starvation.

Cell cycle arrest of *UbK48R* mutants. Ubiquitination has been implicated in cell cycle regulation through degradation of cyclins (25, 30, 40). The role of Lys-48 multiubiquitin chains in cell cycle progression was assessed by examining the morphology of nuclei and spindles in *UbK48R* mutants following a shift to glucose. UbK48R was expressed from the *CUP1* promoter under noninducing conditions. After 6 h in glucose medium, 85% of the *UbK48R* mutant cells displayed large buds and a distinct terminal phenotype of arrest in medial nuclear division (Fig. 6). Nuclei were segregated into two lobes joined through the mother-bud junction. Cells containing lobed nuclei also

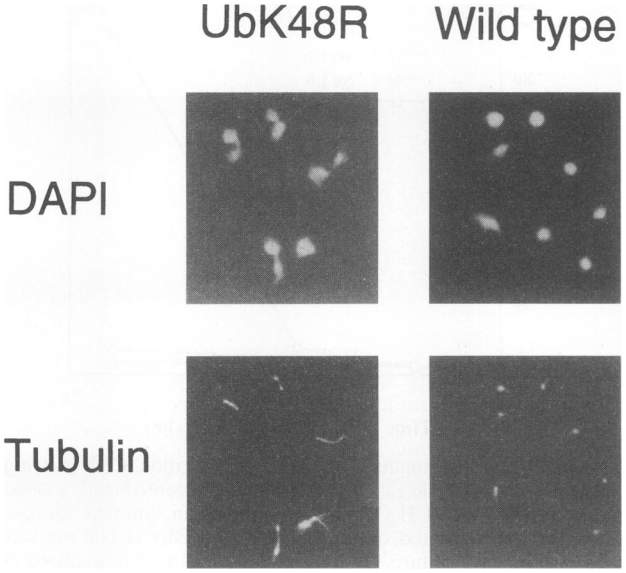

FIG. 7. Induction of cell cycle arrest in medial nuclear division is a recessive phenotype of the *UbK48R* mutation. Cultures growing exponentially in synthetic galactose medium were shifted to synthetic glucose medium for 6 h. Nuclear morphology is shown by the fluorescent DNA-binding dye DAPI, and spindle morphology is visualized with antitubulin antibody. Left, SUB315. Right, SUB313.

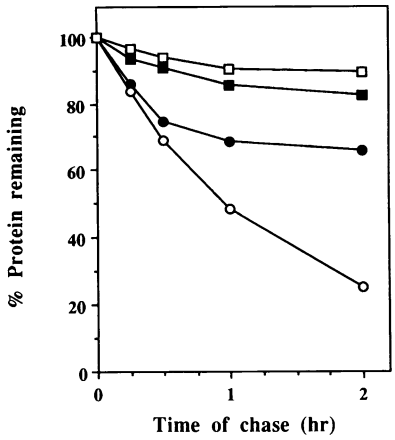

FIG. 9. Recessive effect of the *UbK48R* mutation on the degradation of short-lived and canavanyl proteins. Cultures growing exponentially in synthetic galactose medium were shifted to synthetic glucose medium for 6 h and then exposed to canavanine and labeled with [^{35}S]methionine. Degradation of total cellular protein was measured during the chase incubation. Open circles, wild-type Ub (SUB313) plus canavanine. Open squares, UbK48R (SUB315) plus canavanine. Solid circles, wild-type Ub with no canavanine. Solid squares, UbK48R with no canavanine. Radioactivity in proteins recovered during the chase is expressed as a percentage of that found at the end of the labeling period.

displayed extended spindles, as evidenced by tubulin staining (Fig. 7). The cell cycle arrest observed after 6 h in glucose medium was fully reversible and was not accompanied by a loss of viability. These results suggested that an inability to form Lys-48 multiubiquitin chains results in arrest in late G_2 or M phase of the cell cycle.

After 6 h in glucose medium, approximately 80% of *UbK48R* mutant cells had a 2n DNA content (Fig. 8C). This confirms that *UbK48R* cells arrest predominantly in the G_2 or M phase, although a significant fraction appears to arrest in G_1 as well. Isogenic wild-type control cultures exhibited equivalent numbers of cells with 1n and 2n DNA contents (Fig. 8A). In a third culture, neither ubiquitin nor UbK48R was expressed from the *CUP1* promoter. The shift to glucose induces a simple depletion of free ubiquitin in these cells. This resulted in a less pronounced G_2/M arrest than that seen in *UbK48R* mutants (Fig. 8B).

Effect of the *UbK48R* mutation on degradation of canavanyl proteins. The Δ*ubi1-4* strains provide an experimental system in which the effect of the *UbK48R* mutation on protein turnover in vivo can be tested. One class of substrates shown to be degraded through the ubiquitin pathway in both yeast and mammalian cells is misfolded proteins (10, 51). Such proteins can be produced through translational errors, nonsense mutations, or translational incorporation into protein of amino acid derivatives such as the arginine analog canavanine. In the presence of glucose, the *UbK48R* mutant degrades canavanyl proteins very poorly (Fig. 9). When canavanine is omitted, a smaller fraction of pulse-labeled protein is degraded, and this degradation is less affected in the *UbK48R* mutant. The residual degradation observed in the *UbK48R* mutant may reflect either the continued presence of some wild-type ubiquitin, a Lys-48 independent ubiquitin-dependent pathway, or a ubiquitin-independent pathway of turnover.

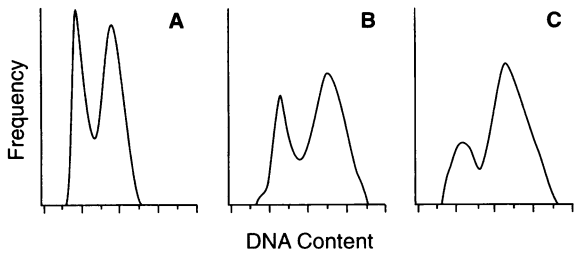

FIG. 8. Arrest of cell cycle progression by the *UbK48R* mutation is associated with a predominant 2n DNA content. Cultures prepared as for Fig. 7 were stained with propidium iodide and analyzed for DNA content with a fluorescence-activated cell sorter analyzer. (A) Wild-type Ub (SUB313). (B) No Ub (SUB314). (C) UbK48R (SUB315).

DISCUSSION

Ubiquitin groups bound to a protein substrate can be distributed in a variety of patterns. For example, more than one lysine residue within the target protein may serve as a ubiquitin acceptor site (28, 31, 32). Such proteins may be subject to multiubiquitination even in the absence of multiubiquitin chain formation. However, it appears that most natural substrates are modified by multiubiquitin chains. One artificial substrate can be targeted for degradation by a single ubiquitin molecule bound to its N terminus (19). In contrast, individual ubiquitin groups fused to the N termini of several other proteins are not sufficient to destabilize them in vivo (5, 36). For example, a fusion of ubiquitin to the N terminus of β-Gal (Ub–Pro–β-Gal) is destabilized only when multiubiquitinated through the Lys-48 residue of the N-terminal ubiquitin (36). Differences between these results may in part reflect differing properties of in vivo and in vitro assay systems.

We have characterized the in vivo effects of preventing Lys-48 multiubiquitin chain formation in *S. cerevisiae*. Previous work has shown that apart from preventing Lys-48 chain

assembly, the *UbK48R* mutation has little effect on the capacity of the protein to serve as a substrate for ubiquitin conjugation. For example, the Michaelis constant (K_m) and turnover number (k_{cat}) values for ATP-PP_i exchange by the ubiquitin-activating enzyme are equivalent for ubiquitin and UbK48R (27). The levels of monoubiquitinated forms of different substrates formed in vitro are also not significantly affected by this mutation (27). Structural studies have shown that the side chain of Lys-48 is hydrogen bonded to the carbonyl group of Ala-46 (55). This interaction may be perturbed by the Arg-48 substitution. However, in the crystal structures of Lys-48-linked diubiquitin and tetraubiquitin (11, 12), this hydrogen bonding interaction has been lost yet the tertiary structure of the molecule is unchanged at Ala-46 and elsewhere, apart from the side chain of Lys-48 itself. The apparent equivalence of ubiquitin and UbK48R in the formation of monoubiquitinated conjugates suggests that the phenotypes associated with UbK48R expression are likely to be causally related to Lys-48 chain assembly. However, it remains possible that the mutation perturbs ubiquitin function in a way that has yet to be detected.

The strains described here may be used to assay the Lys-48 dependence of a variety of proteolytic substrates. The breakdown of canavanyl proteins is strongly inhibited by the *UbK48R* mutation, consistent with a major role of Lys-48 chain formation in protein turnover. The stringency of the requirement for Lys-48 is also reflected by the lethality of the K48R substitution. Lethality may arise from a role of Lys-48 chains in degradative events that are required for cell cycle progression. Two mutations that may affect ubiquitin pathway function have recently been reported to cause arrest of cells with a 2n DNA content. The mutations define the genes *CIM3* and *CIM5*, which appear to encode components of the 26S protease (24). Because these mutations do not result in lobed nuclei, they may arrest cells at a point in the cell cycle earlier than does the *UbK48R* mutant. In *Xenopus laevis* egg extracts, the metaphase-to-anaphase transition can be delayed by adding methylated ubiquitin (34). Multiubiquitination through either Lys-48 or some other residue may therefore play a role in mitosis in *X. laevis* earlier than and additional to that suggested by the postmetaphase arrest of the *UbK48R* mutant.

There is evidence that degradation of B-type cyclins is required for progression through late G_2 or M phase in both yeasts and higher eukaryotes (23, 40). The morphological markers of cell cycle arrest resulting from stabilization of a B-type mitotic cyclin in yeast cells (23) are similar to those seen in *UbK48R* mutants, suggesting that the *UbK48R* mutation may result in an inability to degrade mitotic cyclins. However, it is unlikely that this is the only lethal effect of *UbK48R*, because expression of this mutation also appears to result in a minor population of G_1-arrested cells.

The phenotypic effects of the *UbK48R* mutation are critically dependent on the manner in which they are assayed. Thus, expression of UbK48R complements the canavanine sensitivity of *ubi4* mutants (Table 1) but does not support degradation of canavanyl proteins (Fig. 9). Conversely, the *UbK63R* mutation confers canavanine sensitivity but has no detectable effect on degradation of canavanyl proteins (52). A related problem is presented by the ability of UbK48R to correct the stress sensitivities of *ubi4* mutants even though it has a dominant negative effect on protein turnover and cannot rescue the *ubi1 ubi2 ubi3 ubi4* quadruple mutant. The ability to complement *ubi4* is a specific property of UbK48R, because three other ubiquitin mutants failed to do so.

These paradoxical findings may be explained largely on the basis of effects on free ubiquitin pools. For example, canavanine sensitivity in these strains may result from chronic depletion of free ubiquitin by canavanine rather than an acute inability of the mutant to degrade canavanyl proteins. During a stress such as heat shock at 39°C, free ubiquitin levels decline precipitously if ubiquitin cannot be supplied by the polyubiquitin gene *UBI4* (22). It is likely that under these conditions, free ubiquitin is lost by sequestration into an expanded pool of stable or unstable conjugates (22, 43, 47). Expression of an excess of UbK48R should prevent incorporation of the bulk of wild-type ubiquitin from this pool if two reasonable conditions are met: the pool should be titratable, and the proportional representation of UbK48R within the pool should be comparable to that of wild-type ubiquitin. Also, by capping nascent chains, UbK48R could reduce the total amount of ubiquitin derivatives in the conjugate pool. While this model may explain the ability of UbK48R to complement *ubi4*, we cannot exclude the possibility that under conditions of stress, Lys-48 chain formation is dispensable. To distinguish between these models is not straightforward because of technical difficulties in measuring low levels of wild-type ubiquitin in the presence of high levels of UbK48R.

The model described above proposes that the availability of limiting amounts of wild-type ubiquitin for conjugation may be enhanced when excess amounts of UbK48R are present. Remarkably, this postulated effect of UbK48R may be stronger than its inhibitory effect on chain formation. This is suggested by the potency of UbK48R in complementing the *ubi4* mutation (Fig. 5). High ratios of mutant to wild-type ubiquitin do not result in dramatic inhibition of Lys-48 chain formation (Fig. 1), because of the rapid dynamics of multiubiquitin chains, as revealed by the time-dependent lengthening of multiubiquitin chains in the presence of UbK48R,G76A.

The stable capping of chain growth by UbK48R,G76A indicates that ubiquitin-ubiquitin linkage sites other than Lys-48, such as Lys-63 (52), cannot be efficiently utilized with this substrate, even when competitive effects of the Lys-48 linkage site are eliminated. Thus, the utilization of Lys residues in ubiquitin for chain formation can be a highly specific process. Synthesis of the Lys-48 chains on Lys–β-Gal is catalyzed by Rad6 (Ubc2) in cooperation with the Ubr1 protein (3, 7, 13, 53). In the absence of Ubr1, purified Rad6 (Ubc2) enzyme forms Lys-6-linked multiubiquitin chains on histone H2B (1). Thus, the specificity of linkage site selection is a function not only of the Ubc enzyme that catalyzes the reaction but of other factors. In this case, Ubr1 may direct the linkage specificity, possibly through specific interactions with ubiquitin (46).

The results of this study, and the recent identification of Lys-63 as a multiubiquitination site involved in DNA repair (52), indicate that at least two nonredundant pathways for multiubiquitin chain formation exist, both of which are of functional importance in yeast cells. The degree to which the arrangement of multiple ubiquitin groups into chains may increase the efficiency of substrate degradation remains unclear, as does the significance for degradation of alternative chain linkage sites. Ubiquitin-dependent degradation can be achieved in vitro in the absence of multiubiquitin chain formation, although at reduced rates (19, 28). However, the model substrates used in these studies may be largely unfolded and therefore unusually susceptible to proteolytic cleavage. Consequently, the discriminatory capacity of the system with regard to multiubiquitin chains may be relaxed. Degradation of a protein bearing one ubiquitin group appeared to be deficient in processivity (19). Thus, multiubiquitin chains may serve not only to enhance the rate of substrate breakdown but also to minimize the generation of incompletely degraded proteins, perhaps by increasing the stability of the interaction between the substrate and protease.

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