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The N-end rule is mediated by the UBC2(RAD6) ubiquitin-conjugating enzyme

(protein degradation/yeast/*RAD6*/*UBC2*)

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ABSTRACT **The N-end rule relates the *in vivo* half-life of a protein to the identity of its amino-terminal residue. Distinct versions of the N-end rule operate in all organisms examined, from mammals to bacteria. We show that UBC2(RAD6), one of at least seven ubiquitin-conjugating enzymes in the yeast *Saccharomyces cerevisiae*, is essential for multiubiquitination and degradation of the N-end rule substrates. We also show that UBC2 is physically associated with UBR1, the recognition component of the N-end rule pathway. These results indicate that some of the *UBC2* functions, which include DNA repair, induced mutagenesis, sporulation, and regulation of retrotransposition, are mediated by protein degradation via the N-end rule pathway.**

Selective protein degradation underlies the elimination of damaged or otherwise abnormal proteins and the temporal control of many cellular processes that involve short-lived regulators. At least some proteins are short-lived *in vivo* because they contain sequences (degradation signals) that make these proteins substrates of specific proteolytic pathways. An essential component of one degradation signal is the protein's amino-terminal residue (1). The presence of this signal, named the N-degron (2), is manifested as the N-end rule, which relates the metabolic stability of a protein to the identity of its amino-terminal residue (1). Distinct versions of the N-end rule operate in all organisms examined, from mammals to bacteria (refs. 1–12; J. Tobias, T. Shrader, G. Rocap, and A.V., unpublished data). The eukaryotic N-degron is a bipartite signal, comprising a destabilizing amino-terminal residue (1) and a specific internal Lys residue (6, 8, 9).

The N-end rule is organized hierarchically (Fig. 1). Specifically, amino-terminal Asp and Glu (and Cys in mammalian reticulocytes) are *secondary* destabilizing residues in that they are destabilizing through their ability to be conjugated to Arg, one of the *primary* destabilizing residues (1, 7, 10, 12). Amino-terminal Asn and Gln are *tertiary* destabilizing residues in that they are destabilizing through their ability to be converted, via selective deamidation, into the secondary destabilizing residues Asp and Glu (Fig. 1) (7).

In the yeast *Saccharomyces cerevisiae*, the recognition component of the N-end rule pathway is encoded by the *UBR1* gene (11). The 225-kDa UBR1 protein, named N-recognin [also known as the type 1, 2 E3 protein (5, 7, 13)], selects potential proteolytic substrates by binding to their primary destabilizing amino-terminal residues (Fig. 1) (6, 11). The yeast N-recognin (11, 14) and its mammalian counterparts (5, 7, 13, 16, 18) each possess distinct binding sites for the two classes of primary destabilizing residues. The type 1 binding site is specific for the positively charged amino-terminal residues Arg, Lys, and His. The type 2 binding site is specific for the bulky hydrophobic amino-terminal residues Phe, Trp, Tyr, and Leu (and Ile in yeast) (5, 7, 11, 13).

If a substrate bears both determinants of the N-degron, the binding of N-recognin is followed by formation of a multiubiquitin chain linked to the N-degron's second determinant, a specific internal Lys residue (6–9). The coupling of ubiquitin (Ub) to proteins is catalyzed by a family of Ub-conjugating (UBC) enzymes [also called E2 enzymes (13)] and involves formation of an isopeptide bond between the carboxyl-terminal Gly residue of Ub and the ϵ-amino group of a Lys residue in an acceptor protein (13, 19, 20). In a multiubiquitin chain, Ub itself serves as an acceptor, with several Ub moieties attached sequentially to the initial acceptor protein to form a chain of branched Ub-Ub conjugates (8, 21).

There are seven or more distinct UBC enzymes in *S. cerevisiae*; other eukaryotes appear to have at least as many (20). Two of the yeast UBC enzymes have been identified as the products of the previously known genes *RAD6* (renamed *UBC2*) (22), which participates in DNA repair, induced mutagenesis, sporulation (23–33), and suppression of retrotransposition (34), and *CDC34* (renamed *UBC3*), which is required for the transition from G_1 to S phase of the cell cycle (35). Two of the other yeast UBC enzymes, UBC4 and UBC5, have been shown to be required for most of the Ub-dependent protein degradation in this organism (18, 36).

We now report that UBC2(RAD6) is essential for the degradation of N-end rule substrates and that UBC2 is physically associated with the *UBR1*-encoded N-recognin. Thus, at least one of the multiple functions of *UBC2* is likely to be mediated by protein degradation via the N-end rule pathway.

MATERIALS AND METHODS

Plasmids. The plasmid pSOB44 is a derivative of pSOB37 (11), in which the *UBR1* promoter was replaced with the ≈1.5-kilobase (kb) *Bam*HI fragment from the *ADH1* promoter-containing plasmid pJDcAG1 (37). The plasmid pADHUBC2 was constructed by inserting the ≈1.5-kb *Bam*HI–*Eco*RI fragment from pJDcAG1 into *Bam*HI/*Eco*RI-cut YEplac195 (38). The resultant plasmid was cut with *Eco*RI, and the ≈0.6-kb *Eco*RI fragment containing the *UBC2* coding sequence (22) was inserted. pADHUBC2 fully complemented the radiation sensitivity and slow growth phenotypes of the *ubr1*Δ *ubc2*Δ BBY68 strain (see the legend to Fig. 4).

Coimmunoprecipitation. Exponential yeast cultures (A_{600} < 1) were labeled with [^{35}S]methionine (0.3 mCi/ml; 1 Ci = 37 GBq) for 30 min at 30°C in synthetic medium (11) lacking methionine. The cells were pelleted by centrifugation, resuspended in DB buffer (50 mM NaCl/1 mM Na_2EDTA/50 mM Na Hepes, pH 7.5) containing protease inhibitors leupeptin, pepstatin A, antipain, chymostatin, and aprotinin (each at 20

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Abbreviations: Ub, ubiquitin; UBC, Ub-conjugating; Ub-X-βgal, Ub-X-β-galactosidase.

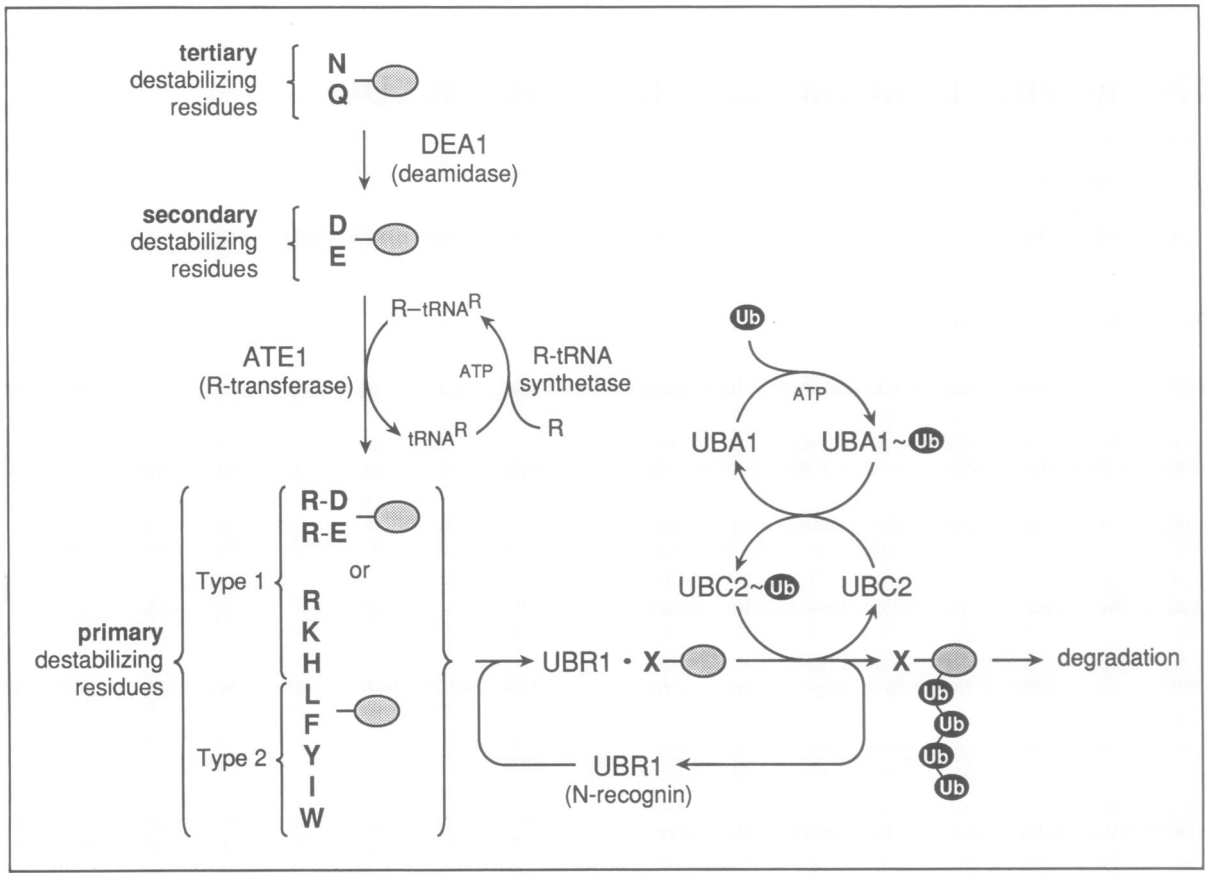

FIG. 1. The N-end rule pathway in *S. cerevisiae*. Primary, secondary, and tertiary destabilizing amino-terminal residues are defined in the main text; they are denoted by boldface letters using the single-letter abbreviations for amino acids. The shaded ovals indicate the rest of a protein substrate. Conversion of the tertiary destabilizing residues **N** and **Q** into the secondary destabilizing residues **D** and **E** (7) is catalyzed by a deamidase (encoded by *DEA1*) specific for amino-terminal **N** and **Q** (R. Baker and A.V., unpublished data). Conjugation of the primary destabilizing residue **R** to the secondary destabilizing amino-terminal residues **D** and **E** (7) is catalyzed by the arginyl-tRNA-protein transferase (R-transferase) encoded by the *ATE1* gene (10). The distinction between type 1 (**R, K, H**) and type 2 (**L, F, Y, I, W**) primary destabilizing amino-terminal residues is based on their binding to distinct sites on the *UBR1*-encoded N-recognin (7, 11, 13, 14). If a substrate bears both determinants of the N-end rule-based degradation signal [the N-degron (2)], the UBR1-associated UBC enzyme encoded by the *UBC2* gene (see main text) uses activated Ub, produced by the *UBA1*-encoded Ub-activating enzyme (15), to catalyze formation of a substrate-linked multiubiquitin chain. The latter is required (8) for the substrate's subsequent degradation by an ATP-dependent protease (13, 16, 17).

μg/ml; Sigma), and lysed by a Vortex treatment with glass beads (11). The extracts were centrifuged at 12,000 × *g* for 10 min. Immunoprecipitation assays were carried out with the supernatants by adding ascitic fluid containing the anti-ha 12CA5 monoclonal antibody (39) to the single or mixed extracts described in *Results*. All samples also contained an ≈10-fold excess of an identically prepared unlabeled extract from untransformed BBY68 cells (see the legend to Fig. 4). The samples were incubated for 2 hr at 0°C; 20 μl of protein A-Sepharose (Repligen) was then added, and the suspensions were incubated with rocking for 1 hr at 4°C, followed by a 3-s centrifugation in a microcentrifuge. Pellets were washed three times with 0.5 ml of DB buffer containing 5% glycerol, resuspended in electrophoretic sample buffer, heated at 100°C for 3 min, and electrophoresed in a 13% polyacrylamide/SDS gel, followed by fluorography.

RESULTS AND DISCUSSION

Ubiquitination and degradation of proteins by the N-end rule pathway were assayed *in vivo* using derivatives of *Escherichia coli* β-galactosidase as model substrates. In eukaryotes, Ub-X-β-galactosidase (Ub-X-βgal) fusion proteins are precisely deubiquitinated by Ub-specific processing proteases to yield X-βgal test proteins bearing the residue X at the amino terminus (1, 7). In contrast to the function of Ub in protein degradation, the role of Ub in these engineered Ub fusions is simply to allow the generation of X-βgals bearing different amino-terminal residues. Depending on the identity of X, the X-βgal proteins are either long-lived or metabolically unstable, with destabilizing amino-terminal residues conferring short half-lives on the corresponding X-βgals (1, 6–11).

Which of the yeast UBC enzymes is specific for the N-end rule pathway? *In vivo* degradation of the N-end rule substrates (X-βgals) was tested in *ubc1*, *ubc4*, and *ubc5* null mutants and found to be either normal or only slightly reduced in comparison to the congenic wild-type strains (data not shown). However, these normally short-lived X-βgals were found to be dramatically stabilized in a null *ubc2(rad6)* mutant.

The X-βgal test proteins used in these experiments had either Arg (a type 1 primary destabilizing residue), Leu (a type 2 primary destabilizing residue), or Met (a stabilizing residue) at their amino termini. Metabolic stabilities of these X-βgals were compared indirectly, by determining their intracellular levels (Fig. 2), and directly, by pulse–chase analyses (Fig. 3). Previous work (10, 11, 14) has shown that the steady-state level of an X-βgal in yeast cells is a function of its metabolic stability; compare, for instance, the levels of Met-βgal [$t_{1/2}$ > 20 hr (1)], Arg-βgal [$t_{1/2}$ ≈ 2 min (1)], and Leu-βgal [$t_{1/2}$ ≈3 min (1)] expressed from identical vectors in

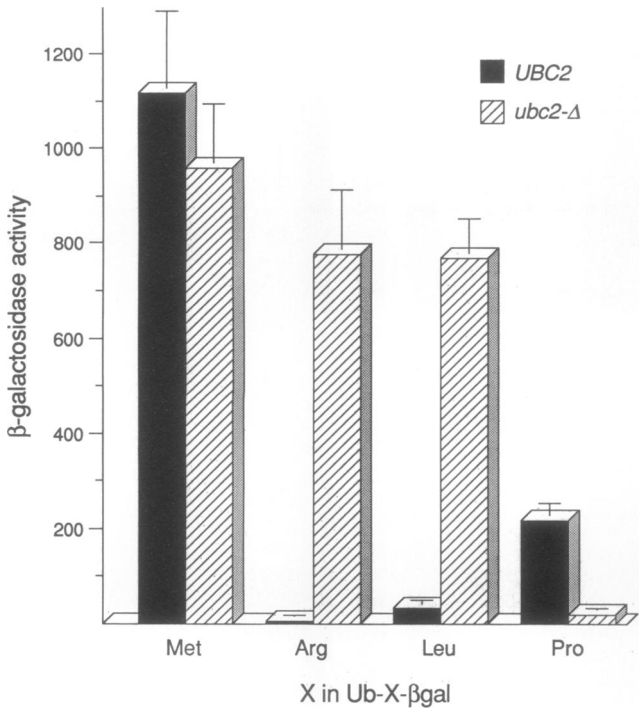

FIG. 2. The N-end rule pathway is inactive in a *ubc2* null mutant. Levels of βgal activity in the wild-type (*UBC2*) *Saccharomyces cerevisiae* strain YPH500 (black bars) and in the congenic *ubc2*Δ strain BBY67 (striped bars) expressing either Ub-Met-βgal, Ub-Arg-βgal, Ub-Leu-βgal, or Ub-Pro-βgal. [Unlike the other 19 Ub-X-βgals, Ub-Pro-βgal is inefficiently deubiquitinated; its metabolic instability is independent of the N-degron (see text).] Values shown are the means of at least four independent measurements. Standard deviations are shown above each bar. The congenic *S. cerevisiae* strains YPH500 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) (40) and BBY67 (a *ubc2*Δ::*LEU2* derivative of YPH500) were transformed with the previously described (1) 2-μm-based vectors expressing Ub-X-βgals from a galactose-inducible promoter. Exponential cultures (A_{600} < 1) growing at 30°C in SD-galactose medium (1, 6) were assayed for β-galactosidase activity as described (41).

wild-type (*UBC2*) cells (Fig. 2, black bars). However, in the congenic *ubc2*Δ cells, which lack the UBC2 Ub-conjugating enzyme, the levels of Arg-βgal and Leu-βgal were greatly increased and became similar to the level of the long-lived Met-βgal (Fig. 2). Lys-βgal, His-βgal, Phe-βgal, Tyr-βgal, Ile-βgal, and Trp-βgal were also tested as described above and yielded results (data not shown) similar to those with Arg-βgal and Leu-βgal.

These findings were confirmed and extended by pulse–chase analysis (Fig. 3). Arg-βgal and Leu-βgal were dramatically stabilized in the *ubc2*Δ background; their half-lives became indistinguishable from that of the long-lived Met-βgal (Fig. 3 *a* and *b*). As expected from the known enzymatic activity of UBC2 (22) and the known requirement for multiubiquitination in the degradation of N-end rule substrates (7, 8), overexposures of the Fig. 3 fluorograms revealed the presence of multiubiquitinated Arg-βgal and Leu-βgal derivatives in the wild-type (*UBC2*) cells (Fig. 3*c*) but not in congenic *ubc2*Δ cells (Fig. 3*d*). We conclude that the UBC2 Ub-conjugating enzyme is essential for the multiubiquitination and degradation of N-end rule substrates.

The conspicuous and pleiotropic phenotype of *ubc2(rad6)* null mutations (22–34) stands in contrast to the relatively mild phenotype of *ubr1* null mutations, which also inactivate the N-end rule pathway (11). Thus, unlike N-recognin, whose known functions are confined to the N-end rule pathway (11), the UBC2 enzyme is an essential component not only of this pathway but at least of one other Ub-dependent pathway as well. At the same time, the absence of a functional UBC2 enzyme does not inactivate the Ub system in general, as indicated by the lack of metabolic stabilization of Ub-Pro-βgal in *ubc2*Δ cells (Fig. 3*b*; compare Fig. 3*a*). In contrast to the other 19 Ub-X-βgals, Ub-Pro-βgal is inefficiently deubiquitinated (1). It is metabolically unstable (1), but its degradation is independent of the N-degron and N-recognin (2, 11) and has recently been shown to be mediated by the amino-terminal Ub moiety of Ub-Pro-βgal (E. Johnson and A.V., unpublished data). Remarkably, Ub-Pro-βgal not only remained multiubiquitinated and short-lived in the *ubc2*Δ cells (Fig. 3*b*) but its half-life in fact *decreased*, from ≈8 min in wild-type (*UBC2*) cells to ≈2 min in *ubc2*Δ cells [Figs. 2 and 3 *a* and *b*; half-life values were determined as described (1, 6)]. Most of the Ub-Pro-βgal degradation is dependent on the previously identified (18, 36) UBC4 enzyme (E. Johnson, B.B., W. Seufert, S. Jentsch, and A.V., unpublished data). One possibility is that the absence of UBC2 results in overexpression of either the UBC4 enzyme or a UBC4-associated recognin.

The UBC2-mediated multiubiquitination of an N-end rule substrate requires, and is preceded by, substrate binding by the *UBR1*-encoded N-recognin. This functional proximity of UBR1 and UBC2 is underscored by their physical association, which was demonstrated by coimmunoprecipitation (Fig. 4). In this experiment, a modified UBR1 protein was used, whose carboxyl terminus was extended with a nine-residue epitope (derived from hemagglutinin (ha) of influenza virus) that is recognized by a monoclonal antibody (39). The epitope-tagged UBR1 (UBR1-ha) was functionally active, in that the *UBR1-ha* gene complemented the *ubr1*Δ mutant to the same extent as the unmodified *UBR1* gene (11). When overexpressed from the *ADH1* promoter on a high copy plasmid, the ≈226-kDa UBR1-ha protein could be immunoprecipitated with the anti-ha monoclonal antibody from [^{35}S]methionine-labeled cell extracts (ref. 11 and Fig. 4, lane c). Extracts were prepared from [^{35}S]methionine-labeled *ubr1*Δ *ubc2*Δ cells transformed with either a control plasmid (vector alone), a plasmid expressing UBR1-ha, or a plasmid expressing UBC2. The control extract (lacking UBR1-ha and UBC2) was processed for immunoprecipitation with the anti-ha monoclonal antibody either alone (Fig. 4, lane a) or after having been mixed with either the UBC2-containing extract (Fig. 4, lane b) or the extract containing UBR1-ha (Fig. 4, lane c). The UBC2-containing extract was also mixed with the extract containing UBR1-ha, and the mixed extract was immunoprecipitated with the anti-ha antibody (Fig. 4, lane d). This antibody precipitated the UBR1-ha protein from samples containing UBR1-ha (Fig. 4, lanes c and d) and did not precipitate a protein the size of UBC2 (19.7 kDa) from samples containing UBC2 but lacking UBR1-ha (Fig. 4, lane b). However, the immunoprecipitation from the sample containing *both* UBR1-ha and UBC2 yielded not only UBR1-ha but also a protein the size of UBC2 (Fig. 4, lane d; compare lane c). We conclude that the UBC2 Ub-conjugating enzyme is physically associated with the *UBR1*-encoded N-recognin. In agreement with this result, Reiss *et al.* (42) have observed, using sedimentation assays, that the partially purified N-recognin (type 1, 2 E3 protein) from rabbit reticulocytes binds to one or more of the UBC enzymes present in reticulocyte extract.

In addition to identifying one specific function of the UBC2 enzyme, the discovery of its role in the N-end rule pathway opens new directions for studies of this pathway. For example, an ≈100-fold increase in the frequency of transposition of the *S. cerevisiae* Ty1 retrotransposon in *ubc2* mutants (34) is consistent with the possibility that the N-end rule pathway is involved in the regulation of retrotransposition. Furthermore, the analysis of targeting and ubiquitination in the N-end rule pathway can now be aided by developing an *in*

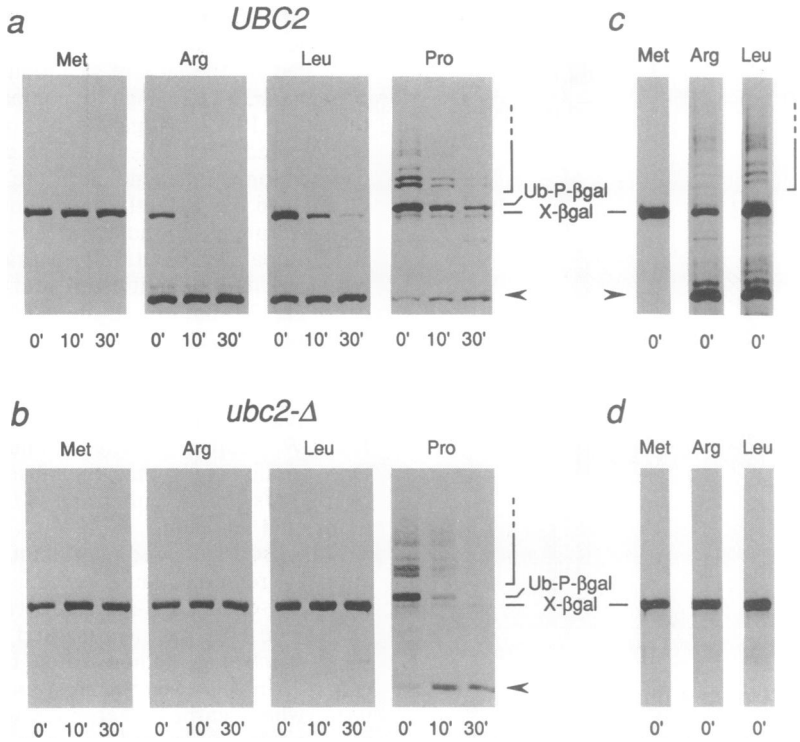

FIG. 3. Pulse–chase analysis of X-βgal degradation in *UBC2* and *ubc2*Δ cells. (*a*) Exponential cultures of the wild-type (*UBC2*) *S. cerevisiae* strain YPH500 (see legend to Fig. 2) expressing either Ub-Met-βgal, Ub-Arg-βgal, Ub-Leu-βgal, or Ub-Pro-βgal were labeled with [^{35}S]methionine for 5 min at 30°C (lanes 0′), followed by a chase in the presence of unlabeled methionine and the translation inhibitors cycloheximide and trichodermin for 10 and 30 min (lanes 10′ and 30′, respectively), extraction, immunoprecipitation, and electrophoretic analysis of X-βgal as described (11). The bands of X-βgals and of (inefficiently deubiquitinated) Ub-Pro-βgal are indicated. An arrowhead denotes an ≈90-kDa long-lived βgal cleavage product specific for short-lived X-βgals and Ub-Pro-βgal (1, 11). Half-open square brackets indicate the bands of multiply ubiquitinated βgal (1, 7). (*b*) Same as *a* but in the congenic *ubc2*Δ strain BBY67. (*c*) Overexposure of the zero-time points from *a* to show multiubiquitination of the short-lived Arg-βgal and Leu-βgal and the absence of this modification in the long-lived Met-βgal. (*d*) Same as *c* but in the *ubc2*Δ cells.

vitro system that contains defined (cloned) components, including the *UBC2* gene product. Other relevant yeast genes, such as *UBA1* [encoding Ub-activating enzyme (15)], *UBR1* [encoding N-recognin (11)], and *UBP1-UBP3* [encoding Ub-specific processing proteases (J. Tobias, R. Baker, and A.V., unpublished data)], have recently become available as well.

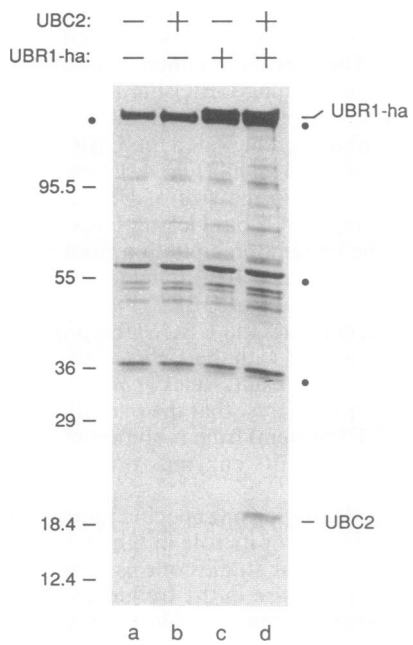

FIG. 4. UBC2 binds to UBR1. Extracts were prepared from [^{35}S]methionine-labeled BBY68 cells [*ubr1*Δ *ubc2*Δ*::LEU2*, a haploid derivative of YPH501 (40)] transformed with either the control high copy plasmid YEplac112 (ref. 38; a vector alone), the plasmid pSOB44 (expressing the epitope-tagged UBR1-ha from the *ADH1* promoter), or the plasmid pADHUBC2 (expressing UBC2 from the *ADH1* promoter). The extract containing UBC2 (7.5×10^4 acid-insoluble cpm) was mixed with either the control extract (1.5×10^5 cpm) lacking UBC2 and UBR1-ha (lane b) or the extract containing UBR1-ha (1.5×10^5 cpm) (lane d). The extract containing UBR1-ha (1.5×10^5 cpm) was also mixed with the control extract (7.5×10^4 cpm) (lane c). These three samples and the control extract alone (2.25×10^5 cpm) (lane a) were immunoprecipitated with a monoclonal antibody to the ha epitope (39), followed by SDS/PAGE and fluorography. The bands of UBR1-ha and UBC2 are indicated. Bands present in all four lanes (major species marked by dots) correspond to proteins that cross-react with the anti-ha monoclonal antibody. Molecular masses (in kDa) of prestained markers (Diversified Biotech) are shown on the left.

Genetic and biochemical approaches will now make possible precise distinctions between those functions of the UBC2 enzyme that are dependent on its interaction with N-recognin (11) (Fig. 4) and those mediated by other, still unknown UBC2-binding recognins specific for signals other than the N-degron. These distinctions will encompass growing and sporulating yeast cells; while both *UBR1* and *UBC2* are expressed during sporulation (ref. 33 and data not shown), the absence of the N-end rule pathway (in *ubr1* mutants) results in a mild sporulation defect (increased content of two-spore asci) (11), whereas the absence of both the N-end rule and other UBC2-dependent pathways (in *ubc2* mutants) either precludes (25, 26) or severely perturbs (31) sporulation.

Counterparts of the yeast *UBR1*-encoded N-recognin (11) and UBC2 Ub-conjugating enzyme (22) exist in mammals (5, 7, 13, 43, 44). Thus, a functional and mechanistic dissection of the yeast UBR1/UBC2 complex identified in the present work should also advance the understanding of the N-end rule pathway in higher organisms.

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