The antizyme family consists of closely homologous proteins believed to regulate cellular polyamine pools. Antizyme1, the first described, negatively regulates ornithine decarboxylase, the initial enzyme in the biosynthetic pathway for polyamines. Antizyme1 targets ornithine decarboxylase for degradation and inhibits polyamine transport into cells, thereby diminishing polyamine pools. A polyamine-stimulated ribosomal frameshift is required for decoding antizyme1 mRNA. Recently, additional novel conserved members of the antizyme family have been described. We report here the properties of one of these, antizyme2. Antizyme2, like antizyme1, binds to ornithine decarboxylase and inhibits polyamine transport. Using a baculovirus expression system in cultured SF21 insect cells, both antizymes were found to accelerate ornithine decarboxylase degradation. Expression of either antizyme1 or 2 in SF21 cells also diminished their uptake of the polyamine spermidine. Both forms of antizyme can therefore function as negative regulators of polyamine production and transport. However, in contrast to antizyme1, antizyme2 has negligible ability to stimulate degradation of ornithine decarboxylase in a rabbit reticulocyte lysate.

The mammalian antizyme (AZ)1 was first described as an inhibitor of ornithine decarboxylase (ODC) (reviewed in Ref. 1). ODC is a key enzyme in polyamine metabolism. It is induced by growth signals, and overexpression is observed in many tumor cells. Further, forced expression of ODC can transform mouse fibroblast cells (2).

AZ is induced when cellular polyamine levels rise. AZ mRNA has two overlapping open reading frames, a short ORF1 and a second ORF2, which encodes most of the AZ protein, but lacks an initiation methionine (3). A +1 translational frameshift, favored by elevated polyamines, aligns the two ORFs, thus producing the full-length functional AZ protein. AZ1 regulates ODC activity by dissociating the enzymatically active ODC dimer, forming the inactive ODC:AZ1 heterodimer (4, 5). ODC is a substrate for degradation by the 26 S proteasome, and is much more efficiently degraded when associated with AZ1 (6, 7). This accelerated form of ODC proteolysis is ATP-dependent but, distinct from most proteasome substrates, does not require ubiquitination (8–10). AZ1 thus takes part in a form of feedback regulation that restricts polyamine pools. Two activities of AZ1 are relevant to its limitation of ODC activity. The first is stoichiometric with respect to ODC, depends on dissociation of the ODC homodimer, and is, in principle, reversible. The second is catalytic with respect to ODC, because the enzyme is destroyed while AZ1 is recycled. In addition to its effects on ODC, AZ1 also negatively regulates polyamine transport into cells (11, 12).

Both the structure of AZ proteins and the polyamine-induced frameshifting mechanism are highly conserved; they have been found in a spectrum of organisms from man to Drosophila. Recently, a second AZ (AZ2) gene has been reported in human and mouse (13, 14); their transcripts also retain the characteristic pseudoknot structure that mediates AZ1 frameshifting (3, 15). GenBankTM search shows the additional existence of a third form of AZ (AZ3) in humans. AZ1 and AZ2 are each more conserved across species than they are within a single species, implying that AZ1 and AZ2 have maintained independent lineages since their divergence from a common ancestral gene. This suggests that they mediate distinct functions. AZ1 is known to limit polyamine accumulation in three ways: 1) it binds to and inactivates ODC; 2) it causes the degradation of ODC; and 3) it inhibits cellular uptake of polyamines. Here we assess the capacity of AZ2 to carry out these activities, using AZ1 as a reference for comparison. We find that AZ2, like AZ1, binds to ODC and inhibits polyamine transport. AZ2 expression accelerates ODC degradation in cultured cells but has negligible degradative activity compared with AZ1 in an in vitro system.

EXPERIMENTAL PROCEDURES

Plasmids and Baculovirus—Throughout this paper, we enumerate the first amino acid or the first nucleotide of AZ ORF1 as position 1. Z1 is a rat AZ1 partial cDNA clone, which lacks the first 45 nucleotides of ORF1 (3). GST-AZ169–227, a fusion of Z1 to GST, has been described previously (16). AZ2 sequences were amplified from a human cDNA library (17). Primers used were upstream primer AZ2a (5′-ccgaggattctgtaacaacc) and downstream primer AZ2b (5′-gctactagaagcc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-cggaattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). Treatment with calf intestinal phosphatase and restriction with EcoRI produced the desired fragment. PCR products were cloned into the vector pCRII (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc). PCR products were cloned into the vector pCR2.1 (Invitrogen) by TA cloning and sequenced. GST-AZ233–189 was made by PCR (primers: 5′-ccgaggattc(EcoRI)gccgctgtagccgctgctc and 5′-cggaattc(EcoRI)ccgggtctctctcttct) and downstream primer AZ2c (5′-ctgatgtcgaccc).
resultant fragment was subsequently inserted into pBacPAKHis1 at KpnI and blunt-ended HindIII sites to make pBacPAKHis.AZ2AT217. To create a baculovirus vector that requires frameshifting for expression of AZ2, AZ2 sequences were excised from pCR2.1 by EcoRV/KpnI digestion and inserted into pBacPAKHis1 digested with HindIII/KpnI to make pBacPAKHis.AZ2. The amino acid tag sequences removed were native AZ2 for His6AZ2: MG(His)6VVCIRLRPM and for His6AZ2AT217: MG(His)6VVDKLCGRSIPRM. The underlined M indicates the native initiation methionine of AZ2. A baculovirus expression vector for His6-tagged mouse ODC, pBacPAKHis.ODC, was made by PCR cloning. An ODC fragment containing the whole ORF (461 amino acid residues) was PCR amplified from pOD48 (20) using the primers 5'-aattctgcatctatagggaccatggatgtccctcacccaccc and 5'-ggggtacc(KpnI)tgktgccceaacagtcc. The product was blunt-ended by treating with T4 DNA polymerase, digested with KpnI and inserted into the PmlI/KpnI sites of pBacPAKHis1 to make pBacPAKHis.AZ2. These constructs were packaged for delivery to Sf21 cells in insect cells as recombinant baculovirus and cells infected following the protocols recommended by CLONTECH. Virus purification, amplification, and expression of heterogeneous proteins were according to standard protocols (21). pBacPAK6 virus encoding Escherichia coli β-galactosidase was purchased from CLONTECH.

**ODC Activity and Inhibition Assay**—GST-AZ169–227 and GST-AZ233–197 were expressed in *E. coli* and purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). Proteins were eluted from the columns with 50 mM Tris-HCl, 120 mM NaCl, 0.5% Nonidet P-40, 200 mM sodium orthovanadate, and 10 μg/ml each of phenylmethylsulfon fluoride, aprotonin, pepstatin A, and leupeptin. Radiolabeled proteins were immunoprecipitated with rabbit polyclonal antibody prepared against recombinant mouse ODC. For Western blotting, cell extracts were fractionated by SDSPAGE, transferred to a nitrocellulose membrane, and probed with peroxidase-conjugated anti-His antibody (Roche Molecular Biochemicals). To assess association of ODC with AZs, in vitro translated and metabolically labeled ODC was incubated with purified GST-fusion proteins bound to glutathione-Sepharose beads in phosphate-buffered saline plus 0.05% Triton X-100. Bound ODC was analysed by SDS-PAGE and autoradiography or by using a PhosphoImager (Molecular Dynamics).

**ODC in Vitro Degradation**—[35S]methionine-labeled ODC and AZ were translated in vitro in the presence of [35S]methionine in rabbit reticulocyte lysate (Promega) as described (22). A DNA template for transcription by T7 RNA polymerase was generated by PCR. Primers used were the following: ODC1–461 (5'-gttaaatgctactatgggactggcagcttactaag and 5'-cggaggactctatacgctatgat), AZ1–233–217 (5'-gttaaatgctactatgggactggcagcttactaag and 5'-ggggtacc(KpnI)tgktgccceaacagtcc), AZ2–233–189 (5'-gttaaatgctactatgggactggcagcttactaag and 5'-ggggtacc(KpnI)tgktgccceaacagtcc) to promote translation of the second ORF. As expected, sequence comparing of AZ1 and AZ2 of rodents and humans show that each is more highly conserved across species lines than are AZ1 and AZ2 when compared within species (Fig. 1) (14). Such conservation of both AZ1 and AZ2 suggests they may have distinct cellular functions. We therefore compared AZ1 with AZ2 to compare which activities of the former are also found in the latter.

**AZ2 Binds to ODC and Inhibits Its Activity in Vitro**—Sequence similarities between AZ1 and AZ2 suggest that AZ2 may also be a negative regulator of ODC. To test this idea, the second ORF of AZ1 and AZ2 were each expressed as GST fusion proteins in *E. coli*. Similar to GST-AZ1 (20–22), GST-AZ2 (23–189) bound to ODC (Fig. 2A). As expected, both AZ1 and AZ2 inactivated ODC enzymatic activity. A parallel dilution series of each fusion protein showed that they were approximately equipotent in inhibiting ODC activity (Fig. 2B). Similar to AZ1, the functional domain of AZ2 for ODC binding and inactivation is within ORF2.

**AZ Activity in Sf21 Cells**—Native AZ proteins are the product of expression of both ORF1 and 2. To test in vivo function of the full proteins in cells, we used a point deletion of a single nucleotide to align the two ORFs. We thereby made expression independent of cellular polyamine status and avoided the re-
requirement for frameshifting, a process that reduces the efficiency of expression. We expressed full-length AZ1 and AZ2 using a baculovirus expression system (21). This system was developed to facilitate high level expression of cloned gene products in insect cells, and uses virus-derived vectors for transient expression. The two ORFs were aligned by a single base deletion, ΔT\textsuperscript{205} for AZ1 and ΔT\textsuperscript{97} for AZ2. Infection of SF21 cells with viruses encoding AZ1 or AZ2 resulted in the cellular production of similar amounts of ODC inhibitory proteins. Titration of extracts prepared from cells infected with AZ1 (AZ1ΔT\textsuperscript{205}) or AZ2 (His6AZ2ΔT\textsuperscript{97}) against active ODC showed that they differed by only 2-fold (AZ1 > AZ2) in inhibitory activity (Fig. 3). Full-length AZ1 (AZ1ΔT\textsuperscript{205}) or AZ2 (His6AZ2ΔT\textsuperscript{97}), when co-expressed with His\textsubscript{6}-tagged ODC in SF21 cells, form a complex (Fig. 4). ODC-AZ co-infected SF21 cells were metabolically labeled and extracts immunoprecipitated with an anti-ODC antibody. Both AZ1ΔT\textsuperscript{205} and His\textsubscript{6}AZ2ΔT\textsuperscript{97} were pulled down together with His\textsubscript{6}ODC by anti-ODC antibody (Fig. 4).

AZ2 Frameshift in Vivo—To test whether AZ2 is capable of frameshifting in vivo, we also expressed AZ2 cDNA in SF21 cells with an N-terminal His\textsubscript{6} tag. As no mutagenesis was performed on this construct to align ORF1 and ORF2, expression of the protein should require an in vivo frameshift. Although expressed at a lower level compared with His\textsubscript{6}AZ2ΔT\textsuperscript{97}, which does have the reading frames artificially aligned, His\textsubscript{6}AZ2 was nevertheless expressed in SF21 cells (Fig. 4). Note that His\textsubscript{6}AZ2 migrated a little faster than His\textsubscript{6}AZ2ΔT\textsuperscript{97} because the frameshift has caused the first native amino acid of AZ2 to be different between the His\textsubscript{6}tag and the frameshift frame. Therefore, the frameshift has produced functional protein was supported by the following evidence. First, it bound to ODC and was immunoprecipitated with ODC by an anti-ODC antibody (Fig. 4). Second, SF21 cells expressing His\textsubscript{6}AZ2 but not cells infected with a control virus expressing His\textsubscript{6}AZ2ΔT\textsuperscript{97} were nevertheless expressed in Sf21 cells (Fig. 4). Note that His\textsubscript{6}AZ2 migrated a little faster than His\textsubscript{6}AZ2ΔT\textsuperscript{97} because the frameshift has caused the first native amino acid of AZ2 to be different between the His\textsubscript{6}tag and the frameshift frame. Therefore, the frameshift has produced functional protein was supported by the following evidence. First, it bound to ODC and was immunoprecipitated with ODC by an anti-ODC antibody (Fig. 4). Second, SF21 cells expressing His\textsubscript{6}AZ2 but not cells infected with a control virus containing ODC inhibitory activity (data not shown). We also constructed a fusion of the AZ2 frameshift region to EGFP such that ORF2 of AZ2 was in frame with the EGFP reading frame. When this construct was transiently expressed in COS-7 or ODC-deficient Chinese hamster ovary C55.7 (27) mammalian cells and protein products detected with an anti-EGFP antibody, protein of the size expected for the frameshift product was observed. Data consistent with this experiment are shown (Fig. 5). In the latter case AZ expression should cause steady-state levels of ODC to decline. Western blotting with an antibody against the His\textsubscript{6}tag at the N terminus of the ODC protein (Fig. 5B) revealed that ODC protein was indeed reduced to an undetectable level by expression of AZ, compared with a minor reduction seen with the control β-galactosidase construct. Similar results were obtained when the anti-ODC antibody was used for Western blotting (data not shown), which ruled out the possibility that the N terminus of His\textsubscript{6}ODC fusion had been cleaved in AZ-infected cells. It is not likely that the reduced ODC level results from reduced replication of the virus encoded by AZ2.
ing ODC in cells infected by the virus encoding AZs. AZ expression had little or no effect on ODC expression as measured by metabolic labeling (Fig. 4), which detects newly synthesized proteins instead of steady state protein levels. Taken together, the data strongly suggest that ODC degradation in Sf21 cells is accelerated by both AZ1 and AZ2. (Pulse-chase experiments were not carried out to confirm this conclusion, as we could not establish effective chase conditions for these cells.) The expression level of AZ2 from His6AZ2ΔT97 in Sf21 cells was much lower than the ODC expression level when cells were infected with ODC baculovirus alone (Fig. 5B). This suggests that in this experimental system, 1 molecule of AZ2 can catalyze the degradation of more than 1 ODC molecule.

AZ2 Does not Cause ODC Degradation in Vitro—Extensive

**Fig. 2. Binding and inhibition of ODC.** AZ binds to and inhibits ODC in vitro. Panel A, 35S-labeled in vitro translated ODC was allowed to interact with immobilized GST, GST-AZ1, or GST-AZ2. The ODC input protein (left lane) and bound protein was visualized by SDS-PAGE and autoradiography. Panel B, titration of ODC activity by GST (●), GST-AZ1 (○), or GST-AZ2 (✕). 4 μg of extract protein from ODC vector-infected Sf21 cells was used per assay point as the source of ODC activity. Data is plotted as a percent of activity present without addition of GST proteins and was approximately 400 nmol/min/mg of protein.

**Fig. 3. ODC inhibitory activity of AZ expressed in Sf21 cells.** ODC activity was titrated by extracts of Sf21 cells expressing AZ1ΔT205 (○), AZ2ΔT97 (✕), or PAK6 encoding β-galactosidase (●). 4 μg of extract protein from ODC-infected Sf21 cells was used per assay point as the source of ODC activity. Data is plotted as a percent of activity present without addition of inhibitory proteins and was approximately 400 nmol/min/mg of protein.

**Fig. 4. ODC and AZ form intracellular complexes.** Sf21 cells were singly or doubly infected with the viruses indicated and metabolically labeled with [35S]methionine. Radiolabeled proteins were immunoprecipitated with an anti-ODC antibody and visualized by SDS-PAGE and autoradiography. Arrows mark the positions of His6ODC and of co-immunoprecipitated proteins encoded by AZ1ΔT205, His6AZ2ΔT97, or His6AZ2 vectors. The position of migration of marker proteins of the indicated molecular masses (kDa) are shown on the left.

**Fig. 5. Reduction of ODC activity and protein level by AZ co-expression in Sf21 cells.** ODC levels were assessed in Sf21 cells infected with ODC alone or in combination with AZ1ΔT205, His6AZ2ΔT97 or PAK6, a β-galactosidase control. A, ODC enzymatic activity; B, steady state level of His6ODC detected by Western blotting with an anti-His6 antibody. Note that AZ1ΔT205 was not detected, because it was not His6-tagged.
subject to 60 min of incubation at 37 °C. The amount of ODC that remained undegraded is and the AZ on ice and addition of an ATP generating system, samples were immediately analyzed (left lanes) or incubated at 37 °C for 60 min before analysis. The amount of ODC that remained undegraded is shown below each lane as a percentage of that present in the sample not subject to 60 min of incubation at 37 °C.

previous studies have used both crude and purified cellular extracts as constituents of an in vitro system for study of ODC degradation (10, 16, 28, 29). Such investigations have shown proteolysis to be independent of ubiquitination and dependent on ATP, AZ1, and the 26 S proteasome. Using a rabbit reticulocyte extract supplemented with an ATP regenerating system, we examined the capacity of proteins corresponding to AZ1 or AZ2 ORF2 to direct degradation of ODC. The AZs and ODC were produced by in vitro translation. They were radiolabeled using incorporation of [35S]methionine to provide a means for following their amount and stability. A semi-quantitative asessment of the relative potencies of AZ1 and AZ2 was obtained by comparing the degradative activity of a dilution series of the two proteins (Fig. 6). Relative intensity of labeling, normalized to the respective methionine content of each protein, was used to estimate relative protein stoichiometry. At the highest concentration of each AZ used in the experiment shown, the AZs and ODC were initially present at approximately equimolar concentrations.

In the case of AZ1, an 8-fold dilution resulted in approximately the same extent of ODC degradation as the highest concentration examined, and a 16-fold dilution produced more ODC degradation than a control with no AZ1 added. In the control with no exogenous AZ1 added, the intensity of the ODC signal was reduced about 2-fold compared with an identical sample, but one not subjected to the 1-h incubation period used to elicit degradation. This “AZ-independent background degradation” is prevented by the proteasome inhibitor N-acetyl-leuc- leu-norleucinal peptide (results not shown) and is probably due to the presence of endogenous AZ in the reticulocyte lysate (28).

The effect of adding AZ2 is very different from that seen with AZ1. AZ2 produced no degradation, even when added at a 1:1 molar ratio with respect to ODC. In fact, even the lowest concentration used provided a modest ODC-protective effect compared with a control sample incubated without either AZ1 or AZ2. These results imply that under the experimental conditions used, AZ2 is at least 16-fold less potent than AZ1 in directing the degradation of ODC.

AZ Inhibits Spermidine Uptake—In addition to regulating ODC activity, AZ1 also inhibits polyamine transport into cells. In mammalian cells transfected with AZ1 under the control of an inducible promoter, polyamine uptake was reduced several-fold when AZ1 was expressed (11, 12). We measured spermi-
AZ2 in Polyamine Metabolism

The opposite degradative properties of AZ2 we observed in two different systems suggest that this function may be subject to physiologic regulation. AZ2 may have the capacity to reversibly inhibit ODC, and thus provide transient inhibitory regulation or a means to store inactive ODC in a form available for rapid use. AZ2 cDNA was originally described in a screen for seizure-inducible brain mRNAs (13). ODC has been found to be present in adult mouse brain in an inactive complex with antizyme that can be activated on further purification (33, 34). The AZ activity present in mouse brain has been found to be unreactive with a series of monoclonal antibodies reactive with rat liver AZ (34), a result consistent with distinctive tissue distributions of the AZs. If the brain ODC complex contains AZ2, it may represent a stored form of ODC available for activation, perhaps by displacing AZ2 with antizyme inhibitor, a catalytically inactive AZ-binding homolog of ODC (35).

Acknowledgments—We thank Sudarsi Desa for technical assistance.

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