RESEARCH COMMUNICATION

Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells

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Antizyme, a spermidine-induced protein that binds and stimulates ornithine decarboxylase degradation, is now shown also to mediate the rapid feedback inhibition of polyamine uptake into mammalian cells. Using a cell line (HZ7) transfected with truncated antizyme cDNA, and mutant ornithine decarboxylase cell lines, we demonstrate that this newly discovered action of antizyme is distinct from its role in modulating polyamine biosynthesis.

INTRODUCTION

Mammalian tissues require substantial levels of the polyamines spermidine and spermine for normal cell growth and function. These can be synthesized from ornithine, through the intermediate putrescine, or, when available, they may be taken up from the cellular environment via a very efficient polyamine-transport system [1–3]. Since excessive polyamines are detrimental to normal cell function [4], both polyamine biosynthesis and transport are tightly feedback-regulated. Substantial progress has been made in understanding feedback repression of polyamine biosynthesis, which appears to centre around the polyamine-stimulated production of a labile protein, antizyme, that binds specifically and reversibly to the initial enzyme in polyamine synthesis, ornithine decarboxylase (ODC) [5–10]. Experiments *in vitro* have revealed that this ODC–antizyme complex is sensitive to rapid degradation by the 26 S proteasome [11,12]. In recent studies Murakami et al. [13] firmly established that the polyamine feedback on ODC activity in intact cells is indeed mediated by antizyme. These workers stably transfected a rat hepatoma (HTC) cell with an expression vector containing a truncated rat antizyme cDNA, Z1, under the control of a glucocorticoid-inducible promoter. Using this transfectant, HZ7, they modified cellular antizyme activity separately from any changes in exogenous polyamine levels, and clearly demonstrated that antizyme induces the rapid degradation of ODC in intact cells.

Much less is known about feedback regulation of the polyamine uptake system. Several investigators have shown that cells fed with spermidine or spermine will rapidly incorporate these until intracellular polyamine levels are slightly above normal (1–2 h), at which time uptake is abruptly terminated [14–17]. We [18] and others [19] have demonstrated that such attenuation of polyamine uptake could be prevented by the simultaneous inhibition of protein synthesis with cycloheximide. Further, transport activity that was lost in response to polyamine addition was regained within a few hours after cycloheximide addition [18]. These data suggested that elevations in cellular polyamine levels stimulate the production of a labile protein that reversibly inactivates the polyamine transporter. We have also reported that the feedback control of polyamine uptake did not function properly in a variant (DH23b) of the HTC cell line that was selected for overproduction of a stable form of ODC [20]. This suggested that the feedback response noted in polyamine transport may share a common intermediate with the response resulting in ODC instability. From these data we hypothesized that the unstable protein that is induced by spermidine and reversibly inactivates polyamine transport is actually antizyme. We now have tested this hypothesis by utilizing the Z1 antizyme-transfected cell line, HZ7. When dexamethasone was used to stimulate antizyme production, polyamine transport was rapidly and reversibly inhibited even under conditions of polyamine deprivation, thereby confirming a role of antizyme in the feedback control of polyamine transport. This establishes a second, and entirely distinct, function for this very unusual regulatory protein.

EXPERIMENTAL

Cell culture

Rat hepatoma (HTC) cells and their ODC-stable variant (HMOA) were grown in monolayer and suspension cultures in Swim's 77 medium containing 10% calf serum. The transfected clone, HZ7, was kindly provided by Dr. S. Matsufuji and Dr. S. Hayashi. As they have described previously [13], a truncated cDNA sequence of the rat liver antizyme gene was inserted downstream of the mouse mammary tumour virus long-terminal-repeat promoter of a pMAMneo vector (Clontech). HTC cells were transfected with this construct and one stable transfectant clone, HZ7, was selected for its abundant production of antizyme in response to dexamethasone. We maintained this line under the same conditions as the parental HTC cells, except for the presence of Geneticin (G418, 500 μg/ml). Transfectant controls were made by inserting the pMAMneo vector alone into HTC cells, by using electroporation (Electro Cell Manipulator 600; BTX Inc.). These clones were also maintained in the presence of Geneticin. In some cases cultures were partially deprived of polyamines by a 48 h exposure to the ODC inhibitor difluoromethylornithine (DFMO, Eflornithine; kindly provided by Marion Merrell Dow Research Institute). ODC-deficient Chinese-hamster ovary cells (C55.7), kindly provided by Dr. Immo Scheffler, were grown in the same media as the HTC cells but with 0.05 mM putrescine added, and 2.5% fetal-calf serum plus 2.5% calf serum.

Abbreviations used: ODC, ornithine decarboxylase; DFMO, difluoromethylornithine (Eflornithine).

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Assays of ODC and antizyme activities

Cell pellets were homogenized by brief sonication in 0.02 M potassium phosphate buffer (pH 7.0) containing 2.0 mM dithiothreitol and 0.2 mM EDTA. ODC activity was assayed by measuring the release of $^{14}CO_2$ from L-[^{14}C]ornithine as described previously [9]. One unit is defined as the release of 1 nmol of $^{14}CO_2$/h. Antizyme activity was estimated as described previously [9], and 1 unit of ODC-antizyme was defined as the amount inhibiting 1 unit of ODC. Total cell protein was measured by the method of Bradford [21].

Polyamine incorporation

The uptake of [^{14}C]polyamines in a 20 min period was measured as described previously [18], and kinetic studies were as detailed by Mitchell et al. [20]. Cell polyamine levels were determined by using a Mono-S (Pharmacia) ion-exchange column and post-column derivative formation as described previously [20].

Antibody preparations

Rabbit antibody to pure ODC was prepared as previously described [22], and the specificity was enhanced for immunoblot experiments by affinity purification using pure ODC bound to Affi-Gel 10 (Bio-Rad). Affinity-purified polyclonal rabbit antibody specific for antizyme was prepared by using an antizyme fusion protein expressed in bacteria. A *Nco*I linker (GATCTC-CATGGA) was constructed, self-hybridized and inserted into the *Bam*HI site of the multiple cloning site of the fusion-protein bacterial expression vector pGEX-3X (Pharmacia). Truncated cDNA coding for rat liver antizyme was obtained in a prokaryotic expression plasmid pTV-Z1NN2 from Dr. S. Matsufuji and Dr. S. Hayashi [23]. The pTV-Z1NN2 was cut with *Nco*I, and the fragment coding for antizyme was ligated into the *Nco*I site of the modified pGEX-3X vector. Direction was confirmed with *Pst*I, and the construct was used to transform *Escherichia coli* strain DH5α. In response to induction by isopropyl β-D-thiogalactoside, clones containing the pGEX-3X/AZ construct produced a fusion protein with a portion of glutathione transferase on the N-terminal end and over 90% of the antizyme sequence on the C-terminus. This fusion protein was purified by affinity to a 2 ml column of GSH–agarose (Sigma) followed by gradient elution from a Mono-Q (Pharmacia) ion-exchange column. The pure fusion protein was used to prepare polyclonal antibody as described previously [22], and immunoreactive rabbit serum was further purified by affinity to fusion protein bound to Affi-Gel 10 (Bio-Rad).

RESULTS AND DISCUSSION

In the absence of dexamethasone, the HZ7 transfected cell line appears to be quite similar to the parental HTC cell type. In particular we have observed that HZ7 cells grown in suspension culture for 48 h contain little antizyme activity (Figure 1a) and respond to the addition of fresh media with a transient increase in ODC activity, reaching a peak between 4 and 6 h (Figure 1b). Consistent with the work of Murakami et al. [13], the addition of 1 μM dexamethasone along with fresh medium induced a rapid increase in antizyme activity (Figure 1a), which resulted in the prevention of any measurable increase in ODC activity (Figure 1b). We evaluated polyamine uptake into these cells under the same induction conditions (Figure 1c). The addition of fresh media did cause a transient increase in the activity of the spermidine transporter, consistent with that generally observed in untransfected HTC cells. In the presence of dexamethasone, however, this increase in transport activity in the HZ7 cells was completely blocked, and the uptake velocity was actually decreased to less than half its initial value within 6 h of dexamethasone addition. By contrast, dexamethasone (up to 10 μM) does not affect spermidine transport in either the parental HTC cells or several clones of this cell that had been stably transfected with the pMAMneo vector alone as controls (results not shown). These results suggest that the Z1 antizyme induced by dexamethasone in HZ7 cells is a causative agent in the inactivation of spermidine transport.

The relationship between antizyme and polyamine transport was further established by studies using cells grown under polyamine-limiting conditions, which inhibit the formation of

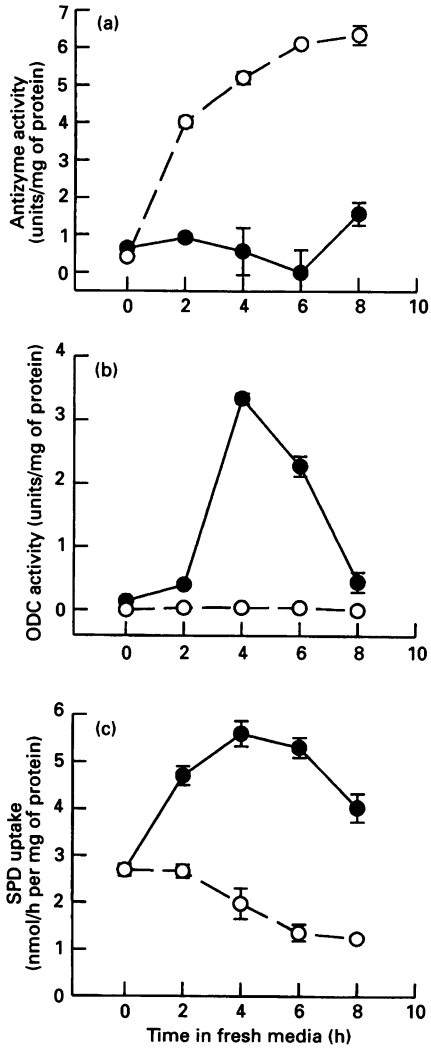

Figure 1 Effect of dexamethasone on antizyme and ODC activity and on polyamine transport in HZ7 cells

Cells of a 48 h suspension culture of the antizyme-gene-transfected clone HZ7 were resuspended in fresh growth media with (○) or without (●) addition of 1 μM dexamethasone. Samples were extracted from these cultures at 2 h intervals and assayed for antizyme activity (**a**), ODC activity (**b**) and spermidine (SPD) uptake (**c**), as described in the Experimental section. In all Figures the data points are means of triplicate assays, with S.D. indicated when it exceeds the size of the plotted symbol. Except where indicated, each experiment shown is representative of the pattern of response observed in three or more separate replicates of a procedure.

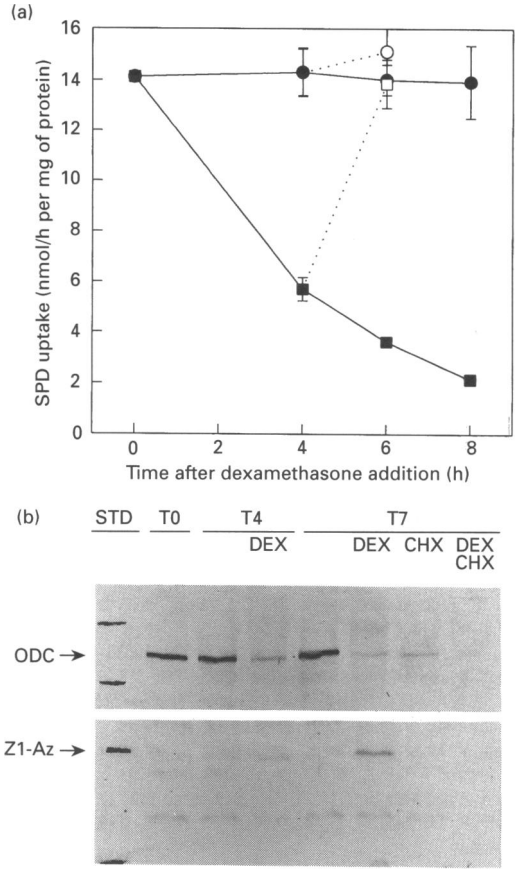

Figure 2 Effect of dexamethasone on HZ7 cells partially deprived of polyamines

A suspension culture of HZ7 cells was grown in the presence of 4 mM DFMO for 48 h and then divided into two flasks, one a control (●) and the other receiving 1 μM dexamethasone (■). After 4 h, cycloheximide (0.2 mM) was added to a portion of each culture (white symbols, dotted lines). (**a**) Cell samples were extracted as indicated and assayed for the velocity of spermidine (SPD) uptake as described in the Experimental section. (**b**) Samples were removed from the cultures at 0, 4 and 7 h, suspended in SDS sample buffer, and applied to a SDS/10%-polyacrylamide gel; 100 μg of protein/lane was used. Protein bands were transferred to a nitrocellulose filter (Hoefer), stained with Ponceau S to reveal the molecular-mass standards (STD) and cut horizontally between the 30 and 45 kDa markers. After blocking with 5% non-fat dried milk, the upper portion was exposed to affinity-purified antibody to ODC and the lower to antibody specific to antizyme. Bands were revealed by Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad). The Z1-antizyme (Z1-Az) runs slightly faster than the 30 kDa standard. The lanes are indicated by time (h; T0, T4 and T7) and by additions to cultures (DEX, dexamethasone; CHX, cycloheximide).

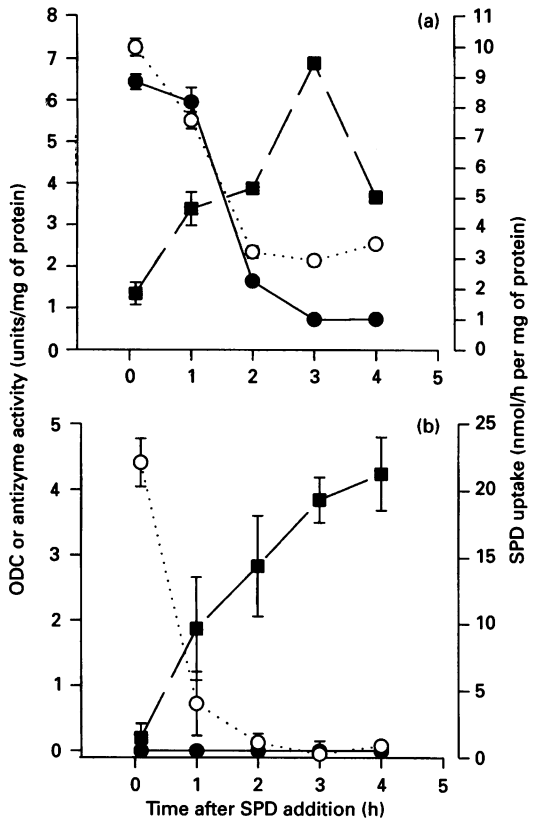

Figure 3 Response of HMOA and C55.7 cells to spermidine addition

(**a**) HMOA cells were suspended in fresh media to induce ODC activity. After 4 h, spermidine (50 μM) was added and samples were subsequently withdrawn and assayed for antizyme (■) and ODC (●) activity, spermidine (SPD) uptake (○), and immunoblot detection of antizyme and ODC protein (results not shown) as described in Figures 1 and 2. (**b**) Suspension cultures of ODC-deficient Chinese-hamster ovary cells (C55.7) were grown in media without putrescine for 48 h to induce a partial polyamine deficiency. Spermidine was then added, and samples were withdrawn and analysed as described in (**a**). Data points in this panel are means ± S.D. of assays combined from duplicate experiments done on separate days.

spermidine-induced antizyme from the native cellular gene. In the experiment shown in Figure 2, HZ7 cells were treated with an irreversible inhibitor of ODC, DFMO (4 mM) for 2 days before testing. This pretreatment decreased putrescine and spermidine levels by over 80% and induced a 5–6-fold increase in the rate of spermidine incorporation into these cells (cf. Figures 1c and 2a). In previous studies with HTC cells, we have shown that this transport increase results partially from the activation of existing transporter protein and partially from the appearance of newly synthesized transporter [18]. Addition of dexamethasone to HZ7 cells containing such induced transport activity resulted in an 85% decrease in this activity within 8 h (Figure 2a). Furthermore, the addition of cycloheximide 4 h after dexamethasone completely reversed the dexamethasone-induced inhibition of transport that had already occurred.

The presence of DFMO in the experiment of Figure 2 precludes the analysis of ODC and antizyme activity in these samples. Instead, changes in the levels of both ODC and antizyme protein were observed by immunoblot analysis as shown in Figure 2(b). A protein band that cross-reacted with antibody specific to antizyme was noted to appear 4 h after dexamethasone addition and to increase in intensity with an additional 3 h treatment by this inducer. We identified this band as Z1 antizyme because of its co-migration with Z1 antizyme produced both by translation *in vitro* and by bacterial expression. Consistent with the known instability of antizyme [5,6], the Z1 band was not found in the 7 h samples of dexamethasone-treated cells that received cycloheximide at 4 h (last lane, Figure 2b). ODC protein levels were visibly diminished in the samples of HZ7 cells exposed to dexamethasone or cycloheximide, and were almost undetectable in samples of cultures receiving both treatments.

The above studies with HZ7 cells show that rapid modulation of polyamine transport is mediated by antizyme and not by a direct effect of cellular polyamines. The following experiments demonstrate that the response of polyamine transport to antizyme is completely independent of any changes in ODC level, and therefore distinct from the function of antizyme that has been reported previously. In the first study (Figure 3a), a sub-line of

HTC cells (HMOA) was used in which a mutant ODC is produced that is quite stable even in the presence of antizyme [24,25]. When spermidine (50 μM) was added to cultures of these cells, antizyme was induced and ODC activity declined by over 90%, yet the elevated levels of immunologically detected ODC protein did not decrease appreciably within 3 h. Despite the continued presence of ODC protein, polyamine transport was inhibited by about 70% in response to polyamine addition. As shown in Figure 3(b), even in the absence of ODC protein the polyamine transporter was very effectively inhibited upon antizyme induction by spermidine addition. The Chinese-hamster ovary cells used in this study, C55.7, contain an altered ODC gene and reportedly produce only very low levels of immunologically detectable ODC protein [26,27]. The appearance of antizyme protein consistent with the increase in antizyme activity, and the absence of ODC protein in this experiment, were confirmed (results not shown) by immunodetection as described in Figure 2(b). Although antizyme is known to diminish rapidly the cellular level of ODC, it is clear from these studies that this interaction with ODC is not necessary for the inhibition of polyamine-transport activity by antizyme.

Although the experiments shown above analysed spermidine uptake velocity, the decrease in putrescine transport activity resulting from a 4 h exposure of HZ7 cells to dexamethasone was found to be identical with that of spermidine. Further, we have analysed the kinetics of spermidine transport before and after this decrease, and found no measurable difference in substrate affinity. Although others have reported the possibility of multiple polyamine transporters with varied specificity in mammalian cells [2,3], our present and past studies [18,20] are consistent with a common polyamine transporter in this cell. If significant levels of more than one polyamine transporter exist in HTC cells, then they all appear to respond equally to the increase in cellular antizyme levels.

The mechanism of antizyme's inhibition of polyamine transport is unknown. This spermidine-induced regulatory protein could bind directly to the transporter or one of its subunits, or it may act through one or more unspecified intermediates. Since the mammalian cell polyamine transporter has not been isolated or identified as yet, it is not possible at present to test whether antizyme has any specific affinity for this protein. However, early studies on cellular distribution of antizyme found a substantial portion of the antizyme activity in unstimulated mammalian cells to be membrane-associated [28]. If antizyme does have a direct affinity for the polyamine transporter as well as ODC, then it would appear that the dynamics of this partitioning of a labile regulatory protein (antizyme) between interactions with a stable membrane protein (transporter) and a labile cytoplasmic enzyme (ODC) would be quite complex. Further, the binding of antizyme to ODC is thought to alter the structure of ODC sufficiently to expose a region near the C-terminal end that stimulates degradation by the 26 S proteasome [10,11,29]. If antizyme also binds a component of the polyamine transporter, then it will be of interest to ascertain whether this interaction has any effect on the half-life of this protein that has previously been shown to be rather stable [17].

Although it is not yet known how antizyme reversibly depresses polyamine transport, the efficiency of having one spermidine-induced factor co-ordinate the modulation of two entirely distinct mechanisms of cellular polyamine accumulation can be easily appreciated.

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