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Degradation of ornithine decarboxylase in *Saccharomyces cerevisiae* is ubiquitin independent☆

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Abstract

Ornithine decarboxylase (ODC), the first rate-limiting enzyme in the polyamine biosynthesis is one of the most rapidly degraded proteins in eukaryotic cells. Mammalian ODC is a notable exception to the widely accepted dogma that ubiquitination is always required for targeting a protein to degradation by the 26S proteasome. However, while it is well established that in mammalian cells degradation of ODC is ubiquitin independent, the requirement of ubiquitination for degradation of ODC in yeast cells remained undetermined. We have investigated ODC degradation in three mutant strains of *Sacharomyces cerevisiae* in which ubiquitin-dependent protein degradation activity is severely compromised. While yeast ODC was rapidly degraded in all these mutant strains the degradation of N-end rule substrates was inhibited. A mutant mouse ODC that fails to interact with Az was rapidly degraded in yeast cells but was stable in mammalian cells suggesting that interaction with a mammalian Az like yeast protein is not necessary for the degradation of ODC in yeast cells. Deletion analysis revealed that sequences from its unique N-terminus are involved in targeting yeast ODC to rapid degradation in yeast cells.

Keywords: Ubiquitin; Proteasome; Polyamines; Antizyme

Ornithine decarboxylase (ODC), a key enzyme in the biosynthesis of polyamines, decarboxylates ornithine to form putrescine [1]. Putrescine is then converted to spermidine and spermine through the concerted action of *S*-adenosylmethionine decarboxylase, and spermidine and spermine sythase [2]. The polyamines spermidine, spermine, and their precursor putrescine are aliphatic polycations demonstrated to be functionally important in fundamental cellular processes such as growth, differentiation, transformation, and apoptosis [1,3–7]. A complex circuit of biosynthesis, catabolism, excretion, uptake, and recycling regulates the cellular pool of polyamines [8]. ODC is regulated at the transcriptional and translational levels and by its rapid degradation [9–13]. In addition to being a regulatory mechanism for irreversible removal of unwanted proteins, rapid intracellular degradation of proteins enables efficient manipulation of gene expression by upstream genetic regulatory mechanisms. ODC was one of the first enzyme proteins demonstrated to decay rapidly due to the relative ease of monitoring its activity [14]. Interestingly, in contrast to the vast majority of cellular proteins that are degraded through the ubiquitin-proteasome pathway, degradation of mammalian ODC is proteasome-dependent but ubiquitin independent [9,12]. Instead, degradation of mammalian ODC requires its prior interaction with a polyamine-induced protein named antizyme (Az) [15]. Az acts as a key regulator in maintaining cellular polyamine homeostasis since it regulates ODC activity through its degradation [15–19], and polyamine transport via yet undetermined mechanism [20,21]. Az expression is regulated by specific +1 translational frameshifting that is induced by polyamines in a concentration-dependent manner [22,23]. In this sense the frameshifting event serves as the cellular sensor for polyamine levels.

The mechanism of translational frameshifting enabling the expression of Az, and the involvement of Az

☆ *Abbreviations:* ODC, ornithine decarboxylase; Az, antizyme; yODC, yeast ODC; mODC, mouse ODC; MTM, mouse ODC with *Trypanosoma brucei* Az binding site

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in mediating ODC degradation are highly conserved in diverse genera from *Schizosaccharomyces pombe* to humans [24]. Various attempts to find homolog of Az in the budding yeast *Sacharomyces cerevisiae* by computational tools did not yield any results. This together with the lack of direct experimental evidence for the presence of a functional analog of Az, has left the mechanism of ODC degradation in the *S. cerevisiae* mostly unresolved. Genetic studies clearly established the role-played by the 26S proteasome in degradation of ODC in yeast cells [25,26]. However, the mechanism responsible for marking ODC to degradation in budding yeast is still unknown. Therefore it was important to investigate possible involvement of the ubiquitin system in the process of yeast ODC degradation.

Two yeast strains harboring two different mutant alleles of the ubiquitin-activating enzyme, E1 [27,28], and a strain with a disrupted allele encoding a deubiquitinating enzyme that is required for rapid degradation of ubiquitin-proteasome pathway substrates [29] were used for testing possible involvement of the ubiquitin system in the degradation of ODC. We demonstrate here that the degradation of yeast ODC in yeast cells is ubiquitin independent. We also show that a mutant mouse ODC that is incapable of binding to mammalian Az is rapidly degraded in yeast cells while being stable in mammalian cells, suggesting that yeast cells mark ODC to degradation differently than mammalian cells. Deletion analysis revealed that a segment from the N-terminus is required for the degradation of yeast ODC.

Materials and methods

Yeast strains. The wild-type strain WCG4a (MATa *ura3 leu2-3, 112 his3-11, 15*) was used to dissect the degradation signal of yeast ODC. The E1 temperature sensitive mutant strain is a derivative of JD77-1A [27] that is *uba1-Δ*::*HIS3*. These cells were maintained by compensating wild-type *UBA1* or temperature sensitive allele *uba1-ts26*, expressed from pRS314 based expression constructs. These cells were kindly provided by Dr. J. Dohmen. The E1 hypomorphic mutant strains MHY1409 (MATa *uba1-2 his3-D200 leu2-3, 112 lys2-801 trp1-1 gal2*) and the compatible wild-type strain MHY501 (MATa *his3-Δ200 leu2-3, 112 ura3-52 lys2-801 trp1-1*) [28] were kindly provided by Dr. M. Hochstrasser (Yale University, New Haven, CT). The Doa4 disrupted strain BY4741 (*MATa, leu2, ura3, his3, lys2 doa4::kanMX4*) was from Euroscarf.

Plasmids. Yeast and mouse ODC cDNAs were cloned as *Sal*I (5′)–*Sac*I (3′) into the pYEP51 yeast expression vector [30] downstream to the inducible GAL10 promoter. Insertions of HA tag and introduction of deletions were performed by the uracil incorporation method of site-directed mutagenesis [31]. The p415-GPD-Ub-L-LacZ expression construct was kindly provided by Swanson and Hochstrasser [28]. The pUB23 construct encoding Ub-R-LacZ [32] was kindly provided by D. Kornitzer. For expression in mammalian cells mouse ODC DNA as a *Eco*RI (5′)–*Xba*I (3′) fragment, and yeast ODC DNA as a *Eco*RI (5′)–*Not*I (3′) fragment were cloned into pCI neo and pAlter-max (Promega), respectively. The generation of mouse ODC Del-6 which lacks the C-terminal destabilizing signal and the MTM mutant which is incapable of interacting with Az is described in [33] and [16], respectively.

In vitro degradation assay. Constructs encoding the relevant proteins were linearized by cutting with restriction enzyme at the 3′ end of the inserted DNA. These linear DNAs were used as templates for transcription in presence of T7 RNA polymerase using an in vitro transcription kit (Promega) as recommended by the manufacturer. The resulting RNA was then used to program the synthesis of proteins in vitro in rabbit reticulocyte lysate (Promega) in the presence of [^{35}S] methionine. In vitro degradation assay was performed in rabbit reticulocyte lysate based degradation mix containing 40 mM Tris, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 0.5 mM ATP, 10 mM creatine phosphate, and 1.6 mg/ml creatine phosphokinase. Degradation reactions were incubated at 37 °C and samples were withdrawn at various time points. The tested proteins were fractionated by SDS–PAGE and visualized by autoradiography.

Degradation in mammalian cells. HEK293 cells were transiently transfected with pCI-mODC, pCI-MTM, and pAlter-max-yODC using calcium-phosphate transfection method. Forty-eight hours post transfection cycloheximide (20 μg/ml) was added and cellular extracts were prepared at the indicated times in NP-40 lysis buffer (100 mM Tris–base, pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.01% Triton-X 100, 1% NP-40, and protease inhibitor cocktail). The level of the ODC proteins was determined by Western blot analysis (see below).

Degradation in yeast cells. Yeast cells were routinely grown at 30 °C. In case of the E1 temperature sensitive strains, the permissive temperature was 26 °C and the restrictive temperature was 37 °C. Cells were grown for 8–12 h in synthetic minimal medium containing yeast nitrogen base supplemented with amino acids and with 2% raffinose, 2% galactose, 2% glycerol, 2% ethanol, and 40 mg/ml aspartate (synthetic galactose medium). Amino acids were omitted according to the genotypic requirement. Expression was induced by galactose and the chase was initiated by transferring the cells to glucose (4%) containing medium. In case of Ub-L-LacZ, which is expressed from a constitutive promoter, chase was performed by adding cycloheximide (0.5 mg/ml) to the growth medium. At the indicated times cellular extracts were prepared using glass beads in NP-40 lysis buffer containing protease inhibitor cocktail. The level of the ODC proteins was determined by Western blot analysis (see below).

Western blot analysis. Protein content in cell-lysates was determined using the Bradford method [34]. Equal amounts of total protein were then fractionated by electrophoresis in SDS polyacrylamide gel (8% or 10%). The gels were blotted onto nitrocellose membranes that were blocked with 5% non-fat milk in PBS-T and probed with specific rabbit polyclonal anti-yeast ODC antibodies (developed in our laboratory) or monoclonal anti-mouse ODC antibodies (Sigma). β-Galactosidase was probed with polyclonal anti-βGal antibodies (Chemicon). Signals were detected using SuperSignal chemiluminescence reagent (Pierce). Quantitation was performed by scanning the X-ray film using UmaxIII scanner and Adobe photoshop 4.0 and measuring band intensities using image gauge software (Fuji).

Results

Degradation of yeast ODC in yeast cells is ubiquitin independent

It is widely believed that substrates of the 26S proteasome must be ubiquitinated as a prerequisite for their degradation. Mammalian ODC is the most notable and the only well-documented exception to the ubiquitin targeting dogma [4,35,36]. However, while it is clear that

in mammalian cells ODC is presented to the 26S proteasome without ubiquitination, it is still unknown how ODC is degraded in the budding yeast *S. cerevisiae*. In the present study we tested the possible involvement of the ubiquitin system in the degradation of ODC in yeast cells. This was done by investigating ODC degradation in strains containing lesions in specific components of the ubiquitin system. In the first strain, deletion of the *UBA1* gene encoding the ubiquitin activating enzyme, E1, is complemented by a plasmid harboring wild type or mutant allele encoding a temperature sensitive E1 [37]. These mutant cells grow at the permissive temperature (26 °C) but their growth is arrested at the restrictive temperature (37 °C) (Fig. 1A). Yeast ODC was expressed in these cells from a galactose inducible promoter. The short-lived N-end rule substrate, Ub-Arginine-LacZ that is degraded via the ubiquitin-proteasome pathway [32,38], served as a positive control. Yeast ODC expression was induced by growth in galactose containing medium at 26 °C (permissive temperature). Following induction the cells were transferred to non-permissive temperature (37 °C) for 2 h before initiating the chase by adding glucose to the growth medium. As shown in Fig. 1B, not only that the degradation of yeast ODC was not inhibited at the non-permissive temperature, this degradation was actually stimulated probably due to enhanced metabolic activity at the elevated temperature. In contrast, at the non-permissive temperature, the degradation of Ub-Arginine-LacZ was stimulated in the wild-type strain but was inhibited in the mutant strain.

The second strain, in which ODC degradation was tested, contains a recently described hypomorphic allele of *UBA1* [28]. This allele, uba1-2 contains mTN3-based transposon inserted 84 bp upstream to the translational start site of *UBA1*. This leads to a viable mutant phenotype in which the coding sequence for the Uba1 protein is intact but the ubiquitin-proteasome degradation pathway is markedly affected probably due to insufficient amounts of otherwise normal Uba1 protein. The degradation of yeast ODC was tested in these mutant cells, in parallel to the rapidly degraded ubiquitin-proteasome pathway substrate Ub-Leucine-LacZ, which served as a positive control. It is shown in Fig. 2, that yeast ODC is efficiently degraded both in the wild type and in uba1-2 cells. In contrast, the degradation of Ub-Leucine-LacZ is markedly impaired in the mutant cells.

The third strain in which ODC degradation was tested lacks the Doa4 deubiquitinating enzyme that was demonstrated to play a central role in ubiquitin-dependent proteolysis [29,39]. It seems that Doa4 is required for recycling ubiquitin from ubiquitinated substrates targeted to degradation in the proteasome or in vacuoles. In the *doa4* mutant, free ubiquitin is depleted and ubiquitin dependent degradation is inhibited [29,40,41]. We expressed yeast ODC and Ub-Arginine-LacZ in wild type and Δdoa4 cells and followed their degradation. While yeast ODC was rapidly degraded in both strains, the degradation of N-end rule substrate was inhibited in the mutant cells (Fig. 3).

Thus the above observations clearly indicate that the degradation of ODC in yeast cells is ubiquitin

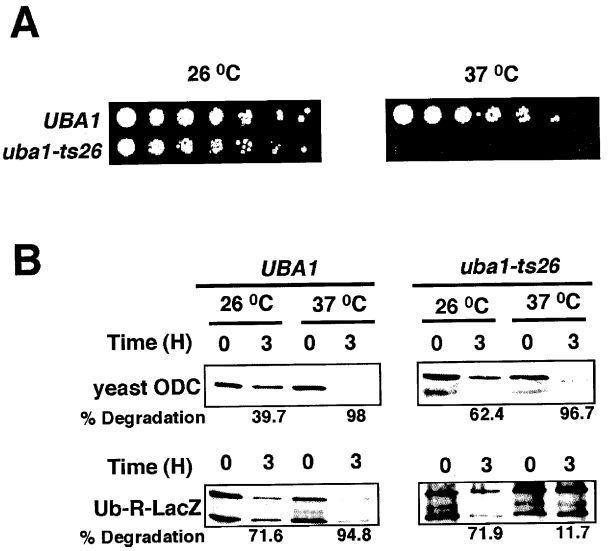

Fig. 1. Yeast ODC is rapidly degraded in yeast cells containing mutant ubiquitin activating enzyme. (A) Fivefold dilutions of wild-type (UBA1) and *uba1-ts26* cells expressing a temperature sensitive E1 were spotted on YPD plates and incubated at the permissive (26 °C) or non-permissive (37 °C) temperature. (B) Yeast ODC and the N-end rule substrate Ub-Arginine-LacZ were expressed in wild type and *uba1-ts26* cells from a galactose inducible promoter. Following induction half of the cells were transferred to the non-permissive temperature for 2 h to inactivate E1. Chase was initiated by the addition of glucose to the growth medium. Cellular extracts were prepared at the indicated times and the level of the tested proteins was determined by Western blot analysis.

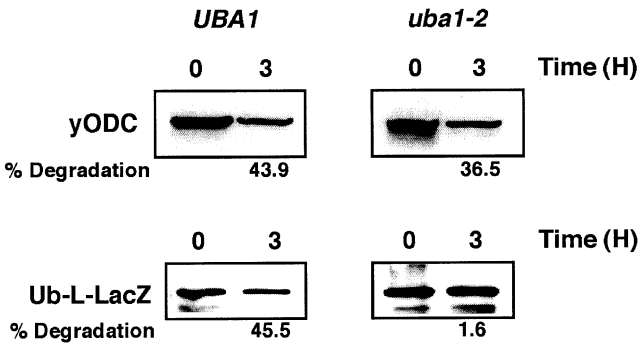

Fig. 2. Yeast ODC is rapidly degraded in yeast cells containing the hypomorphic uba1-2 allele. Yeast ODC and the N-end rule substrate Ub-Leucine-LacZ were expressed in wild type and in mutant cells containing the *uba1-2* allele that express restricted amount of the ubiquitin activating enzyme. Cellular extracts were prepared at the indicated times of chase and the level of these proteins was determined by Western blot analysis.

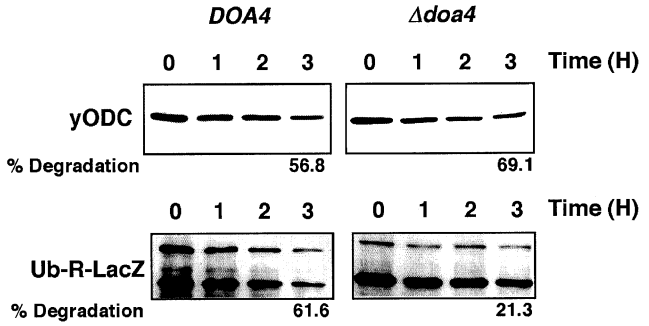

Fig. 3. Yeast ODC is rapidly degraded in ΔDoa4 yeast mutant cells containing limited amount of free ubiquitin. Yeast ODC and the N-end rule substrate Ub-Arginine-LacZ were expressed in wild type and *Δdoa4* mutant cells from vectors containing a galactose inducible promoter. Cellular extracts were prepared at the indicated times after initiating the chase by the addition of glucose. The level of the two proteins was determined by Western blot analysis.

independent, as is the degradation of ODC in mammalian cells.

Differential requirements for the degradation of ODC proteins in mammalian and yeast cells

In mammalian cells the ubiquitin independent degradation of ODC is mediated by interaction with the polyamine-induced protein, Az [4,15,16,35]. Therefore our present demonstration of ubiquitin independent degradation of ODC in budding yeast raises the possible involvement of Az in this degradation process. However, while Az mediated degradation of ODC is now well established in *S. pombe* [24,42], Az-like protein has never been demonstrated in *S. cerevisiae*. The efficient degradation of mammalian ODC in yeast cells was exploited as a tool for investigating the possible presence of Az like protein in yeast cells. For this purpose we have used a mutant mouse ODC termed, MTM, whose Az binding site was mutated to abolish its ability to bind to Az [16]. The degradation of this mutant was tested in vitro in a reticulocyte lysate based degradation mix, in human embryonal kidney 293 cells and in yeast cells. Wild-type mouse and yeast ODCs and the stable mouse ODC mutant, Del-6, lacking the C-terminal destabilizing segment [33], served as controls. The MTM mutant which was stable in mammalian cells (Fig. 4A and B) was rapidly degraded in yeast cells (Fig. 4C). All control proteins behaved as expected namely; in both mammalian systems, mouse ODC was rapidly degraded while the two mutant mammalian enzymes (MTM and Del-6) and yeast ODC were stable (Fig. 4A and B). In yeast cells both wild-type mouse and yeast ODC were rapidly degraded while the Del-6 mutant was stable (Fig. 4C). The rapid degradation of the ODC mutant MTM in yeast cells indicates that interaction with a mammalian Az like yeast protein is not required for the degradation of mouse ODC in yeast cells.

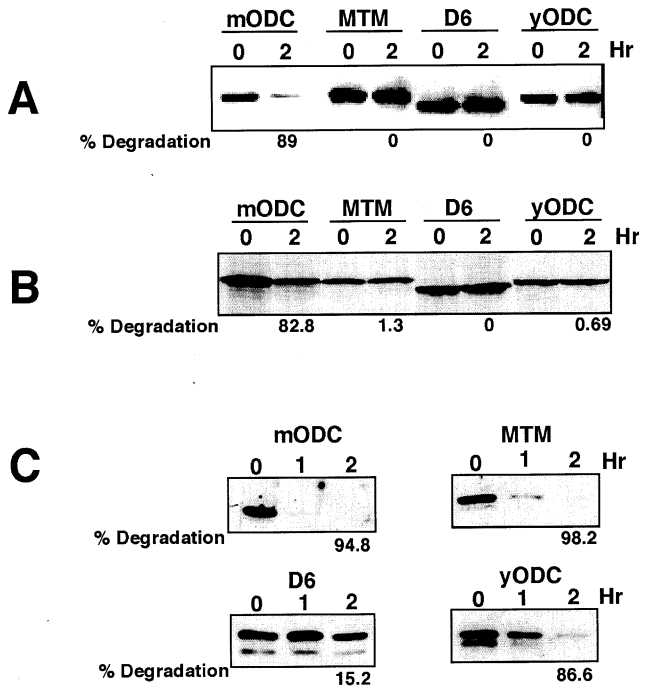

Fig. 4. The mouse ODC mutant, MTM, that does not interact with mammalian Az is stable in mammalian cells but rapidly degraded in yeast cells. cDNAs encoding mouse ODC (mODC), the mouse ODC mutant, MTM that does not bind mammalian Az [16], the mouse ODC mutant, Del-6 lacking the C-terminal destabilizing segment [33] and yeast ODC (yODC) were cloned into mammalian or yeast expression vectors. The mammalian constructs were either transfected into 293 cells (A) or transcribed in vitro and translated in reticulocyte lysate (B). The yeast expression constructs were transformed into wild-type yeast cells (C). Chase in mammalian cells was initiated by the addition of cycloheximide (20 μg/ml) to the growth medium, and in yeast cells by their transfer from galactose containing medium to glucose containing medium. The level of the relevant proteins was determined by Western blot analysis.

Yeast ODC is recognized by the degradation machinery through N-terminal destabilizing sequence

As demonstrated above despite naturally lacking the C-terminal destabilizing signal, yeast ODC is rapidly degraded in yeast cells while the mouse ODC mutant Del-6, was stable. These results suggest that different sequence(s) target yeast ODC to rapid degradation in yeast cells. Sequence comparison between mammalian and yeast ODC demonstrates the presence of unique 44 amino acids at the N-terminus of the yeast enzyme. We therefore tested the possible involvement of this segment in targeting ODC to degradation. Incorporation of HA tag adjacent to the initiation codon of yeast ODC resulted in significant stabilization (Fig. 5A). Incorporation of the HA tag at the C-terminus of yeast ODC did not affect its degradation (Fig. 5A). Similar incorporation of HA tag at the N-terminus of the mouse enzyme did not inhibit its rapid degradation (Fig. 5A). These results suggested that the N-terminal

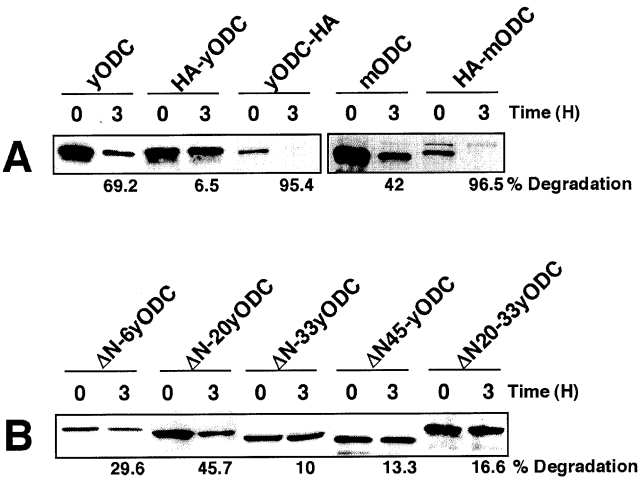

Fig. 5. N-terminal segment marks yeast ODC to rapid degradation in yeast cells. (A) DNAs encoding wild-type yeast ODC (yODC), yeast ODC containing HA tag at its N-terminus (HA-yODC), or at its C-terminus (yODC-HA), wild-type mouse ODC (mODC), or mouse ODC containing HA at its N-terminus (HA-mODC) were cloned in the expression vector pYEP51 and expressed in wild-type yeast cells. (B) In frame deletions were introduced into the N-terminus of yeast ODC and the resulting mutant proteins were expressed in wild-type yeast cells. Expression was induced by growth in galactose containing medium and chase was initiated by the addition of glucose. Cellular extracts were prepared at the indicated times and the level of the various ODC proteins was determined by Western blot analysis.

segment might be involved in targeting yeast ODC to rapid degradation. Indeed, deletion of N-terminal 44 amino acids greatly stabilized yeast ODC (Fig. 5B). We have further dissected this 44 amino acid segment by introducing frame deletions into this region. Deletions of the first 6 and 20 amino acids resulted in partial inhibition of the degradation, while deletion of first 33 amino acids stabilized the enzyme (Fig. 5B). These results suggest that the N-terminus, especially the segment encompassing amino acids 20–33 may constitute a degradation signal. In agreement with this hypothesis, deletion of amino acids 20–33 stabilized yeast ODC (Fig. 5B). We therefore conclude that the N-terminal segment that is unique to yeast ODC acts similar to the C-terminal segment of the mammalian enzyme in mediating its recognition by the proteolytic machinery.

Discussion

The main goal of the present study was to determine whether the degradation of ODC in yeast cells requires ubiquitination or whether as demonstrated in mammalian cells it is ubiquitin independent but requires interaction with antizyme [9,12,15]. Three yeast mutant strains with impaired ubiquitination activity were used in order to test whether ubiquitination is required to target ODC to degradation in yeast cells. In all three strains yeast ODC was rapidly degraded while the degradation of N-end rule β-galactosidase substrates was markedly inhibited. We therefore conclude that as established in mammalian cells [9,12], also in yeast cells the degradation of ODC is ubiquitin independent.

In mammalian cells the ubiquitin independent targeting of ODC to degradation by the proteasome requires prior interaction of ODC with the polyamine-induced protein, Az. Although polyamines were demonstrated to regulate ODC decay in yeast cells similar to the way they regulate ODC decay in mammalian cells [43,44], presence of Az or Az like protein has not been demonstrated in *S. cerevisiae*. It can be speculated that Az like protein may be present in the budding yeast but that it is divergent enough from the mammalian Az to escape homology database searches and hybridizations. Several lines of evidence suggest possible involvement of Az or Az like protein in the degradation of ODC in yeast cells; (1) Addition of polyamines to the growth medium accelerated ODC degradation [43–45]. Cycloheximide prevented induction of ODC degradation by polyamines [46]. (2) Studies performed in vitro demonstrated that both mammalian and yeast 26S proteasomes degrade mouse ODC in an Az dependent manner [47]. (3) Recent study claimed that increased expression of Az in yeast cells reduced steady state levels of co-expressed FLAG tagged mouse ODC [35]. However, Az expression and mouse ODC degradation were not directly demonstrated in that study. Here we have tested the possible existence of Az in yeast cells by yet additional approach. We have monitored the degradation of a mouse ODC mutant termed MTM, whose Az binding site was altered to the parallel sequence of trypanosome ODC that does not bind mammalian Az [16]. We show here that while the ability to bind Az is essential for the degradation of mammalian ODC in mammalian cells, it is not required for the degradation of this protein in yeast cells. Therefore, our present result suggests that either Az is not required for the degradation of ODC in yeast cells, or that a paralog protein fulfills an Az like role in yeast cells. However the mode of action of such paralog may be distinct from that of the mammalian Az.

The gene encoding *S. cerevisiae* ODC, originally characterized by Fonzi et al. [48], displays 40% identity to the mammalian enzyme. However, it has some distinct differences. Most notably, it lacks the 38 amino acids C-terminal region that constitutes the degradation signal of mammalian ODC, and has a unique 44 amino acids N-terminal extension. Regardless of the identity of the cellular machinery that marks ODC to rapid degradation in yeast cells, it is clear that yeast ODC contains distinct sequence motifs that mediate such recognition. In mammalian ODC, two segments were demonstrated to be important for degradation, a C-terminal destabilizing segment (amino acids 423–461) [33,49], whose explicit mode of action is still unknown,

and the Az binding site encompassing amino acids 117–140 [17]. We demonstrate here that the unique N-terminal segment of yeast ODC may serve as the destabilizing signal. Since yeast ODC is stable in mammalian cells such a signal is specific to yeast cells. This is in contrast to the C-terminal destabilizing segment of the mammalian enzyme which functions both in mammalian and in yeast cells.

Based on our present and previous studies [25,26], it appears that there are distinct differences in the degradation of mouse and yeast ODC in mammalian and yeast cells although in both cases the degradation is ubiquitin independent. The degradation signals of mouse and yeast ODC are different being located at the C- and N-terminus, respectively. Different subunits of the yeast proteasome are implicated in the degradation of yeast and mouse ODC in yeast cells [25,26]. Binding to Az is essential for the degradation of mammalian ODC in mammalian cells but not in yeast cells. Now that the possible involvement of the ubiquitin system in the degradation of ODC in yeast cells is ruled out studies must be conducted to test whether yeast cells contain mammalian-Az like protein or whether they utilize a completely alternative strategy for degrading ODC.

Acknowledgments

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