Forced expression of antizyme abolishes ornithine decarboxylase activity, suppresses cellular levels of polyamines and inhibits cell growth

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Ornithine decarboxylase (ODC) is a key enzyme in polyamine biosynthesis. It is a short-lived protein and negatively regulated by its products, polyamines. Its degradation is accelerated by the binding of antizyme, an ODC-inhibitory protein induced by polyamines. To evaluate the physiological importance of antizyme we examined the effect of forced expression of antizyme on cellular ODC and polyamine levels and cell growth. Antizyme almost completely abolished the induction of ODC by growth

stimuli. This may have been caused by antizyme-induced rapid degradation of newly synthesized ODC, since the half-life of ODC complexed with antizyme was less than 5 min. Forced expression of antizyme caused reductions of cellular putrescine and spermidine levels, and inhibited cell growth, which was partially restored by the addition of putrescine. These observations suggested a critically important role of antizyme in polyamine metabolism.

INTRODUCTION

Polyamines are indispensable for animal cell growth [1–3]. Ornithine decarboxylase (ODC), the initial and key enzyme in polyamine biosynthesis, has a very short half-life of 10–90 min [4,5]. The induction of ODC is a prerequisite for cell proliferation [6,7], and has recently been shown to be critical for cell transformation [8]. On the other hand, ODC activity is down-regulated by increased cellular polyamines. The polyamine-induced down-regulation has been thought to include inhibition of translation, acceleration of ODC degradation and induction of an ODC-inhibitory protein, antizyme [3,9,10]. Antizyme binds to ODC with high affinity, forming an inactive complex [11–13]. Forced expression of the antizyme gene elicited destabilization of ODC in cultured cells [14], and antizyme markedly accelerated ATP-dependent ODC degradation *in vitro* [15–18]. On the other hand, specific binding of antizyme to ODC is required for polyamine-regulated degradation of ODC [19]. These findings indicate that antizyme mediates polyamine function by feedback regulation of ODC degradation. To what extent does antizyme affect cellular levels of ODC and polyamines? To evaluate the physiological importance of antizyme we examined the effects of forced expression of antizyme on cellular levels of ODC and polyamines and on cell growth.

EXPERIMENTAL

Cell culture, ODC induction, determinations of ODC activity, antizyme activity, ODC–antizyme complex and polyamines, and materials

HZ7 cells, transfectants having antizyme cDNA driven by a glucocorticoid-inducible promoter, and its control HC4 cells were cultured as described previously [14]. ODC was induced by changing culture medium for fresh medium [14]. ODC activity, antizyme activity and polyamine concentrations were determined as described previously [20]. The amount of ODC–antizyme complex was determined as the increase in ODC activity caused by an addition of an excess amount of antizyme inhibitor, which specifically binds to antizyme with high affinity and thus can release active ODC from the complex [20]. Total ODC (free

ODC plus ODC complexed with antizyme) was determined by measuring ODC activity in the presence of antizyme inhibitor. α -Difluoromethylornithine (DFMO) was a generous gift of Merrell Dow Research Institute (Cincinnati, OH, U.S.A.).

Determination of the rate of synthesis of ODC protein in cells

The apparent rate of synthesis of ODC protein was determined by measuring the incorporation of [35 S]methionine into the enzyme. Cells were cultured in fresh medium with or without dexamethasone (1 μ M) for 2.5 h. Cells were then washed with warm fresh medium containing the same components except for the absence of L-methionine and L-cysteine, and incubated for 10 min in the same medium with or without dexamethasone. Then a mixture of L-[35 S]methionine and L-[35 S]cysteine (Expre 35 S 35 S, Dupont–New England Nuclear) was added (25 μ Ci/ml) to the medium and after 10 min of incubation cells were harvested. Labelled ODC in cell extracts was immunoprecipitated and analysed as described previously [21]. Radioactivity of ODC bands was determined by a laser-image analysing system (FUJIX BAS 2000) and the relative values are shown as photo-stimulated luminescence (PSL).

Synthesis of ODC in a reticulocyte lysate

Plasmid pODC20.7 containing mouse ODC cDNA [22] was kindly supplied by P. Coffino (University of California, San Francisco, CA, U.S.A.) and pODC24 plasmids [23] derived from pODC20.7 were kindly supplied by K. Igarashi (Chiba University, Chiba, Japan). Capped transcript was synthesized with SP6 RNA polymerase from pODC24 which had been linearized with *Bam*H1 [23]. Translation was performed in a total volume of 15 μ l containing 25 μ Ci of [35 S]methionine (1000 Ci/mmol), 10.5 μ l of rabbit reticulocyte lysate with translation mixture (Wako Chemicals), RNasin (1 unit/ μ l) and 0.2 μ g of mRNA with 1 μ l of recombinant antizyme (41 units) or vehicle. The antizyme was prepared as described previously [24] and dialysed against 20 mM Hepes/KOH buffer containing 1 mM dithiothreitol, pH 7.6.

Selection of antizyme- and ODC-overproducing variant cells

Antizyme-overproducing Chinese hamster ovary (CHO) cells were isolated from an ODC-overproducing CHO cell line, DF3 (kindly supplied by I. E. Scheffler [25]), using the suicide selection method introduced by Steglich and Scheffler [26]. For mutagenesis, the parental DF3 cells were treated with ethylmethane sulphate (440 $\mu\text{g/ml}$) for 2 days. After being washed, the cells were cultured for 3 days and reseeded at 1×10^5 cells/100 mm diam. plate. Thereafter, the cells were cultured for 4.5 h in the presence of putrescine (10 mM) for induction of antizyme. Then the cells were cultured for 4 h in fresh medium without putrescine for induction of ODC. Finally, cells were cultured in the presence of [^3H]ornithine (5 $\mu\text{Ci/ml}$) for 4 h. A plate was then washed and stored at -70°C for 2 weeks as described by Steglich and Scheffler. After the second round of [^3H]ornithine suicide, the survivors were plated in microwells at 1 cell/well in growth medium [Dulbecco's modified Eagle's medium with 2% (v/v) fetal-calf serum, 4% (v/v) newborn-calf serum and non-essential amino acid mixture]. Antizyme activity of cells was examined after incubation with putrescine (10 mM) for 6.5 h. Several cell lines showed specific antizyme activity of about 100 units/mg of protein. Although the specific activity was only 3–5-fold of that in parent cells, it was enough for our purpose, and the 6F cell line was selected for further use.

RESULTS AND DISCUSSION

Effect of forced expression of antizyme on ODC induction

ODC is known to be induced by various growth stimuli including change of culture medium. During the process of ODC induction both the synthesis rate and the degradation rate change, often causing a pulse-like increase of ODC activity [21]. To examine the effect of antizyme on ODC induction we replaced culture medium of HZ7 cells with fresh medium with or without dexamethasone. Previously we confirmed that dexamethasone increased the amount of antizyme mRNA (Z1 mRNA) and induced active antizyme in HZ7 cells [14]. As shown in Figure 1(b), dexamethasone completely inhibited the increase of ODC activity induced by medium change and suppressed pre-existent ODC activity in HZ7 cells. Similar effects of dexamethasone were observed on total levels of ODC (sum of free ODC and inactive ODC complexed with antizyme). Such effects of dexamethasone were not observed in control HC4 cells (Figure 1a). Half-lives of total ODC of HZ7 cells were determined at indicated times. As expected from previous results [14], dexamethasone shortened the half-lives of ODC at all time points (parentheses in Figure 1b). These results indicated the important contribution of ODC degradation and its regulation by antizyme in determining the cellular level of ODC. So did antizyme affect only ODC degradation? In the steady state, total ODC activity differed 25-fold in the presence and absence of dexamethasone, whereas the half-life of ODC changed by approx. 6-fold. This suggested that antizyme also affected ODC synthesis. We therefore examined the effect of antizyme expression on ODC synthesis.

Effect of antizyme on ODC synthesis

To induce ODC activity, cells were fed fresh medium with or without dexamethasone for 2.5 h. Then incorporation of [^3S]methionine into ODC in the cells was measured for a labelling period of 10 min as described in the Experimental section. Dexamethasone inhibited the incorporation of labelled methionine into ODC protein in HZ7 cells but not in control cells (Table 1). This result suggested the direct inhibition of ODC

Figure 1 Effect of dexamethasone on ODC induction by change of medium

The growth medium of about 50%-confluent cells was replaced with fresh medium with or without dexamethasone (1 μM) to induce ODC, and cells were harvested at the indicated times thereafter. Cytosolic extracts were prepared and assayed for ODC activity. The ODC activity and total ODC content (free ODC plus ODC-antizyme complex) were determined in the absence and presence of antizyme inhibitor respectively. (a) HC4 cells; (b) HZ7 cells. Symbols: circles, no addition; triangles, dexamethasone was added when medium was changed. Open symbols show free ODC and closed symbols show total ODC. Figures in parentheses show half-lives (in min) of total ODC determined at the indicated times. To determine the half-life of total ODC, cycloheximide (50 $\mu\text{g/ml}$) was added to three dishes at the indicated times and harvested 0, 30, and 60 min later and cytosolic extracts were assayed for ODC activity in the presence of antizyme inhibitor. Data were the results of a single experiment. Essentially similar results were obtained in four other experiments.

synthesis by antizyme. We, however, could not exclude the possibility that the inhibition is indirect, owing to accelerated degradation of newly synthesized ODC by antizyme. If direct inhibition is the case, antizyme is likely to inhibit translation but not transcription, since polyamines are known not to affect the cellular level of ODC mRNA [27–31]. Therefore, we examined whether antizyme inhibits translation of ODC mRNA in a reticulocyte lysate. We used the 5' truncated mODC24 [23] which

Table 1 Effect of dexamethasone on incorporation of [35 S]methionine into ODC protein (mean $\pm$ S.D., $n = 3$)

Cells were cultured in fresh medium with or without dexamethasone (Dex) ($1 \mu\text{M}$) for 2.5 h and then exposed to [35 S]methionine for 10 min. The radioactivities in ODC and soluble protein were determined. Results are means $\pm$ S.D. for three dishes

Cell line	Addition	^{35}S incorporation		Rate of ODC synthesis (%)
		ODC (c.p.m./mg of protein)	Total protein (10^4 c.p.m./mg)	
HC4	None	232 $\pm$ 44	273 $\pm$ 30	0.0085 $\pm$ 0.0013
HC4	Dex	270 $\pm$ 41	250 $\pm$ 12	0.0109 $\pm$ 0.0020
HZ7	None	193 $\pm$ 68	307 $\pm$ 51	0.0062 $\pm$ 0.0014
HZ7	Dex	64 $\pm$ 5	261 $\pm$ 12	0.0025 $\pm$ 0.0001

Figure 2 Half-life of complexed ODC in CHO 6F cells

CHO 6F cells were cultured for 4 h in the presence of 10 mM putrescine to induce antizyme. The cells were washed with warm fresh medium without putrescine and cultured in the fresh medium for the indicated time to induce ODC, and then cycloheximide ($50 \mu\text{g/ml}$) was added. The cells were harvested at 0, 5, 10, 15 min or 0, 10, 20, and 30 min later. Cytosolic extracts were assayed for antizyme activity (O) and ODC activity in the presence (▲) and absence (Δ) of antizyme inhibitor. Half-lives (in min) of total ODC and free ODC at indicated times are shown in parentheses. Data are the results of a single experiment. Essentially similar results were obtained in two more experiments.

contained 24-bp 5'-untranslated region (UTR), the coding region and the 3' UTR of the natural ODC mRNA, since truncation of 5' UTR increases translation efficiency of ODC mRNA [23,32]. Antizyme did not affect translation of ODC mRNA *in vitro*. Relative incorporation of ^{35}S into ODC protein during translation in the reticulocyte lysate with or without antizyme was 35 ± 4 and 36 ± 2 (mean $\pm$ S.D., $n = 3$) respectively. This result suggested that previous inhibition of incorporation of labelled methionine into ODC (Table 1) was not attributed to translational inhibition of ODC synthesis but to accelerated degradation of newly synthesized ODC. The labelling period of 10 min was almost the same as the half-life of total ODC in dexamethasone-treated cells (Figure 1). Therefore, if ODC degradation is random, the incorporation of [35 S]methionine into ODC molecules should reflect closely the true rate of synthesis.

Figure 3 Effects of dexamethasone on cell growth

(a) HZ7 cells were plated in 35-mm-diam. dishes at 5.4×10^4 cells/dish and cultured with no additive (Δ) or with addition of $1 \mu\text{M}$ dexamethasone ($\blacktriangle$). Four days later the cells were washed and cultured with fresh medium with ($\square$) or without (Δ , $\blacktriangle$) $1 \mu\text{M}$ dexamethasone. (b) HZ7 cells were plated in 35-mm-diam. dishes at 4.3×10^4 cells and cultured with no additive (Δ), $1 \mu\text{M}$ dexamethasone ($\square$), $5 \mu\text{M}$ DFMO ($\circ$), dexamethasone plus $0.5 \mu\text{M}$ putrescine ($\blacksquare$) or DFMO plus putrescine ($\bullet$). The growth medium was changed with each fresh medium 3 days after the plating. Data are the results of a single experiment. Essentially similar results were obtained in two more experiments.

However, antizyme prefers to bind to newly synthesized ODC owing to its monomer structure [33] rather than to pre-existing ODC; the former ODC is likely to be immediately trapped by antizyme under the experimental conditions, where antizyme is continuously synthesized owing to the presence of dexamethasone. Thus the half-life of newly synthesized ODC may be as short as that of ODC complexed with antizyme.

Half-life of ODC complexed with antizyme

The half-life of ODC complexed with antizyme can be determined under conditions where all ODC molecules are bound with antizyme. Thus it is equal to the half-life of the ODC-antizyme complex determined in the presence of an excess amount of antizyme compared with ODC. To determine the half-life precisely, we tried to isolate cells which overproduce both ODC and antizyme by using an enrichment-by-suicide technique developed for the isolation of an ODC-deficient mutant [26]: the mutant was selected from a mutagenized population of ODC-overproducing CHO cells by putrescine pretreatment followed by a suicide enrichment with [^3H]ornithine. Parental ODC-overproducing mutant CHO cells (DF3) could be killed efficiently by [^3H]ornithine. If these cells had been pretreated with polyamines, antizyme-overproducing cells among them could inactivate ODC and would presumably be spared from radiation suicide. We obtained cells (6F) which produced antizyme at approx. 5-fold of the level of untreated cells and kept the ODC-overproducing ability. Antizyme was induced in 6F cells by putrescine, and then ODC was induced by fresh medium lacking putrescine. Half-lives of ODC were determined at indicated times. When antizyme remained in excess relative to ODC, the half-life of the ODC-antizyme complex, namely the half-life of ODC complexed with antizyme, was very short (shorter than 5 min), as expected (Figure 2). These results indicated that the suppression of incorporation of labelled methionine into ODC protein (Table 1) may be explained by acceleration of the degradation of newly synthesized ODC, suggesting a critically important role for

Figure 4 Effect of dexamethasone on cellular polyamine levels

Cellular polyamine levels were determined in HZ7 cells which were cultured in the experiment shown in Figure 3(b). Symbols are the same as in Figure 3. (a) Putrescine, (b) spermidine, (c) spermine. Data are from a single experiment. Essentially similar results were obtained in two more experiments.

antizyme in the down-regulation of ODC. This conclusion is consistent with recent negation of translational regulation by polyamines [34].

Effects of forced expression of antizyme on cell growth and polyamine levels

Polyamines are essential for cell growth. Therefore if the contribution of antizyme is important in the regulation of whole polyamine metabolism, its expression should affect cellular polyamine levels and cell growth. HZ7 and HC4 cells were cultured in the presence and absence of dexamethasone. DFMO, an irreversible inhibitor of ODC, was also added for comparison. Dexamethasone did not affect the growth of HC4 cells, but inhibited that of HZ7 cells. The growth inhibition was completely reversed by withdrawal of dexamethasone (Figure 3a), but it was only partially recovered by simultaneous addition of putrescine, which was in contrast with the growth inhibition caused by DFMO (Figure 3b). Similar results were obtained with other antizyme transfectants, HZ3 or HZ8 cells. Cellular polyamine levels were examined in HZ7 cells (Figure 4). Dexamethasone depleted putrescine levels, severely decreased spermidine, but did not affect spermine levels. Simultaneous addition of putrescine recovered the cellular putrescine level completely and the spermidine level partially. In contrast, simultaneous addition of putrescine completely recovered both putrescine and spermidine levels suppressed by DFMO. Thus the effects of forced expression of antizyme on cell growth and polyamine levels were not the same as, though similar to, that of DFMO, a potential cancer chemotherapeutic agent [35].

Recently we showed that ODC is degraded ATP- and antizyme-dependently and ubiquitin-independently by 26S proteasome [16], which is widely viewed as a major ATP-dependent protease specific for ubiquitin-conjugated proteins [36]. The protease is known to be abundant in cells. Since ubiquitination is unnecessary for ODC degradation and binding between ODC and antizyme is instantaneous, the rate-limiting factor for rapid ODC degradation may be the availability of antizyme. Consistent with this, forced expression of antizyme caused ODC degradation,

resulting in depletion of ODC (Figure 1), putrescine and spermidine in cells and inhibition of cell growth (Figure 3). This suggested possible development of anti-proliferative drugs based on acceleration of ODC degradation. For this, it is important to examine further whether or not the role of antizyme is restricted to acceleration of ODC degradation. In this connection, it is interesting to note that antizyme may be an inhibitory regulator of the polyamine-transport system [37].

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