Amino Acids (2012) 42:539–547
DOI 10.1007/s00726-011-1031-y

ORIGINAL ARTICLE

Differential expression of ornithine decarboxylase antizyme inhibitors and antizymes in rodent tissues and human cell lines

Bruno Ramos-Molina · Andrés J. López-Contreras · Asunción Cremades · Rafael Peñafiel

Received: 17 March 2011 / Accepted: 30 May 2011 / Published online: 4 August 2011

Abstract Ornithine decarboxylase antizyme inhibitors, AZIN1 and AZIN2, are regulators and homologous proteins of ornithine decarboxylase (ODC), the rate limiting enzyme in the biosynthesis of polyamines. In this study, we have examined by means of real-time RT-PCR the relative abundance of mRNA of the three ODC paralogs in different rodent tissues, as well as in several cell lines derived from human tumors. With the exception of mouse and rat testes, ODC mRNA was the most expressed gene in all tissues examined (values higher than 60%). AZIN2 was more expressed than AZIN1 in testis, epididymis, brain, adrenal gland and lung, whereas the opposite was found in liver, kidney, heart, intestine and pancreas, as well as in all the cell lines examined. mRNA abundance of the three antizymes (AZ1, AZ2 and AZ3) that interact with ODC and antizyme inhibitors was also analyzed. AZ1 and AZ2 mRNA were ubiquitously expressed, AZ1 mRNA being more abundant than that of AZ2, although the ratio was dependent on the mouse tissue. In carcinoma-derived cells AZ1 was more expressed than AZ2, whereas in neuroblastoma-derived cells AZ2 mRNA was much more abundant than that of AZ1. AZ3 was expressed exclusively in rodent testes, where it was the most abundant of the three antizymes (~80%). This study is the first comparative-quantitative analysis on the expression of antizymes and antizyme inhibitors in different types of mammalian cells.

Keywords Antizymes · Antizyme inhibitor · AZIN2 · Ornithine decarboxylase · Polyamines · RT-PCR

Abbreviations

AZ1	Antizyme 1
AZ2	Antizyme 2
AZ3	Antizyme 3
AZs	Antizymes
AZIN1	Antizyme inhibitor 1
AZIN2	Antizyme inhibitor 2
AZINs	Antizyme inhibitors
DMEM	Dulbecco's modified Eagle medium
ERGIC	Endoplasmic reticulum–Golgi intermediate compartment
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
MMLV	Moloney murine leukemia virus
ODC	Ornithine decarboxylase
ORF	Open reading frame
RT-PCR	Reverse transcription polymerase chain reaction

Introduction

Polyamines are ubiquitous small basic molecules that are essential for normal as well as neoplastic cell proliferation (Thomas and Thomas 2001; Gerner and Meyskens 2004). Cellular polyamine contents are tightly regulated by different processes that include polyamine biosynthesis, catabolism, uptake and excretion (Pegg 2009). In mammals, polyamines act as regulators of both their biosynthesis and

B. Ramos-Molina · A. J. López-Contreras · R. Peñafiel (✉)
Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Medicine, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain
e-mail: rapegar@um.es

A. Cremades
Department of Pharmacology, Faculty of Medicine, University of Murcia, Murcia, Spain

uptake by stimulating the synthesis of a family of small proteins termed antizymes (AZs), formed by at least three different members, named AZ1, AZ2 and AZ3 (Mangold 2005). AZ1, the first characterized AZ, binds and inhibits ornithine decarboxylase (ODC), the first and rate limiting enzyme in the polyamine biosynthetic pathway, and directs ODC to the degradation by the proteasome, through a process independent of ubiquitination (Hayashi et al. 1996; Coffino 2001). The other two antizymes also bind and inhibit ODC, but apparently only AZ2 promotes ODC degradation (Snapir et al. 2009). The three AZs also inhibit polyamine uptake by affecting a not yet well-characterized mammalian transport system (Mangold 2005). Whereas AZ1 and AZ2 are ubiquitously expressed, AZ3 is testis-specific (Ivanov et al. 2000; Tosaka et al. 2000). AZ synthesis is mainly controlled at the translational level by a ribosomal frameshifting mechanism stimulated by polyamines (Rom and Kahana 1994; Matsufuji et al. 1995), which is conserved in the three types of AZs (Ivanov et al. 1998, 2000). AZs are themselves regulated by ODC-related proteins known as antizyme inhibitors (AZINs), which bind to AZs and negate the inhibitory effects of the AZ on ODC and polyamine uptake (Mangold 2006; Kahana 2009; Lopez-Contreras et al. 2010). The first AZIN characterized (Fujita et al. 1982), now known as AZIN1, is considered as an important regulator of polyamine metabolism and cell growth (Mangold 2006; Kahana 2009). In fact, mice with homozygous AZIN1 gene disruption died on the day of birth with abnormal liver morphology (Tang et al. 2009). AZIN2 is mostly expressed in testes (Pitkanen et al. 2001; Lopez-Contreras et al. 2006), and more specifically in the haploid germinal cells, which suggests that it may have a role in spermiogenesis (Lopez-Contreras et al. 2009a). Despite the high homology of AZINs to ODC, they lack enzymatic activity (Murakami et al. 1996; Lopez-Contreras et al. 2006), and unlike ODC they are degraded in a ubiquitin-dependent manner in a reaction inhibited by AZs (Bercovich and Kahana 2004; Snapir et al. 2008).

Apart from the established role of AZs and AZINs in the regulation of polyamine metabolism, several studies have provided evidence that these proteins may interact with other cellular proteins such as cyclin D1 and Aurora-A, two growth-related proteins (Newman et al. 2004; Kim et al. 2006; Lim and Gopalan 2007). These findings, together with the reported localization of AZ1 and AZIN1 at the centrosomes (Mangold et al. 2008; Murakami et al. 2009; Kasbek et al. 2010) reinforce the relevance that AZs and AZINs may have in cell division. In addition, recent data showing that AZIN2 is located on specific mast secretory cells and neurons (Kanerva et al. 2009; Mäkitie et al. 2010) open the possibility that this protein may participate in the modulation of secretory pathways (Kanerva et al. 2010).

Most of the studies dealing with the expression of AZINs and AZs have determined mRNA levels using Northern blot or dot-blot analyses. Since in some tissues the levels of certain messengers could be very low, in this work we have analyzed mRNA abundance of AZIN1 and AZIN2 using real-time reverse transcription polymerase chain reaction (RT-PCR), in order to quantify more precisely their levels in different mouse tissues and cell lines derived from human tumors. In addition, AZ mRNAs were also evaluated and compared with those of AZINs, with the aim to get a better understanding of the tissue expression of all these related genes.

Materials and methods

Materials

Moloney murine leukemia virus (MMLV) reverse transcriptase, RNAlater®, RNase free water and GenElute mammalian total RNA Miniprep kit were purchased from Sigma (St. Louis, MO). SYBR Green® PCR Master Mix was from Applied Biosystems (Warrington, UK). Primers were purchased from Sigma-Aldrich (Cambridge, UK). Dulbecco's Modified Eagle Medium (DMEM), glutamine, fetal bovine serum and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA).

Animals

Adult male Swiss CD1 mice and Sprague–Dawley rats were used in these experiments. Animals were fed standard chow and water and libitum. Animals were maintained at 22°C ambient temperature and 50% relative humidity under a controlled 12:12 h light–dark cycle. Animals were killed by cervical dislocation after ether anesthesia, and the tissues were quickly removed, weighed and processed. To manipulate the tissues surgical material and micropipettes were treated with RNAse Zap (Ambion). Procedures involving animals and their care were conducted in conformity with institutional guide-lines that comply with Spanish and European laws and policies for the Care and Use of Laboratory Animals.

Cell lines

Cell lines were obtained from the Servicio de Cultivos Celulares (University of Murcia) and were cultured in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown to about 80% confluence and were collected for RNA extraction.

Quantitative real-time RT-PCR

Total RNA was extracted from cells and tissues (fresh or kept in RNAlater at 4°C) with GenElute mammalian total RNA Miniprep kit following the manufacturer's instructions. First-strand cDNA was obtained from total RNA using MMLV reverse transcriptase. One to 5 μg of total RNA was reverse-transcribed using 1 μl oligo (dT) as primer, 1 μl of 10 mM dNTP Mix, 1 μl of MMLV reverse transcriptase, 2 μl of buffer (containing 500 mM Tris-ClH pH 8.3, 50 mM KCl, 3 mM $MgCl_2$ and 5 mM DTT) and nuclease-free water up to 20 μl. RNA, oligo dT and dNTPs were mixed and incubated at 70°C for 10 min. Mixture was put on ice for a few minutes and MMLV reverse transcriptase was added. After incubation at 37°C for 1 h, the transcriptase was denatured by heating at 90°C for 10 min.

PCR amplification was carried out using a SYBR Green® PCR Master Mix (Applied Biosystems) and a 7500 Real-Time instrument (Applied Biosystems). Different sets of primers and cDNA were used and the fluorescence data were collected and analyzed using 7500 SDS software (Applied Biosystems). The expression level of each gene was normalized against beta-actin. In some cases, L17 ribosomal protein or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also used for comparison. Prior to the analysis of the different genes, the efficiency of the amplification for each pair of primers and template was tested by using known amounts of recombinant pcDNA3 plasmid containing as insert the ORF of each particular gene studied, which had been generated as previously described (Lopez-Contreras et al. 2006). The values obtained for each gene were used to adjust the mRNA abundance. The following primers were used: mouse β-actin (forward, 5′-GATTACTGCTCTGG CTCCTAGCA-3′; reverse, 5′-GCTCAGGAGGAGCAATG ATCTT-3′); rat β-actin (forward, 5′-GATTACTGCTCTG GCTCCTAGCA-3′; reverse, 5′-GCTCAGGAGGAGCAA TGATCTT-3′); human β-actin (forward, 5′-GATCAC TGCCCTGGCACCCAGC-3′; reverse, 5′-GCTCAGGAGG AGCAATGATCTT-3′); mouse and rat ODC (forward, 5′-A TGGGTTCCAGAGGCCAAA-3′; reverse, 5′-CTGCTTCA TGAGTTGCCACA- TT-3′); human ODC (forward 5′-ATG GCTTCCAGAGGCCGAC-3′; reverse, 5′-TTGCTGCATG AGTTGCCACGCA-3′); mouse AZIN1 (forward, 5′-CTT TCCACGAACCATCTGCT-3′; reverse, 5′- TTCCAGCA TCTTGCATCTCA-3′); rat AZIN1 (forward, 5′-CTTT GCACGGACCGTCTGCT-3′; reverse, 5′-TTCCAGCAT CTTGCATCTCA-3′); human AZIN1 (forward, 5′-CTT TCCATGAACCATCTGCT-3′; reverse, 5′-TTCCAGC ATCTTGCATCTCA-3′); mouse and rat AZIN2 (forward, 5′-GCTTAGAGGGAGCCAAAGTG-3′; reverse, 5′-CTC AGCAAGGATGTCCACAC-3′); human AZIN2 (forward, 5′-GCCACCACGGACGAGGTA-3′; reverse, 5′-TCACT ATGGCACCCAGGTCAG-3′); mouse and rat AZ1 (forward, 5′-GAGTTCGCAGAGGAGCAACT-3′; reverse, 5′-CCAAGAAAGCTGAAGGTTCG-3′); human AZ1 (forward, 5′-GGAGTTCGCTGAGGAGCAGC-3′; reverse, 5′-GGAGTTCGCTGAGGAGCAGC-3′); mouse and rat AZ2 (forward 5′-AGTAAGTGTCCCCAGCTCCA-3′; reverse, 5′-ATCTTCGACAGTGGGTGAGG-3′); human AZ2 (forward 5′-GTAACTGTCCCCAGCTCCAG-3′; reverse 5′-AT CTTCGACAGTGGGTGAGG-3′); mouse AZ3 (forward, 5′-CCAGGTGGGTAGGAGCACT-3′; reverse 5′-AAGCA GGGGGTCAGTTGATA-3′); rat AZ3 (forward, 5′-CCA GGTGGGTAGGAGCACT-3′; reverse, 5′-AAGCAGG GGGTCGGCTGATA-3′); human AZ3 (forward 5′-CAG GAGGGCAAAAGCACC-3′; reverse, 5′-GAGCAGGG GGTCAGTAGCCA-3′); mouse GAPDH (forward, 5′-CC TGCGACTTCAACAGCAAC-3′; reverse, 5′-TCCACCA CCCTGTTGCTGTA-3′); human GAPDH (forward 5′-CCTCTGACTTCAACAGCGAC-3′; reverse 5′-TCCAC CACCCTGTTGCTGTA-3′); mouse ribosomal protein L19 (forward 5′-GGCTTGCCTCTAGTGTCCTC-3′; reverse 5′-CTGATCTGCTGACGGGAGTT-3′). The data are presented as mean ± SEM.

Results

Previous studies on the expression of AZIN2 based on dot-blot and semiquantitative RT-PCR analyses revealed that, in humans and mice, AZIN2 transcripts were most abundant in testis and brain (Pitkanen et al. 2001; Lopez-Contreras et al. 2006). In order to quantify more accurately mRNA levels, we analyzed using real-time RT-PCR the expression pattern of AZIN2 and other related genes in several mouse tissues and in cell lines derived from different human tumors.

Figure 1a shows the abundance of AZIN2 mRNA in mouse tissues normalized against beta-actin. Testes presented the highest values among several tissues analyzed. The expression of AZIN2 in the testis was about 30-fold higher than in the brain. Similar values to those found in brain were observed in epididymis and pancreas, whereas lower values were found in other tissues, including lung, heart, kidney and liver. The levels of AZIN1 mRNA were also determined and they are shown in Fig. 1b. It can be seen that AZIN1 mRNA was more abundant in pancreas and testis, followed by kidney and liver. Lower amounts were detected in other tissues. In general, these results agree with previous reports on the expression of AZIN1 in different tissues both from rats and 3 week mouse embryos (Murakami et al. 1996; Tang et al. 2009). Figure 1c shows the ratio between AZIN2 and AZIN1 levels in the tissues studied. AZIN2 was more highly expressed than AZIN1 in testis, adrenal gland, lung, brain and epididymis, this ratio being the highest for testis (about tenfold). On the other

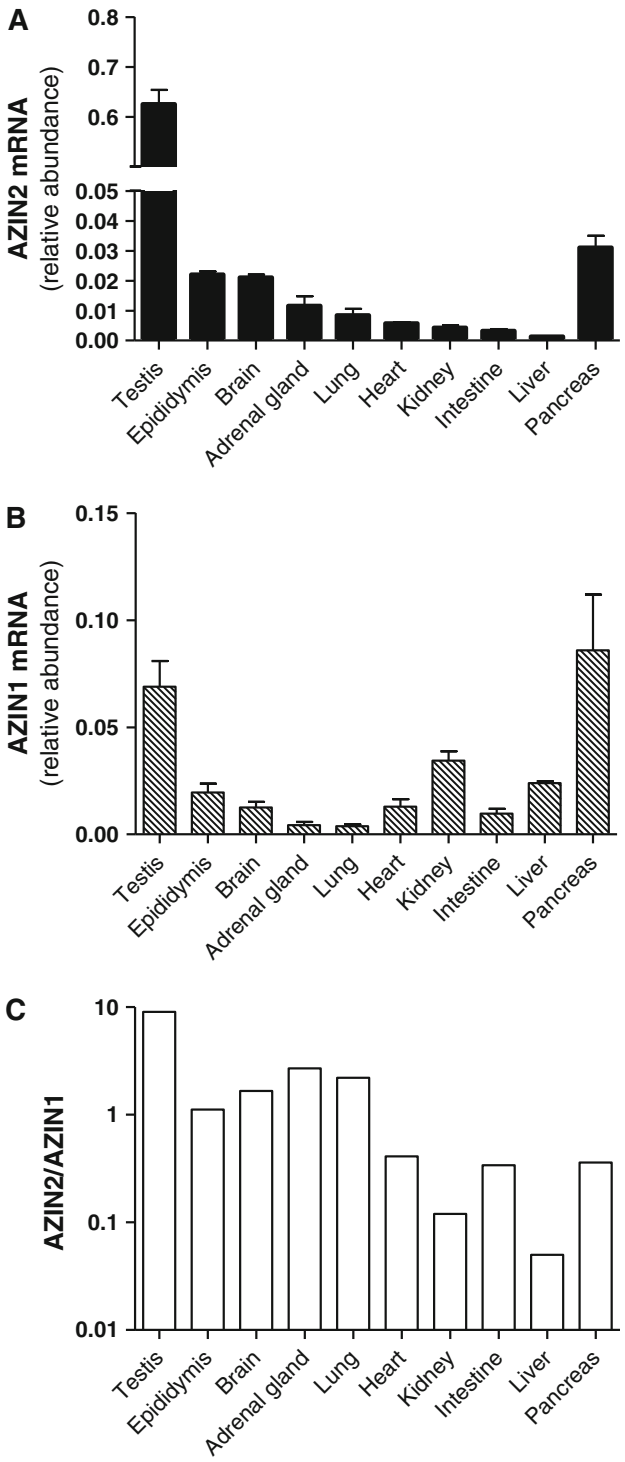

Fig. 1 Quantitative real-time RT-PCR analysis of antizyme inhibitors in different tissues of adult male mice. AZIN2 mRNA values (**a**) and AZIN1 mRNA values (**b**) are normalized against beta-actin. Results are the mean ± SEM of triplicate determinations of RNA samples from 4 to 8 adult male mice. **c** AZIN2:AZIN1 ratio calculated from the values plotted in (**a**) and (**b**)

hand, in kidney and liver the expression of AZIN1 was considerably higher than that of AZIN2, with values about 8- and 25-fold, respectively. In order to compare the expression of antizyme inhibitors with their paralog ODC, the levels of ODC mRNA were also determined and the percentages of each paralog were calculated. The results are shown in Fig. 2. In all tissues studied, the levels of ODC mRNA were considerably higher than those of their paralogs AZIN1 and AZIN2, especially in lung, kidney, intestine and heart (higher than 80%). The highest percentage of AZIN2 was observed in testes (45%), followed by brain (25%) and adrenal glands (16%). The tissue exhibiting the highest proportion of AZIN1 mRNA was the liver followed by pancreas and brain (about 15% each).

Since the three ODC paralogs are functionally related to AZs, we next examined the transcript levels of the three ODC antizymes expressed in mammalian tissues. AZ1 and AZ2 were expressed in all the tissues studies, and their absolute values (normalized to beta actin) were much higher than those found for AZINs in the same type of tissue (Fig. 3). In agreement with previous studies (Ivanov et al. 2000; Tosaka et al. 2000), AZ3 was almost exclusively expressed in the testes. The measured value of AZ3 in the mouse testis was 4.63 ± 0.51 and it was the AZ most highly expressed in that tissue (85% of all AZs). The presence of AZ3 mRNA in the RNA isolated from the epididymis (about 4%) may originate from residual expression of this gene in the spermatozoa stored in the cauda region of the epididymis. In the other tissues examined, AZ1 mRNA was predominant with most values higher than 90% in most of them (Fig. 3c). The ratio between values of AZ1 and AZ2 varied from 2 (in testis) to 41 (in lung). These data are in agreement with results from dot-blot analysis of human tissues, where AZ1 was found to be overall 16 times more abundant than that of AZ2 (Ivanov et al. 1998).

Since the expression of AZIN2 and AZ3 has been almost exclusively studied in murine and human testes (Ivanov et al. 2000; Tosaka et al. 2000; Lopez-Contreras et al. 2006), we analyzed the expression of both genes and their corresponding paralogs also in rat testes. As shown in Fig. 4a, the relative expression of ODC paralogs in mouse and rat testes was similar. AZIN2 mRNA was more abundant than that of AZIN1, but in the rat testis the two AZINs were overall more highly expressed than ODC, in contrast with mice. As shown in Fig. 4b, in the rat testis AZ3 mRNA was again the most abundant of the three AZs. In contrast to mice testis, AZ2 mRNA values in the rat testis were higher than those of AZ1.

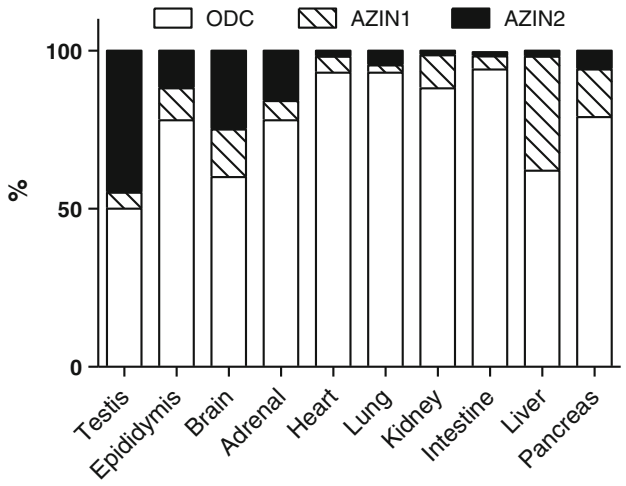

Fig. 2 Relative expression of the three ODC paralogs in male mouse tissues. Data are given as the percentage of ODC, AZIN1 and AZIN2 on total expression and were calculated from the means of triplicate determinations of RNA samples from 4 to 8 adult male mice

Whereas in tumor cells, ODC expression has been widely studied (Shantz and Levin 2007), data on the expression of AZs and AZINs are scarce. So, we decided to measure mRNA levels of AZs and AZINs in different tumor cell lines and in HEK293 cells. ODC was the most abundant paralog expressed in all types of cells studied (data not shown). In addition, the absolute values of AZIN2 were extremely low, and consequently the AZIN1:AZIN2 ratio was much higher that found in any of the mouse tissues examined (data not shown). Regarding AZs, no significant expression of AZ3 was found in any of the tumor cells. The relative expression of AZ1 and AZ2 was dependent on the type of cells analyzed (Fig. 5). Of note is that AZ2 was predominantly expressed in cells derived from neuroblastomas, such as Kelly, NB69, U373 or SH-SY5Y cells and also in U973 cells derived from a histiocytic lymphoma. In contrast, AZ1 was more expressed in carcinoma-derived cells such as HepG2 (hepatic carcinoma), Caco-2 cells (colon carcinoma), HeLa cells (cervical carcinoma), Kato III cells (gastric carcinoma), HBL (melanoma) or DMS3 cells (small cell lung carcinoma).

Discussion

AZs and AZINs are important regulators of ODC and polyamine uptake (Mangold 2005; Kahana 2009; Lopez-Contreras et al. 2010). Whereas it is well known that ODC is regulated at transcriptional, translational and post-translational levels (Pegg 2006), less is known about the

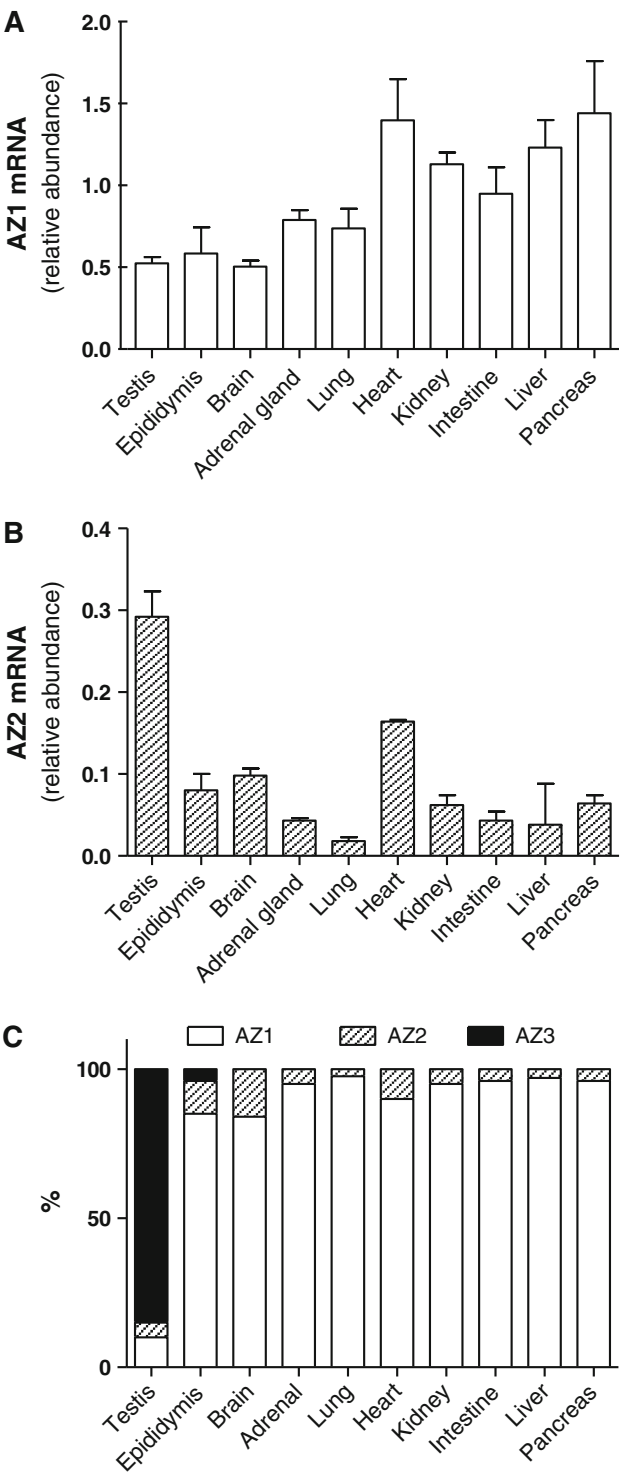

Fig. 3 Quantitative real-time RT-PCR analysis of antizymes in male mouse tissues. **a** AZ1 mRNA; **b** AZ2 mRNA. Absolute values were normalized to beta-actin. Results are the mean ± SEM of triplicate determinations of RNA samples from 4 to 8 adult mice. The mean value of AZ3 in the testis was 4.63 ± 0.51. **c** Relative expression of the three AZs in tissues of adult mice

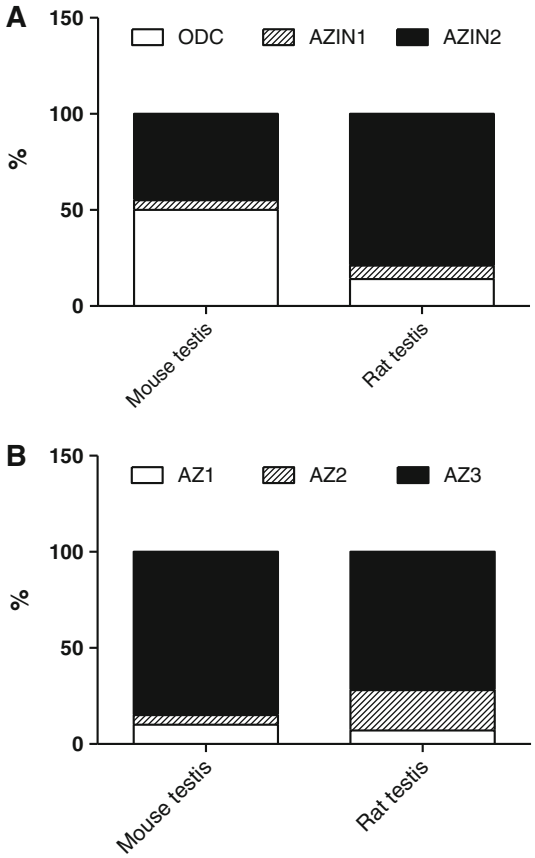

Fig. 4 Comparative expression of ODC and AZ paralogs between rat and mouse testis. **a** Percentage of expression of ODC, AZIN1 and AZIN2 on total value. **b** Percentage of expression of AZ1, AZ2 and AZ3. Rat data were calculated from duplicate determinations of RNA samples from four 80 day-old male rats

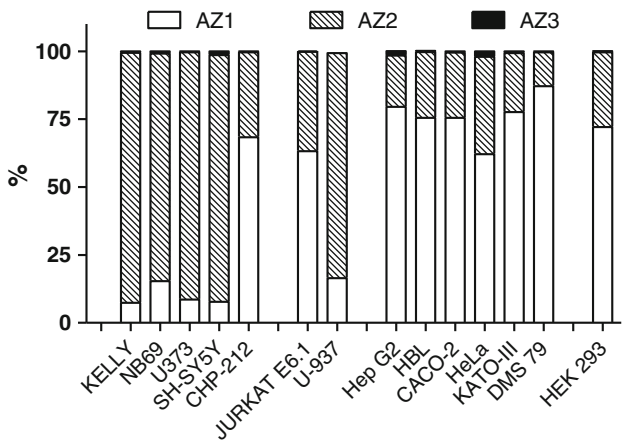

Fig. 5 Quantitative real-time RT-PCR analysis of AZ paralogs in cultured cells derived from human neoplasms. The relative abundance was calculated from the absolute values normalized to beta-actin. Results are the means of representative data from two independent experiments (% variation 3–15%). The origin of each cell line is given in "Results". Cells were grown up to approximately 80% of confluency

regulation of AZs and AZINs. In the case of AZs, polyamines are positive regulators by stimulating a +1 ribosomal frameshifting at the level of AZ mRNA translation (Rom and Kahana 1994; Matsufuji et al. 1995) and by decreasing the degradation of AZ protein by the proteasome (Palanimurugan et al. 2004). Early studies demonstrated that the induction of AZ1 in the rat by polyamines was not dependent on mRNA synthesis (Hayashi et al. 1996). However, in murine cells reductions in the levels of AZ1 mRNA in response to polyamine depletion, produced by difluoromethylornithine, were also reported (Nilsson et al. 1997). In addition, previous studies indicated that AZ1 and AZ2 mRNAs were present in all tissues examined, whereas the expression of AZ3 was exclusive of the testis (Ivanov et al. 2000; Tosaka et al. 2000). On the other hand, limited information is available about the regulation of AZIN1 and AZIN2, and data about their tissue distribution are scarce. In the rat, AZIN1 transcripts were found in different tissues, being AZIN1 mRNA increased by isoproterenol treatment in the heart (Murakami et al. 1996). In mouse fibroblasts, AZIN1 mRNA was enhanced by growth-stimulating factors (Nilsson et al. 2000) and mouse embryos contain AZIN1 mRNA in many different organs (Tang et al. 2009). In most of these studies, mRNA abundance was estimated by semiquantitative Northern blot or dot-blot analyses. Regarding AZIN2, although the expression of the gene is the mouse testis is regionally and temporally controlled (Lopez-Contreras et al. 2009a), there are no data about the factors that may participate in its regulation. Up-regulation of AZIN2 mRNA upon activation of mast cells with phorbol esters and the calcium ionophore A23187 has been recently reported (Kanerva et al. 2009).

Here, we have used real-time RT-PCR analysis to obtain more precise information of mRNA levels, in order to get an efficient comparison about the relative expression of all these genes in mouse tissues and in several cell lines derived from human tumors. Note that, although ODC and AZ protein levels are clearly dependent on translational control mechanisms, mRNA levels also have a clear influence on the amount of protein synthesized, and the analysis of mRNA may give important information about tissue distribution of the different paralogs. Our results clearly indicated that ODC mRNA is much more abundant than its corresponding paralogs in most tissues and cell lines analyzed. This is in agreement with studies on mice fibroblasts or mammary glands, which showed that the levels of AZIN1 mRNA were always lower than those of ODC (Nilsson et al. 2000; Murakami et al. 2010). With respect to AZIN2, the only exception was found in the mouse testis, where AZIN2 mRNA was present at levels comparable to those of ODC. Previous studies demonstrated that AZIN2 is mainly expressed in the testicular

haploid cells, suggesting that this gene may be implicated in spermatogenesis (Lopez-Contreras et al. 2009a). On the other hand, and in agreement with earlier studies (Pitkanen et al. 2001; Lopez-Contreras et al. 2006), AZIN2 transcripts were also found in the brain, but their levels were about twofold lower than those of ODC. Recently, it has been described that in the human brain, AZIN2 protein is present in the neuron axons of both white and gray matter and in the somas of selected cortical pyramidal neurons (Mäkitie et al. 2010). Moreover, in the same work, accumulation of AZIN2 in brains affected by Alzheimer's disease was also identified. Although it has been postulated that AZIN2 may participate in the regulation of the intracellular vesicle trafficking (Kanerva et al. 2010), the physiological role of AZIN2 in the central nervous system is far from being fully understood. Our results, showing that the levels of AZIN2 mRNA in epididymis, pancreas and adrenal glands were close to those found in brain, together with the fact that in other tissues AZIN2 transcripts were also detectable, indicate that AZIN2 is more widely expressed than previously thought. Moreover, the comparison with the expression of AZIN1 indicates that AZIN2 is present in the same tissues as AZIN1, although the ratio between the two messengers depends on the type of tissue. The highest AZIN2:AZIN1 ratio was found in testes, whereas the lowest one was in kidney and liver. At present, it is unknown whether in a particular tissue AZIN1 and AZIN2 are simultaneously expressed in the same type of cells. The recent finding showing that AZIN2 is expressed in human mast cells, specifically in serotonin-containing granules (Kanerva et al. 2009), together with the presence of AZIN2 in the haploid testicular cells (Lopez-Contreras et al. 2009a) and in specific groups of neurons (Mäkitie et al. 2010), would support the contention that AZIN2 is expressed in specific differentiated cells. However, this possibility cannot exclude the co-expression with AZIN1. In functional transfection assays of AZIN1 and AZIN2 in HEK293 cells, both genes exerted similar effects on ODC activity and polyamine uptake (Lopez-Contreras et al. 2006, 2008), and both proteins were able to interact with the three AZs (Mangold and Leberer 2005; Lopez-Contreras et al. 2006). Besides, studies on the subcellular localization of AZIN1 and AZIN2 have revealed that AZIN1 is located in centrosomes (Mangold et al. 2008) and that it can be translocated between cytosol and nucleus during the cell cycle (Murakami et al. 2009). However, AZIN2 is mainly located in vesicular-like structures in the ERGIC (Lopez-Contreras et al. 2009b) and in the trans–Golgi network (Kanerva et al. 2010).

An important aspect of AZINs is their possible role in cell proliferation and tumorigenesis. Regarding AZIN1, it was shown that the overexpression of this gene enhanced cell proliferation and promoted cell transformation (Keren-Paz et al. 2006). Moreover, AZIN1 mRNA was elevated in gastric tumors in comparison with adjacent normal tissue (Jung et al. 2000), and the silencing of AZIN1 expression reduced cell proliferation (Choi et al. 2005). Although the influence of AZIN1 on cell growth has been related to its ability to bind AZs and hence to affect polyamine metabolism, other studies have presented evidence suggesting that the effect of AZIN1 on cell proliferation may be partially independent from the ability of AZIN1 to interact with AZs (Kim et al. 2006). On the other hand, mouse fibroblasts stably transfected with AZIN2 also showed a growth advantage but to a lower extent than AZIN1 (Snapir et al. 2008). We show here that in the tumor-derived cell lines the levels of AZIN1 mRNA were on average much higher than those of AZIN2 mRNA. This suggests that it is unlikely that AZIN2 may have a prominent role in cell proliferation.

Of the three AZs conserved among mammals, AZ1 and AZ2 have a broad distribution, while AZ3 is testis specific (Ivanov et al. 2000; Tosaka et al. 2000). Semiquantitative estimation of mRNA abundance in different human tissues indicated that AZ1 mRNA is overall 16 times more abundant than AZ2 mRNA (Ivanov et al. 1998) The results shown here indicate that although in mouse tissues AZ1 mRNA is more abundant than AZ2 mRNA, the ratio is dependent on the type of tissue, varying from a ratio of about 2 in the testis to 41 in the lung. Interestingly, AZ2 was much more highly expressed than AZ1 in most cell lines derived from neuroblastomas, whereas the opposite was found in carcinoma-derived cells. In this context, high AZ2 mRNA expression has been correlated with increased survival in patients suffering from neuroblastoma (Geerts et al. 2010). Although both AZs appear to share a role in regulating ODC activity and polyamine uptake, differences related to their biological functions cannot be excluded. In fact, both antizymes exhibit different subcellular localization (Murai et al. 2009) and, as shown here, they have a different expression pattern. Our results also corroborate that AZ3 is the antizyme most expressed in the testes. Although the exact role of AZ3 in testis physiology has not been exactly defined, AZ3 knockout male mice present abnormal spermatozoa and are infertile (Tokuhiro et al. 2009). The abundant expression of AZ3 and AZIN2 in the same type of testicular cells, and the existing parallelism between both genes in the testis along postnatal ontogeny (Lopez-Contreras et al. 2009a) suggested that they are specific partners, which can be required for the fine control of polyamine levels during the second half of the spermatogenesis. The finding that the expression profiles of AZs and AZINs in rat and mouse tissues are similar, and that in both cases AZ3 and AZIN2 are mainly expressed in this tissue, reinforces the idea that they may play a relevant role in male reproduction.

Overall, our results have revealed that, with the exception of AZ3, all AZs and AZINs are expressed in all mouse tissues studied, although for each gene its expression is dependent on the type of tissue. The possible relevance of a particular pattern of expression of these genes in cell physiology remains to be elucidated.

Acknowledgments This work was supported by Grants 08681/PI/08 from Seneca Foundation (Autonomous Community of Murcia), and SAF2008-03638 from the Spanish Ministry of Science and Innovation and FEDER funds from The European Community.

Conflict of interest The authors declare that they have no conflict of interest.

References

Bercovich Z, 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

Choi KS, Suh YH, Kim WH, Lee TH, Jung MH (2005) Stable siRNA-mediated silencing of antizyme inhibitor: regulation of ornithine decarboxylase activity. Biochem Biophys Res Commun 328:206–212

Coffino P (2001) Regulation of cellular polyamines by antizyme. Nat Rev Mol Cell Biol 2:188–194

Fujita K, Murakami Y, Hayashi S (1982) A macromolecular inhibitor of the antizyme to ornithine decarboxylase. Biochem J 204: 647–652

Geerts D, Koster J, Albert D, Koomoa DL, Feith DJ, Pegg AE, Volckmann R, Caron H, Versteeg R, Bachmann AS (2010) The polyamine metabolism genes ornithine decarboxylase and antizyme 2 predict aggressive behavior in neuroblastomas with and without MYCN amplification. Int J Cancer 126:2012–2024

Gerner EW, Meyskens FL Jr (2004) Polyamines and cancer: old molecules, new understanding. Nat Rev Cancer 4:781–792

Hayashi S, Murakami Y, Matsufuji S (1996) Ornithine decarboxylase antizyme: a novel type of regulatory protein. Trends Biochem Sci 21:27–30

Ivanov IP, Gesteland RF, Atkins JF (1998) A second mammalian antizyme: conservation of programmed ribosomal frameshifting. Genomics 52:119–129

Ivanov IP, Rohrwasser A, Terreros DA, Gesteland RF, Atkins JF (2000) Discovery of a spermatogenesis stage-specific ornithine decarboxylase antizyme: antizyme 3. Proc Natl Acad Sci 97:4808–4813

Jung MH, Kim SC, Jeon GA, Kim SH, Kim Y, Choi KS, Park SI, Joe MK, Kimm K (2000) Identification of differentially expressed genes in normal and tumor human gastric tissue. Genomics 69:281–286

Kahana C (2009) Antizyme and antizyme inhibitor, a regulatory tango. Cell Mol Life Sci 66:2479–2488

Kanerva K, Lappalainen J, Makitie LT, Virolainen S, Kovanen PT, Andersson LC (2009) Expression of antizyme inhibitor 2 in mast cells and role of polyamines as selective regulators of serotonin secretion. PLoS One 4(8):e6858

Kanerva K, Mäkitie LT, Bäck N, Andersson LC (2010) Ornithine decarboxylase antizyme inhibitor 2 regulates intracellular vesicle trafficking. Exp Cell Res 316:1896–1906

Kasbek C, Yang C, Fisk HA (2010) Antizyme restrains centrosome amplification by regulating the accumulation of Mps1 at centrosomes. Mol Biol Cell 21:3878–3889

Keren-Paz A, Bercovich Z, Porat Z, Erez O, Brener O, Kahana C (2006) Overexpression of antizyme-inhibitor in NIH3T3 fibroblasts provides growth advantage through neutralization of antizyme functions. Oncogene 25:5163–5172

Kim SW, Mangold U, Waghorne C, Mobascher A, Shantz L, Banyard J, Zetter BR (2006) Regulation of cell proliferation by the antizyme inhibitor: evidence for an antizyme-independent mechanism. J Cell Sci 119:2853–2861

Lim SK, Gopalan G (2007) Antizyme1 mediates AURKAIP1-dependent degradation of Aurora-A. Oncogene 26:6593–6603

Lopez-Contreras AJ, Lopez-Garcia C, Jimenez-Cervantes C, Cremades A, Peñafiel R (2006) Mouse ornithine decarboxylase-like gene encodes an antizyme inhibitor devoid of ornithine and arginine decarboxylating activity. J Biol Chem 281:30896–30906

Lopez-Contreras AJ, Ramos-Molina B, Cremades A, Penafiel R (2008) Antizyme Inhibitor 2 (AZIN2/ODCp) stimulates polyamine uptake in mammalian cells. J Biol Chem 283: 20761–20769

Lopez-Contreras AJ, Ramos-Molina B, Martínez de la Torre M, Peñafiel-Verdú C, Puelles L, Cremades A, Peñafiel R (2009a) Expression of antizyme inhibitor 2 in male haploid germinal cells suggests a role in spermiogenesis. Int J Biochem Cell Biol 41:1070–1107

Lopez-Contreras AJ, Sánchez-Laorden BL, Ramos-Molina B, de la Morena E, Cremades A, Peñafiel R (2009b) Subcellular localization of antizyme inhibitor 2 in mammalian cells: influence of intrinsic sequences and interaction with antizymes. J Cell Biochem 107:732–740

Lopez-Contreras AJ, Ramos-Molina B, Cremades A, Peñafiel R (2010) Antizyme inhibitor 2: molecular, cellular and physiological aspects. Amino Acids 38:603–611

Mäkitie LT, Kanerva K, Polvikoski T, Paetau A, Andersson LC (2010) Brain neurons express ornithine decarboxylase-activating antizyme inhibitor 2 with accumulation in Alzheimer's disease. Brain Pathol 20:571–580

Mangold U (2005) The antizyme family: polyamines and beyond. IUBMB Life 57:671–676

Mangold U (2006) Antizyme inhibitor: mysterious modulator of cell proliferation. Cell Mol Life Sci 63:2095–2101

Mangold U, Leberer E (2005) Regulation of all members of the antizyme family by antizyme inhibitor. Biochem J 385:21–28

Mangold U, Hayakawa H, Coughlin M, Munger K, Zetter BR (2008) Antizyme, a mediator of ubiquitin-independent proteasomal degradation and its inhibitor localize to centrosomes and modulate centriole amplification. Oncogene 27:604–613

Matsufuji S, Matsufuji T, Miyazaki Y, Murakami Y, Atkins JF, Gesteland RF, Hayashi S (1995) Autoregulatory frameshifting in decoding mammalian ornithine decarboxylase antizyme. Cell 80:51–60

Murai N, Shimizu A, Murakami Y, Matsufuji S (2009) Subcellular localization and phosphorylation of antizyme 2. J Cell Biochem 108:1012–1021

Murakami Y, Ichiba T, Matsufuji S, Hayashi S (1996) Cloning of antizyme inhibitor, a highly homologous protein to ornithine decarboxylase. J Biol Chem 271:3340–3342

Murakami Y, Suzuki J, Samejima K, Kikuchi K, Hascilowicz T, Murai N, Matsufuji S, Oka T (2009) The change of antizyme inhibitor expression and its possible role during mammalian cell cycle. Exp Cell Res 315:2301–2311

Murakami Y, Suzuki J, Samejima K, Oka T (2010) Developmental alterations in expression and subcellular localization of antizyme and antizyme inhibitor and their functional importance in the murine mammary gland. Amino Acids 38:591–601

Newman RM, Mobascher A, Mangold U, Koike C, Diah S, Schmidt M, Finley D, Zetter BR (2004) Antizyme targets cyclin D1 for

degradation. A novel mechanism for cell growth repression. J Biol Chem 279:41504–41511

Nilsson J, Koskiniemi S, Persson K, Grahn B, Holm I (1997) Polyamines regulate both the transcription and translation of the gene encoding ornithine decarboxylase antizyme in mouse. Eur J Biochem 250:223–231

Nilsson J, Grahn B, 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

Palanimurugan R, Scheel H, Hofmann K, Dohmen RJ (2004) Polyamines regulate their synthesis by inducing expression and blocking degradation of ODC antizyme. EMBO J 23:4857–4867

Pegg AE (2006) Regulation of ornithine decarboxylase. J Biol Chem 281:14529–14532

Pegg AE (2009) Mammalian polyamine metabolism and function. IUBMB Life 61:880–894

Pitkanen LT, Heiskala M, Andersson LC (2001) Expression of a novel human ornithine decarboxylase-like protein in the central nervous system and testes. Biochem Biophys Res Commun 287:1051–1057

Rom E, Kahana C (1994) Polyamines regulate the expression of ornithine decarboxylase antizyme in vitro by inducing ribosomal frame-shifting. Proc Natl Acad Sci USA 91:3959–3963

Shantz LM, Levin VA (2007) Regulation of ornithine decarboxylase during oncogenic transformation: mechanisms and therapeutic potential. Amino Acids 33:213–223

Snapir Z, Keren-Paz A, Bercovich Z, Kahana C (2008) ODCp, a brain- and testis-specific ornithine decarboxylase paralogue, functions as an antizyme inhibitor, although less efficiently than AzI1. Biochem J 410:613–619

Snapir Z, Keren-Paz A, Bercovich Z, Kahana C (2009) Antizyme 3 inhibits polyamine uptake and ornithine decarboxylase (ODC) activity, but does not stimulate ODC degradation. Biochem J 419:99–103

Tang H, Ariki K, Ohkido M, Murakami Y, Matsufuji S, Li Z, Yamamura K (2009) Role of ornithine decarboxylase antizyme inhibitor in vivo. Genes Cells 14:79–87

Thomas T, Thomas TJ (2001) Polyamines in cell growth and cell death: molecular mechanisms and therapeutic applications. Cell Mol Life Sci 58:244–258

Tokuhiro K, Isotani A, Yokota S, Yano Y, Oshio S et al (2009) OAZ-t/OAZ3 is essential for rigid connection of sperm tails to heads in mouse. PLoS Genet 5(11):e1000712

Tosaka Y, Tanaka H, Yano Y, Masai K, Nozaki M, Yomogida K, Otani S, Nojima H, Nishimune Y (2000) Identification and characterization of testis specific ornithine decarboxylase antizyme (OAZ-t) gene: expression in haploid germ cells and polyamine-induced frameshifting. Genes Cells 5:265–276