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The short-lived MATα2 transcriptional regulator is ubiquitinated *in vivo*

(ubiquitin/protein degradation/*Saccharomyces cerevisiae*/α2 repressor/epitope tagging)

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ABSTRACT **The substrates of ubiquitin-dependent proteolytic pathways include both damaged or otherwise abnormal proteins and undamaged proteins that are naturally short-lived. Few specific examples of the latter class have been identified, however. Previous work has shown that the cell type-specific MATα2 repressor of the yeast *Saccharomyces cerevisiae* is an extremely short-lived protein. We now demonstrate that α2 is conjugated to ubiquitin *in vivo*. More than one lysine residue of α2 can be joined to ubiquitin, and some of the ubiquitin moieties form a Lys^{48}-linked multiubiquitin chain. Overexpression of degradation-impaired ubiquitin variants was used to show that at least a significant fraction of α2 degradation is dependent on its ubiquitination.**

The temporal control of many cellular processes involves short-lived regulators. Proteins that are either conditionally or constitutively short-lived *in vivo* include cell cycle regulators such as the cyclins (1) and proteins controlling cell differentiation and embryonic development (2–5).

A distinct class of intracellular proteolytic pathways involves the covalent ligation of a 76-residue protein, ubiquitin (Ub), to the ε-amino groups of acceptor proteins, which are thereby marked for degradation (6). Ub-protein conjugation is catalyzed by a family of Ub-conjugating enzymes, also called E2 enzymes (7). For many short-lived intracellular proteins, their conjugation to Ub is apparently essential for their degradation (8, 9). At the same time, the only specific examples of naturally short-lived proteins whose degradation appears to involve ubiquitination are the phytochrome photoreceptor of plants (10) and cyclins, a family of cell cycle regulators (11).

Our recent studies of short-lived proteins included the cell type-specific transcriptional repressor MATα2 of the yeast *Saccharomyces cerevisiae* (3). This eukaryote has two haploid cell types, **a** and α, which can mate to form an **a**/α diploid (12). Ultimately, cell identity is determined by the mating-type, or *MAT*, locus. In homothallic *S. cerevisiae* strains, unexpressed **a** or α information at two other genomic sites is copied into the *MAT* locus during a mating-type switch. Interconversion between **a** and α cell types can occur in <90 min. Selective protein degradation might underlie the mating-type switch by causing rapid disappearance of the regulatory proteins involved once their synthesis has ceased.

Indeed, the α2 repressor, encoded by the *MATα* locus and involved in the repression of **a** cell-specific genes in α cells and of haploid cell-specific genes in **a**/α diploids, has an *in vivo* half-life of only ≈5 min at 30°C (3). Both the amino-terminal and the carboxyl-terminal domains of α2 contain regions that can act as autonomous degradation signals; these signals operate via genetically distinguishable pathways (3). We now demonstrate, using epitope-tagged forms of ubiquitin, that α2 is ubiquitinated *in vivo*. We also show that ubiquitination of α2 contributes to its rapid degradation.

MATERIALS AND METHODS

Yeast Strains and Media. The *S. cerevisiae* strains used were DBY1705 (*MATα leu2-3,112 ura3-52 lys2-801 gal2*), DBY1829 (*MATα leu2-3,112 ura3-52 lys2-801 his3-Δ200 trp1-1*), HR125-5Dalf [Δ(*MATα*)::*CAN1-14 leu2-3,112 ura3-52 trp1 his3 his4*] (3), and YPH500 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) (13). Yeast media were prepared as described (3). For induction of *CUP1* promoter-dependent Ub alleles, $CuSO_4$ was added (0.1 mM) 4–7 hr before radiolabeling.

Plasmids. The plasmid used for constructing site-specific Ub mutants was YEp96, a 2-μm-based *S. cerevisiae–Escherichia coli* shuttle vector that contains a synthetic version of the 76-residue yeast Ub coding sequence under the control of the copper-inducible *CUP1* promoter (14). YEp105, encoding a c-myc epitope attached to the amino terminus of Ub, will be described elsewhere (M.J.E. and M.H.; see Fig. 1*B*). YEp110 encodes yeast Ub with an arginine (instead of lysine) at position 48 (Ub-R48). YEp112, encoding an epitope from the hemagglutinin (ha) of influenza virus (15) attached to the amino terminus of Ub (Fig. 1*B*), was constructed as follows. Complementary 55-mer synthetic oligodeoxynucleotides encoding the ha extension were annealed to generate a double-stranded fragment with *Eco*RI and *Bgl* II adhesive ends that could be ligated into the *Eco*RI/*Bgl* II-cut Ub gene of YEp96. The synthetic fragment contained an *Nhe* I site to facilitate screening for the fragment's insertion. YEp113 was constructed similarly to YEp112 except that the parental plasmid was YEp110, resulting in a ha-tagged, Arg^{48}-substituted Ub variant (haUb-R48). For unknown reasons, the ha-tagged Ub was expressed at lower levels than the myc-tagged Ub (data not shown). Under the conditions described below and in ref. 3, 40–60% of the ha-tagged Ub conjugates were precipitated from yeast extracts with the anti-ha monoclonal antibody. In the experiments shown in Fig. 3, the human Ub gene (in YEp90) and its Ub-R48 allele (in YEp94) were used. Qualitatively similar results were obtained with the corresponding yeast Ub alleles (data not shown).

The mutant α2 repressor, with Lys → Arg substitutions at positions 107 and 109, was produced by oligonucleotide-directed mutagenesis of the *MATα2* gene using M13mp19 (16). The mutated gene was transferred into YEplac195 (17), which was then transformed into HR125-5Dalf *S. cerevisiae* that also harbored either YEp96 or YEp105.

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Abbreviations: Ub, ubiquitin; ha, hemagglutinin; βgal, β-galactosidase.

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Immunoprecipitation and Pulse–Chase Assays. For immunoprecipitation of α2, an anti-α2 antibody affinity-purified against α2 produced in *E. coli* was used (3). The 12CA5 monoclonal antibody against the ha epitope and the monoclonal antibody to *E. coli* β-galactosidase (βgal) have been described (15, 18). Exponentially growing yeast cells were labeled for 5 min at 30°C with Tran^{35}S-label (ICN) [for the experiment in Fig. 1*A*, [^{3}H]-leucine (Amersham) was used], harvested by centrifugation, and disrupted by mixing with an equal volume of 2% SDS/30 mM dithiothreitol/90 mM Na Hepes, pH 7.5, and incubating for 3 min at 100°C. In the experiments with Ub-Pro-βgal, cells were disrupted by vortex mixing with glass beads (3). Other aspects of the procedures used for cell lysis, immunoprecipitation, and pulse–chase analysis were as described (3). In the experiments with α2 described here, cells carried *MATα* on a high-copy plasmid. For the serial immunoprecipitations in Fig. 2, proteins of the first immunoprecipitate were eluted from the protein A-agarose beads (Repligen) by incubating the beads (5 μl) in 50 μl of 1% SDS/50 mM Na Hepes, pH 7.5, at 100°C for 2 min. The eluate was diluted 20-fold with 1% (vol/vol) Triton X-100/0.15 M NaCl/5 mM Na EDTA/50 mM Na Hepes, pH 7.5, containing protease inhibitors (see ref. 3). Ascitic fluid (2 μl) containing the 12CA5 antibody to the ha epitope was then added, and ha-containing proteins were precipitated (3). Unless stated otherwise, α2 and Ub-Pro-βgal were analyzed by electrophoresis in 12% and 6% polyacrylamide/SDS gels, respectively.

RESULTS

The α2 Repressor Is Ubiquitinated *in Vivo*. Ub-dependent degradation of a protein involves the formation of transient Ub-protein conjugates (6). When the short-lived α2 repressor was pulse-labeled and immunoprecipitated with an affinity-purified anti-α2 antibody (3) from SDS extracts of *S. cerevisiae* cells that expressed α2 from a high-copy plasmid, one or more minor labeled species larger than α2 could be detected in the precipitate (Fig. 1*A*, arrows). That these high molecular mass species contained α2 was indicated by the correlation of their levels with the level of α2 in the cell and by their absence from anti-α2 precipitates produced with extracts from a cells (data not shown). In addition, mutation of certain residues in α2 altered the electrophoretic pattern of the high molecular mass species (see below). Although these putative Ub-α2 conjugates could not be detected by immunoblot analysis with polyclonal antibodies to Ub (data not shown), it remained possible that this method was insufficiently sensitive to detect short-lived Ub-α2 conjugates.

We previously constructed a Ub mutant in which the amino terminus of yeast Ub was extended by a 13-residue peptide containing an epitope from the human c-myc protein (20). This Ub variant (mycUb) is indistinguishable from wild-type Ub in its ability to be enzymatically conjugated to and cleaved from acceptor proteins (M.J.E. and M.H., unpublished data). The mycUb is ≈1.5 kDa larger than wild-type Ub. If the high molecular mass species present in the α2 immunoprecipitates (Fig. 1*A*) were Ub-α2 conjugates, *in vivo* substitution of Ub with the larger mycUb should lead to a decrease in electrophoretic mobility of these species but not of unmodified α2.

Fig. 1*C* shows SDS/PAGE patterns of α2 immunoprecipitates from pulse-labeled cells that carried either YEp96 (wild-type Ub) or YEp105 (mycUb). Levels of mycUb expressed from the *CUP1* promoter of YEp105 in cells grown in the absence or presence of added copper ions were ≈2-fold and ≈100-fold higher, respectively, than the levels of wild-type Ub expressed in the same cells from the four genes *UBI1–UBI4* (21) (M.J.E. and M.H., unpublished data). Comparison of lanes 2 and 3 in Fig. 1*C* showed that the putative monoubiquitinated α2 species was largely replaced by an α2-containing protein ≈1.5 kDa larger when mycUb was expressed at high levels (Ub-α2 vs. mUb-α2). Moreover, a second, larger species, presumed to be a doubly ubiquitinated α2 (Ub_2-α2), also migrated more slowly when derived from cells overproducing mycUb (mUb_2-α2; Fig. 1*C*, lanes 2 and 3). These and other experiments (see Fig. 5) indicated that overexpression of mycUb was accompanied by increases in size of at least three higher molecular mass, α2-containing species (or groups of species; see below). We conclude that the α2 repressor is ubiquitinated *in vivo* and that it is conjugated to multiple Ub moieties. [These experiments

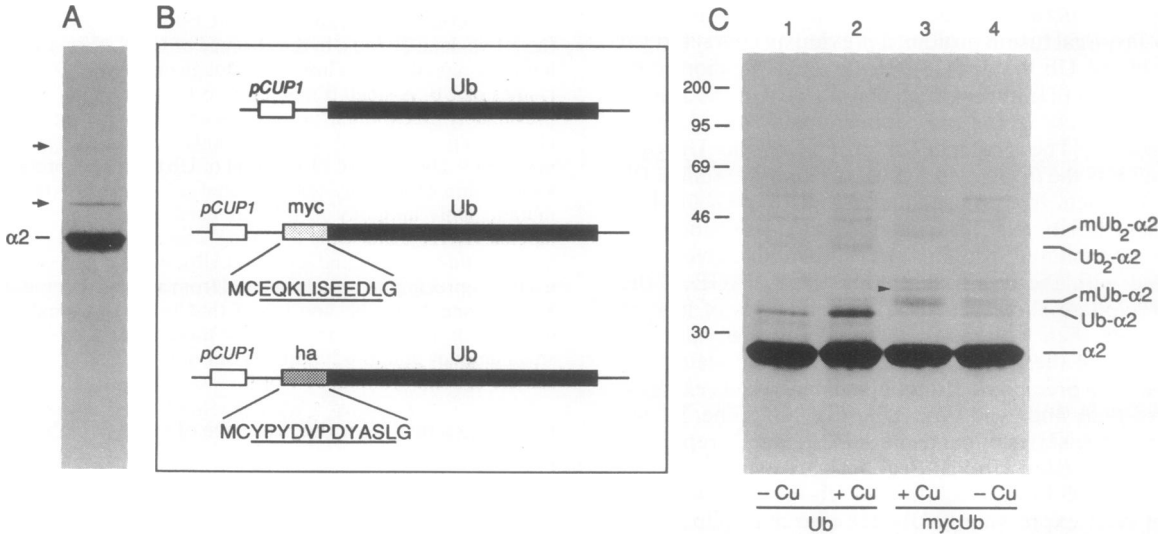

FIG. 1. The α2 repressor is ubiquitinated *in vivo*. (*A*) Immunoprecipitation with antibody to α2 yields, in addition to α2, a set of higher molecular mass species (arrows). DBY1705 cells carrying a high-copy *MATα* plasmid were pulse-labeled for 5 min, followed by extraction, immunoprecipitation, and SDS/15% PAGE. (*B*) Epitope-tagged forms of ubiquitin used to examine α2 ubiquitination. The epitopes from human c-myc and influenza virus hemagglutinin (ha) are underlined. (*C*) Expression of mycUb (mUb) in DBY1829 cells leads to an increase in the apparent molecular masses of the putative Ub-α2 conjugates seen in *A*. The anomalously slow migration of the doubly ubiquitinated species (Ub_2-α2) is similar to that seen with other proteins modified by a multi-Ub chain (19; Fig. 2B). Heterogeneity of the ubiquitinated α2 species is also evident; a clear example is the minor mycUb-α2 band marked by an arrowhead in lane 3. Molecular masses (in kilodaltons) of marker proteins are indicated.

made use of $\alpha 2$ expressed from a high-copy *MAT*α plasmid; similar results were obtained with $\alpha 2$ expressed from the single-copy chromosomal *MAT*α locus (data not shown)].

Another feature of Fig. 1*C* (lane 4) is the presence of approximately equal amounts of the mycUb-$\alpha 2$ and Ub-$\alpha 2$ species (as well as mycUb$_2$-$\alpha 2$ and Ub$_2$-$\alpha 2$) in uninduced cells, which express comparable amounts of wild-type Ub and mycUb. Thus, the relative levels of the Ub- and mycUb-containing $\alpha 2$ conjugates approximated the relative levels of Ub and mycUb in the cell. Note that the set of Ub-$\alpha 2$ conjugates is more heterogeneous than is apparent from the above discussion; see, for instance, the additional, unassigned mycUb-$\alpha 2$ species indicated by an arrowhead in Fig. 1*C*, lane 3. This heterogeneity could be due to incomplete denaturation of conjugates by SDS and/or to the presence of minor alternative sites of Ub attachment within $\alpha 2$ (see below).

Multiply Ubiquitinated $\alpha 2$ Contains a Lys^{48}-Linked Multi-Ub Chain. Multiple Ub moieties could be joined to the $\alpha 2$ protein in several distinct ways, illustrated in Fig. 2*A* for a Ub$_3$-$\alpha 2$ conjugate. Experiments with an *in vitro* proteolytic system from rabbit reticulocytes have shown that degradation of proteins by the N-end rule pathway [a Ub-dependent pathway (18) whose substrates do not include $\alpha 2$ (3)] requires formation of a multi-Ub chain on a targeted protein (19). In this chain, several Ub moieties attach sequentially to an initial acceptor protein to form a branched Ub-Ub structure in which the carboxyl-terminal Gly^{76} of one Ub is joined to the ε-amino group of the Lys^{48} in the adjacent Ub moiety. Substitution of an arginine residue for Lys^{48} in Ub does not perturb the ability of Ub-R48 to be conjugated to other proteins but renders it unable to serve as an acceptor protein within the multi-Ub chain (19). The Ub-R48 mutant is therefore an efficient terminator of multi-Ub chain formation.

To ascertain whether the multiply ubiquitinated $\alpha 2$ derivatives contain a Lys^{48}-linked multi-Ub chain, we first tagged both wild-type Ub and its Ub-R48 variant with an epitope from the ha of influenza virus (Fig. 1*B Bottom*). This epitope is recognized by the high-avidity monoclonal antibody 12CA5 (15). (An alternative epitope tag was necessary because of inefficient precipitation of myc-tagged proteins by the anti-myc antibody.) The utility of the ha-tagged Ub variants for analyzing the structure of Ub-protein conjugates was tested with a Ub-Pro-βgal fusion protein, a previously characterized substrate of the Ub system (18). Ub-Pro-βgal is short-lived due to formation of a multi-Ub chain on Lys^{48} of the amino-terminal Ub moiety (ref. 19; E. Johnson and A.V., unpublished results). [The presence of proline at the Ub–βgal junction inhibits the *in vivo* removal of the amino-terminal Ub (18, 19).] Extracts from pulse-labeled cells expressing Ub-Pro-βgal and haUb were precipitated with an antibody to βgal; the precipitated proteins were then dissolved and precipitated with the anti-ha antibody. SDS/PAGE of the second precipitate showed it to contain a series of labeled species larger than Ub-Pro-βgal itself (Fig. 2*B*, lane 2). Because none of these species were seen in the identically obtained anti-ha precipitate from pulse-labeled cells expressing Ub-Pro-βgal and wild-type Ub (Fig. 2*B*, lane 3), we conclude that the series of bands in Fig. 2*B*, lane 2, represent multiply ubiquitinated Ub-Pro-βgal derivatives.

Formation of multiply ubiquitinated Ub-Pro-βgal was suppressed in cells expressing haUb-R48 (the multi-Ub chain-terminating Ub variant; see above) (Fig. 2*B*, lane 1), while levels of the unmodified Ub-Pro-βgal were virtually the same in all of these experiments (data not shown). The Ub moieties of the multiply ubiquitinated Ub-Pro-βgal have been shown, by direct structural analysis, to form a Lys^{48}-linked multi-Ub chain (19). The suppression of multi-Ub chain formation by the haUb-R48 mutant (Fig. 2*B*, lane 1), in addition to supporting the earlier conclusions (19), validates the use of this mutant as a probe for the presence of multi-Ub chains in other proteins.

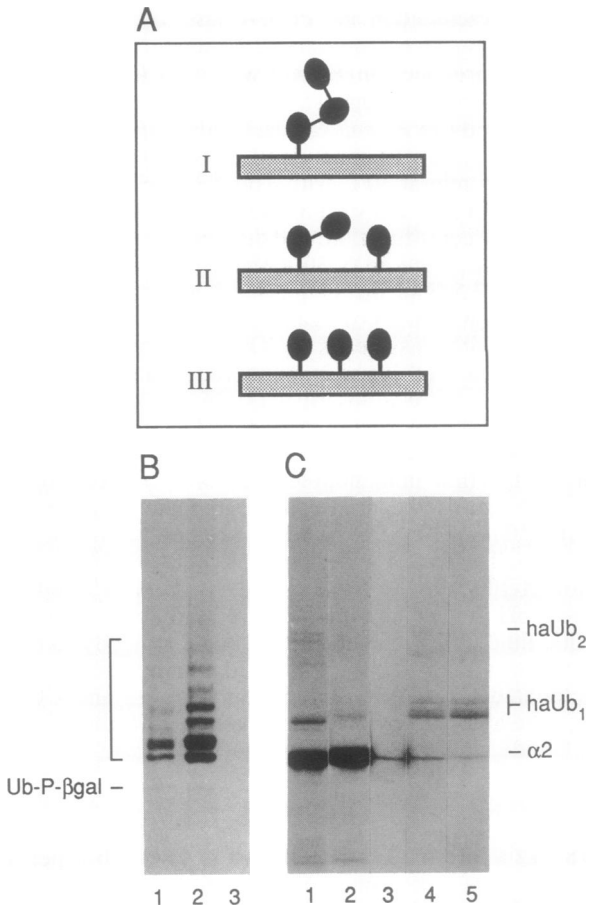

FIG. 2. Evidence for a branched multi-Ub chain in the multiply ubiquitinated $\alpha 2$ protein. (*A*) Three ways in which three Ub molecules (black ovals) could be conjugated to an acceptor protein (rectangle). (*B*) Use of ha-tagged Ub to mark ubiquitinated derivatives of Ub-Pro-βgal (Ub-P-βgal). Proteins from extracts of pulse-labeled YPH500 cells were precipitated with an antibody to βgal, dissolved, and reprecipitated with an anti-ha antibody. Extracts were made from cells expressing Ub-Pro-βgal and haUb-R48 (lane 1), haUb (lane 2), or Ub (lane 3). The minor multiubiquitinated species that did form in cells expressing haUb-R48 had aberrant mobilities (lane 1 vs. lane 2); thus, in the absence of Lys^{48}, formation of Ub-Ub linkages may occur at a low rate through one or more of the other six lysines of Ub. A small fraction of Ub-Pro-βgal is visible on the gel, presumably precipitated by anti-βgal antibody that survived the SDS elution step. Multiply ubiquitinated haUb$_n$-(Ub-Pro-βgal) species are marked by a bracket. (*C*) Detection of Ub in anti-$\alpha 2$ precipitates and suppression of multiply ubiquitinated $\alpha 2$ species by the haUb-R48 mutant. Only antibody to $\alpha 2$ was used with extracts from pulse-labeled HR125-5Dalf cells (carrying a high-copy *MAT*α plasmid) in lane 1 (wild-type Ub) and lane 2 (haUb), while anti-ha antibody was used to reprecipitate proteins eluted from anti-$\alpha 2$ precipitates in lane 3 (Ub), lane 4 (haUb), and lane 5 (haUb-R48). A small fraction of unmodified $\alpha 2$ present in the latter three lanes was due to either a small amount of anti-$\alpha 2$ antibody surviving the SDS elution step or sample spillage from lanes 1 or 2.

Experiments identical in design to those with Ub-Pro-βgal were performed with the $\alpha 2$ repressor and yielded similar results. Specifically, when $\alpha 2$-expressing cells contained exclusively wild-type Ub, no $\alpha 2$-containing species could be precipitated (from a dissolved $\alpha 2$ immunoprecipitate) by the anti-ha antibody (Fig. 2*C*, lane 3). However, an otherwise identical experiment with cells expressing high levels of haUb yielded a number of species larger than $\alpha 2$ after precipitation by the anti-ha antibody from the dissolved $\alpha 2$ immunoprecipitate (Fig. 2*C*, lane 4). These results provided independent

evidence that the high molecular mass, α2-containing proteins were Ub conjugates, a conclusion reached above through the use of the myc-tagged Ub (Fig. 1). When cells expressed both α2 and high levels of haUb-R48, formation of the doubly ubiquitinated α2 species was suppressed relative to that of the monoubiquitinated α2 (Fig. 2*C*, lanes 4 vs. 5), indicating the presence of Ub-Ub linkages in the doubly ubiquitinated species. We conclude that at least some of the Ub moieties in multiply ubiquitinated α2 derivatives occur as a Lys^{48}-linked multi-Ub chain.

Ubiquitin and α2 Degradation. Earlier *in vitro* evidence indicated that formation of a multi-Ub chain is required for the degradation of substrates of the N-end rule pathway (19). To address a possible role for the multi-Ub chain in the degradation of α2 [which is not an N-end rule substrate (3)], we measured the effect of the chain-terminating Ub-R48 mutant on the metabolic stability of α2. The *in vivo* half-life of α2 was increased ≈2-fold in cells expressing high levels of Ub-R48 (in addition to wild-type Ub, expressed from the chromosomal *UBI1–UBI4* genes) (Fig. 3). Control experiments (data not shown) indicated that the inhibition of α2 degradation by Ub-R48 was not due to the $CuSO_4$ added to induce the *CUP1* promoter-mediated expression of Ub-R48 (see also Fig. 4). Furthermore, expression of Ub-R48 resulted in a >2-fold increase in the relative amount of ^{35}S in the pulse-labeled monoubiquitinated α2 species (from <1% to nearly 2% of ^{35}S in the unmodified α2 species) (compare Fig. 3 *A* and *B*). A similar increase was seen in the analogous experiments with the ha-tagged Ub-R48 mutant (Fig. 2*C*, lane 4 vs. lane 5). Thus, while monoubiquitination of α2 is unimpaired in the presence of Ub-R48, further Ub additions are inhibited, as is the degradation of α2.

We recently found that myc-tagged Ub (mycUb) also inhibits Ub-dependent protein degradation. However, in contrast to Ub-R48, which is a multi-Ub-chain terminator, mycUb acts by inhibiting the degradation of multi(mycUb)-chain-bearing substrates (M.J.E. and M.H., unpublished results). We therefore examined the effect of mycUb on α2 degradation (Fig. 4). The half-life of α2 increased almost 2-fold (from ≈6 min to ≈11 min) in cells expressing high levels of mycUb (Fig. 4). Since in all of these experiments cells also contained considerable amounts of wild-type Ub, the inhibition of α2 degradation by either Ub-R48 or mycUb was expected to be "leaky." Thus, the observed degree of inhibition of α2 degradation by either mycUb or Ub-R48 suggests that at least a significant fraction of α2 turnover occurs via one or more Ub-dependent pathways.

Multiple Ubiquitination Sites in α2. The data of Figs. 1*C* and 2*C* suggested that more than one lysine of α2 can be joined to Ub. Cyanogen bromide peptide mapping of Ub-α2 conjugates (M.H., unpublished data) suggested that at least one Ub attachment site is located in the region linking the two globular domains of the α2 protein. A double Lys → Arg substitution in this region (positions 126 and 129) had no effect on α2 ubiquitination or degradation (data not shown). Substitution of arginines for the remaining two lysines within the linker region (positions 107 and 109) eliminated a minor Ub-α2 species seen with wild-type α2 (Fig. 5). Thus, either Lys^{107} or Lys^{109} is likely to serve as a minor ubiquitination site in α2. The function, if any, of this site is unknown. Its elimination did not alter the half-life of α2, and cells expressing the mutant α2 protein (α2-R107,109) continued to mate as α cells; the resultant diploids sporulated normally (data not shown).

DISCUSSION

We have shown that the MATα2 repressor of *S. cerevisiae* is multiply ubiquitinated *in vivo*, that some of the Ub moieties form a Lys^{48}-linked multi-Ub chain, and that ubiquitination of α2 contributes to its metabolic instability. Although the set of naturally short-lived proteins known to be degraded via Ub-dependent pathways is likely to grow, at present it is limited to the α2 repressor; the phytochrome photoreceptor of plants, whose light-induced degradation is accompanied by ubiquitination (10); and cyclins, whose periodic destruction controls transitions in the cell cycle (11). One difficulty in establishing whether the degradation of a short-lived protein requires its conjugation to Ub stems from the fact that, depending on the relative rates of ubiquitination, deubiquitination, and degradation of the Ub-containing protein, the steady-state levels of ubiquitinated species may range from negligible to readily detectable (18). For instance, while the levels of Ub-phytochrome conjugates in plant cells are high during phytochrome degradation (10), the steady-state levels of Ub-α2 conjugates in yeast cells are very low and required special techniques, such as epitope tagging of Ub, for their identification. It is therefore possible that other naturally short-lived proteins whose degradation appears to be independent of Ub by criteria such as immunoblotting (4) may eventually be found to require Ub for proteolysis.

The α2 repressor joins a growing list of known *in vivo* acceptors of ubiquitin. These include mammalian histones H2A and H2B, the actin of insect flight muscle, several plasma membrane proteins (e.g., the growth hormone receptor), and the plant protein phytochrome (reviewed in refs. 7 and 10). With the exception of α2 and phytochrome, it

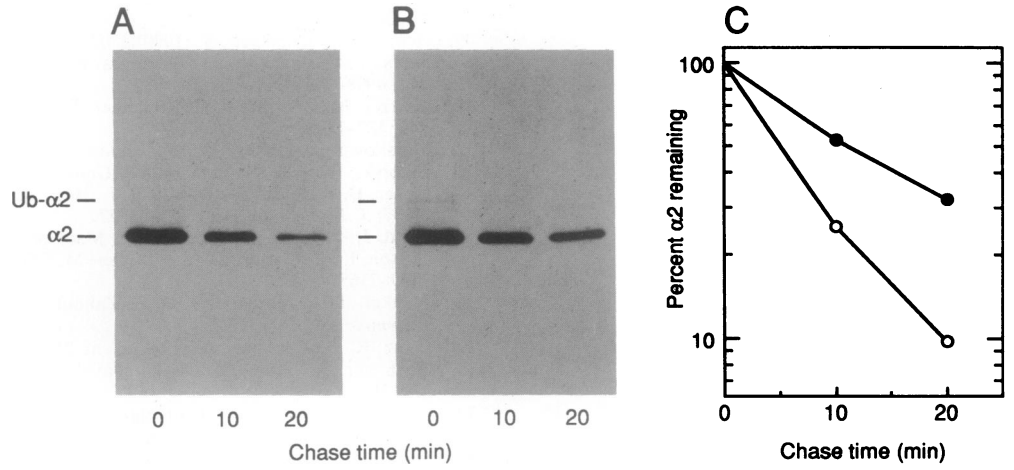

FIG. 3. Overexpression of Ub-R48 inhibits α2 degradation. (*A*) Pulse–chase analysis of α2 degradation in DBY1829 cells expressing high levels of wild-type Ub. (*B*) Same as in *A* except that cells expressed high levels of the Ub-R48 mutant. (*C*) Quantitation of an independent set of pulse–chase experiments. ●, Copper-induced cells expressing the Ub-R48 mutant; ○, copper-induced cells expressing wild-type Ub.

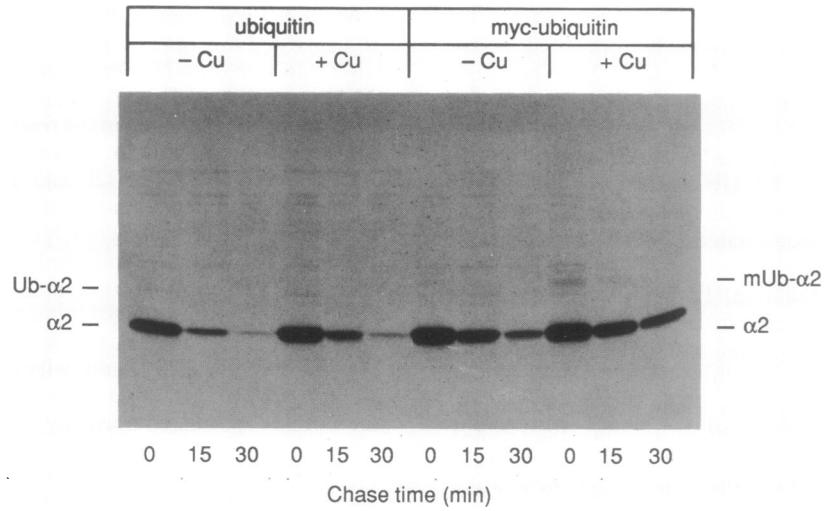

FIG. 4. An amino-terminally extended variant of Ub that inhibits Ub-dependent protein degradation also inhibits degradation of α2. Pulse–chase analysis was performed as described (3). Proteins were extracted from DBY1829 cells carrying plasmids expressing either wild-type Ub (YEp96) or mycUb (mUb; YEp105) and were precipitated with antibody to α2.

remains unclear whether ubiquitination of these proteins influences their metabolic stability.

The α2 repressor contains two autonomous degradation signals that function via genetically distinct proteolytic pathways (3). Ubiquitination of α2 has been shown to be essential for maximal rates of α2 degradation (see *Results*). Null mutations in several of the *UBC* genes, which encode Ub-conjugating enzymes, inhibit α2 turnover (M.H., S. Jentsch, and A.V., unpublished results). Use of the methods developed in the present work and the previously isolated *S. cerevisiae* mutants defective in α2 degradation (3) should make it possible to determine whether conjugation of Ub to α2 is required for all or only some of the α2-degrading pathways.

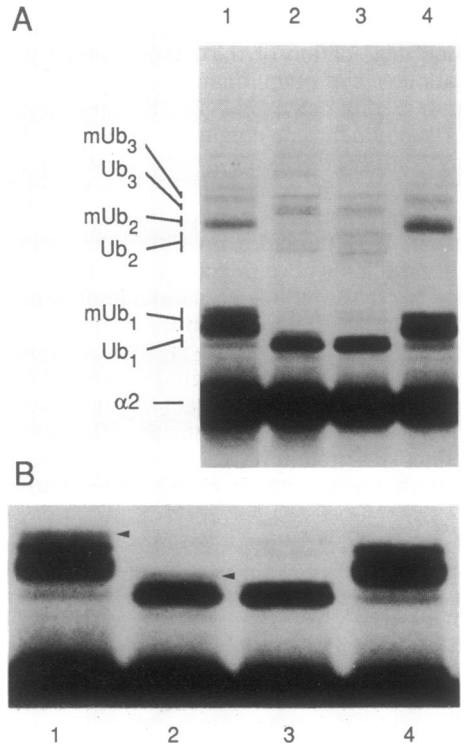

FIG. 5. Evidence for multiple ubiquitination sites in α2. (*A*) HR125-5Dalf cells expressing either wild-type Ub (lanes 2 and 3) or mycUb (mUb; lanes 1 and 4) were pulse-labeled, and extracts were precipitated with antibody to α2. These cells also carried a high-copy plasmid encoding either wild-type α2 protein (lanes 1 and 2) or α2 with Lys → Arg substitutions at positions 107 and 109 (lanes 3 and 4). (*B*) An enlargement of a portion of *A* that highlights the three monoubiquitinated species of the wild-type α2, observed with either wild-type Ub (lane 2) or mycUb (lane 1). Only two of these species are seen with the mutant α2 protein (lanes 3 and 4). The more slowly migrating species missing in the samples of mutant α2 are indicated with arrowheads in lanes 1 and 2.

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