Overproduction of stable ornithine decarboxylase and antizyme in the difluoromethylornithine-resistant cell line DH23b

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DH23b cells, a variant of the HTC line selected for their resistance to difluoromethylornithine, exhibit defective feedback regulation of ornithine decarboxylase (ODC) stability and polyamine transport, and accumulate ODC protein to > 1000 times normal concentrations. The components of the polyamine feedback regulation system have been examined in an attempt to understand these unusual responses. Southern-blot analysis revealed an amplification (approx. 10-fold) in ODC DNA sequence without any concomitant increase in antizyme. Moreover, the amplified ODC sequence contains a single base substitution that results in the conversion of Cys-441 into Trp. This modification has previously been shown to cause ODC stability in HMOA cells. Although antizyme activity has not been noted in DH23b cells, Western-blot analysis revealed the accumulation of antizyme protein to > 50 times that induced in parental HTC cells. This increase is consistent with a 6–9-fold increase in the half-life of antizyme in these cells, a consequence of the inability of the mutant ODC–antizyme complex to be degraded by 26 S proteasome. Associated with the stabilization of antizyme in both DH23b and HMOA cells is the appearance of two additional forms of antizyme protein with apparent molecular masses of 22 and 18.5 kDa. It is suggested that these result from proteolytic removal of discrete fragments from the N-terminal end of antizyme, perhaps an indication of an initial step in rapid antizyme turnover.

INTRODUCTION

In mammalian cells ornithine decarboxylase (ODC; EC 4.1.1.17), the initial enzyme in polyamine biosynthesis, is very tightly regulated at the transcriptional, translational and post-translational levels [1,2]. Most striking is the very rapid degradation of existing ODC protein in response to elevations in the cellular concentrations of spermidine or spermine. Recent studies have shown a unique mechanism whereby these higher polyamines stimulate translation of a regulatory protein, antizyme, that specifically binds ODC and facilitates its degradation by the 26 S proteasome ([3,4]; for a review, see [2]). Relatively little is known, however, about the interaction between antizyme and ODC, and how this interaction stimulates ODC degradation.

Antizyme has an extremely high affinity for ODC, and two regions of the ODC protein have been identified as being essential for this interaction [5–7]. Further, the antizyme protein has distinct sites for ODC binding and destabilization [8,9]. Li and Coffino [10] suggested a model, based on antibody recognition studies, in which the binding of antizyme to ODC induces exposure of the C-terminal end of the ODC protein, which then may serve as a target recognized by the 26 S proteasome. It is not clear what happens to the antizyme in this process. Tokunaga et al. [11] showed that antizyme does not need to be degraded in order to facilitate ODC proteolysis; however, others [2,12] have presented evidence suggesting that some antizyme may be lost in the process, which is consistent with early work showing that antizyme is a labile cellular protein [13]. The fate of antizyme during ODC degradation is of enhanced importance because we and others have recently shown that this regulatory protein is also responsible for the rapid and reversible modification of the polyamine transport system [14,15].

One approach to increasing our understanding of the mechanism of rapid degradation of ODC in response to elevations in cellular polyamines is to examine variant cell lines in which this process is disrupted. Miyazaki et al. [16] performed such a study in HMOA cells, in which the half-life of ODC is reported to be 10-fold greater than that in the parental HTC cell line. In these HMOA cells the antizyme sequence was found to be unaltered, and the antizyme protein was equivalent to the wild-type in its ability to stimulate normal ODC degradation. These workers demonstrated that the inability of antizyme to induce rapid degradation of HMOA cell ODC was associated with a change near the C-terminal end of the ODC protein itself, a single amino acid replacement, Cys-441 to Trp.

Previously we reported the isolation and characterization of an ODC-overproducing cell variant, DH23b, in which ODC levels were also not diminished by elevations in intracellular polyamines [17,18]. Unlike HMOA cells, which were sensitive to a concentration of 0.1 mM of the irreversible inhibitor α-difluoromethylornithine (DFMO), the DH23b cells grew best in levels of this ODC inhibitor exceeding 10 mM. The half-life of ODC was estimated to be somewhat greater than that in HMOA cells, but the major difference noted was the accumulation of approx. 1000 times the normal level of ODC protein. It is not at all clear why the accumulation of stable ODC should confer resistance to this inhibitor, as virtually all of this enzyme would be immediately inactivated by the DFMO. Moreover, a mere 10-fold increase in ODC synthesis is definitely not sufficient to allow growth in a medium containing 10 mM DFMO. These cells were also found to lack any apparent feedback control of polyamine uptake [18], a response that we have shown is normally mediated by antizyme. In the studies reported here we have investigated the mechanism of ODC overproduction and stability in DH23b cells, and the apparent absence of antizyme activity. We also report the discovery of greatly elevated levels of antizyme protein, and at least two additional antizyme forms, in this variant cell line. These abnormalities increase our understanding of the intricacies of the mechanisms controlling cellular polyamine levels.

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

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EXPERIMENTAL

Cell culture

Rat hepatomal (HTC) cells and their ODC-stable variant (HMOA) were grown in monolayer and suspension cultures in Swim's 77 medium containing 10 % (v/v) calf serum as previously described [14]. DH23b cells were selected from HTC cells as described previously [17] and cultures maintained as for the parental line, except for the presence of 10 mM DFMO (Eflornithine; kindly provided by the Marion Merrell Dow Research Institute, Cincinnati, OH, U.S.A.) in the medium. HZ7 cells were kindly provided by Dr. S. Matsufuji and Dr. S. Hayashi (Jikei University School of Medicine, Tokyo, Japan), and contain a truncated cDNA sequence of the rat liver antizyme gene inserted downstream of the mouse mammary tumour virus long-terminal-repeat promoter of a pMAMneo vector (Clontech) [19]. We maintained this line under the same conditions as for the parental HTC cells, except for the presence of Geneticin (G418; 400 μg/ml).

Southern-blot analyses

Genomic DNA was extracted from cultured cells by guanidine thiocyanate/2-methylpropan-1-ol fractionation. Samples of DNA (20 μg) were digested with the indicated restriction endonucleases, fractionated by electrophoresis in 0.9 % agarose for 20 h, transferred to a Zeta-probe blotting membrane (Bio-Rad) by capillary transfer and fixed with UV light. According to the manufacturer's instructions, the blot was prehybridized for 5 min at 65 °C in 1 mM EDTA, 0.25 M Na_2HPO_4 (pH 7.2) and 7 % SDS, and hybridized with radiolabelled probe with 10 % (w/v) poly(ethylene glycol) (8000 Da) as an enhancer for 24 h at 65 °C. The blots were washed twice at 65 °C for 60 min in 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2) and 5 % SDS, followed by two washes with 1 mM EDTA, 40 mM Na_2HPO_4 (pH 7.2) and 1 % SDS at 65 °C for 60 min.

The ODC probe was a gel-purified 1409 bp *Bam*HI fragment containing the coding region from pSVL-ODC kindly supplied by Dr. R. Autelli (University di Torino, Torino, Italy) [20]. DNA (25 ng) was labelled by the Random Primers DNA Labeling System, using the conditions recommended by the supplier (Gibco-BRL). A probe for the antizyme sequence was prepared in a similar manner using an 820 bp *Hin*dIII/*Xba*I fragment from pBluescript II sk-AZ plasmid. Z1-antizyme sequence obtained from Drs. Matsufuji and Hayashi [21] was cut from the pTV-Z1NN2 plasmid using *Nco*I and inserted into the *Bam*HI site of pBluescript II sk using a linker.

PCR enhancement of ODC sequences

Genomic DNA was isolated using proteinase K and phenol/chloroform extraction. A sample of 0.02 μg of genomic DNA was amplified by *Taq* polymerase (Promega), using 0.09 μg of the following primers: sense, 5′ GTTAGCATATGTGACCTGTGGGTTC 3′; antisense, 5′ GCAGGTAAGAGCTACAAGAATGGCA 3′. Following precipitation with ethanol, the PCR products were digested with *Apa*I and fractionated on agarose gel as indicated in Figure legends. Some preparations of the PCR-amplified genomic ODC sequences were cloned into a pGemT vector and the sequences of several of these were determined using a USB Sequenase 2.0 kit.

RNA was prepared from DH23b and HTC cells using guanidinium thiocyanate, and mRNA was isolated using oligo(dT)–cellulose. cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase (Gibco-BRL). Primers were constructed to allow PCR enhancement of 778 bases of the 3′ end of the ODC coding region using *Taq* polymerase (Promega). Some of the reverse transcription-PCR product was digested directly with *Apa*I, as indicated in the text.

Antibody preparation for Western-blot analysis

Affinity-purified polyclonal rabbit antibody specific for antizyme was prepared as described previously by using an antizyme fusion protein (pGEX-AZ) expressed in bacteria [14].

In vitro transcription and translation

pBluescript II sk-AZ was linearized by *Acc*I or *Hin*dIII and transcribed by T3 RNA polymerase (Promega) for truncated and intact antizyme mRNAs respectively. ^{35}S-labelled truncated and intact antizyme proteins were produced *in vitro* in a rabbit reticulocyte lysate (Promega) using the *in vitro* transcribed RNAs, and analysed by SDS/PAGE followed by Western blotting and autoradiography.

RESULTS

The initial experiments were designed to explain three, possibly interrelated, unusual aspects of DH23b cells that were noted previously: (1) overexpression of the ODC gene; (2) stability of the ODC protein; and (3) the apparent absence of antizyme activity [14,17,18].

ODC and antizyme gene expression in DH23b cells

Overexpression of the ODC gene was observed in [^{35}S]methionine labelling experiments, where the rate of ODC synthesis in DH23b cells was found to be about 10-fold greater than that in parental HTC cells [17]. To understand the basis for this increase in ODC expression, DNA was extracted from the DH23b and HTC cells, digested with restriction enzymes, separated on agarose gels, and the resulting blots probed with a rat ODC cDNA sequence. As shown in Figure 1(A), fragments cut with either *Eco*RI or

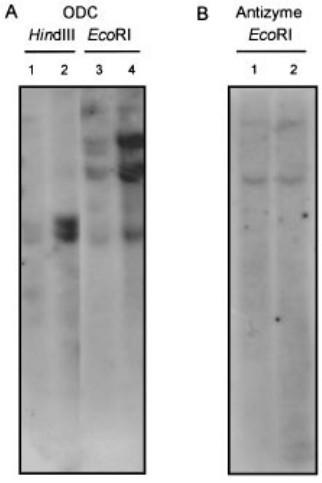

Figure 1 Southern-blot analysis of ODC and antizyme gene abundance in DH23b cells

(**A**) Equal amounts of genomic DNA from HTC (lanes 1 and 3) and DH23b (lanes 2 and 4) cells were digested with *Hin*dIII (lanes 1 and 2) or *Eco*RI (lanes 3 and 4) and probed with rat ODC cDNA as indicated in the Experimental section. (**B**) The DNA preparations cut with *Eco*RI in (**A**) were also size fractionated and probed with a *Hin*dIII/*Xba*I antizyme fragment from Z1 antizyme (lane 1, HTC; lane 2, DH23b).

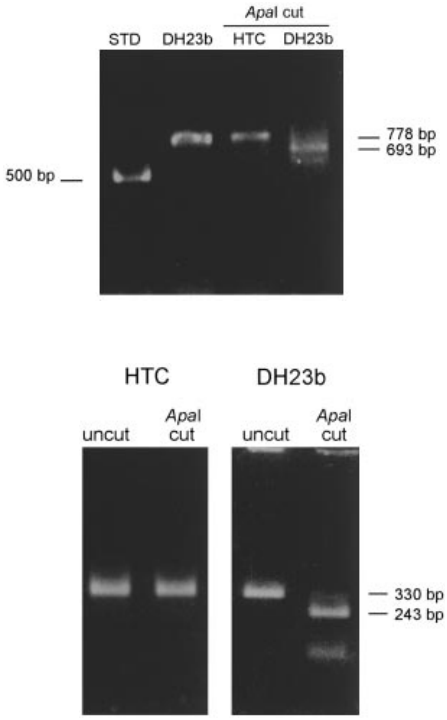

Figure 2 *Apa*I digests of amplified ODC genomic and cDNA from HTC and DH23b cells

Upper panel: the ODC cDNA sequence coding for the C-terminal end was amplified by PCR and completely digested with *Apa*I. The intact (778 bp) and cut (693 bp) fragments were separated by agarose-gel electrophoresis and visualized with ethidium bromide. Lane STD contains a molecular size standard. Lower panel: regions of the genomic DNA coding for the C-terminal end of ODC were amplified from HTC and DH23b cells by PCR. Portions of the resulting 330 bp fragments were completely digested or not with *Apa*I as indicated.

*Hin*dIII revealed approx. 10-fold more ODC DNA in DH23b cells, suggesting that the enhanced ODC synthesis is directly related to an increase in ODC gene copy number in the variant. By comparison, the same DNA preparations were probed for antizyme sequence, and there appeared to be no difference between the cell variants (Figure 1B).

ODC stability in DH23b cells

Several groups have shown the C-terminal end of ODC to be necessary for rapid proteolytic degradation of this enzyme. To check for possible variations in this region of ODC in DH23b cells, segments of genomic DNA coding for ODC were amplified by PCR, and multiple clones of the C-terminal end of the coding region were constructed and sequenced. In all clones sequenced the amplified genomic DNA of DH23b cells was found to differ from that of parental HTC cells by a single base change resulting in the replacement of Cys-441 with Trp. This modification is identical to the point mutation found to be responsible for stable ODC in the HMOA cell variant, which had been selected from the same parental HTC cells [16].

In sharp contrast to the situation in HMOA cells, where wild-type and mutant ODCs were expressed equally, the ODC mRNA expressed in DH23b cells appeared to be almost entirely of the mutant form. The modification in the ODC sequence can be easily detected by the appearance of a new *Apa*I site in the DNA for this region of the gene. As shown in Figure 2 (upper panel), when appropriate primers were used to PCR-amplify this region

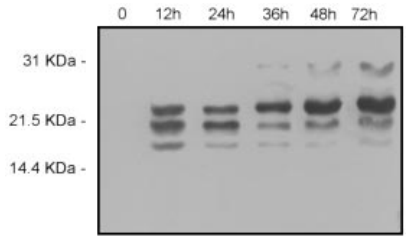

Figure 3 Increase in antizyme protein in DH23b cells after removal from DFMO

DH23b cells grown in 10 mM DFMO were washed free of this inhibitor, and cell samples were removed at the indicated time points and suspended in SDS sample buffer. Equal portions (20 μg of cell protein) were fractionated by SDS/PAGE and blotted to nitrocellulose. The nitrocellulose was stained with Ponceau S to reveal the molecular-mass standards (kDa) as indicated. After blocking with 5% non-fat dried milk, the lower half of the blot was exposed to affinity-purified antibody to antizyme. Bands were revealed using the Amplified Alkaline Phosphatase Goat Anti-Rabbit Immuno-Blot Assay Kit (Bio-Rad).

of reverse transcription cDNA from HTC cells there was no cutting by *Apa*I, compared with essentially complete cutting of that prepared from DH23b cells. The observation that the great majority of the genomic ODC sequences also contain this mutation, as indicated by *Apa*I susceptibility (Figure 2, lower panel), is consistent with the abundance of mutant, relative to wild-type, ODC mRNA in DH23b cells. Thus it appears that the only ODC sequence amplified in DH23b cells is that coding for the stable, mutant, form of ODC.

Antizyme expression in DH23b cells

Antizyme activity has never been observed in DH23b cells either in the presence or in the absence of DFMO in the media. Further, attempts to induce antizyme activity in these cells by exposure to exogenous putrescine or spermidine resulted in cell lysis, and appeared to indicate a lack of sufficient antizyme activity to modulate the polyamine transporter [14,15,18]. Therefore we were surprised to find that our Western-blot analysis of DH23b cells indicated the presence of substantial quantities of antizyme protein during their growth on 10 mM DFMO. Moreover, once the cells were removed from DFMO the levels of antizyme protein appeared to climb sharply, eventually reaching concentrations 50–100-fold above that normally inducible in the parental HTC cells (Figure 3). This major increase in antizyme level appears to follow the pattern of enhanced production of putrescine in DH23b cells that has been shown to occur shortly after their removal from DFMO [22]. The peak levels of antizyme protein were approximated by band intensity comparisons with purified (glutathione S-transferase)–Z1 fusion protein, and antizyme was found to be induced to about 0.15 μg/mg of protein in the same samples where ODC protein was estimated at 5 μg. The absence of any measurable free antizyme activity is consistent with the persistence of such a large excess of ODC protein.

The Western blots of antizyme present in DH23b cells were also surprising in the number of different forms of the protein that they revealed. As shown in Figure 4, Western-blot analysis of HTC cell extracts generally reveals two bands. Presumably these represent variable use of the two alternate start sites during antizyme translation [3,4]. Both the 29.5 and 24 kDa forms are present in extracts of DH23b cells. Additionally, this variant cell type exhibits inconstant amounts of two apparently smaller proteins, of approx. 22 and 18.5 kDa. We suggest that these

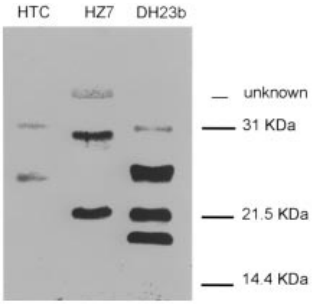

Figure 4 Comparison of antizyme bands present in extracts of HTC, HZ7 and DH23b cells

Pellets of DH23b cells that had been removed from DFMO treatment for 12 h were compared with HTC cells treated with 0.5 mM putrescine for 4 h and HZ7 cells stimulated with dexamethasone for 6 h. Chromatography and Western-blot procedures were as indicated in the legend to Figure 3. Positions of molecular mass standards (kDa) are indicated. Protein contents of samples applied were 110 μg of HTC, 45 μg of HZ7 and 10 μg of DH23b cell extract.

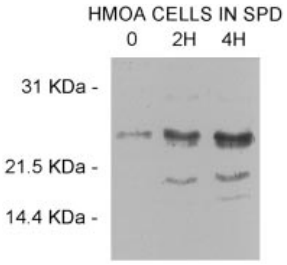

Figure 5 Appearance of antizyme protein forms in HMOA cells

Cultures of HMOA cells were induced for ODC by suspension in fresh culture medium. After 4 h, antizyme was induced by the addition of 50 μM spermidine and 3 mM aminoguanidine, and samples were subsequently removed to SDS sample buffer at the indicated times (h). Chromatography and Western-blot procedures were as indicated in the legend to Figure 3. Positions of molecular mass standards (kDa) are indicated. Samples of 60 μg of cell protein were applied per lane.

bands also represent antizyme fragments, as opposed to non-specific contaminating proteins, because: (1) the bands were specifically visualized by antibodies prepared in three different rabbits, and each preparation had been further purified by affinity chromatography; (2) HTC cells did not show these bands even though 10 times as much protein was applied to the SDS/PAGE gel; (3) like the larger antizyme forms, they co-purified with the ODC protein; and (4) the appearance and disappearance of these bands was correlated with the induction and disappearance of the main antizyme forms.

In HZ7 cells, another variant derived from the HTC line, a Z1 antizyme sequence has been placed under the control of a dexamethasone-inducible promoter [19]. In this construct a new translation initiation site has been placed in-frame with the antizyme coding region, obviating the requirement for spermidine induction of antizyme translation. The location of this initiation codon is between the alternate initiation codons in the wild-type mRNA. As anticipated, the mass of the dexamethasone-induced band is about 28 kDa, distinct from either of the two forms found in the parental HTC cells. However, as seen in Figure 4, in addition to the expected band there frequently appears to be two other cross-reacting bands, one that is clearly too large to be encoded by the Z1 antizyme sequence and one that migrates as if it were a protein of 22 kDa. The latter band has been observed, but not discussed, previously [14,23]. Paradoxically, although the smaller band (appearing at about 22 kDa) is about the same size as one of the extra bands seen in DH23b cells, neither seem to correlate with the induction of the authentic Z1 antizyme band.

The appearance of the two smaller antizyme forms in DH23b cell extracts may be associated with the inability of the ODC–antizyme complex to be degraded rapidly in these cells. As shown in Figure 5, induction of antizyme in HMOA cells, which contain some of the same stable ODC mutant, will also result in the appearance of these two smaller antizyme forms. In this cell line, however, the apparent amount of these forms remains low compared with that of the 24 kDa antizyme band.

It is unlikely that any of these unidentified bands are produced by proteolysis during cell extraction, as either quick-frozen or fresh cell pellets were lysed directly in SDS sample buffer. They may, however, be the products of specific proteolytic cleavage of antizyme within the intact cell. If so, then it must be the N-terminal end that is being removed, as these forms bind to ODC normally, whereas the C-terminal amino acids are absolutely necessary for antizyme's affinity for ODC [8,9]. Secondly, our affinity-purified antibody preparations used to reveal these forms in Western blots are specific for the C-terminal end of antizyme. As seen in Figure 6, *in vitro* synthesized Z1 antizyme, which is identical to wild-type antizyme except in its N-terminal end, is clearly identified by our antibody to antizyme, whereas Z1 synthesized without 13 of its C-terminal amino acids is not recognized at all.

Antizyme stability in DH23b cells

The striking accumulation of antizyme in DH23b cells removed from DFMO is unexpected. As shown in Figure 1(B), the antizyme sequence was not amplified along with the mutant ODC gene in this cell variant. Therefore, even though polyamine levels are somewhat elevated in DH23b cells removed from DFMO, this would not be expected to yield $>$ 50-fold more antizyme protein than is induced in HTC cells. Instead, as shown in Figure 7, antizyme profusion may be the result of increased stability of this protein. In this experiment DH23b cells were washed free of DFMO and antizyme was allowed to accumulate for 12 h. Following inhibition of protein synthesis by cyclo-

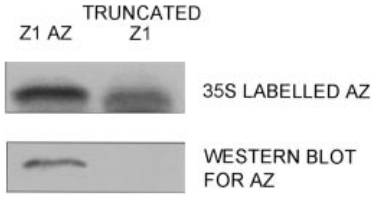

Figure 6 Selectivity of our immunodetection procedures for the C-terminal end of antizyme

^{35}S-labelled Z1 antizyme (AZ) was synthesized *in vitro* from full-length Z1 mRNA or mRNA truncated to produce antizyme missing approx. 13 amino acids from its C-terminal end. The translation products were chromatographed on SDS/PAGE and transferred to nitrocellulose as in Figure 3. Antizyme protein was detected by immunoreactivity with our affinity-purified antibody to antizyme and visualization using the SuperSignal CL-HRP Substrate System (Pierce). Identical blots were autoradiographed to reveal the presence of antizyme translation products. Equal amounts of mRNA were used in these *in vitro* translation reactions. The appearance of a lesser amount of [^{35}S]methionine in the truncated antizyme band is due to the fact that one of the two methionine residues in antizyme is within the region removed by this deletion.

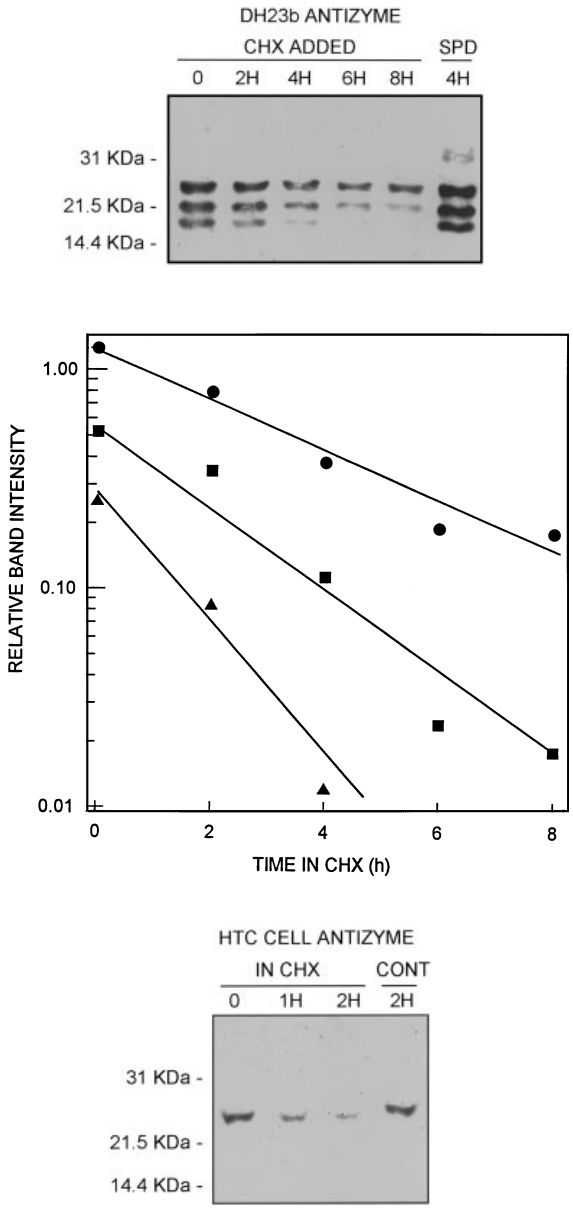

Figure 7 Comparison of the half-lives of antizyme protein in DH23b and HTC cells

Top panel: antizyme was induced in DH23b cells by culturing in the absence of DFMO for 12 h. After this time cycloheximide (CHX; 0.2 mM) was added to an aliquot of the cells and samples were withdrawn at the indicated times (h) and treated as described in Figure 3. For comparison, 50 mM spermidine (SPD) was added to another portion of the culture and a sample extracted 4 h later. Positions of molecular mass standards (kDa) are indicated. Middle panel: to determine the relative intensities of the antizyme bands, the blots were first scanned using Photoshop 3.0 and an Envisions 8100 scanner. The images were then analysed using Optimus 4.02. Relative band intensities were then plotted with respect to time after cycloheximide (CHX) addition: ●, 24 kDa band; ■, 22 kDa band; ▲, 18.5 kDa band. Bottom panel: antizyme was induced in HTC cells by the addition of 50 μM spermidine to cultures 4 h after their last media change. After an additional 4 h of incubation, further antizyme synthesis was prevented by the addition of 0.2 mM cycloheximide (CHX). Samples taken at 0, 1 and 2 h later were analysed for antizyme protein as described in the legend to Figure 3. For comparison, the last lane contains cell extract of a paired culture untreated with cycloheximide (CONT).

heximide, the levels of antizyme in the cells were monitored every 2 h by Western-blot analysis (Figure 7, top panel). The middle panel shows a typical quantification of the band intensities. The main antizyme band, at 24 kDa, was found to be degraded with a half-life of approx. 3 h. A similar analysis of antizyme from HTC cells (bottom panel) confirmed earlier reports [13] that antizyme in these parental cells is rapidly degraded with a half-life of 20–30 min. It is interesting to note that the 22 kDa and 18.5 kDa antizyme bands of the DH23b cells appeared to be less stable than the main 24 kDa band, with half-lives of about 1.8 and 1 h respectively.

DISCUSSION

Many cell lines have been selected for resistance to DFMO in their media. In almost all instances the cells achieve tolerance of this irreversible inhibitor by extreme overproduction of ODC, in some cases exceeding $> 10\,\%$ of total protein synthesis [24–26]. In the DFMO-resistant variant DH23b, however, synthesis of ODC is only increased about 10-fold [17], a difference that can now be explained by an equivalent enhancement in the prevalence of the ODC genomic sequence in this cell (Figures 1 and 2). Further, the sequence that is amplified in DH23b cells differs from wild-type in a single base substitution, such that Cys-441 is changed to Trp in the resulting ODC protein. This same mutation in the C-terminal end of ODC was found to be responsible for ODC stability in HMOA cells [16]. In that cell variant, unlike the DH23b cells, equal amounts of wild-type and mutant ODC forms appear to be produced. It should be noted that HMOA cells were selected for resistance to the reversible inhibitor α-methylornithine [27] and will not proliferate in the presence of even 0.1 mM DFMO (results not shown).

How does the accumulation of stable, DFMO-inactivated, ODC allow the DH23b cells to tolerate 10 mM DFMO in their medium? In a previous study we have presented evidence that the inhibition of ODC activity by DFMO may be slowly reversed under certain reaction conditions [28]. If removal of the DFMO metabolite from ODC does occur in the intact cell, even to a minute extent, then the 1000-fold excess of DFMO-inactivated ODC in DH23b cells may be expected to yield a constant supply of active enzyme sufficient to produce the required levels of putrescine.

The 50–100-fold increase in antizyme protein in the DH23b cells is surprising (Figure 3), especially in view of the fact that there is no demonstrable increase in the prevalence of the genomic sequence for antizyme in this variant (Figure 1). Instead of accelerated synthesis of antizyme, the major factor causing the increased levels in DH23b cells may be the approx. 6–9-fold increase in the half-life of the antizyme protein (Figure 7). Some stabilization of antizyme was observed in early studies on HMOA cells [13]. In that report evidence was presented that free antizyme in these cells was subject to rapid degradation (as we have shown in Figure 7 for HTC cells), while ODC-bound antizyme was somewhat more stable. It appears that the modification of Cys-441 in ODC results in an ODC–antizyme complex that is a relatively poor substrate for the 26 S proteasome. As a consequence, the antizyme in this stable complex would also be protected from degradation. Since DH23b cells contain greatly elevated levels of the stable Trp-441 ODC, it follows that they will also accumulate antizyme. Though elevated, antizyme levels never exceed or even approach ODC concentrations, thereby accounting for the absence of detectable antizyme activity or antizyme-mediated feedback of the polyamine transport system in this variant.

Western-blot analysis of accumulated antizyme protein in DH23b and HMOA cells revealed the existence of two, apparently smaller, forms of antizyme that were not previously noted (Figure 3–5). These are not likely to be products of

alternate initiation codons, like the 29.5 and 24 kDa bands, as the only other in-frame AUG is very near the 3′ end of the message. It is possible that these are major fragments resulting from specific proteolytic cleavages of antizyme. In this case, clearly it must be the N-terminal end of the protein that is lost because the fragments still bind ODC and are recognized by an antibody specific to the C-terminal end (Figure 6). These proteolytic products should still be active, as deletion studies have shown that up to 113 of the N-terminal amino acids of antizyme can be removed without affecting either ODC binding or destabilization [8,9].

Since the 18.5 and 22 kDa antizyme bands were only obvious in cells in which the normal degradation of antizyme protein had been inhibited, it is tempting to speculate that these may be intermediates in the antizyme degradation process, which would normally be present in concentrations too low to be detected. At this point nothing is known about the mechanism of rapid antizyme degradation in intact cells, and conditions for studying this phenomenon *in vitro* have not been identified as yet. The protein seems curiously stable even in proteolytic systems that degrade ODC [8,29]. Thus antizyme stability due to overexpression of stable ODC in DH23b cells renders this variant cell line an excellent candidate for future studies involved in elucidating the mechanism of control of this most important regulatory protein.

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