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Stress Resistance in *Saccharomyces cerevisiae* Is Strongly Correlated with Assembly of a Novel Type of Multiubiquitin Chain

TERRA ARNASON AND MICHAEL J. ELLISON*

Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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The covalent attachment of ubiquitin (Ub) to short-lived or damaged proteins is believed to be the signal that initiates their selective degradation. In several cases, it has been shown that the proteolytic signal takes the form of a multi-Ub chain in which successive Ub molecules are linked tandemly at lysine 48 (K-48). Here we show that Ub molecules can be linked together in vivo at two other lysine positions, lysine 29 (K-29) and lysine 63 (K-63). The formation of these alternative linkages is strongly dependent on the presence of the stress-related Ub conjugating enzymes UBC4 and UBC5. Furthermore, expression of Ub carrying a K-63 to arginine 63 substitution in a strain of *Saccharomyces cerevisiae* that is missing the poly-Ub gene, *UBI4*, fails to compensate for the stress defects associated with these cells. Taken together, these results suggest that the formation of multi-Ub chains involving K-63 linkages plays an important role in the yeast stress response. In broader terms, these results also suggest that Ub is a versatile signal in which different Ub chain configurations are used for different functions.

In 1980, Hershko et al. (15) proposed that the selective conjugation of ubiquitin (Ub) to short-lived proteins marked these substrates for degradation. From a mechanistic standpoint, this proposal has given rise to two distinct variations: either Ub itself is recognized directly by some component of the proteolytic apparatus, or Ub acts indirectly by changing the structure of the targeted proteins to a proteolytically labile form (12).

In recent years, both of these ideas have been reiterated in modified forms (6, 10, 23) that account for findings that strongly correlate the turnover of damaged and short-lived proteins with the assembly of a multi-Ub chain onto the targeted protein (reviewed in references 7, 14, 20, and 29). Within this chain, Ub molecules are linked to each other by isopeptide bonds that connect lysine 48 (K-48) of one Ub molecule to the carboxy terminus of another (2). To date, this particular chain configuration has been found to be associated with the turnover of several ubiquitinated proteins, including the yeast transcriptional repressor MATα2 (18), cyclins (9, 16), and various substrates associated with the N-end rule pathway (1, 2, 10).

Evidence which supports the hypothesis that Ub functions as a direct signal in proteolysis comes from the effect of epitope-tagged Ub expression on protein turnover in *Saccharomyces cerevisiae* (6). In that study it was found that tagged Ub could be targeted correctly to a short-lived β-galactosidase fusion protein but that conjugation resulted in strong inhibition of its turnover. From this observation, it was suggested that the amino-terminal tag interfered with an important determinant within Ub that was involved in protease-conjugate recognition. It was also suggested that chain assembly served to amplify this determinant, thereby increasing the efficiency of the protease-conjugate interaction. Despite these findings, the function of the multi-Ub chain remains an open question.

In this work, we show that multi-Ub chain configurations that use lysine linkages other than K-48 can be formed in vivo. One of these novel configurations appears to play an important role in the response of *S. cerevisiae* to environmental stress.

* Corresponding author. Mailing address: Department of Biochemistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Phone: (403) 492-5839. Fax: (403) 492-0886.

MATERIALS AND METHODS

Plasmids and yeast strains. Each of the Ub derivatives described in Fig. 1 to 3 was expressed from a high-copy-number yeast plasmid that is identical to the *TRP1* copper-inducible Ub expression plasmid YEp96 (described in reference 6), with the exception of the specific changes noted below. (i) All K-to-R substitutions listed for the various Ub derivatives shown in Fig. 1 to 3 were made by replacing K codons with the R codon AGA. (ii) The DNA sequence of the N-terminally Myc-tagged Ub (mUb) is identical to that described for YEp104 (6). (iii) The DNA sequence of the mUb.K_0 gene is identical to mUb except for the seven K-to-R codon changes shown in Fig. 1. (iv) In genes encoding C-terminally Myc-tagged Ub (Ubm), the G-76 codon of Ub has been replaced with a DNA segment encoding the peptide sequence APCEQKLISEEDL (containing the Myc epitope). The AP portion of this peptide (occupying amino acid positions 76 and 77) was included to prevent cleavage by the Ub processing enzymes (21). (v) In UbΔ genes, the C-terminal codons G-75 and G-76 have been deleted. (vi) The donor Ub derivatives used for Fig. 1 and 2 are contained on a common high-copy-number *URA3* vector that was created by positioning the *Bam*HI-*Pst*I expression cassette from YEp96 (Ub), YEp104 (mUb) (6), or the mUb.K_0 equivalent of YEp104 between the *Bam*HI and *Pst*I sites of the high-copy-number *URA3* plasmid YEp352 (17). The accompanying *TRP1* sequence of the cassette was subsequently removed from the resulting vector as a *Cla*I-*Nar*I fragment and the plasmid was religated. The *URA3*-UBC4 expression plasmid used for Fig. 2 is identical to the YEp352 Ub plasmids described above except that the UBC4 coding sequence has replaced the Ub coding sequence. The DNA sequences of all plasmid-borne Ub genes were verified by DNA sequencing with an automated DNA sequencer (Applied Biosystems) operated by the Department

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
SUB60	*MATa his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ubi4-Δ2::LEU2*	8
MHY501	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1*	3
MHY508	*MATα his3-Δ200 leu2-3,112 ura3-52 lys2-801 trp1-1 ubc4-Δ1::HIS3 ubc5-Δ1::LEU2*	3

of Biochemistry DNA Sequencing and Synthesis Facility at the University of Alberta. Details relating to the construction of any plasmid described here are available on request.

For experiments described in the legends to Fig. 1, 3, and 4, plasmids were introduced into the yeast strain SUB60, which carries a deletion for the polyubiquitin gene *UBI4* (8). For experiments described in the legend to Fig. 2, plasmids were introduced into the *UBC4 UBC5* wild-type strain MHY501 (3) or the *ubc4 ubc5*Δ deletion strain MHY508 (3). The genotypes of SUB60, MHY501, and MHY508 are described in Table 1. In MHY508, free Ub levels are elevated by the constitutive expression of *UBI4* (24). In order to compensate for this elevation, wild-type Ub was coexpressed in MHY501 from the YEp352 Ub plasmid described above. The level of Ub produced from this plasmid typically exceeded the Ub levels in the *ubc4 ubc5* mutant by an order of two- to threefold (unpublished data). The various combinations of coexpressed plasmids in Fig. 1 and 2 are summarized in Table 2.

Western (immunoblot) analysis of Myc-tagged Ub. Yeast strain growth conditions, extract preparation, electrophoresis of total yeast protein, and immunoblot analysis have been previously described (19), with the following exceptions. Cells were grown to early exponential phase at 30°C in synthetic defined (SD) media (25) supplemented with selected amino acids critical for growth. Cultures were diluted to 3.0×10^6 cells per ml in the presence of $CuSO_4$ (100 μM) to induce protein synthesis and were allowed to grow for five generations prior to harvesting. Pelleted cells were resuspended directly into electrophoresis load mix. Sample supernatants were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel (18% acrylamide, 0.09% bisacrylamide). After transfer, and following anti-Myc antibody treatment, filters were washed in 1× Tris buffer saline plus Tween (TBS+Tween; 10 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.05% Tween 20) three times and then incubated in 50 ml of TBS+Tween containing 17 μl of goat anti-mouse immunoglobulin G horseradish peroxidase conjugate (Bio-Rad) for 45 min at 4°C. Filters were then washed as described above, and bands were visualized with X-ray film, using the enhanced chemiluminescence system (ECL; Amersham). The quantitation of Myc-tagged Ub and its conjugates was performed by selecting film exposures that fell within the linear range of film density, followed by the densitometric scanning of appropriate bands, using a Joyce-Loebl Chromoscan 3 scanning densitometer.

Phenotype analysis. Survival of cells after treatment with canavanine, chronic heat, and UV radiation was determined as previously described (19). Cell survival in the presence of canavanine was determined by plating exponentially growing yeast cells (previously induced for 2 generations with $CuSO_4$ [100 μM]) on supplemented minimal medium plates containing 2 μg of canavanine per ml and $CuSO_4$ (100 μM). Colonies were counted after 6 days of incubation at 30°C. Chronic heat stress survival was determined as for canavanine survival, except that canavanine was omitted from the medium and plates were incubated at 38.5°C for 24 h, followed by a shift to 22°C for 4 days to allow for colony development. UV survival was determined as for canavanine survival, except that canavanine was omitted from plates, and cells were irradiated with 200 J of UV (254 nm) per m^2 immediately following plating as previously described (5). In the same experiment, survival of a *rad6* (*ubc2*) mutant was less than 0.01%.

Bulk protein turnover. Protein turnover measurements were determined essentially as described by Hodgins et al. (19) by the method of Seufert and Jentsch (24). Yeast cells (SUB60) carrying either the Ub or Ub.RRR expression plasmids were grown to a cell density of 2×10^6 cells per ml at 30°C in liquid SD medium (25) supplemented with histidine (10 mg/liter), lysine (40 mg/liter), and uracil (2.4 mg/liter). Ub and Ub.RRR gene expression was induced for one doubling period by the addition of $CuSO_4$ to a final concentration of 100 μM. After this period, cultures were divided into two, and to one half was added canavanine (20 μg/ml). Following a second doubling period, cells were labeled with [^{35}S]methionine (75 μCi/ml of culture medium) for 5 min, chased with cold methionine (10 mM final concentration), and washed several times to remove unincorporated label. Protein turnover was determined by measuring the release by cells of radioactivity into the medium as a function of time, as previously described (19).

RESULTS

K-29, K-48, and K-63 are targeted for ubiquitination. Although the existence of multi-Ub chains in which Ub molecules were linked through residues other than K-48 have been

TABLE 2. Ubiquitin plasmid coexpression combinations[a]

Figure	Strain	Acceptor		Donor	
		Construct	Marker	Construct	Marker
1A	SUB60	Ubm	*TRP1*	wt Ub	*URA3*
1B	SUB60	UbΔ	*TRP1*	mUb	*URA3*
1C	SUB60	wt Ub	*TRP1*	mUb.K_o	*URA3*
2	MHY501	Ubm	*TRP1*	wt Ub	*URA3*
	MHY508	Ubm	*TRP1*		
	MHY501 + pUBC4	Ubm	*TRP1*		

[a] Summarized here are the characteristics of plasmids used in the coexpression experiments whose results are shown in Fig. 1 and 2. "Acceptor" refers to the Ub derivative that serves as the substrate in the conjugation reaction, whereas "donor" refers to the Ub derivative that is conjugated to the substrate. wt, wild type.

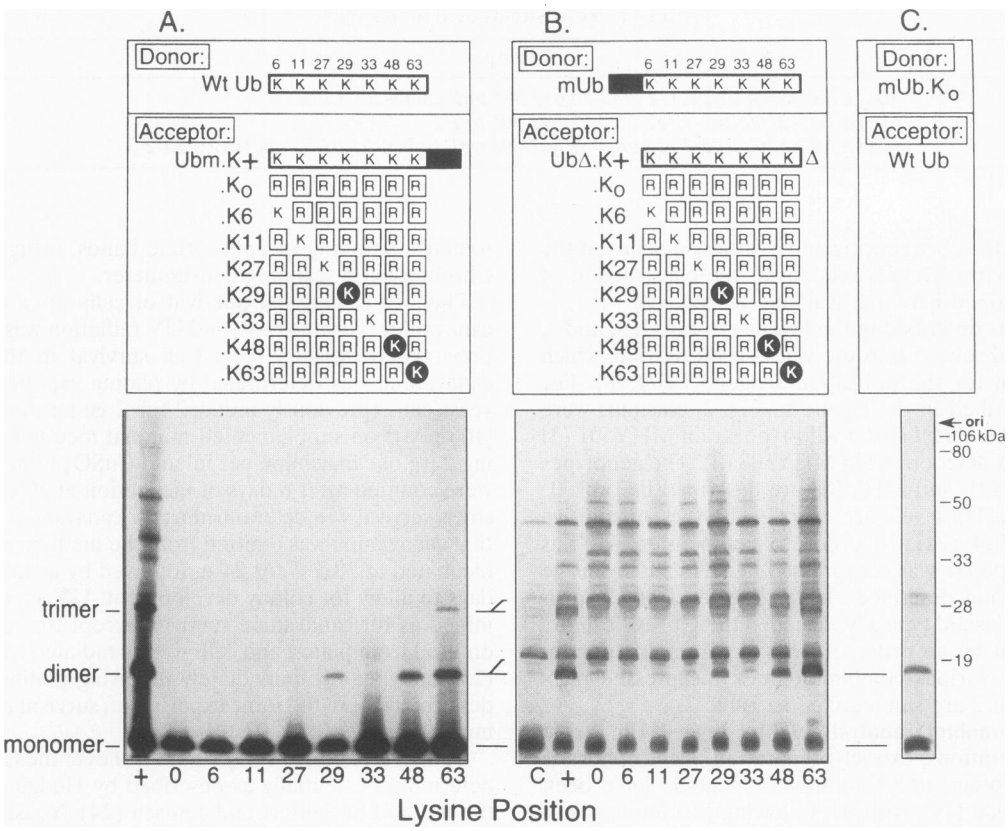

FIG. 1. K-29, K-48, and K-63 are targeted for ubiquitination. Shown is an SDS Western blot of total protein from yeast cells coexpressing tagged Ub derivatives in combination with untagged derivatives and probed with the anti-Myc tag antibody. Schematics indicate the pairs of Ub derivatives expressed in each experiment (A to C) (also see Table 2). Donor, the Ub moiety that contributes its C terminus to conjugate formation; acceptor, the targeted derivative. Numbers mark the position of lysines (K) present in the wild-type sequence (Wt Ub). Lysine-to-arginine (R) replacements for each derivative are boxed. Lysines empirically determined to be sites of ubiquitination are circled. The Myc epitope tag is shown as a black box. For gels, positions of dimeric and trimeric Ub conjugates containing tagged Ub are indicated by lines. Gel lane numbers indicate the lysine positions of each acceptor. (A) Wild-type Ub coexpressed with C-terminally Myc-tagged Ub (Ubm). Lanes: +, Ubm.K+ (all lysines present); O, Ubm.K_o (all lysines absent). (B) N-terminally Myc-tagged Ub (mUb) coexpressed with Ub with G-75 and G-76 deleted (UbΔ). Control lane (lane C) is mUb expressed without its UbΔ counterpart. (C) Wt Ub coexpressed with N-terminally Myc-tagged Ub in which all lysines have been substituted (mUb.K_o).

reported (13, 18, 21), in no instance was the nature of the linkage or its biological function determined. To establish which of the seven lysines contained within Ub could serve as targets for Ub chain assembly, we created a set of yeast plasmids, each expressing a Ub derivative in which all but one lysine were replaced with arginines. Each Ub derivative varied from the others in the position of its single remaining lysine. Detection of these derivatives and their conjugates was facilitated by fusing a noncleavable Myc epitope sequence (22) to the carboxy-terminal codon of each construct (Ubm). Similar Ub-peptide fusions fail to function as a usable source of cellular Ub owing to their blocked C termini. They do, however, function as substrates for Ub conjugation (21).

Three of these Ub derivatives were ubiquitinated when expressed in *S. cerevisiae*: Ubm.K29, Ubm.K48, and Ubm.K63 (Fig. 1A). In contrast, derivatives with either no lysines or single lysines situated at positions other than 29, 48, or 63 failed to be ubiquitinated. The simplest conclusion that can be drawn from these results is that Ubm.K29, Ubm.K48, and Ubm.K63 are each ubiquitinated at K-29, K-48, and K-63, respectively. It was also possible, although unlikely, that ubiquitination was occurring at the α-amino group of the amino-terminal methionine in an unobvious manner that required the presence of either K-29, K-48, or K-63, but not all three. The Ub-Ubm linear fusion resulting from N-terminal targeting would position a methionine at the junction between the Ub and Ubm moieties. This possibility was ruled out on the basis of the inability to cleave Ub-Ubm conjugates into their monomeric components, using the methionine-specific reagent cyanogen bromide (data not shown). It was also possible, although unlikely, that ubiquitination was occurring at residues with nucleophilic side chains other than lysines in a manner that required the presence of K-29, K-48, or K-63, but not all three. In the case of Ub, such residues include serine, threonine, and tyrosine and would result in the formation of a labile ester linkage (28). The possibility of ester formation was eliminated on the basis of the complete resistance of these conjugates to treatment at 100°C in the presence of 0.2 N NaOH (results not shown). Taken together, these results provide convincing evidence that K-29, K-48, and K-63 were the sole residues targeted for ubiquitination.

The predominant Ub conjugate in each case was the Ub-Ubm dimer accompanied by lower levels of the trimeric species Ub_2-Ubm. Whereas the position that links the first Ub in the chain to Ubm is defined by the single available lysine in Ubm, the lysine position on the Ub moiety of Ub-Ubm that links subsequent Ub monomers cannot be determined from this experiment.

In view of the generally accepted role of the Ub system in the turnover of damaged proteins, it was possible that the ubiquitination of Ubm.K29, Ubm.K48, and Ubm.K63 resulted from the recognition of structural damage that might be induced by the various modifications made to the Ub polypeptide sequence. Two lines of evidence argue against this possibility. First, selective ubiquitination at positions 29, 48, and 63 occurred regardless of the presence (Fig. 1A) or absence (Fig. 1B) of the carboxy-terminal tag on the accepting Ub derivative. In the case of Fig. 1B, a derivative was used as the acceptor (UbΔ) that lacked both the C-terminal Myc tag and the two C-terminal glycines of Ub; therefore, while this derivative can function as a substrate for ubiquitination, it cannot act as a cellular source of Ub. Since in this case the acceptor is not tagged, detection of its conjugates by Western analysis was facilitated by the coexpression of N-terminally tagged mUb as the donor. The ability of mUb to form conjugates with a range of protein substrates in addition to the UbΔ acceptor resulted in a more complicated banding pattern than observed for the Ubm conjugates shown in Fig. 1A. Among the prominent conjugates detected in addition to those containing UbΔ is the mUb-mUb dimer which, as a consequence of the additional tag, migrates at a slightly greater molecular weight than the mUb-UbΔ dimer. Significantly, the expression of UbΔ in combination with mUb results overall in higher levels of conjugate formation relative to that of cells expressing mUb alone. Similarly, the levels of mUb-UbΔ dimers formed by ubiquitination of K-29, K-48, and K-63 are considerably greater than those of their Ubm counterparts shown in Fig. 1A. The significance of these observations is discussed below.

In another experiment (Fig. 1C), we assessed the effect of complete lysine replacement on the integrity of the various derivatives used for Fig. 1 by determining whether or not mUb carrying mutations at all seven lysine positions (mUb.K_o) could be activated and conjugated to Ub. As expected, the banding pattern resulting from the coexpression of mUb.K_o and Ub is different compared with that of cells expressing only mUb. However, the observation that mUb.K_o-Ub dimer levels are similar to mUb-mUb dimer levels relative to their respective monomeric forms argues that there is no obvious conjugation defect associated with complete lysine replacement. In other experiments we observed that Ub.K_o exhibited the same resistance to trypsin cleavage as Ub and that Ub.K_o conjugated to a test substrate in vitro with an efficiency that was indistinguishable from that of Ub (unpublished data). Taken together with the in vivo data discussed above, these results indicated that ubiquitation at K-29 and K-63 did not result from the recognition of these substrates as damaged proteins.

On the basis of the above observation, we concluded that the targeting of K-29, K-48, and K-63 resulted from recognition of a native Ub structure. This conclusion was reinforced upon examination of the conjugation properties of Ubm derivatives containing only two lysine-to-arginine replacements at any two of the three positions 29, 48, and 63 (Fig. 2). As expected, a derivative carrying substitutions at all three positions failed to serve as a substrate for conjugation, whereas conjugation was restored upon reintroduction of a single lysine at any of the three positions. Thus, the results from this limited substitution experiment paralleled the results of Fig. 1.

Ubiquitination of K-29 and K-63 depends on UBC4. The dramatic increase in overall conjugate formation that results when UbΔ is expressed in combination with mUb relative to that in cells expressing mUb alone (Fig. 1B) suggested to us that UbΔ expression was somehow increasing the levels of one or more Ub conjugating activities. The particularly dramatic increase in the amount of the mUb-UbΔ conjugate linked at K-63 suggested that this same activity is capable of catalyzing a K-63 linkage. In unrelated work, we have found that the expression of UbΔ in a yeast strain that was wild type for the stress-inducible poly-Ub gene, *UBI4*, actually induced *UBI4* transcription substantially, suggesting that these cells were under stress (unpublished data). Since the stress-inducible Ub conjugating enzymes UBC4 and UBC5 are induced under conditions similar to those for UBI4, we reasoned that induced levels of UBC4 and UBC5 synthesis may be responsible for the increased ubiquitination observed in Fig. 1B and for the catalysis of K-63 Ub-Ub linkages.

We tested this idea by examining the types of Ub-Ubm conjugates formed in cells either wild type for *UBC4 UBC5* expression or carrying deletions for both alleles (*ubc4 ubc5*Δ) (Fig. 2). The Ubm derivatives used in this experiment carried only two lysine-to-arginine mutations situated at any two of the targeted positions, leaving the third available for conjugation. Significantly, both K-29 and K-63 conjugates were dramatically reduced in cells with the stress Ub conjugate enzyme genes *UBC4* and *UBC5* deleted. Quantitation of the dimer percentage (relative to monomer) revealed an approximate 16-fold drop in the percentage of the K-63 conjugate observed in mutant cells (1.4%) versus wild-type cells (22%). By comparison, the percentage of the K-48 conjugate was virtually indistinguishable between mutant (14%) and wild-type (15%) cells. Furthermore, reintroduction of a *UBC4* expression plasmid into the *ubc4 ubc5*Δ strain restored conjugation at positions 29 and 63, indicating that conjugation at these sites is indeed dependent on *UBC4* and not the result of a cryptic mutation acquired by the strain. While this experiment does not directly address the role of *UBC5* in K-29 and K-63 targeting, the degree of similarity exhibited between *UBC4* and *UBC5* (95% identity) coupled with the fact that they are interchangeable for function when present at similar concentrations (data not shown) makes it a more than reasonable proposition.

A K-63 mutation effects stress resistance. The requirement of UBC4 and UBC5 (UBC4/5) for conjugating Ub to K-29 and K-63, coupled with the role of these enzymes in stress resistance, raised the question of whether these linkages are necessary for stress resistance. We tested this idea by making use of the fact that, like *ubc4 ubc5*Δ mutants, deletion of the stress inducible poly-Ub gene, *UBI4*, results in extreme sensitivity to chronic heat or the amino acid analog canavanine (8). This phenotype was postulated to arise from the inability of *ubi4*Δ mutant cells to satisfy the increased demand for Ub under adverse conditions, since the overexpression of Ub from a plasmid restores the wild-type phenotype. If a given Ub-Ub linkage were important to this process, then the expression of a Ub derivative that could not make this linkage should not be able to suppress the *ubi4*Δ phenotype. Within this context, the expression of untagged Ub with replacements at either position 29 (Ub.R29) or 48 (Ub.R48) or both was almost as effective as wild-type Ub in restoring viability to *ubi4*Δ cells exposed to canavanine or chronic heat (Fig. 3). Ub.R63, however, failed to restore viability with respect to either type of stress. Notably, *ubi4*Δ cells expressing Ub.R63 displayed none of the obvious phenotypes associated with other Ub-related disorders, including DNA damage sensitivity (Fig. 3) or slow growth (results not shown). These results demonstrate that of the three targeted positions, only K-63 plays a critical role in restoring stress resistance to a *ubi4*Δ mutant.

Effect of the K-63 mutation on protein turnover. In addition to defects in stress resistance, *ubc4 ubc5*Δ mutant cells are defective in their ability to degrade canavanyl proteins (24). In view of the involvement of UBC4 in the formation of K-63

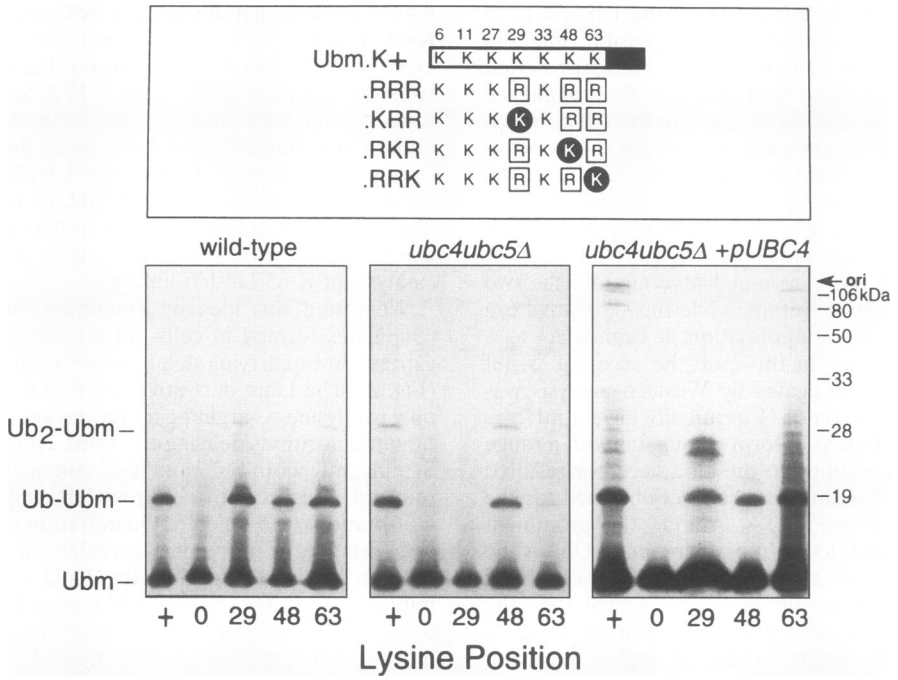

FIG. 2. K-29 and K-63 ubiquitination is dependent on *UBC4* expression. The experimental design is similar to that described for Fig. 1A, except that Ubm derivatives contain lysine-to-arginine substitutions only at the three targeted positions, 29, 48, and 63 (Ubm.RRR), or at any two of the three positions (Ubm.KRR, Ubm.RKR, and Ubm.RRK). Ubm derivatives are expressed in either wild-type cells or in the *ubc4 ubc5*Δ double mutant with, or without, the *UBC4* expression plasmid p*UBC4*. Wild-type Ub is coexpressed with the various Ubm derivatives in wild-type cells in order to compensate for the elevated level of Ub observed in the *ubc4 ubc5*Δ double mutant (see Materials and Methods and Table 2). "Ub-Ubm" and "Ub_2-Ubm" mark the positions of the tagged dimeric and trimeric conjugates, respectively. Lanes: +, 0, 29, 48, and 63, lysines available for ubiquitination for Ubm derivatives K+, RRR, KRR, RKR, and RRK, respectively.

Ub-Ub linkages and the apparent importance of K-63 in conferring stress resistance to cells carrying the stress-sensitive *ubi4*Δ defect, it was of interest to determine if a *ubi4*Δ mutant expressing Ub.R63 also displayed a general defect in proteolysis. The results of such an experiment are shown in Fig. 4. In this experiment, *ubi4*Δ mutants overexpressing either wild-type Ub or the linkage-defective Ub derivative, Ub.RRR, were compared for their abilities to degrade bulk protein in the presence or absence of canavanine. As seen in Fig. 4, the rate of bulk protein turnover is relatively unaffected by either the presence of canavanine or the expression of a Ub derivative that is defective for linkage formation at K-29, K-48, and K-63. It would appear, then, that the stress sensitivity associated with either the *ubi4*Δ mutation or the Ub.R63 mutation is unrelated to the protein turnover defect associated with a *ubc4 ubc5*Δ mutation.

DISCUSSION

The results of the preceding section establish that (i) in addition to K-48, K-29 and K-63 also serve as sites that link Ub

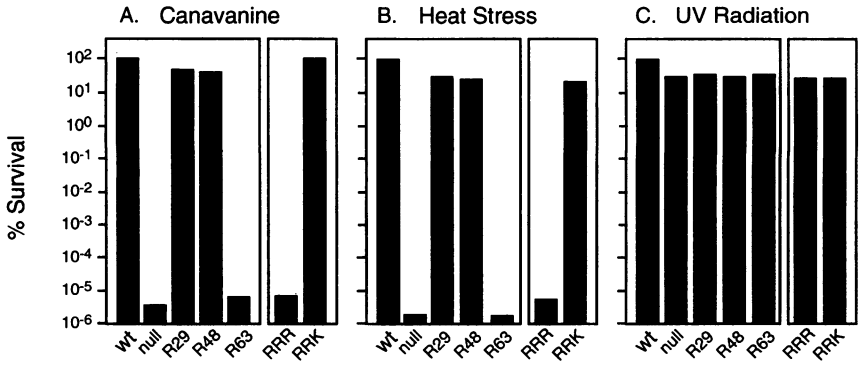

FIG. 3. K-63 is required for Ub complementation of the stress defects associated with a poly-Ub gene mutant. Survival of yeast cells expressing different untagged Ub derivatives is represented as a percentage of survivors relative to that of cells expressing wild-type Ub (wt) as determined in plating assays under the conditions indicated and described in Materials and Methods. All derivatives are expressed from plasmids in the *ubi4*Δ strain, SUB60. R29, R48, and R63 are Ub derivatives with R replacements at positions 29, 48, and 63, and RRK has replacements at positions 29 and 48. "Null" refers to cells containing a non-Ub plasmid, serving as a negative control.

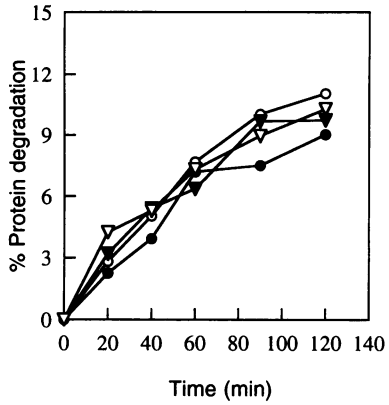

FIG. 4. Bulk protein turnover in a yeast strain expressing a Ub derivative with a defect in multi-Ub chain assembly. Protein turnover in yeast cells expressing wild-type Ub (circles) or Ub.RRR (triangles) was measured in the presence (open symbols) or absence (closed symbols) of canavanine as described in Materials and Methods. Protein degradation is expressed as a percentage of the total incorporated radioactivity released from cells as a function of time.

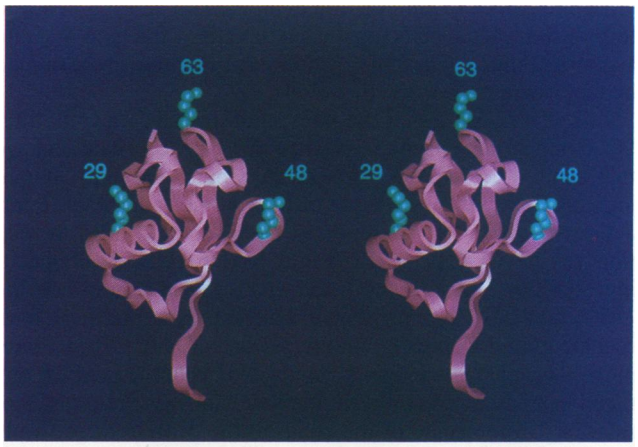

FIG. 5. Stereo three-dimensional structure of ubiquitin showing the positions of K-29, K-48, and K-63.

to Ub; (ii) the ubiquitination of K-29 and K-63 is dependent on UBC4 and probably UBC5, two enzymes that are essential for stress survival; and (iii) replacement of K-63 with a residue that cannot be ubiquitinated has a profound effect on stress survival. The simplest and most logical interpretation of these results is that ubiquitination of K-63 is essential for stress survival, whereas the ubiquitination of K-29 plays a nonessential role in an unknown function.

Another interpretation of these results, however, is that the biological effects arising from K-63 replacement result not from a failure to ubiquitinate this position but from a subtle perturbation of Ub structure that reduces its activity with respect to particular functions. It is fair to say that this argument extends beyond a discussion of K-63 and includes those studies that address the function of K-48 multi-Ub chains by using mutated or chemically modified Ub. In light of this argument, evidence supporting the role of any chain configuration in one function or another remains necessarily correlative.

Nonetheless, there are several arguments with respect to the present work that run counter to the structural perturbation viewpoint. First, the crystal structures of Ub (30) and the recently determined Ub_2 dimer (4) show that the K-63 side chain makes no contact with the rest of the Ub molecule (Fig. 5); therefore, a simple K-to-R replacement should have no effect on overall structure. This conclusion is consistent with the observation that Ub.R63 is assembled into K-48 multi-Ub chains in vitro at a rate that is indistinguishable from that of wild-type Ub (results not shown).

Although K-63 replacement has no apparent effect on Ub structure, it is possible that K-63 makes a critical contact with another protein that is affected upon its replacement. In this light, the R-63 mutation could manifest itself in either of two ways. In the first instance, expression of Ub.R63 could produce a dominant-negative effect by titrating a protein that is necessary for stress resistance into an unproductive complex. Such a phenotype would be expected to be more severe than even the null situation. The fact that the null mutant shows similar levels of stress sensitivity relative to Ub.R63 expression argues against this possibility. In the second instance, K-63 replacement may weaken an interaction with a protein that plays a critical role in stress resistance. Furthermore, such an interaction defect might be expected to show temperature sensitivity. While the loss of a key interaction might explain the effect of K-63 substitution under conditions of chronic heat stress, it has difficulty accounting for the extreme canavanine sensitivity exhibited by the mutant at temperatures as low as 22°C. In addition, a K-to-R replacement at position 63 extracts a rather small thermodynamic price, and any postulated loss of affinity in a hypothesized interaction should at least be partially overcome by an increase in the intracellular concentration of Ub.R63. Yet overexpression of Ub.R63 has absolutely no positive effect on stress survival relative to the null situation. In conclusion, while we cannot entirely rule out the possibility that the effect of K-63 substitution is unrelated to its ubiquitination, we feel that this explanation is unlikely.

A commonly held opinion is that resistance to stress requires the turnover of damaged proteins and that the stress sensitivity associated with mutations in genes such as *UBI4* and *UBC4/5* results from a failure to degrade damaged proteins. This viewpoint is reinforced by the observation that the stress-sensitive *ubc4 ubc5*Δ mutant is also defective in the turnover of bulk canavanyl proteins (24). The results from Fig. 4, however, raise the possibility of another interpretation. First, stress-sensitive *ubi4*Δ cells expressing linkage defective Ub (Ub.RRR) and exposed to canavanine show no obvious defect in bulk protein turnover when compared with *ubi4*Δ cells rendered stress resistant by wild-type Ub overexpression. Therefore, unlike the *ubc4 ubc5*Δ mutation, stress sensitivity of the *ubi4*Δ mutation is not correlated with an obvious general defect in proteolysis. Yet despite this apparent contradiction, the stress phenotypes associated with the *ubi4*Δ and *ubc4 ubc5*Δ mutations are nonetheless mechanistically related in so far as K-63 ubiquitination is dependent on the presence of UBC4/5, and K-63 in turn is essential for restoring stress resistance to a *ubi4*Δ mutant by Ub overexpression. On the basis of this relationship it may be that, like *ubi4*Δ, the general protein turnover phenotype associated with the *ubc4 ubc5*Δ mutation is unrelated to its stress phenotype. On the basis of these ideas, we suggest that in addition to its postulated role as a general scavenger of damaged proteins (24), UBC4/5 may function in a more specific manner by targeting a regulator of the stress response for modification.

From a mechanistic standpoint, the ability of UBC4/5 to participate in the formation of different chain linkages is

intriguing. Since the interaction of an E2 with an E3 is believed to govern target specificity, it is possible that the type of chain assembled onto a particular protein is ultimately programmed by its associated E3.

It is also possible that the interaction of UBC4/5 with other Ub conjugating enzymes governs the type of chain linkage that can be formed. Genetic evidence indicating that Ub conjugating enzymes are capable of both homo- and heterointeractions in vivo was first reported by Silver et al. (26) in relation to the CDC34 (UBC3) and RAD6 (UBC2) conjugating enzymes and later by Chen et al. (3) in relation to the UBC6 and UBC7 conjugating enzymes. Furthermore, we have recently found a direct homointeraction between UBC4 monomers in vivo (11). A model for multi-Ub assembly in which one member of an interacting enzyme pair catalyzes the transfer of the first Ub molecule of the chain to the protein target while the other member catalyzes the transfer of the second Ub molecule to the first Ub molecule has been previously proposed (26). On the basis of this model, the type of Ub-Ub linkage that is formed might be determined by the particular E2 partner UBC4 has selected and whether or not UBC4 catalyzes the first or second Ub transfer.

It is presently unclear whether K-63-linked chains function like K-48 chains in marking protein targets for degradation. This issue will remain unclear until natural targets of K-63 chain assembly have been identified. Our present suspicion, however, is that the K-63 chain configuration does not function as a proteolytic signal. This bias is based on our previous finding that steric interference of the amino-terminal region of Ub by the addition of an epitope tag blocks the proteolysis of N-end rule-dependent test proteins (6). In light of this evidence, we proposed that the amino-terminal region of Ub constituted an important determinant in protease-conjugate recognition and that the assembly of a K-48 chain could serve to amplify this determinant. By comparison, K-63 lies close to the amino terminus of Ub (Fig. 5), and therefore the repeated linkage of Ub through K-63 represents no further advantage in signal amplification than the addition of a single Ub molecule to the targeted substrate.

Spence and Finley have independently found evidence for the involvement of K-63 multi-Ub chain formation in DNA repair (27); therefore, it would appear that this signaling mechanism extends beyond stress resistance. Our failure to detect a relationship between DNA repair and K-63 chain formation may simply reflect the possibility that the Ub-dependent component of DNA repair is less perturbed by Ub.R63 overexpression than the Ub-dependent component associated with stress resistance.

K-29, K-48, and K-63 are separated from one another by a considerable distance on the surface of the Ub molecule (Fig. 5); therefore, the structures of Ub chains composed of different linkages will vary from one another markedly. A sense of the structural complexity that becomes possible with these three coupling positions is illustrated by the five classes of chain configurations in Fig. 6. The most complex configurations naturally arise from the combination of different linkages within the same chain or from the possibility that one Ub molecule can accommodate more than one linkage. Experiments are under way to test these two possibilities. The observed and potential variations in chain configurations, combined with the notion that different Ub chains perform different functions, highlight the versatility of Ub conjugation as a posttranslational signal.

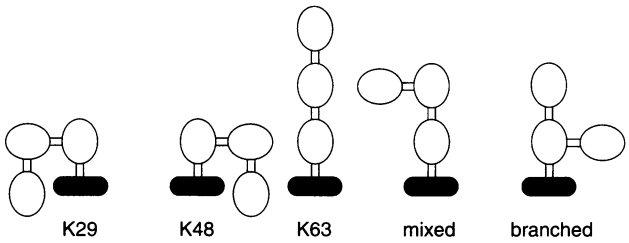

FIG. 6. Possible multi-Ub chain configurations based on the lysine geometry shown in Fig. 5.

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