

Regulation of cell proliferation by the antizyme inhibitor: evidence for an antizyme-independent mechanism

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Summary

The antizyme inhibitor was discovered as a protein that binds to the regulatory protein antizyme and inhibits the ability of antizyme to interact with the enzyme ornithine decarboxylase (ODC). Blocking antizyme activity subsequently leads to increased intracellular levels of ODC and increased ODC enzymatic activity. We now report that antizyme inhibitor is a positive modulator of cell growth. Overexpression of antizyme inhibitor in NIH-3T3 mouse fibroblasts or in AT2.1 Dunning rat prostate carcinoma cells resulted in an increased rate of cell proliferation and an increase in saturation density of the cultured cells. This was accompanied by an increase in intracellular levels of the polyamine putrescine. In AT2.1 cells, antizyme inhibitor overexpression also increased the ability of the cells to form foci when grown under anchorage-independent conditions. In order to determine the role of antizyme on antizyme inhibitor activity we created an antizyme inhibitor mutant, AZI $\Delta_{117-140}$, which lacks the

putative antizyme-binding domain. We show that this mutant fails to bind to antizyme, but remains capable of inducing increased rates of cell proliferation, suggesting that antizyme inhibitor has antizyme-independent functions. Silencing antizyme inhibitor expression leads to diminished levels of cyclin D1 and to reduced cell proliferation. Antizyme inhibitor is capable of preventing cyclin D1 degradation, and this effect is at least partially independent of antizyme. We show that wild-type antizyme inhibitor and the AZI $\Delta_{117-140}$ mutant are capable of direct interaction with cyclin D1 suggesting a potential mechanism for the antizyme-independent effects. Together, our data suggest a novel function for antizyme inhibitor in cellular growth control.

Key words: Antizyme inhibitor, Proliferation, Cyclin D1, Growth regulation, Ornithine decarboxylase, Neoplasm, Polyamine

Introduction

Levels of the enzyme ornithine decarboxylase (ODC) are tightly regulated during the cell cycle. The expression of ODC is Myc dependent (Bello-Fernandez et al., 1993). ODC activity levels are activated in a biphasic manner during the cell cycle with a first activation phase in late G1, which is followed by a rapid decline in the half-life of ODC (Oredsson, 2003). This rapid degradation of ODC is mediated by the regulatory protein antizyme (Murakami et al., 1992). Antizyme binds directly to ODC, blocking its enzymatic active site (Heller et al., 1976; Kern et al., 1999). Antizyme also increases the affinity of ODC for the 26S proteasome, where ODC is degraded in a ubiquitin-independent process (Bercovich et al., 1989; Glass and Gerner, 1987; Murakami et al., 1992). Although first thought to bind only to ODC, antizyme has been shown recently to bind and facilitate the ubiquitin-independent degradation of other small proteins, including Smad1 (Gruendler et al., 2001), Snip1 (Lin et al., 2002) and cyclin D1 (Newman et al., 2004).

Antizyme acts as a negative regulator of cell growth (Murakami et al., 1994) and as a tumor suppressor (Feith et al.,

2001; Iwata et al., 1999). Antizyme upregulation or overexpression correlates with growth suppression in several model systems including H-Ras transformed NIH-3T3 cells (Iwata et al., 1999), malignant hamster keratinocytes (Tsuji et al., 1998) and polyamine-treated prostate carcinoma cells (Koike et al., 1999). Antizyme overexpression also induces terminal differentiation in hamster keratinocytes (Tsuji et al., 2001). Furthermore, antizyme overexpression in transgenic models results in decreased cell proliferation, increased apoptosis and suppression of carcinogen-induced tumor growth (Feith et al., 2001; Fong et al., 2003). In addition, antizyme inhibits polyamine uptake and stimulates polyamine secretion (He et al., 1994; Mitchell et al., 1994; Mitchell et al., 1995; Sakata et al., 2000).

Together, these results demonstrate a role for antizyme in the negative regulation of cell proliferation and tumorigenesis, probably as a result of modulation of polyamine levels and facilitated degradation of growth regulatory molecules such as ODC and cyclin D1.

The endogenous antizyme inhibitor (AZI) was first discovered as a protein that binds to antizyme and inhibits

several of its functions (Fujita et al., 1982). AZI blocks the ability of antizyme to promote ODC degradation (Murakami et al., 1993) and to inhibit ODC enzymatic activity (Fujita et al., 1982). AZI shares 65% homology with ODC (Murakami et al., 1996) and conserves the majority of residues that are required for ODC activity, but AZI itself does not exhibit any known enzymatic activity (Murakami et al., 1996). AZI binds antizyme with high affinity (Kitani and Fujisawa, 1989; Murakami et al., 1989) preventing formation of the antizyme-ODC complex and consequently suppressing ODC degradation (Murakami et al., 1996). Antizyme binding does not accelerate AZI degradation (Murakami et al., 1996) but instead stabilizes AZI (Bercovich and Kahana, 2004). Interactions of AZI with proteins other than members of the antizyme family have not previously been described.

ODC is a positive regulator of cell growth and ODC overexpression can induce hyperproliferation and elements of the transformed phenotype (Auvinen et al., 1992); upregulation of ODC in Myc-overexpressing transgenic mice is also correlated with development of B-cell tumors (Nilsson et al., 2005). The ability of AZI to reverse antizyme-mediated ODC degradation led us to examine whether AZI could also influence cell proliferation. Like ODC, AZI levels are elevated soon after growth factor stimulation of mouse fibroblasts (Nilsson et al., 2000) and then drop as antizyme levels rise. AZI is also upregulated in gastric tumor cells (Jung et al., 2000), suggesting a possible correlation between AZI and malignancy. We now present evidence for a positive correlation between AZI expression and cell proliferation. Surprisingly, this effect appears to be partially independent of the ability of AZI to interact with antizyme. We show further that AZI is capable of a novel interaction with cyclin D1, suggesting a potential mechanism for the antizyme-independent effects on cell proliferation.

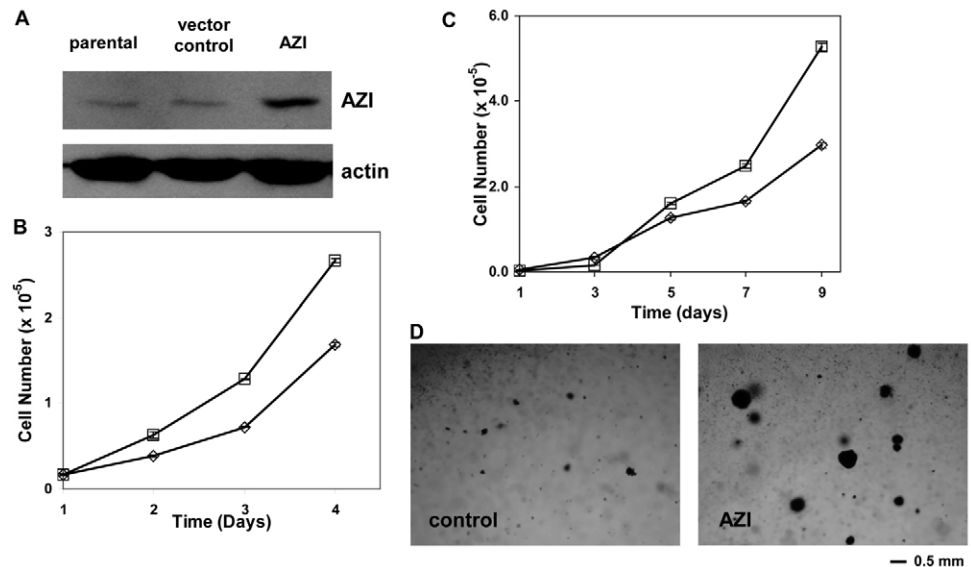
Results

Effect of AZI overexpression on cell proliferation and transformation

In order to study the effect of AZI on cell proliferation, AT2.1 rat prostate carcinoma cells were transfected with AZI or vector control and selected for stable expression. AT2.1 cells expressing AZI at five times endogenous levels (Fig. 1A) grew more rapidly in medium containing 1% serum compared with the vector control cells, with a decrease in doubling time from 22.5 ± 1.3 (s.e.m.) to 18.7 ± 0.7 hours (Fig. 1B). Three additional AT2.1 AZI-overexpressing clones were also examined and these also demonstrated increased proliferation rates compared with vector control cells (data not shown). There was also a decrease in the length of the lag phase in AZI-overexpressing cell lines and these cells attained higher final cell densities (Fig. 1B and data not shown). When cell proliferation was measured in medium containing 10% serum, the differences in proliferation rate were less pronounced but still significant (Fig. 1C). Reduced sensitivity to contact inhibition suggested a transformed phenotype. We therefore tested the AZI-overexpressing cells for their ability to grow under anchorage-independent conditions. Our results revealed that AT2.1 prostate cancer cells overexpressing AZI have an enhanced ability to grow in soft agar. AZI overexpression resulted in an increased number of foci greater than 0.5 mm (53.9 ± 14.4 versus 0.2 ± 0.1 in the vector controls) (Fig. 1D). Thus, anchorage-independent growth in AT2.1 cells is enhanced after transfection with AZI.

To investigate whether AZI was sufficient for cellular transformation in non-tumor cells, we next investigated the effect of AZI on NIH-3T3 cells. NIH-3T3 cells expressing 2.5 times more AZI than vector controls (Fig. 2A), grew well in reduced-serum (0.5%) medium that did not support the growth of vector control cells (Fig. 2B). In medium containing 10% serum, AZI-overexpressing cells also showed a higher

Fig. 1. Effect of AZI on proliferation and anchorage-independent growth in AT2.1 prostate cancer cells. (A) 50 μ g total cell lysate from AT2.1 cells stably transfected with pcDNA3 or pcDNA3-AZI constructs were analyzed for AZI and actin expression by western blotting. The stable AZI cell line used in these studies showed a fivefold increase in AZI expression relative to endogenous levels. (B) Proliferation of control (\diamond) and AZI-overexpressing AT2.1 cells (\square) in low serum (1% FBS). Cells were plated at a density of 1×10^4 cells in 12-well plates and the total number of cells per well were counted. Error bars indicate standard error of the mean (s.e.m.). (C) Proliferation of control (\diamond) and AZI-overexpressing AT2.1 cells (\square) grown in high serum (10% FBS) was assessed as described above. (D) Phase-contrast micrographs of control and AZI-overexpressing AT2.1 cells comparing colony formation in 0.45% noble agar, as described in the Materials and Methods. Colonies were visualized after 2 weeks using 0.02% Giemsa stain. AZI-overexpressing cells formed more colonies with diameters greater than 0.5 mm. Similar results were seen with a second clone that expressed AZI at twice the wild-type level (data not shown).



proliferation rate with an average doubling time of 18.0 ± 0.8 hours relative to 24.0 ± 0.6 hours for vector control cells. In addition, AZI-overexpressing cells reached a higher saturation density in 10% serum relative to the vector control cells (Fig. 2C). In contrast to the transforming effect of ODC overexpression in NIH-3T3 cells (Auvinen et al., 1992; Moshier et al., 1993) however, NIH-3T3 cells overexpressing AZI did not show an increased capacity to grow in soft agar (data not shown).

Relationship between AZI overexpression and ODC activity

The principal known function of AZI is to bind and sequester antizyme, leading to elevated levels and activity of ODC (Murakami et al., 1996; Murakami et al., 1989). To determine whether AZI overexpression correlates with increased ODC functional activity, we measured basal ODC enzymatic activity in AZI-overexpressing cells. Our results demonstrate

that ODC activity is markedly increased by AZI overexpression in both AT2.1 (Fig. 3A) and NIH-3T3 cells (Fig. 3B). Measurement of polyamine levels in AT2.1 cells after 72 hours in low serum showed an increased level of putrescine in the AZI-overexpressing cells but little change in either spermidine or spermine levels, as shown in Table 1. Since antizyme is able to downregulate the polyamine transport system we tested whether AZI, which inhibits antizyme activity, might influence polyamine uptake. However, we did not observe any increase in spermine uptake in the AZI-overexpressing AT2.1 cells (Fig. 3C).

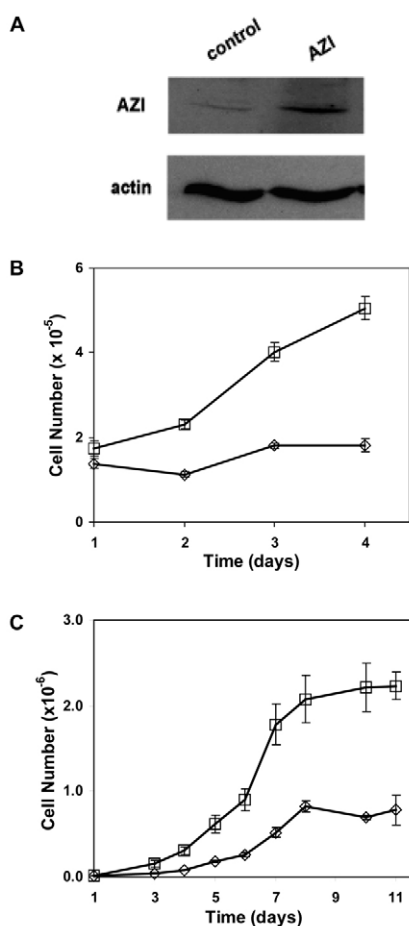


Fig. 2. Effect of AZI on proliferation of non-transformed NIH-3T3 fibroblasts. (A) Western blot analysis of 50 µg of total cell lysate from NIH-3T3 cells stably infected with retroviral vectors pWZL or pWZL-AZI. (B) Proliferation of control (◇) and AZI-overexpressing NIH-3T3 cells (□) grown in low serum (0.5% BCS). Cells were plated at a density of 5×10^4 cells in 12-well plates and the total number of cells per well were counted. Error bars indicate s.e.m. (C) To assess saturation density, control (◇) and AZI-overexpressing NIH-3T3 cells (□) were plated at 1×10^4 cells in six-well plates in media containing 10% BCS and cell number assessed over 11 days.

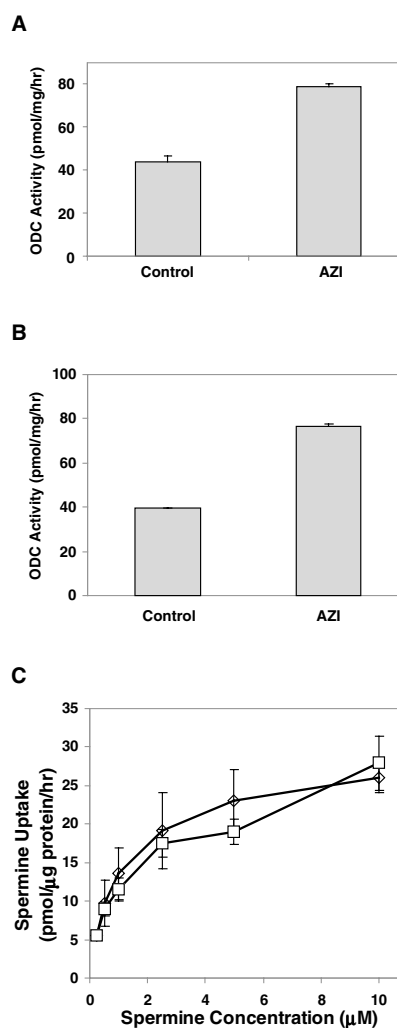


Fig. 3. AZI expression increases ODC activity in AT2.1 and NIH-3T3 cells. (A) ODC activity (pmol ¹⁴CO₂/mg protein/hr) was measured in control and AZI-overexpressing AT2.1 cells, using [¹⁴C]-L-ornithine as the substrate. Total cell lysates were collected 24 hours after plating 5×10^5 cells in medium containing 10% serum. Error bars indicate s.e.m. (B) Effect of AZI overexpression on cellular ODC activity in NIH-3T3 fibroblasts. ODC activity was measured as above. (C) Polyamine uptake by AZI-overexpressing cells was measured following a 15 minute incubation with 0.25-10 µM [¹⁴C]spermine, as described in the Materials and Methods. No significant difference was observed in spermine uptake between AT2.1 vector control (◇) and AZI-overexpressing cells (□). Error bars indicate s.e.m.

Table 1. Cellular polyamine levels in AZI-overexpressing and vector control cells

	Polyamine level (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
AT2.1-pcDNA3	1.23±0.07	7.40±0.22	7.12±0.55
AT2.1-pcDNA3-AZI	4.79±0.51	7.80±0.92	6.93±1.05

Polyamine levels in AT2.1 cells were measured, as described in the Materials and Methods. Mean values from triplicate samples ± s.d. are shown.

AZI promotes cell proliferation in the absence of antizyme-binding activity

To investigate whether the proliferative effect of AZI is dependent on its ability to interact with antizyme we generated an AZI mutant that is no longer able to bind antizyme. A domain of 24 amino acids has been described in ODC that mediates binding to antizyme (Li and Coffino, 1992). A similar domain (ASQIKYAAKVGVNIMTCDNEVELK), sharing 63% identity with ODC, exists in AZI. To determine the role of antizyme binding in AZI activity, we constructed an AZI mutant, AZI Δ ₁₁₇₋₁₄₀, in which this putative antizyme-binding region was deleted (Fig. 4A) and tested its ability to bind to

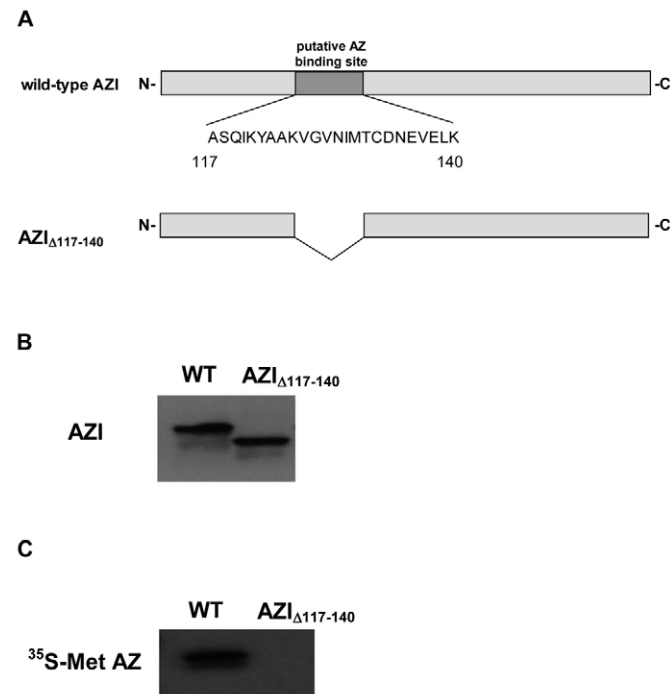


Fig. 4. Deletion of the putative antizyme-binding site and effect on antizyme binding. (A) Schematic of wild-type AZI and mutant AZI Δ ₁₁₇₋₁₄₀. The putative antizyme (AZ)-binding site, designated residues 117-140, was deleted using overlap extension PCR. (B) Western blot analysis of wild-type AZI and AZI Δ ₁₁₇₋₁₄₀ using AZI antibody. Proteins were synthesized using in vitro transcription and translation from pTriEx-Hygro-AZI and TriEx-Hygro- AZI Δ ₁₁₇₋₁₄₀ constructs as S-tagged fusion proteins. (C) Loss of antizyme-binding by AZI Δ ₁₁₇₋₁₄₀. The ability of S-tagged AZI or AZI Δ ₁₁₇₋₁₄₀ to associate with radiolabeled antizyme was examined by affinity purification using S-protein agarose beads, followed by SDS-PAGE analysis and autoradiography. The mutant AZI Δ ₁₁₇₋₁₄₀ was unable to bind antizyme.

native antizyme. Comparable quantities of mutant and wild-type AZI were produced as S-tag fusion proteins by in vitro transcription and translation, (Fig. 4B) and independently combined in solution with radiolabeled antizyme. AZI-antizyme complexes were isolated on columns containing S-protein agarose to capture S-tagged AZI. Our results reveal that deletion of the putative antizyme-binding domain (AZI Δ ₁₁₇₋₁₄₀), results in loss of the capacity of AZI to bind antizyme (Fig. 4C).

As antizyme binding is considered to be an essential component of AZI activity (Murakami et al., 1996; Murakami et al., 1989), we next determined the effect of the AZI Δ ₁₁₇₋₁₄₀ deletion on AZI functional activity. Our results show that AT2.1 cells expressing the antizyme non-binding mutant AZI Δ ₁₁₇₋₁₄₀ display increased proliferation relative to control cells, although the response is slightly attenuated compared with cells overexpressing similar levels of wild-type AZI (Fig. 5A). Because elevated wild-type AZI levels are accompanied

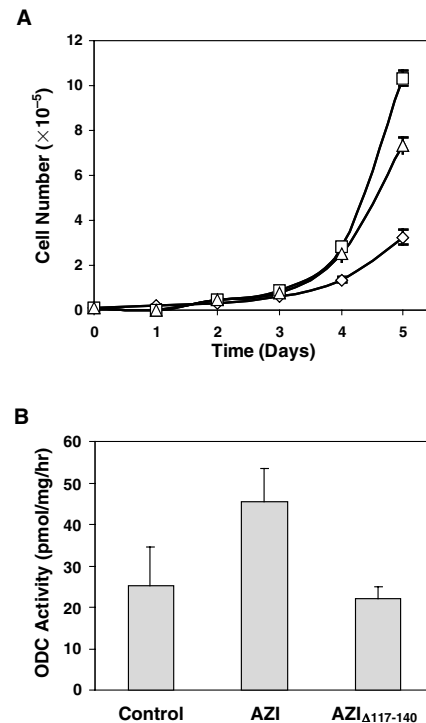


Fig. 5. Overexpression of AZI Δ ₁₁₇₋₁₄₀ increases proliferation of AT2.1 cells without increasing ODC activity in AT2.1 cells. (A) Proliferation of AT2.1 cells stably transfected with pTriEx-Hygro, pTriEx-Hygro-AZI, or pTriEx-Hygro-AZI Δ ₁₁₇₋₁₄₀, was measured as described in Materials and Methods, in media containing 1% FBS. Error bars indicate s.e.m. \diamond , Vector control AT2.1 cells; \square , AZI-overexpressing AT2.1 cells; \triangle , AZI Δ ₁₁₇₋₁₄₀ overexpressing AT2.1 cells. The proliferation rate of AZI Δ ₁₁₇₋₁₄₀ overexpressing cells decreased compared with wild-type AZI-overexpressing cells, but still demonstrated increased proliferation relative to vector controls. An average doubling time of 22.8 ± 1.6 (s.d.) for control cells; 15.6 ± 1.7 for AZI-overexpressing cells; and 18 ± 1.7 for AZI Δ ₁₁₇₋₁₄₀ overexpressing cells was determined from two separate experiments. (B) ODC activity was measured in control, AZI and AZI Δ ₁₁₇₋₁₄₀-overexpressing AT2.1 cells. 5×10^5 cells were plated in RPMI supplemented with 10% FBS, and ODC activity assessed as described in the Materials and Methods. Error bars indicate s.e.m.

Table 2. Polyamine levels in AZI $\Delta_{117-140}$ -expressing cells

	Polyamine level (nmol/mg protein)		
	Putrescine	Spermidine	Spermine
AT2.1 pTEH	4.17 \pm 0.07	13.22 \pm 0.09	7.99 \pm 0.21
AT2.1 pTEH-AZI $\Delta_{117-140}$	3.11 \pm 0.15	15.35 \pm 0.71	9.22 \pm 0.49

AT2.1pTEH and AT2.1pTEH-AZI $\Delta_{117-140}$ -expressing cells were grown in 0.5% FBS-containing media for 72 hours before cells were collected and polyamine levels measured, as described in the Materials and Methods. Results are mean \pm s.d. from triplicate samples. Increased putrescine levels were not observed in the AT2.1pTEH-AZI $\Delta_{117-140}$ cells, in contrast to results observed for wild-type AZI overexpression (Table 1).

by an increase in ODC activity (Fig. 3A,B), we also examined the effect of the mutant AZI $\Delta_{117-140}$ on ODC activity. ODC activity has been shown to be regulated by antizyme (reviewed by Coffino, 2001). In the absence of antizyme binding in AZI $\Delta_{117-140}$ -overexpressing cells, ODC activity was unchanged relative to that of vector control cells (Fig. 5B). In addition, putrescine levels did not increase in cells expressing AZI $\Delta_{117-140}$ compared with vector control cells (Table 2). This is in contrast to cells overexpressing wild-type AZI, where putrescine levels were affected by AZI. There were also only minor differences in spermidine or spermine levels between

AZI $\Delta_{117-140}$ and vector control cells after growth in low serum for 72 hours. These results suggest that the increase in putrescine levels observed in the wild-type AZI-overexpressing cells may not be wholly responsible for their increased proliferation. Taken together, these data imply that the ability of AZI to alter the growth properties of AT2.1 cells does not exclusively rely on the ability of AZI to bind and inactivate antizyme or on the ability of AZI to increase ODC activity.

Effects of silencing AZI using siRNA

Since overexpression of AZI led to an increase in cell proliferation we speculated that silencing AZI would lead to a decrease in cell proliferation. Although the silencing of AZI by RNAi was transient, we observed a twofold decrease in cell number in AZI RNAi-treated cells relative to cells treated with scrambled control siRNA (Fig. 6A,B).

Although AZI is rapidly induced in growth-stimulated cells before upregulation of ODC (Nilsson et al., 2000), there have been no reports indicating that AZI can directly influence cell-cycle proteins. We recently reported that antizyme can mediate degradation of the G1 cell-cycle protein cyclin D1 (Newman et al., 2004), just as it does for ODC (Murakami et al., 1992). We therefore used gene silencing to investigate the effect of

AZI depletion on levels of key cell-cycle proteins. AZI siRNA was transfected into AT2.1 cells to diminish AZI expression and cell lysates were analyzed for levels of cell-cycle proteins after 48 hours of gene silencing. Treatment with AZI-specific siRNA resulted in a marked reduction of AZI in AT2.1 cells (Fig. 6C). Interestingly, cyclin D1 was also diminished in these cells whereas cyclin A levels were not affected. Similar results were found in rat HTC cells (data not shown), a cell line commonly used for studies on the antizyme and AZI pathways (Mitchell et al., 2002; Murakami et al., 1996). Thus, levels of cyclin D1 decrease when AZI expression is suppressed.

Since we reported that antizyme can promote the degradation of cyclin D1 we next investigated whether AZI, which inactivates antizyme, can attenuate cyclin D1 degradation. We performed an *in vitro* degradation assay using rabbit reticulocyte lysate with cyclin D1, wild-type AZI and AZI $\Delta_{117-140}$ synthesized by *in vitro* transcription and translation. As shown in Fig. 7, cyclin D1 was stabilized in the presence of wild-type AZI. Surprisingly, AZI $\Delta_{117-140}$ was also able to attenuate cyclin D1 degradation to a similar extent as wild-type AZI (Fig. 7). This suggests that AZI can stabilize cyclin D1 through an antizyme-independent mechanism.

We next investigated whether a direct interaction exists between AZI and cyclin D1. Following transfection of HEK 293 cells with antizyme, S-tagged AZI and cyclin D1, we found a novel association between AZI and cyclin D1 (Fig. 8A). The interaction between AZI and cyclin D1 was specifically lost in the presence of antizyme suggesting that the affinity of AZI for antizyme is greater than the affinity of AZI for cyclin D1.

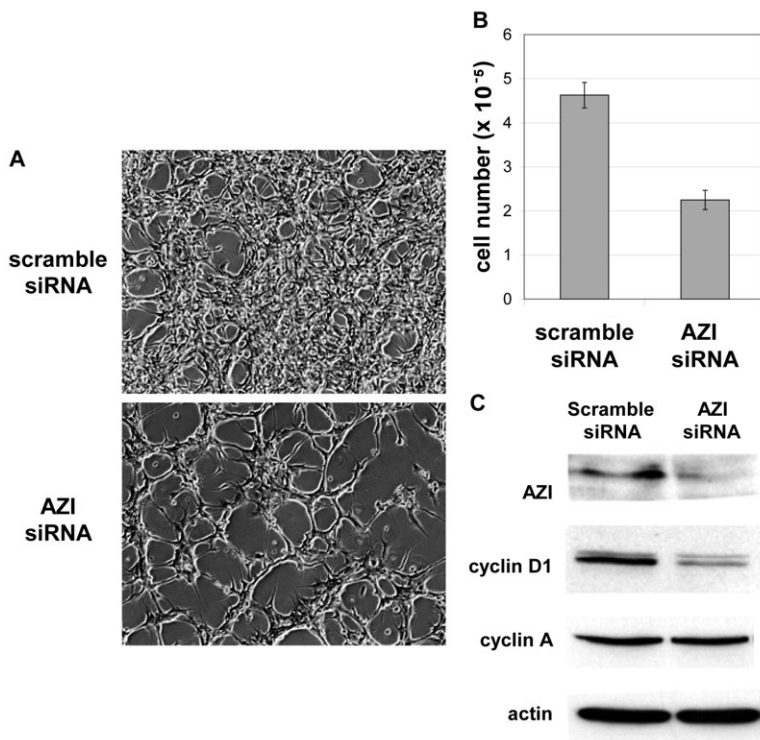


Fig. 6. Effect of AZI RNAi on cell proliferation and cell-cycle protein levels. (A) Phase-contrast micrographs of AT2.1 cells transfected with control or AZI siRNA, 48 hours post transfection. (B) Quantification of cell number after scramble- or AZI-siRNA transfection. At 48 hours post transfection, cells were trypsinized and the number of cells per condition was quantified using a Coulter counter. (C) Western blot analysis of cell lysates from control or AZI siRNA-transfected AT2.1 cells, collected 48 hours following transfection. AZI siRNA-transfected AT2.1 cells exhibited decreased levels of AZI (62 \pm 6% decrease) and cyclin D1 (56 \pm 4%, both mean \pm s.e.m. from three independent experiments), but not of cyclin A or actin.

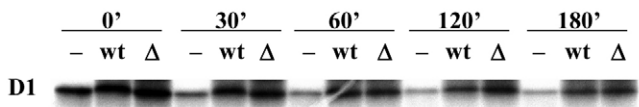
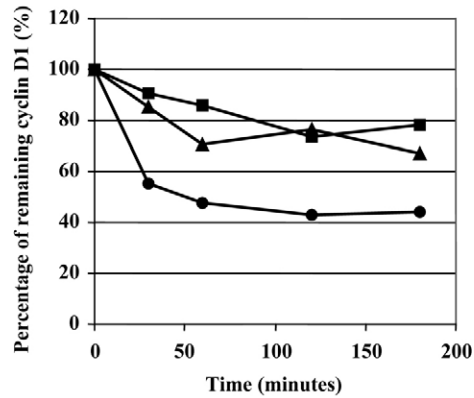


Fig. 7. AZI shows ability to attenuate cyclin D1 degradation. Cyclin D1, wild-type AZI and AZI Δ ₁₁₇₋₁₄₀ were synthesized *in vitro* transcription and translation in separate reactions. All three proteins were radiolabeled with [³⁵S]methionine. Translated proteins were combined and incubated in an ATP-regenerating buffer for 0, 30, 60, 120 and 180 minutes. The amount of cyclin D1 remaining at the indicated time points was assessed by SDS-PAGE and PhosphorImager analysis (lower panel). Results are representative of two independent experiments. ●, cyclin D1 alone; ▲, Cyclin D1 + wild-type AZI; ■, % Cyclin D1 + AZI Δ ₁₁₇₋₁₄₀.

We further investigated the interaction of AZI and cyclin D1 using the AZI Δ ₁₁₇₋₁₄₀ mutant, which fails to bind antizyme. AZI Δ ₁₁₇₋₁₄₀ demonstrated binding to cyclin D1 equivalent to that of wild-type AZI, implying that the interaction between AZI and cyclin D1 relies on a domain distinct from the antizyme-binding site of the AZI molecule (Fig. 8B). In further support of this, the interaction between AZI Δ ₁₁₇₋₁₄₀ and cyclin D1 was not disrupted by antizyme (Fig. 8B).

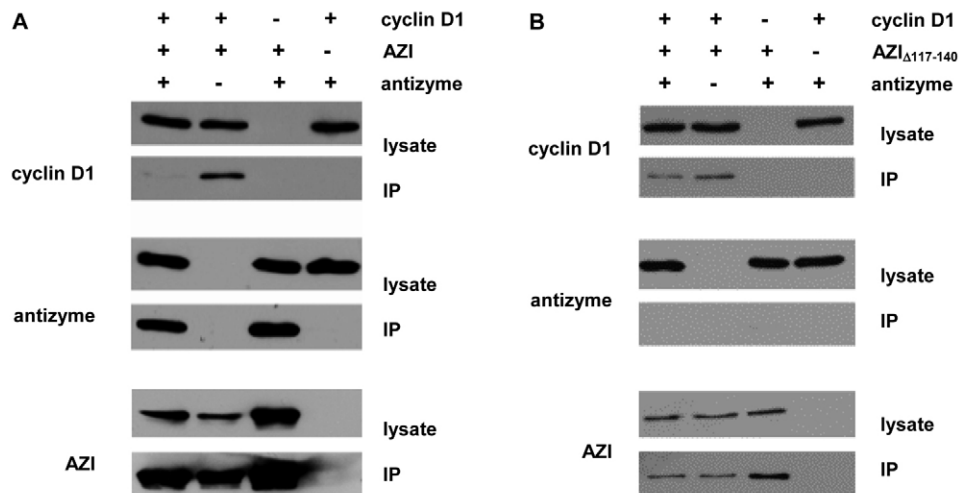
Discussion

The regulatory protein antizyme has received considerable attention both for its unusual post-transcriptional regulation by frame-shifting (Matsufuji et al., 1995) and for its ability to degrade selected proteins in the absence of ubiquitylation (Lin et al., 2002; Murakami et al., 1992; Newman et al., 2004). Because ODC was the first target shown to be degraded in association with antizyme, much of the focus of antizyme studies has been on its role in modulating ODC and the polyamine pathway. ODC has been established as an oncogene, capable of increasing both proliferation and transformation (Auvinen et al., 1992; Moshier et al., 1993). Consistent with this hypothesis, antizyme is downregulated in certain carcinomas (Koike et al., 1999; Tsuji et al., 1998), and its upregulation has been associated with cell-cycle arrest and with differentiation of carcinoma cells (Koike et al., 1999; Tsuji et al., 2001). Recent evidence, however, suggests that the antizyme-mediated degradation pathway may have additional targets (Lin et al., 2002; Newman et al., 2004).

In contrast to the considerable interest in antizyme, its endogenous inhibitor, AZI, is less well studied. Because AZI has homology to ODC, including an antizyme-binding sequence, it has been assumed that the central function of AZI is to bind and sequester antizyme, leading to higher levels of ODC owing to reduced antizyme-mediated ODC degradation. One consequence of this activity might be increased cellular proliferation because ODC activity is increased. Higher AZI levels would be expected to sequester antizyme, resulting in greater ODC levels and activity as well as enhanced polyamine uptake. While this manuscript was under review, Choi et al. reported that silencing AZI led to decreased cell proliferation of A549 lung carcinoma cells as well as a concomitant reduction of ODC activity and polyamine levels (Choi et al., 2005). We now show evidence that AZI can influence cell proliferation in both an antizyme-dependent and -independent mechanism. We also report that antizyme is not the sole binding partner for AZI and that interactions between AZI and the cell-cycle regulatory protein cyclin D1 may also contribute to the activity of AZI.

Our results demonstrate that AZI overexpression leads to increased rates of cell proliferation in both rat carcinoma cells

Fig. 8. Interaction of AZI and cyclin D1 *in vitro* and effect of AZI Δ ₁₁₇₋₁₄₀ mutation. (A) 293 cells were transfected with combinations of plasmids expressing S-tagged AZI, FLAG-tagged antizyme and HA-tagged cyclin D1. After 48 hours, lysates were collected and incubated with S-protein agarose. AZI-interacting proteins were identified by western blot using anti-HA, anti-FLAG or anti-AZI antibodies. (B) Immunoprecipitation of transfected S-tagged AZI Δ ₁₁₇₋₁₄₀, FLAG-tagged antizyme and HA-tagged cyclin D1 as described above. AZI Δ ₁₁₇₋₁₄₀ interacted with cyclin D1 but not antizyme.



and mouse fibroblasts. This difference was most apparent in low serum concentrations, suggesting that AZI can overcome restrictions posed by growth factor limitation – a factor in tumor formation. These results suggest that high AZI levels could give cells a growth advantage under conditions where nutrients or oxygen are limited such as in avascular early tumors or in late-stage necrotic tissues. Differences were also observed in the ability of the AZI-overexpressing cells to have a shortened lag phase, to reach higher saturation densities *in vitro* and, in the case of the AT2.1 Dunning carcinoma cells, to enhance anchorage-independent cell growth. Because AT2.1 cells already exhibit a limited ability to form small colonies in semi-solid medium, these cells may not require many additional changes to increase that oncogenic capability. Importantly, NIH-3T3 cells can be transformed by ODC (Auvinen et al., 1992; Moshier et al., 1993) but not by AZI. One possible explanation is that the increase in ODC activity by AZI is not sufficient to promote transformation. Unlike the fourfold increase in ODC activity in ODC transformants (Moshier et al., 1993), ODC activity was increased only twofold in the AZI-overexpressing cells. Furthermore, there may be other functions of ODC which have not yet been fully elucidated, such as the ability of ODC to modulate downstream kinases and transcription factors (Gilmour et al., 1999; Kielosto et al., 2004).

The total effects of wild-type AZI on cell proliferation are likely to be mediated by multiple mechanisms. Our work suggests that increased AZI activity leads to elevated polyamine levels in AT2.1 cells mainly by antagonizing antizyme-mediated degradation of ODC and not by influencing polyamine uptake. We cannot, however, exclude the proposals that the effect on polyamine uptake is cell-type specific and that AZI could affect polyamine uptake in other cells such as CHO, which has been reported recently (Mitchell et al., 2004). Altered polyamine levels have been shown previously to affect cell proliferation and this mechanism may explain part of the proliferative effects induced by altering AZI expression. Here we suggest the existence of a second pathway for the proliferation enhancing effect of AZI. Intriguingly, overexpression of the non-antizyme-binding mutant AZI $_{\Delta 117-140}$ led to increased cell proliferation, although not to the same extent as the wild-type AZI. In addition, the AZI $_{\Delta 117-140}$ mutant was able to attenuate cyclin D1 degradation to a similar extent as wild-type AZI. These results demonstrate the presence of at least two potential mechanisms for AZI function in AT2.1 cells: an antizyme-dependent mechanism and an antizyme-independent mechanism. Taken together, our results dispel the assumption that cells use AZI purely for its ability to inhibit antizyme and to increase ODC stability and activity.

In an attempt to ascertain the mechanism underlying the antizyme-independent effects of AZI on cyclin D1 stability, we asked whether AZI might bind directly to this cell-cycle regulatory protein. We were able to show that both AZI wild-type and the non-antizyme binding mutant AZI $_{\Delta 117-140}$ interact with cyclin D1. Inhibition of the association between cyclin D1 and AZI occurs in the presence of antizyme, suggesting that AZI may have a stronger affinity for antizyme than for cyclin D1. This suggests that both antizyme (Newman et al., 2004) and AZI may contribute in a separate way to the control of cyclin D1 levels. The relative importance of these two cyclin

D1 modulators remains to be determined, as does their role in modulating the normal cell cycle. In addition, cyclin D1 levels may also be influenced by alterations in cell proliferation that result from changes in polyamine levels.

The finding that AZI overexpression results in increased cell growth as well as the induction of the transformed phenotype suggests that AZI may act to participate in maintaining the transformed phenotype in concert with other activated oncogenes. Interestingly, we find that AZI levels are increased in NIH-3T3 cells expressing the activated Ras oncogene, showing a potential link between AZI and oncogene function (unpublished data). AZI is upregulated in gastric cancer compared with normal tissue (Jung et al., 2000) as well as in several cancers, as revealed by exploration of the National Cancer Institute's Cancer Genome Anatomy Project (CGAP) and ONCOMINE (<http://www.oncomine.org>) databases (Rhodes et al., 2004).

Although originally described as an inhibitor of antizyme, our results demonstrate that AZI has additional functions. The proliferation-enhancing effects of AZI may consequently be mediated by multiple mechanisms. In one case, the sequestration of antizyme by AZI may lead to stabilization of ODC and cyclin D1 and to increased ODC activity and polyamine levels (Fujita et al., 1982; Murakami et al., 1996; Murakami et al., 1989; Newman et al., 2004). Secondly, our new finding that AZI has antizyme-independent effects and binds directly to the cell-cycle regulator cyclin D1 suggests an additional potential mechanism for the growth promoting activity of AZI. This work adds to the growing data that the antizyme-AZI pathway is an important means of modulating cell proliferation and oncogenesis.

Materials and Methods

PCR amplification of rat AZI

Rat AZI was amplified from a rat liver cDNA library (gift from M. Klagsbrun, Children's Hospital, Boston, MA) using 5' Fwd1 primer 5'-GGGGTACCG-CCACCATGAAAGGATTTATTGACG and 3' Rev1 primer 5'-GCTCTAGAA-AGAAGCGTTAATGCCTGTTT (Custom primers from Invitrogen, Carlsbad, CA). The PCR product was first ligated into pCRII-TOPO and then digested with *KpnI* and *XbaI*. AZI was then ligated into pcDNA3 vector (Invitrogen) and cloned into the retroviral pWZL vector (gift from C. Furman, Dana Farber Cancer Institute, Boston, MA) using *BamHI* and *XhoI* sites. AZI was also amplified using a new set of primers to introduce in-frame *BamHI* and *PstI* sites to clone into pTriEx-4 Hygro (Novagen, Madison, WI) which added an S-tag to the N-terminus of AZI. The forward primer, mutFwd1, was 5'-CGGGATCCGATGAAAGGATTTATTGAC-GATGAAA, and the reverse primer, mutRev1, was 5'-AACTGCAGAGCTT-CAGTGAGAAGTTGTCTTCTGGCT. AZI was then cloned into pTriEx-4 Hygro.

Construction of AZI $_{\Delta 117-140}$

The AZI deletion mutant, AZI $_{\Delta 117-140}$, was created using site-specific mutagenesis by fusion PCR of overlapping DNA fragments. Two PCR reactions were set up to synthesize the 5' and 3' ends of AZI $_{\Delta 117-140}$ from pTriEx-4 Hygro-AZI. The primers, Fwd1 and Δ RevM 5'-CCTCGCAATTTTGTGCTTACAAGGACTTG, were used to synthesize the 5' end. To create the 3' end, Δ FwdM2 primer 5'-CCTTGTA-AGCAAAAATTGCGAGGAATCAC and Rev1 were used. To produce AZI $_{\Delta 117-140}$, the products of these two PCR reactions were denatured and allowed to anneal as they share an overlapping region. This new product was used as a template to amplify the resulting deletion mutant in a final PCR reaction using Fwd1 and Rev1 primers. The final deletion mutant was then re-cloned into the original pTriEx-4 Hygro (Novagen) and sequenced to ensure absence of any mutations introduced by PCR.

AT2.1 cell transfection and stable cell line production

AT2.1 cells derived from the Dunning rat prostate carcinoma (Isaacs et al., 1986) (gift of J. Isaacs, Johns Hopkins University, Baltimore, MD) were maintained in RPMI media (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1% glutamine/penicillin/streptomycin (GPS, Invitrogen) and 250 nM dexamethasone (Sigma, St Louis, MO). Stable transfectants were produced with Lipofectamine Plus

(Invitrogen) according to the manufacturer's instructions, using pcDNA3 and pTriEx-4 Hygro constructs. The pcDNA3 stable transfectants were selected in 0.5 mg/ml G418 and the pTriEx-4 Hygro transfectants selected in 0.4 mg/ml hygromycin B. Single pcDNA3 clones were isolated, expanded and analyzed for AZI expression levels. Pooled pTriEx-4 Hygro stable transfectants were also collected and analyzed. Western blots of lysates from AZI-overexpressing stable cell lines were quantified for AZI expression using Imagequant software (Amersham Biosciences, Piscataway, NJ).

NIH-3T3 transfection and stable cell line production

NIH-3T3 cells (ATCC, Manassas, VA) were maintained in DMEM supplemented with 10% calf serum (HyClone, Logan, UT), and 1% penicillin/streptomycin. Stable NIH-3T3 cell lines were created by retroviral transfection using the pWZL constructs. First, HEK 293 EBNA cells (gift from C. Furman, Department of Biology, MIT, Cambridge, MA) were plated in medium containing heat-inactivated calf serum and 0.4 mg/ml G418. Cells were transfected simultaneously with three plasmids: one containing the GAG and POL genes; one containing the VSVG envelope behind an EBNA origin; and one containing AZI using Fugene 6 (Roche, Mannheim, Germany) as the transfection reagent. After 2 to 3 days of virus production, the conditioned media was collected and filtered. At 40% confluence, NIH-3T3 cells were infected with the conditioned media and 8 μ g/ml polybrene (Abbott Laboratories, Abbott Park, IL). The infected cells then were split at 1:20 and selected with 5 μ g/ml blastocidin for 1 week. AZI NIH-3T3 transfectants expressed AZI at levels 2.5 times greater than the vector control transfectants.

Anti-AZI siRNA design and transfection

To silence AZI gene expression, we designed siRNA against a 21-nucleotide sequence of rat AZI. The siRNA duplex, synthesized by Dharmacon Research (Lafayette, CO) was designed to target the mRNA sequence AAGAUCGUG-AAGAAGCACAGU. This region corresponds to nucleotides 139-159 of AZI. The siRNA scramble II duplex (Dharmacon) was used as a control. To perform gene silencing, 1×10^5 cells were plated per well in a six-well plate. 6 μ l Lipofectamine 2000 (Invitrogen) was used as the transfection reagent to deliver 1.2 μ mol of siRNA duplex in 206 μ l per well. AT2.1 cells were washed first with serum-free antibiotic-free media. The mix containing siRNA and Lipofectamine 2000 was then added to the same media. After 4 hours of incubation, serum was added to the media. Cell lysates were collected 2-3 days post-transfection to determine diminished AZI levels. Phase-contrast photographs were taken and cells counted using a Coulter particle counter at 48 hours post-transfection.

Proliferation assays

Cells were grown in media containing 10%, 1% or 0.5% serum. On the days indicated, cells were trypsinized and counted using a Coulter particle counter (Beckman Coulter, Fullerton, CA). Each condition was carried out in triplicate. Population doubling times were determined by linear regression analysis of the raw data from at least three separate experiments using Sigma Plot software (Systat Software, Point Richmond, CA).

Soft-agar transformation assay

The soft agar assay was based on a modified protocol as described (LaMontagne et al., 1998). Briefly, 1×10^4 viable cells were plated in 0.45% noble agar in 10% FBS-containing medium per 60 mm² plate overlaid onto a lower layer containing 0.7% noble agar in culture medium. Colonies whose diameter measured greater than 0.5 mm were quantified after two weeks at 5% CO₂ in a humidified incubator. The foci were visualized after staining with 0.02% Giemsa. Colony formation was measured using a micrometer under light microscopy.

Measurement of ODC activity

ODC activity was measured as described (Coleman and Pegg, 1998). Briefly, 2.5×10^5 cells were plated per 60 mm² dish. After 24 hours, the cells were rinsed with cold PBS and the lysates collected in extraction buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM dithiothreitol and 0.1 mM EDTA). Protein concentration was assayed for each sample by BCA assay (Pierce, Rockford, IL). The sample was incubated with L-[1-¹⁴C]ornithine hydrochloride (Amersham Bioscience) at 37°C. Radiolabeled CO₂ was absorbed by hyamine hydroxide, dissolved in 10 ml scintillation fluid and measured in a scintillation counter (Beckman Coulter, Fullerton, CA).

Measurement of polyamine levels and polyamine transport assay

Polyamine content was determined using an ion-pair reverse-phase high-performance liquid chromatography method as described previously (Pegg et al., 1989) and normalized to mg protein/sample. To assay polyamine transport, 1×10^4 AT2.1 cells were plated per well in a 96-well plate and incubated overnight. Cells were rinsed with modified Hank's balanced salt solution (MHBS, 0.01 M HEPES, HBSS, pH 7.4). To measure spermine uptake, 50 μ l ¹⁴C-radiolabeled spermine solution was distributed to each well. Final concentrations of [¹⁴C]spermine solutions were 0.25, 0.5, 1.0, 2.5, 5.0 and 10.0 μ M. After a 15 minute incubation

at 37°C, cells were rinsed with ice-cold PBS four times. Protein concentration was first quantified in each well using Bio-Rad dye reagent (Bio-Rad, Hercules, CA) and measuring absorbance at 620 nm. Spermine uptake was then measured by adding 100 μ l formic acid (25 M) to lyse the cells and the contents of each well were transferred to mini β -vials and radioactivity levels measured using a scintillation counter. Data are expressed as pmol/ μ g protein/hour.

Association of AZI with antizyme and cyclin D1

The interactions between AZI, antizyme and cyclin D1 were tested in vitro in HEK 293 cells grown in DMEM, 10% FBS. First 3×10^5 HEK 293 cells were plated per 30 mm² dish. The cells were transfected with AZI, antizyme and cyclin D1 using Fugene 6 following the manufacturer's protocol. Flag-tagged rat antizyme 1 and HA-tagged human cyclin D1 were prepared as described previously (Newman et al., 2004). 36 hours post transfection, the cells were lysed in lysis buffer (60 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100) at 4°C. Immunoprecipitation was carried out in the same buffer, using S-protein agarose (Novagen). AZI-associated interactions were examined by SDS-PAGE and subsequent western blot analysis.

Immunoblotting

The protein concentration of lysates was measured using Bio-Rad protein assay reagent and the BCA assay (Pierce). Equal protein amounts were loaded on polyacrylamide gels. Samples were transferred onto 0.2 μ m nitrocellulose (Protran, Schleicher & Schuell, Keene, NH) for antizyme blotting, or onto 0.45 μ m PVDF Immobilon-P (Millipore, Billerica, MA). Total protein was probed with rabbit polyclonal antizyme antibody (1:2000, gift from J. Mitchell, Northern Illinois University), mouse monoclonal AZI antibody (1:2000, gift from S. Matsufuji, Jikei University) or rabbit polyclonal ODC antibody (1:500, Progen, Heidelberg, Germany; M1B6, 1:1000, gift from P. Coffino, University of California at San Francisco, CA). Other proteins were analyzed using antibodies against cyclin D1 (HD-11, 1:500; 72-136G, 1:500) or cyclin A (C19, 1:500), from Santa Cruz Biotech (Santa Cruz, CA) and actin (C4, 1:2500, Roche; MAB 1501, 1:5000, Chemicon).

In vitro degradation assay of cyclin D1

Cyclin D1, wild-type AZI, and AZI_{Δ117-140} were synthesized in rabbit reticulocyte lysates (Promega, Madison, WI) by in vitro transcription and translation in separate reactions. All three proteins were radiolabeled with [³⁵S]methionine (Perkin Elmer, Boston, MA). Translated proteins were combined and incubated in an ATP-regenerating buffer (60 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 4 mM DTT, 2 mM ATP, 20 mM creatine phosphate, 3.2 μ g/ml phosphocreatine kinase) at 37°C. The amount of cyclin D1 remaining at the indicated time points was assessed by SDS-PAGE and PhosphorImager analysis.

Antizyme-AZI association assay

S-tagged wild-type and mutant forms of AZI were synthesized separately in rabbit reticulocyte lysates (Promega, Madison, WI) with cold methionine. Rat antizyme 1 cloned into pET33b+ as described (Newman et al., 2004) was synthesized in the presence of EasytagTM Express Protein Labeling mix containing L-[³⁵S]methionine (Perkin Elmer, Boston, MA). The synthesized proteins were incubated together at 4°C for 1 hour in association buffer (20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 0.5% Triton X-100, 5 mM EDTA and 3 mM MgCl₂) containing 5% milk and a cocktail of protease inhibitors (Complete; Roche, Mannheim, Germany). The mix was precleared for 30 minutes at 4°C with protein A and protein G agarose beads (Santa Cruz Biotech). The supernatant was then transferred to a fresh tube containing S-protein agarose beads (Novagen), which bind to the S-tag on AZI protein. After overnight incubation at 4°C, the beads were washed three times with association buffer containing Complete, EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). Samples were boiled for 5 minutes in SDS sample buffer and loaded onto 10% SDS polyacrylamide gels.

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