Mammalian antizyme (mAz) is a central element of a feedback circuit regulating cellular polyamines by accelerating ornithine decarboxylase (ODC) degradation and inhibiting polyamine uptake. Although yeast antizyme (yAz) stimulates the degradation of yeast ODC (yODC), we show here that it has only a minor effect on polyamine uptake by yeast cells. A segment of yODC that parallels the Az binding segment of mammalian ODC (mODC) is required for its binding to yAz. Although demonstrating minimal homology to mAz, our results suggest that yAz stimulates yODC degradation via a similar mechanism of action. We demonstrate that interaction with yAz provokes degradation of yODC by yeast but not by mammalian proteasomes. This differential recognition may serve as a tool for investigating proteasome functions.

Cellular proteins are in a continuous state of synthesis and degradation. The intracellular concentration of proteins is determined by the balance between synthesis and degradation (1). Proteins are usually tagged for selective degradation via the 26 S proteasomes by ubiquitination (2). However, some proteins may be degraded by the proteasome without requiring ubiquitination. These include the cyclin-dependent kinase inhibitor p21Cip1 (1), the α subunit of the T-cell antigen receptor (3, 4), IκBα (5), troponin (6), and ODC, the first enzyme in the polyamine biosynthesis pathway (7–9). ODC is the best-studied example of a protein degraded in a ubiquitin-independent manner. ODC differs from the other proteins, because an alternative mechanism for its presentation to the proteasome was provided.

ODC is the first and rate-limiting enzyme in the biosynthesis pathway of polyamines. ODC decarboxylates ornithine to form putrescine, which is further converted to spermidine and spermine via the action of spermidine and spermine synthase, respectively (10). Polyamines are small aliphatic charged molecules that play an important role in various cellular functions and are essential for the process of cellular proliferation (10–13). Polyamine depletion results in growth cessation (14–16), whereas excessive intracellular accumulation of polyamines is cytotoxic (17, 18), calling for strict regulation of the intracellular polyamines pools.

An efficient autoregulatory circuit affecting both synthesis and uptake controls cellular levels of polyamines. The central element of this circuit is a polyamine-induced protein termed antizyme (Az). Az serves as a cellular polyamine sensor, because it is synthesized from two open reading frames via a polyamine-stimulated ribosomal frameshifting (19, 20). Interaction between ODC and Az results in inhibition of ODC activity due to trapping of transient ODC subunits into inactive ODC/Az heterodimers, and in presenting these ODC monomers to ubiquitin independent degradation by the 26 S proteasome (8, 21–23). It was suggested that interaction with Az exposes the C-terminal degradation signal of mODC (24), which serves as the proteasome recognition element (25). In addition to regulating ODC activity and degradation, mAz also regulates polyamine transport via a yet unresolved mechanism (26, 27).

Similar to its mammalian counterpart, yODC is degraded in a ubiquitination-independent manner in yeast cells (28). Early studies demonstrated that, like in mammalian cells, in yeast cells polyamines induced rapid ODC degradation, suggesting the yeast cells contain a functional Az orthologue (29, 30). Indeed, after a lengthy time of unfruitful searching for Az in Saccharomyces cerevisiae using standard methods, yAZ was recently identified and cloned using a bioinformatic approach (31). The yeast Az orthologue resembles mAz in its mode of synthesis and in its ability to promote ODC degradation.

In the present study we demonstrate that, in contrast to efficiently regulating yODC activity and degradation, yAz exerts only a mild effect on the process of polyamine uptake. Therefore, the growth of yeast cells is inhibited by overexpressed yAz, predominantly via inhibiting ODC activity and mediating its degradation, leading to reduction of the intracellular polyamine pools. We show that the stability of yODC in mammalian cells is not a result of the absence of a compatible Az or lack of a C-terminal destabilizing signal found on the mammalian enzyme, but is rather a result of the inability of the mammalian proteasome to degrade yODC.
Polyamine Uptake Assay—The assay was performed essentially as described (39). Cells were grown to the mid-logarithmic phase, washed three times in glucose-citrate buffer (50 mM sodium citrate (pH 5.5), 2% d-glucose), and resuspended in the same buffer at a concentration of 10⁶ cells/ml. Transport was initiated by adding 0.2 volume of [¹⁴C]spermine (10 Ci/mol at 100 μM, from Amersham Biosciences), and the cells were incubated at 30 °C with mild shaking. Uptake was terminated by transferring 100-μl aliquots into 1 ml of ice-cold stop buffer (glucose-citrate buffer containing 2 mM unlabeled spermine). The cells were then layered onto cellulose-acetate filters (0.45-μm pore size) that were washed three times with stop buffer, and the retained radioactivity was determined by liquid scintillation spectrometry.

Growth Assays—Growth curves were generated by diluting yeast overnight cultures to A₆₀₀ = 0.05, and grown in microplate optical reader (GENios, Tecan) in 30 °C with continuous shaking. Growth curves were generated by automatic measurement of A₆₀₀ performed every 10 min.

Polyamine Analysis—The assay was performed according to Madhubala et al. (40). Yeast cultures were grown overnight, and 15 optical density units were collected from each sample. The samples were centrifuged for 30 s at 10,000 × g, washed with phosphate-buffered saline, and re-centrifuged. The cells were resuspended in 100 μl of phosphate-buffered saline, 4.5 μl of 70% perchloric acid was added, and the samples were vortexed and centrifuged for 5 min at 13,000 × g. Markers were prepared using equal amounts of putrescine, spermidine, and spermine. Then, 200 μl of Dansyl chloride (3 mg/ml in acetone) and 10 mg of Na₂CO₃ were added to 100 μl of each sample. The samples were incubated overnight in the dark. Next, 10 mg of proline was added, and after 1 h of incubation, 250 μl of toluene were added, and the samples were centrifuged for 30 s at 13,000 × g. Five microliters of markers and 100 μl of each sample were spotted onto a TLC plate (AALOGRAM SIL G/UV, from Macherey-Nagel, Germany), and resolved by chromatography in ethyl acetate:cyclohexane (2:3, v/v). Polyamines were visualized using a gel imager with a UV filter.

In Vivo Degradation Assay—293HEK cells were transiently transfected with yODC, mODC or the chimera proteins together with either yAz or mAz. Chase was initiated by the addition of cycloheximide (20 μg/ml) to the growth medium, and the cells were harvested at the indicated times thereafter. Cells were lysed, and aliquots containing equal amounts of protein were fractionated by SDS-PAGE and examined by Western blot analysis.

In Vitro Translation and Degradation Assay—The relevant proteins were translated in vitro for 1 h at 30 °C using TnT reaction mix (Promega) in the presence of [³⁵S]methionine. When required, the amount of the tested proteins was normalized by dividing the radioactivity in the relevant band to the number of their methionine residues. When it was important that ubiquitin be completely absent from the degradation reaction, the in vitro translated proteins were fractionated on ion exchange column. The tested proteins were incubated in a reticulocyte lysate-based degradation mixture (40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine (Sigma), 1.6 mg/ml creatine phosphoki-
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FIGURE 1. Overexpression of yAz in yeast cells results in polyamine depletion and growth inhibition. Yeast cells transformed with empty pAD54 (pAD empty), pAD54 encoding mammalian Az (pAD-mAz), pAD54 encoding yeast Az (pAD54-yAz), and cells deleted of their Az gene (Oaz1Δ) were diluted to A600 = 0.05 and plated in six replicates in a 96-well plate in synthetic medium with and without 0.1 mM spermidine (spd). The plates were grown in GENius microplate optical reader for 72 h at 30 °C with constant agitation. A600 was measured automatically every 10 min. The inset presents the polyamine content of the tested cells (put = putrescine, spd = spermidine, and spm = spermine).

RESULTS

Overexpression of mAz in mammalian cells leads to growth arrest due to polyamine depletion, being an outcome of reduced ODC activity and polyamine uptake (43). To determine whether yAz exerts a similar effect on growth of yeast cells, we compared the growth rate of yeast cells overexpressing yAz and of oaz1Δ cells lacking yAz. Interestingly, despite their slightly increased uptake activity, cells overexpressing yAz show slower growth rate, whereas deletion of the yAz gene did not affect growth (Fig. 1). To determine whether the reduced growth rate of yAz-overproducing cells is a result of decreased cellular polyamine concentration, we determined the polyamine content of these cells. As shown in the insert of Fig. 1, reduced intracellular polyamine levels accompanied the slower growth rate of yAz-overexpressing cells. Deletion of yAz resulted in a ~20-fold increase in putrescine content, as expected from the large increase of ODC activity in these cells. This increase in putres-

nase (Roche Applied Science), 6 μl of reticulocyte lysate (Promega), and when supplemented with 1.5 μg of purified yeast or rat 26 S proteasome at a total volume of 15 μl and incubated at 37 °C for the indicated times. To test for ATP requirement, ATP, phosphocreatine, and creatine phosphokinase were omitted and 2-deoxyglucose (20 mM) and hexokinase requirement, ATP, phosphocreatine, and creatine phosphokinase were added. Yeast cells were lysed using the Cellytic reagent (Sigma). Samples containing 40–50 μg of protein were resolved by fractionation in a 12.5% polyacrylamide-SDS gel, and the radioactivity of the individual proteins determined using the Fuji Bas2500 phosphorimaging device.

Isolation of Yeast and Mammalian Proteasome—Yeast proteasome was purified according to the conventional protocol as described by Leggett et al. (41). Mammalian proteasome was kindly provided by Dr. Ami Navon (Weizman Institute of Science, Rehovot, Israel). It was purified using a procedure described in (42).

Co-immunoprecipitation Assay—In vitro translated [35S]methionine-labeled WT yODC and the yODC mutant lacking the putative yAz binding site (yODCΔyAzBS) were incubated in Nonidet P-40 buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40) for 12 h either alone or together with HA-tagged yAz and with α-HA beads. The beads were collected by centrifugations and washed four times with Nonidet P-40 buffer, and the bound material was eluted in Lammli sample buffer and fractionated by polyacrylamide-SDS gel electrophoresis. Radioactive bands were visualized using a Fuji Bas2500 phosphorimaging device.

Western Blot Analysis—Mammalian Cells were washed with cold phosphate-buffered saline, harvested, and lysed in radio-immune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 0.5% Nonidet P-40, 1% Triton X-100, 1% sodium deoxycholate). Yeast cells were lysed using the Celllytic reagent (Sigma). Samples containing 40–50 μg of protein were resolved by 12% SDS-PAGE and blotted onto nitrocellulose membranes. The primary antibodies used were anti-HA monoclonal antibody (1:2000, BabCO), anti-yeast ODC polyclonal antibody (1:300, produced in our laboratory), anti-mouse ODC polyclonal antibody (1:300, produced in our laboratory), anti-rat antizyme (1:500, produced in our laboratory). Goat anti-mouse or anti-rabbit immunoglobulin G-horseradish peroxidase conjugate (1:10,000, Jackson) were used as a secondary antibody. Signals were detected using the enhanced chemiluminescence system (Pierce).
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The C-terminal Destabilizing Segment of mODC Does Not Target yODC to Degradation in Mammalian Cells—Two segments of mODC were demonstrated important for its rapid degradation. The first, containing amino acids 117–140 is required for Az binding, which stimulates degradation (21, 22). The second, encompassing the 37 most C-terminal amino acids, which constitutes the proteasome recognition signal is essential for ODC degradation (25, 44–46). yODC lacks a C-terminal segment that parallels the C-terminal degradation signal of mODC. Trypanosome (Trypanosome brucei) ODC, which also lacks a C-terminal degradation signal, is stable when expressed in mammalian cells but is converted into a rapidly degraded protein when the C terminus of mODC was appended to it (44). Furthermore, the C-terminal segment of mODC targeted heterologous proteins to proteasomal degradation (47–49). In these cases the degradation of the chimeric proteins did not require interaction with Az. We therefore next tested whether the stability of yODC in mammalian cells is a result of the absence of an appropriate destabilizing signal. To this end, two chimerical proteins were constructed in which the segment encompassing the 37 most C-terminal amino acids of mODC was appended either to the C terminus of yeast ODC, or replacing its N-terminal degradation signal encompassing amino acids 1–47 (28). The stability of the resulting proteins was tested in transfected 293 cells (Fig. 2C) and in vitro in the reticulocyte lysate-based degradation system (Fig. 2D). Both proteins remained stable when incubated alone or together with yAz (Fig. 2, C and D), suggesting that yeast ODC is incompatible with the mammalian degradation machinery even when containing the mammalian degradation signal.

Yeast ODC Is Recognized and Degraded by Yeast but Not by the Mammalian Proteasome—It is possible that degradation of yODC in the mammalian system requires other unknown yeast factors, or that the mammalian proteasome is incapable of processing the yeast Az-ODC complex. To test the latter possibility, we examined yODC degradation in the presence of highly purified yeast 26 S proteasome. Addition of yeast proteasome to the degradation mix induced efficient degradation of yODC (Fig. 3A). Equal amounts of mammalian proteasome (normalized by activity against a fluorogenic peptide) failed to provoke yODC degradation, suggesting that yAz mediates recognition of yODC by yeast but not by mammalian proteasome. This degradation of yODC by the yeast proteasome is Az-dependent (Fig. 3B) and requires metabolic energy in the form of ATP (Fig. 3C). Next we have tested whether under these conditions the C terminus of mODC can function as a yODC-targeting signal, because mouse ODC is rapidly degraded in yeast cells in a C terminus-dependent manner (28, 50, 51). For this purpose the C-terminal segment of mODC was appended at the N or C terminus of the stable yODC mutant lacking its N-terminal destabilizing segment and the stability of the resulting proteins tested in a reaction containing yeast proteasome and yAz. Although the chimera containing the C terminus of mODC at the C terminus of the yeast enzyme remained stable, the one containing the mouse C-terminal segment at the N terminus was rapidly degraded (Fig. 3D). As previously demonstrated (49, 52), converting cysteine 441 of the mouse degradation signal to alanine interfered with the ability of this segment to confer lability on yODC (Fig. 3D). These results demonstrate that yODC is recognized for degradation by the yeast but not by the mammalian proteasome and could suggest that yAz may act similarly to mammalian Az in exposing the degradation signal.
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Identification of the Az Binding Site of yODC—The mammalian ODC segment encompassing amino acids 117–140 was suggested to function as the putative Az binding site (21). Best fit alignment between yeast and mouse ODC revealed that the region encompassing amino acids 164–187 of the yeast enzyme corresponds to the putative Az binding segment of mODC (Fig. 4A). To determine whether this segment of yODC is required for Az binding, it was deleted from yeast ODC incubated together with yAz in an in vitro degradation reaction supplemented with either mammalian or yeast proteasome (mProt and yProt, respectively). B, yODC lacking the first N-terminal 47 amino acids (ΔNyODC), this protein containing the C-terminal degradation signal of mODC at either its C terminus (ΔNyODC/CmODC) or to its N terminus (CmODC/ΔNyODC) and (CmODC/C441A/ΔNyODC, in which cysteine 441 of the mouse degradation signal was converted to alanine) were incubated in an in vitro degradation reaction supplemented with yProt and yAz (the two later proteins were purified on DEAE column as in A). As in A, the tested proteins and yAz were purified on DEAE column. At the end of the incubation period the tested proteins were resolved by SDS-PAGE and visualized using the Fuji Bas2500 Bioimager.

Characterization of yAz Sequences That Are Important for Inhibiting ODC Activity and Promoting ODC Degradation—Previous studies have demonstrated that large C-terminal segment of mAz is required for ODC binding and inactivation, whereas a smaller more N-terminal segment, which does not affect binding or ODC inactivation, is required for targeting ODC to degradation (22, 53, 54). To characterize functional domains of yAz, 14 in-frame deletions were introduced into yAz, and the ability of the resulting mutants to inhibit yODC activity and to promote yODC degradation was examined. As shown in Fig. 5, the vast majority of the mutants lost the ability to inhibit ODC activity and to promote ODC degradation, probably reflecting their inability to bind to yODC. There were no mutants that significantly inactivated yODC but failed to promote its degradation.

DISCUSSION

The mechanism by which Az controls cellular polyamine levels appears to be conserved among mammalian species. This includes inhibition of ODC activity and induction of ODC degradation, along with an inhibitory effect on the mechanistically yet unresolved process of polyamine uptake. Some of these features are also shared by the long sought Az orthologue of the budding yeast S. cerevisiae (31). Although yAz displays minimal homology to mAz, it retains the ability to efficiently stimulate ODC degradation. However, its ability to affect polyamine uptake was not tested. We demonstrate here that the effect yAz exerts on polyamine uptake in yeast cells is minor compared with that exerted by mammalian Az in mammalian cells. Despite the minor increase of polyamine uptake in yAz-overproducing yeast cells their polyamines content was actually reduced, suggesting that the dominant effect of yAz is on ODC activity and degradation. Indeed, although its effect on uptake was in the range of 20%, yAz deletion led to 50-fold increase in ODC activity. It is possible that the effect yAz exerts on polyamines uptake is rather indirect, reflecting attempts mediated by other regulatory mechanisms to compensate for the dramatic reduction in polyamines production. As in mammalian cells, the large increase in ODC activity leads to dramatic increase in the intracellular putrescine concentration. However, in contrast to the toxic effect the accumulated putrescine caused in mammalian cells (18), no such effect was observed in yeast cells.

A segment of mODC encompassing amino acids 117–140 seems to function as the potential Az binding site (21). Best-fit alignment between yeast and mouse ODC revealed that amino acids 164–187 of yODC correspond to this putative Az binding site. Despite displaying only 50% homology to the parallel mouse segment, deletion of this segment of yODC yielded a mutant that displays poor Az binding and is therefore stabilized. Although our analysis implicates the segment containing amino acids 164–187 as the putative yAz binding site, further analysis will be required to determine whether this segment constitutes the entire yAz binding site and which individual amino acids contribute to binding.

Two types of domains were observed in mammalian Az. The C-terminal half of the molecule is required for binding to and inactivating ODC, whereas a smaller N-terminal segment that
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Previous studies have demonstrated that the C-terminal segment of mammalian ODC encompassing the 37 most C-terminal amino acids is essential for its degradation (45, 46), and that it constitutes a dominant degradation signal as it can be transferred to ODC related and unrelated stable proteins (44, 45, 48, 49, 56). It was suggested that interaction with Az exposes the C-terminal degradation signal of mODC (24). Interestingly, while being degraded in mammalian cells in an Az- and C terminus-dependent manner, in yeast cells mODC is degraded in a C terminus-dependent but Az-independent manner (28, 50, 51). In contrast, yODC is rapidly degraded in yeast cells (28, 50, 51) but is a stable protein when expressed in mammalian cells (28). yODC lacks a C-terminal segment that corresponds to the C-terminal degradation signal of the mammalian enzyme. As we show here, yODC remained stable when incubated in a reticulocyte lysate-based in vitro degradation reaction together with yAz, or when co-expressed in transfected cells together with yAz, even when the C-terminal degradation signal of mODC was appended to it. Addition of purified yeast 26 S proteasomes but not of mammalian 26 S proteasome to the in vitro degradation reaction promoted yAz-dependent yODC degradation. This result suggests that, although the yeast proteasome efficiently recognizes both yeast and mouse ODC (as reflected by the rapid degradation of mODC in yeast cells (50, 51)), yODC is recognized efficiently by the yeast proteasome, but is either poorly or not recognized by the mammalian proteasome. This difference in the recognition ability of the two proteasomes may be useful for the identification of the specific proteasomal subunit that recognizes ODC and perhaps also the one recognizing polyubiquitin chains.

In light of the high homology between yeast and mammalian ODCs, it is rather remarkable that mammalian and S. cerevisiae Azs are so divergent. Although this difference may underlie the poor recognition of the yAz·yODC complex by the mammalian proteasome, it is quite striking that its mechanism of action yAz resembles mAz. It was suggested that interaction with mAz imposes a conformational change on mODC, resulting in the exposure of its C-terminal proteasome recognition signal (24). yODC lacks a C-terminal segment that corresponds to the C-terminal destabilizing segment of the mouse enzyme, and its degradation is mediated by an N-terminal segment (28). As we demonstrate here, the C-terminal destabilizing segment of mODC can replace the destabilizing segment of yODC only when appended to the N terminus, but not when appended to the C terminus of a stable yODC N-terminal deletion mutant. This suggests that, like mAz, also yAz may impose a conformational change on yODC, however, in this case the conformational alteration exposes the N terminus.

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