

# Amino Acids Regulate Expression of Antizyme-1 to Modulate Ornithine Decarboxylase Activity\*

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**Background:** Amino acids regulate ornithine decarboxylase (ODC) activity through antizyme-1 (AZ1).

**Results:** Asparagine or glutamine prevents AZ1 expression in response to amino acid starvation. AZ1 synthesis by amino acids is regulated by a differential activation of mTORC1/2.

**Conclusion:** The inhibition of mTORC1 and activation of mTORC2 during amino acid starvation induces AZ1 synthesis.

**Significance:** Putrescine and amino acids regulate ODC activity by stimulating AZ1 synthesis via mTORC2.

In a glucose-salt solution (Earle's balanced salt solution), asparagine (Asn) stimulates ornithine decarboxylase (ODC) activity in a dose-dependent manner, and the addition of epidermal growth factor (EGF) potentiates the effect of Asn. However, EGF alone fails to activate ODC. Thus, the mechanism by which Asn activates ODC is important for understanding the regulation of ODC activity. Asn reduced antizyme-1 (AZ1) mRNA and protein. Among the amino acids tested, Asn and glutamine (Gln) effectively inhibited AZ1 expression, suggesting a differential role for amino acids in the regulation of ODC activity. Asn decreased the putrescine-induced AZ1 translation. The absence of amino acids increased the binding of eukaryotic initiation factor 4E-binding protein (4EBP1) to 5'-mRNA cap and thereby inhibited global protein synthesis. Asn failed to prevent the binding of 4EBP1 to mRNA, and the bound 4EBP1 was unphosphorylated, suggesting the involvement of the mammalian target of rapamycin (mTOR) in the regulation of AZ1 synthesis. Rapamycin treatment (4 h) failed to alter the expression of AZ1. However, extending the treatment (24 h) allowed expression in the presence of amino acids, indicating that AZ1 is expressed when TORC1 signaling is decreased. This suggests the involvement of cap-independent translation. However, transient inhibition of mTORC2 by PP242 completely abolished the phosphorylation of 4EBP1 and decreased basal as well as putrescine-induced AZ1 expression. Asn decreased the phosphorylation of mTOR-Ser<sup>2448</sup> and AKT-Ser<sup>473</sup>, suggesting the inhibition of mTORC2. In the absence of amino acids, mTORC1 is inhibited, whereas mTORC2 is activated, leading to the inhibition of global protein synthesis and increased AZ1 synthesis via a cap-independent mechanism.

The polyamines, spermidine and spermine and their diamine precursor putrescine, are required for the proliferation of

eukaryotic cells and the growth of tissues (1–3). In addition, polyamines are involved in the regulation of a variety of other cellular functions, such as transmembrane ion flux, apoptosis, and migration (4, 5, 6). The importance of polyamines to cellular function has been demonstrated in both normal and cancer cells from a variety of tissues (7, 8). The intracellular levels of polyamines are closely controlled and primarily depend on the activity of ornithine decarboxylase (ODC<sup>2</sup>; EC 4.1.17), which catalyzes the first rate-limiting step in polyamine synthesis. ODC activity itself is highly regulated and varies in response to many different stimuli in different tissues and cells. In general, activity increases dramatically and transiently in response to trophic hormones, growth factors, other proliferative stimuli, and damage (1, 9–11). ODC activity has one of the shortest half-lives of any mammalian enzyme (12, 13). The intracellular concentration of polyamines is controlled at several steps, including their uptake and their biosynthesis. The latter is predominantly achieved by controlling ODC activity by a mechanism involving ODC antizyme (AZ) (14, 15). AZ was first identified in mammals and exists in several isoforms (AZ1, AZ2, AZ3, and AZ4) (16–18). AZ1 prevents the formation of active ODC homodimers by forming ODC-AZ heterodimers (19, 20). Furthermore, AZ1 binding mediates ODC degradation by the 26 S proteasome (20–22). AZ levels increase with the elevation in intracellular concentration of polyamines. The induction of AZ by polyamines thus constitutes a feedback control in polyamine homeostasis. AZ expression is regulated by polyamines in a unique manner during translation of the AZ mRNA, which involves a programmed +1 frameshift, resulting in the expression of the functional full-length AZ protein (18, 23). AZ binds to and accelerates the degradation of ODC (24) and other proteins shown to regulate proliferation and cell death, such as Aurora-A, cyclin D1, and Smad1 (25–28).

Among normal dietary constituents, certain amino acids markedly increase ODC activity and mucosal growth when

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<sup>2</sup> The abbreviations used are: ODC, ornithine decarboxylase; AZ, antizyme; EBSS, Earle's balanced salt solution; mTOR, mammalian target of rapamycin; eIF4E and eIF4G, eukaryotic initiation factor 4E and 4G, respectively; 4EBP1, eIF4E-binding protein; p70S6K, p70-S6 ribosomal protein kinase; EGFR, EGF receptor; MPER, mammalian protein extraction reagent; DPBS, Dulbecco's PBS; AZin, AZ inhibitor; CHX, cycloheximide; AIB,  $\alpha$ -aminoisobutyric acid; AA– and AA+, without and with amino acids, respectively; ORN, ornithine.

administered intragastrically (29, 30). The effects of amino acids on ODC activity have been shown to occur in a wide variety of tissues and cells. Fausto (1) found that some amino acids could increase ODC activity in regenerating liver. Chen and Canellakis (13) and Chen and Chen (31) examined the ability of amino acids to induce ODC activity in cells incubated in a simple salt-glucose solution (Earle's balanced salt solution; EBSS), thus eliminating stimulation from growth factors present in serum or other amino acids and nutrients normally found in incubation medium. They showed that ODC activity was increased in neuroblastoma cells by asparagine (Asn) and glutamine. Additional studies by the same group (32–34) found that, although Asn was the most effective, other amino acids transported by one of the  $\text{Na}^+$ -dependent systems could also induce ODC activity. Whereas induction of ODC activity was dependent on  $\text{Na}^+$ , it was independent of metabolism or the ability of the amino acid to be incorporated into protein. However, other amino acids like lysine (Lys) and valine (Val) were unable to induce ODC in EBSS. Of particular interest was the finding, in a variety of cell types, that the induction of activity by hormones and growth factors required the presence of Asn or a similar amino acid (32, 33).

In previous work (35), our laboratory reported that Asn but not EGF could stimulate ODC activity in intestinal epithelial cells incubated in EBSS. EGF elicited a potentiated response in the presence of Asn. Although induction of ODC activity did not involve the synthesis of new ODC protein, it did require both transcription and translation (35). These data indicate that amino acids either induced or repressed a specific regulator of ODC enzyme activity.

Recent studies have shown that the mammalian target of rapamycin (mTOR) regulates the translation machinery by controlling the phosphorylation of both eukaryotic initiation factor 4E-binding protein (4EBP1) and p70-S6 ribosomal protein kinase (p70S6K) via mTORC1 and mTORC2 (36, 37). Gln has been shown to be essential for the metabolism of intestinal mucosa and for proliferation of colon cancer cells (38). In most studies, cellular deprivation of a single essential amino acid or branched chain amino acid decreased mTOR signaling and inhibited protein translation. However, the effect of Asn and Gln on mTOR signaling is unknown.

The significance of the current study is the finding of a unique mode of regulation of AZ1 expression. Generally, amino acid starvation inhibits protein synthesis, and the addition of amino acids restores it. However, AZ1 synthesis occurred in the absence of amino acids, and its synthesis was prevented by the addition of specific amino acids. This makes sense because AZ inhibits ODC and in the absence of amino acids ODC activity is unable to stimulate or support growth. Therefore, this study provides an important insight into the complexity of amino acid signaling and, thereby, its role in the regulation of various cellular processes. We show that Asn stimulated ODC activity by preventing the expression of AZ1 without a change in the level of ODC protein. The disappearance of antizyme is essential for EGF to increase the activity of the enzyme. Asn significantly decreased the levels of AZ1 mRNA and protein. Although amino acids normally regulate protein translation via the mTOR pathway, AZ1 synthesis occurred independently of

mTORC1 but depended on mTORC2 signaling. Asn-mediated inhibition of AZ1 synthesis involved the dephosphorylation of 4EBP1 by mTORC2. This is the first demonstration of the mechanism whereby Asn and related amino acids regulate ODC activity.

## EXPERIMENTAL PROCEDURES

**Chemicals and Supplies**—Disposable culture ware was purchased from Corning Glass. Tissue culture media and dialyzed fetal bovine serum (FBS) were from Invitrogen. Biochemicals were purchased from Sigma. L-[1- $^{14}\text{C}$ ]ornithine (specific activity 51.6 mCi/mmol) and [ $^{35}\text{S}$ ]methionine/cysteine (specific activity 1175.0 Ci/mmol) were purchased from PerkinElmer Life Sciences. Rabbit polyclonal and mouse monoclonal antibodies against AZ1 and antizyme inhibitor (AZin) were a gift from Dr. Senya Matsufuji (26, 27). ODC antibody and AZ siRNAs (AZ1 and AZ2) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). EGFR, Tyr(P) $^{1173}$  EGFR, ERK, phospho-ERK, phospho-p70S6K, phosphoribosomal S6 protein, phospho-4EBP1, total 4EBP1, and eukaryotic initiation factor 4E (eIF4E) antibodies and rapamycin were purchased from Cell Signaling Technology (Beverly, MA). PP242 was purchased from Tocris Biosciences (Ellisville, MO). Anti-actin antibody was purchased from Millipore (Billerica, MA). EGF was purchased from Invitrogen. Mammalian protein extraction reagent (MPER) and Halt protease mixture reagents were purchased from Thermo Scientific (Rockford, IL). Phosphatase inhibitor tablets (PhosStop) were from Roche Applied Science. The Western blot detection system (SuperSignal ELISA Femto Maximum Sensitivity Substrate) was purchased from Thermo Scientific. Moloney murine leukemia virus reverse transcriptase, PCR nucleotide mix, and random primers were purchased from Promega (Madison, WI). SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA). All other chemicals were of the highest purity commercially available. PCR primers were designed using Primer 3 software from the Whitehead Institute (Cambridge, MA).

**Plasmids**—Three AZ1 siRNA oligonucleotide sequences were designed and cloned into a plasmid vector (pcDNA6.2-GW/EmGFP-MiR) and confirmed by sequencing using appropriate primer pairs. Selected clones for the vector (MiR-LacZ-EGFP) and AZ1 (MiR-LacZ-AZ1-EGFP) were used to prepare plasmid DNA for the transfection of IEC-6 cells using the Endo-Free Plasmid Maxi kit from Qiagen.

**Cell Culture**—The IEC-6 cell line was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. The stock was maintained in T-150 flasks in a humidified, 37 °C incubator in an atmosphere of 90% air, 10%  $\text{CO}_2$ . The medium consisted of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) with 5% heat-inactivated FBS, 10  $\mu\text{g}$  of insulin, and 50  $\mu\text{g}/\text{ml}$  gentamicin sulfate. The stock was passaged weekly and fed three times per week. Passages 15–20 were used in the experiments.

**Experimental Protocol**—For most experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . They were counted and plated (day 0) at  $6.25 \times 10^4$  cells/ $\text{cm}^2$  in DMEM plus 5% dialyzed FBS, 10  $\mu\text{g}$  of insulin, and 50  $\mu\text{g}$  of gentamicin

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sulfate/ml. The cells were fed on day 2. On day 3, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with serum-free DMEM with 10  $\mu\text{g}$  of insulin and 50  $\mu\text{g}$  of gentamicin sulfate/ml. On day 4, the medium was removed, the cells were washed once with Hanks' balanced salt solution, and the medium was replaced with EBSS containing whatever additions were required by the experiment.

**ODC Assay**—The activity of the enzyme ODC was assayed with a radiometric technique in which the amount of  $^{14}\text{CO}_2$  liberated from DL-[1- $^{14}\text{C}$ ]ornithine was estimated (39). Briefly, after experimental treatment, the dishes were placed on ice; the monolayers were washed three times with cold Dulbecco's PBS (DPBS), and 0.5 ml of 1 mM Tris buffer (pH 7.4) containing 1 mM EDTA, 0.05 mM pyridoxal phosphate, and 5 mM dithiothreitol was added. The cells were frozen at  $-80^\circ\text{C}$  until assayed. At the time of assay, the cells were thawed on ice, scraped into Microfuge tubes, sonicated, and centrifuged at  $12,000 \times g$  at  $4^\circ\text{C}$  for 10 min. The ODC activity of an aliquot of supernatant was incubated in a stoppered tube in the presence of 6.8 nmol of [ $^{14}\text{C}$ ]ornithine for 15 min at  $37^\circ\text{C}$ . The  $^{14}\text{CO}_2$  liberated by the decarboxylation of ornithine was trapped on a piece of filter paper impregnated with 20  $\mu\text{l}$  of 2 N NaOH, which was suspended in a center well above the reaction mixture. The reaction was stopped by the addition of TCA to a final concentration of 5%. The  $^{14}\text{CO}_2$  trapped in the filter paper was measured by liquid scintillation spectroscopy. Aliquots of the supernatant were assayed for total protein with the method described by Bradford (40). Enzymatic activity is expressed as pmol of  $\text{CO}_2$ /h/mg of protein.

**RNA Extraction, Reverse Transcription, and Real-time PCR**—Serum-starved cells incubated in EBSS with or without Asn for 4 h were washed twice with DPBS followed by the addition of TRIzol reagent for the extraction of total RNA following the manufacturer's (TRIzol) instructions. The yield and quality of total RNA was measured by absorbance at 260/280 nm. One  $\mu\text{g}$  of total RNA and 0.5  $\mu\text{g}$  of the random primers were used for reverse transcription with Moloney murine leukemia virus reverse transcriptase following the manufacturer's instructions. The resulting cDNA was diluted to 100  $\mu\text{l}$  with Diethylpyrocarbonate treated water and used as a template for real-time PCR. Briefly, PCR primers were designed with a melting temperature ( $T_m$ ) of  $59-61^\circ\text{C}$ . Amplicon size was 50–150 bases. Forward and reverse primers spanning exon-exon junctions were selected to avoid amplification of genome sequences (for AZ1, 5'-ACTGCTTCGCCAGAGAGAA (forward primer) and ATCTTCAGGGGTGGGTGAG-3' (reverse primer); for actin, 5'-GCTGAAAAGATGACCCAGATCA (forward primer) and CACAGCCTGGATGGCTACGT-3' (reverse primer). PCRs were performed using an ABI Prism 7700 sequence detection system (Applied Biosystems). The reaction contained 25  $\mu\text{l}$  of SYBR reagent, 5  $\mu\text{l}$  of diluted cDNA, and 300–900 nM primers in a 50- $\mu\text{l}$  volume. The thermal cycling conditions involved an initial denaturing step at  $95^\circ\text{C}$  for 15 s and an annealing and extension steps at  $60^\circ\text{C}$  for 1 min. Quantitative values were obtained from the threshold cycle value ( $C_t$ ), which is the point where a significant increase of fluorescence is first detected. The transcript number of rat  $\beta$ -actin was

quantified as an internal RNA control, and each sample was normalized on the basis of its  $\beta$ -actin content. The relative gene expression level of each sample was then normalized to the EBSS (control). Final results, expressed as  $n$ -fold difference in gene expression relative to  $\beta$ -actin and the control group, termed  $n$ , were calculated as  $n = 2^{(\Delta C_{t_{\text{sample}}} - \Delta C_{t_{\text{calibrator}}})}$ , where  $C_t$  values of the sample and calibrator were determined by subtracting the average  $C_t$  value of a target gene from the corresponding  $C_t$  value of the  $\beta$ -actin gene.

**siRNA Transfection**—AZ1- and AZ2- specific siRNA (20–25 nucleotides) derived from *Rattus norvegicus* (Santa Cruz Biotechnology, Inc.) were used for the transient transfection of IEC-6 cells. FuGENE-HD transfection reagent was mixed with 10  $\mu\text{M}$  siRNA (3:5  $\mu\text{l}$ ) in serum-free medium to a total volume of 100  $\mu\text{l}$  and incubated for 45 min at room temperature. IEC-6 cells at a relatively early passage were grown to 60% confluence in 6-well plates. For transfection, the cell monolayer was rinsed with serum-free medium, and the siRNA/FuGENE-HD mixture was added drop by drop onto the cell monolayer and incubated for 24 h followed by incubation in serum containing medium for an additional 24 h at  $37^\circ\text{C}$ . Transiently transfected cells were serum-starved for 24 h and rinsed with Hanks' balanced salt solution and further incubated with serum-free medium or EBSS for 3 h. Cells were washed with DPBS and lysed with MPER containing protease and phosphatase inhibitors to determine the expression of AZ1.

AZ1 siRNA sequence was also cloned in MiR-LacZ-EGFP vector as per the manufacturer's instructions for the stable transfection of cells to knock down AZ1. Empty vector (MiR-LacZ-EGFP) or AZ1 (MiR-AZ1-EGFP) plasmid DNA (10  $\mu\text{g}$ ) was transfected as described above. The success of transfection was ascertained by monitoring GFP expression, and stable clones were selected using blasticidin. Isolated clones were characterized by determining the expression of AZ1 and induction of ODC activity by Asn.

**[ $^{35}\text{S}$ ]Methionine/Cysteine Incorporation and AZ1 Synthesis**—Confluent serum-starved IEC-6 cells grown in 6-well plates (triplicate for each treatment) or T-75 flasks were incubated in EBSS with or without Asn in the presence of 10  $\mu\text{Ci/ml}$  [ $^{35}\text{S}$ ]methionine/cysteine for 2 and 4 h. Six-well plates were washed five times, and cells were lysed using MPER containing protease inhibitors. The supernatant was used to determine the protein concentration by the BCA method. An equal amount of protein from each sample was precipitated using TCA and resuspended in SDS sample buffer at a concentration of 1  $\mu\text{g}/\mu\text{l}$ . Incorporation of [ $^{35}\text{S}$ ]methionine/cysteine was measured by liquid scintillation spectroscopy. Equal amounts of protein (30  $\mu\text{g}$ ) were resolved on 12% SDS-PAGE and analyzed by Western blotting for AZ1 expression.

Cell extracts prepared from T-75 flasks (900  $\mu\text{g}$  of protein) were incubated with protein AG plus agarose beads (25  $\mu\text{l}$ ) for 2 h at  $4^\circ\text{C}$  on a rocker followed by centrifugation. Pre-cleared cell extracts were incubated with AZ1 antibody (3  $\mu\text{g}$ ), followed by the addition of protein AG plus agarose beads (25  $\mu\text{l}$ ), and processed as mentioned above. Agarose beads were washed twice, resuspended in SDS sample buffer, and resolved on 12% SDS-PAGE. Immunoprecipitated AZ1 transferred on PVDF



membrane was sprayed with Enhance reagent and exposed to x-ray film overnight at  $-70^{\circ}\text{C}$ .

**Cap Binding Assay**—Cells treated as indicated were washed with DPBS and lysed in binding buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM EDTA, containing phosphatase and protease inhibitor mixtures. Protein (500  $\mu\text{g}$ ) from the lysates in 500  $\mu\text{l}$  of cap binding buffer was added to 50  $\mu\text{l}$  of prewashed 7-methyl GTP-Sepharose beads (illustra<sup>TM</sup> from GE Healthcare UK Ltd.), and slurry was incubated on a rocker for 2 h at room temperature. Samples were washed twice with cap binding buffer, followed by the addition of sample buffer, and boiled, and equal amounts were resolved by SDS-PAGE and transferred onto PVDF membrane. Blots were probed for the indicated antibodies.

**Western Blot Analysis**—After experimental treatments, IEC-6 cells were washed twice with DPBS. The DPBS was removed, 500  $\mu\text{l}$  of MPER containing protease and phosphatase inhibitors was added, and the plates were frozen overnight and scraped. The cell lysate was centrifuged at 10,000 rpm for 10 min. The supernatant was used to determine the protein concentration by the BCA method. Total cell protein (30  $\mu\text{g}$ ) was separated on 10% SDS-PAGE and transferred to a PVDF membrane for Western blotting with a specific primary antibody and appropriate secondary antibody labeled with horseradish peroxidase (HRP). Immune complexes were detected by a chemiluminescence system.

**Statistics**—All data are expressed as means  $\pm$  S.E. Densitometric analysis of Western blots from three different experiments was performed. Analysis of variance and appropriate post hoc testing determined the significance of the differences between means. Values of  $p < 0.05$  were considered significant.

## RESULTS

**Expression of AZ1, AZin, and ODC**—Based on the known steps in the regulation of ODC activity, we hypothesized that the effects of Asn on ODC activity might be due to the inhibition of AZ or stimulation of AZin expression. IEC-6 cells grown to confluence and serum-starved for 24 h were used throughout the investigation. Serum-starved cells were incubated in EBSS with or without additives for the indicated period. When IEC-6 cells were incubated in EBSS for 3 h, a 20-kDa isoform of AZ1 was detected (Fig. 1A) using a specific antibody generated against rat AZ1 protein (41). The appearance of AZ1 was prevented by Asn. To confirm the involvement of AZ1, IEC-6 cells transiently transfected with AZ1 and AZ2 specific siRNA were exposed to serum-free DMEM or EBSS for 3 h, and the levels of AZ1 protein were determined by Western blotting. Results in Fig. 1, B and C, show that AZ1-specific siRNA significantly decreased basal AZ1 as well as the expression of AZ1 induced during the incubation with EBSS. However, AZ2 siRNA failed to affect the expression of AZ1. The failure of AZ2 siRNA to alter the expression of AZ1 despite sharing high amino acid homology with the AZ1 indicates that AZ siRNAs had no off-target effects. Furthermore, transfection of plasmid-based AZ1 siRNA decreased AZ1 expression in EBSS and significantly increased ODC activity compared with that induced in vector-transfected cells in response to Asn (Fig. 1, D and E), confirming

our siRNA results shown in Fig. 1B. These results indicate that EBSS induced the expression of AZ1.

We determined whether the regulation of AZ1 expression by Asn is unique to IEC-6 cells or is a more general phenomenon by measuring the ODC activity and AZ1 levels in NIH3T3 cells. The addition of Asn to EBSS significantly increased ODC activity in both cell types, although the increase was significantly higher in NIH3T3 cells (Fig. 1F). The basal level of AZ1 in EBSS was lower in NIH3T3 cells compared with that observed in IEC-6 cells (Fig. 1F). The addition of Asn significantly decreased AZ1 expression in both cell types (Fig. 1F). Therefore, despite differences in levels of ODC activity and protein, Asn had a similar effect in both cell lines. The addition of 10 mM Asn in EBSS decreased AZ1 protein (Fig. 1A). The effects of EGF and Asn + EGF on AZ1 expression were more or less similar to those of EBSS and EBSS + Asn, respectively. ODC and AZin proteins were unchanged in response to EBSS, Asn, EGF, or Asn + EGF (Fig. 1A). Thus, Asn appears to regulate AZ1 expression, and EGF may have a secondary role in ODC activation.

**Effect of Asn on Expression of AZ1 and ODC Activity**—In order to gain insight into the mode of action of amino acids, we examined the effectiveness of Asn in a short term and extended time course study. In the presence of Asn, ODC activity was detected within 60 min and continued to increase for at least 180 min, which correlated with the absence of AZ1 (Fig. 2, A and B). Furthermore, the levels of AZ1 protein continue to increase in EBSS through 6 h (Fig. 2, C and D). Although Asn prevented the expression of almost all AZ1, a significant amount of AZ1 was detected after 4 h of incubation compared with 0 h (Fig. 2, C (lanes 8 and 9) and D). Asn-induced ODC activity increased rapidly for 4 h and then began to decline, indicating a regulatory role of AZ1 in the activation of ODC by Asn. Furthermore, it is apparent that the very low levels of AZ1 expression at 4 and 6 h efficiently reduced ODC activity (Fig. 2E). The levels of ODC protein remained unchanged throughout the EBSS and Asn treatment (Fig. 2C, top). Thus, AZ1 appears to be a high affinity modulator of ODC activity. To test this notion, cells were incubated in EBSS for 2, 30, and 60 min to allow accumulation of low levels of AZ1 and were exposed to EBSS or Asn for 3 h. ODC activity in response to Asn was significantly decreased in cells preincubated with EBSS for 30 and 60 min (Fig. 3A). These observations were confirmed by measuring the ODC activity of Asn-stimulated cell extract combined with the extract of cells treated with EBSS for 3 and 6 h. EBSS cell extract significantly decreased Asn-induced ODC activity (Fig. 3B). These data indicate that ODC activity largely depends on the level of AZ1 protein and that its expression is regulated by Asn in IEC-6 cells.

Polyamines are known to stimulate AZ expression by a frame shifting mechanism (23, 41, 43, 44). We used putrescine to augment AZ1 expression in EBSS. Fig. 3C shows that putrescine (10  $\mu\text{M}$ ) significantly increased AZ1 compared with that seen in EBSS (Fig. 3C). AZ1 immunoprecipitated from extracts of cells treated with EBSS and EBSS plus putrescine significantly inhibited ODC activity induced by Asn (Fig. 3, C (top) and D). The Coomassie Brilliant Blue-stained gel shows the level of immunoprecipitated AZ1, which was higher in EBSS plus putrescine compared

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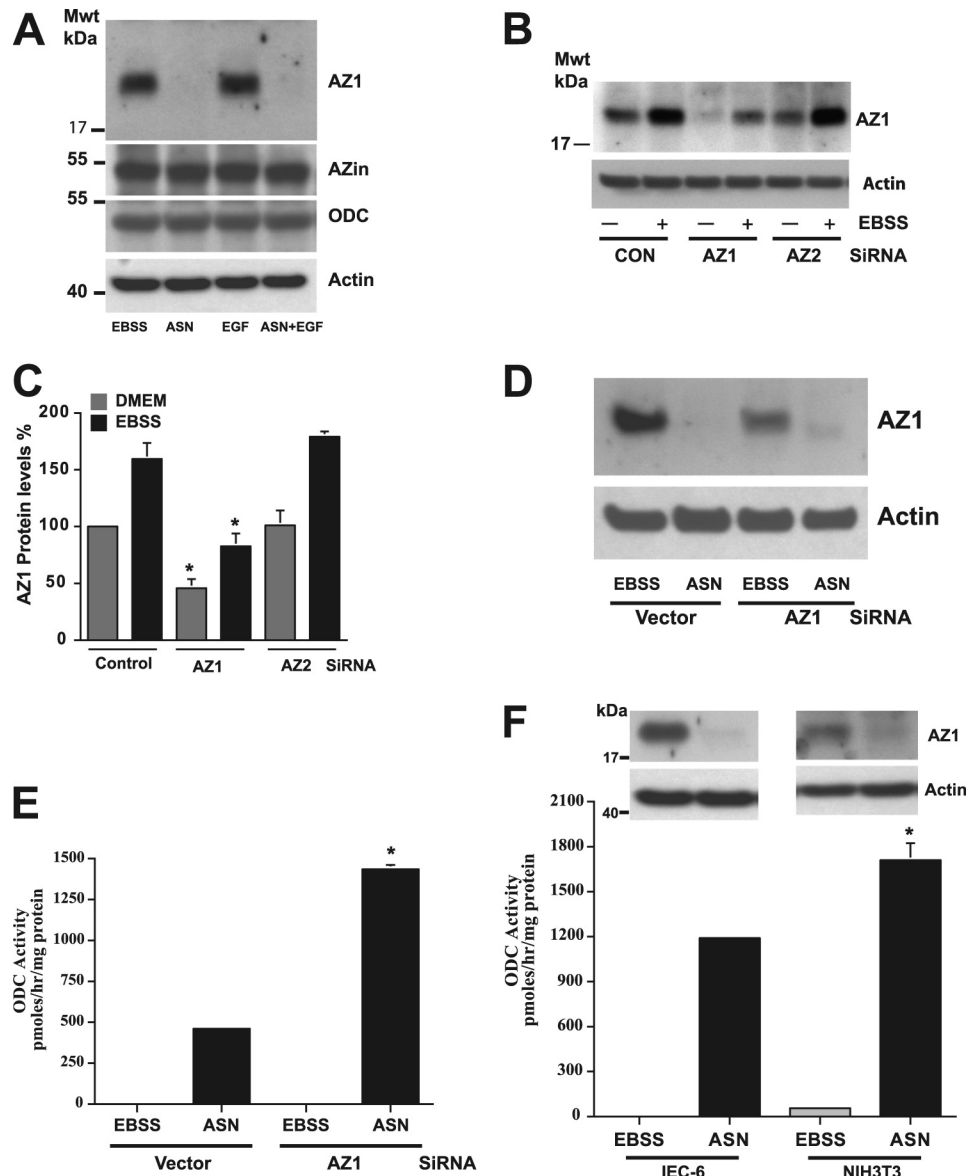


FIGURE 1. A, Asn was added to EBSS at a concentration of 10 mM in the presence and absence of EGF (30 ng/ml) and incubated for 3 h. Cell extracts were analyzed by Western blot to determine the levels of AZ1, AZin, ODC, and actin. IEC-6 cells transfected with AZ1- or AZ2-specific siRNA as described under "Experimental Procedures" were incubated with serum-free DMEM or EBSS for 3 h. B, cell extracts were analyzed by Western blot to determine the levels of AZ1. C, densitometric analysis of the AZ1 from Western blot (B) of siRNA-transfected cells. Cells transfected with MiR-LacZ-EGFP (Vector) or MiR-AZ1-EGFP (AZ1) specific siRNA as described under "Experimental Procedures" were incubated with EBSS in the presence or absence of 10 mM Asn for 3 h. Cell extracts were analyzed by Western blot to determine the levels of AZ1 (D) and ODC activity (E). IEC-6 and NIH3T3 cells were exposed to EBSS or EBSS + Asn for 3 h. ODC activity and AZ1 levels were determined as described under "Experimental Procedures" (F). Values are means  $\pm$  S.E. (error bars);  $n = 3$  cultures. \*,  $p < 0.05$  compared with corresponding control values. Representative Western blots from three observations are shown.

with EBSS alone (Fig. 3D, right). The extent of ODC inhibition correlated with the level of AZ1 (Fig. 3D). These results confirmed the role of AZ1 in the regulation of ODC activity.

To identify the mechanism by which Asn regulates AZ1 expression, we allowed AZ1 to accumulate in EBSS-treated cells for 3 h. We then incubated cells for an additional 3 h in the presence of Asn and studied the disappearance of AZ1 (Fig. 4, A and B). The prolonged incubation in EBSS (total 6 h) significantly increased AZ1 protein levels. The addition of Asn after 3 h of incubation in EBSS nearly eliminated the accumulated AZ1 within 1 h (Fig. 4, A (lane 7) and B). Thus, the effectiveness of Asn in eliminating AZ1 appears to depend on the level of preexisting AZ1 protein. Prolonging the incu-

bation in EBSS beyond 6 h caused AZ1 protein to decrease spontaneously within 1.5 h (Fig. 4, C (lanes 3 and 4) and D). The addition of Asn after 6 h of incubation in EBSS further decreased AZ1 (Fig. 4, C (lane 7) and D). The decrease in AZ1 in EBSS was about 45%, and Asn accelerated it to about 80% (Fig. 4B, lanes 4 and 7).

We determined the stability of AZ1 in EBSS for 4 h to avoid the spontaneous degradation phase (6 h) and then added cycloheximide (CHX), Asn, or Asn + CHX and monitored AZ1 levels over the course of 120 min (Fig. 5A). In EBSS, the level of AZ1 increased for 120 min, after which the addition of CHX caused a rapid decrease resulting in the loss of about 50% of AZ1 protein within 75 min, which was similar to the decrease

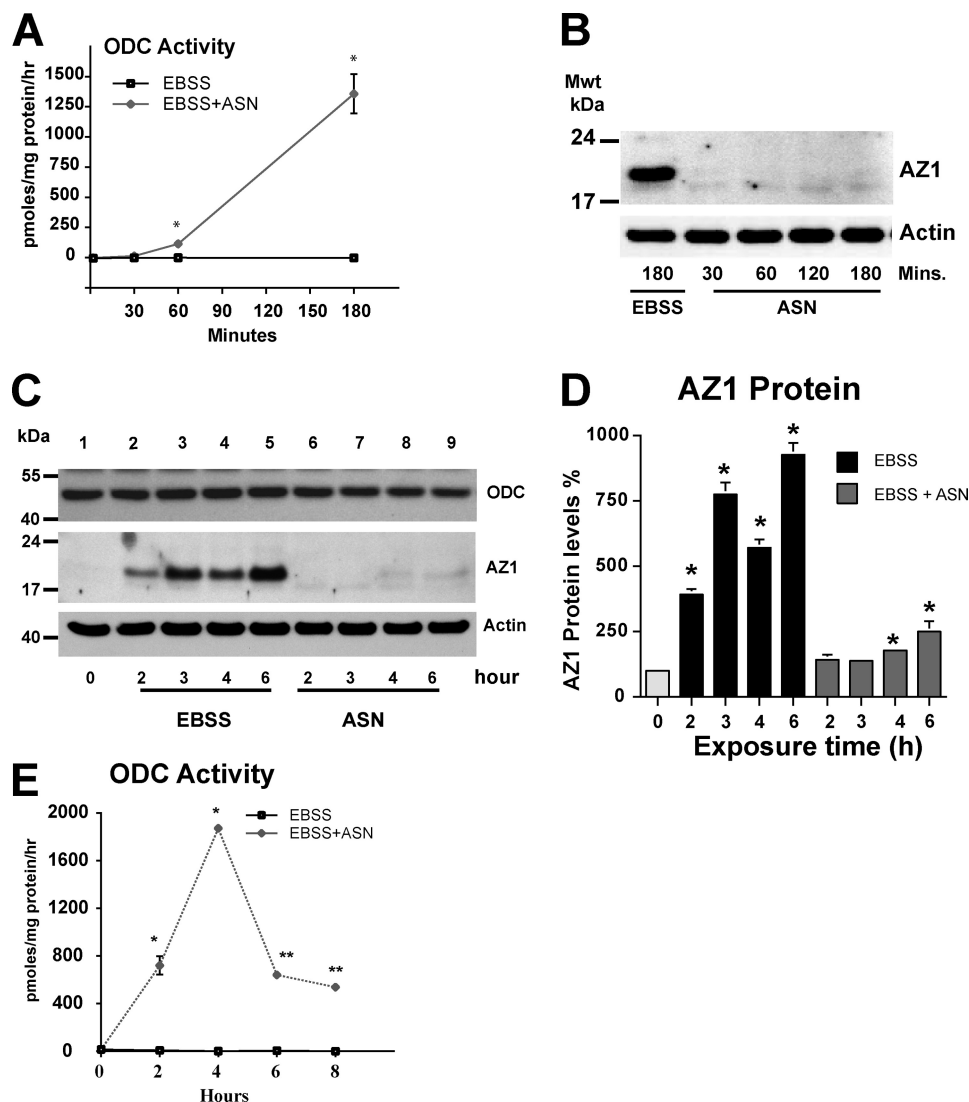


FIGURE 2. *A*, Asn was added to EBSS at a concentration of 10 mM and incubated for the indicated time, and ODC activity was determined after 3 h. Asn was added to EBSS at a concentration of 10 mM and incubated for the indicated time. *B*, cell extracts were analyzed by Western blot to determine the levels of AZ1 and actin. Asn was added to EBSS at a concentration of 10 mM and incubated for the indicated time, and AZ1 levels (*C*) and densitometry of AZ1 (*D*) and ODC activity (*E*) were determined as described earlier. A representative Western blot from three observations is shown. Values are means  $\pm$  S.E. (error bars);  $n = 3$  cultures. \*,  $p < 0.05$  compared with the corresponding control value.

observed with EBSS + Asn. The addition of CHX to the EBSS + Asn group did not significantly affect the decrease in AZ1 compared with EBSS + Asn alone (Fig. 5*B*). Asn significantly decreased the expression of AZ1 mRNA as measured by real-time PCR (Fig. 5*C*). Inhibition of transcription by actinomycin D (*ACT-D*) failed to prevent AZ1 synthesis in EBSS (Fig. 5*D*), indicating that ongoing transcription may not control the synthesis of AZ1. Actinomycin D completely prevented the expression of p21<sup>Waf1/Cip1</sup> protein within 1 h and  $\beta$ -actin after 3 h, indicating an effective inhibition of transcription (data not shown). Therefore, we used Coomassie Brilliant Blue staining to confirm equal loading of the samples.

In order to confirm the role of protein synthesis in the expression of AZ1, we measured [<sup>35</sup>S]methionine/cysteine incorporation into proteins synthesized in EBSS with and without Asn for 2 and 4 h. First, we found that the addition of methionine/cysteine in EBSS had no effect on AZ1 synthesis (data not shown). We then analyzed equal amounts of protein

for the incorporation of [<sup>35</sup>S]methionine/cysteine by scintillation spectroscopy. Fig. 6*A* shows that Asn significantly decreased the incorporation of radioactive amino acids into newly synthesized proteins compared with that seen in EBSS. TCA-precipitated proteins from <sup>35</sup>S-labeled samples were resolved by SDS-PAGE, transferred to a PVDF membrane, and analyzed by Western blotting. The membrane was washed, dried, sprayed with the Enhance reagent, and exposed to x-ray film as described under "Experimental Procedures." The results in Fig. 6*B* show that in the presence of Asn, there was no detectable AZ1 expression at 2 h and only a slight increase at 4 h (Fig. 6*B*, middle). A band from the superimposed Western blot and <sup>35</sup>S-labeled film shows decreased labeling of the band corresponding to AZ1, which began to appear at 4 h in the presence of Asn. However, increasing intensity of the band was noted at 2 and 4 h in the presence of EBSS (Fig. 6*B*, top). We further confirmed these observations by immunoprecipitation of AZ1 from the extracts. The newly synthesized AZ1 increased

## Amino Acids and Antizyme-1 Expression

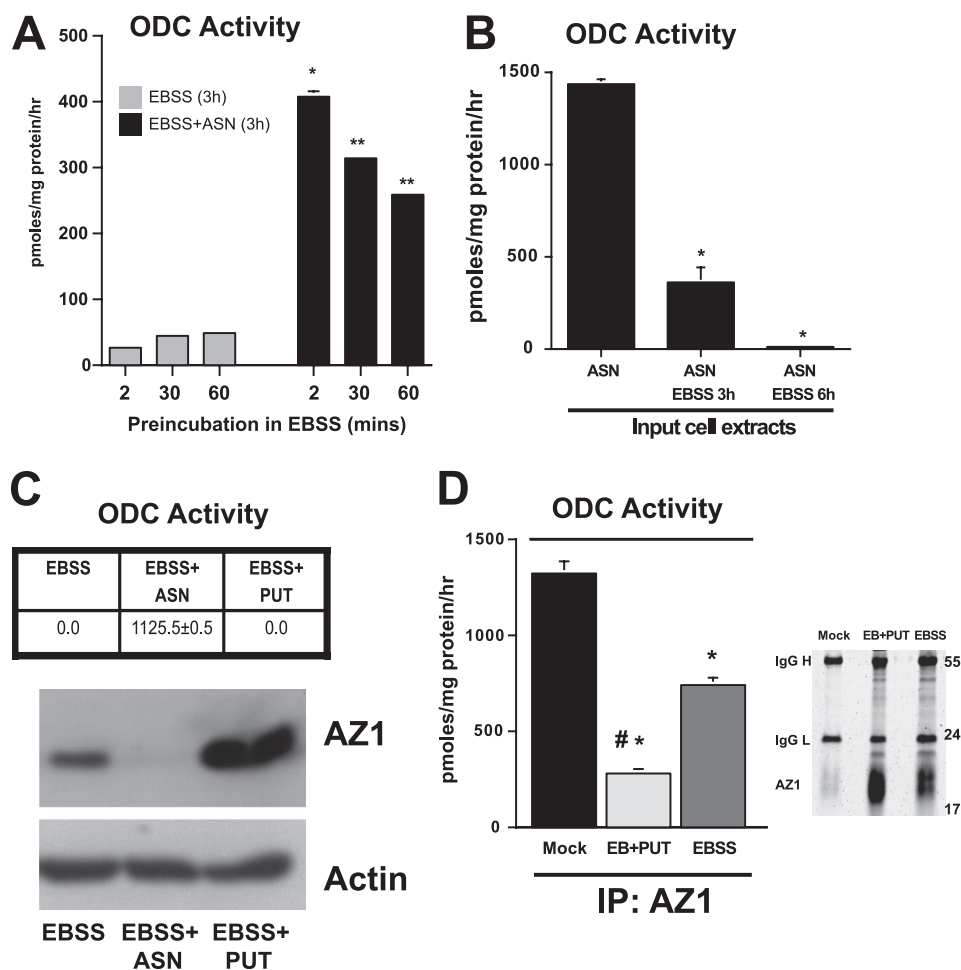


FIGURE 3. *A*, cells were exposed to EBSS, and Asn was added after 2, 30, and 60 min and further incubated for 3 h. ODC activity was determined as described under "Experimental Procedures." Values are means  $\pm$  S.E.;  $n = 3$  cultures. \*,  $p < 0.05$  compared with corresponding control value. *B*, confluent serum-starved cells were exposed to EBSS or EBSS + Asn (ASN) for 3 h, and cell extracts were prepared in ODC buffer. Asn extract and Asn extract + EBSS extract (1:1) were used for the determination of ODC activity. Values are means  $\pm$  S.E.;  $n = 3$  cultures. \*,  $p < 0.05$  compared with Asn. *C*, cells exposed to EBSS, EBSS + Asn, or EBSS + 10  $\mu$ M putrescine (PUT) for 3 h were analyzed by Western blot to determine the levels of antizyme AZ1 and ODC activity. Values are means  $\pm$  S.E. A representative Western blot from three observations is shown. *D*, equal amounts of proteins from EBSS and EBSS + putrescine cell extracts subjected to immunoprecipitation (IP) using AZ1 antibody were used to determine their inhibitory effect on EBSS + Asn-induced ODC activity (left). Immunoprecipitated proteins resolved by SDS-PAGE were stained using Coomassie Brilliant Blue stain (right) to ascertain the presence of AZ1. IgG H and IgG L, heavy and light chains, respectively, of AZ1 antibody. Values are means  $\pm$  S.E. (error bars);  $n = 3$  cultures. \*,  $p < 0.05$  compared with corresponding control value (Mock). #,  $p < 0.05$  compared with EBSS.

in a time-dependent manner in EBSS, whereas it was reduced in the presence of Asn (Fig. 6C). The band marked by the *asterisk* showing a pattern identical to that of AZ1 might be a degradation product generated during storage and immunoprecipitation. It is known that AZ1 is highly unstable and requires a high concentration of salt and/or Triton X-100 for stabilization (45). We had not attempted to stabilize AZ1 to avoid effects on immunoprecipitation. These results support the conclusion that Asn modulated AZ1 levels by regulating its synthesis.

**Effect of Amino Acids on Expression of AZ1**—Previous studies, including our own (35), showed that, like Asn, glutamine (Gln) and  $\alpha$ -aminoisobutyric acid (AIB) increased and lysine (Lys) and valine (Val) failed to increase ODC activity. These results led us to conclude that transport systems A and N type amino acids are inducers, whereas other amino acids do not induce ODC activity. Asn, Gln, and AIB prevented AZ1 expression (Fig. 7A). Interestingly, Val and Lys, non-inducers of ODC activity, increased AZ1 expression, although the effect of

Val was weaker compared with Lys (Fig. 7B). Both Lys and Val increased AZ1 protein in a time-dependent manner (Fig. 7B). Val significantly increased AZ1 levels compared with EBSS within 2 h, and Lys induced AZ1 within 1 h and caused strong increases at 2 and 3 h (Fig. 7C). These data suggest that Lys and Val inhibit ODC activity via AZ1.

Lys, Val, and ornithine (ORN) significantly decreased (40%) ODC activity stimulated by Asn, which correlated with levels of AZ1 (Fig. 8, A and B). Val and ORN added together almost completely inhibited ODC activity. However, ODC protein levels were unchanged in response to the addition of these amino acids to EBSS (Fig. 8B, top). Thus, it is clear from these results that Lys, Val, and ORN oppose the effect of Asn in the clearance of AZ1. The growth medium, DMEM, contains amino acids, including Gln (3.9 mM), Lys (1.0 mM), and Val (0.8 mM), shown to have opposing effects on AZ1 expression. Therefore, the amino acid composition of culture medium should influence ODC activity. The addition of 10 mM Asn to DMEM in the absence of amino acids increased ODC activity 3-fold com-



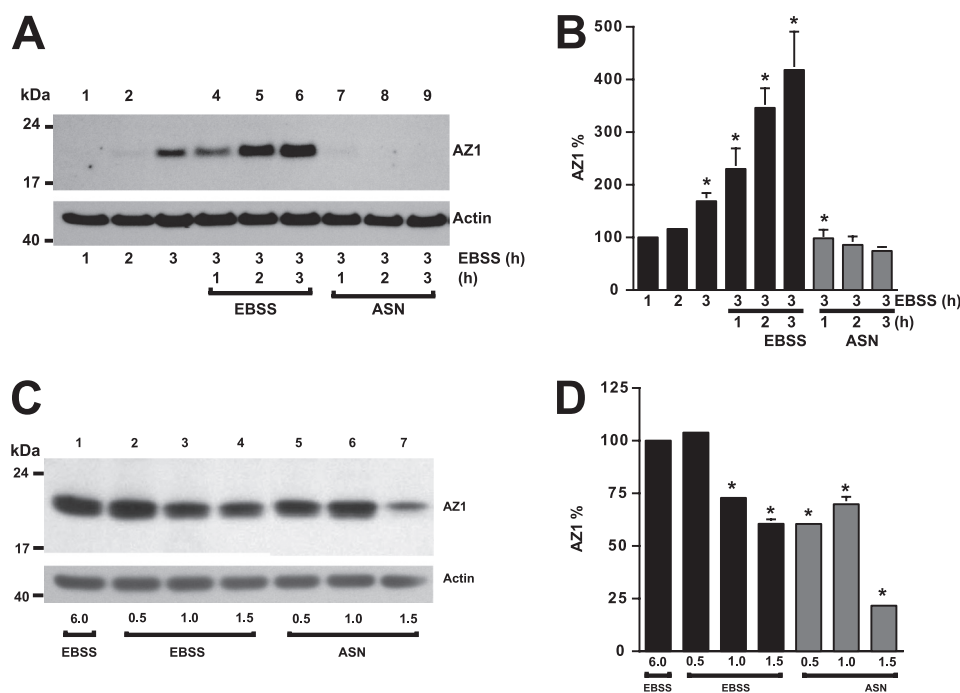


FIGURE 4. A and C, cells were exposed to EBSS for 3 h (A) or 6 h (C), followed by further incubation in the presence or absence of Asn for the indicated time, and cell extracts were analyzed by Western blot to determine the levels of AZ1 and actin. Bar graphs B and D show densitometry of blots from A and C, respectively. Representative Western blots from three observations are shown. \*,  $p < 0.05$  compared with the corresponding control value. Error bars, S.E.

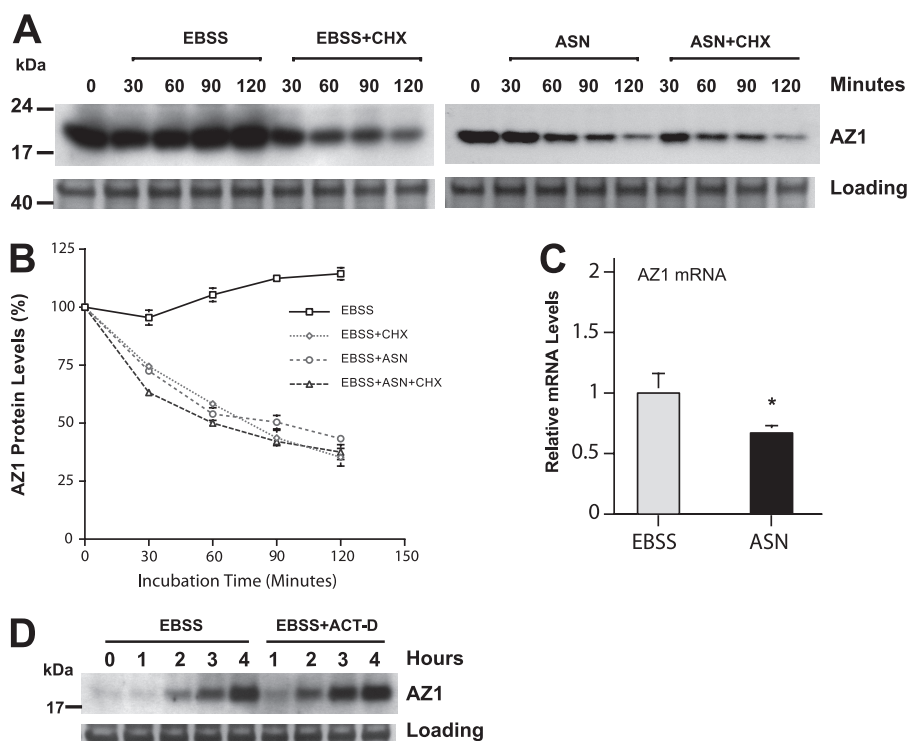


FIGURE 5. A, confluent serum-starved cells were exposed to EBSS for 4 h, followed by the addition of Asn in the presence and absence of CHX and incubated for the indicated time period. Cell extracts were analyzed by Western blot to determine the levels of AZ1. Representative Western blots for CHX (A) and densitometry of Western blots of CHX treatment (B) from three observations are shown. C, total mRNA extracted from confluent serum-starved cells exposed to EBSS or EBSS + Asn (ASN) for 3 h were analyzed by real-time PCR for the quantitative assay of AZ1 mRNA using a gene-specific primer pair as described under "Experimental Procedures." \*,  $p < 0.05$  compared with corresponding control value. D, confluent serum-starved cells were exposed to EBSS for the indicated time period in the presence and absence of actinomycin-D (ACT-D), and cell extracts were analyzed by Western blot to determine the levels of AZ1. A representative Western blot from three observations is shown. Membranes stained with Coomassie Brilliant Blue to ensure equal loading are shown (Loading). Error bars, S.E.

pared with that observed in DMEM containing amino acids (Fig. 8C). Cells exposed to DMEM containing amino acids and EBSS for 3 h showed significantly higher levels of AZ1 com-

pared with that in DMEM (Fig. 8D). These results indicate that the amino acid composition of growth medium may affect ODC induction by regulating AZ1 levels.



## Amino Acids and Antizyme-1 Expression

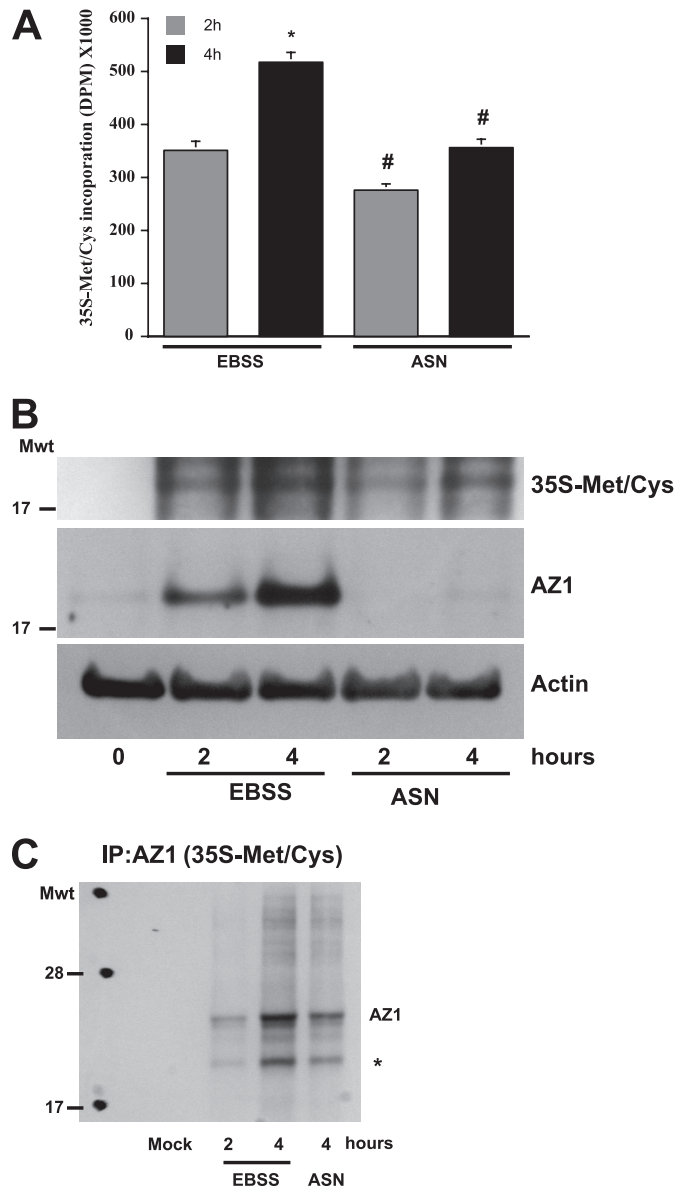


FIGURE 6. *A*, confluent serum-starved cells were exposed to EBSS in the presence and absence of Asn for the indicated time in the presence of 10  $\mu$ Ci/ml [ $^{35}$ S]methionine/cysteine. Cell extracts were prepared as described under "Experimental Procedures." Equal amounts of protein were precipitated by TCA, and 10  $\mu$ g of protein from each sample was used to quantify incorporation of [ $^{35}$ S]methionine/cysteine by liquid scintillation spectroscopy. *B*, equal amounts of protein (30  $\mu$ g) were resolved on 12% SDS-PAGE and analyzed by Western blotting for AZ1 and actin. Equal amounts (900  $\mu$ g) of protein from the above samples were subjected to immunoprecipitation (IP) using AZ1 antibody and resolved on SDS-PAGE followed by transfer to PVDF membrane. Then the membrane was dried, sprayed with the Enhance reagent, and exposed to x-ray film as described under "Experimental Procedures" (*C*). Values are means  $\pm$  S.E. (error bars);  $n = 3$  cultures. \* and #,  $p < 0.05$  compared with corresponding control values. Representative Western blots from three observations are shown.

**Effect of Amino Acids on mTOR and AZ1**—Nutrient deprivation decreases the rate of global protein synthesis by regulating the levels or activities of the components of protein translation machinery. 4EBP1 plays a crucial role in nutrient-sensing mechanisms in eukaryotic systems (46). 4EBP1 binds eIF4E and prevents its association with eIF4G and, thereby, prevents translation. 4EBP1 is inactivated by phosphorylation, allowing eIF4E to initiate translation (46). The 7-methyl-GTP-Sephar-

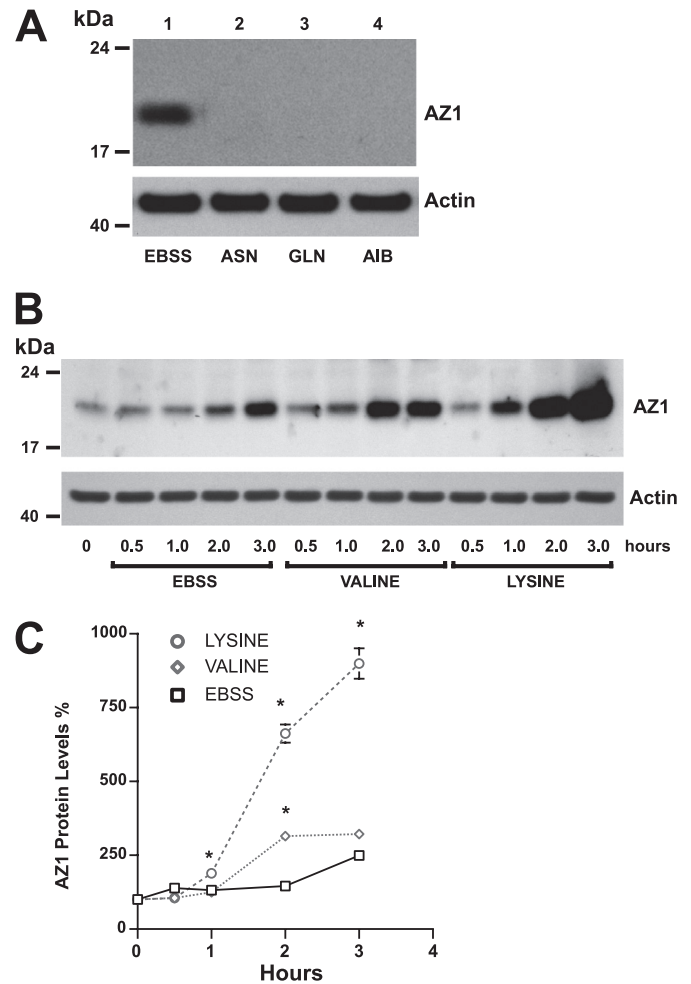
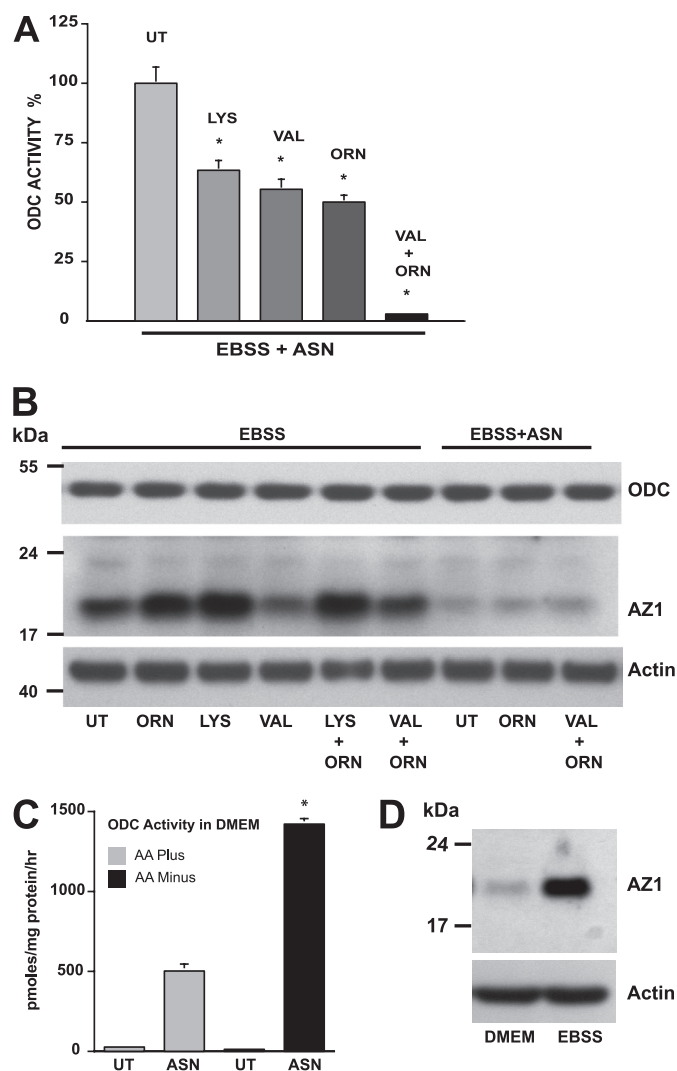


FIGURE 7. *A*, confluent serum-starved cells were exposed to EBSS in the presence and absence of Asn, Gln, and AIB for 3 h, and cell extracts were analyzed by Western blot to determine the levels of AZ1 and actin. Representative Western blots from three observations are shown. *B*, confluent serum-starved cells were exposed to EBSS in the presence and absence of Val or Lys, and at timed intervals, cell extracts were prepared and analyzed by Western blot to determine the levels of AZ1 and actin. *C*, the line graph shows the densitometry of Western blot (*B*). Representative Western blots from three observations are shown. \*,  $p < 0.05$  compared with corresponding control value. Error bars, S.E.

ose binding assay showed slightly lower binding of eIF4E in cells incubated in DMEM without amino acids (AA $-$ ) compared with that seen in DMEM with amino acids (AA $+$ ), and the addition of Asn to the DMEM (AA $-$ ) restored the binding of eIF4E to m7-GTP cap (Fig. 9A). Furthermore, m7-GTP cap bound 4EBP1 levels were undetectable compared with that seen in the DMEM without amino acids (AA $-$ ) and Asn-added DMEM (AA $-$ ). Interestingly, the levels of phosphorylated 4EBP1 were higher in DMEM (AA $-$ ) compared with that seen in DMEM (AA $+$ ) and DMEM (AA $-$ ) to which Asn was added (Fig. 9A). However, the increase in phospho-4EBP1 in the absence of amino acids is slight in comparison with the increase in 4EBP1 itself. It is also possible that the phospho-4EBP1 is hypophosphorylated and may not dissociate from eIF4E. These results indicate that the absence of amino acids inhibits the translation of mRNA containing 5'-m7-GTP cap and that the addition of Asn failed to reverse the inhibition of translation initiation. Furthermore, the phosphorylation of 4EBP1 was



**FIGURE 8.** *A*, confluent serum-starved cells were exposed to EBSS + Asn in the presence and absence of Lys, Val, ORN, or Val + ORN, and ODC activity was determined after a 3-h incubation. *B*, confluent serum-starved cells exposed to EBSS or EBSS + Asn were incubated with the indicated combination of amino acids for 3 h, and cell extracts were prepared and analyzed by Western blot to determine the levels of AZ1, ODC, and actin. *C*, confluent serum-starved cells were exposed to DMEM (AA+) or amino acid free DMEM (AA-) in the presence and absence of Asn. ODC activity was determined after 3 h of incubation. *D*, confluent serum-starved cells were exposed to DMEM or EBSS for 3 h, and cell extracts were prepared and analyzed by Western blot to determine the levels of AZ1. Values are means  $\pm$  S.E. (error bars);  $n = 3$  cultures. \*,  $p < 0.05$  compared with corresponding control value. Representative Western blots from three observations are shown.

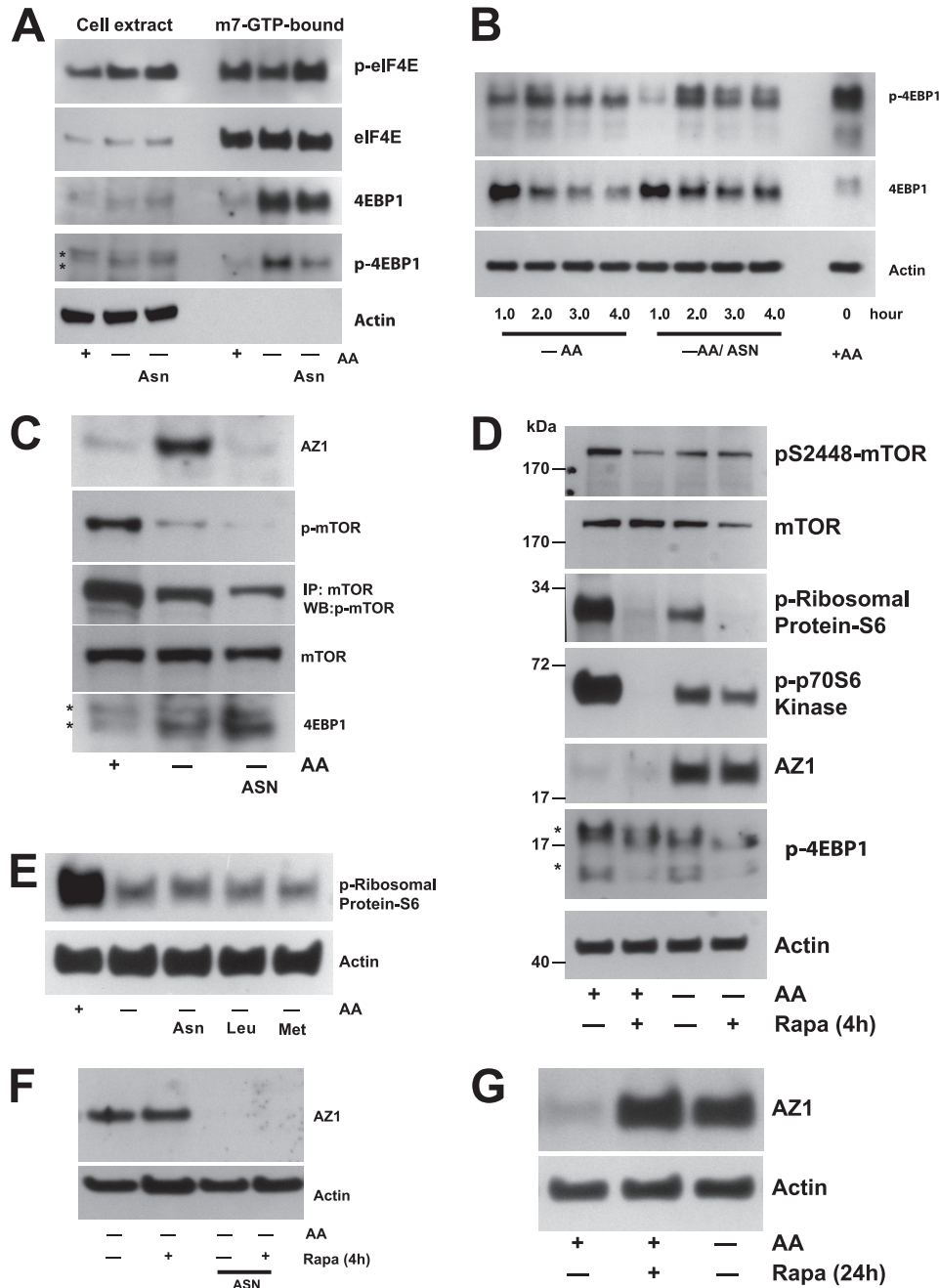
decreased in the DMEM (AA-) compared with DMEM (AA+). However, the levels of total 4EBP1 protein decreased in a time-dependent fashion in DMEM (AA-). The addition of Asn to DMEM (AA-) prevented the rapid decrease in the 4EBP1 protein seen in DMEM (AA-). Overall, the total amounts of 4EBP1 were higher in the DMEM (AA-) to which Asn was added (Fig. 9B). The increase in unphosphorylated 4EBP1 and inhibition of AZ1 expression in response to Asn suggested the involvement of the mTOR pathway. The activation of mTOR signaling was higher in the medium containing amino acids compared with that seen in the medium lacking amino acids. Asn further decreased active mTOR protein compared with AA+ and AA- conditions (Fig. 9C). Immunopre-

cipitation of mTOR further confirmed the phosphorylation state of mTOR in these conditions. In order to ascertain the role of mTOR on AZ1 expression, we determined the effect of rapamycin on AZ1 expression and p70S6K activity in serum-starved cells incubated for 4 h in DMEM containing a full set of amino acids and DMEM lacking amino acids (Fig. 9D). Rapamycin indeed inhibited mTOR phosphorylation and p70S6K activity, as evidenced by the complete inhibition of ribosomal protein-S6 phosphorylation in the cells incubated with amino acid-containing medium. Interestingly, rapamycin prevented the phosphorylation of ribosomal protein S6 in both the presence and absence of amino acids, and the activity of p70S6K decreased to a lower extent in the absence of amino acids. Furthermore, rapamycin failed to alter the levels of AZ1 protein in medium without amino acids (Fig. 9D). Because AZ1 synthesis is undetectable in DMEM containing amino acids, the effect of rapamycin on AZ1 expression in this condition cannot be predicted. The addition of Asn, Leu, or Met to DMEM (AA-) failed to restore p70S6K activity to the levels seen in the presence of a full set of amino acids (Fig. 9E). Rapamycin treatment for 4 h had no effect on the expression of AZ1 in the presence or absence of Asn in the DMEM without amino acids (Fig. 9F). However, prolonging the treatment with rapamycin for 24 h induced AZ1 expression in DMEM (AA+) comparable with that seen in DMEM (AA-) (Fig. 9G). These results suggest that the mTORC1 inhibition induces AZ1 synthesis. Because rapamycin-independent mTOR signaling is mediated by mTORC2, we determined the involvement of mTORC2 in AZ1 expression. PP242, an inhibitor of mTOR having a more pronounced effect on mTORC2, inhibited AKT-Ser<sup>473</sup>, a downstream target of mTORC2, and ribosomal protein S6 phosphorylation and decreased AZ1 expression (Fig. 10A). Because putrescine induces AZ1 translation by ribosomal frameshifting, we determined the effect of rapamycin and PP242 on putrescine-induced AZ1 expression. Putrescine increased AKT-Ser<sup>473</sup> phosphorylation and AZ1 levels, which were not altered by rapamycin, suggesting the activation of mTORC2 but not mTORC1 (Fig. 10B). However, PP242 decreased both the basal and putrescine-induced AZ1 levels (Fig. 10B). Furthermore, PP242 completely inhibited AKT-Ser<sup>473</sup> and 4EBP1 phosphorylation with concomitant accumulation of total 4EBP1 protein. These findings indicate a role for mTORC2 in the regulation of AZ1 translation. Thus, Asn appears to inhibit AZ1 by decreasing its frameshift-induced translation via mTORC2. Asn-decreased putrescine induced AZ1 in a time-dependent fashion, correlating with a decrease in AKT-Ser<sup>473</sup> and ribosomal protein S6 phosphorylation (Fig. 10C). These results suggest that mTORC2 regulates AZ1 synthesis and that Asn decreases mTORC2 activity in the absence of amino acids.

## DISCUSSION

Polyamines are essential for normal growth and differentiation, but they also play an important role in the development of cancer. The overexpression of ODC in NIH3T3 cells causes neoplastic transformation (47). Furthermore, sustained activation of ODC is associated with neoplastic transformation caused by oncogenic Ras (48), Src (49), activated RhoA (50), and forced expression of eIF4E (51). Antizymes are crucial, negative

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**FIGURE 9.** *A*, confluent serum-starved cells were exposed to DMEM without amino acids (AA<sup>-</sup>) in the presence or absence of Asn and DMEM with amino acids for 4 h. Protein (500  $\mu$ g) from cell extracts was subjected to an m7-GTP pull-down assay and analyzed by Western blot to determine the levels of phospho-eIF4E, phospho-4EBP1, and total 4EBP1. Protein (15  $\mu$ g) from whole cell extract were also loaded on the gel. Representative Western blots from three observations are shown. *B*, confluent serum-starved cells were exposed to DMEM without amino acids (AA<sup>-</sup>) in the presence or absence of Asn for the indicated time, and total and phosphorylated 4EBP1 were determined by Western blot analysis. *C*, confluent serum-starved cells were exposed to EBSS in the presence and absence of Asn for 4 h, and cell extracts were analyzed by Western blot to determine phospho-mTOR and total mTOR, 4EBP1, and AZ1; DMEM served as a positive control (AA<sup>+</sup>). mTOR immunoprecipitated from 500  $\mu$ g of protein was probed with Ser(P)<sup>2448</sup>-mTOR antibody. *D*, confluent serum-starved cells were exposed to DMEM containing amino acids (AA<sup>+</sup>) or without amino acids (AA<sup>-</sup>) in the presence or absence of rapamycin (20 nM) for 4 h, and cell extracts were analyzed by Western blot to determine the levels of phospho-mTOR and total mTOR, phospho-p70S6K, phosphoribosomal protein S6, AZ1, and actin. *E*, confluent serum-starved cells were exposed to EBSS in the presence and absence of Asn, Leu, and Met for 3 h, and cell extracts were analyzed by Western blot to determine the levels of phosphoribosomal protein S6 and actin. DMEM served as a positive control (AA<sup>+</sup>). *F*, confluent serum-starved cells were exposed to DMEM without amino acids (AA<sup>-</sup>) in the presence or absence of Asn followed by the addition of rapamycin (20 nM) to appropriate groups for 4 h, and cell extracts were analyzed by Western blot to determine the levels of AZ1 and actin. Representative Western blots from three observations are shown. *G*, confluent cells were exposed to 20 nM rapamycin (*Rapa*) during serum starvation (24 h) followed by incubation in DMEM (AA<sup>+</sup>) in the presence or absence of rapamycin or DMEM (AA<sup>-</sup>) for 4 h, and cell extracts were analyzed by Western blot to determine the levels of AZ1 and actin. Representative Western blots from three observations are shown.

modulators of intracellular polyamine levels. Antizyme-1 interacts with ODC, leading to ubiquitin-independent degradation by the 26 S proteasome. A single molecule of AZ1 can cause the

degradation of several ODC molecules. Furthermore, AZ1 also regulates the entry and export of polyamines across the plasma membrane and thereby maintains intracellular polyamine lev-



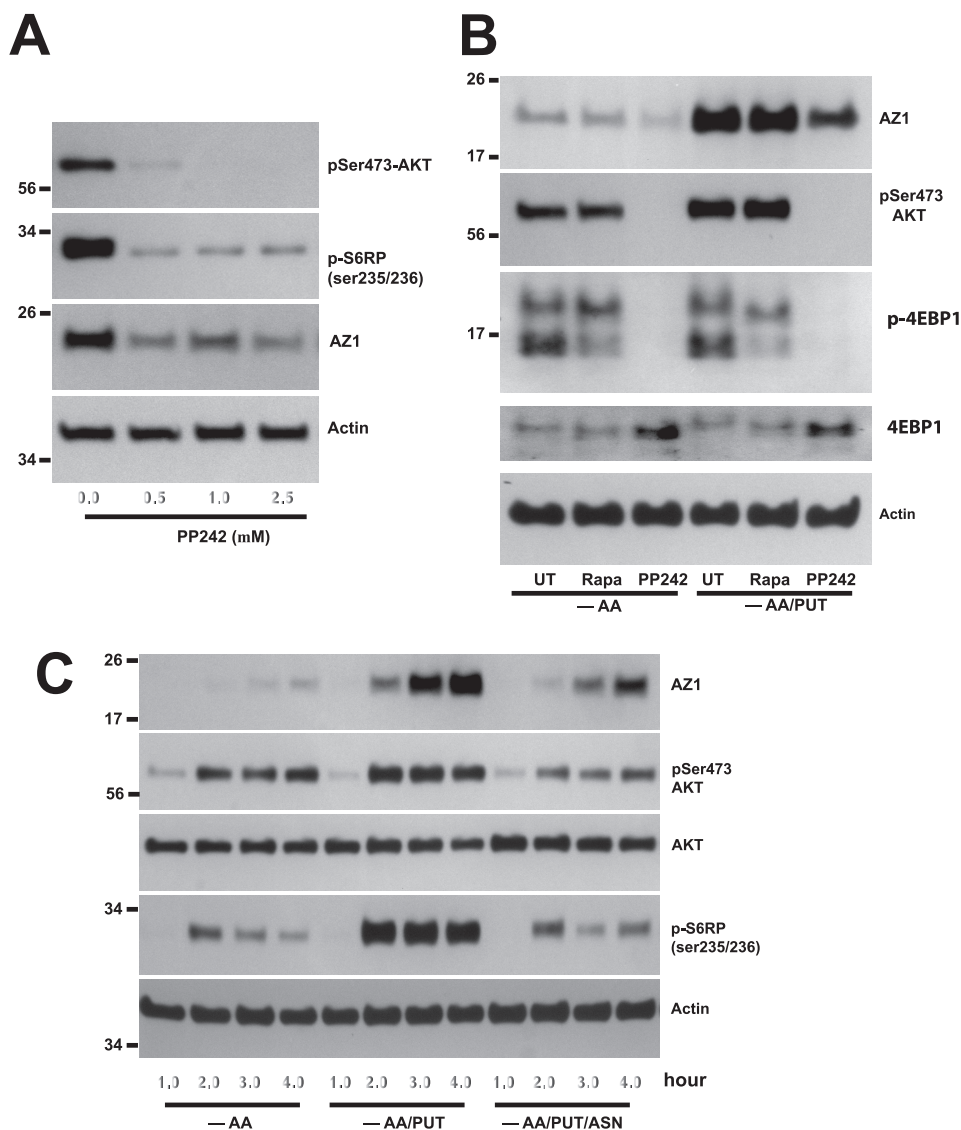


FIGURE 10. *A*, serum-starved cells were incubated with the indicated concentration of PP242 for 4 h, and cell extracts were analyzed by Western blot to determine the levels of Ser(P)<sup>473</sup>-AKT, Ser(P)<sup>235/236</sup>-S6 ribosomal protein, AZ1, and actin. *B*, serum-starved cells incubated in DMEM (AA<sup>-</sup>) in the presence or absence of putrescine (10 μM) were treated with or without mTOR inhibitors (rapamycin, 20 nM; PP242, 2.5 μM) for 4 h. Cell extracts were analyzed by Western blot to determine the levels of AZ1, Ser(P)<sup>473</sup>-AKT, total 4EBP1, phospho-4EBP1, and actin. *C*, serum-starved cells were incubated in DMEM (AA<sup>-</sup>) in the presence and absence of 10 μM putrescine (PUT) in the presence and absence of 10 mM Asn for the indicated time. Cell extracts were analyzed by Western blot to determine the levels of AZ1, Ser(P)<sup>473</sup>-AKT, Ser(P)<sup>235/236</sup>-S6 ribosomal protein, and actin. Representative Western blots from three observations are shown.

els. Several studies have shown that overexpression of AZ1 prevents tumor growth (52), inhibits cell proliferation, and causes cell cycle arrest analogous to polyamine depletion by DFMO, a suicide inhibitor of ODC activity. An ODC-like protein, called AZin, increases the activity of ODC by binding to AZ. Serum and phorbol ester increase AZin expression earlier than ODC activity and promote growth and tumor formation, respectively (53). Thus, AZ and AZin play crucial roles in maintaining the homeostatic balance of polyamines by regulating ODC activity and thereby the normal growth of cells.

Because transport systems A and N type amino acids are required for the induction of ODC activity by growth factors (35), we postulated that Asn might affect ODC activity by regulating the expression of antizyme or antizyme-inhibitor proteins. One of the most interesting findings in this study was the observation that amino acid starvation increased the expres-

sion of AZ1 in IEC-6 cells (Fig. 1). ODC induction correlated with a decrease in AZ1 in cells incubated with Asn or Asn + EGF. However, the levels of AZin remained unchanged in response to Asn, EGF, or Asn/EGF compared with EBSS, indicating that Asn induces ODC activity by regulating AZ1 (Fig. 1A). As mentioned earlier, AZ targets ODC protein for degradation; thus, constant ODC protein levels indicate that AZ1 may not control ODC protein degradation. However, the degradation of ODC by AZ1 cannot be ruled out because the rates of degradation of ODC may be similar among the treatment groups. AZ1 accumulation over 6 h of incubation in EBSS did not result in the degradation of ODC protein (Fig. 2, *C* and *D*). Because AZ1 shares 50% homology with AZ2, we speculated that AZ1-specific antibody might recognize AZ2. However, transfection of cells by AZ1 or AZ2 siRNA clearly demonstrated that AZ1, induced in response to amino acid starvation,

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is a product of AZ1 mRNA and excluded the involvement of AZ2. Furthermore, anti-AZ1 antibody immunoprecipitated AZ1 from the extracts of cells exposed for 4 h to EBSS or EBSS plus putrescine (Fig. 3D). These results suggest that AZ1 inhibits ODC activity by binding to ODC and preventing the formation of active ODC dimers. A time-dependent increase in AZ1 in response to amino acid starvation (EBSS) was prevented by Asn, leading to elevated ODC activity, indicating a role for Asn in either preventing the expression or increasing the turnover of AZ1 (Figs. 2 and 4).

In an extended time course study, a sharp decline in Asn-induced ODC activity after 4 h correlated with low but detectable levels of AZ1 (Fig. 2, D (lanes 8 and 9) and E). A decrease in intracellular Asn due to metabolic conversion might be responsible for the increase in AZ1. The activity of the system A amino acid transporter increases in response to prolonged amino acid starvation. This mechanism, called adaptive regulation was described in mesenchymal cells of avian origin (54), but it has also been found in many other models (55). Transport systems A and N type amino acids induce ODC activity in various cell types (32–34), and our results show that amino acid starvation induces AZ1 in both IEC-6 and NIH3T3 cells. Because Asn prevents AZ1 induction (Fig. 1F), this suggests a common mechanism regulating AZ1 expression by amino acids in mammalian cells. The undetectable levels of AZ1 in the presence of Asn at earlier time points (30 min) and the onset of ODC induction after 60 min (Fig. 2, A and B) suggest that an undetectable amount of AZ1 protein might be sufficient for the inhibition of ODC activity. Preincubation of cells in EBSS for 30 and 60 min significantly decreased Asn-induced ODC activity (Fig. 3A), and *in vitro* inhibition of Asn-induced ODC activity by extracts of cells incubated for 3 h in EBSS (Fig. 3B) supports the notion that low levels of AZ1 can inhibit ODC activity. This was further confirmed by the inhibition of ODC activity *in vitro* by immunoprecipitated AZ1 from extracts of cells incubated in EBSS or EBSS plus putrescine (Fig. 3D). The effects of putrescine on AZ1 expression in EBSS support previous reports showing that putrescine stimulates AZ1 expression by a +1 frameshifting mechanism (23, 41, 43). However, it is unclear whether Asn prevents the synthesis or the accumulation of AZ1 protein. The levels of AZ1 decrease after 6 h of incubation in the absence of amino acids (EBSS) (Fig. 4, C and D). The decline in AZ1 may be due to the lack of amino acids in the EBSS, resulting in a decrease in the rate of protein synthesis. Asn addition along with EBSS or its addition after 3 h of incubation in EBSS completely eliminated AZ1 levels within 1 h. However, Asn addition after 6 h in EBSS required 1.5 h to significantly decrease AZ1 protein. These results clearly show that the effectiveness of Asn in decreasing AZ1 depends on the level of the protein.

Earlier reports showed that transport systems A and N type amino acids, including Asn, Gln, and AIB (a nonmetabolizable analog of Asn), induced ODC activity in EBSS, whereas Lys, Val, and other amino acids failed to do so (35). Thus, amino acids unable to activate ODC were considered non-inducer amino acids. Our results confirmed that ODC-inducing amino acids (Gln and AIB) prevent AZ1 accumulation similar to Asn (Fig. 7A) and that amino acids unable to induce ODC activity (Lys, Val, and ORN) fail to decrease AZ1 protein (Figs. 7 and 8).

Because AZ1 increases in EBSS in a time-dependent manner, it is also possible that Lys, Val, and ORN may not modulate AZ1 expression but may stabilize AZ1 or its interaction with ODC or AZin. However, a time course study clearly showed that both Lys and Val increased AZ1 compared with EBSS (Fig. 7B). Furthermore, the increases in AZ1 began earlier in Lys and Val compared with EBSS, indicating an active role for these amino acids in suppressing ODC activity. These observations are supported by the fact that Asn-induced ODC activity significantly decreases in the presence of either Lys or Val (Fig. 8A). Although Val induces AZ1 to a lesser extent compared with Lys, its suppression of Asn-induced ODC activity is similar to that of Lys. It has been shown that Val decreases intracellular ORN, thereby depleting ODC substrate (56). The decrease in Asn-induced ODC activity in the presence of Val (Fig. 8) could be due to the depletion of ORN. In that case, the addition of ORN should reverse the Val suppression of Asn-induced ODC activity. Surprisingly, the addition of ORN along with Val significantly inhibits Asn-induced ODC activity, and ORN itself significantly decreases Asn-induced ODC activity by increasing the levels of AZ1 similar to Lys. Furthermore, Val and ORN had an additive inhibitory effect on ODC activity. Although Asn significantly decreased AZ1 induced by these amino acids, ODC induction was significantly lower, affirming that almost complete elimination of AZ1 is required for the full induction of ODC activity. Our results confirm that the amino acids Asn and Gln repress and Lys, Val, and ORN induce AZ1 expression and show that these amino acids play an important role in the regulation of ODC activity. These results also suggest that the amino acid composition of a culture medium is an important factor in the regulation of polyamine levels during the normal growth of cells. Asn (10 mM) induces ODC activity in amino acid-containing medium (DMEM) and in DMEM without amino acids. However, Asn induced significantly higher levels of ODC activity in DMEM alone compared with DMEM containing other amino acids (Fig. 8D). The decrease in Asn-induced ODC activity in amino acid-containing medium can be attributed to the presence of AZ1-inducing amino acids like Val and Lys. Furthermore, comparison of AZ1 expression in DMEM and EBSS showed that amino acid starvation specifically induces AZ1. In addition, putrescine increased AZ1 severalfold over that observed in EBSS (Fig. 3C).

Several features of antizyme induction suggest regulation at the level of translation. Antizyme synthesis is inhibited by cycloheximide but not by actinomycin D (16, 57). Although only a minute amount of antizyme is present in mammalian tissues (about 1–2 ppm of total soluble protein), its mRNA level is relatively high and not further elevated by polyamines (23). The addition of Asn along with EBSS or following the accumulation of AZ1 (prolonged incubation in EBSS) significantly decreased AZ1, indicating that Asn prevented the translation of or augmented the degradation of AZ1 and, thus, allowed the efficient homodimerization of ODC monomers. If Asn modulates AZ1 degradation, inhibition of protein synthesis should have an additive effect, leading to rapid elimination of AZ1 in the presence of Asn. Inhibition of protein synthesis by CHX alone or in combination with Asn decreased AZ1 levels at the same rate as Asn alone, suggesting that both are acting via the

same mechanism (Fig. 5, A and B). Incorporation of [<sup>35</sup>S]methionine/cysteine confirmed that the observed increase in AZ1 in EBSS is indeed due to synthesis of AZ1. Asn caused a generalized decrease in ongoing protein synthesis, including AZ1 (Fig. 6). Although Asn significantly decreased AZ1 mRNA (Fig. 5C), the failure of actinomycin D (*ACT-D*) to prevent the accumulation of AZ1 indicates that translation of preexisting AZ1 mRNA was sufficient to sustain the level of AZ1 (Fig. 5D). Thus, it is evident from the above results that Asn decreases AZ1 primarily by regulating its translation.

Several studies have demonstrated that limiting amino acids, inhibition of protein synthesis, and serum starvation block cell cycle progression (58). However, more recent studies indicate that amino acids can act as signaling molecules, suggesting that amino acids control the cell cycle by modulating the expression of specific genes independently of effects on global protein synthesis. Induction of p21 and p27 expression by amino acid deprivation in HepG2 human hepatoma cells involved mRNA stabilization (59). Nakajo *et al.* (38) showed that Gln was required for the growth of rat intestinal epithelial cells and that it inhibited mTOR activity induced in response to arginine and proposed a differential role for individual amino acids rather than more general effects on gut homeostasis.

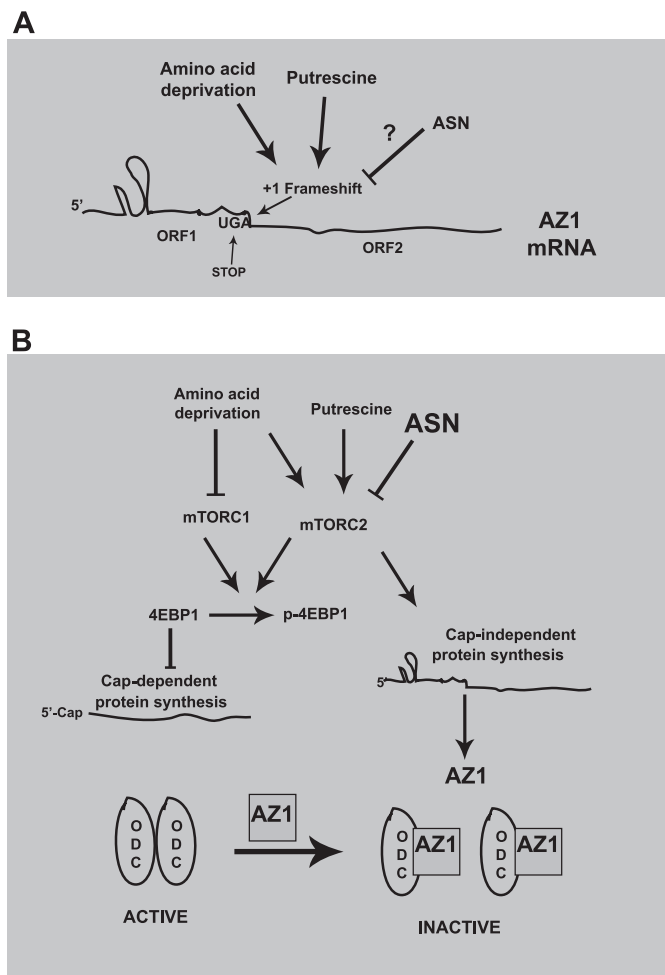
Generally, amino acid limitation results in dephosphorylation of p70S6K and 4EBP1, whereas the addition of amino acids leads to rapid TORC1/2-dependent phosphorylation of these molecules (60, 61). eIF4E plays a key role in the regulation of mRNA translation and cellular physiology. Overexpression of eIF4E can lead to cell transformation, unregulated cell growth, and enhanced translation of mRNA containing 5'-untranslated regions (62). Binding of 4EBP1 to eIF4E prevents the interaction with other partners and blocks cap-dependent mRNA translation initiation. 4EBP1 undergoes multiple phosphorylations, which disrupt its ability to bind eIF4E, leading to the release of eIF4E, which then binds eIF4G and initiates protein translation (63–65). The phosphorylation of 4EBP1 is strongly modulated by the phosphoinositide 3-kinase (PI3K)/Akt pathway and by nutrients/amino acids. In addition to mTOR, other factors that have been implicated in the regulation of 4EBP1 phosphorylation/activation states include the kinases pim-2 (66), PKC $\delta$ , and c-Abl (67) and phosphatases, such as serine/threonine protein phosphatase 2A (68–70). Thus, a complex system of signaling molecules, composed of scaffold proteins, kinases, and phosphatases, appears to control the phosphorylation/activity of 4EBP1. eIF4E binds the 5'-cap structure of mRNA, forms complexes with other initiation factors, and facilitates the binding of the 40 S subunit to mRNA and the identification of the AUG start codon. Thus, amino acid deprivation should mimic the effect of rapamycin, a specific inhibitor of mTOR.

The absence of all of the constituent amino acids in DMEM decreased mTOR signaling, as evidenced by the decreased phosphorylation of p70S6K and its downstream substrate, ribosomal protein S6, and increased binding of 4EBP1 to the mRNA cap (Fig. 9). It is evident from the mTOR activity in DMEM (AA+) and DMEM (AA–) that the activation of mTOR signaling requires the presence of other amino acids besides asparagine. However, AZ1 synthesis is inhibited in DMEM (AA+).

Thus, AZ1 synthesis occurs on a background of low mTOR activity. Short term inhibition (4 h) of mTOR activity by rapamycin failed to induce AZ1 synthesis in DMEM (AA+), and rapamycin had no effect on AZ1 induced in response to amino acid starvation (AA–). However, prolonged inhibition of mTOR by rapamycin treatment for 24 h induced AZ1 expression in DMEM (AA+), thus mimicking the absence of amino acids (Fig. 9G). The addition of single amino acids (Asn, Leu, or Met) to DMEM (AA–) did not restore the phosphorylation of ribosomal protein S6 to levels observed in DMEM (AA+), suggesting that a full complement of amino acids is necessary to activate mTOR. These results clearly established that the decreased mTOR activity in response to amino acid starvation inhibits cap-dependent mRNA translation. However, increased AZ1 synthesis under these conditions suggests a cap-independent mRNA translation. The 5' pseudoknot structure in the AZ1 mRNA, and a distinct +1 ribosomal frameshifting mechanism are involved in decoding AZ1 mRNA, further suggesting the involvement of cap-independent translation. The fact that polyamines induce the frameshifting (17, 18) suggests that AZ1 synthesis might be regulated by an additional translational control (Fig. 10). Spermine, for example, has been shown to regulate synthesis of mitochondrial proteins by stabilizing a small subunit of the ribosome and fMet-tRNA complex (71). Furthermore, recently Choo and Blenis (72) reported that rapamycin inhibited S6K in various cell lines. However, prolonging the treatment resulted in the hyperphosphorylation of 4EBP1. This led to rapamycin-resistant cap-dependent translation. These results suggest that additional mechanisms regulating the phosphorylation state of 4EBP1 might be involved. We have also observed 4EBP1 phosphorylation with a low mTOR background (AA–; Fig. 9).

The mTOR pathway forms two functionally distinct protein complexes, mTORC1 and mTORC2. Decreased mTORC1 activity in the absence of amino acids might lead to an increase in mTORC2 activity in order to sustain translation of stress response proteins. AZ1 is induced by stress (19) and, in addition to regulating ODC activity, is involved in the regulation of cyclin D activity (73) and polyamine transport (23, 24, 44), suggesting involvement of the mTORC2 pathway in the regulation. The fact that rapamycin does not inhibit phosphorylation of AKT-Ser<sup>473</sup>, a downstream target of mTORC2, allowed us to differentiate between the contributions of these complexes in the regulation of AZ1. Inhibition of mTORC2 by PP242, as judged by the decrease in AKT-Ser<sup>473</sup> phosphorylation, decreased the levels of AZ1 (Fig. 10). Although polyamines regulate AZ1 translation by ribosomal frameshifting, AZ1 translation induced by putrescine also increased AKT-Ser<sup>473</sup> phosphorylation, suggesting the involvement of mTORC2 in AZ1 translation (Fig. 10). Asn decreased putrescine-induced AKT-Ser<sup>473</sup> phosphorylation with a concomitant decrease in AZ1 expression. PP242 completely inhibited 4EBP1 phosphorylation and caused the accumulation of 4EBP1. Analogous to PP242, Asn also caused the accumulation of 4EBP1 and decreased AZ1. These results suggest that Asn decreased transcription of AZ1 mRNA and prevented its translation by directly inhibiting mTORC2 activity and/or the ribosomal frameshifting.





**FIGURE 11. Amino acids activate ODC by regulating synthesis of AZ1.** *A*, schematic representation of AZ1 mRNA organization and translation. *B*, inhibition of mTORC1 in the absence of amino acids decreased 4EBP1 phosphorylation, which prevented cap-dependent mRNA translation. The absence of amino acids and putrescine increased mTORC2 activity and AZ1 synthesis. Asn decreased AZ1 mRNA and inhibited both the mTORC2 activity and AZ1 synthesis. Thus, in the absence of amino acids activation of mTORC2 allowed the translation of AZ1 mRNA by a cap-independent mechanism. AZ1 binds ODC monomers, preventing the formation of active dimers.

In the presence of Asn or a stimulating amino acid, the normal signaling pathways initiated by EGF result in the potentiation of ODC activity compared with that elicited by Asn acting alone. Several complex 5'-UTR mRNAs involved in cell growth, cell cycle progression, and angiogenesis are selectively translated via eIF4E, including ODC (42, 74). The amount of eIF4E is much lower compared with other translation factors and is activated by mitogenic stimuli (42). The translation of mRNA containing complex 5'-UTR (pseudoknot structure), internal ribosome entry site, and 5'-terminal oligopyrimidine tract (5'-TOP) also occurs independently of classical 5'-cap-dependent mechanisms. In confluent serum-starved IEC-6 cells incubated in EBSS, the addition of Asn or EGF failed to stimulate ODC synthesis (35), suggesting that eIF4E may be necessary but not sufficient to support the translation of ODC mRNA. Although eIF4E stimulates translation of multiple mRNAs, the availability of translatable mRNA in confluent serum-starved cells may be a limiting factor. In fact, decreased AZ1 mRNA in the presence of Asn resulted in the decreased availability of the trans-

latable pool of AZ1 mRNA (Fig. 5C). This decreased mRNA level could possibly contribute to decreased AZ1 synthesis.

Fig. 11A shows that the AZ1 is decoded from two open reading frames (ORFs). Translation is initiated at a start codon for ORF1, and subsequent +1 translational frameshifting at the end of ORF1 (stop codon, UGA) is necessary for the synthesis of functional AZ1. Putrescine increases the efficiency of the obligatory +1 frameshifting (17, 18). Amino acid deprivation induced AZ1 synthesis. Asn prevented AZ1 synthesis in response to amino acid deprivation and putrescine. Our previous studies and present results suggest a scheme (Fig. 11B) showing that the decreased mTOR activity in the absence of amino acids inhibits cap-dependent translation of mRNA. However, translation of AZ1 mRNA containing complex 5'-UTR (pseudoknot structure) occurs in a cap-independent fashion. Furthermore, polyamine-induced mTORC2 activity stimulated AZ1 translation. Analogous to PP242, Asn inhibits mTORC2 and prevents AZ1 mRNA translation. Decreased AZ1 expression allows the homodimerization of ODC monomers, leading to the formation of active ODC.

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