ODCp, a brain- and testis-specific ornithine decarboxylase paralogue, functions as an antizyme inhibitor, although less efficiently than AzI1

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INTRODUCTION

Polyamines are natural polycations that are essential for various cellular processes including cellular proliferation [1–4]. Intracellular polyamine levels are tightly regulated, since at the lower limit their depletion leads to growth arrest [2,4–6] and at the upper limit they exert a cytotoxic effect [7–9]. Multiple mechanisms, including synthesis, catabolism and transport, regulate cellular polyamine levels.

In mammalian cells, both polyamine synthesis and polyamine transport are part of an autoregulatory circuit mediated by a polyamine-induced protein termed Az (antizyme). Az is synthesized from two open reading frames via a polyamine-stimulated ribosomal frameshifting [10,11]. Az binds and inactivates ODC (ornithine decarboxylase), the first and rate-limiting enzyme in the polyamine biosynthesis pathway. Az then facilitates the recognition of ODC by the 26S proteasome, leading to its rapid ubiquitin-independent degradation [12–15]. Increased intracellular Az concentration also decreases polyamine uptake and stimulates polyamine excretion via a yet uncharacterized mechanism [16,17].

There are several forms of Az, Az1 being the most abundant one. While all forms are capable of inhibiting ODC [18], it seems that Az2 is incapable of promoting ODC degradation [19]. Az3 expression is restricted to the testis and, to a lesser extent, to the brain [20–22].

An ODC-related protein was demonstrated to inhibit Az functions. This protein, termed AzI (Az inhibitor), is highly homologous with ODC but retains no ornithine-decarboxylating activity [23]. AzI binds Az with higher affinity than ODC and, as a result, it is capable of rescuing ODC from interaction with Az and from degradation [23,24]. Several lines of evidence suggest that AzI has a physiological role in regulating polyamine metabolism and cellular growth. AzI mRNA increases rapidly following growth stimulation of quiescent cells [24]. siRNA (small interfering RNA) to AzI reduces ODC activity and cellular proliferation [25], whereas AzI overexpression increases ODC activity, polyamine uptake and cell proliferation [26,27]. The human AzI gene is found at a chromosomal location that is amplified in several tumours [28,29], and AzI overexpression was demonstrated in gastric tumours [30]. In line with having a regulatory role, AzI has a very short half-life [31]. Interestingly, in contrast with the ubiquitin-independent degradation of ODC that is mediated by Az, AzI degradation is ubiquitin-dependent and is inhibited by Az [31]. AzI disrupts the interaction between all four forms of mammalian antizyme and ODC [32].

Recently, another ODC homologous protein was identified in humans and mice and it was termed ODCp (ODC paralogue) [21,33]. mRNA for ODCp was found in testis and brain, compatible with the expression pattern of Az3. Studies with both mouse and human ODCp showed that they lack ornithine- and arginine-decarboxylating activity and that they can rescue ODC from degradation. It was therefore suggested that ODCp functions as AzI and it was named AzI2 [33,34]. Based on in vitro degradation studies, it was demonstrated that, like AzI, ODCp is also degraded in a ubiquitin-dependent manner [34].

In the present study, we further substantiated the suggestion that ODCp functions as AzI. We demonstrate that ODCp is degraded via the ubiquitin system, as its degradation is inhibited by inactivation of a thermosensitive ubiquitin-activating enzyme, E1. We also show that Az inhibits ODCp degradation by interfering with its ubiquitination. We demonstrate that although ODCp is capable of inhibiting the Az-mediated degradation of ODC, it does so less efficiently than AzI. Stable overexpression of ODCp is also demonstrated using an in vitro degradation assay. We show that the basis for the inferiority of ODCp as an AzI is its lower affinity towards Az (AzI1 and AzI3). Further, we show here that ODCp, like AzI, is degraded in a ubiquitin-dependent manner, in a reaction that does not require either interaction with Az or the integrity of its C-terminus. Interaction with Az actually stabilizes ODCp by interfering with its ubiquitination. This results in sequestration of Az into a stable complex with ODCp, which is the central feature contributing to the ability of ODCp to function as AzI.

Key words: antizyme inhibitor (AzI), ornithine decarboxylase (ODC), ODC paralogue (ODCp), polyamine, protein degradation, ubiquitin.
in cells increases their ODC activity, their polyamine uptake and their growth rate. However, it does so less efficiently than AzI1. Our results demonstrate that the basis for the inferiority of ODCp as an Az-inhibitory protein is its lower affinity towards Az.

**EXPERIMENTAL**

**Cell lines, culture and transfection conditions**

The cells used in the present study were: HEK-293 cells (human embryonic kidney cells), NIH 3T3 mouse fibroblasts and A31N-ts20, a Balb/c mice-derived mouse cell line that harbours a temperature-sensitive ubiquitin-activating enzyme (E1) [35]. HEK-293 and NIH 3T3 cells were cultured at 37°C in a humidified incubator with 5% CO2, in Dulbecco’s Modified Eagle’s medium containing 10% (v/v) foetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. A31N-ts20 cells were cultured at 32°C and transferred to 39°C for E1 inactivation. HEK-293 cells were transiently transfected by the calcium phosphate method [36]. Stable transfection of NIH 3T3 and A31N-ts20 cells was performed using jetPEI (Polyplus Transfection) according to the manufacturer’s instructions. Stable transfectants were selected for their ability to grow in the presence of puromycin.

**Cloning and plasmids**

Az3 and ODCp were cloned by RT (reverse transcription)–PCR using RNA isolated from mouse testis. AzI1, ODCp, ODC, Az1 and Az3 (the latter two as clones from which one nucleotide was deleted so that they will not require frameshifting for expression), and HA (haemagglutinin)-tagged ubiquitin were amplified by PCR and cloned between the XhoI and NotI sites of the bicistronic vector pEFIRES [37]. HA and FLAG tags were added by PCR.

**Mutagenesis**

Mutations and deletions were introduced using the overlap extension method [38,39]. The sequences of the primers used are available on request.

**Immunoblot analysis**

Cellular extracts were prepared by lysing cells in lysis buffer (50 mM Tris/HCl, pH 8, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT (dithiothreitol) and 1 µg/ml each of leupeptin, aprotinin and pepstatin). Insoluble material was removed by centrifugation. Equal amounts of protein were mixed with Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.123 M Tris/HCl, pH 6.8), heated for 5 min at 95°C, and fractionated by SDS/PAGE. The resolved proteins were electroblotted on to a nitrocellulose membrane. The membrane was incubated with the indicated antibodies followed by horseradish peroxidase-conjugated anti-IgG antibodies. The antibodies used were: mouse monoclonal anti-FLAG, anti-ODC, anti-actin and anti-HA (Sigma), and rabbit polyclonal anti-Az [13]. Signals were quantified using ImageJ software.

**In vivo degradation assay**

Chase was initiated by the addition of cycloheximide (20 µg/ml) to the growth medium of transiently transfected cells or cells stably expressing the proteins of interest, and the cells were harvested at the indicated times thereafter. Cells were lysed and aliquots containing equal amounts of protein were fractionated by SDS/PAGE and examined by Western-blot analysis.

**In vivo ubiquitin conjugation assay**

At 24 h after co-transfection with constructs encoding the FLAG-tagged tested protein and HA–ubiquitin, the cells were treated for 4 h with 100 µM MG-132 (the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-leucinal), and cellular extracts were prepared. Aliquots containing equal amounts of proteins were immunoprecipitated with anti-FLAG beads (Sigma). The immunoprecipitated material was resolved by SDS/PAGE, transferred on to nitrocellulose and probed with anti-HA antibodies.

**In vitro translation and degradation assay**

The tested proteins were translated in vitro for 1 h at 30°C using TNT reaction mix (Promega) in the presence of [35S]methionine and incubated in a reticulocyte lysate-based degradation mixture [40 mM Tris/ HCl, pH 7.5, 5 mM MgCl2, 2 mM DTT, 0.5 mM ATP, 10 mM phosphocreatine (Sigma), 1.6 mg/ml creatine kinase (Roche Applied Science) and 6 µl of reticulocyte lysate (Promega)] at 37°C for the indicated times. When required, the molar amounts of the tested proteins were normalized by dividing the radioactivity of the relevant band by the number of their methionine residues. At the end of the incubation period, the proteins were resolved by SDS/PAGE (12.5% gel) and the radioactivity of the individual proteins was determined using a Fuji Bas2500 phosphoimager.

**Determination of growth rate**

Cells were plated in 12-well plates in a medium containing 10% foetal bovine serum. At the indicated times, cells were trypsinized and counted using a Bright Line Counting Chamber (Hauser Scientific, Horsham, PA, U.S.A.).

**Determination of ODC activity**

ODC activity assay was performed as previously described [40]. Briefly, 100 µg portions of cellular extracts were mixed with 100 µl of ODC buffer (25 mM Tris/HCl, pH 7.5, 2.5 mM DTT, 0.1 mM EDTA, 0.2 mM pyridoxal phosphate and 0.33 mM L-ornithine), containing 0.5 µCi L-[14C]ornithine. The reaction mixture was incubated at 37°C in a 96-well plate for 4 h. The liberated [14C]CO2 was trapped in a covering 3-mm paper soaked with saturated barium hydroxide solution. The paper was washed with acetone, dried and the results were quantified using a Fuji Bas2500 phosphoimager.

**Determination of polyamine uptake**

Cells (2 × 104) were plated on to a 96-well plate. After 24 h, the cells were washed with Hanks balanced salt solution and incubated for 15 min with 50 µl of 5 µM [3H]spermidine (36 Ci/mmole) suspended in the same solution. The cells were then washed three times and their protein content was determined using a protein assay kit from Bio-Rad. The cells were dissolved in formic acid and transferred into vials containing 3 ml of UltimaGold LCS cocktail and radioactivity was determined using a liquid-scintillation counter.
RESULTS

ODCp is degraded by the proteasome without requiring its C-terminus or Az binding

A human ODC-like protein, originally identified as an ODCp specific for the central nervous system and testis [33], was recently proposed to be a novel Az1 (AzI2) [21,34]. We have previously shown that AzI is rapidly degraded by the proteasome in a ubiquitin-dependent manner [31]. To determine the stability of ODCp, we have generated an NIH 3T3-derived cell line stably overproducing ODCp. An NIH 3T3 cell line overexpressing AzI served as a control [31]. Cellular extracts were prepared at various times following the addition of cycloheximide to the growth medium and the levels of ODCp and AzI were determined by Western-blot analysis. Figure 1(A) shows that ODCp is degraded by the proteasome, as its degradation is inhibited by the proteasome inhibitor MG-132. Interestingly, ODCp is less labile than AzI.

ODC requires its C-terminal destabilizing segment and its Az binding segment for degradation [12,41–44]. We have recently demonstrated that unlike ODC, the degradation of AzI occurs efficiently without its C-terminal segment and without its putative Az-binding site [31]. To determine whether the parallel segments of ODCp are required for its degradation, two mutants were prepared, one lacking the C-terminal 40 amino acids and the second with a deletion of amino acids 118–141 that correspond to the putative Az-binding site of ODC (amino acids 117–140) [12]. To determine their stability, these proteins were transiently expressed in HEK-293 cells, cycloheximide was added after 24 h, and cellular extracts prepared at the indicated times were analysed by Western-blot analysis. Both mutants were rapidly degraded (Figures 1B and 1C), demonstrating that like the degradation of AzI, that of ODCp is also independent of the C-terminus and does not require Az binding.

ODCp is degraded in a ubiquitin-dependent manner in a reaction inhibited by Az

The above results demonstrated the similarity of ODCp degradation to that of AzI and prompted us to test whether like AzI [31], ODCp is also degraded in a ubiquitin-dependent manner. To this end, we have utilized A31N-ts20 cells harbouring a thermosensitive ubiquitin-activating enzyme, E1 [35], to establish cell lines stably overproducing ODCp or AzI. These cells were maintained either at the permissive temperature (32°C) or transferred to the non-permissive temperature (39°C) for 24 h. Cellular extracts were prepared and the levels of ODCp and AzI were determined by Western-blot analysis. Similarly to AzI, ODCp accumulated at the non-permissive temperature (Figure 2A), suggesting that the degradation of ODCp is ubiquitin-dependent.

We have previously demonstrated that not only is AzI not required for AzI1 degradation, but also that AzI1 is actually stabilized by AzI1 [31]. To determine whether AzI exerts a similar effect on ODCp, ODCp was transiently expressed either alone or together with AzI in HEK-293 cells, and its stability was determined. As shown in Figure 2(B), ODCp was stabilized when co-expressed with AzI1. Az3, which was first identified as a spermatogenesis stage-specific novel Az [45], was recently described also in the brain [21]. ODCp is expressed specifically in the central nervous system and testis [33]. Since ODCp and Az3 are expressed in the same tissues, it is feasible that there is a specific interaction between these two proteins. However, it was demonstrated that AzI1 can interact with AzI1, Az2 and Az3 and is able to disrupt the interaction between each of these Az forms and ODC [32]. We therefore set out to examine the effect of Az3 on the degradation of ODCp and AzI. For this purpose, ODCp and AzI were transiently expressed in HEK-293 cells alone or together with AzI1. Cycloheximide (CHX) was added for the indicated times and the levels of the tested proteins were determined by Western-blot analysis using anti-FLAG, anti-Az and anti-actin antibodies. Each of the above experiments was repeated three times, yielding similar results.
Az1 (serum control) and HA–ubiquitin were co-expressed in HEK-293 cells with or without Az1. Cellular extracts were prepared and immunoprecipitated with anti-FLAG antibodies. The precipitated material was resolved by electrophoresis and probed with anti-HA antibodies. As shown in Figure 2(D), Az inhibited the conjugation of ubiquitin to both ODCp and Azl. Since Az1 did not inhibit conjugation of ubiquitin to the mutant ODCp lacking its Az-binding site, together with HA-tagged ubiquitin and in the presence or absence of Az. Cellular extracts were prepared 24 h post-transfection, and subjected to immunoprecipitation with anti-FLAG antibodies. The immunoprecipitated material was fractionated by SDS/PAGE, blotted to nitrocellulose and probed with anti-HA antibodies (upper panel). In parallel, total cellular extract was subjected to Western-blot analysis and probed with anti-FLAG, anti-Az or anti-actin antibodies (the three lower panels respectively). Each of these experiments was repeated at least twice, yielding similar results.

An ODCp chimaera containing the C-terminal degradation signal of ODC is still stabilized by Azl

Despite the high homology between ODC and Az1, they differ in their mechanism of degradation [31]. Our results support a recent study that demonstrated that ODCp, which is highly homologous with both ODC (48% identity) and Az1 (44% identity) [21], is degraded via the ubiquitin system [34]. The Az-dependent degradation of ODC requires interaction with Azl [12], as well as the C-terminal degradation segment that serves as the proteasome-recognition signal [46], which is exposed by Azl [47]. Since ODCp binds Azl, but its C-terminus differs from that of ODC, we next tested whether the degradation of ODCp can be subverted to the Az-dependent pathway by replacing its C-terminus with that of ODC. The chimaera ODCp–ODC was expressed in HEK-293 cells alone or together with Azl, and its stability was determined. As reported for the Azl–ODC chimaera [31], the ODCp–ODC chimaera also remained a substrate of the ubiquitin system, as reflected by its stabilization by co-expressed Azl (Figure 3).

ODCp provides cells with growth advantage, but to a lesser degree than Azl

Having demonstrated that ODCp resembles Azl in its mode of degradation, we next set out to determine whether ODCp can in fact function as Azl. We have previously demonstrated that Azl has two distinct functions. It saves ODC from Azl-induced degradation and it increases polyamine uptake [26]. Both effects are an outcome of its ability to bind to Azl and neutralize its functions, and together they provide cells with a growth advantage. In order to determine whether ODCp has the ability to provide cells with a growth advantage, we have established an NIH 3T3-derived cell line stably overexpressing ODCp (NIH 3T3-ODCp) (Figure 4A). These ODCp-overexpressing cells displayed elevated ODC and polyamine uptake activity as compared with control cells stably transfected with an empty vector (NIH 3T3-E) (Figures 4B and 4C). Although this was a significant increase (P < 0.08 for ODC and P < 0.0001 for uptake, as determined by t test), it was less profound than that observed in cells stably overexpressing Azl (NIH 3T3-Azl). In agreement, overexpressed ODCp provided a clear growth advantage, although to a lesser extent than Azl-overproducing cells (Figure 4D). Altogether our

Figure 2 The degradation of ODCp is ubiquitin-dependent and is inhibited by both Az1 and Azl

(A) Constructs encoding Azl and ODCp were stably transfected into A31N-ts20 cells that contain a thermosensitive ubiquitin-activating enzyme, E1. Extracts were prepared from cells grown at the permissive temperature (32°C) and from cells grown for 24 h at the restrictive temperature (39°C). The levels of the tested proteins were determined by Western-blot analysis using anti-FLAG and anti-actin antibodies. (B, C) FLAG-tagged Azl and ODCp were transfected into HEK-293 cells together with Azl (B) or FLAG-tagged Az1 (C). Cycloheximide (CHX) was added 24 h post-transfection, cellular extracts were prepared at the indicated times, and the levels of the tested proteins were determined by Western-blot analysis using anti-FLAG, anti-Az and anti-actin antibodies. (D) HEK-293 cells were transfected with FLAG-tagged Az1, ODCp and a mutant ODCp lacking the Az-binding site, together with HA–ubiquitin and in the presence or absence of Az. Cellular extracts were prepared 24 h post-transfection, and subjected to immunoprecipitation with anti-FLAG antibodies. The immunoprecipitated material was fractionated by SDS/PAGE, blotted to nitrocellulose and probed with anti-HA antibodies (upper panel). In parallel, total cellular extracts were subjected to Western-blot analysis and probed with anti-FLAG, anti-Az or anti-actin antibodies (the three lower panels respectively). Each of these experiments was repeated at least twice, yielding similar results.
ODCp protects ODC from Az-stimulated degradation less efficiently than AzI1 due to its lower affinity towards Az

Our results demonstrated that although it functions as AzI, ODCp is less potent than AzI1. In order to understand the basis for this difference, we compared the ability of ODCp and AzI1 to inhibit Az-stimulated ODC degradation in vitro. Using a similar assay, it was recently reported [34] that both proteins display a comparable ability to negate Az and stabilize ODC. As we show here, ODCp was less efficient in interfering with Az-stimulated ODC degradation (Figure 5A). It should be noted in this respect that Kanerva et al. [34] used a huge excess of AzI over Az (14:1) compared with the 5:1 ratio used in our study. In agreement with our results, co-immunoprecipitation analysis demonstrated that ODCp interacts with both AzI1 and AzI3 less efficiently than does AzI (Figure 5B).

DISCUSSION

Recent studies have demonstrated that human and mouse cells contain an ODC paralogue termed ODCp [21,34]. Human and mouse ODCp lack both ornithine- and arginine-decarboxylating activity, and its tissue distribution in both species is restricted to brain and testis.

We have previously demonstrated that AzI negates both functions of Az: stimulation of ODC degradation and inhibition of polyamine uptake [31]. Mouse and human ODCp elevate ODC activity by inhibiting ODC degradation both in vitro and in vivo in transiently transfected cells [21,34]. Therefore it was suggested that ODCp acts as an Az inhibitory protein and it was termed AzI2. We show here that stable overexpression of ODCp in NIH 3T3 mouse fibroblasts results in elevated ODC and polyamine uptake activity. Furthermore, as with AzI-overexpressing cells [26,27], overexpression of ODCp also provides cells with a growth advantage. Taken together, these results provide direct and significant evidence that ODCp may indeed play a physiological role in regulating cellular polyamine metabolism and cellular growth and therefore can be termed AzI2.

In order to function as an AzI, it is not enough for AzI2 to bind Az and, as a result, save ODC from Az-stimulated degradation. It is also not sufficient that Az does not induce AzI2 degradation. As we have demonstrated in the case of AzI1, the most important characteristic of a bona fide AzI is its stabilization by Az, which results in sequestration of Az into a stable complex [31]. We show here that, as with AzI1, not only is Az unnecessary for AzI2 degradation, but interaction with Az actually stabilizes AzI2. As we recently demonstrated [34], we show here that AzI2 undergoes ubiquitin-dependent degradation. Moreover, we demonstrate that Az inhibits AzI2 degradation by interfering with its ubiquitination. The inhibition of AzI2 ubiquitination and degradation is directly related to its ability to bind Az, as Az

Results suggest that ODCp is a member of the AzI family and therefore can be named AzI2.
failed to stabilize a mutant AzI2 with compromised Az-binding ability.

Based on the ability of AzI2 to negate Az’s inhibition of ODC activity and promotion of its proteasomal degradation, Kanerva et al. [34] concluded that AzI2 functions as efficiently as AzI1 as an inhibitor of Az functions. We show here that AzI2 is less potent than AzI1 in its Az-inhibitory activity. We demonstrate that stable transfectants expressing AzI2 display lower ODC activity, polyamine uptake and growth rate compared with cells overexpressing even lesser amounts of AzI1. Also, our in vitro degradation studies demonstrated a higher efficiency of AzI1 in protecting ODC from Az-stimulated degradation. We show here that the basis for the functional difference between these two Az-inhibitory proteins is the lower affinity of AzI2 towards Az.

The overlapping expression pattern of AzI2 and Az3 suggests that they are specific partners and therefore it could be expected that they will interact with high affinity. We show here that this is actually not the case, because Az3 binds AzI1 with higher affinity than it binds AzI2. It therefore seems that their specificity might be solely a result of their spatial co-expression.

In light of the lower efficiency of AzI2, it is not clear why both AzI1 and AzI2 are needed. It was recently demonstrated that AzI1 and AzI2 are closely related, and that they were evolved from single gene duplication [48]. While retaining the ubiquitous expression pattern of ODC, AzI1 evolved much higher affinity for Az, thus making it an efficient regulator of Az. In contrast, AzI2 retained the relatively lower affinity for Az, but probably evolved a means for regulated expression, restricting it to testis and brain, paralleling the expression pattern of Az3. This possibly provides an additional level of regulation that is specific for Az3. It will be of interest to determine the precise expression pattern of Az3 and AzI2 and determine the underlying mechanism.

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