Antizyme Targets Cyclin D1 for Degradation: A Novel Mechanism for Cell Growth Repression

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Summary

Overproduction of the ornithine decarboxylase (ODC) regulatory protein ODC-antizyme (AZ) has been shown to correlate with cell growth inhibition in a variety of different cell types. Although the exact mechanism of this growth inhibition is not known, it has been attributed to the effect of antizyme on polyamine metabolism. Antizyme binds directly to ODC, targeting ODC for ubiquitin-independent degradation by the 26S proteasome. We now show that antizyme induction also leads to degradation of the cell cycle regulatory protein cyclin D1. We demonstrate that antizyme is capable of specific, non-covalent association with cyclin D1 and that this interaction accelerates cyclin D1 degradation in vitro in the presence of only antizyme, cyclin D1, purified 26S proteasomes, and ATP. In vivo, antizyme upregulation induced either by the polyamine spermine or by antizyme overexpression, causes reduction of intracellular cyclin D1 levels. The antizyme-mediated pathway for cyclin D1 degradation is independent of the previously characterized phosphorylation- and ubiquitination-dependent pathway, because antizyme upregulation induces the degradation of a cyclin D1 mutant (T286A) that abrogates its ubiquitination. We propose that antizyme-mediated degradation of cyclin D1 by the proteasome may provide an explanation for the repression of cell growth following antizyme upregulation.
Introduction

The regulatory protein antizyme (AZ) has been studied primarily in the context of its role in facilitating degradation of the enzyme ornithine decarboxylase (ODC), which catalyzes the rate-limiting step in polyamine synthesis (reviewed in 1-3). AZ is therefore thought to be dedicated principally to the feedback regulation of polyamine levels. AZ synthesis is controlled via an unusual mechanism of translational frameshifting. The ribosomal frameshift required for the translation of full-length AZ is directly induced by polyamines when their levels rise (4,5). Polyamines can thus inhibit their own synthesis via AZ-mediated down-regulation of ODC. This mechanism serves to prevent extreme fluctuations in polyamine levels, which are thought to be toxic (6-8). Despite this homeostatic mechanism, the levels of polyamines and ODC vary markedly during the cell cycle, indicating that additional factors control polyamine levels and suggesting a role for this pathway in the regulation of cell proliferation (9).

Overproduction of AZ in a variety of cell types, including malignant oral keratinocytes, hepatoma cell lines and prostate cancer cells, coincides with growth inhibition (10-12) and cell cycle arrest in the G1 phase (13,14). Furthermore, overexpression of antizyme in both mouse skin cancer and gastric epithelia models has been shown to result in tumor suppression (15,16). These and other observations of antiproliferative effects of antizyme prompted us to test whether antizyme may have a specific role in cell cycle regulation, thereby accounting for its potential role as a tumor suppressor.
The cell cycle arrest previously seen in prostate carcinoma and malignant oral keratinocyte models and the growth inhibition seen in a variety of cell types upon antizyme overexpression could be explained if antizyme modulated intracellular levels of cell cycle regulatory proteins such as cyclins, as it does for ODC. We previously showed that treatment of cells with the polyamine spermine resulted in both antizyme upregulation and cell cycle arrest (14). In the current study, we have studied the ability of antizyme to complex with and degrade cyclin D1. We report that antizyme binds to cyclin D1 and facilitates its proteasomal degradation. Using purified components, we find that antizyme-mediated degradation of cyclin D1 proceeds in the absence of ubiquitin. As the degradation of this cyclin has been previously characterized and found to be dependent on the SCF family of ubiquitin-protein ligases (17,18), our data indicate that the same protein may be delivered to the proteasome via two distinct targeting mechanisms. More generally, our results suggest that antizyme may indeed act as a tumor suppressor by controlling progression through the G1 phase of the cell cycle by a novel mechanism.

Experimental Procedures

Plasmid construction

The rat antizyme 1 gene was PCR-amplified from plasmid GST-AZΔT (gift of T. Tsuji) using primers AZHis-FWD (5’-gcgtgacatatgtgaatctcc-3’) and AZHis-REV (5’-gcacaatcatgactgaggacaaccc-3’). The PCR product was digested with Nde1 and Xho1 enzymes, and ligated into plasmid pET-33b(+) (Novagen) digested with the same. The
AZ PCR product was also cloned into pCMV-Flag using the same restriction enzymes. To construct plasmids HKT-D1, HKT-ODC, cDNAs encoding human cyclin D1 and ODC were PCR-amplified from plasmids pRcCMVD1wt and pODC10/2H respectively (gifts of M. Ewen and T. Tsuji) using primers D1-FWD (5’-cgaggatccaatggaacaccagctcctgtgtctg-3’) and D1-REVB (5’-cgaagctttcagatgtccacgtcccgcacgtc-3’) for cyclin D1 and primers RM-ODC1 (5’-ggaacagagctcaatcatgaacaac-3’) and RM-ODC2 (5’-cagctactcagtgctatctacac-3’) for ODC. The cyclin D1 PCR product was digested with BamH1 and HindIII and subcloned into plasmid pET-33b(+) while the ODC PCR product was digested with Sac1 and Xho1 prior to subcloning into the same vector. Human antizyme 1 cDNA was amplified from a human cDNA library using primers AZHis-FWD and AZHis-REV and cloned into plasmid pQE30 (Qiagen). The cyclin D1 mutant T286A was generated by site directed mutagenesis using the Stratagene QuikChange mutagenesis kit according to the manufacturer’s protocol. As template the pRMD1-HA construct (human Cyclin D1 with C-terminal HA-tag cloned into pcDNA3) was used. The mutagenic primer were 5’-ACCTGGCTTTGCGCACCCACCGAC-3’ (T286A.For) and 5’-CGTCGGGTGGCGCGCAAGCCAGGT-3’ (T286A.Rev).

Cell culture and transfection
The AT2.1 cell line derived from the Dunning rat prostate carcinoma (obtained from Dr. John Isaacs) was maintained as described previously (19). For spermine treatment, cells were plated at a density of 2 X 10^5 cells/10 cm^2 dish in RPMI 1640 (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL) and 250 nM dexamethasone
(Sigma), then incubated overnight. Cells were subsequently washed twice in serum-free RPMI 1640 containing 5 ng/ml human recombinant epidermal growth factor, 5 µg/ml bovine insulin, 5 µg/ml transferrin, and 5 ng/ml sodium selenite (Sigma). Cells were then incubated in the above media without serum in the presence or absence of 10 µM spermine tetrahydrochloride (Sigma). For immunoblot analysis, 2.5 x 10^5 AT2.1 were plated in 60mm dishes. After 16-24 hrs cells were transiently transfected with 1.5ug pcDNA3, pcDNA3-HA-CD1, pcDNA3-HA-CD1 T286A, respectively using Fugene 6 (Roche). After an additional 16-24 h cells were treated with 10 µM spermine for 8 h.

The rat hepatoma HTC cell line (obtained from Dr. John Mitchell) was maintained in DMEM containing 10% FBS. For transient transfection for pulse-chase experiments, cells were plated at a density of 2 X 10^5 cells/10 cm^2 dish one day prior to transfection. On the day of transfection, cells were transfected with 7 µg of either FLAG vector or FLAG-AZ plasmid DNA using Lipofectamine Plus (Invitrogen) transfection reagent according to manufacturer recommendations. For additional pulse-chase experiments, HTC cells were transiently transfected with either 7 µgs FLAG vector or FLAG-AZ plasmids DNA in combination with 7 µgs D1(T286A) plasmid DNA. For transient transfection for immunoblot analysis, 2.5 x 10^5 AT2.1 cells were plated in 60mm dishes. After 16-24 hrs cells were transiently transfected with 1.5ug pcDNA3, pcDNA3-HA-CD1, pcDNA3-HA-CD1 T286A, respectively using Fugene 6 (Roche). After an additional 16-24 h cells were treated with 10 µM spermine for 8 h.
Stable transfectants of the hamster malignant oral keratinocyte cell line HCPC containing either pcDNA3 vector alone or pcDNA3/Hamster AZ were obtained from Dr. Takanori Tsuji. Cells were maintained in DMEM containing 10% FBS and 200 µg/ml G418.

**Immunoblotting**

Cells were washed twice in phosphate buffered saline (PBS), and lysed in lysis buffer A (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 50 mM Tris-HCl [pH 8.0]). Equal amounts of total protein were separated by SDS-PAGE, electrotransferred to nitrocellulose membrane (Schleicher and Schuell) and probed with either cyclin D1 (HD-11; 1:500), cyclin A (C19; 1:500), cdk2 (M2; 1:500) (all from Santa Cruz Biotech), pRb (1:500, Pharmingen), ODC (1:500, gift from P. Coffino) or (1:1000, Progen), actin (C4; 1:2500, Roche) or antizyme (polyclonal, 1:2000, gift from J. Mitchell).

**Immunoprecipitation**

For immunoprecipitation of *in vitro* translated proteins, human cyclin D1 and rat antizyme were individually transcribed and translated *in vitro* from plasmids pD1HA (gift of M. Ewen), and pHKTAZ respectively in the presence of $^{35}$S-methionine, using the reticulocyte-based TNT Transcription/Translation System (Promega). The transcription/translation reaction was stopped by the addition of 20 mM EDTA, which also served to prevent protein degradation by inhibiting any remaining ATP-dependent proteasome activity in the reaction mixture. Extracts were mixed in proportions yielding equivalent radioactivity among the *in vitro* translated proteins. Proteins were then allowed to interact in IP Buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM MgCl$_2$, 1 mM dithiothreitol) for 60 min at 4°C prior to the addition of antibody against antizyme or
cyclin D1. After an additional 60-min incubation at 4°C, immune complexes were isolated by the addition of agarose beads conjugated to either protein A or protein G (Santa Cruz Biotech.) and additional incubation for 60 min at 4°C. The beads were harvested by centrifugation at 8,000 x g for 5 min, resuspended in sample buffer and boiled prior to separation of proteins by SDS-PAGE.

For IP-Westerns of FLAG-hAZ or HA-hCyclin D1, 3.5 x 10^5 293 cells were transfected with 1 µg of each plasmid (pcDNA3HA-D1, pCMVFlag-AZ1 or empty vector) using Fugene 6 transfection reagent according to the manufacturer's instructions. Cells were harvested 36h after transfection and lysed by vortexing on ice. The lysis buffer contained 50 mM Tris [pH 7.4], 50 mM NaCl, 5 mM MgCl₂, 0.1% Triton X-100 supplemented with Protease Inhibitor Cocktail (Roche). The cell lysate (1ml) was incubated with 4µg of antibody against the Flag-epitope of AZ (Sigma, M2, mouse monoclonal) for 2h at 4°C. The antibody was precipitated at 4°C for an additional 2h using 30 µl of Protein A beads (Pierce). The beads were washed 3 times in lysis buffer, resuspended in 60 µl Laemmli loading buffer and boiled. Samples were applied to SDS-PAGE and analyzed by immunoblot using antibody against the HA-tag of cyclin D1. 10 µl of pre-IP cell lysate was also analyzed by SDS-PAGE and immunoblot as a transfection control.

**Antisense inhibition of antizyme upregulation in vivo**

AT2.1 cells were cultured for spermine treatment as described above. Prior to treatment, 0.5 mM of standard control (5'-cctcttacctcagttacaatttata-3') or antizyme specific (5'-gggtccgaaaccagcaaaaacctcc-3') FITC-conjugated antisense morpholino oligonucleotides
(GeneTools) were delivered into cells using Special Delivery Reagent (GeneTools) according to the manufacturer's protocol. 24 hours after morpholino delivery, cells were treated with spermine as described above for an additional 24 hours prior to lysis and immunoblotting.

To silence AZ gene expression in HCPC-1 or HTC cells, a 21-nucleotide sequence was designed to target the mRNA sequence 5'-AACCUUCAGCUUUCUUGGCUU-3'. The AZ specific siRNA and negative control siRNA (Scramble II duplex) were from Dhharmacon Research. 2 X 10^5 (HCPC-1) or 3.5 X 10^5 (HTC) cells were plated per 35 mm well. For each well, 6 µl LipofectAMINE 2000 (Invitrogen) was used as transfection reagent to deliver 400 pmole of siRNA duplex. Cells were washed first with serum-free, antibiotic-free media. The mix containing RNAi and LF2000 was then added. After four hours of incubation, serum was added to the media. Cell lysates were collected 2 days after transfection.

**35S labeling and pulse-chase**

For short metabolic labeling, AT2.1 cells were treated with spermine as described above. 1, 4, 8 and 12 h after spermine addition, cells were washed, starved for 30 min in methionine-free, cysteine free RPMI (Invitrogen). Subsequently, cells were washed twice in PBS, then incubated for 10 minutes in the above media containing 10% dialyzed FBS (Invitrogen) and 100 mCi/ml 35S EXPRESS Labeling Mix (Perkin-Elmer). After labeling cells were washed 4 times in PBS, lysed in lysis buffer A (see above). Subsequently, lysates were used for immunoprecipitation using antibody against cyclin
D1. Purified complexes were separated by SDS/PAGE and visualized using a phosphoimaging system (Imagequant).

For pulse-chase analysis, HTC cells were cultured and transiently transfected as described above. Cells were then starved for 30 min in methionine free, cysteine free DMEM (Invitrogen). Subsequently, cells were washed twice with PBS, then incubated for 30 min. in the above media containing 10% dialyzed FBS and 100 µCi/ml $^{35}$S EXPRESS Labeling Mix. After labeling, cells were washed twice in PBS. The media was then replaced with growth medium containing 50 µg/ml cysteine (Gibco/BRL), and 50 µg/ml methionine (Gibco/BRL). After rinsing with PBS, cells were lysed in lysis buffer A (see above) 0, 10, 20, 40, 60, and 80 minutes after addition of the chase media. The lysates were then used for immunoprecipitation as described above using antibody against the FLAG epitope of AZ, cyclin D1, or β-actin. Proteins were separated by SDS-PAGE, visualized and quantitated using a phosphoimaging system. The signal intensity of each band was normalized against that of β-actin.

**Complex formation using purified proteins**

His-tagged human cyclin D1 and ODC proteins were expressed in *E. coli* strain BL21(DE3). Proteins were purified by nickel chelate affinity chromatography (Qiagen) following the manufacturer’s instructions. Rat GST-AZ, was expressed in *E. coli* strain BL21(DE3) and purified with glutathione-Sepharose resin (Pharmacia) according to manufacturer’s instructions. To assay complex formation using GSH resin (Pharmacia), 31 µg GST or 56 µg rat GST-AZ proteins were incubated with a 3-fold molar excess of
cyclin D1, ODC, or citrate synthase (CS, Roche Biochemicals) in binding buffer (50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 4 mg/ml BSA) for 45 min at 30°C in a volume of 1 ml. To show competition between cyclin D1 and ODC for AZ-binding, AZ was preincubated with a 2 or 10-fold molar excess of ODC for 15 min. The reaction mixture was then applied to a GSH-resin column at 4°C for 1 h and subsequently washed with binding buffer lacking BSA. Bound proteins were eluted with buffer containing 50 mM Tris-HCl [pH 7.8], 150 mM NaCl, 10% glycerol, and 20 mM reduced glutathione. Proteins were separated by SDS/PAGE and analyzed by Coomassie brilliant blue staining or immunoblotting.

**In vitro protein degradation in reticulocyte lysates**

Human cyclin D1 and rat antizyme were synthesized in vitro using reticulocyte lysates as described above. Equal amounts of 35S-labeled cyclin D1 were incubated with antizyme in ATP-regenerating buffer (30 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 2 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 1.6 mg/ml phosphocreatine kinase) for 90 min at 37°C. The reaction was stopped by the addition of an equal volume of 2X SDS-PAGE sample buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 0.2% bromophenol blue, 10% glycerol, 100 mM dithiothreitol), then boiled prior to analysis by SDS-PAGE.

**In vitro degradation assay using purified proteasomes**

Human 26S proteasomes were purified from red blood cells as described previously (20). Purified protein substrates, rat antizyme and purified proteasomes were incubated in degradation buffer (50 mM Tris [pH 7.2], 0.01% Tween 20, 1 mM DTT, 400 µg/ml BSA,
1 mM EDTA, 5 mM MgCl₂, 100 mM NaCl, 10% glycerol), and an ATP regenerating system containing phosphocreatine (10 mM), ATP (2 mM) and creatine phosphokinase (3 U) for 0, 2 and 4 h at 30°C. Z-Ile-Glu-(Obut)-Ala-Leu-H aldehyde (0.2 mM; Peptide Institute) and apyrase (0.6 U; Sigma) were added where indicated. The reaction volume was 25 µl. After incubation, the reaction was stopped by the addition of SDS-PAGE sample buffer, then boiled prior to analysis by SDS-PAGE.

Results

Upregulation of endogenous antizyme in prostate cancer cells results in degradation of cell cycle regulatory proteins

Addition of exogenous polyamines has been shown to upregulate endogenous antizyme levels in a variety of different cell lines. We previously showed that induction of antizyme within two hours after addition of the polyamine spermine is followed by G1 growth arrest in prostate carcinoma cell lines (14). As cyclins are key determinants of cell cycle transitions, we investigated whether growth arrest was due to a change in the levels of specific cyclins responsible for the G1-S transition. Cyclin levels were measured in cell lines grown in defined media in the presence or absence of 10 µM spermine. After 24 hours we observed a marked decrease in the levels of cyclin D1, in spermine-treated cells relative to untreated controls (Fig. 1a). Under the conditions of this experiment, antizyme is efficiently induced, as previously shown (14). As expected, ODC levels are reduced following antizyme induction (Fig. 1a). Levels of other cell cycle regulatory proteins, such as cdk2, and cyclin A, remained unchanged, as did the level of
actin (Fig. 1a). Transcriptional regulation could not account for the decrease in cyclin D1 levels in these cell lines, since treated and untreated cells contained equivalent levels of mRNA encoding this protein (Fig. 1b). Furthermore, loss of cyclin D1 could not be attributed to a block in translation as synthesis of cyclin D1 protein remained constant during spermine treatment in these cell lines (Fig. 1c).

A critical function of the cyclin D1/CDK complex is phosphorylation of the retinoblastoma protein (pRb), which is required for entry into the S phase of the cell cycle (21-23). After spermine-induced loss of cyclin D1, pRb was found in a hypophosphorylated form (Fig. 1d). By this functional criterion, it is likely that cyclin D1/CDK kinase complexes are effectively inhibited by spermine treatment. The loss of G1-specific cyclin D1/CDK complexes and the resulting decrease in Rb phosphorylation may account for the predominant G1 arrest seen in these cells after spermine treatment (14).

**Antizyme is required for the destruction of cyclin D1**

The loss of cyclin D1 seen in spermine-treated prostate carcinoma cells could be a consequence of spermine-induced cell cycle arrest rather than a consequence of antizyme upregulation. To determine if antizyme alone is sufficient to promote degradation of cyclin D1, we looked at the effect of blocking antizyme synthesis on the levels of cyclin D1 following spermine treatment. Non-specific and antizyme-specific antisense morpholino oligonucleotides were introduced into rat prostatic carcinoma cells and
incubated for 24 hours. Cells were then incubated in the presence or absence of spermine prior to analysis of antizyme and cyclin D1 levels. As expected, after 24 hours in the presence of spermine we observed a marked increase in the levels of antizyme in those cells containing the control morpholino (Fig. 2a). In contrast, addition of the antizyme-specific antisense morpholino inhibited antizyme upregulation relative to the control. Coincident with this decrease in antizyme levels we observe increased levels of cyclin D1 in spermine-treated cells in the presence of the anti-antizyme morpholino. Levels of cdk2 were unaffected under all conditions tested. These results suggest that degradation of cyclin D1 in these cells is not a non-specific effect of spermine treatment, but does depend on the presence of increased levels of antizyme.

While our data indicate that spermine-mediated induction of antizyme leads to degradation of cyclin D1, levels of polyamines and antizyme have been shown to fluctuate even in cells growing under normal conditions. To determine if antizyme has a role in the degradation of cyclin D1 in the absence of spermine treatment, we looked at the effect of blocking endogenous antizyme synthesis on the levels of cyclin D1 in untreated cells. HTC hepatoma cells were grown in routine cell culture media and were transfected with either scrambled or AZ-specific siRNA and incubated for 24 h. Analysis of the endogenous levels of AZ along with cell cycle regulators indicated that suppression of antizyme expression again coincided with stabilization of cyclin D1 (Fig 2b). Levels of other cell cycle regulators such as cdk4 or cyclin A were not affected by ablation of AZ, indicating that antizyme specifically promotes degradation of cyclin D1 even in cells grown under normal cell culture conditions. As expected, levels of ODC, a
known target of AZ-mediated degradation, were increased in HCPC malignant oral keratinocytes transfected with AZ-specific siRNA (Fig. 2c).

**Antizyme overexpression leads to loss of cyclin D1**

Similar to the effects seen in prostate carcinoma cells, stimulation of antizyme expression in rat hepatoma cells has been shown to result in growth inhibition (12). Additionally, overexpression of antizyme in malignant oral keratinocytes and prostate carcinoma has previously been shown to lead to G1 growth arrest and a reversal of the malignant phenotype (13,14). We overexpressed antizyme in rat hepatoma cells (Fig. 3a) and malignant oral keratinocytes (Fig. 3b) to investigate whether the growth inhibition previously seen in these cell types could be correlated with a change in cyclin D1 levels. Full-length antizyme is efficiently expressed in these transfectants. We observed a marked decrease in the levels of cyclin D1 in cell lines overexpressing AZ as compared to control cells. Levels of another cell cycle regulatory protein, cdk2, remained unchanged, as did the level of actin. These results show that overexpression of antizyme alone is sufficient to promote specific degradation of cyclin D1 in these cell types.

To further investigate the ability of AZ to degrade cyclin D1 and to dissociate this process from the well-studied-ubiquitin dependent degradation pathway, we employed a mutant form of cyclin D1 (T286A) that cannot be degraded via the ubiquitin pathway (24,25). As shown in figure 4, levels of this mutant are stable in control AT2.1 cells, but fell significantly following spermine-induced upregulation of antizyme. These results
add support for the hypothesis of an alternate pathway for an ubiquitin-independent pathway of cyclin D1 turnover.

Taken together, our antizyme ablation and overexpression results show that alteration of antizyme levels leads to changes in cyclin D1 levels consistent with the hypothesis that antizyme influences cyclin D1 levels in vivo whether levels of AZ are modulated by overexpression, by gene silencing or by polyamine-mediated induction. Our results in untreated HTC cells confirm a role for endogenous AZ in modulating cyclin D1 levels. During normal cell cycle progression, similar results were obtained using a variety of cell types. This indicates that even low, endogenous levels of antizyme have some capacity to affect cyclin D1 levels in the cell types tested.

The proteasome mediates down-regulation of cyclin D1 via accelerated breakdown in a ubiquitin-independent manner

To show directly that enhanced protein degradation mediates the decreased levels of cyclin D1 in a ubiquitin-independent manner, we used AZ-overexpressing cells to determine the half-life of the mutant form of cyclin D1 (T286A) that cannot be degraded via the ubiquitin pathway. Hepatoma cells transiently transfected with a plasmid vector expressing the T286A cyclin D1 mutant along with either empty vector or full-length AZ were pulsed with $^{35}$S-methionine and then chased with media containing excess unlabeled methionine. Lysates prepared 0, 10, 20, and 40 minutes after addition of chase media were subject to immunoprecipitation using antibodies against cyclin D1. Degradation of the T286A mutant was seen only in cells expressing AZ and the overexpressed protein
was stable in control cells (Fig. 5a, b). Because control cells showed no alteration in the rate of degradation of the T286A cyclin D1 mutant, enhanced degradation of the protein seen in AZ overexpressing cells could be attributed directly to AZ. These results confirm the ability of AZ to mediate cyclin D1 degradation in the absence of ubiquitination. To investigate this relationship further, we employed a variety of in vitro assays to study whether AZ can bind directly to cyclin D1 and influence cyclin D1 stability.

**Antizyme complexes with cyclin D1 *in vitro***

The principal known function of antizyme is to bind to ornithine decarboxylase (ODC) and increase the affinity of ODC for the proteasome, where it is degraded in an ubiquitin-independent manner. The finding that cyclin D1 degradation correlates with increased antizyme levels led us to test whether antizyme can directly target additional regulatory proteins for degradation. A prediction of this model is that antizyme should be capable of complex formation with proteins whose degradation it stimulates. Here we use three independent methods to show that antizyme is capable of direct binding to cyclin D1 both in vitro and in vivo.

First, the cyclin D1 and antizyme proteins were synthesized in $^{35}$S-labeled form by *in vitro* translation in rabbit reticulocyte lysates. After terminating translation, lysates containing cyclin D1 and antizyme were mixed and incubated together in the presence of EDTA (which inhibits proteasomal activity) for one hour to allow complex formation. Subsequent immunoprecipitation, using antibodies against either cyclin D1 or antizyme,
show evidence of an antizyme-cyclin D1 complex (Fig. 6a). Control experiments indicated that all antibodies used are non-crossreactive (Fig. 6b).

Secondly, we assessed complex formation between antizyme and cyclin D1 by co-immunoprecipitation of overexpressed proteins from mammalian cell lysates. Direct binding of antizyme to cyclin D1 was confirmed by the ability of antibodies against antizyme to isolate a complex of antizyme and cyclin D1 (Fig. 6c).

Finally, complex formation between antizyme and cyclin D1 was assayed using purified recombinant proteins. Direct binding of antizyme to purified cyclin D1 was confirmed using GSH beads to isolate proteins bound to a GST-antizyme fusion protein (Fig. 6d). Specificity of this interaction is suggested by the fact that antizyme fails to bind negative control proteins such as citrate synthase (CS) (Fig. 6d). Furthermore, ODC effectively inhibits the interaction between cyclin D1 and antizyme (Fig. 6e), indicating that AZ may have a higher affinity for ODC as compared to cyclin D1.

**Antizyme promotes proteasomal degradation of cyclin D1 *in vitro***

Our initial experiments indicated that cells containing high levels of antizyme have reduced levels of cyclin D1. We therefore examined the effect of antizyme on cyclin D1 protein stability in an *in vitro* degradation assay using rabbit reticulocyte lysate as a source of proteasomes. Antizyme and cyclin D1 were individually synthesized and $^{35}$S-labeled in reticulocyte lysates, then incubated in the presence of an ATP-regenerating system for 1.5 hours at 30°C. Both proteins were stable under these conditions (Fig. 7a).
In contrast, when antizyme-containing lysate was mixed with cyclin D1-containing lysate, efficient degradation of cyclin D1 was observed. These data thus indicate that the ability of antizyme to facilitate protein degradation is not limited to ODC and SMAD1 but may be a more general mechanism for ubiquitin-independent degradation. The application of this mechanism to cell cycle regulatory proteins suggests a novel means of modulating the cell cycle through antizyme-mediated protein degradation.

It is unclear from the above studies whether antizyme binding alone is sufficient to target proteasomal degradation of cyclin D1. In particular, cyclin D1 can be modified by ubiquitination, thus raising a question as to whether the antizyme-dependent pathway of cyclin D1 turnover is independent of the ubiquitin-dependent pathway. To confirm that antizyme alone can promote degradation of cyclin D1 in an ubiquitin independent manner, we reconstituted cyclin D1 degradation \textit{in vitro} using purified components. Purified human proteasomes alone do not degrade cyclin D1 (Fig. 7c). However, cyclin D1 was unstable in the presence of both antizyme and proteasomes (Fig. 7b). When proteasomes were omitted from the reaction, cyclin D1 was stabilized (Fig. 7d). In support of the conclusion that antizyme targets cyclin D1 for degradation by the proteasome, the reaction was sensitive to both proteasome inhibitors and ATP-depletion (Fig. 7e, f). It should be noted that the degradation experiments were carried out at 30°C rather than 37°C, which may account for the slower progression of cyclin D1 degradation than has been previously reported. These characteristics of antizyme-dependent cyclin D1 degradation closely mimic those of ODC degradation (26). Given that purified
proteins were used in these experiments, no factors other than antizyme, ATP and the proteasome appear to be strictly required for cyclin D1 degradation by this pathway.

Discussion

Studies from several laboratories have shown that perturbation of the levels of antizyme, ODC, or polyamines can affect cell cycle progression (9,14,27,28). However, the mechanisms by which antizyme may influence the cell cycle have remained unclear. It has often been assumed that the effects of antizyme are mediated via enhanced ODC degradation, and consequently, suppression of polyamine levels. Our data suggest that the effects of antizyme can also be mediated through a pathway that involves a direct interaction between antizyme and cell cycle regulators. This is consistent with the report that ectopic overexpression of antizyme in malignant oral keratinocytes results in an increased doubling time coupled with G1 arrest (13). Murakami and colleagues have also shown that AZ overexpression inhibits growth of HTC cells (29). Addition of putrescine does not completely reverse that growth inhibition as it does for cells treated with the ODC inhibitor DFMO (10). Similar results were obtained with S. pombe antizyme overexpression (30). The growth phenotype is apparently not due simply to reduced polyamine levels, because it is only partially relieved by the addition of polyamines to the growth medium (30). These data imply that direct regulation of the cell cycle by antizyme is an evolutionarily conserved biological mechanism.

Our results suggest the possibility that altered antizyme regulation could lead to improperly regulated cell growth in human malignancy or other diseases characterized by
abnormal cell proliferation. This view is consistent with our previous finding that spermine negatively regulates prostate cancer cell growth (31) and that spermine-mediated growth arrest of prostate cancer cells is apparently dependent on the ability of those cells to upregulate antizyme after spermine exposure (14). Our results may provide an explanation for the previous findings that overexpression of antizyme in ras-transformed NIH3T3 cells as well as in malignant oral keratinocytes inhibits cell growth and tumor formation both in vitro and in vivo (11,13). Additionally, overexpression of antizyme in a mouse skin cancer and gastric epithelia models was shown to inhibit tumor formation (16). These findings suggest that antizyme may have a role in retarding tumor development.

Whether antizyme can target the degradation of G1 cell cycle regulators in other cell types and cancer models besides hepatoma cells, malignant oral keratinocytes and in prostate carcinoma cells remains to be determined. However, the required components of this pathway are ubiquitously expressed. Additionally, loss of antizyme expression has been reported in the progression of prostatic carcinoma (14), as well as for progression of keratinocyte-derived oral tumors (32). Considerable work has been done to develop polyamine analogs that suppress cell growth and induce apoptosis (33). Consistent with the work of Mitchell et al (12), our work suggests that antizyme may be a principal target through which such drugs exert their antiproliferative effects and provides a potential mechanism for their action.
Our results document a new pathway for cyclin D1 degradation, which differs from previously described cyclin degradation pathways in being independent of ubiquitination as demonstrated by cyclin D1 degradation in vitro in the presence of only antizyme and purified proteasomes. In addition, overexpression of antizyme mediates degradation in vivo of a cyclin D1 mutant that is not susceptible to ubiquitin-mediated degradation. In the alternative cyclin degradation pathway we have described, antizyme replaces ubiquitin as the activity responsible for delivering the protein substrate to the proteasome for degradation. We suggest that antizyme has the ability to form complexes with cyclin D1 and that binding of antizyme to this target protein does not require additional factors. This is consistent with the demonstrated mechanism for antizyme-mediated degradation of ornithine decarboxylase (1,2,26). Evidence in support of this model has been obtained in hepatoma cells, malignant oral keratinocytes, in reticulocyte extracts, and in purified systems. In vivo, the ability of antizyme to induce degradation of cyclin D1 is not contingent on its artificial overexpression, but is observed when endogenous antizyme is induced through the physiological mode of increased polyamine levels. There is some variability of response when a large population of cells is tested and we believe that this is due, in part, to differing levels of the endogenous antizyme inhibitor (AZI) in these cells. Further studies will be required to determine whether other cellular properties also influence the ability of antizyme to facilitate cyclin D1 degradation. Additionally we show that even the low endogenous levels of antizyme present in the cells may also contribute to fluctuations in cyclin D1 levels.
During the cell cycle, cyclin D1 is phosphorylated at Thr-286 by the glycogen synthase kinase 3β (GSK-3β) and subsequently polyubiquitinated and degraded by the 26S proteasome. Our data shows that this phosphorylation-ubiquitination pathway is dispensable for AZ-mediated proteasomal degradation. However, in addition to promoting the ubiquitination and degradation, phosphorylation of cyclin D1 at Thr-286 also mediates nuclear export of cyclin D1 to the cytoplasm. This redistribution of cyclin D1 is dependent on the nuclear exportin CRM1 (34). The non-phosphorylatable D1-T286A mutant has been shown to remain in the nucleus throughout the cell cycle (34). Therefore, in order for antizyme to promote degradation of D1-T286A it may be required to enter the nucleus. Intriguingly, localization of both antizyme and ODC to the nucleus has been demonstrated during mouse development (35), and nuclear localization signals have been identified in the antizyme protein (36). Like cyclin D1, AZ has been shown shuttle between the nucleus and cytoplasm in a CRM-1 dependent manner (36). Furthermore, antizyme is able to enter the nucleus in response to bone morphogenic protein type-1 receptor activation by forming a complex with the Smad1 transcription factor and the proteasome subunit HsN3. It has been suggested that AZ could bind to ODC and other proteins in the nucleus and escort them to the cytoplasm for degradation (36). Taken together these finding strengthen a role for AZ in regulation of cyclin D1 levels in the absence of ubiquitin.

The implication of our results is that alterations in antizyme levels may provide one form of cell cycle regulation that operates by influencing intracellular levels of cyclin D1. Recent evidence that antizyme may also interact with SMAD1 indicates that ubiquitin-
independent regulation of protein turnover may be a more prevalent mechanism of protein degradation than previously thought (37). It is also worth noting that an endogenous inhibitor of antizyme (antizyme inhibitor, AZI) has been identified (38-41) and that intracellular levels of AZI will likely influence the ability of antizyme to regulate protein degradation. The ability of antizyme to suppress cyclin D1 levels provides one explanation for the previous observations that antizyme upregulation results in cell cycle arrest in a variety of cell types. Because AZ levels are low in certain late stage cancers, it is conceivable that antizyme may act as a tumor suppressor by helping to finely regulate cyclin levels in vivo.
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Figure Legends

Figure 1  Loss of cyclins and CDKs in spermine-treated prostate carcinoma cells

a, Immunoblot analysis of whole-cell extracts from AT2.1 cells treated for 24 h with 10 µM spermine. 24 hours prior to spermine addition, AT2.1 cells were plated at a density such that 60% confluence is achieved at t=0. After spermine was added, cells were allowed to grow for an additional 24 hours. Equal amounts of protein from total cell extracts were loaded in each lane, and the blots were probed with antibodies to various proteins as indicated.

b, Northern blot analysis showing that cyclin D1 transcript levels are not significantly affected by spermine after a 24-hour treatment. Ethidium bromide staining of 18S ribosomal RNA shows equal loading of RNA in each lane.

c, AT2.1 cells were treated with spermine as described previously. 0, 4, 8, and 12 hours after spermine addition, cells were metabolically labeled for 10 minutes. Cells were then washed extensively in PBS prior to lysate preparation. The amount of cyclin D1 present at each time point was assessed by co-immunoprecipitation, SDS/PAGE and phosphorimager analysis.

d, Effect of spermine treatment on hyperphosphorylation of the retinoblastoma protein pRb. Immunoblot analysis of whole cell extracts from AT2.1 cells treated with spermine, as in a.
Figure 2  Antizyme is required for degradation of cyclin D1 in spermine-treated AT2.1 cells

a, Immunoblot analysis of whole cell extracts from AT2.1 cells treated for 24h with 10 µM spermine. 24 hours prior to spermine addition, standard control antisense morpholino oligonucleotide (C) or antizyme-specific antisense morpholino oligonucleotides (AZ) were delivered into AT2.1 cells. After spermine was added, cells were allowed to grow for an additional 24 hours. Equal amounts of protein from total cell extracts were loaded in each lane, and the blots probed with antibody against antizyme, cyclin D1, cdk2 or actin.

b-c, Immunoblot analysis of whole cell extracts from HTC (b) or HCPC (c) cells transfected with either scrambled or AZ-specific siRNA and incubated for 48 hours. Equal amounts of protein from total cell extracts were loaded in each lane and the blots probed with antibody against antizyme, cyclin D1, cdk4, cyclin A, ODC, or actin.

Figure 3  Loss of cyclin D1 in carcinoma cells overexpressing antizyme

Immunoblot analysis of whole-cell extracts from rat hepatoma cells or malignant oral keratinocytes cells overexpressing antizyme. a, Rat hepatoma cells were either mock transfected (C) or transiently transfected with pCMV-FLAG-AZ (AZ). b, Stable transfectants of malignant oral keratinocytes containing either empty vector (C) or pcDNA3-AZ (AZ) were analyzed. Equal amounts of protein from total cell extracts were loaded in each lane, and the blots were probed with antibodies to various proteins as indicated.
Figure 4  Loss of wild type and T286A mutant cyclin D1 in spermine-treated cells

Immunoblot analysis of AT2.1 cells transiently transfected with empty pcDNA3HA vector, or vector expressing either wild-type cyclin D1 or the T286A cyclin D1 mutant which is not able to be ubiquitinated. 24 hours after transfection, cells were treated with 10 μM spermine for 8 h. Equal amounts of protein from total cell extracts were loaded in each lane, and the blots probed with antibody against antizyme, the HA-tag, cyclin D1, or actin.

Figure 5  Pulse-chase analysis of wild-type and T286A mutant cyclin D1

a-b, HTC cells were transiently transfected with T286A mutant cyclin D1 plasmid along with either pCMV-Flag vector or with pCMV-FlagAZ. 24 hours after transfection, cells were labeled with $^{35}$S for 30 min prior to addition of chase media. Lysates were prepared 0, 10, 20, and 40 minutes after addition of chase media and the amount of cyclin D1 and AZ remaining at each time point was assessed by co-immunoprecipitation, SDS-PAGE, and phosphorimager analysis.

Figure 6  Interaction of AZ with cyclin D1

a-b, Co-immunoprecipitation of antizyme with cyclin D1. $^{35}$S-labeled proteins were produced individually by in vitro translation in reticulocyte extracts. After translation was terminated, extracts containing different $^{35}$S-labeled proteins were mixed and incubated for 1 h. Complex formation was assessed by immunoprecipitation, SDS-PAGE, and fluorography (a). Antibody specificity of cyclin D1 and AZ antibodies was demonstrated
by co-immunoprecipitation experiments (described in a) using $^{35}$S-labeled cyclin D1 and AZ (b).

c, Total cell extracts from 293 cells transiently transfected with HA-cyclin D1 and FLAG-AZ constructs were used for immunoprecipitation using antibody against the FLAG epitope tag of AZ. Complex formation was assessed by immunoblot analysis using antibody against the HA epitope of cyclin D1.

d-e, Purified, His-tagged cyclin D1, His-tagged ODC, or CS was incubated with a 3-fold molar excess of GST or GST-AZ (d). His-tagged cyclin D1 was incubated with GST-AZ in the presence of increasing amounts of His-tagged ODC (e). Proteins bound to the glutathione resin were visualized by SDS/PAGE and immunoblot analysis using anti-6X His (d), anti CS (d) or anti-cyclin D1 antibody (e).

**Figure 7 Antizyme targets cyclin D1 for degradation in a purified system**

a, Antizyme and cyclin D1 were individually synthesized and in vitro transcription/translation in rabbit reticulocyte extracts. The reaction was stopped and equal amounts of the antizyme and $^{35}$S labeled cyclin D1 containing extracts were mixed and incubated with an ATP-regenerating system for 1.5 hours at 30°C. Samples were then analyzed by SDS-PAGE and fluorography. Experimental details as in figure 6a.

b-f, Purified His-tagged cyclin D1 (10 µg) was incubated with purified His-tagged antizyme (AZ; 10 µg) and purified proteasomes (20 µg) for 0, 2 and 4 h at 30°C (b), with proteasome alone (e) or with antizyme alone (d). e, as in b, but with proteasome inhibitor
Z-Ile-Glu-(Obut)-Ala-Leu-H-aldehyde (0.2 mM) added. f, apyrase (0.6 U) added. The amount of cyclin D1 remaining at the indicated time points was assessed by immunoblot analysis using an anti-cyclin D1 antibody.
Figure 1

(a) Spermine effects on time (h) of D1, ODC, A, cdk2, and actin.

(b) Spermine effects on 18S RNA.

(c) Time (h) effects on D1.

(d) Spermine effects on ppRb and pRb.
Figure 2

(a) morpholino spermine
- C C AZ AZ

(b) siRNA C AZ
AZ D1 cdk4 cyclin A

(c) siRNA C AZ
ODC AZ actin
Figure 4

|      | Vector | HA-D1 Wild-type | HA-D1 T286A |
|------|--------|-----------------|-------------|
| Spermine | -      | -               | -           |
|       | +      | +               | +           |

\(\alpha\)-AZ
\(\alpha\)-HA
\(\alpha\)-D1
\(\alpha\)-actin
Figure 5

(a) Western blot analysis of Vector and AZ samples at different time points (0, 10, 20, 40 minutes). The blots were probed with antibodies against AZ, D1/T286A, and Actin.

(b) Graph showing the percentage of D1(T286A) remaining over time. The graph compares AZ minus (open circles) and AZ plus (filled squares) conditions.
### Figure 6

**a**

| 35S-label     | D1+AZ          |
|---------------|----------------|
| IP antibody   | D1            |
| AZ            | D1            |

**b**

| 35S-label     | AZ | D1 | AZ | D1 |
|---------------|----|----|----|----|
| IP antibody   | AZ | D1 | D1 | AZ |

**c**

| lysate       | IP:αAZ |
|--------------|--------|
| D1+AZ        | D1+AZ  |
| D1           | D1     |

**d**

| 10% input    | GST | ODC | CS |
|--------------|-----|-----|----|
| D1           |     |     |    |

**e**

| no ODC | 2x ODC | 10x ODC |
|--------|--------|---------|
|        |        | D1      |

**WB:αD1**
Figure 7

(a) D1

AZ  -  +

(t = 0 h)

(t = 1.5 h)

(b) (c) (d) (e) (f)

% D1 Remaining

0 2 4

Time (h)
Addendum 1

Pulse-chase analysis of cyclin D1 in spermine-treated cells

AT2.1 cells were labeled with 35S methionine for 2h then treated with 10 mM spermine during the chase period. Lysates were prepared 0, 10, 20, 40, 60, and 120 min. after spermine addition and the amount of cyclin D1 remaining at each time point was assessed by co-immunoprecipitation, SDS-PAGE separation and phosphorimager analysis.
Antizyme targets cyclin D1 for degradation: A novel mechanism for cell growth repression
Ruchi M. Newman, Arian Mobascher, Ursula Mangold, Chieko Koike, Sri Diah, Marion Schmidt, Daniel Finley and Bruce R. Zetter

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