Human ornithine decarboxylase paralogue (ODCp) is an antizyme inhibitor but not an arginine decarboxylase

Kristiina KANERVA*, Laura T. MÄKITIE*, Anna PELANDER†, Marja HEISKALA‡ and Leif C. ANDERSSON*§[1]

*Department of Pathology, Haartman Institute, University of Helsinki, Helsinki, Finland, †Department of Forensic Medicine, University of Helsinki, Helsinki, Finland, ‡Schering-Plough Research Institute, Kenilworth, NJ 07033, U.S.A., and §HUSLAB, Helsinki, Finland

ODC (ornithine decarboxylase), the rate-limiting enzyme in polyamine biosynthesis, is regulated by specific inhibitors, AZs (antizymes), which in turn are inhibited by AZI (AZ inhibitor). We originally identified and cloned the cDNA for a novel human ODC-like protein called ODCp (ODC paralogue). Since ODCp was devoid of ODC catalytic activity, we proposed that ODCp is a novel form of AZI. ODCp has subsequently been suggested to function either as mammalian ADC (arginine decarboxylase) or as AZI in mice. Here, we report that human ODCp is a novel AZI (AZIN2). By using yeast two-hybrid screening and *in vitro* binding assay, we show that ODCp binds AZ1–3. Measurements of the ODC activity and ODC degradation assay reveal that ODCp inhibits AZ1 function as efficiently as AZI both *in vitro* and *in vivo*. We further demonstrate that the degradation of ODCp is ubiquitin-dependent and AZ1-independent similar to the degradation of AZI. We also show that human ODCp has no intrinsic ADC activity.

Key words: antizyme (AZ), antizyme inhibitor (AZI), arginine decarboxylase (ADC), ornithine decarboxylase (ODC), ODC paralogue (ODCp), polyamine.

INTRODUCTION

We originally identified a novel human ODC (ornithine decarboxylase)-like protein called ODCp (ODC paralogue) [1]. The *odcp* gene encodes a 50 kDa protein comprising several motifs and domains conserved in ODC, including PLP (pyridoxal-5-phosphate)-binding site, membrane translocation motif p47^{phox} [2] and AZ (antizyme)-binding site [1,3]. The key residues important for the decarboxylase activity of ODC are, however, mutated in ODCp, as is the PEST (Pro-Glu-Ser-Thr) domain essential for the rapid turnover of ODC. Therefore we suggested that ODCp is a novel tissue-specific AZI (AZ inhibitor) [1]. The tissue distribution of ODCp is restricted, with high expression in brain and testis.

Two different roles have subsequently been proposed for ODCp. Zhu et al. [4] reported that ODCp is a mammalian ADC (arginine decarboxylase), which catalyses the decarboxylation of L-arginine to agmatine. Micro-organisms and plants contain ADC, and they produce putrescine from both agmatine and ornithine [5,6]. In mammals that receive agmatine from their diet, the existence of ADC has not been demonstrated previously. Later studies have, however, been unable to verify the ADC activity of ODCp [7,8]. López-Contreras et al. [8] recently reported that the murine paralogue of ODCp acts as an AZI. They showed that interaction between AZ1–3 and mouse ODCp leads to increased ODC activity and decreased rate of ODC degradation.

The family of AZs in mammals includes at least three members. AZ1 and AZ2 are expressed ubiquitously in all cell types, but the expression levels of AZ2 are lower [9]. AZ3 is a testis-specific paralogue, the expression of which is restricted to certain stages of spermatogenesis: in late spermatocytes and early spermatids [10]. All AZs have been shown to inhibit the decarboxylase activity of ODC [10–12]. AZ1 is also able to induce ODC degradation via 26S proteasomes but it is still unclear whether the other AZs display this function [8,11]. All AZs are inactivated by AZI [12], a protein that displays sequence homology to ODC, but lacks decarboxylating activity [13]. AZI binds AZs with higher affinity than does ODC, and releases active ODC from the catalytically inactive ODC–AZ complex [14].

In the present study, we addressed the question of whether human ODCp functions as AZI or ADC. Here, we report that ODCp acts as a regulator of ODC activity and inhibits its proteasomal degradation. ODCp is degraded by ubiquitination like AZI. We also demonstrate that ODCp does not act as mammalian ADC. Our results reveal that human ODCp is a novel AZI.

EXPERIMENTAL

Materials

Oligonucleotides were purchased from Sigma-Proligo. Phosphocreatine was obtained from Fluka, and IPTG (isopropyl β-D-thiogalactoside) from Fermentas. All the other chemicals were obtained from Sigma.

Plasmid construction

The cDNAs encoding ODCp, AZ1 and AZ3 were amplified from human QUICK Clone cDNA libraries (Clontech) using whole brain library as a template for AZ1 and ODCp, and a testis library for AZ3. IMAGE cDNA clones from the RZPD German Resource Center for Genome Research were used as templates for the amplification of human AZ2 (clone ID IRAKp961E02134Q2) and AZI (clone ID IRAUp969Bo377D6). To clone AZs, the ribosomal frame-shifting site was deleted as described previously [12]. The resulting AZ1–3 fragments, AZI and ODCp were subcloned into the pCDNA3.1 vector (Invitrogen) under the control of the T7 promoter. The human ODC cDNA (a gift from Dr Erkki

Abbreviations used: ADC, arginine decarboxylase; AZ, antizyme; AZI, AZ inhibitor; ESI, electrospray ionization; F-II, fraction II of rabbit reticulocyte lysate; GST, glutathione transferase; LC, liquid chromatography; ODC, ornithine decarboxylase; ODCp, ODC paralogue.

[1] To whom correspondence should be addressed, at Department of Pathology, Haartman Institute, P.O. Box 21 (Haartmaninkatu 3), FIN-00014 University of Helsinki, Helsinki, Finland (email leif.andersson@helsinki.fi).

Hölttä, Department of Pathology, Haartman Institute, University of Helsinki, Helsinki, Finland) was cloned into the pCIneo vector (Promega). For transient transfection experiments, ODCp and ODC cDNAs were cloned into the p3xFLAG-CMV-10 vector (Sigma), and AZI into the pAMC expression vector (a gift from Dr Tatiana Petrova, Molecular and Cancer Biology Program, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland) to produce N-terminally c-Myc-tagged AZI.

Cell culture and transient transfections

COS-7 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) fetal bovine serum, 1 mM L-glutamine, 50 mg/ml penicillin and 50 mg/ml streptomycin. Transient transfections of COS-7 cells, used in the ODC activity assay, were performed with FuGENE™ HD (Roche). For ADC activity measurements, COS-7 cells were transfected with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions.

Yeast two-hybrid screening

The yeast two-hybrid screening was performed using Matchmaker™ Two-hybrid System 3 (Clontech). Briefly, the full-length human ODCp cDNA was cloned in-frame with the GAL4 DNA-binding domain of pGBKT7 vector and used as a bait construct for the analysis. The bait was transformed into yeast strain AH109, which was mated with strain Y187 carrying the prey library (pretransformed human bone marrow Matchmaker™ cDNA library, catalogue number HY4053AH; Clontech) in pACT2 vector. No autonomous activation of the reporter genes was observed with the bait construct. The positive clones on selective media were further confirmed by monitoring the expression of the reporter genes, and the inserts of the positive clones were amplified by PCR and sequenced.

In vitro translation

L-[^{35}S]methionine (Amersham Biosciences)-labelled AZ1–3, AZI, ODCp and ODC were synthesized *in vitro* with the T7 TNT system (Promega) from the corresponding pCDNA3.1 and pCIneo constructs. The protein amounts of different *in vitro* translation samples were determined and equalized prior to assays by SDS/PAGE and fluorography.

GST (glutathione transferase) fusion proteins and pull-down assay

The full-length human ODCp was cloned into the pGEX-4T-3 vector (Amersham Biosciences). GST and GST–ODCp fusion proteins were expressed in *Escherichia coli* BL21. The fusion proteins were purified from crude bacterial lysates with glutathione–Sepharose 4B beads (Amersham Biosciences) and analysed by Western blotting, using the anti-GST antibody (Serotec) to equalize the protein amounts. Glutathione–Sepharose-bound GST and GST–ODCp were incubated with *in vitro* translation products in binding buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100 and protease inhibitors) at room temperature (22 °C). After 1 h, the beads were washed at least five times with binding buffer. The bound proteins were eluted in Laemmli sample buffer and resolved by SDS/PAGE (13 % gel) and visualized by fluorography.

In vitro protein degradation assay

In vitro-translated [^{35}S]methionine-labelled proteins were combined in the proportion 2:1:14 (ODC:AZ1:ODCp/AZIN2) and incubated in a reticulocyte lysate-based degradation mixture with ATP-regenerating buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 10 mM phosphocreatine and 1.6 μg/ml creatine kinase) at 37 °C for the indicated times. Equal amounts of aliquots of the reaction were stopped by addition of Laemmli sample buffer. The amount of proteins remaining undegraded was determined by SDS/PAGE and fluorography. The intensities of the bands were analysed also by scanning the dried, fluorographed gel with a PhosphoImager (BAS-1500; Fuji). In the *in vitro* degradation assay with fraction II, 10 μl of the buffer was replaced by fraction II of rabbit reticulocyte (Calbiochem). Otherwise the assay was performed as mentioned above.

Ubiquitin conjugation assay

In vitro-translated [^{35}S]methionine-labelled AZI, ODCp and ODC were incubated at 37 °C for the indicated times in reaction buffer (20 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.5 mM ATP, 0.5 μg of ubiquitin aldehyde and 5 μg of ubiquitin). The degradation of equal-sized aliquots was determined by SDS/PAGE, fluorography and phosphoimager scanning. When the accumulation of high-molecular-mass ubiquitin conjugates of the proteins was analysed, proteasome inhibitors [150 μM MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal) and 150 μM clasto-lactacystin β-lactone] were added in the reaction buffer.

ODC assay

The ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described previously [1]. For the assay, *in vitro*-translated proteins were combined in the proportion 2.5:1:7 (ODC:AZ1:ODCp/AZIN2), and the assay was repeated three times. To measure the ODC activity *in vivo*, COS-7 cells were transiently transfected with FLAG–ODCp, c-Myc-AZI or the empty p3xFLAG-CMV-10 vector. After 24 h, the cells were collected as described earlier [1], and ODC activity was measured. Transfection experiment was performed in duplicate, and all the measurements were performed in triplicate.

ADC assay

The ADC activity measurement was performed essentially as described previously [4]. A 10 μl portion of *in vitro*-translated ODCp and ODC was diluted directly to the ADC assay reaction buffer whose pH was either 7.4 or 8.25. In the former pH, the assay was carried out at 37 °C and in the latter at 30 °C. The empty pCDNA3.1 vector was used as a negative control and *E. coli* ADC (0.021 unit/reaction; Sigma) was used as a positive control. The assay was repeated three times under both conditions. COS-7 cells, transfected with FLAG–ODCp, FLAG–ODC or p3xFLAG-CMV-10 vector, were lysed in the sample buffer (10 mM Tris/HCl, pH 7.4 or 8.25, and protease inhibitors) by sonicating, and centrifuged at 1000 ***g*** for 10 min at 4 °C. The supernatant was re-centrifuged at 12 000 ***g*** for 20 min at 4 °C to obtain cytosolic and crude mitochondrial fractions. The mitochondrial pellet was resuspended in the sample buffer (pH 8.25). The ADC assay of the cytosolic fraction was performed under both conditions mentioned above but the mitochondrial fraction was analysed only in pH 8.25 at 30 °C. All the measurements were performed in triplicate, and the expression of ODC and ODCp was verified by Western blotting with anti-FLAG M2 antibody (Sigma).

Measurement of agmatine by chromatography and MS

ODCp was expressed in COS-7 cells for 24–36 h. Untransfected cells cultured for 2 h in the presence of 10 mM agmatine were used as a positive control. Agmatine analysis was performed by

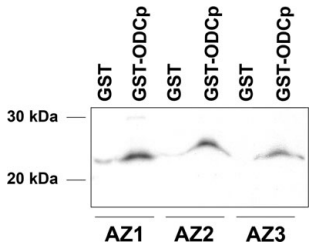

Figure 1 Human ODCp binds to AZ1–3

A GST pull-down assay to test the binding of ODCp to antizymes. *In vitro*-translated AZ1–3 were pulled down with glutathione–Sepharose-bound GST or GST–ODCp. The bound material was separated by SDS/PAGE and fluorographed.

ESI (electrospray ionization) LC (liquid chromatography)/TOF (time-of-flight) MS. The identification of agmatine was based on accurate mass determination. For MS, the dialysed samples were diluted in 0.1 % formic acid–acetonitrile. The liquid chromatograph was an Agilent (Waldbronn) 1100 series instrument. The separation was performed with a Phenomenex (Torrance) Luna C-18(2) 100 mm × 2 mm (3 μm) column with a 21 min gradient at 40 °C at a flow rate of 0.3 ml/min. The eluent components were 5 mM ammonium acetate in 0.1 % formic acid and acetonitrile. The injection volume was 20 μl. The mass analyser was a Bruker Daltonics (Bremen) MicrOTOF with an orthogonal ESI source operated in the positive ion mode with an m/z range of 50–800. The capillary exit voltage was set at 70 V, the hexapole RF (relative fluorescence) at 50 V, and the transfer time at 35 μs. The nebulizer gas flow was 1.6 bar (1 bar = 100 kPa), the drying gas flow was 8 litres/min, and the drying temperature was 200 °C. The external and post-run calibrations were performed with sodium formate clusters in quadratic mode with six calibration points.

RESULTS AND DISCUSSION

Human ODCp interacts with AZ1, AZ2 and AZ3

We screened a yeast two-hybrid library of human bone marrow cDNA using the full-length human ODCp as a bait. Twenty-six clones were isolated and sequenced. A GenBank® search revealed that 21 of these clones encoded human ODC AZ1 (GenBank® accession no. NM_004152). The interaction between ODCp and AZ1 was further confirmed by the GST pull-down assay. GST pull down was also used to test whether ODCp interacts with AZ2–3. *In vitro*-translated, ^{35}S-labelled AZ1–3 were incubated with immobilized GST or GST–ODCp. AZ1, AZ2 and AZ3 bound to GST–ODCp fusion protein, but not to GST alone (Figure 1).

The binding of ODCp to AZ1–3 thus supported our original hypothesis that ODCp is a tissue-specific AZI [1]. Moreover, Kidron et al. [3] recently reported a close relationship between ODCp and AZI sequences in the phylogenetic tree. They also showed that human ODCp is not significantly homologous with ADCs of other species, and concluded that it is very unlikely that human ODCp could account for any ADC activity. Our results are also in agreement with the report of López-Contreras et al. [8] who, using co-immunoprecipitation, showed that mouse ODCp interacts with AZ1–3. We used a different method to show the interaction between ODCp and AZ1–3, and actually two independent methods confirmed that ODCp binds to AZ1. Our results thus demonstrate that human ODCp binds AZ1–3, as does the mouse orthologue of ODCp.

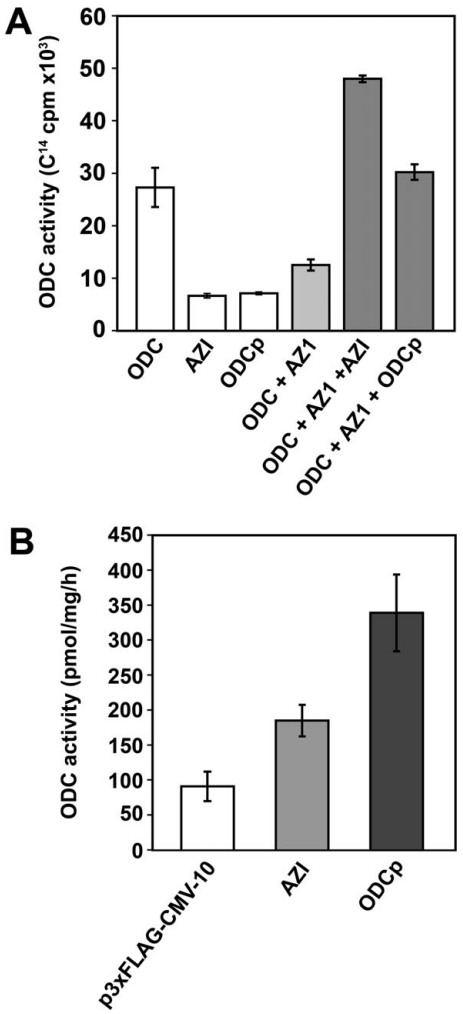

Figure 2 Human ODCp acts as an AZI both *in vitro* and *in vivo*

(**A**) Effect of ODCp on the activity of ODC *in vitro*. ODC, ODCp, AZI and AZ1 were *in vitro* translated and combined as shown in the Figure, after which the ODC activity of the reactions was determined. The experiment was performed in triplicate and the error bars refer to the S.D. of the results. (**B**) Influence of ODCp on ODC activity in COS-7 cells. FLAG-tagged ODCp, the c-Myc-tagged AZI and the empty FLAG vector were expressed in COS-7 cells. After 24 h, the ODC activity of cell lysates was determined. The bars represent the mean values for two simultaneous transfections. The measurements were performed in triplicate and the error bars refer to the S.E.M.

Human ODCp acts as an AZI

To study the AZI activity of human ODCp, we combined *in vitro*-translated ODC with AZ1 and AZI or ODCp, and measured the ODC activity of the reactions. We also measured the activity of AZI and ODCp alone to ensure that they by themselves did not contribute to ODC activity. As shown in Figure 2(A), ODC activity increased in the reaction mixtures containing ODC with either ODCp or AZI together with AZ1, compared with the control reaction containing ODC with AZ1. This indicates that, *in vitro*, human ODCp functions as an AZI. To investigate this possibility also *in vivo*, we transfected COS-7 cells with cDNA encoding FLAG–ODCp, c-Myc–AZI or empty p3xFLAG-CMV-10 vector, after which the ODC activity was measured. In the cells transfected with ODCp, a rise in ODC activity similar to that observed in the AZI transfectants took place (Figure 2B). Our findings suggest that ODCp activates ODC at least as efficiently as AZI. In our study, the increase in ODC activity was in fact slightly

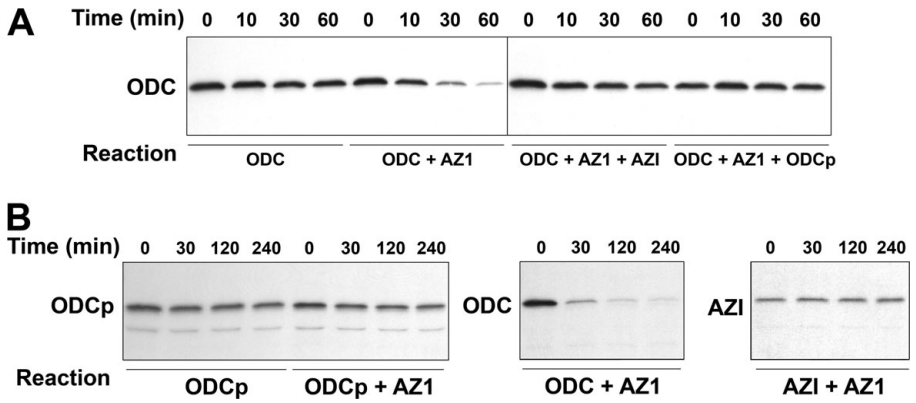

Figure 3 Human ODCp inhibits AZ1-induced degradation of ODC

(**A**) *In vitro* degradation assay to study the effect of ODCp on the AZ1-induced degradation of ODC. *In vitro*-translated proteins were combined as indicated and incubated in ATP-regenerating buffer as described in the Experimental section. The reactions were resolved by SDS/PAGE and fluorographed. Because the molecular masses of ODC, AZI and ODCp are nearly equal, AZI and ODCp were produced as unlabelled to avoid mixing them up with ODC. The amount of labelled AZ1 (results not shown) was used to control the amount of protein loaded on to the gel in the parallel samples. (**B**) *In vitro* degradation assay of ODCp in F-II that lacks ubiquitin. *In vitro*-translated proteins were combined as shown in the illustration, incubated in a buffer with F-II and resolved by SDS/PAGE and fluorography.

higher in the ODCp transfectants than in the AZI transfectants. This might be due to a difference in expression level of ODCp and AZI. Nevertheless, it would be interesting to examine further whether ODCp acts more efficiently in activation of ODC than AZI.

The ODCp-induced activation of ODC does not by itself prove that ODCp acts as an AZI. The interaction of ODCp with AZ1 could also have other functionality than regulation of ODC activity since it has been shown that AZ1 can interact and regulate several additional proteins besides ODC [15,16]. Since there is only a limited amount of AZ1 molecules in the reaction mixture, ODCp could sequester all AZ1, thereby competing with ODC for AZ1 binding without still having AZI activity as a main function itself. To ultimately function as AZI, ODCp should also inhibit the AZ1-dependent degradation of ODC, but on the other hand, the interaction between ODCp and AZ1 should not induce degradation of ODCp itself. To examine whether ODCp competes with ODC for the binding of AZ1 and inhibits ODC degradation, we used an *in vitro* protein degradation assay with rabbit reticulocyte lysate as a source of proteasomes. As reported earlier by Li and Coffino [17], ODC alone is not degraded in reticulocyte lysate in the timeframe used in the experiment, whereas in the presence of AZ1, the degradation occurs rapidly (Figure 3A). When AZI or ODCp was added to the reaction, the AZ1-induced degradation of ODC was prevented. We noted that the *in vitro* degradation assay was sensitive to the relative amounts of the proteins. Hence, López-Contreras et al. [8] did not find that murine AZI adequately prevented the AZ1-induced degradation of ODC. This may be due to their assay based on co-transfections, the effect of which is difficult to control, and thus does not provide ultimate support to their conclusion. Our results quantitatively demonstrate that the binding of ODCp to AZ1 is not only competitive to ODC, but that ODCp also inhibits AZ1 function on ODC degradation.

The interaction with AZ does not induce degradation of AZI [13,18]. Thus we wanted to find out whether the binding of ODCp to AZ1 leads to the degradation of ODCp similarly as it does with ODC. On the other hand, if AZ1 has no influence on the degradation of ODCp, the function of ODCp is to act as an AZI. To study this, we used the *in vitro* degradation assay based on F-II (fraction II of rabbit reticulocyte lysate), which is devoid of ubiquitin. Therefore, if any degradation of ODCp was seen in F-II, it is solely induced by the binding of ODCp to AZ1. In the reaction containing ODCp alone or with AZ1, no degradation of ODCp was observed (Figure 3B). As Bercovich et al. [19] reported earlier, in reactions containing ODC and AZ1, ODC was efficiently degraded; providing a positive control for the assay. AZI combined with AZ1 was used as a negative control, and the protein remained undegraded (Figure 3B). We concluded that the interaction between ODCp and AZ1 does not address ODCp itself to degradation. Instead, the function of ODCp binding to AZ1 is to inhibit the effects of AZ1 on ODC degradation and activity. Consequently, ODCp activates ODC and inhibits its degradation, which emphasizes the role of ODCp as an AZI. The corresponding functional interactions between ODCp and AZ2 or AZ3 remain to be elucidated.

Degradation of ODCp is ubiquitin-dependent

AZI is degraded via the ubiquitin–proteasome pathway, as are most of the cellular proteins [18], whereas ODC is degraded in an ubiquitin-independent manner mediated by AZ1 [20]. To investigate whether ODCp is degraded by ubiquitination, we conducted an ubiquitin conjugation assay with *in vitro*-translated ^{35}S-labelled proteins. As ubiquitin conjugation plays no part in the turnover of ODC, it was used as a negative control. The use of ODC also monitored the presence of endogenous AZ1 in the lysate. The amounts of both ODCp and AZI diminished as the assay progressed (Figure 4A). As expected, ODC remained undegraded: this also verifies that the reticulocyte lysate itself does not contain enough AZ1 to induce the degradation of ODC at a selected time-scale. It should be noticed that ODC can also be degraded without AZ [21], although this happens at a lower rate and thus was not significant in the assay employed. We deduced that ubiquitin conjugation stimulated the degradation of ODCp in reticulocyte lysate. To further confirm this observation, we repeated the ubiquitin conjugation assay in the presence of proteasome inhibitors to see whether high-molecular-mass ubiquitin conjugates of ODCp accumulated in the reaction mixture. Ubiquitinated ODCp was clearly observed (Figure 4B). As expected, AZI was also ubiquitin-conjugated, whereas ODC remained unubiquitinated. This ultimately demonstrated that ODCp degradation is ubiquitin-dependent, as is the degradation of AZI. The finding also demonstrated that ODCp and AZI have

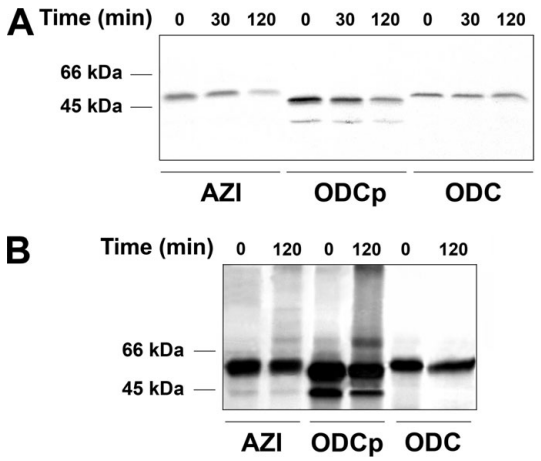

Figure 4 Degradation of ODCp is ubiquitin-dependent

(**A**) Ubiquitin conjugation assay of ODCp used to test the degradation of ODCp. *In vitro*-translated AZI, ODCp and ODC were incubated in a buffer containing the complete ubiquitin-conjugating system. The samples were fractionated by SDS/PAGE and fluorographed. (**B**) Ubiquitin-conjugation assay with proteasome inhibitors. ^{35}S-labelled AZI, ODCp and ODC were incubated in an ubiquitin-conjugating buffer with MG-132 and clasto-lactacystin β-lactone. Ubiquitination of equal-sized aliquots was determined by SDS/PAGE and fluorography.

similar impacts on the regulation of ODC activity and ODC degradation. This result together with the degradation assays really proves that human ODCp functions as an AZI. We conclude that human ODCp counteracts AZ1 as efficiently as AZI both *in vitro* and *in vivo*, and thus represents a novel AZI.

ODCp does not function as a mammalian ADC

To examine whether ODCp displays L-arginine decarboxylating activity, we used the ADC assay developed by Zhu et al. [4]. As the conditions of the assay were quite extreme (30 °C, pH 8.25), we repeated the assay also under more physiological conditions (37 °C, pH 7.4). Under neither conditions was ADC activity detected with *in vitro*-translated ODCp or ODC (Figure 5A). *E. coli* ADC was used as a positive control, and as shown in Figure 5(A), the assay performed better in pH 7.4 at 37 °C. To investigate the ADC activity of ODCp also *in vivo*, we transfected COS-7 cells with FLAG-tagged ODCp, ODC or empty p3xFLAG-CMV-10 vector. As the ADC activity in rodents was originally measured from mitochondria [22–24], we separated cytosolic and crude mitochondrial fractions from transfectants and measured the ADC activity of them under conditions mentioned above. We also confirmed with anti-FLAG Western blot that the transfectants expressed the fusion proteins (results not shown). No ADC activity was, however, detected in ODCp transfectants under either conditions of the assay (Figure 5B). As already known, ODC is able to release small quantities of $^{14}CO_2$ from L-arginine converted into ornithine [25], demonstrating that the sensitivity of the assay was sufficient. ADC of *E. coli* was used as a positive control to show that the assay conditions were permissive for ADC activity.

To further investigate the L-arginine decarboxylating ability of ODCp, we used LC and MALDI–TOF-MS (matrix-assisted laser desorption ionization–time-of-flight MS) to quantify agmatine from ODCp-transfected COS-7 cells. No generation of agmatine was detected in ODCp-expressing COS-7 cells and the sample was negative for agmatine (*m*/*z* 131.1291). The limit of detection for agmatine in the spiked sample was 1.5 nmol/ml. The procedure easily measured agmatine from COS-7 cells cultured for 2 h in the presence of 10 mM agmatine, used as a positive control. The

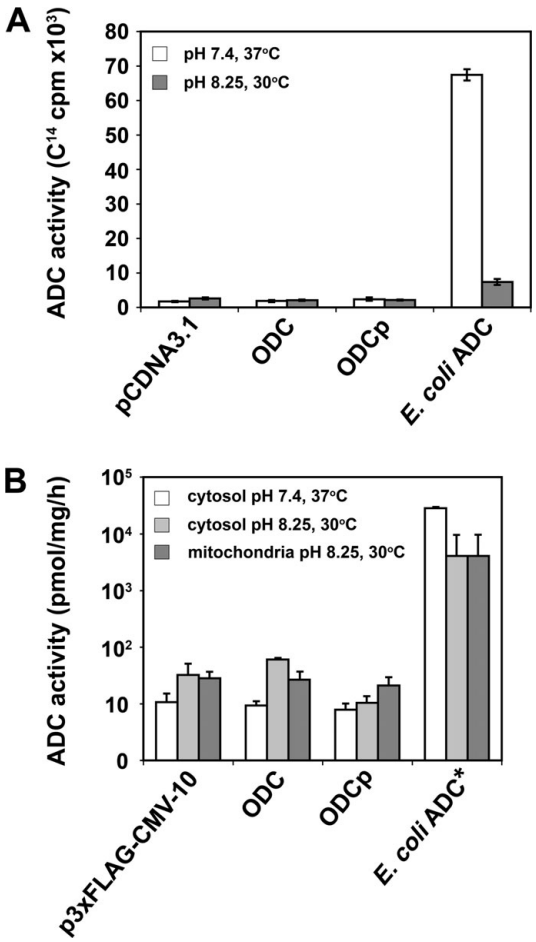

Figure 5 Human ODCp has no intrinsic ADC activity

(**A**) The ADC activity measurement of *in vitro* translation products. ODC, ODCp and the empty pCDNA3.1 vector were *in vitro* translated and the ADC activity of the samples was determined in pH 7.4 at 37 °C or in pH 8.25 at 30 °C. *E. coli* ADC was used as a positive control. Under both conditions, the experiment was performed in triplicate and the error bars refer to the S.E.M. (**B**) Activity of ADC in COS-7 cells expressing FLAG–ODC, FLAG–ODCp or the empty p3xFLAG-CMV-10 vector. Cytosolic and crude mitochondrial fractions of the transfectants were obtained as described in the Experimental section and the ADC activity was measured in pH 7.4 at 37 °C or in pH 8.25 at 30 °C. The measurement was performed in triplicate and the error bars refer to the S.E.M. Note the logarithmic scale on the vertical axis. *The activity of *E. coli* ADC, serving as a positive control, is indicated in C-14 c.p.m. · unit^{-1} · h^{-1}.

theoretically accurate mass of protonated agmatine is 131.1291, and the mass accuracy of the sample analysed was 0.9 mDa. The agmatine concentration of the sample was approx. 0.15 μmol/ml. For comparison, elevated levels of polyamines were readily detected from ODC-transfected COS-7 cells (results not shown).

As the elusive mammalian ADC activity has been suggested to localize in mitochondria [22–24], we also investigated whether expressed FLAG–ODCp travels to the mitochondria. Double immunofluorescent stainings with anti-FLAG and MitoTracker in COS-7 and Paju cells (a human neural crest-derived cell line) transfected with FLAG–ODCp cDNA did not reveal any co-localization (results not shown).

Iyo et al. [26] reported decreased agmatine concentrations in rat neurons transfected with siRNA (small interfering RNA) directed against ODCp. Our present results indicate the binding of ODCp to AZ1–3, which can lead to a decrease in the pool of free AZs known to enhance the polyamine uptake [27,28]. Since agmatine is transported by the same mechanism [29,30],

overexpression of ODCp might lead to an increase in agmatine content. Accordingly, down-regulation of ODCp might decrease the content of agmatine. It is thus likely that ODCp influences agmatine concentrations via AZs without any intrinsic ADC activity. The term ADC should now be removed from sequence data banks, since the combined knowledge demonstrates that ODCp has no intrinsic arginine-decarboxylating activity. Instead, ODCp is a new member of the AZI family. We agree with López-Contreras et al. [8] that ODCp should be replaced by the term AZIN2 (antizyme inhibitor 2). AZI could thus be renamed as AZIN1 (antizyme inhibitor 1).

ODC and AZIN1 are known to be expressed in all types of cells, whereas the expression of ODCp/AZIN2 is predominant in brain and testis. The differences in the localization of the two AZI species suggest some functional difference. Assuming that both of these molecules, AZIN1 and ODCp/AZIN2, are expressed in the same cell, it is intriguing why some cells need such multilevel regulation of ODC activity. It is possible that the function of ODCp/AZIN2 is restricted to certain subcellular locations in highly specialized cells in brain and testis.

We thank Tiiu Arumäe, Anu Harju and Anna Wilenius for technical assistance. This work was supported by grants from the Academy of Finland, the Finnish Cancer Organisations, the Sigrid Jusélius Foundation and the Research Foundation of the University of Helsinki.

REFERENCES

1 Pitkanen, L. T., Heiskala, M. and Andersson, L. C. (2001) Expression of a novel human ornithine decarboxylase-like protein in the central nervous system and testes. Biochem. Biophys. Res. Commun. **287**, 1051–1057
2 Heiskala, M., Zhang, J., Hayashi, S., Holtta, E. and Andersson, L. C. (1999) Translocation of ornithine decarboxylase to the surface membrane during cell activation and transformation. EMBO J. **18**, 1214–1222
3 Kidron, H., Repo, S., Johnson, M. S. and Salminen, T. A. (2007) Functional classification of amino acid decarboxylases from the alanine racemase structural family by phylogenetic studies. Mol. Biol. Evol. **24**, 79–89
4 Zhu, M. Y., Iyo, A., Piletz, J. E. and Regunathan, S. (2004) Expression of human arginine decarboxylase, the biosynthetic enzyme for agmatine. Biochim. Biophys. Acta **1670**, 156–164
5 Martin-Tanguy, J. (2001) Metabolism and function of polyamines in plants: recent development (new approaches). Plant Growth Regul. **34**, 135–148
6 Nakada, Y. and Itoh, Y. (2003) Identification of the putrescine biosynthetic genes in *Pseudomonas aeruginosa* and characterization of agmatine deiminase and *N*-carbamoylputrescine amidohydrolase of the arginine decarboxylase pathway. Microbiology **149**, 707–714
7 Coleman, C. S., Hu, G. and Pegg, A. E. (2004) Putrescine biosynthesis in mammalian tissues. Biochem. J. **379**, 849–855
8 Lopez-Contreras, A. J., Lopez-Garcia, C., Jimenez-Cervantes, C., Cremades, A. and Penafiel, R. (2006) Mouse ornithine decarboxylase-like gene encodes an antizyme inhibitor devoid of ornithine and arginine decarboxylating activity. J. Biol. Chem. **281**, 30896–30906
9 Ivanov, I. P., Gesteland, R. F. and Atkins, J. F. (1998) A second mammalian antizyme: conservation of programmed ribosomal frameshifting. Genomics. **52**, 119–129
10 Ivanov, I. P., Rohrwasser, A., Terreros, D. A., Gesteland, R. F. and Atkins, J. F. (2000) Discovery of a spermatogenesis stage-specific ornithine decarboxylase antizyme: antizyme 3. Proc. Natl. Acad. Sci. U.S.A. **97**, 4808–4813
11 Zhu, C., Lang, D. W. and Coffino, P. (1999) Antizyme2 is a negative regulator of ornithine decarboxylase and polyamine transport. J. Biol. Chem. **274**, 26425–26430
12 Mangold, U. and Leberer, E. (2005) Regulation of all members of the antizyme family by antizyme inhibitor. Biochem. J. **385**, 21–28
13 Murakami, Y., Ichiba, T., Matsufuji, S. and Hayashi, S. (1996) Cloning of antizyme inhibitor, a highly homologous protein to ornithine decarboxylase. J. Biol. Chem. **271**, 3340–3342
14 Nilsson, J., Grahn, B. and Heby, O. (2000) Antizyme inhibitor is rapidly induced in growth-stimulated mouse fibroblasts and releases ornithine decarboxylase from antizyme suppression. Biochem. J. **346**, 699–704
15 Lin, Y., Martin, J., Gruendler, C., Farley, J., Meng, X., Li, B. Y., Lechleider, R., Huff, C., Kim, R. H., Grasser, W. A., Paralkar, V. and Wang, T. (2002) A novel link between the proteasome pathway and the signal transduction pathway of the bone morphogenetic proteins (BMPs). BMC Cell Biol. **3**, 15
16 Newman, R. M., Mobascher, A., Mangold, U., Koike, C., Diah, S., Schmidt, M., Finley, D. and Zetter, B. R. (2004) Antizyme targets cyclin D1 for degradation. A novel mechanism for cell growth repression. J. Biol. Chem. **279**, 41504–41511
17 Li, X. and Coffino, P. (1993) Degradation of ornithine decarboxylase: exposure of the C-terminal target by a polyamine-inducible inhibitory protein. Mol. Cell. Biol. **13**, 2377–2383
18 Bercovich, Z. and Kahana, C. (2004) Degradation of antizyme inhibitor, an ornithine decarboxylase homologous protein, is ubiquitin-dependent and is inhibited by antizyme. J. Biol. Chem. **279**, 54097–54102
19 Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. and Kahana, C. (1989) Degradation of ornithine decarboxylase in reticulocyte lysate is ATP-dependent but ubiquitin-independent. J. Biol. Chem. **264**, 15949–15952
20 Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature **360**, 597–599
21 Murakami, Y., Matsufuji, S., Tanaka, K., Ichihara, A. and Hayashi, S. (1993) Involvement of the proteasome and antizyme in ornithine decarboxylase degradation by a reticulocyte lysate. Biochem. J. **295**, 305–308
22 Li, G., Regunathan, S., Barrow, C. J., Eshraghi, J., Cooper, R. and Reis, D. J. (1994) Agmatine: an endogenous clonidine-displacing substance in the brain. Science. **263**, 966–969
23 Lortie, M. J., Novotny, W. F., Peterson, O. W., Vallon, V., Malvey, K., Mendonca, M., Satriano, J., Insel, P., Thomson, S. C. and Blantz, R. C. (1996) Agmatine, a bioactive metabolite of arginine. Production, degradation, and functional effects in the kidney of the rat. J. Clin. Invest. **97**, 413–420
24 Regunathan, S. and Reis, D. J. (2000) Characterization of arginine decarboxylase in rat brain and liver: distinction from ornithine decarboxylase. J. Neurochem. **74**, 2201–2208
25 Osterman, A. L., Kinch, L. N., Grishin, N. V. and Phillips, M. A. (1995) Acidic residues important for substrate binding and cofactor reactivity in eukaryotic ornithine decarboxylase identified by alanine scanning mutagenesis. J. Biol. Chem. **270**, 11797–11802
26 Iyo, A. H., Zhu, M. Y., Ordway, G. A. and Regunathan, S. (2006) Expression of arginine decarboxylase in brain regions and neuronal cells. J. Neurochem. **96**, 1042–1050
27 Mitchell, J. L., Judd, G. G., Bareyal-Leyser, A. and Ling, S. Y. (1994) Feedback repression of polyamine transport is mediated by antizyme in mammalian tissue-culture cells. Biochem. J. **299**, 19–22
28 Sakata, K., Fukuchi-Shimogori, T., Kashiwagi, K. and Igarashi, K. (1997) Identification of regulatory region of antizyme necessary for the negative regulation of polyamine transport. Biochem. Biophys. Res. Commun. **238**, 415–419
29 del Valle, A. E., Paz, J. C., Sanchez-Jimenez, F. and Medina, M. A. (2001) Agmatine uptake by cultured hamster kidney cells. Biochem. Biophys. Res. Commun. **280**, 307–311
30 Satriano, J., Isome, M., Casero, Jr, R. A., Thomson, S. C. and Blantz, R. C. (2001) Polyamine transport system mediates agmatine transport in mammalian cells. Am. J. Physiol. Cell Physiol. **281**, C329–C334

Received 26 July 2007/20 September 2007; accepted 27 September 2007
Published as BJ Immediate Publication 27 September 2007, doi:10.1042/BJ20071004