Ubiquitin-independent Mechanisms of Mouse Ornithine Decarboxylase Degradation Are Conserved between Mammalian and Fungal Cells

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Received for publication, November 20, 2002, and in revised form, January 30, 2003
Published, JBC Papers in Press, January 31, 2003, DOI 10.1074/jbc.M211802200

The polyamine biosynthetic enzyme ornithine decarboxylase (ODC) is degraded by the 26 S proteasome via a ubiquitin-independent pathway in mammalian cells. Its degradation is greatly accelerated by association with the polyamine-induced regulatory protein antizyme 1 (AZ1). Mouse ODC (mODC) that is expressed in the yeast Saccharomyces cerevisiae is also rapidly degraded by the proteasome of that organism. We have now carried out in vivo and in vitro studies to determine whether S. cerevisiae proteasomes recognize mODC degradation signals. Mutations of mODC that stabilized the protein in animal cells also did so in the fungus. Moreover, the mODC degradation signal was able to destabilize a GFP or Ura3 reporter in GFP-mODC and Ura3-mODC fusion proteins. Co-expression of AZ1 accelerated mODC degradation 2–3-fold in yeast cells. The degradation of both mODC and the endogenous yeast ODC (yODC) was unaffected in S. cerevisiae mutants with various defects in ubiquitin metabolism, and ubiquitinylated forms of mODC were not detected in yeast cells. In addition, recombinant mODC was degraded in an ATP-dependent manner by affinity-purified yeast 26 S proteasomes in the absence of ubiquitin. Degradation by purified yeast proteasomes was sensitive to mutations that stabilized mODC in vivo, but was not accelerated by recombinant AZ1. These studies demonstrate that cell constituents required for mODC degradation are conserved between animals and fungi, and that both mammalian and fungal ODC are subject to proteasome-mediated proteolysis by ubiquitin-independent mechanisms.

The 26 S proteasome is the major neutral protease of the cytoplasmic and nuclear compartments of eukaryotic cells. It is a multisubunit, ATP-dependent, protease composed of at least two subcomplexes: a barrel-shaped 20 S core particle that sequesters the proteolytic active sites from the intracellular environment, and a 19 S regulatory particle that contains activities required for recognition, unfolding, and translocation of substrates to the interior of the core particle (1). Its substrate proteins include major regulators of cell growth and differentiation, and also proteins that fail to fold into a native conformation. The proteasome needs therefore to recognize appropriate substrates accurately to support proper timing of cellular events and to eliminate proteins that have failed to follow normal folding pathways. Many of these short-lived proteins are targeted to the proteasome by the conjugation of ubiquitin, an evolutionarily conserved 76-amino acid globular protein (2). The efficient recognition of ubiquitinylated substrates by the proteasome requires, minimally, the formation of a tetraubiquitin chain on the substrate protein (3). The polyubiquitin signal is in turn recognized by a receptor site that includes the Rpt5/S6 ATPase subunit of the regulatory particle (4). Additionally, the proteasome has the capacity to recognize a class of substrates that do not require ubiquitin modification for their regulated degradation (5). These include the cyclin-dependent kinase inhibitor p21 and ornithine decarboxylase (ODC),1 which catalyzes the initial step in polyamine biosynthesis. In most eukaryotic cells, ODC is subject to regulation by spermidine and spermine, the end products of the polyamine biosynthetic pathway. These act not through allosteric feedback regulation, the usual mechanism, but by changing the abundance of the ODC protein (6). This form of feedback regulation is accomplished by an autoregulatory circuit composed of ODC, polyamines, and the regulatory protein antizyme 1 (AZ1) (7). Excess polyamines induce a +1 translational frame-shift of the AZ1 mRNA that is required to align a short upstream open reading frame (ORF) with a downstream ORF encoding the functional protein (8). AZ1 binds the ODC monomer, dissociating the enzymatically active ODC homodimer and thereby inhibiting its activity (9). AZ1 binding exposes a COOH-terminal degradation signal in the ODC protein that leads to an increased rate of degradation of ODC by the proteasome (10). The AZ1-dependent degradation of ODC by the proteasome is remarkable in that it occurs in mammalian cells independently of the ubiquitinylation of ODC. Both in vivo data (11, 12) and in vitro data (13, 14) support this conclusion.

The ODC-AZ regulatory circuit has been best characterized in mammalian cells, but functional AZ homologs have also been described in a number of other organisms, including the nematode Caenorhabditis elegans (15), the fly Drosophila melanogaster (16), several filamentous fungi, and the fission yeast Schizosaccharomyces pombe (15, 17). However, no AZ homolog is evident in the genome sequence of the budding yeast Saccharomyces cerevisiae (18). In this organism, polyamine regu-

1 The abbreviations used are: ODC, ornithine decarboxylase; AZ1, antizyme 1; GFP, Aequorea victoria green fluorescent protein; HA, hemagglutinin; mODC, Mus musculus ornithine decarboxylase; ORF, open reading frame; yODC, Saccharomyces cerevisiae ornithine decarboxylase; E1, ubiquitin-activating enzyme; Ub, ubiquitin.

‡ Supported by National Research Service Award Postdoctoral Fellowship GM20527 from the National Institutes of Health.
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This work was supported in part by Grant GM45335 from the National Institutes of Health (to P. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The on-line version of this article (available at http://www.jbc.org) contains Fig. 1.
lution of ODC resembles that of mammalian cells in that excess polyamines increase the rate of ODC degradation (19). Polyamine regulation in S. cerevisiae also involves de novo protein synthesis (20), a requirement of an AZ-like regulatory mechanism, but not of polyubiquitinylation. Both yeast and mouse ODC are rapidly degraded by the 26 S proteasome in S. cerevisiae (19, 21).

Proteasome structure is highly conserved between mammals and fungi, and so too is the general use of ubiquitin modification as a mechanism for recognition of substrates. We asked whether, and to what extent, mammalian and yeast proteasomes also share the capacity to recognize and respond to the structural hallmarks that are used in mammalian cells for the constitutive and regulated degradation of ODC. We show here that for both yeast and mouse ODC, degradation in yeast cells does not depend on ubiquitin. We found that recognition of mouse ODC requires similar structural and functional elements, regardless of the source of the proteasome, implying that ubiquitin-independent substrates, like those that depend on ubiquitin, must utilize a conserved discriminatory capacity of the proteasome.

EXPERIMENTAL PROCEDURES

Yeast Strains—Yeast strains were maintained and manipulated using standard protocols (22). For analysis of FL* mODC and yODC FL* FLAG turnover (reported in Figs. 1, 3, and 4), the appropriate expression vectors were transformed into strain Y13 (MATa his3 leu2 trp1 ura3 spe4::HIS3 trp1::URA3) (23), a polyamine auxotroph (19). Growth in defined medium of polyamine auxotrophic strains was maintained by addition of 0.1 μm spermidine, a concentration insufficient for repression of endogenous yODC; the level of spermidine in the medium was increased to 1 mM to induce repression of yODC. Vectors for the expression of GFP or Ura3 fusion proteins (Fig. 2) were transformed into strain MHY7402 (MATa his3 leu2-3,112 lys2-801 trp1-1 ura3-52 XbaI/EcoRI site following the stop codon of the mODC ORF) into the similarly digested ex-}

Conservation of ODC Degradation Mechanisms

GFP-mODC fusions were constructed by splice overlap extension PCR (SOE-PCR) using a GFPGuv vector (BD Sciences Clontech) as a template. A PCR fragment containing the COOH-terminal half of the GFP ORF was generated by a sense primer overlapping the XhoI site within the GFPGuv coding region, and an antisense primer complementary to the COOH terminus of GFPGuv and amino acids 425–461 of mODC. A second PCR fragment was generated by amplification of the COOH terminus of the mODC or mODC Δ444 coding region using a sense primer complementary to the antisense primer used to generate the GFP fragment and an antisense primer that appended an EcoRI site following the stop codon of the mODC ORF. Following SOE-PCR, the resulting DNA fragments were digested with XhoI and EcoRI, and ligated into a similarly digested pGFPGuv vector from which the fragment containing the COOH terminus of GFPGuv was removed. The ORFs encoding GFPGuv and GFP-mODC fusion proteins were subcloned from the pGFPGuv-based vectors into yeast expression vectors as XbaI-EcoRI fragments.

Ura3-mODC fusions were constructed similarly to the GFP-mODC fusions by SOE-PCR. The coding region of URA3 was amplified from the pRS306vector (34) using a sense primer that appended an NH2-terminal FLAG epitope tag and a flanking BamHI site, and an antisense primer complementary to the COOH terminus of URA3 and amino acids 425–461 of mODC. A DNA fragment containing the COOH terminus of mODC was generated using a sense primer complementary to the antisense primer used to amplify the URA3 coding region and an antisense primer that appended an EcoRI site following the stop codon of the mODC ORF. Following SOE-PCR, the DNA fragment encoding Ura3-mODC was digested with BamHI and EcoRI and ligated into similarly digested yeast expression vectors.

Metabolic Labeling, Immunoprecipitations, and Immunoblotting— Pulse-chase analysis was carried out similarly to the protocol described by Suzuki and Varehovsky (35). 10 ml cultures of yeast transformants were grown to midexponential growth phase (A600 0.5–1). Cells were harvested by centrifugation (3 min, 2000 × g), and washed twice in 1 ml of SD medium lacking methionine (SD –Met). The cells were resuspended in 0.4 ml of SD –Met and labeled for 5 min at 30 °C with 200 μCi of a L-[35S]methionine and L-cysteine mixture (Exper 35S35S protein labeling kit, Amersham Pharmacia Biotech). To monitor the degradation of GFP-mODC fusions were constructed by splice overlap extension PCR (SOE-PCR) using a pGFPuv vector (BD Sciences Clontech) as a template. A PCR fragment containing the COOH-terminal half of the GFP ORF was generated by a sense primer overlapping the XhoI site within the GFPGuv coding region, and an antisense primer complementary to the COOH terminus of GFPGuv and amino acids 425–461 of mODC. A second PCR fragment was generated by amplification of the COOH terminus of the mODC or mODC Δ444 coding region using a sense primer complementary to the antisense primer used to generate the GFP fragment and an antisense primer that appended an EcoRI site following the stop codon of the mODC ORF. Following SOE-PCR, the resulting DNA fragments were digested with XhoI and EcoRI, and ligated into a similarly digested pGFPGuv vector from which the fragment containing the COOH terminus of GFPGuv was removed. The ORFs encoding GFPGuv and GFP-mODC fusion proteins were subcloned from the pGFPGuv-based vectors into yeast expression vectors as XbaI-EcoRI fragments.

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col. Filters were blocked in Tris-buffered saline + 1% Triton X-100 (TBS-T) including 5% powdered low-fat milk and 5% bovine serum albumin, and washed 3 times for 5 min with TBS-T. FLAG-tagged proteins were detected with mouse anti-FLAG M2 antibody (Sigma, 1:2000 dilution) and sheep anti-mouse IgG-horseradish peroxidase conjugates (Amersham Biosciences, 1:1500 dilution).

For detection of ubiquitin conjugates, cells were grown and harvested as described above, but lysis and immunoprecipitations were carried out in buffers containing 50 mM N-ethylmaleimide. Hemagglutinin-ubiquitin conjugates were detected, following fractionation by SDS-PAGE and transfer to nitrocellulose, by incubation with anti-IA-horse-radish peroxidase conjugates (Santa Cruz Biotecnology, 1:1000 dilution).

Proteasome Purification—Proteasomes were affinity purified from RJ1144 cells as described (28). Normally, this purification protocol involves separating tagged proteasome complexes from extracts using anti-FLAG antibody conjugated to agarose beads followed by elution of the complexes with FLAG peptide. We have found that the FLAG peptide causes activation of the normally gated 20 S core particle that results in a reproducible ATP-independent degradation of mODC. To avoid exposure to the FLAG peptide, we used proteasome complexes still associated with the antibody-agarose, a form active for ATP-de-
dependent proteolysis of mODC. Briefly, cells were grown to early sta-
tionary phase (A600 2–3) in 2 liters of SD medium and harvested by centrifugation (2000 g, 20 min). The cells were pooled and washed in 100 ml of ice-cold water. The cell pellet was resuspended in an equal volume of buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCl2, 10% glycerol) containing 5 mM ATP and drop frozen in liquid N2 in a mortar resting in a bed of dry ice. The cells were ground with a pestle for 30 min with periodic additions of liquid N2 to keep the material frozen. The ground cells were thawed and most cell debris was removed from the lysate by centrifugation (4000 g, 20 min). The supernatant was removed and released counts were measured in a liquid scintillation counter. Percentage degradation of radiolabeled mODC proteins throughout a 1-h chase period following metabolic

epitope tag to the NH2 terminus of mouse ODC (FLAGmODC). The tagged mODC was expressed from yeast centromeric expression vectors (33) in a spe1Δspe2Δ transformants. Wild type FLAGmODC was expressed from the ADH (PADH) or GPD (PGPD) promoters in p414 vectors. COOH-terminal mutants were all expressed from the ADH promoter of p414ADH. Epitope-tagged mODCs were immunoprecipitated with anti-FLAG antibody.

Mouse ODC expressed in yeast cells is rapidly degraded by the 26 S proteasome (21). We compared, in yeast transfor-
mants, the degradation of FLAG-tagged wild type mODC expressed from either the yeast ADH promoter or the stronger GPD promoter. We found that wild type FLAGmODC expressed from the ADH promoter was easily detectable by immunoblotting (Fig. 1A) and rapidly degraded in spe1Δspe2Δ cells with a half-life of ~10 min, as measured by pulse-chase analysis (Fig. 1B). The expression of FLAGmODC protein from a vector contain-
ing the more active GPD promoter led to a 3-fold stabilization of FLAGmODC in those transformants (half-life = ~30 min, Fig. 1B), suggesting that overexpression interferes with normal degradation of mODC as it does with turnover of endogenous yODC (19, 36, 37).

We used vectors containing the ADH promoter to compare turnover of wild type and mutant forms of mODC in yeast cells. The mODC mutants analyzed included truncated forms lacking the COOH-terminal 5 or 37 amino acids of the mODC, and full-length mODC containing a Cys441 to Ala mutation. The COOH-terminal 37 amino acids of mODC (residues 425–461) contains a signal required for its rapid degradation in animal cells or in vitro (10, 38), and both Cys441 and the 5 terminal residues in this region are critical for its function (31, 39). Truncation of 5 or 37 residues from the COOH terminus, or the mutation of Cys441 to Ala, led to greater expression of these proteins than of the wild type FLAGmODC in exponentially growing cultures (Fig. 1A), and significant stabilization of these proteins throughout a 1-h chase period following metabolic

RESULTS

Carboxyl-terminal Recognition Elements of Mouse ODC Are Recognized in Yeast—To facilitate studies of mODC degrada-
tion in S. cerevisiae, we appended a single copy of the FLAG

Fig. 1. COOH-terminal mODC mutants are stable in yeast cells. A, immunoblot analysis with anti-FLAG antibody (Sigma) of spe1Δspe2Δ cells transformed with p414ADH vectors expressing wild type FLAGmODC or COOH-terminal mutants. B, pulse-chase analysis of FLAGmODC in spe1Δspe2Δ transformants. Wild type FLAGmODC was expressed from the ADH (PADH) or GPD (PGPD) promoters in p414 vectors. COOH-terminal mutants were all expressed from the ADH promoter of p414ADH. Epitope-tagged mODCs were immunoprecipitated with anti-FLAG antibody.

* M. A. Hoyt and P. Coffino, unpublished results.
* M. Zhang, C. M. Pickart, and P. Coffino, EMBO J., in press.

The fluorescence of GFPuv and GFP-
Conservation of ODC Degradation Mechanisms

Fig. 2. GFP- and Ura3-mODC fusion proteins are unstable in yeast cells. A, immunoblot analysis with anti-GFP antibody of MHY501 transformed with p416ADH vectors expressing wild type GFPuv or a GFPuv fusion to amino acids 425–461 of mODC. The GFP fluorescence of transformants expressing the corresponding proteins is indicated in arbitrary fluorescence units. B, pulse-chase analysis of GFPuv and GFP-mODC fusion protein turnover in yeast transformants. GFPuv and GFP-mODC fusions were immunoprecipitated with anti-GFP antibody and protein G-Sepharose. *Arrow* indicates the position of the appropriate proteins. C, pulse-chase analysis of FLAG-Ura3-mODC and FLAG-Ura3-mODC(C441A) fusion protein turnover in yeast transformants. FLAG-Ura3-mODC fusions were immunoprecipitated with anti-FLAG M2 affinity gel. D, immunoblot analysis with anti-GFP antibody of a pre1-1 pre2-2 mutant (WC44-1122a) or a congenic *PRE1* *PRE2* wild type strain (WC44a) transformed with GFPuv and GFPuv-mODC expression vectors. Extracts from mutant and wild type cells were prepared and analyzed in parallel and immunobots were identically exposed.

Labeling (Fig. 1B). Thus, COOH-terminal mutations that alter mODC degradation in animal cells similarly affect this process in yeast cells.

The Carboxyl Terminus of Mouse ODC Is a Portable Proteosome Recognition Element in Yeast—Because mODC is unstable in yeast cells, and truncation of its COOH terminus leads to stabilization, we asked whether this region of mODC conferred rapid turnover when attached to either a heterologous or endogenous protein expressed in *S. cerevisiae*. The fusion of the COOH-terminal 37 amino acids of mODC to the COOH terminus of *Trypanosoma brucei* GFP or GFP leads to the rapid turnover of these otherwise stable proteins in mammalian cells (40, 41). We attached the COOH-terminal 37 amino acids of mODC (425–461) to the COOH terminus of either GFP from *Aequorea victoria*, or orotidine-5'-monophosphate decarboxylase encoded by the *URA3* gene of *S. cerevisiae*. We constitutively expressed the GFP-mODC fusion protein and unmodified GFP in yeast transformants from the *ADH1* promoter. Immunoblot analysis with an anti-GFP antibody (Fig. 2A) showed that the steady-state level of GFP-mODC was greatly reduced compared with that of unmodified GFP in the yeast transformants. GFP fluorescence was similarly reduced in cells expressing the GFP-mODC fusion (Fig. 2A).

We measured the degradation of GFP and the GFP-mODC fusion in yeast cells directly by pulse-chase analysis. Whereas unmodified GFP was stable throughout the 30-min chase period, the GFP-mODC fusion was degraded with a half-life of ~10 min (Fig. 2B), confirming that the COOH-terminal 37 amino acids of mODC behaved similarly as a degradation signal in fungal and mammalian cells. We conclude that the decreased expression of GFP, to which the COOH-terminal portion of mODC has been appended, was caused by its increased rate of degradation. We found similar effects when we attached these mODC sequences to the COOH terminus of the *S. cerevisiae* URA3 ORF, which encodes a normally stable protein (Fig. 2C). Thus, the mODC COOH terminus appears capable of acting in cis to impart instability to a number of unrelated proteins in yeast cells.

Whereas appending the COOH terminus of mODC could import a cis-acting degradation signal to GFP (or Ura3) functionally akin to that present in native mODC, it is possible that the attachment of these sequences could result in some other effect, such as misfolding, which could result in accelerated degradation. To rule this out, we introduced a C441A mutation in the GFP- and Ura3-mODC fusion proteins. Pulse-chase analysis demonstrated that the C441A mutation led to the stabilization of both fusion proteins (Fig. 2, B and C). This mutation should prevent degradation effects specific to the mODC COOH terminus, but not more general effects, such as perturbation of structural integrity, which could lead to rapid degradation of the fusion protein. Additionally, the GFP-mODC fusion protein was fluorescent (Fig. 2A), and the Ura3-mODC fusion protein supported the growth of a ura3 mutant (data not shown), indicating that the mODC extensions did not perturb the normal functions of these proteins. We therefore conclude that the destabilization of the fusion proteins is because of the presence of the mODC COOH terminus specifically, rather than misfolding or other artificial characteristics of the fusion protein.

To confirm that the reduction in steady-state levels of the fusion proteins was because of increased degradation by the 26 S proteasome, we examined expression of the GFP-mODC fusion protein in a *pre1-1 pre2-2* mutant. This mutant carries mutations in the β4 and β5 subunits of the 20 S core particle and shows marked defects in the degradation of proteosome substrates (24). The GFP-mODC fusion protein accumulated to a significantly higher steady-state level in the *pre1-1 pre2-2* mutant when compared with the congenic wild type strain, whereas the abundance of the GFP control was unchanged (Fig. 2D). In summary, these results demonstrate that, as in mammalian cells, the COOH terminus of mODC is sufficient to confer proteosome-mediated proteolysis on an otherwise stable protein in *S. cerevisiae*, and that Cys441 is critical for this function.

Effects of Polyamine Pool Repletion and Antizyme on Turnover of Mouse and Yeast ODC—in mammalian cells, increases in cellular polyamine pools leads to the rapid degradation of mODC, a process mediated by *de novo* synthesis of the regulatory protein AZ1 in response to polyamines. In *S. cerevisiae* a similar mechanism apparently functions in the regulation of endogenous yODC (20), although no AZ1 homolog has yet been identified in this organism (18). We asked whether this *S. cerevisiae* polyamine-responsive regulatory factor acted on mODC that was expressed in yeast cells, and if heterologous expression of a mammalian AZ1 further accelerated mODC degradation in these cells. We compared the effect of polyamine administration on the expression of FLAg-mODC and COOH-terminal tagged yODCFLAg, also expressed from a centromeric vector in the *spe1Δspe2Δ* mutant (NH2-terminal epitope tags interfere with expression and regulation of yODC) (4). The *spe1Δspe2Δ* mutant, by precluding polyamine biosynthesis, allows manipulation of cellular polyamine pools by addition of exogenous spermidine. The addition of excess exogenous spermidine had no effect on the steady-state level of FLAg-mODC protein (Fig. 3A) or its activity (data not shown). Measurements of FLAg-mODC turnover by pulse-chase analysis in the presence or absence of exogenous spermidine confirmed that polyamines did not accelerate the degradation of FLAg-mODC in

*4* F. Merz and P. Coffino, unpublished results.
yeast cells (Fig. 3B). In contrast, excess polyamine administration greatly reduced the abundance of γODCFLAG (Fig. 3A), indicating that the γODC regulatory factor was indeed induced, and that the epitope tag had no effect on polyamine regulation of γODC. We conclude that the endogenous γODC regulatory system does not act on mODC.

We next determined the effect of expression of AZ1 on the degradation of FLAMOΔODC in yeast cells. For these studies, we used a rat AZ1 cDNA in which the two partial ORFs comprising full-length AZ1 had been aligned by a single base deletion (8). This AZ1 ORF (AZ1ΔT) obviated the polyamine-induced frameshifting otherwise required for the translation of AZ1. In initial experiments we expressed the AZ1ΔT ORF from multicopy vectors with promoters of various strengths (33) in yeast transformants also expressing FLAMOΔODC from a multicopy vector (Fig. 4A). The steady-state levels of FLAMOΔODC in these transformants exhibited a modest decrease in response to expression of AZ1 from vectors with increasing promoter strength. This decrease was presumably because of increased levels of AZ1 expression, an assumption corroborated by the almost complete inhibition of ODC enzymatic activity in the transformants also expressing AZ1 from stronger promoters (data not shown).

To confirm AZ1 expression directly, we appended a single copy of the FLAMOΔODC epitope to the N-terminus of AZ1ΔT, and expressed this ORF (FLAMOΔODC AZ1ΔT) and FLAMOΔODC from centromeric yeast expression vectors. We compared the steady-state level expression of FLAMOΔODC from the ADH promoter in yeast transformants also carrying FLAMOΔODC AZ1ΔT expressed from the stronger GPD promoter, or an empty vector. In agreement with the previous result, expression of FLAMOΔODC AZ1ΔT reduced the steady-state level of FLAMOΔODC compared with the empty vector control (Fig. 4B).

To verify that the decreased levels of FLAMOΔODC in transformants in which AZ1 was also expressed were because of accelerated degradation, we measured the effects of AZ1 co-expression on FLAMOΔODC turnover by pulse-chase analysis. The results showed that the half-life of FLAMOΔODC decreased from ~10 min in a transformant carrying an empty vector control (Fig. 4C, top panel) to ~4 min in transformants co-expressing FLAMOΔODC AZ1 (Fig. 4, C, bottom panel, and D). We conclude that AZ1 expression causes a modest, but reproducible acceleration of mODC turnover in yeast cells. In contrast with the effect of AZ1 on mODC in mammalian cells, in which a small amount of AZ1 relative to mODC produces a large effect on turnover, an excess of AZ1 leads to a modest increase in turnover of mODC in S. cerevisiae.

Turnover of Mouse and Yeast ODC Is Independent of Ubiquitin—In both cultured mammalian cells (12), and in vitro (14), AZ1-dependent degradation of mODC by the proteasome is independent of the ubiquitin system. To determine whether ubiquitylated forms of FLAMOΔODC were present in yeast cells, FLAMOΔODC was immunoprecipitated from yeast transformants expressing either ubiquitin (Ub) or a hemagglutinin epitope-tagged form of ubiquitin (HAUb). As a positive control, transformants expressing Leu-β-galactosidase and Ub or HAUb were similarly analyzed in parallel. Leu-β-galactosidase is a substrate of the N-end rule-mediated ubiquitylation pathway, and is rapidly degraded in yeast cells because of the presence of a destabilizing Leu residue at its NH2 terminus (43). We expressed both FLAMOΔODC and Leu-β-galactosidase from vectors bearing the ADH promoter. To further enhance the detection of transient polyubiquitylated forms, we utilized a proteasome inhibitor and carried out expression in a strain deleted for RPN4, a transcriptional activator of genes encoding proteasomal subunits. We confirmed a previous report (44) that cells with an rpn4Δ allele, unlike cells with wild type RPN4, are sensitive to the effects of proteasome inhibitors (data not shown). Proteasome function in these transformants was inhibited by treatment with 100 μM MG132, a peptide aldehyde inhibitor of the proteasome (45). In cells expressing both Leu-β-galactosidase and HAUb, HAUb-Leu-β-galactosidase conjugates were detectable with anti-HA antibodies following immunoprecipitation with anti-β-galactosidase antibody (Fig. 4B).
Conservation of ODC Degradation Mechanisms

Ub-mODC conjugates are not detectable in yeast cells. Vectors (Yep96 or Yep112 (59)) bearing Ub or HA-Ub expressed from the Cu²⁺-inducible CUP1 promoter were co-transformed into MHY74 rpm4Δ mutant cells with either p415ADE vectors expressing FLAVG-mODC (lanes 1–4) or Leu-β-galactosidase (lanes 5 and 6). Following induction with 100 μM CuSO₄, and inhibition of proteasome-specific proteolysis with 100 μM MG132, cell extracts were immunoprecipitated (IP) with the indicated antibody, fractionated by SDS-7.5% PAGE, transferred to nitrocellulose, and probed with anti-HA-horseradish peroxidase conjugates. The positions of FLAVG-mODC and Leu-β-galactosidase following fractionation by SDS-7.5% PAGE are indicated.

Fig. 5. Ub-mODC conjugates are not detectable in yeast cells.

Fig. 6. Mouse ODC degradation is not impaired in yeast mutants defective for ubiquitin metabolism. Pulse-chase analysis of FLAVG-mODC, yODCFLAG, and Leu-β-galactosidase expressed in MMY501 (wild type), MMY1409 (ubα1-2), or MMY823 (doa4Δ) transformants. FLAG-tagged mODC and yODC were expressed from p414ADE and p414SPE1 vectors, respectively, and immunoprecipitated with anti-FLAG affinity gel. Leu-β-galactosidase was expressed from p415ADH and p414SPE1 vectors, respectively, and immunoprecipitated with anti-β-galactosidase antibody. Transformants expressing yODCFLAG were treated with 1 μM spermidine. The positions of the relevant band in each panel are marked (†).

Using the criteria described above for FLAVG-mODC, we asked whether ubiquitin plays a role in the degradation of yODCFLAG in response to excess polyamines. We measured the half-life of yODCFLAG in uba1-2 and doa4Δ mutants and the congenic wild type strain by pulse-chase analysis following treatment with excess spermidine (Fig. 6, right panels). This analysis indicated that yODCFLAG was degraded with similar kinetics in wild type and mutant strains. We also found that polyamine regulation of endogenous yODC activity in these mutants was indistinguishable from that of the congenic wild type strain (data not shown). These results suggest that both the constitutive rapid degradation of mODC and the polyamine-induced degradation of endogenous yODC occur independently of ubiquitinylation in S. cerevisiae.

In Vitro Degradation by Yeast Proteasomes Reproduces Salient Characteristics of in Vivo Degradation—Both the AZ1-dependent (14) and AZ1-independent (49) degradation of mODC can be reconstituted in vitro with 26 S proteasomes from mammalian sources. We used affinity-purified yeast proteasomes to determine whether ubiquitin-independent mODC degradation could be replicated in a yeast in vitro system. Yeast proteasomes were isolated from a strain carrying a COOH-terminal FLAG-His₆ tag of the Prol β subunit of the 20 S core particle. We prepared agarose beads loaded with either the 26 S holoenzyme or 20 S core particle of the proteasome. We verified the composition of the immunopurified complexes by SDS-PAGE of a portion of the preparations eluted under denaturing conditions or following elution with FLAG peptide (Fig. 7A). The proteins visualized following Coomassie staining of these gels showed the expected patterns for 20 S and 26 S proteasomes (28, 49).

Using the immunoaffinity purified yeast proteasomes and recombinant mODC produced in E. coli as substrate, we verified that degradation of mODC was linear with time and increasing proteasome concentrations, and inhibited >90% by epoxomicin (Supplemental Fig. 1). Epoxomicin is a specific inhibitor of the proteasome that covalently binds to its catalytic subunits (50). As expected for a substrate of the 26 S proteasome, mODC degradation was ATP-dependent, and required association of the 19 S regulatory particle with the 20 S core (Fig. 7B). We compared the effects of mODC mutations that limit its degradation in cells on degradation in vitro using either yeast or rat proteasomes (Fig. 7C). Mutations that truncated the last 5 amino acids of the mODC COOH terminus or altered Cys⁴⁴¹ had similar inhibitory effects on
surprisingly, the addition of AZ1, even in large stoichiometric excess, had no stimulatory effect on mODC degradation by yeast 26 S proteasomes (Fig. 7D). Under similar assay conditions, AZ1 stimulated the degradation of mODC by rat liver proteasomes ~6.5-fold (Fig. 7D). Both yeast and rat proteasomes showed similar degradative activity toward mODC in the absence of AZ1, but AZ1 significantly stimulated only degradation by the mammalian protease. We conclude that mODC degradation by the yeast 26 S proteasome can be reconstituted in vitro, in the absence of the ubiquitinylating of mODC. However, degradation in the purified system differed from both in vivo degradation in yeast cells and in vitro degradation using mammalian-derived proteasomes in that AZ1 had no stimulatory effect on mODC degradation.

**Discussion**

For the majority of labile proteins, post-translational modification by polyubiquitin constitutes the necessary and sufficient marker for proteasomal recognition. The addition of polyubiquitin chains depends on a complex series of enzymes (2). The task of substrate identification and marking is thus removed from the proteasome itself and devolves instead on a series of ubiquitin activators and transferases. Such delegation of executive authority seems to limit the proteasome to a straightforward binary decision: if and only if a protein bears a polyubiquitin marker of the requisite size, it is to be recognized, unfolded, inserted into a hollow catalytic chamber, and there hydrolyzed to peptides.

This view of proteasome function likely understates its discriminatory capacity. Both prokaryotes and eukaryotes contain compartmentalized proteases that accomplish substrate recognition by association with regulatory complexes. The prokaryotic *E. coli* Clp/Hsl proteolytic complexes are composed of two stacked hexameric rings of a protease subunit flanked on one or both sides by hexamers of a regulatory ATPase subunit. These ATPases each recognize distinct and limited sets of substrate proteins (51). The greater structural complexity of the eukaryotic proteasome 19 S regulator suggests that it is capable of more elaborate forms of substrate recognition and processing. The 19 S complex includes two subassemblies. The base is juxtaposed to the ends of the 20 S core complex and contains six ATPase proteins plus two additional proteins. The lid, hinged to the base and positioned distally, contains 12–14 other proteins. The mammalian proteasome 19 S regulatory complex, in addition to making a binary decision based on the presence or absence of a ubiquitin chain, has the capacity to edit polyubiquitin chains, perhaps performing thereby a proofreading function (52). The proteasome also has direct interactions with the enzymes that carry out late steps in the process of adding ubiquitin chains (28, 53). Additionally, the proteasome has the capacity to recognize a class of substrates that do not require ubiquitin chains, those associated with ubiquitin-independent degradation signals, those associated with mODC.

Mamroud-Kidron and Kahana (21) showed that mODC was rapidly degraded in yeast and provided genetic evidence that the 26 S proteasome was the protease responsible. We show now that mODC degradation has similar structural requirements in yeast and animal cells, namely its COOH-terminal five amino acids and Cys441, that these elements can be exported to an otherwise stable protein, that degradation occurs without prior ubiquitinylation in yeast as well as animal cells, and that purified yeast proteasomes have similar capabilities. The present studies therefore demonstrate both in vivo and in vitro that *S. cerevisiae* proteasomes conserve salient characteristics of mODC recognition.
Our present studies contribute to answering a further question. Can degradation of mODC take place without AZ1? In vivo evidence has been difficult to gather because it is hard to fully exclude the action of residual AZ1 in animal cells. Using yeast as a foreign host resolves this issue. There is no obvious AZ1 homolog in S. cerevisiae, and the unidentified yeast ODC regulatory protein does not act on mODC when induced by polyamine addition, yet mODC is rapidly degraded in these cells. This degradation shows the same structural requirements as in animal cells. Thus, in a cell type lacking any obvious AZ1 or other polyamine-responsive regulatory activity that can act on mODC, this protein is subject to rapid degradation by the proteasome.

In vitro evidence for AZ1-dependent degradation of mODC has also been difficult to obtain, likely because of the use of assay methods insufficiently sensitive to accurately measure limited substrate degradation. We have now demonstrated in a purified system that the degradation of mODC by yeast or rat proteasomes occurs in vitro in the absence of AZ1. Given the relatively low levels of AZ1-independent mODC degradation using our in vitro assays (<10%), only the combined use of a released radiolabel assay, a substrate of high specific radioactivity, and concentrated proteasomes allow its detection. Because the occurrence of AZ1-independent degradation of ODC has previously been hard to establish, the question of whether it requires ubiquitin has also been difficult to answer. This question is now clearly resolved: regardless of whether AZ1 is or is not present, degradation of ODC takes place without the participation of ubiquitin.

Although AZ1 is dispensable for mODC degradation in yeast cells, AZ1 expression does produce an increase in the rate of mODC degradation. This 2–3-fold stimulation of mODC degradation is more modest than the ~10-fold effect seen in animal cells (55). However, the 10-min half-life of mODC in yeast is also more rapid than in the ~60-min half-life of this protein in animal cells in the absence of AZ1 induction. Perhaps the more rapid basal degradation of mODC in yeast cells mutates its response to AZ1. Strikingly, the AZ1 stimulation of mODC turnover is lost in our purified in vitro system, but retained in purified systems utilizing proteasomes from mammalian sources (14).

The structural features of mammalian ODC that are required for its degradation are not present in the otherwise highly conserved yeast homolog. When the two proteins are aligned, mODC is seen to contain a COOH-terminal extension, not present in yODC. Conversely, yODC contains an NH2-terminal extension not found in the mammalian enzyme. The mODC degradation signal resides in its COOH terminus (38, 40), and the NH2 terminus of yODC is required for its rapid degradation (42). Although the mODC degradation signal is recognized by both mammalian and yeast proteasomes, yODC is stable in mammalian cells (42), suggesting either that its degradation signal is specific for yeast proteasomes, or that interaction with the yeast polyamine regulator is indispensable for yODC degradation.

Even though no AZ homolog has yet been identified in S. cerevisiae, a polyamine-ODC-AZ regulatory circuit may yet exist in this organism. Several observations strengthen this conjecture. Polyamines increase the turnover of ODC in yeast cells (19), this regulation requires new protein synthesis (20), and, as shown here, the regulated turnover of yODC is ubiquitin-independent. It has also been recently reported that the basal turnover rate of yODC (in the absence of excess polyamines) is not impaired in mutants that alter ubiquitin metabolism (42). The only other substrate of the proteasome shown to be degraded in S. cerevisiae independently of ubiquitinylation is Rpn4. Rpn4 is a transcriptional activator of genes encoding subunits of the proteasome as well as other genes (56, 57). The Rpn4 protein is short-lived and interacts with the Rpn2 subunit of the base of the 19 S regulatory particle (58).

The finding that yeast provides an appropriate milieu for studies of mammalian ODC degradation by the 26 S proteasome will facilitate future studies. For example, the apparent discrepancy between the capacity of AZ1 to accelerate degradation in vivo but not in vitro suggests the participation of additional components of the degradative system that are excluded upon purification. Utilizing the genetic methods available in yeast should help to reveal the identity and roles of ancillary factors that influence degradation.

Acknowledgments—We are grateful to Hui Chen and Frieder Merz for constructing plasmids used in this study, and Robert Swanson (University of Chicago, Chicago, IL) Mark Hochstrasser (Yale University, New Haven, CT), and Rati Verma and Raymond Deshaies (California Institute of Technology, Pasadena, CA) for strains, plasmids, and helpful advice.

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Conservation of ODC Degradation Mechanisms

Ubiquitin-independent Mechanisms of Mouse Ornithine Decarboxylase Degradation Are Conserved between Mammalian and Fungal Cells
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doi: 10.1074/jbc.M211802200 originally published online January 31, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211802200

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