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Distinct Domains of Antizyme Required for Binding and Proteolysis of Ornithine Decarboxylase

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Selective degradation by proteasomes of ornithine decarboxylase, the initial enzyme in polyamine biosynthesis, is mediated by the polyamine-inducible protein antizyme. Antizyme binds to a region near the N terminus of ornithine decarboxylase (X. Li and P. Coffino, Mol. Cell. Biol. 12:3556-3562, 1992). This interaction induces a conformational change in ornithine decarboxylase that exposes its C terminus and inactivates the enzyme (X. Li and P. Coffino, Mol. Cell. Biol. 13:1487-1492, 1993). Here we show that the C-terminal half of antizyme alone can inactivate ornithine decarboxylase and alter its conformation, but it cannot direct degradation of the enzyme, either in vitro or in vivo. A portion of the N-terminal half of antizyme must be present to promote degradation.

The rapid turnover of proteins is important in regulating cell growth and metabolism. The proteasome is a proteolytic machine that selectively degrades ubiquitinated and nonubiquitinated proteins (6, 26). 20S and 26S forms of the proteasome, each with multiple subunits, have been isolated from eukaryotic cells. The mechanism whereby proteasomes distinguish appropriate substrate proteins is poorly understood (7), although some studies have shown that ubiquitination (9) or exposure of internal structural elements (such as PEST sequences [25]) can provide degradation signals.

Ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, is among the most labile of cellular proteins. It provides a unique system with which to study regulated stability: polyamine levels within cells strongly influence the stability of ODC (18, 31). By promoting the destruction of the enzyme, polyamines limit their own accumulation. This form of feedback inhibition utilizes irreversible breakdown of ODC rather than, as in allosteric regulation, reversible inhibition. The polyamine-inducible agent of destruction of ODC is a protein termed antizyme (AZ). AZ has a high affinity for ODC (11). Association of the two entrains the following series of events: ODC becomes catalytically inactive (but activity can still be restored, by dissociation of the complex); the C terminus becomes more accessible (13); in a latter step, this one irreversible and dependent on ATP, ODC is degraded by the 26S proteasome (21).

A more comprehensive description of this process requires an understanding of the structural elements of both proteins that participate in their association, the effect of that association on each participant, and the functional domains, if any, of AZ required for subsequent proteolysis of ODC. Information of this kind would help to resolve the following question: Does binding of AZ to ODC simply change the conformation of the latter, making it more available as a substrate for degradation, or does AZ carry out some additional action, perhaps delivering ODC to the proteolytic apparatus or activating proteolysis?

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One strategy for defining the structural elements that sustain distinct functions is to examine the activities of genetically altered forms of ODC and AZ. We earlier performed such studies on ODC (12), using deletions of mouse ODC and chimeras made between mouse and trypanosome ODC. (The latter is 69% identical to the mouse enzyme within a common core region but is stable and unreactive with AZ [24].) We showed that two elements must be present within mouse ODC for AZ-mediated proteolysis. One region, near the N terminus, is required for high-affinity binding of AZ (12). A second, at the C terminus of ODC, is required for AZ-mediated degradation (3, 4, 13). This region is made more readily reactive with C-specific antibody when AZ is present. The C terminus is not required for the enzymatic activity of ODC (16). Furthermore, truncation of the C terminus does not prevent the binding of AZ nor abolish one functional consequence of AZ binding: reversible inhibition of enzymatic activity (12). Functional studies of the mutant forms of ODC were greatly facilitated by the use of an in vitro system derived from rabbit reticulocytes in which degradation of ODC is dependent on AZ (13, 21).

We now extend these studies to the other participant in the ODC-AZ interaction. Using deleted recombinant forms of AZ, we find that one region is required for association with and inhibition of ODC and that another is needed to direct degradation. The C-terminal half of AZ can bind ODC and abolish ODC activity, but it cannot promote degradation. This requires the involvement of a distinct region of AZ outside the C terminus.

MATERIALS AND METHODS

AZ cDNA and expression vectors. The rat AZ lambda gt11 partial cDNA clone Z1 (17, 19) and the dexamethasone-inducible vector pMAMneoZ1 (22) were obtained from S. Hayashi. Z1 and pMAMneoZ1 have a 212-amino-acid open reading frame. Throughout this report, we use a numbering convention that specifies the start of this reading frame to be amino acid 1 of AZ.

Construction of plasmids encoding AZ-GST fusions and preparation of AZ fusion proteins. Rat AZ cDNA Z1 (17) was expressed as a fusion protein C terminal to glutathione *S*-transferase (GST) by cloning into the *Bam*HI-*Eco*RI sites

of the pGEX-3 vector (30). Deletions within the AZ Z1 212-amino-acid open reading frame were made by PCR, using oligonucleotides that included *Bam*HI or *Eco*RI sites. The resulting amplified PCR fragments were restricted and ligated into the pGEX-3 vector to create fusion proteins with various deletions in the AZ portion. To express the fusion proteins, *Escherichia coli* carrying these recombinant plasmids was induced with isopropylthiogalactopyranoside, (IPTG), and the proteins were then purified with glutathione-Sepharose 4B beads (Pharmacia). For use in the degradation assay, proteins were eluted from beads with 0.1 M glycine and 0.1 M NaCl (pH 2.5), and the eluates were neutralized with 1.5 M Tris-HCl (pH 8.8).

In vitro translation. Plasmid DNA was used as a template to amplify chimeric ODC cDNA or AZ cDNA by PCR, using as the 5′ oligonucleotide a primer containing a T7 RNA polymerase recognition site, and the amplified DNA was then transcribed into cRNA with T7 RNA polymerase (12). cRNA was translated in vitro by using a rabbit reticulocyte lysate (Promega). The mouse ODC, radiolabeled with [^{35}S]methionine, was used for a binding assay or, in unlabeled form, for an inhibition assay.

Assays of AZ binding and inhibitory activity. Ten microliters of translation lysate containing [^{35}S]methionine-labeled mouse ODC was added to 500 μl of phosphate-buffered saline containing 0.05% Triton X-100 and 15 μl of glutathione-Sepharose 4B beads (Pharmacia) bearing the fusion proteins. After shaking at 4°C for 1 h, the beads were washed three times with phosphate-buffered saline. The proteins associated with the beads were made soluble in sodium dodecyl sulfate (SDS) sample buffer, and the radiolabeled ODC was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. For the inhibition assay, 10 μl of beads bearing the fusion proteins was mixed with 10 μl of in vitro-translated mouse ODC and incubated on ice for 5 min. ODC activity of the mixture was then determined as described previously (12).

In vivo regulation by AZ 106-212. DNA encoding AZ amino acids 106 to 212 (AZ 106-212) was amplified by PCR, using the rat AZ lambda gt11 cDNA clone Z1 as the template. An *Nhe*I restriction site and start codon ATG were incorporated into the 5′ oligonucleotide primer, and a *Sal*I site was incorporated into the 3′ primer. The PCR fragment so generated was then cloned into the *Nhe*I and *Sal*I sites of the dexamethasone-inducible expression vector pMAMneoZ1 (22) to generate pMAMneoAZ106-212. Plasmid pMAMneoAZ106-212 is identical to pMAMneoZ1 except that it encodes a truncated form of AZ composed of amino acids 106 to 212 in place of amino acids 1 to 212. Transformants were selected with G418. Pools of stably transformed clones were plated (approximately 10^6 cells per 100-mm-diameter Falcon dish) and incubated at 37°C for 16 h. Dexamethasone was added to the cell culture to a final concentration of 1 μM to induce AZ or AZ 106-212 protein. Cell lysates were prepared after the indicated time of treatment and assayed for ODC activity as described previously (12).

Immunoprecipitation of ^{35}S-labeled ODC. AZ or AZ 106-212 stably transfected HTC cells were plated at a density of about 10^6 cells per 100-mm-diameter dish (Falcon) and grown overnight. The cells were treated with 1 mM dexamethasone or were left untreated for 4 h and then labeled with [^{35}S]methionine for 30 min. Cells were collected in Nonidet P-40 lysis buffer (150 mM NaCl, 1% Nonidet P-40, 50 mM Tris [pH 8.0]). Cell lysates containing 5 × 10^6 acid-precipitable cpm were immunoprecipitated (8) with anti-mouse ODC-specific polyclonal rabbit antiserum. Immunoreactive protein was collected by using Pansorbin and subjected to SDS-PAGE and autoradiography.

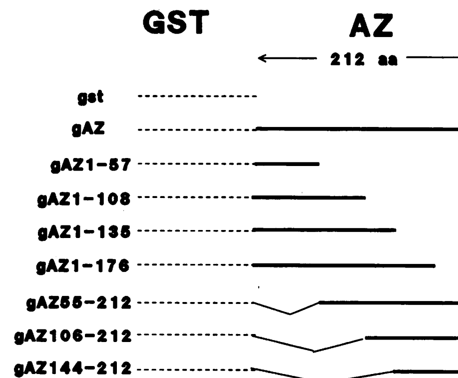

FIG. 1. Structure of the GST-AZ fusion protein (gAZ) and deletions within the AZ moiety.

RESULTS

The 106 C-terminal amino acids of AZ suffice for ODC binding and inhibition. Polyamines induce AZ without increasing the amount of its mRNA (17). This suggests the possibility that polyamines modulate translation of AZ mRNA. Consideration of the DNA sequences of rat AZ cDNA and genomic DNA (17, 19; cDNA sequence confirmed in this laboratory) reveals a single long open reading frame (325 codons), but this sequence includes no suitably placed methionine (AUG) initiation codon. Translation must either start at an AUG within a shorter, partially overlapping reading frame and then shift frame or start at a non-AUG codon lying near the 5′ end of the longest reading frame. Therefore, the primary structure of the amino terminus of rat AZ can not be inferred from DNA sequence alone, and direct protein sequencing has not been done. Nonetheless, we can carry out informative experiments with recombinant AZ, because, as shown below, all of the known cellular and biochemical functions of the protein are executed by an AZ consisting of the 212 C-terminal amino acids of the long open reading frame. These functions include the ability to bind and inhibit the enzymatic activity of ODC, alter its conformation, and direct its destruction in vivo and in vitro (12, 13, 21, 22, 23).

We used as the starting point a recombinant construct, termed gAZ, encoding the 212 carboxy-terminal amino acids of AZ fused, for ease of manipulation, to GST. We made a series of deletions within the AZ moiety of this fusion protein (Fig. 1). Deletions impinged on either end, one set (gAZ 1-57, gAZ 1-108, gAZ 1-135, and gAZ 1-176) removing C-terminal amino acids and the other set (gAZ 55-212, gAZ 106-212, and gAZ 144-212) removing N-terminal amino acids. gAZ 1-108 and gAZ 106-212 are reciprocal truncations, as are gAZ 1-57 and gAZ 55-212. gAZ 55-212 contains the PEST region (25) of AZ (amino acids 85 to 98), but gAZ 106-212 does not. The fusion proteins were produced in *E. coli* and purified by affinity chromatography with glutathione-Sepharose beads. SDS-PAGE showed the various proteins to have the expected mobilities (data not shown).

To determine the ODC binding domain of AZ, glutathione-Sepharose beads with the various gAZ proteins bound were mixed with ^{35}S-labeled mouse ODC and collected by cen-

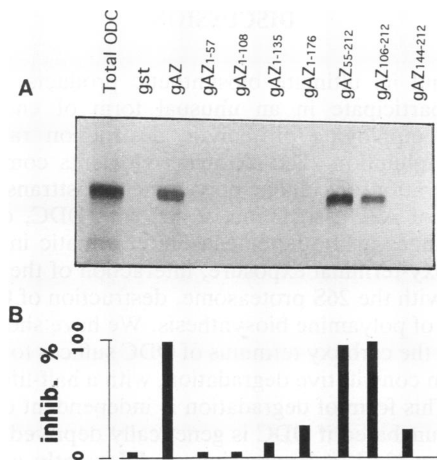

FIG. 2. Binding and inhibition of mouse ODC with gAZ and deletion mutants. (A) ^{35}S-labeled mouse ODC was used as a target for an AZ binding assay by mixing with gAZ and deleted gAZ proteins bound to Sepharose beads. The ODC bound by each gAZ fusion protein, as well as the total labeled ODC available for binding, was analyzed by SDS-PAGE. (B) Unlabeled mouse ODC was mixed with gAZ or its deletion mutants, and the ODC activity was assayed as described in the text. The inhibition activity is expressed as a percentage of that found with gAZ.

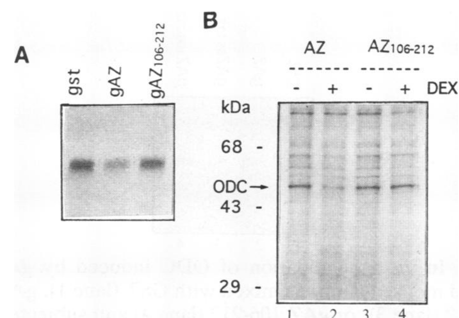

FIG. 3. In vitro and in vivo degradation of mouse ODC by gAZ and gAZ 106-212. (A) ^{35}S-labeled mouse ODC was incubated with GST, gAZ, or gAZ 106-212, and ODC degradation was initiated by adding the ATP-regenerating system for 1 h at 37°C. The remaining intact ODC was analyzed by SDS-PAGE. (B) HTC cells stably transformed with glucocorticoid-inducible constructs expressing AZ (lane 1 and 2) or AZ 106-212 (lane 3 and 4) were induced for 4 h with dexamethasone (lane 2 and 4) or were untreated (lane 1 and 3) and then labeled with [^{35}S]methionine. Cell lysates were subjected to immunoprecipitation with antibodies for mouse ODC, which reacts efficiently with the rat ODC of HTC cells. Immunoprecipitates were examined by SDS-PAGE and autoradiography.

trifugation. ODC associated with beads was then determined by SDS-PAGE and autoradiography (Fig. 2A). ODC bound efficiently to the GST-AZ fusion protein gAZ and not to the GST control, indicating that the interaction between AZ and ODC is specific. All C-terminal truncations of AZ tested (gAZ 1-57, gAZ 1-108, gAZ 1-135, and gAZ 1-176) abolished the interaction with ODC. Two truncations of the N terminus (gAZ 55-212 and gAZ 106-212) maintained the interaction, but a larger truncation, gAZ 144-212, destroyed the ability of AZ to bind to ODC. Therefore, we concluded that the N terminus of AZ is not required for ODC binding and that the binding domain is at the C terminus. The final 107 amino acids (those in gAZ 106-212) suffice for binding, but the final 69 amino acids (in gAZ 144-212) do not. The same deletion proteins were used to analyze inhibition of ODC activity (Fig. 2B). Inhibition and binding results were concordant for all of the deletion proteins, suggesting that the regions of AZ needed to bind and inhibit ODC overlap or are identical.

The ODC binding region of AZ is insufficient to promote degradation. We have previously used an in vitro assay to show that the gAZ protein can promote the degradation of ODC (13). We examined whether gAZ 106-212, the smallest AZ fragment we tested that binds ODC, can do so as well. The gAZ and gAZ 106-212 proteins were first titrated to determine their inhibitory activity for ODC, using conditions (limitation of ATP) that do not permit degradation. The degradation assay was then carried out by using an amount of each AZ protein fivefold in excess of that just sufficient to abolish ODC activity fully. For the degradation assay, ^{35}S-labeled ODC was first mixed with gAZ or gAZ 106-212. Degradation was carried out by adding an ATP-regenerating system and incubating the mixture at 37°C. The ODC remaining undegraded was examined by SDS-PAGE. As shown in Fig. 3A, gAZ 106-212, unlike gAZ, did not cause degradation. This result suggested that the ODC binding/inhibition domain of AZ is not sufficient to direct degradation.

We also tested whether amino acids 106-212 of AZ can promote ODC degradation in vivo. Murakami et al. have demonstrated that forced expression of AZ promotes ODC degradation in vivo (22). They transfected HTC cells with a vector expressing AZ from a glucocorticoid-inducible promoter; glucocorticoid treatment led to a decline of ODC activity (22). To assess its capacity to induce ODC degradation, AZ 106-212 was cloned into the identical pMAMneo glucocorticoid-inducible vector and transfected into HTC cells. As a positive control, cells were similarly transfected with the construct encoding intact AZ (amino acids 1 to 212). Transformants were selected with G418, and pools of stable transformants were used for the studies. A decline of ODC activity was observed after glucocorticoid induction of cells expressing AZ, as previously reported, and also in those expressing AZ 106-212. Induction of the AZ construct for 4 h reduced ODC activity to 33% of that in the untreated AZ control cells; the corresponding figure was 25% for AZ 106-212 (after 16 h of induction, 21 and 24%, respectively). The reduction of activity caused by the truncated AZ could be explained as the result of either inhibition of activity or accelerated degradation of ODC. To distinguish between these possibilities, we compared the effects of expressing AZ and AZ 106-212 on the abundance of ODC. HTC cells transformed with each construct were treated with glucocorticoid for 4 h and metabolically labeled with [^{35}S]methionine during the last 30 min of this treatment period. Cellular extracts were prepared, and ODC was immunoprecipitated. Glucocorticoid treatment markedly reduced the amount of labeled ODC in cells expressing AZ but not in cells expressing AZ 106-212 (Fig. 3B). This finding implies that in vivo expression of AZ increases the turnover of ODC, but that expression of the deleted form of AZ does not. These results are consistent with the conclusion that both in vivo and in vitro AZ 106-212 can inhibit ODC activity but cannot activate its destruction.

We previously proposed a model whereby AZ induces proteolysis by exposing the C terminus of ODC, based on the observation that gAZ caused an enhanced reaction with an antibody directed against the 37 C-terminal amino acids of

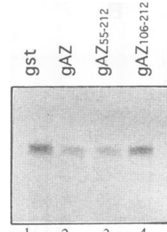

FIG. 4. In vitro degradation of ODC induced by gAZ 55-212. ^{35}S-labeled mouse ODC was mixed with GST (lane 1), gAZ (lane 2), gAZ 55-212 (lane 3), or gAZ 106-212 (lane 4) and subjected to the in vitro degradation assay. Induced degradation was assessed as in Fig. 3A.

mouse ODC (1, 13). We examined whether gAZ 106-212 could act similarly. Like gAZ, gAZ 106-212 enhanced the reaction of ODC with the antibody (results not shown). We conclude that the region missing in gAZ 106-212 does not govern the interaction of AZ with ODC but is required for degradation.

A portion of the N terminus of AZ participates in degradation. To better define the extent of the N-terminal functional region, we next examined the capacity of gAZ 55-212 to promote degradation. As shown in Fig. 4, gAZ 55-212 induced degradation as effectively as gAZ. Amino acids 55 to 105 are redundant for binding and inhibition of ODC but are required for its degradation. Amino acids 85 to 98 of this region of AZ constitute a PEST motif. Because PEST sequences are common in unstable proteins (27), we tested the possibility that the PEST sequence of AZ is responsible for the degradative function. The PEST motif (amino acids 85 to 98) was deleted by the homology extension PCR procedure as described previously (10). The capacity of the ΔPEST AZ mutant to inhibit ODC activity and direct degradation of ODC was then tested. The PEST deletion changed neither the inhibitory activity of AZ (results not shown) nor its capacity to promote degradation of ODC (Fig. 5A). The PEST region was similarly deleted from AZ 57-212, and this deletion also did not alter degradation of ODC (data not shown). AZ itself was little degraded in the in vitro assay, regardless of the presence or absence of the PEST motif (Fig. 5B) or of ODC (results not shown). AZ has a PEST region, but its presence destabilizes neither the protein in which it resides nor ODC.

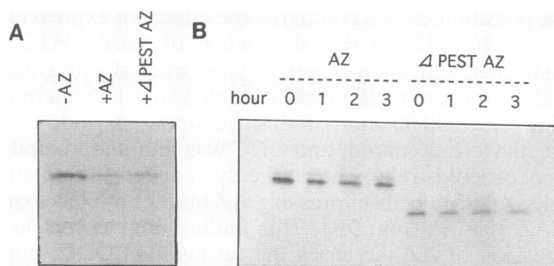

FIG. 5. Stability of the ΔPEST mutant of AZ and its ability to promote ODC degradation. (A) [^{35}S]methionine-labeled mouse ODC was mixed with either in vitro-translated AZ or the ΔPEST mutant and subjected to the degradation assay for 1 h. (B) [^{35}S]methionine-labeled AZ and the ΔPEST mutant were made by in vitro translation subjected to in vitro degradation. The undegraded labeled proteins were immunoprecipitated with polyclonal rabbit antiserum to AZ and examined by SDS-PAGE and autoradiography.

DISCUSSION

ODC and its ultimate biosynthetic products, the polyamines, participate in an unusual form of end product feedback, employing proteolytic destruction rather than allosteric inhibition. The following elements compose this cycle: elevation of cellular polyamines, posttranscriptional induction of AZ, association of AZ with ODC, conformational changes in the latter causing enzymatic inactivation and carboxy-terminal exposure, interaction of the ODC-AZ complex with the 26S proteasome, destruction of ODC, and reduction of polyamine biosynthesis. We have shown previously that the carboxy terminus of ODC suffices to confer on the protein constitutive degradation, with a half-life of 1 h or less (4). This form of degradation is independent of AZ and proceeds unabated if ODC is genetically deprived of an AZ binding site (3, 12). Expression of AZ greatly accelerates degradation, to the extent that newly synthesized ODC is destroyed within minutes of synthesis (31), and ODC pools that preexist AZ induction can be made to disappear sevenfold more rapidly (20). Both an AZ binding site and the carboxy terminus of ODC (or a substitute region, amino acids 376 to 425 of mouse ODC [13]) must be present to elicit AZ-accelerated destruction.

These observations were consistent with the view that AZ acts merely to augment exposure of the ODC carboxy terminus. According to this formulation, AZ merely disturbs the equilibrium between two conformers of ODC (13). The present results show this model to be insufficient: AZ does something more. A truncated form of AZ (amino acids 107 to 212) can change the accessibility of the carboxy terminus and inhibit enzymatic activity but cannot direct degradation. A more extended form of AZ (amino acids 55 to 212) can direct degradation of its target protein, both in vitro and in vivo. What action does this further 52-amino-acid region carry out? We do not as yet know. It may promote association of the AZ-ODC complex with the proteasome or activate the proteasome or change its specificity. It may do these things directly or through other cellular elements.

The polyamine-ODC-AZ proteolytic feedback loop has many parts, most of them imperfectly understood. Both opaque and intriguing is the means by which polyamines induce AZ. Western blot (immunoblot) analysis shows the level of AZ to increase (15), but the induction is clearly not associated with a change in AZ mRNA level (17). Thus, the process is likely translational. There is at present uncertainty about the primary structure of the N terminus of AZ. Two open reading frames of significant size are present in rat AZ mRNA (19). One, counting from its first AUG initiation codon, is 204 nucleotides long, and a second, which contains no AUG codon near its beginning, is 675 nucleotides long. The two overlap by 200 nucleotides; in principle, translation could initiate within this overlap in either reading frame. Translation of AZ mRNA may thus afford unusual opportunities for regulation. The second open reading frame encompasses 225 amino acids and is therefore of the right size to encode AZ, which is about 26 kDa in molecular mass, but lacks the near-universal AUG initiation codon. The first is far too small, but initiates with an AUG. The clone that we used in these studies is the Z1 clone, which contains most of the longer open frame sequence, i.e., the last 212 amino acids. Fortunately, an AZ containing amino acids 55 to 212 has all of the known functions of AZ. Therefore, while the nature of the translational control that produces AZ in response to cellular polyamines and the structure of the

translation product remain open questions, their resolution will not alter the conclusions described here.

The presence within a protein of a PEST region, one rich in the amino acids proline, aspartic acid, glutamic acid, serine, and threonine, correlates well with protein lability (27). This circumstantial association has been tested genetically, with mixed results. Appending the PEST-rich 37 amino acids to dihydrofolate reductase converts this enzyme from a stable to an unstable protein, but only in the context of additional structural modifications (14). Deletion of the same 37-amino-acid region from ODC stabilizes that protein (4), as do less extensive internal deletions that impinge on the PEST-rich moiety, but truncation of the terminal 5 amino acids, well outside the PEST residues, is also stabilizing (3). The presence of a PEST region within AZ demonstrates that the PEST motif may be implicated in degradation without covalent association with the target. In the present case, association does not imply action: deletion of the PEST motif from AZ (amino acids 85 to 98) leaves degradation unimpaired. The PEST region of AZ, like one of the two PEST regions of ODC (3, 4, 28), seems a reliable witness of protein degradation but not an agent.

The functionally distinct domains observed in AZ are seen as well in the viral E6 protein, which directs the degradation of p53. The C terminus of E6 suffices for p53 binding, but its N terminus must also be present for degradation to occur (2). A recombinant form of the E6 protein bearing a retinoblastoma protein binding domain directs the destruction of its new binding partner (29), suggesting that binding and degradation domains can be freely rearranged in a modular fashion. However, ODC failed a similar test of modularity: a series of recombinant constructs consisting of E6 equipped with the ODC binding region of AZ did not direct the in vitro degradation of ODC (data not shown). Ubiquitination precedes the degradation of both p53 and the retinoblastoma protein but not that of ODC. Perhaps modularity of functional domains cannot bridge such a mechanistic gap. Reverse genetics has revealed the surprising presence within AZ of a region of unknown biochemical function that is needed to elicit degradation. It remains now to determine the partners with which it interacts and the ambit and consequences of those interactions.

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