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Inhibition of the N-end rule pathway in living cells

(protein degradation/ubiquitin/yeast/peptides as inhibitors)

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ABSTRACT **The N-end rule relates the metabolic stability of a protein to the identity of its amino-terminal residue. Previous work, using amino acid derivatives such as dipeptides to inhibit N-end rule-mediated protein degradation in an extract from mammalian reticulocytes, has demonstrated the existence of specific N-end-recognizing proteins in this *in vitro* system. We now show that these nontoxic amino acid derivatives, when added to growing cells of the yeast *Saccharomyces cerevisiae*, are able to inhibit the degradation of proteins by the N-end rule pathway *in vivo*. Moreover, this inhibition is shown to be selective for the two distinct classes of destabilizing amino-terminal residues in substrates of the N-end rule pathway.**

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). This degradation signal is manifested as the N-end rule, which relates the metabolic stability of a protein to the identity of its amino-terminal residue (1–8). Similar but distinct versions of the N-end rule operate in mammals (2, 5, 8–11), yeast (1–5, 7, 12), and bacteria (J. Tobias and A.V., unpublished results).

The N-end rule-based degradation signal in eukaryotes comprises a *destabilizing* amino-terminal residue and a specific internal lysine residue (1–5, 8). The set of destabilizing amino-terminal residues is organized hierarchically. 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, a *primary* destabilizing residue (1, 5–7). 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 (5).

Previous work (1) has predicted the existence of "N-end-recognizing" proteins that select potential proteolytic substrates by binding to their amino-terminal residues. N-end-recognizing proteins have recently been detected in an *in vitro* ubiquitin-dependent proteolytic system derived from mammalian reticulocytes, and identified as the E3 proteins that were previously shown to bind proteolytic substrates prior to their ubiquitination by a subset of ubiquitin-conjugating (E2) enzymes (5, 9, 10, 12, 13). Three distinct types of E3 activity have been detected in the reticulocyte-derived *in vitro* system by using assays based on selective inhibition of the degradation of specific proteins by dipeptides bearing different destabilizing amino-terminal residues. The type I E3 activity is specific for the positively charged destabilizing amino-terminal residues Arg, Lys, and His (5, 9). The type II activity is specific for the bulky hydrophobic destabilizing amino-terminal residues Phe, Trp, Tyr, and Leu (and Ile in yeast) (5, 9). The type III activity is specific for the amino-terminal residues Ala, Ser, and Thr, which share the properties of small size and lack of charge (5). Ala, Ser, and Thr are destabilizing in reticulocytes but stabilizing in yeast, implying that *Saccharomyces cerevisiae* lacks type III E3 activity (1, 5).

The efficient and selective inhibition of the N-end rule pathway by amino acid derivatives in reticulocyte extract has prompted us to ask, using *S. cerevisiae* as a model system, whether a similar approach is feasible with intact cells. The ability to perturb the N-end rule pathway *in vivo* would provide a powerful tool for studies of this pathway, especially in organisms where genetic analysis is difficult, and might also prove useful in pharmacological and biotechnological applications. We show that the *in vivo* inhibition of the N-end rule pathway with amino acid derivatives is feasible and should be generally applicable.

METHODS

Amino Acid Derivatives, Yeast Strains, and Expression Vectors. Amino acid derivatives were obtained from Bachem Bioscience or Sigma and stored as either 0.2 M (dipeptide) or 1 M [leucine methyl ester (Leu-OMe)] solutions in synthetic defined (SD)-galactose medium [2% galactose, 0.67% yeast nitrogen base without amino acids (Difco), adenine (10 μg/ml), and other auxotrophic nutrients (14)] containing in addition 0.2 M potassium phosphate buffer (pH 7.0). In the inhibition experiments, control yeast cultures received equivalent amounts of potassium phosphate buffer in SD-galactose; the addition of buffer alone did not affect the levels of Xaa-β-galactosidase (X-βgal) proteins (data not shown). The L-Arg-D-Ala dipeptide was custom-synthesized and purified by Bachem Bioscience.

S. cerevisiae strain BWG1-7a (*MATa his4 ura3 ade1 leu2*) (15) transformed with 2μ-based vectors expressing ubiquitin-X-βgal (Ub-X-βgal) fusion proteins from the galactose-inducible *GAL1* promoter (1–3) was grown at 30°C in SD-galactose medium (14).

βgal Assay. Yeast cells were pelleted by centrifugation at 12,000 × *g* for 20 sec and resuspended in an equal volume of Z buffer (35 mM 2-mercaptoethanol/10 mM KCl/0.1 M potassium phosphate, pH 7.0). Aliquots from the suspension were diluted to 0.5 ml with Z buffer, followed by the addition of 20 μl of $CHCl_3$ and 20 μl of 0.1% SDS. The suspension was mixed with a Vortex mixer at top speed for 10 sec; βgal activity assays were then initiated (at 30°C) by adding 0.1 ml of *o*-nitrophenyl β-D-galactoside (4 mg/ml) and terminated by adding 0.5 ml of 1 M Na_2CO_3. The samples were centrifuged at 12,000 × *g* for 2 min and the OD_{420} of the supernatants was determined. βgal activity was calculated from OD_{420} readings normalized to cell density and the length of assay (16).

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Abbreviations: βgal, β-galactosidase; X-βgal, βgal bearing an amino-terminal residue X; Ub-X-βgal, ubiquitin-X-βgal; Leu-OMe, leucine methyl ester.

Pulse–Chase Analysis. Pulse labeling with [^{35}S]methionine, nonradioactive chase, extraction, immunoprecipitation with a monoclonal antibody to βgal, and electrophoretic analysis were carried out as described (1) except that cells were harvested by centrifugation at 2000 × *g* for 5 min, rather than by filtration, and that exponentially growing cultures (OD_{600} < 1) were used. To determine the degradation kinetics of X-βgal proteins, their electrophoretic bands were excised from scintillant-impregnated gels, and ^{35}S in the bands was determined.

RESULTS AND DISCUSSION

Effects of Amino Acid Derivatives on the *in Vivo* Levels of X-βgal Proteins. Preliminary experiments showed that, as expected (17), yeast cells can take up amino acid derivatives from their growth medium, because histidine in SD medium (14) could be replaced by an equimolar amount of the dipeptide His-Ala (all amino acids are L stereoisomers unless stated otherwise) to support the growth of histidine auxotrophs. Similarly, either Leu-Leu or Leu-OMe could substitute for leucine in supporting the growth of leucine auxotrophs (data not shown), implying that amino acid derivatives are converted, presumably after uptake, into the corresponding amino acids.

We next asked whether amino acid derivatives, once taken up by the cell, could inhibit the N-end rule pathway *in vivo*. The model substrates used were derivatives of *Escherichia coli* βgal. In eukaryotes, Ub-X-βgal fusion proteins are precisely deubiquitinated either *in vivo* or in cell-free extracts by an endogenous Ub-specific processing protease to yield X-βgal proteins bearing the residue X at the amino terminus (1, 5). 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-βgal proteins (1–8).

In preliminary experiments, yeast expressing various Ub-X-βgal proteins were plated on SD-galactose medium containing amino acid derivatives and the chromogenic βgal substrate 5-bromo-4-chloro-3-indolyl β-D-galactoside (see *Methods* and ref. 14). The results (not shown) were consistent with the possibility, to be confirmed below, that certain amino acid derivatives (but not equimolar amounts of their constituent free amino acids) could increase steady-state levels of normally short-lived X-βgal proteins. These effects were quantified by measuring the enzymatic activity of intracellular X-βgal proteins as a function of either the intracellular concentration of an amino acid derivative or the elapsed incubation time. An initial experiment (Fig. 1*A*) indicated that the effect of Leu-OMe (10 mM) on the level of Leu-βgal was stronger with exponentially growing cells (OD_{600} < 1). Further analyses were carried out with such cells.

The maximal effect of Leu-OMe (after 1 hr of incubation) on the levels of X-βgal proteins with type II (bulky hydrophobic) destabilizing amino-terminal residues (5, 9) was reached at ≈10 mM Leu-OMe (Fig. 1*B*). This maximally effective concentration was approximately the same for all of the type II destabilizing residues [Leu, Trp, and Tyr (Fig. 1*B*); Phe and Ile (data not shown)]. Notably, the level of Arg-βgal, which bears a type I (positively charged) destabilizing amino-terminal residue (5, 9), was unaffected by Leu-OMe (Fig. 1 *A* and *B*). At 10 mM, neither Leu-OMe nor dipeptides (see below) influenced the appearance (under a phase-contrast microscope) or growth rate of yeast cells (data not shown).

The level of Leu-βgal in cells was found to increase, over a period of at least 8 hr, in direct proportion to the length of incubation with Leu-OMe at 10 mM (Fig. 1*C*). As a result, after 8 hr of incubation with Leu-OMe, the level of Leu-βgal

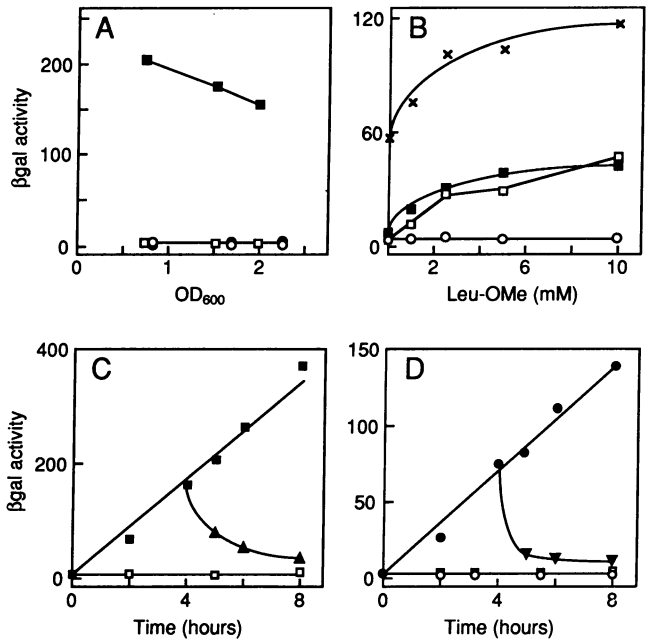

FIG. 1. Inhibition of the N-end rule pathway *in vivo*. (*A*) Levels of Leu-βgal and Arg-βgal as functions of cell density in *S. cerevisiae* cultures in the presence or absence of Leu-OMe. Strain BWG1-7a expressing Ub-Leu-βgal (□, ■) or Ub-Arg-βgal (○, ●) was grown in SD-galactose at 30°C. At different cell densities, Leu-OMe was added to half of each culture (■, ●) to a final concentration of 10 mM, incubation was continued for 3 hr, and βgal activity was determined and plotted against the final OD_{600} (see *Methods*). (*B*) Effect of varying extracellular Leu-OMe concentration on steady-state levels of X-βgal. Strain BWG1-7a expressing Ub-Arg-βgal (○), Ub-Leu-βgal (■), Ub-Trp-βgal (□), or Ub-Tyr-βgal (×) was grown as in *A* to OD_{600} of ≈1.0. Leu-OMe was then added to yield the indicated concentration, incubation was continued for 1 hr, and βgal activity was determined. (*C*) Time dependence and reversibility of the Leu-OMe effect on the level of Leu-βgal. Strain BWG1-7a expressing Ub-Leu-βgal was grown as in *A* to OD_{600} of ≈0.5. Leu-OMe was then either added to 10 mM (■) or not added (□), and βgal activity was determined over the next 8 hr of growth in the same medium. After 4 hr of growth, cells from half the culture containing Leu-OMe were collected by centrifugation, washed, resuspended, and grown further in SD-galactose lacking Leu-OMe (▲). (*D*) Time dependence and reversibility of the effect of Arg-containing dipeptides on the level of Arg-βgal. Strain BWG1-7a expressing Ub-Arg-βgal was grown as in *A* to OD_{600} of ≈0.5, followed by the addition of either Arg-Ala (to 10 mM; ●), Ala-Arg (to 10 mM; ○) or peptide-free buffer (see *Methods*) (□), and further growth for 8 hr. Cells from half the culture containing Arg-Ala were collected by centrifugation, washed, resuspended, and grown further in SD-galactose lacking Arg-Ala (▼).

in cells was ≈70-fold higher than that in cells from a parallel culture grown in the absence of Leu-OMe (Fig. 1*C*). The rate of increase in the level of Leu-βgal became progressively slower after 8 hr in the presence of Leu-OMe (data not shown).

The amount of Arg-βgal in cells increased ≈55-fold over its normal steady-state level after 8 hr of incubation with Arg-Ala dipeptide at 10 mM (Fig. 1*D*). Crucially, Ala-Arg, a dipeptide of the same composition as Arg-Ala but with a stabilizing amino-terminal residue, had no effect on the *in vivo* level of Arg-βgal (Fig. 1*D*), in agreement with the earlier observations in reticulocyte extract (5, 9) that the identity of the amino-terminal residue in a dipeptide determines its specificity and activity in the inhibition of the N-end rule pathway. [That Ala-Arg had actually been taken up by cells was indicated by its ability to support the growth of an arginine auxotroph (data not shown).] The *in vivo* inhibition of X-βgal degradation by amino acid derivatives was completely reversible: removal of an inhibitor from the medium

caused a gradual decrease of the previously elevated levels of either Leu-βgal or Arg-βgal down to the levels in control cultures (Fig. 1 *C* and *D*).

The addition of the Arg-Ala dipeptide to growing yeast cells metabolically stabilized Arg-, Lys-, and His-βgal but not the X-βgal proteins bearing bulky hydrophobic (type II) destabilizing amino-terminal residues (Leu, Phe, Trp, Tyr, or Ile). No X-βgal proteins were stabilized by the Ala-Arg dipeptide (Fig. 2). The Trp-Ala dipeptide, similarly to Leu-OMe (Fig. 1*B*), metabolically stabilized Leu-, Phe-, Trp-, Tyr-, and Ile-βgal but did not have an effect on any other X-βgal tested, including Arg-, Lys-, and His-βgal (Fig. 2). No X-βgal proteins were stabilized by the Ala-Trp dipeptide (Fig. 2). These results provided *in vivo* evidence for the existence of type I and type II primary destabilizing residues, a feature of the N-end rule pathway originally defined in reticulocyte extract (5, 9). [Ile is a "borderline" stabilizing residue in the reticulocyte N-end rule but a type II primary destabilizing residue in the yeast N-end rule (1, 3, 5). See ref. 5 for a discussion of the distinction between stabilizing and destabilizing residues in the N-end rule.]

The recent cloning of the gene (*UBR1*) for an N-end-recognizing (E3) protein of *S. cerevisiae* has made possible *in vitro* tests of the recognition specificity of this 225-kDa protein (12). UBR1 specifically binds *in vitro* to proteins bearing either type I or type II primary destabilizing amino-terminal residues but does not bind to otherwise identical proteins bearing stabilizing amino-terminal residues (12). Similar conclusions have been reached from the analysis of the partially purified counterpart of UBR1 from rabbit reticulocytes (18). Since the type I and type II binding activities can be inhibited independently in the yeast UBR1 *in vivo* (Fig. 2) and in a mammalian counterpart of UBR1 *in vitro* (5, 9, 18), it is likely that the corresponding binding sites are distinct in both of these proteins.

The Arg-Ala dipeptide, while increasing the levels of X-βgal proteins bearing type I (basic) destabilizing amino-terminal residues (Fig. 2), decreased the levels of X-βgal proteins bearing type II (bulky hydrophobic) destabilizing amino-terminal residues. For instance, after 3 hr in the presence of Arg-Ala, the levels of Tyr-βgal and Leu-βgal were decreased, respectively, to ≈80% and ≈30% of their levels in the absence of the dipeptide (Fig. 2 and data not shown). The "converse" effect was not observed, however: Trp-Ala, while increasing the levels of X-βgal proteins bearing type II destabilizing residues, did not affect the levels of X-βgal proteins bearing type I destabilizing residues (Fig. 2). These *in vivo* data with yeast cells corroborated the analogous *in vitro* findings with reticulocyte extracts (5, 9). One interpretation of these results is that dipeptides with type I (basic) amino-terminal residues inhibit the degradation of endogenous substrates with basic amino termini in both yeast cells and reticulocyte extract and thereby reduce competition for some limiting component of the N-end rule pathway. Since binding sites for the type I and type II primary destabilizing amino-terminal residues are located within the same protein (see above), an alternative possibility is that occupation of a type I binding site in either a yeast or a reticulocyte N-end-recognizing protein increases the efficiency of recognition by that protein of type II proteolytic substrates, whereas occupancy of the type II binding site does not enhance the recognition of type I substrates.

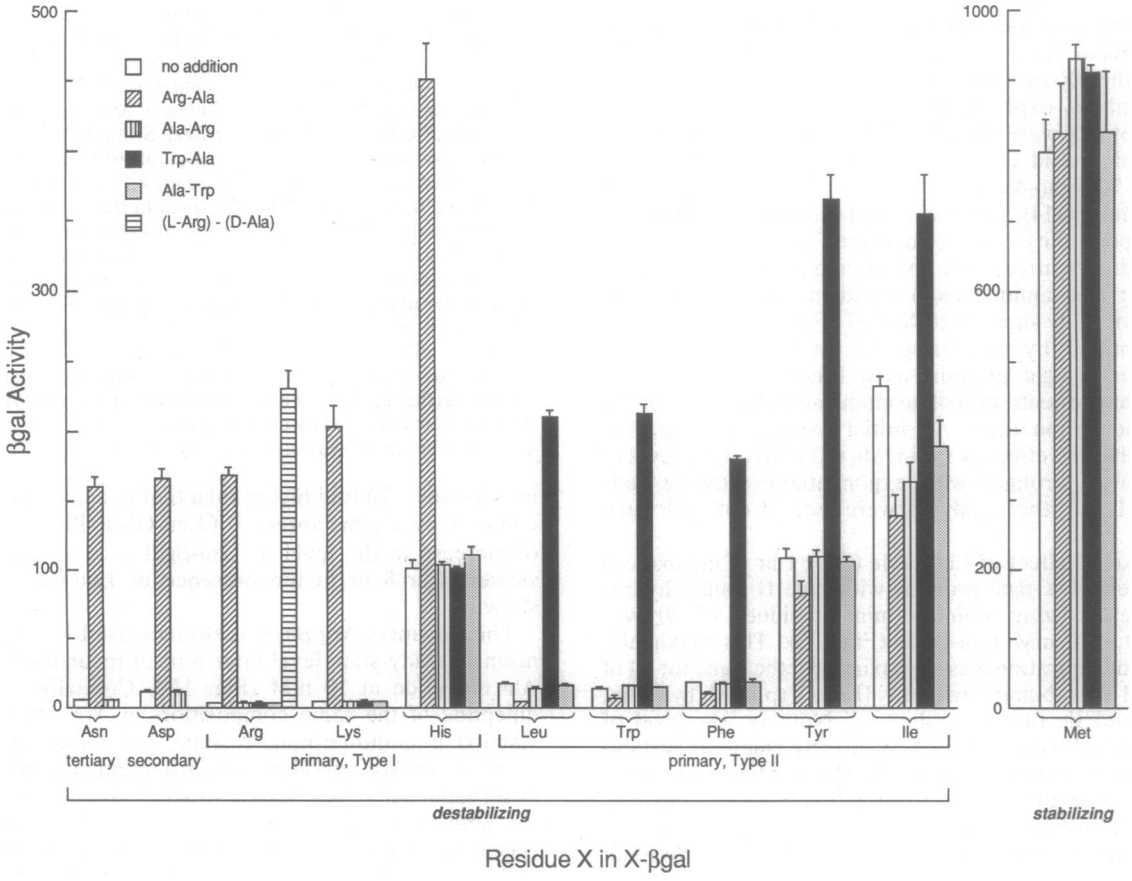

FIG. 2. Effects of dipeptides on the levels of X-βgal proteins in *S. cerevisiae*. Cells expressing various Ub-X-βgal proteins were grown at 30°C in SD-galactose to OD_{600} of ≈0.5. An indicated dipeptide was then added to the medium to a final concentration of 10 mM. Extracts were prepared from cells 3 hr later and assayed for βgal activity. Values shown are the means of at least three independent measurements. Standard deviations are shown above the bars.

In addition to stabilizing Arg-, Lys-, and His-βgal, the Arg-Ala (but not Ala-Arg) dipeptide metabolically stabilized Asp-βgal and Asn-βgal, which bear, respectively, a secondary and a tertiary destabilizing amino-terminal residue (Fig. 2; see Introduction for terminology). This result provided an *in vivo* confirmation of the hierarchical organization of the N-end rule, in which a secondary (and after deamidation, a tertiary) destabilizing residue is conjugated, via Arg-tRNA–protein transferase, to arginine, a type I primary destabilizing residue (5–7).

Independent *in vivo* confirmation of these aspects of the N-end rule was provided by the recent cloning of the *S. cerevisiae* genes *ATE1* and *DEA1*, which encode, respectively, Arg-tRNA–protein transferase (7) and a deamidase specific for amino-terminal Asn and Gln (unpublished results). As expected from the hierarchical structure of the N-end rule (5), null *dea1* mutants are unable to degrade substrates that start with tertiary destabilizing residues (Asn or Gln), whereas null *ate1* mutants are unable to degrade substrates that start with either tertiary (Asn or Gln) or secondary (Asp or Glu) destabilizing residues (ref. 7 and unpublished data).

Recently, Kopitz *et al.* (19) reported that a short-lived enzyme, ornithine decarboxylase, could be arginylated in extracts from rat hepatocytes by an endogenous Arg-tRNA–protein transferase, and furthermore, that the *in vivo* degradation of ornithine decarboxylase in hepatocytes could be partially suppressed by the tripeptide Glu-Val-Phe, a substrate of Arg-tRNA–protein transferase. While still indirect, these results suggest that ornithine decarboxylase is a substrate of the N-end rule pathway that bears a secondary or a tertiary destabilizing amino-terminal residue. If so, it should now be possible to use amino acid derivatives to inhibit, *in vivo*, both the amino-terminal modification of such proteins and their subsequent recognition by E3.

One parameter that is expected to determine the effectiveness of an amino acid derivative as an *in vivo* inhibitor of the N-end rule pathway is the derivative's metabolic stability. To see whether making a dipeptide more resistant to proteolytic cleavage would augment its inhibitory effect, we tested the dipeptide L-Arg-D-Ala, whose second residue is a D-stereoisomer, and found that this dipeptide was more effective in raising steady-state levels of the relevant short-lived X-βgal proteins than was the Arg-Ala dipeptide in which both residues are L-stereoisomers (Fig. 2). Whether the greater effectiveness of L-Arg-D-Ala is due to its (expected) longer half-life *in vivo* or to other reasons, such as higher rate of uptake, remains to be determined.

Metabolic Stabilization of X-βgal Proteins *in Vivo*. Pulse–chase analysis was used to follow the degradation of newly formed X-βgal proteins in the presence or absence of amino acid derivatives (Figs. 3 and 4). As noted previously (1, 3), the *in vivo* degradation of X-βgal proteins did not obey first-order kinetics but instead slowed with time. More precisely, the kinetics of degradation deviated from first order when sufficiently long chase periods (>10 min at 30°C) were used (Fig. 4*A*). For chases shorter than ≈10 min (with 1-min pulses), the degradation kinetics were close to first order for all of the short-lived X-βgal proteins tested (Fig. 4 *B* and *C*; data not shown).

The data of Figs. 3 and 4 confirmed that the increases in steady-state levels of X-βgal proteins in the presence of relevant amino acid derivatives (Fig. 2) were due to metabolic stabilization of normally short-lived X-βgals. For instance, the half-life of Leu-βgal during the first 10 min of chase was ≈3 min in the absence of Leu-OMe and ≈30 min in its presence (Fig. 4*B*). Similarly, averaging over the first 10 min of chase for Arg-βgal (its *in vivo* degradation reproducibly deviated from first-order kinetics even earlier than the degradation of Leu-βgal) yielded half-lives of ≈2 min in the absence of the Arg-Ala dipeptide and ≈20 min in its presence (Fig. 4*C*). [Northern hybridization analyses (data not shown) indicated that the addition of amino acid derivatives did not change the levels of mRNAs encoding the corresponding X-βgal proteins.] Pulse–chase analysis also detected two further changes that have previously been shown to accompany metabolic stabilization of X-βgal proteins: the diminished accumulation of an ≈90-kDa βgal cleavage product specific for short-lived X-βgal species (1, 4), and the appearance of an ≈95-kDa βgal cleavage product specific for long-lived X-βgal species (4) (Fig. 3).

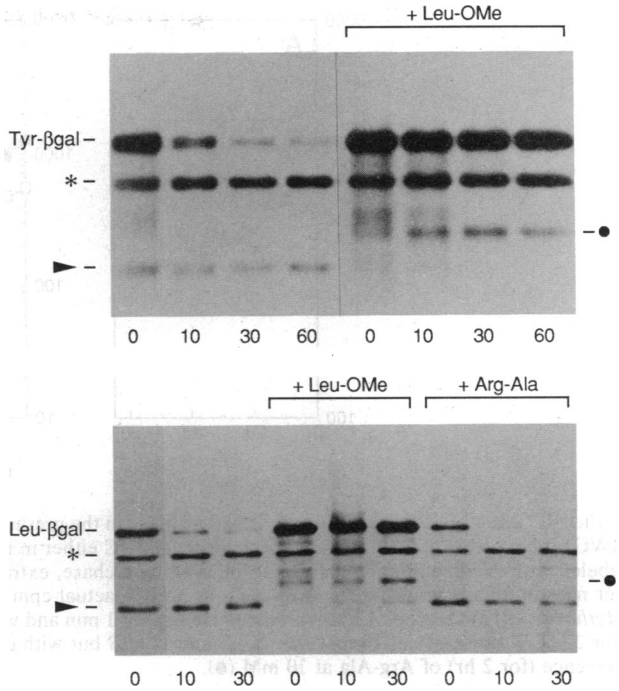

FIG. 3. Pulse–chase analysis of X-βgal degradation in the presence of amino acid derivatives. Exponential cultures of *S. cerevisiae* strain BWG1-7a expressing Ub-Tyr-βgal or Ub-Leu-βgal were labeled with [^{35}S]methionine for 5 min at 30°C (lanes 0) followed by a chase in the presence of unlabeled methionine (10 mM) 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 (see *Methods*). Where indicated, a 60-min (lanes 60) chase point was also taken. In the experiments where amino acid derivatives were present (at 10 mM) during growth, labeling, and chase, their names are shown above the lanes. Identities of X-βgal proteins are indicated at left. Arrowhead denotes an ≈90-kDa long-lived βgal cleavage product specific for short-lived X-βgal proteins (1, 4). Filled circle (at right) denotes an ≈95-kDa long-lived βgal cleavage product specific for long-lived X-βgal proteins (4). Asterisk denotes an unknown *S. cerevisiae* protein that crossreacts with the monoclonal antibody to βgal (1).

Another effect of the amino acid derivatives was an increase in the amount of ^{35}S in the pulse-labeled X-βgal species (a "zero-time" increase) that was especially pronounced for the shorter-lived proteins such as Leu- and Arg-βgal (Fig. 3 and Fig. 4 *B* and *C*). The zero-time increase was expected because metabolic stabilization of a protein will result in its greater net incorporation of label during a pulse of constant duration, since a smaller fraction of the stabilized protein is degraded during this time.

We do not understand the reasons for non-first-order kinetics of X-βgal degradation in yeast (Fig. 4*A*, refs. 1 and 3, and data not shown). It was due neither to the use of translation inhibitors during the chase [because similar results were obtained in the absence of inhibitors (M. Hochstrasser, personal communication)] nor to other features of the degradation assay, because in a similar *in vivo*

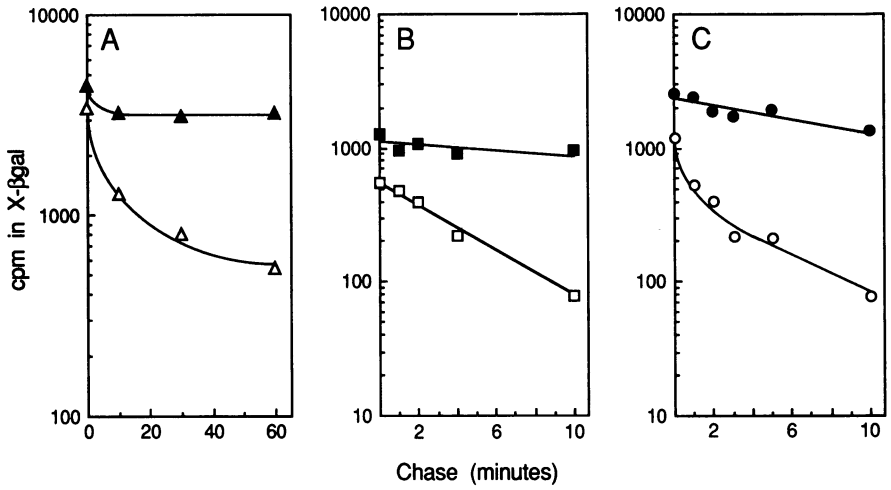

FIG. 4. Metabolic stabilization of X-βgal proteins in the presence of amino acid derivatives. (*A*) Exponential cultures of *S. cerevisiae* strain BWG1-7a expressing Ub-Tyr-βgal and growing at 30°C either in the absence (△) or in the presence (for 2 hr) of Leu-OMe at 10 mM (▲) were labeled with [^{35}S]methionine for 5 min, followed by a chase, extraction, immunoprecipitation, and electrophoretic analysis of X-βgal. Counts per minute (cpm) shown on the ordinate axis are the actual cpm (minus background) in slices of a gel containing the bands of Tyr-βgal (see *Methods*). (*B*) Same as in *A* but with the pulse time of 1 min and with cells expressing Ub-Leu-βgal either in the absence (□) or in the presence (for 2 hr) of Leu-OMe at 10 mM (■). (*C*) Same as in *B* but with cells expressing Ub-Arg-βgal and growing either in the absence (○) or in the presence (for 2 hr) of Arg-Ala at 10 mM (●).

assay, the short-lived MATα2 repressor was degraded in yeast with first-order kinetics (20). Among potential explanations of the non-exponential X-βgal degradation is a hypothesis that the assembly and "maturation" of an X-βgal tetramer make it progressively more resistant to targeting and/or degradation by the N-end rule pathway. Another possible explanation is that an essential targeting component of the N-end rule pathway has affinity for ribosomes, so that a newly formed substrate of the N-end rule pathway, being in the immediate vicinity of a ribosome, has a greater chance of being targeted for degradation than the same substrate at later times and farther away from the sites of translation.

Irrespective of its specific explanations, the non-first-order kinetics of X-βgal degradation makes it possible to account for the apparent discrepancy between, for instance, an ≈10-fold metabolic stabilization of Leu-βgal by Leu-OMe in the 1-min pulse/10-min chase assay (Fig. 4*B*), and an ≈70-fold increase in the level of the same Leu-βgal after 8 hr of incubation with Leu-OMe (Fig. 1*C*). Indeed, since the *in vivo* degradation of an X-βgal slows with time (Fig. 4*A*), a partial metabolic stabilization by an amino acid derivative of more recently formed (and hence shorter-lived) X-βgal molecules would increase the steady-state level of an X-βgal to a greater extent than the same degree of metabolic stabilization of "older" (and hence longer-lived) X-βgal molecules. This argument accounts for the relative increase of an "early" half-life of an X-βgal (Fig. 4 *B* and *C*) being lower than the relative increase of its steady-state level in the presence of an amino acid derivative (Fig. 1 *C* and *D*). (For a first-order decay in the absence of changes in the rate of protein synthesis, the relative increase of a protein's half-life equals the relative increase in its maximal steady-state concentration.)

The ability to inhibit the N-end rule pathway *in vivo* may have practical applications and, in addition, provides a powerful tool for studies of this pathway.

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