Degradation of Antizyme Inhibitor, an Ornithine Decarboxylase Homologous Protein, Is Ubiquitin-dependent and Is Inhibited by Antizyme*

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Ornithine decarboxylase (ODC) is the most notable example of a protein degraded by the 26 S proteasome without ubiquitination. Instead, ODC is targeted to degradation by direct binding to a polyamine-induced protein termed antizyme (Az). Antizyme inhibitor (AzI) is an ODC-related protein that does not retain enzymatic activity yet binds Az with higher affinity than ODC. We show here that like ODC, AzI is also a short-lived protein that undergoes proteasomal degradation. However, in contrast to ODC degradation, the degradation of AzI is ubiquitin-dependent and does not require interaction with Az. Moreover, Az binding actually stabilizes AzI by inhibiting its ubiquitination. Substituting the C terminus of AzI with that of ODC, which together with Az constitutes the complete degradation signal of ODC, does not subvert AzI degradation from the ubiquitin-dependent mode to the Az-dependent mode, suggesting dominance of the ubiquitination signal. Our results suggest opposing roles of Az in regulating the degradation of AzI and ODC.

The polyamines spermine, spermidine, and their precursor putrescine are natural organic cations that play a crucial role in regulating fundamental cellular processes such as proliferation, differentiation, transformation, and apoptosis (1–8). The range of cellular polyamines is limited at the lower level by the essentiality for cellular proliferation and other fundamental cellular processes and at the higher level by their cytotoxicity. Therefore, it is not surprising that cellular polyamines are highly regulated by multiple pathways such as biosynthesis, catabolism, uptake, and excretion. ODC is a key enzyme in the polyamine biosynthesis pathway, and its degradation constitutes an important regulatory mechanism that controls cellular polyamines. ODC is actually one of the most rapidly degraded proteins in eukaryotic cells, and its degradation is unique in being manifested without ubiquitination (9, 10). Instead, interaction with Az, a polyamine-induced protein (11, 12), is required to target ODC for degradation by the 26 S proteasome (13). Interaction of Az with ODC subunits has two outcomes: 1) ODC is inactivated due to trapping of transient ODC monomers by Az to form enzymatically inactive heterodimers (14) and 2) the Az/ODC heterodimers are presented to the proteasome, and ODC is degraded (13–16). Az was also demonstrated to regulate the process of polyamine transport across the plasma membrane via a yet unresolved mechanism (17, 18). Az is rapidly degraded by the proteasome (19); however, its degradation does not occur while presenting ODC to the proteasome (19). Furthermore, Az degradation requires the presence of an active ubiquitin system in the cells (19). The ability of Az to regulate cellular polyamines by regulating both polyamine synthesis and uptake, together with the ability of polyamines to regulate its synthesis, suggest that Az synthesis functions as the cellular polyamine sensing mechanism and that Az is a central regulator of cellular polyamine metabolism.

Mammalian cells contain yet another protein termed antizyme inhibitor (AzI), which may be of great relevance to the regulation of cellular polyamines. AzI displays extensive homology to ODC but lacks decarboxylating activity (20). AzI binds Az with higher affinity than ODC binds to Az; thus, it may save ODC from Az-mediated proteasomal degradation (20, 21). AzI is induced in cells and tissues following growth stimulation (21–23), and is elevated in gastric tumors (24). The potential ability to regulate Az suggests that AzI may play an important role in regulating cellular polyamine levels and cell growth.

In the current study, we have studied the degradation of AzI. We show that AzI is degraded rapidly by the proteasome. In contrast to ODC degradation, which is greatly stimulated by Az, AzI degradation is inhibited by interaction with Az. Genetic and biochemical analysis reveal that AzI is degraded via the ubiquitin system and that interaction with Az interferes with its ubiquitination.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The cell lines used were: 293 human embryonic kidney (293 HEK), NIH3T3 mouse fibroblasts, and A31N-ts20, a BALB/c mouse cell line that harbors a temperature-sensitive E1 ubiquitin-activating enzyme, and its parental cell line A31N (25). Cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin and cultured at 37 °C in a humidified incubator with 5.6% CO2. A31N and A31N-ts20 were cultured at 32 °C and transferred to 39 °C to inactivate the E1 ubiquitin-activating enzyme in A31N-ts20 cells (25).

Plasmids—DNA encoding AzI, Az (a clone that does not require frameshifting for expression), ODC, and HA-tagged ubiquitin were PCR-amplified using specific primers to which XhoI sites (5’-O’-thiotriphosphate); HA, hemagglutinin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptidase ligase.

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† To whom correspondence should be addressed. Tel.: 972-8-9342745; Fax: 972-8-9344199; E-mail: chaim.kahana@weizmann.ac.il.
‡ The abbreviations used are: ODC, ornithine decarboxylase; Az, antizyme; AzI, Az inhibitor; Ub, ubiquitin; ATP-8, adenosine 5’-O’-thiotriphosphate); HA, hemagglutinin; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptidase ligase.
Degradation of AzI Is Ubiquitin-dependent and Inhibited by Az

**Immunoblot Analysis**—Cell extracts were prepared by lysing phosphate-buffered saline-washed cells in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin (Sigma mixture)). Extracts were prepared in radioimmune precipitation lysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8, 1 mM dithiothreitol, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin (Sigma mixture)). Aliquots containing equal amounts of proteins were immunoprecipitated with antibodies to ODC, AzI, and developed. The antibodies used were: mouse monoclonal anti-ODC (Sigma), rabbit polyclonal anti-Az (14), and rabbit polyclonal anti-AzI (prepared in our laboratory), and anti-p53 (kindly provided by M. Oren). The conjugation assay was performed for 60 min at 37 °C (when AzI was tested) or 30 °C (when p53 was tested). When the ATP requirement for conjugation was tested, 10 mM 2-deoxyglucose and 150 units of hexokinase were added prior to the addition of the labeled protein.

**RESULTS**

AzI Is Rapidly Degraded by the Proteasome—AzI is a recently identified protein that shares high homology with ODC.
Degradation of AzI Is Ubiquitin-dependent and Inhibited by Az

As stated above, alignment of the sequences of AzI and ODC reveals extensive differences in their C-terminal segments (Fig. 2C). Since minor changes within the C-terminal segment of ODC inhibited degradation, these differences by themselves suggest that the C-terminal segment of AzI is not required for its degradation. To test this directly, the C-terminal segment of AzI was deleted, and the stability of the resulting mutant was determined in transfected 293 cells. As shown in Fig. 2D, the AzI mutant lacking the C-terminal segment (amino acids 418–448) was rapidly degraded, whereas the parallel ODC mutant (lacking amino acids 423–461) was significantly stabilized. We therefore conclude that Az binding and the C-terminal segment are not required for AzI degradation.

AzI Degradation Requires Ubiquitination—Since we demonstrate here that AzI degradation does not require interaction with Az and that its C-terminal segment does not serve as a degradation signal, we set out to investigate the possibility that AzI is degraded via the ubiquitin system, as is the degradation of the majority of cellular proteins. The following four experimental systems were used to determine whether AzI degradation requires ubiquitination. 1) AzI was transiently transfected into A31N-ts20 cells (25) that contain a thermosensitive ubiquitin-activating enzyme, E1, and into their parental A31N cells, and the transfected cells were maintained at the permissive temperature (32 °C). Twenty-four hours after transfection, half of the cells were transferred to the restrictive temperature (39 °C) for 24 h. Cellular extracts were then prepared, and the level of AzI was determined by Western blot analysis. AzI accumulated only in A31N-ts20 cells, upon their transfer to the non-permissive temperature (39 °C) (Fig. 3A). 2) AzI was translated in vitro in reticulocyte lysate in the presence of 35S-methionine and subjected to degradation in a reticulocyte lysate-based degradation mix or in fraction II of reticulocyte lysate (F-II) that is depleted of ubiquitin. AzI was degraded in reticulocyte lysate-based degradation mix (Fig. 3B) but remained stable when incubated in fraction II (Fig. 3B). As reported before (9), ODC was efficiently degraded in fraction II of reticulocyte lysate (Fig. 3B, F-II). 3) FLAG-tagged AzI or GFP was transfected into 293 cells together with HA-tagged ubiquitin. Cellular extracts were prepared and subjected to immunoprecipitation with anti-FLAG antibodies, and the precipitated material was subjected to Western blot analysis using anti-HA antibodies. High molecular weight Ub conjugates were clearly observed in AzI-transfected cells but not in the GFP-transfected cells (Fig. 3C). 4) AzI, ODC, and p53 were synthesized in vitro in reticulocyte lysate in the presence of 35S-methionine. Conjugation to ubiquitin was performed in rabbit reticulocyte lysate in the presence of 35S-methionine, γ-S-ATP, and Ub-aldehyde (see “Experimental Procedures” for details). Ubiquitinated AzI was clearly observed (Fig. 3D). As expected, ODC remained unubiquitinated, whereas p53 was efficiently conjugated to Ub (Fig. 3D). The formation of both p53 and AzI conjugates required metabolic energy in the form of ATP (Fig. 3D). Based on these four independent criteria, we concluded that in contrast to ODC that is degraded by the proteasome without ubiquitination, AzI is degraded in a Ub-dependent manner.

Interaction with Az Stabilizes AzI—Although the above experiments suggested that interaction with Az is not required
for AzI degradation, Az is present in cells and binds AzI with high affinity (20). Therefore, we next set out to determine whether there will be any effect resulting from the interaction of AzI with Az. For this purpose, AzI and ODC were each expressed together with Az in 293 cells, and their level was determined. As expected, Az destabilized ODC so efficiently that ODC was practically undetectable in the transfected cells (Fig. 4A). In sharp contrast, AzI was dramatically stabilized when co-expressed with Az (Fig. 4A). No inhibition was observed when the AzI-MTM mutant was co-expressed with Az (Fig. 4A). Our results therefore demonstrate that Az stabilizes AzI and that binding to AzI is required for this stabilization. Interestingly, Az is also stabilized due to its interaction with AzI as demonstrated by its increased accumulation when it is co-transfected with increasing amounts of AzI (Fig. 4B).

Az Inhibits AzI Ubiquitination—Having demonstrated that Az inhibits AzI degradation and that AzI degradation is Ub-dependent, we next set out to determine whether there will be any effect resulting from the interaction of AzI with Az. For this purpose, AzI and ODC were each expressed together with Az in 293 cells, and their level was determined. As expected, Az destabilized ODC so efficiently that ODC was practically undetectable in the transfected cells (Fig. 4A). In sharp contrast, AzI was dramatically stabilized when co-expressed with Az (Fig. 4A). No inhibition was observed when the AzI-MTM mutant was co-expressed with Az (Fig. 4A). Our results therefore demonstrate that Az stabilizes AzI and that binding to AzI is required for this stabilization. Interestingly, Az is also stabilized due to its interaction with AzI as demonstrated by its increased accumulation when it is co-transfected with increasing amounts of AzI (Fig. 4B).

**Fig. 3. AzI is targeted for degradation by ubiquitination.** A, a construct encoding AzI was transiently transfected into A31A (WT) and A31N-ts20 (ts20) harboring a temperature-sensitive Ub-activating enzyme. Twenty-four hours after transfection, half of the cells were transferred from the permissive temperature (32 °C) to the restrictive temperature (39 °C) for 24 h. Cellular extracts were prepared, and equal portions of proteins were fractionated by electrophoresis in an SDS-polyacrylamide gel and blotted to nitrocellulose. The level of AzI was determined using anti-AzI antibodies. B, in vitro translated 35S-labeled AzI and ODC were incubated either in a complete rabbit reticulocyte lysate-based degradation mix (RRL) or in fraction II of reticulocyte lysate (F-II) that lacks Ub. Aliquots were removed at the indicated times, fractionated by electrophoresis in an SDS-polyacrylamide gel, and the proteins were visualized by the Fuji BAS2500 phosphorimaging device. C, FLAGAzI or FLAGGFP were transiently expressed in 293 cells together with HA-Ub. Twenty-four hours after transfection, cellular extracts were prepared and subjected to immunoprecipitation (IP) with anti-FLAG antibodies. The precipitated material was fractionated by electrophoresis in an SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-HA antibodies. D, 35S-labeled AzI, ODC, and p53 were synthesized in vitro by translation in reticulocyte lysate and incubated in a conjugation reaction as outlined under “Experimental Procedures.” After 60 min of incubation, the material was fractionated by electrophoresis in an SDS-polyacrylamide gel, and radioactivity was visualized using the Fuji BAS2500 phosphorimaging device.

**Fig. 4. Interaction with Az stabilizes AzI.** A, AzI, ODC, and AzI-MTM were expressed alone or co-expressed with Az in 293 cells. Twenty-four hours after transfection, cycloheximide (CHX, 20 μg/ml) was added, and cellular extracts were prepared at the indicated times. Equal portions of proteins were fractionated by electrophoresis in an SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-AzI and anti-ODC antibodies. B, the AzI-encoding construct was transfected into 293 cells either alone or together with increasing concentrations of the AzI-expressing construct. The total amount of transfected DNA was normalized with empty vector DNA. Cellular extracts were prepared 24 h after transfection, and equal portions of proteins were fractionated by electrophoresis in an SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-AzI antibodies. NS, nonspecific.
Degradation of AzI Is Ubiquitin-dependent and Inhibited by Az

We show here that AzI, an ODC-related protein, is rapidly degraded in a Ub-dependent manner. Although Ub dependence is a standard feature in cellular protein degradation, Ub dependence in the case of AzI degradation is rather surprising since ODC is the most notable example of a short-lived cellular protein that undergoes Ub-independent degradation and since Az shares significant homology with ODC. Although retaining strong binding affinity to Az, the C-terminal segment of AzI differs significantly from the sequence of the C-terminal degradation signal of ODC (Fig. 1A). It was therefore remarkable to find out that AzI was degraded even more rapidly than ODC. Moreover, mutant AzI lacking the entire C-terminal segment was still rapidly degraded, suggesting that the mechanism of AzI degradation is different from that of ODC degradation. It is interesting to note in this respect that yeast and trypanosome ODC, which naturally lack the C-terminal degradation signal, are both stable when expressed in mammalian cells (32, 33).

Az-mediated degradation is a unique process for the degradation of ODC, although it has recently been demonstrated that Az may also mediate the degradation of cyclin D1 (34). The majority of cellular proteins need to be polyubiquitinated prior to their recognition and degradation by the 26 S proteasome. We show here that in contrast to ODC degradation, AzI degradation requires polyubiquitination. This conclusion is based on results we obtained using several parallel experimental approaches. AzI accumulates in cells harboring temperature-sensitive ubiquitin activating enzyme when these cells are incubated at the non-permissive temperature. AzI is stable in fraction II of reticulocyte lysate, which lacks Ub, whereas ODC is efficiently degraded in this fraction. AzI-Ub conjugates are readily observed after co-expression of AzI and HA-Ub in transfected cells or when AzI and Ub are co-incubated in vitro in reticulocyte lysate. Interestingly, AzI was degraded in a Ub-dependent manner even when its C terminus was replaced by the C-terminal destabilizing signal of ODC, suggesting preferred recognition and channeling of AzI degradation to the Ub-dependent pathway.

Degradation of mammalian ODC is greatly stimulated by Az via a unique mechanism of targeting to the 26 S protea-

Fig. 6. The C-terminal degradation signal of ODC does not divert AzI degradation to the Az-dependent pathway. A, a schematic presentation of the AzI chimera that contains the C-terminal degradation signal of ODC (amino acids 423–461) instead of the C terminus of AzI (amino acids 418–448). B, the AzI-ODC chimera was expressed in 293 cells with or without Az, and its stability was determined as described in the legend for Fig. 1C. CHX, cycloheximide.

DISCUSSION

the amount of AzI in the presence of Az due to its stabilization (Fig. 5D).

AzI-ODC Chimera That Contains the C-terminal Destabilizing Signal of ODC Is Degraded in a Ubiquitin-dependent Manner—We demonstrate here that AzI and ODC are two highly homologous related proteins that are recognized for proteasomal degradation by two different mechanisms. We therefore use these two related proteins to determine which mode of degradation, Ub-dependent or Az-dependent, will prevail. As demonstrated above, AzI possesses the segment required for Az binding but lacks a C-terminal degradation signal. We therefore decided to append the C-terminal destabilizing segment of ODC in place of the C-terminal segment of AzI and test whether this change will divert AzI degradation from the Ub-dependent pathway to the Az-dependent pathway. To do so, an NcoI site was introduced into the C terminus of AzI in a position that parallels the NcoI site of ODC. Next, the C-terminal segment of AzI was replaced with that of ODC (Fig. 6A). The resulting protein, denoted AzI-ODC, was expressed in 293 cells alone or together with Az. AzI-ODC behaved as AzI;

Fig. 5. Az inhibits AzI ubiquitination. A, FLAGAzI was transiently expressed in 293 cells together with HA-Ub and with or without Az. The formation of AzI-Ub conjugates was determined as described in the legend for Fig. 3C. B, p53, mdm2, and HA-Ub were co-transfected into 293 cells with or without Az. Cellular extracts were immunoprecipitated with anti-p53 antibody followed by Western blot analysis as described in the legend for Fig. 3C. C, wild-type AzI (AzI wt) and the AzI-MTM mutant were expressed in 293 cells with and without Az. The formation of AzI-Ub conjugates was determined as described in the legend for Fig. 3C. D, cellular extracts analyzed in C were directly fractionated by electrophoresis in SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with anti-AzI antibodies.

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some (for a review, see Ref. 35). We show here not only that AzI degradation does not require Az but also that Az binding actually stabilizes AzI. This negating effect of Az on ODC and AzI may represent an important aspect of the polyamine regulating circuit. Since AzI can sequester Az, it is possible that AzI provides fine-tuning in the ability of Az to stimulate ODC degradation and inhibit polyamine uptake. For instance, the observation that AzI is induced during growth stimulation similarly to ODC (21) suggests that AzI may buffer Az, which appears upon increase of the intracellular concentration of polyamines. Our results demonstrate that both AzI and Az are stabilized upon their interaction. Therefore, it will be of interest to determine whether their association/dissociation is a regulated process and whether this process is affected by the intracellular polyamines. The ability of AzI to sequester Az and neutralize its ability to stimulate ODC degradation and inhibit polyamine uptake suggests that it may act as a central regulator of cellular polyamine homeostasis. Indeed, our recent studies demonstrate growth advantage of AzI overexpressing cells.

We show that the inhibitory effect of Az on the degradation of AzI is exerted by inhibition of AzI ubiquitination. The ability of Az to prevent Ub-dependent degradation appears to be specific to AzI, as Az did not affect the ubiquitination of p53. It has been suggested that Az stimulates ODC degradation by imposing conformational changes that result in exposing its C-terminal degradation signal (36). It is possible that similarly, AzI inflicts conformational changes on AzI that hide its degradation signal. Alternatively, it is possible that Az competes with an E3 on binding to AzI. It will be of interest to identify the components of the Ub system, e.g. the E2 and E3 that mediate the ubiquitination of AzI as well as the signal that mediates the recognition of AzI by the Ub system.

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