RESEARCH COMMUNICATION

Cloning and expression of two ornithine decarboxylase forms from HMO_A cells

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In HMO_A cells [Mamont, Duchesne, Grove and Tardif (1978) *Exp. Cell Res.* **115**, 387–393] the half-life of ornithine decarboxylase (ODC) is 8–14 h instead of 15 min as in the Hepatoma Tissue Culture parental cells, due to a single amino acid substitution [Miyazaki, Matsufuji, Murakami and Hayashi (1993) *Eur. J. Biochem.* **214**, 837–844]. We demonstrate for the first time that HMO_A cells possess two forms of ODC mRNA

that are translated into two proteins differing greatly in turnover rates. We have cloned and transfected the cDNAs for the two ODC forms into COS-1 cells for a direct measurement of their turnover rate. The variant ODC form was much more stable than the wild-type protein, with a half-life of 14 h as compared with 2.5 h.

INTRODUCTION

Ornithine decarboxylase (ODC; E.C. 4.1.1.17) catalyses the first step in the polyamine biosynthetic pathway [1,2] and has been widely studied, since its activity is important in the regulation of proliferation and differentiation of normal and neoplastic cells [3–5]. In addition, ODC is one of the most short-lived proteins in mammalian cells [2,6,7], which makes it an interesting model for studies on intracellular proteolysis.

ODC is a protein of 461 amino acids [8], containing two 'PEST' regions between amino acids 298–333 and 423–449 [9]. PEST sequences are assumed to play a role in the rapid degradation of short-lived proteins, although they have also been found in some long-lived proteins [9]. The second PEST region identified in mammalian ODC seems to play a stronger destabilizing role. Deletion of the last 5–37 amino acids at the C-terminus stabilizes ODC [10,11] and, conversely, these amino acids can confer lability on a stable protein [12].

An ODC form of unusual stability has been described in HMO_A cells, which are derived from the Hepatoma Tissue Culture (HTC) line by selection with the competitive ODC inhibitor α -methylornithine [13]. As compared with the parental line, these cells show an increase in ODC activity and half-life, with no evident alterations of the biochemical and biophysical properties of the ODC protein [14]. Since other short-lived proteins (*S*-adenosylmethionine decarboxylase and tyrosine aminotransferase) are degraded in HMO_A cells at the same rates as in the HTC parental line, the difference in ODC turnover is not related to changes in the proteolytic machinery [14]. Instead, sequencing of a cDNA coding for HMO_A ODC has revealed a point mutation that renders the enzyme more stable in an *in vitro* degradation system [15].

In the present work we have further investigated the molecular basis of the unusual ODC stability in HMO_A cells. Sequencing of cloned cDNAs revealed that HMO_A cells express two forms of

ODC, one containing a point mutation identical to that previously reported [15], and one identical to normal rat liver ODC. That the single amino acid substitution resulting from the point mutation markedly stabilized the enzyme against degradation was demonstrated in a mammalian expression system.

EXPERIMENTAL

Cell lines

HMO_A cells, kindly provided by Dr. P. S. Mamont (Marion Merrell Dow Research Institute, Strasbourg, France), and HTC parental cells were grown in Swins's 77 medium supplemented as described [13]. COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, 2 mM glutamine and antibiotics (100 units/ml penicillin and 100 μ g/ml streptomycin).

RNA extraction and first-strand cDNA synthesis

Total RNA was extracted from HMO_A cells [16] and 10 μ g was used for first-strand synthesis with avian myeloblastosis virus reverse transcriptase (Pharmacia-LKB) in the presence of 50 μ g/ml oligo-dT in a total volume of 20 μ l.

Amplification of the ODC cDNA and cloning strategies

An aliquot (1 μ l) of the first-strand cDNA synthesis mixture was used to amplify ODC cDNA by PCR. The primers used were 5'-CGGGATCCGACCATGGGCAGCTTTACT-3' and 5'-CGGGATCCGCAAGAATGGCATCTATACAT-3'. Amplification was performed by using the GeneAmp PCR Core reagent kit (Perkin Elmer Cetus) with 1 min denaturation at 95 °C, 1 min annealing at 52 °C and 1 min extension at 72 °C for 35 cycles. The 1.4 kb PCR product was first amplified in the pUC18 plasmid and then subcloned in the pSVL expression plasmid (Pharmacia).

Transfection procedure

COS-1 cells were transfected using Lipofectin (Gibco-BRL), with 10 μ g of each plasmid and then grown for 12 h with 4 ml of OptiMem (Gibco-BRL). The medium was then changed to normal Dulbecco's modified Eagle's medium and the cells were grown for an additional 48 h. Thereafter the cells were split into several 100 mm-diam. plates for ODC half-life determination or pulse-labelling experiments.

Determination of ODC half-life

ODC turnover was determined by following the decay of enzymic activity after cycloheximide treatment (50 μ g/ml). ODC activity was assayed by measuring the production of $^{14}\text{CO}_2$ from L-[1- ^{14}C]ornithine (0.25 μ Ci/assay, 0.5 mM final concentration) in the presence of 10 μ M 5'-pyridoxal phosphate.

Metabolic labelling and immunoprecipitation

HTC and HMO_A cells were pulse-labelled for 60 min, 48 h after reseeding, and COS-1 cells for 120 min, 72 h after transfection, with 30 μ Ci/ml of a mixture of [^{35}S]methionine and [^{35}S]cysteine (Tran ^{35}S -Label, ICN Biochemicals). After harvesting, the cells were sonicated in 0.5 ml of 0.1 M Tris/HCl (pH 7.5)/0.1 mM EDTA/2.5 mM DTT/20 mM unlabelled methionine and cysteine, and centrifuged at $6 \times 10^6 g$ to obtain a cytosolic extract. Aliquots of the cytosolic extract, with equal amounts of acid-insoluble radioactivity, were immunoprecipitated with an anti-ODC antibody [17], and the precipitated proteins were resolved by SDS/PAGE (10% gel). The gel was then fixed, treated with Amplify (Amersham), dried and exposed to Hyperfilm (Amersham) for fluorography.

Determination of the nucleotide sequence

The nucleotide sequence was determined by the dideoxy method of Sanger [18], using the AmpliTaq Cycle Sequencing Kit (Perkin Elmer Cetus).

RESULTS AND DISCUSSION

ODC turnover in HTC and HMO_A cells

It has been previously demonstrated [10,19] that, in different cell lines, changes in ODC activity strictly correlate with changes in the amount of the ODC protein. We thus measured the half-life of ODC in HTC and HMO_A cells by following the decay of the enzymic activity after inhibition of protein synthesis with cycloheximide (Figure 1). In HTC cells ODC activity decreased down to 55% of the t_0 value after 15 min and to about 20% after 30 min; the estimated half-life was about 18 min. In HMO_A cells the disappearance of ODC activity after inhibition of protein synthesis occurred in two steps: a rapid decay was observed during the first 15 min and thereafter the decay rate was drastically reduced with no significant change in enzyme activity during a period of 3 h. This decay profile of ODC activity was confirmed in several independent experiments. Having ruled out any resumption of protein synthesis (results not shown), the biphasic decay of ODC activity indicated the presence of two forms of ODC with different degradation kinetics: one, with a measured half-life of about 18 min, similar to that of HTC wild-type protein, and one that was degraded much more slowly. Although ODC turnover in HMO_A cells has been studied previously [14], this is the first report describing a rapid component in ODC degradation in these cells. Since most of the wild-type form is lost in less than 30 min, the fast component of the

Figure 1 ODC turnover in HTC and HMO_A cells

Cells were treated with cycloheximide (50 μ g/ml) and harvested for ODC determination at the times indicated. ODC activity at time 0 was about 0.2 U/mg protein for HTC cells (●) and 5 U/mg protein for HMO_A cells (○). 1 U is defined as the amount of enzyme producing 1 nmol of $^{14}\text{CO}_2$ /h. Values are means $\pm$ S.D. ($n = 3$).

Figure 2 PCR amplification of ODC cDNA from HMO_A cells

Primers used for amplification are described in the Experimental section. Lane 1, *Syl*-digested lambda DNA; lane 2, PCR product; arrow indicates the 1.4 kb PCR product.

ODC activity decay can easily be overlooked when longer sampling times are used, such as in previous studies.

Cloning and sequencing of ODC cDNAs from HMO_A cells

cDNA was obtained from total RNA of HMO_A cells and then amplified by PCR using primers corresponding to the N- and C-terminal regions of the ODC protein in order to obtain the full coding region of ODC cDNA (see the Experimental section). The 1.4 kb band obtained by PCR was in agreement with the expected size of an amplified ODC fragment (Figure 2). The amplified cDNA fragment was subcloned into a pUC18 vector, and individual clones were used for sequence determination. The sequencing revealed the presence of two mRNA forms in the HMO_A cells, differing in only a single base: a T to G transversion

Figure 3 ODC cDNA sequence from HTC and HMO_A cells

Sequence of wild-type (a) and HMO_A (b) ODC. Arrow indicates the T-G base transversion.

(arrow in Figure 3). This finding was confirmed by several independent mRNA retrotranscriptions and cDNA amplifications to exclude artifacts, particularly those due to infidelity of the *Taq* DNA polymerase [20]. Since cloned rat liver cDNA has been demonstrated to contain a T at this position, it can be assumed that the T-containing form represents wild-type ODC and the G-containing form is a variant. This mutation, which substitutes the cysteine residue in position 441 with a tryptophan, is identical to that reported by Miyazaki et al. [15]. No other differences in the ODC coding region were found, suggesting that HMO_A cells express two forms of ODC mRNA with translation products only differing in one amino acid. The biphasic decay of ODC activity observed after cycloheximide treatment indicates a major difference in turnover rate between the two ODC forms.

Turnover of ODC in transfected COS-1 cells

The cDNAs for the wild-type and mutated forms of HMO_A ODC were subcloned in the correct orientation in the expression vector pSVL and then transfected into COS-1 cells. The vector containing the SV40 late promoter gave rise to a very high transient expression in COS-1 cells. As a further control, pSVL plasmid carrying the cDNA for the normal human ODC was transfected into COS-1 cells. The turnover of the enzyme molecules encoded by such cDNAs was followed by measuring the decay of ODC activity after treatment with cycloheximide (Figure 4). Wild-type ODC activity declined with a half-life of about 2.5 h, while that of the variant form decayed more slowly, with a half-life of about 14 h. In the same system, human ODC was degraded at a rate (half-life = 2.5 h) similar to that for HMO_A wild-type ODC. Thus, these measurements directly show that the two forms of HMO_A ODC are characterized by a marked difference in turnover. In COS-1 cells, however, wild-type ODC was more stable than in the parental cells. The reason for such a discrepancy is unknown, but it might be related to the high level of ODC protein expressed in COS-1 cells, which may saturate the degradation pathways. As shown in the present study, the molecular basis for the increased half-life of variant ODC clearly resides in the amino acid substitution, with tryptophan replacing cysteine in position 441. Substituting Cys-441 with alanine has previously been shown to bring about a stabilization of the enzyme in an *in vitro* degradation system [15],

Figure 4 Degradation of ODC in transfected COS-1 cells

COS-1 cells were transfected with cDNAs coding for wild-type (●), variant HMO_A (○) or human ODC (▽). The decay of enzymic activity after treatment with cycloheximide was followed for up to 4 h.

Figure 5 ODC expression in HTC, HMO_A and transfected COS-1 cells

HTC, HMO_A and transfected COS-1 cells were labelled with [³⁵S]methionine/cysteine for 1 or 2 h. ODC was immunoprecipitated and electrophoresed on a 10% polyacrylamide gel. Lanes are: 1, HTC cells; 2, HMO_A cells; 3, COS-1 cells transfected with wild-type ODC; 4, COS-1 cells transfected with variant HMO_A ODC; 5, control COS-1 cells; 6, ¹⁴C-labelled molecular-mass markers. Arrow indicates ODC protein.

suggesting that it is the loss of cysteine, rather than the kind of substituent residue, that is critical for the marked stabilization of the variant ODC. It is noteworthy that the T-G transversion observed was the only difference in the ODC nucleotide sequence in the coding region. No changes were found in the amino acids of the PEST sequence at the C-terminus, which, theoretically, could give rise to a marked stabilization of ODC. Nevertheless, the substitution Cys-441 → Trp might still affect the tridimensional organization of the C-terminus, and consequently of the PEST sequence, in such a way as to affect its function as a molecular determinant of instability.

Metabolic labelling of ODC

ODC was immunoprecipitated from HTC and HMO_A cells after labelling with [³⁵S]methionine/cysteine. Figure 5 shows that

ODC from HTC cells migrated as a single well-defined band by SDS/PAGE (10% gel). By contrast, the enzyme from HMO_A cells was resolved in two bands of different mobility: one migrating as HTC ODC, the other with a faster mobility. To confirm that these two bands represent the two forms of ODC, we pulse-labelled COS-1 cells transfected with either wild-type or variant ODC cDNA from the HMO_A cells. Both the transfected proteins migrated as single bands, but with different mobilities (Figure 5). The wild-type protein migrated as control ODC from HTC cells or the 'slow' component observed in the HMO_A bands, whereas the variant ODC migrated as the 'fast' component of the HMO_A doublet.

A similar difference in electrophoretic mobility was also observed in a system comparing rat wild-type ODC with an enzyme in which Cys-441 was replaced by a tryptophan using site-directed mutagenesis [15]. Thus since there is no evidence so far that mutant and wild-type ODC differ in molecular mass, it appears that the substitution of Cys-441 with tryptophan significantly affected the mobility of the enzyme by SDS/PAGE.

Interestingly, the antibody precipitated several peptides of lower molecular masses than ODC from transfected COS-1 cells (Figure 5). Although much less intense, some of these peptides could also be seen in the immunoprecipitates from HTC and HMO_A cells (Figure 5). They may represent partially degraded ODC or products from downstream initiation or premature termination.

Taken together, the present data clearly indicate that HMO_A cells express ODC from two genes, in all likelihood two active alleles of the same locus. The resistance of the HMO_A cells to the antiproliferative effect of the ODC inhibitor α -methylornithine is accounted for by a point mutation in one of the alleles. This point mutation introduces a single amino acid substitution that renders the enzyme more stable against proteolytic degradation and thus results in an increased cellular ODC level.

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