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Subcellular Localization of Antizyme Inhibitor 2 in Mammalian Cells: Influence of Intrinsic Sequences and Interaction With Antizymes

Andrés J. López-Contreras,[1] Berta L. Sánchez-Laorden,[1] Bruno Ramos-Molina,[1] María E. de la Morena,[1] Asunción Cremades,[2] and Rafael Peñafiel[1*]

[1]*Department of Biochemistry and Molecular Biology B and Immunology, University of Murcia, Murcia, Spain*

[2]*Department of Pharmacology, Faculty of Medicine, University of Murcia, Murcia, Spain*

ABSTRACT

Ornithine decarboxylase (ODC) and the antizyme inhibitors (AZIN1 and AZIN2), regulatory proteins of polyamine levels, are antizyme-binding proteins. Although it is widely recognized that ODC is mainly a cytosolic enzyme, less is known about the subcellular distribution of AZIN1 and AZIN2. We found that these proteins, which share a high degree of homology in their amino acid sequences, presented differences in their subcellular location in transfected mammalian cells. Whereas ODC was mainly present in the cytosol, and AZIN1 was found predominantly in the nucleus, interestingly, AZIN2 was located in the ER-Golgi intermediate compartment (ERGIC) and in the *cis*-Golgi network, apparently not related to any known cell-sorting sequence. Our results rather suggest that the N-terminal region may be responsible for this particular location, since its deletion abrogated the incorporation of the mutated AZIN2 to the ERGIC complex and, on the other hand, the substitution of this sequence for the corresponding sequence in ODC, translocated ODC from cytosol to the ERGIC compartment. Furthermore, the coexpression of AZIN2 with any members of the antizyme family induced a shift of AZIN2 from the ERGIC to the cytosol. These findings underline the complexity of the AZs/AZINs regulatory system, supporting early evidence that relates these proteins with additional functions other than regulating polyamine homeostasis. J. Cell. Biochem. 107: 732–740, 2009.

KEY WORDS: ANTIZYMES; ANTIZYME INHIBITOR 2; ERGIC; ORNITHINE DECARBOXYLASE; POLYAMINES

Polyamines (putrescine, spermidine, and spermine) are ubiquitous aliphatic amines that regulate different processes in the cell, including nucleic acids and protein synthesis, and modulate ion channels and receptors, making these molecules essential for cellular growth and differentiation [Tabor and Tabor, 1984; Cohen, 1998; Igarashi and Kashiwagi, 2000; Childs et al., 2003; Wallace et al., 2003]. Polyamine levels are tightly regulated by different mechanisms, including biosynthesis, degradation, uptake and secretion [Pegg, 1986; Heby and Persson, 1990; Seiler et al., 1996; Janne et al., 2004; Seiler, 2004]. Moreover, in malignant cells polyamine homeostasis is deregulated having the transformed cells increased polyamine levels due to the activation of both biosynthesis and uptake processes [Pegg, 1988; Casero and Marton, 2007].

In the biosynthetic pathway, ornithine decarboxylase (ODC) is a key enzyme, catalyzing the conversion of ornithine into putrescine, diamine that serves as a precursor of the other polyamines. Ornithine decarboxylase activity is highly regulated at transcriptional, translational, and post-translational levels by different stimuli [Pegg, 2006]. An important factor regulating ODC activity is the intracellular concentration of polyamines. High levels of polyamines stimulate the synthesis of a regulatory protein named antizyme (AZ) by the induction of translational frameshifting [Rom and Kahana, 1994; Matsufuji et al., 1995]. Antizyme binds to ODC, inhibiting the enzyme and directing it to the 26S proteasome for its degradation by a ubiquitin-independent process [Murakami et al., 1992, 1996; Coffino, 2001]. Three antizyme isoforms have been described, AZ1, AZ2, and AZ3, and all of them are also able to inhibit the uptake of extracellular polyamines [Murakami et al., 1992, 1996; Coffino, 2001; Mangold, 2005; Lopez-Contreras et al., 2008], and in consequence, the antizymes negatively regulate both polyamine synthesis and uptake. To add further complexity, another

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*Correspondence to: Dr. Rafael 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

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protein called antizyme inhibitor (AZIN) can counteract the actions of the antizymes [Fujita et al., 1982; Murakami et al., 1996; Mangold, 2006].

Recent studies from our laboratory have demonstrated the existence of a new antizyme inhibitory protein named AZIN2, which can be considered as another player in polyamine metabolism in mammalian cells, since it affects not only polyamine biosynthesis but also polyamine uptake [Lopez-Contreras et al., 2006, 2008]. AZIN2 is mainly expressed in the testes, where it might participate in the process of spermiogenesis [Lopez-Contreras et al., 2009]. This protein was previously known as ODC-like or ODCp due to its high homology with ODC [Pitkanen et al., 2001]. In fact, AZIN2, ODC and the first characterized antizyme inhibitor, AZIN1, are homologous proteins highly conserved among mammals, and it is thought they diverged from a single ancient gene. The three proteins share the capacity to bind to antizymes, but during evolution the AZINs have lost their enzymatic capacity. However, whereas ODC and AZIN1 are ubiquitously expressed, AZIN2 is mainly restricted to testis and brain [Pitkanen et al., 2001; Lopez-Contreras et al., 2006]. Moreover, in cell fractionation experiments AZIN2, in contrast to ODC that is mainly a cytosolic enzyme, was fundamentally found in the mitochondrial-membranous fraction [Lopez-Contreras et al., 2006]. Differences in the subcellular location of AZIN1 and AZIN2 may have physiological relevance, not only for its property of regulating polyamine distribution but also for its possible effects on proteasome degradation pathways.

The aim of the present work was to elucidate the precise subcellular location of AZIN2, which may have implications on the regulation of intracellular polyamine distribution, or on the function of other proteins regulated by AZs, especially in male germinal haploid cells, where it is highly expressed. For that purpose, different constructs of AZIN2, AZIN1, and ODC fused to green fluorescent protein (GFP) or FLAG epitope were generated, transiently expressed in HEK293T and COS7 cells and the subcellular distribution analyzed by confocal microscopy.

EXPERIMENTAL PROCEDURES

MATERIALS

Moloney murine leukemia virus reverse transcriptase, *Taq* DNA polymerase, anti-FLAG M2 monoclonal antibody peroxidase conjugate, anti-FLAG M2 monoclonal antibody, anti-HA monoclonal antibody, protease inhibitor mixture (4-(2-aminoethyl) benzenesulfonyl fluoride, EDTA, bestatin, E-64, leupeptin, aprotinin) and Igepal CA-630 were purchased from Sigma. *Pfu* DNA polymerase was obtained from Biotools (Madrid, Spain). Restriction endo-nucleases were from Fermentas Life Sciences (Vilnius, Lithuania). Lipofectamine 2000 Transfection Reagent, Alexa 568-conjugated anti-mouse antibody and *MitoTracker® Red 580* were purchased from Invitrogen (Carlsbad, CA). QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Anti Erk2 polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). ECL+ detection reagent, developing reagents and films were from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Primers were purchased from Sigma Genosys (Cambridge, UK).

CLONING AND GENE REACTION OF TRUNCATED AZIN2 FORMS AND OTHER CONSTRUCTS

ODC, AZIN1, AZIN2, AZ1, AZ2, and AZ3 mouse genes were cloned into the expression vector pcDNA3 (Invitrogen) following standard procedures and using the primers described elsewhere [Lopez-Contreras et al., 2006]. Antizyme constructs with an appropriate deletion of one nucleotide in the frameshifting site, for full-length and functional expression, were obtained by the QuickChange site-directed mutagenesis kit [Lopez-Contreras et al., 2006].

Ornithine decarboxylase and AZIN2 with the FLAG epitope fused to its N-terminus were generated as described in Lopez-Contreras et al. [2006]. Cloning of AZIN1 with the FLAG fused to its C-terminus is described in Lopez-Contreras et al. [2008]. AZIN2 truncated forms cloning was also previously described in Lopez-Contreras et al. [2008].

The enhanced green fluorescent protein sequence (GFP) was added to the C-terminal of ODC, AZIN1, and AZIN2 by subcloning in the vector pEGFP-N2 (BD Biosciences Clontech), between the restriction sites *Eco*RI and *Bam*HI, using as forward primer the original ODC, AZIN1, or AZIN2 cloning primer and as reverse primers one of the following: ODC-GFP, 5′-ATGGGATCC (*Bam*HI)ACACATTGATCCTAGCAGAAG-3′; AZIN1-GFP, 5′-TGCGG-ATCC(*Bam*HI)AAGCTTCAGTGGAAAAGCTGTC-3′; AZIN2-GFP, 5′-G-GCGGATCC(*Bam*HI)ACATGATGCTTGCTGGGGTG-3′. The quimeric fusion protein ^{113}N AZIN2-ODC was generated by the amplification of the AZIN2 N-terminus introducing a *Not*I restriction site in the 3′ end, and the amplification of the corresponding C-terminal sequence in ODC, introducing a *Not*I restriction site in the 5′ end. The N-terminal AZIN2 fragment was digested with the restriction enzymes *Eco*RI and *Not*I, and the C-terminal ODC fragment was digested with *Not*I and *XbaI*, and then a triple ligation with the pcDNA3 containing the FLAG epitope was performed resulting in the quimeric AZIN2-ODC construct. The primers used were: N-AZIN2, forward [see original cloning primer in Lopez-Contreras et al., 2006], reverse, 5′-CAGGCGGCCGC(*Not*I)ACAGATGATCT-TACTGGCAGG; C-ODC, forward, 5′-ATCGCGGCCGC(*Not*I)TCCTTG-TAAACAAGTCTCTC, reverse (see original cloning primer). KDELR-GFP, GFP-Rab1 and MC1R-TAG plasmids were kindly provided by Dr. García-Borrón. The identity of the constructs was ascertained by DNA sequencing of the cloned inserts by means of an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) at the "Servicio de Apoyo a las Ciencias Experimentales," University of Murcia.

CELL CULTURE AND TRANSIENT TRANSFECTION

HEK 293T cells and COS7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2. Cells were grown to about 80% confluence. Transient transfections were carried out with Lipofectamine 2000 transfection reagent as described elsewhere [Lopez-Contreras et al., 2006]. In some experiments cells were incubated with the inhibitor of subcellular vesicle trafficking brefeldin A (1 μg/ml) for 30 min before carrying out the staining.

WESTERN BLOTTING

Cells collected and washed in PBS were homogenized in ice-cold Tris/sucrose buffer using a Polytron homogenizer. The composition of the homogenizing buffer (buffer A) was as follows: 10 mM Tris-HCl (pH 7.2), 0.1 mM pyridoxal phosphate, 0.2 mM EDTA, 1 mM di-thiothreitol, 0.25 M sucrose, 200 μM 4-(2-aminoethyl)benzene-sulfonyl fluoride, 13 μM bestatin, 1.4 μM E-64, 100 μM leupeptin, 30 nM aprotinin. The cell homogenate was centrifuged at 500*g* for 10 min to obtain a post-nuclear supernatant that was centrifuged at 12,000*g* for 20 min to collect a post-mitochondrial supernatant (S12) and a crude mitochondrial pellet (P12). Equal amounts of protein were mixed with Laemmli sample buffer, heated at 95°C for 5 min and fractionated by electrophoresis in 10% polyacrylamide–SDS gels. The resolved proteins were electroblotted to polyvinylidene difluoride membranes, blocked with 5% non-fat dry milk in PBS, and incubated overnight at 4°C with the anti-FLAG antibody peroxidase-labeled (1:5,000). Immunoreactive bands were detected by using ECL+ as detection reagent and commercial developing reagents and films. Erk2 was used as a loading control.

CONFOCAL MICROSCOPY

Cells grown on coverslips were transfected with ODC, AZIN1, or AZIN fused to the green fluorescent protein (GFP) or with ODC, AZIN1, AZIN2, or mutant AZIN2 constructs labeled with the FLAG epitope. In some experiments, cells were cotransfected with the ERGIC/Golgi markers KDELR-GFP, GFP-Rab1, or with the ER retained protein MC1R-TAG. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Igepal. For detection of FLAG labeled proteins, cells were incubated with an anti-FLAG M2 monoclonal antibody (1:7,000), followed by an Alexa 568-conjugated secondary antibody (1:400). For co-localization of AZIN2-GFP and MC1R-TAG, cells treated as above were incubated with anti-HA monoclonal antibody (1:7,000), followed by Alexa 568-conjugated anti-mouse antibody. For the staining of mitochondria cells were loaded with *MitoTracker® Red 580* at 500 nM for 30 min during which time it passively diffuses across the plasma membrane and accumulates in active mitochondria. Then, cells were treated as described above. Finally, samples were mounted by standard procedures using a mounting medium from Dako (Carpinteria) and examined with a Leica laser scanning confocal microscope.

RESULTS

SUBCELLULAR LOCALIZATION OF AZIN2 AND OTHER AZ-BINDING PROTEINS IN TRANSFECTED CELLS ANALYZED BY CONFOCAL MICROSCOPY

In order to assess the subcellular location of AZIN2 and to compare this with that of other AZ-binding proteins, such as ODC and AZIN1, different constructs were generated. They included ODC, AZIN1, and AZIN2, fused to the green fluorescent protein (GFP) (ODC-GFP, AZIN1-GFP, and AZIN2-GFP) or tagged with the FLAG epitope (ODC-FLAG, AZIN1-FLAG, and AZIN2-FLAG). These constructs were used to transfect HEK 293T and COS7 cells. The confocal microscopy analysis revealed that for each AZ-binding protein, the subcellular location of the GFP or FLAG tagged proteins was independent of the type of tag (Fig. 1). As expected, ODC was predominantly found in the cytosol, whereas AZIN1 was mainly found in the nuclei (Fig. 1, left and middle columns). However, AZIN2 showed a different staining pattern, with a strong accumulation in perinuclear structures and a weaker signal detected in cytosol (Fig. 1, right column). These results are compatible with our previous work on cell fractionation, using differential centrifugation, which showed that ODC-FLAG was fundamentally located in the cytosolic fraction, whereas AZIN2-FLAG was mainly present in the mitochondrial-membrane pellet [Lopez-Contreras et al., 2006]. The subcellular pattern of each of these proteins was similar in both COS7 and HEK 293T cells.

CO-LOCALIZATION STUDIES OF AZIN2 WITH DIFFERENT SUBCELLULAR MARKERS USING CONFOCAL MICROSCOPY

In order to obtain more precise information about the intracellular localization of AZIN2, co-localization experiments were performed using different specific organelle markers. As a mitochondrial marker, we used the commercial dye *MitoTracker® Red 580*, a derivative of X-rosamine that is accumulated by active mitochon-

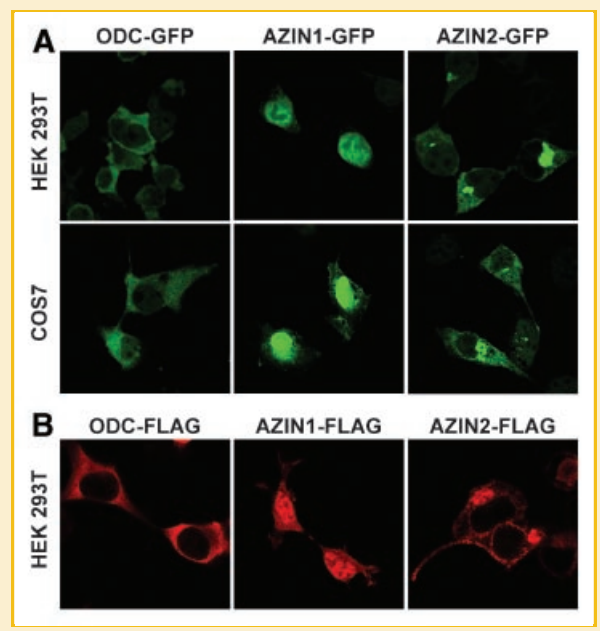

Fig. 1. Subcellular location of AZIN2, ODC, and AZIN1 in transfected cells. A: Laser scanning confocal micrographs of HEK 293T or COS7 cells transfected with ODC, AZIN1, or AZIN2 fused to the fluorescent protein GFP. After transfection, cells were fixed and examined in a confocal microscope. Left column, ODC-GFP (shown in green) is mainly located in the cytosol. Middle column, AZIN1-GFP is shown in green and it is mainly found in the nuclei of cells Right column AZIN2-GFP is shown in green. Note the higher signal in perinuclear structures. B: Laser scanning confocal micrographs of HEK 293 transfected with ODC, AZIN1, or AZIN2 fused to the FLAG epitope. After corresponding transfections, cells were fixed, permeabilized and stained with anti-FLAG antibody and ALEXA anti-mouse, and then examined in a confocal microscope. Left column, ODC-FLAG shown in red, is mainly expressed in the cytosol. Middle column, AZIN1-FLAG is shown in red and it is mainly found in the nuclei of cells. Right column, AZIN2-FLAG is shown in red. Note the higher expression in perinuclear structures.

dria and well retained during cell fixation. Figure 2 (left column) shows that AZIN2 did not co-localize at all with this marker, indicating that AZIN2 is not located in the mitochondria. Next, we tested whether AZIN2 co-localizes with the human melanocortin 1 receptor (MC1R), tagged with three hemagglutinin epitopes and a polyhistidine sequence in its C terminus (MC1R-TAG). MC1R belongs to the G protein coupled receptor family that is located in the plasma membrane but that when fused to TAG epitope is totally retained in the endoplasmic reticulum (ER) [Sanchez-Laorden et al., 2006]. The analysis of HEK 293T cells co-transfected with MC1R-TAG and AZIN2-GFP revealed that although these proteins did not co-localize, they showed a complementary staining pattern (Fig. 2, middle column), suggesting that AZIN2 may be located in the proximity of the ER. Furthermore, AZIN2 did not co-localize with calnexin, a well-known marker of ER (Fig. 2, right column). To gain more information, we tested the possible co-localization of AZIN2 with Rab1, a small GTPase that regulates forward traffic from the ER to the Golgi [Pind et al., 1994], that can be used as a marker of the ER-Golgi intermediate compartment (ERGIC) and of the *cis* side of the Golgi. For that purpose, HEK 293T cells were co-transfected with GFP-Rab1 and AZIN2-FLAG and sequentially scanned in the laser confocal microscope. Figure 3 (left column) shows that AZIN2 did not co-localize with Rab1, although the staining pattern of both proteins was similar and likely complementary, suggesting that

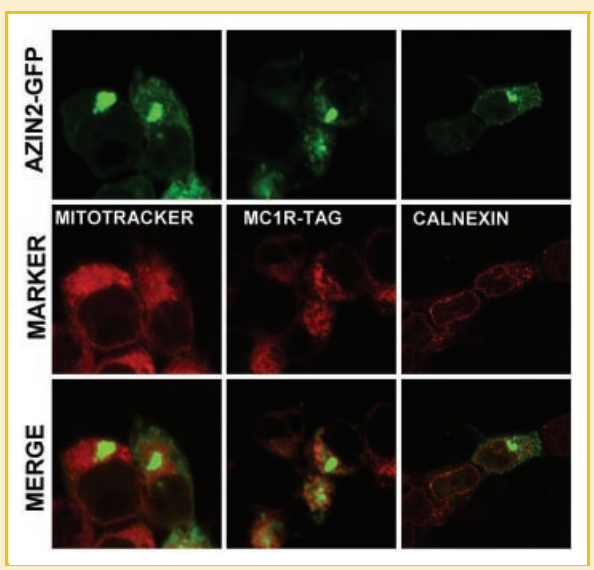

Fig. 2. Co-localization studies of AZIN2 with subcellular markers. HEK 293T cells transfected with AZIN2-GFP were fixed, permeabilized, stained when needed and examined in a confocal microscope. Left column, HEK 293T cells transfected with AZIN2-GFP (shown in green) were loaded with *MitoTracker® Red 580* (shown in red) at 500 nM for 30 min. Then cells were fixed and analyzed in a confocal microscope. Merged images show that AZIN2-GFP is not present in mitochondria. Middle column, cells were cotransfected with AZIN2-GFP (shown in green) and MC1R-TAG (protein retained in Endoplasmic Reticulum) (shown in red). Merged images show the absence of co-localization of the signals of both proteins. Right column, cells were transfected with AZIN2-GFP (shown in green) and stained with anti-calnexin antibody (shown in red). Merged images show no co-localization.

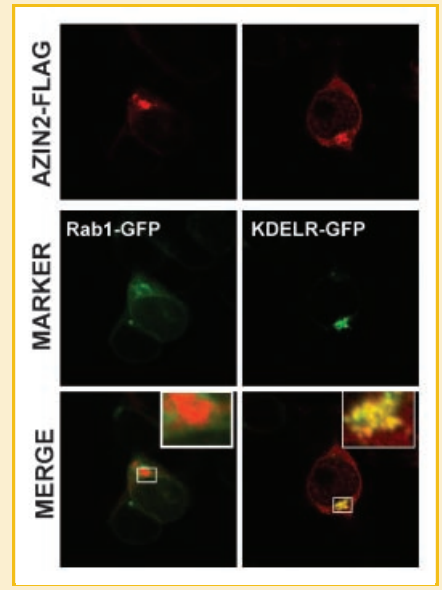

Fig. 3. Co-localization of AZIN2 and KDELR. HEK 293T cells transfected with AZIN2-FLAG were fixed, permeabilized, stained and examined in a confocal microscopy. AZIN2-FLAG (shown in red) was cotransfected with Rab1-GFP (shown in green) or KDELR-GFP (shown in green). In merged images (lower row) co-localization is shown in yellow. Note the lack of co-localization of AZIN2-FLAG and Rab-1-GFP in contrast to KDELR-GFP that shows a strong cosegregation with AZIN2-FLAG (details in the upper right square).

AZIN2 may be located nearby to Rab1. Finally, as shown in Figure 3 (right column) we found that AZIN2-FLAG strongly co-localized with the KDEL receptor fused to GFP (KDELR-GFP) in co-transfected HEK 293T cells. Since KDEL receptor is a *cis*-Golgi/ERGIC located integral membrane protein [Singh et al., 1993], the results suggest that AZIN2 may be located in some part in the cis-Golgi-ERGIC complex. This co-localization of AZIN2-FLAG with KDELR-GFP was also found in COS7 transfected cells (results not shown). The lack of co-localization of AZIN2 with galactosyl transferase-GFP, an enzyme localized in the *trans*-Golgi, excludes the presence of AZIN2 in that compartment (results not shown). To check the association of AZIN2 to Golgi related structures, we used brefeldin A, an inhibitor of subcellular vesicle trafficking. Whereas in control cells AZIN2 showed a very concentrated staining pattern, in treated cells there was a change in that pattern, confirming the association of AZIN2 with Golgi-related structures (results not shown).

INFLUENCE OF ANTIZYMES ON THE SUBCELLULAR LOCALIZATION OF AZIN2 IN HEK 293 CELLS

Although previous studies had demonstrated that AZIN2 was able to interact with all members of the antizyme family, affecting not only ODC activity but also polyamine uptake [Lopez-Contreras et al., 2006, 2008], no data were available about the possible influence of this interaction on the subcellular localization of AZIN2. Since it has been proven that AZs bind to both ODC and AZIN1, affecting their degradation by the proteasome [Murakami et al., 1992; Coffino, 2001; Bercovich and Kahana, 2004], it could be likely that the overexpression of AZs could affect the subcellular localization of

AZIN2. To test this possibility, HEK 293T cells were co-transfected with AZIN2 and mutated forms of the three antizymes, lacking the early stop codon. This mutation eliminates the need for ribosomal frameshifting and thus permitted the synthesis of functional antizymes [Lopez-Contreras et al., 2006]. Confocal microscopy analysis revealed that in the double transfectants AZIN2 was mainly located in the cytosol (Fig. 4A), giving a staining pattern similar to that of ODC. This suggests that the three AZs are able to induce a subcellular shift of AZIN2 from the ERGIC related structures to the cytosol. This result was corroborated by western blot analysis that showed that the AZIN2 protein markedly increased in the cytosolic fraction isolated from co-transfected cells (Fig. 4B).

SEARCHING FOR THE REGION IN AZIN2 RESPONSIBLE FOR ITS ACCUMULATION IN ERGIC STRUCTURES

To assess the importance of the different regions of AZIN2 protein on the translocation to the ERGIC complex, several deletions of AZIN2-FLAG were generated (Fig. 5A), and the subcellular localization of these mutated proteins in transfected HEK 293T cells was studied by confocal microscopy and quantified by Western blotting. The deletion of the 45 or 162 C-terminal amino acids of AZIN2-FLAG ($\Delta^{416-459}$ and $\Delta^{298-459}$) did not affect the distribution pattern, since these AZIN2-truncated proteins still remained in the ERGIC complex and co-localized with the KDELR protein in cotransfected cells, and the ratio between cytosolic AZIN2 (S12) and membranous-like protein (P12) was almost similar to that of wild type AZIN2 (Figs. 5B and 6). The mutant $\Delta^{117-140}$-AZIN2-FLAG, lacking the sequence corresponding to the putative antizyme binding domain (AZBE), identified in ODC [Li and Coffino, 1992] and in AZIN1 [Bercovich and Kahana, 2004; Kim et al., 2006], showed the same subcellular pattern as AZIN2 (Figs. 5B and 6). Moreover, immunoprecipitation assays indicated that this truncated form did not interact with AZs and, accordingly, $\Delta^{117-140}$-AZIN2 was not shifted to the cytosol in co-transfections with AZs (results not shown). On the other hand, with truncated AZIN2 protein, lacking the 39 residues at the N terminus (Δ^{1-39}), the relative accumulation in the pellet with respect to cytosol was lower than in the wild-type (Fig. 5B), but a strong fluorescence signal in the corresponding ERGIC related structures was still observed (Fig. 6). Significantly, all these AZIN2-truncated proteins co-localized with the KDELR protein in cotransfected cells (Fig. 6). Interestingly, the removal of the 113 N-terminal amino acids of AZIN2 (Δ^{1-113}-AZIN2) caused a dramatic loss in the accumulation of this truncated protein in ERGIC related structures, which was associated to a lack of co-localization with KDELR (Fig. 6), and with a marked decrease in the pellet/cytosol ratio of the mutated AZIN2 (Fig. 5B). These results suggested that the 113 N-terminal residues of AZIN2 are essential for the ERGIC specific subcellular location of this protein, different from that of those highly homologues ODC and AZIN1. To corroborate this role of the N-terminal region of AZIN2, a quimeric form of ODC was generated, in which the 113 N-terminal residues of AZIN2 substituted the equivalent region of ODC. Figure 6 shows that this quimeric AZIN2-ODC protein was mainly found in the ERGIC compartment, in contrast to ODC that was mainly found in the cytosol (Figs. 1 and 5B).

DISCUSSION

In the present work we have analyzed the subcellular localization of AZIN2, and of its homologues ODC and AZIN1, using transient expression in mammalian cells and confocal microscopy. Our results clearly show that although these three AZ binding proteins (ODC,

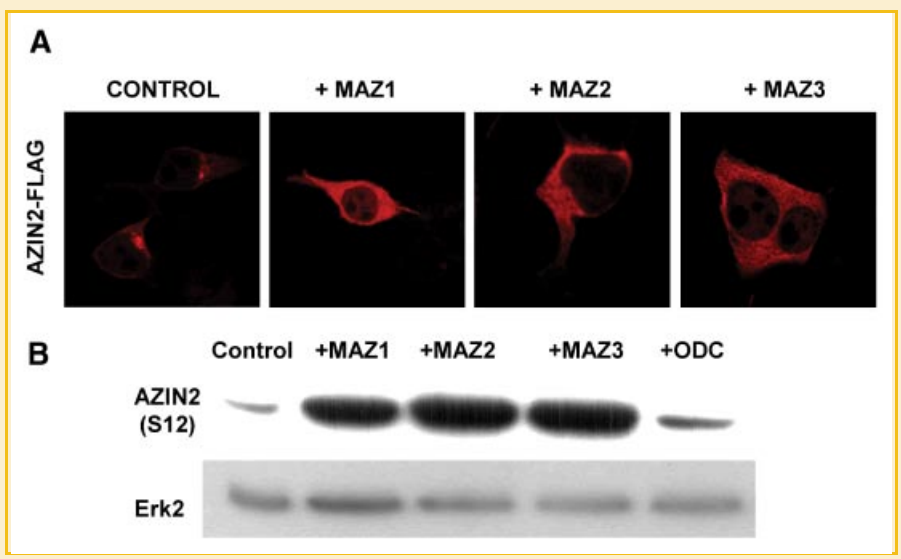

Fig. 4. Influence of antizymes on the subcellular location of AZIN2. A: Laser scanning confocal micrographs of HEK 293T cells transfected with AZIN2-FLAG alone (left), or cotransfected with MAZ1, MAZ2, and MAZ3 indicated on top of each column. The images show a shift in the intracellular staining pattern from an accumulated pattern in cells transfected with AZIN2-FLAG alone to a cytosolic pattern when cotransfected with MAZs. B: Western blot of the cytosolic fraction prepared from HEK293T cells transfected with AZIN2-FLAG alone or in combination with any of the mutated (without the early stop codon) antizymes (MAZ1, MAZ2, MAZ3) or ODC. AZIN2-FLAG protein was assayed with anti-FLAG antibody. Erk2 was used as a loading control.

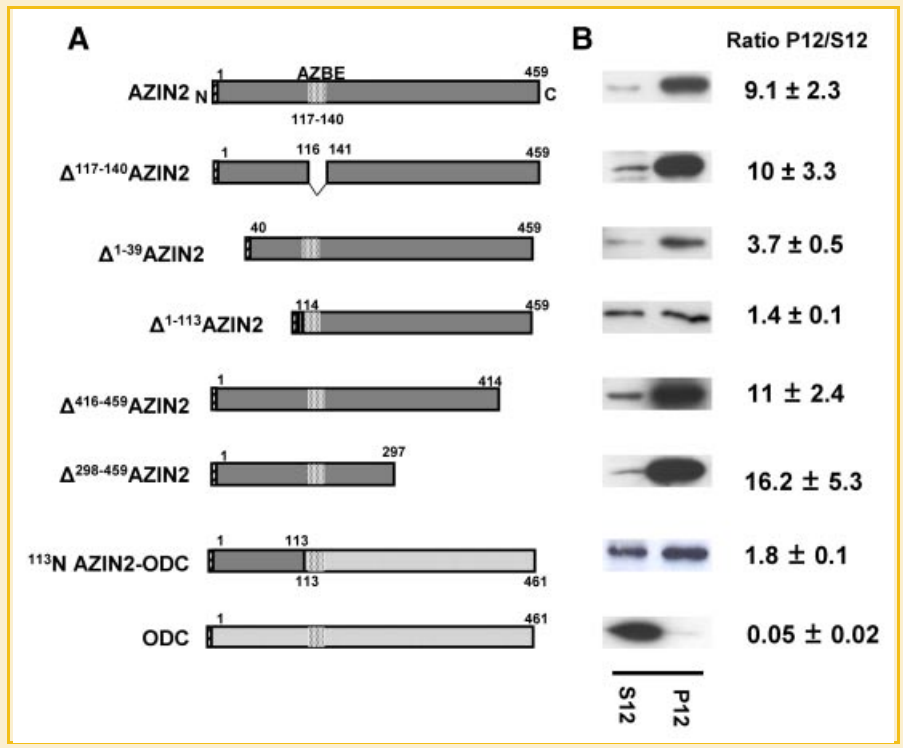

Fig. 5. Influence of AZIN2 domains on the subcellular location. A: Schematic representation of the AZIN2 and ODC constructs. N-terminal deletions (Δ^{1-39}; Δ^{1-113}), C-terminal deletions ($\Delta^{416-459}$; $\Delta^{298-459}$), antizyme binding site deletion of AZIN2 ($\Delta^{117-140}$) and the quimeric AZIN2-ODC protein (^{113}N AZIN2-ODC) were obtained by PCR and subcloning into the pcDNA3 plasmid containing the FLAG epitope (see Experimental procedures Section). B: Cells were transfected with the wild-type AZIN2, ODC or the different constructs generated. After 24 h cells were homogenized in an isotonic buffer. The post-nuclear fraction was centrifuged at 12,000*g* to obtain a cytosolic fraction (S12) and a mitochondria-membrane pellet (P12). Western blots were carried out using anti-FLAG antibody. The P12/S12 ratios for each construct were calculated by dividing the intensity of the P12 band (estimated by image densitometric analysis) by that of the S12 band.

AZIN1, and AZIN2) have a high sequence homology, their subcellular localization was totally different. It should be noted that although heterologous expression systems were used, the results obtained were similar in HEK293T and COS7 cells, and they were also independent from the type of tag used. Moreover, the cytosolic localization found for ODC-GFP and ODC-FLAG is in agreement with early cell fractionation studies from different types of mammalian tissues [McCormick, 1977; Grillo and Fossa, 1983] or with immunocytochemical studies [Schipper et al., 2004]. Similarly, although AZIN2 showed a dual localization, the protein was mainly located in perinuclear structures that may co-sediment in the mitochondrial-membrane fraction, in agreement with our previous results [Lopez-Contreras et al., 2006]. On the other hand, AZIN1 was detected mainly in the nucleus. This is basically in accord with recent findings that located AZIN1 in the nucleus, in Golgi-like structures and associated to AZ1 in centrosomes [Mangold et al., 2008]. These authors postulated that both proteins may have importance in regulating the ubiquitin-independent proteasomal degradation of centrosome-associated proteins.

In contrast to these experimental results, in silico analyses, using different subcellular prediction program (*PSORTII*, http://www.psort.org; *Predotar*, urgi.versailles.inra.fr/predotar/predotar.html; *TargetP 1.1*, http://www.cbs.dtu.dk/ services/TargetP; *SOSUI 1.11*, http://bp.nuap.nagoya-u.ac.jp/sosui) indicated a cytosolic localization for all of these paralogue proteins (ODC, AZIN1, and AZIN2). The reasons for these discrepancies are mostly unknown. One possibility is that AZIN1 and AZIN2 could possess sequences, different to the orthodox cell-sorting sequences, which can direct them to non-cytosolic locations. An alternative possibility is that these proteins could interact with other partners that direct them to specific subcellular compartments.

In accordance with the latter possibility are our results on AZIN2 that demonstrated that the expression of any of the three antizymes can affect AZIN2 localization, shifting the protein from the perinuclear membranous structures to the cytosol. Moreover, for this translocation the presence of the AZBE in the AZIN2 was required, since AZIN2 mutants lacking this region were not translocated to the cytosol in the presence of increased amount of antizymes. On the other hand, our results using different mutated AZIN2 forms also revealed that sequences in the N-terminal region of AZIN2 are more important than sequences in the C-terminal region for the localization of AZIN2 in non-cytosolic compartments. The fact that the substitution of the N-terminal region of ODC by the corresponding region of AZIN2, shifted ODC from the cytosol to the perinuclear location characteristic of AZIN2 suggests that the region from positions 39 to 113 of AZIN2 can be relevant for the subcellular protein localization. Taking into account these considerations, it is likely that the presence of AZIN2 in one or other compartment could

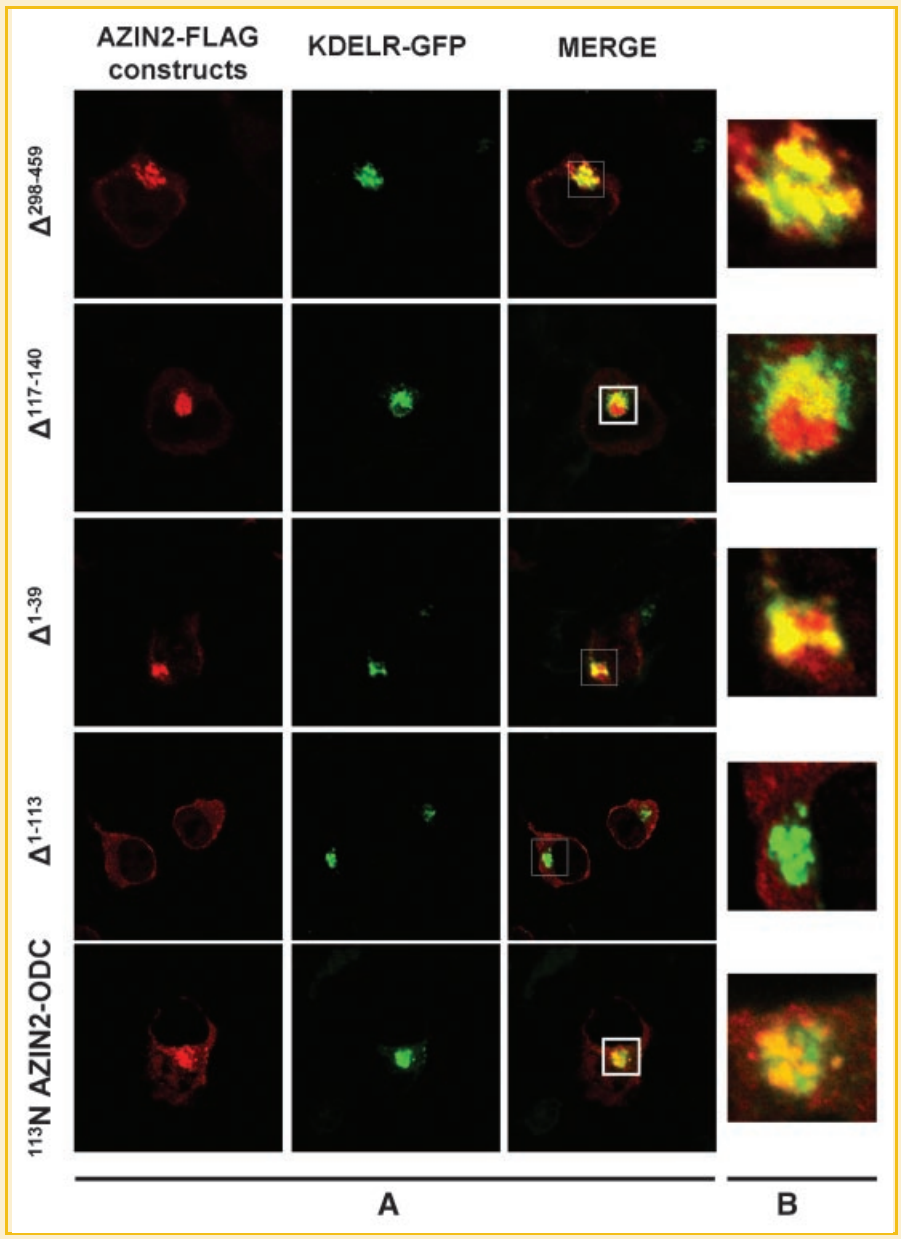

Fig. 6. Co-localization of AZIN2-truncated forms with KDELR. HEK 293T cells were cotransfected with AZIN2-FLAG truncated forms ($\Delta^{298-459}$, $\Delta^{117-140}$, Δ^{1-39}; Δ^{1-113}, or ^{113}N AZIN2-ODC) and KDELR-GFP. After corresponding transfections, cells were fixed, permeabilized, stained with anti-FLAG and examined in a confocal microscope. A: Left column, AZIN2-FLAG truncated forms or the N-terminus-FLAG of AZIN2 fused to ODC are shown in red. Middle column, KDELR-GFP is shown in green. Right column, merged images of red and green signals (B) 4.5× magnification of selected images in the right column. Note that all AZIN2 truncated forms and the N-terminus fused to ODC show a strong co-localization with the KDELR, except for Δ^{113}N AZIN2 that did not colocalize.

be affected by the state of growth of the cells or by the levels of other components, such as polyamines, which may affect the concentration of antizymes of other proteins able to interact with AZIN2. In this regard, although ODC has been found mainly in the cytosol, under certain conditions it has been found in the nucleus [Snyder and Russell, 1970; Bitonti and Couri, 1981; Schipper et al., 2004] or in the plasma membrane [Heiskala et al., 1999].

The dual location of proteins is an important and emergent question in cell biology since it is known that the subcellular location may affect the physiological function of the protein. In this regard, ODC, AZIN1, and also AZs, are good examples of proteins located in several organelles, although its biological significance is far from being understood. Whereas it is clear that ODC activity in the cytosol is related to polyamine biosynthesis, its presence in the nucleus is still a controversial matter, where it has been postulated that it could participate in the regulation of gene expression or merely be present to be degraded by nuclear proteasomes [Schipper et al., 2004].

The precise location of AZIN2 was determined by confocal microscopy experiments. We found that the strongest signal of this

protein corresponded to structures related with the ER-Golgi Intermediate Compartment (ERGIC) and with the *cis*-Golgi network, according to a high co-localization with the KDELR-GFP, a transmembrane integral protein resident in these structures [Singh et al., 1993]. However, AZIN2 did not co-localize with Rab1-GFP, another marker protein resident in ERGIC structures and *cis*-Golgi [Pind et al., 1994], but was detected nearby. This could indicate that AZIN2 could be present in vesicles or stacks in the ERGIC, but not in all of them. The absence of ER signal sequence in AZIN2, the co-localization with KDEL-R and its induced-translocation to the cytosol by antizymes, suggest that AZIN2 could be located in the cytosolic side of the vesicular tubular clusters.

The unexpected localization of AZIN2 in the ERGIC network may extend our understanding on the AZ/AZIN regulatory system. It is known that the ERGIC is the site for the anterograde and retrograde vesicular transport of proteins between the ER and the cis-Golgi network, which also contributes to the concentration, folding and quality control of newly synthesized proteins [Appenzeller-Herzog and Hauri, 2006]. To start discussing the potential role of AZIN2 in ERGIC related structures, we should take into consideration that this protein is specifically expressed in the haploid cells of the testis [Lopez-Contreras et al., 2009]. Although no studies on the subcellular localization of AZIN2 in the male haploid cells are available, our previous results, based on the biochemical cell fractionation of testicular extracts, indicated that this protein was mainly located in membranous structures [Lopez-Contreras et al., 2009]. Since in spermatids and spermatozoa there are specific organelles, the acrosomes, which are very important for the physiology of these cells, one can speculate that AZIN2 could be located in these organelles. This assumption is based on several facts. First, the acrosome is a Golgi-derived secretory granule formed during spermiogenesis [Clermont et al., 1993]. Second, immunocytochemical studies using a polyclonal anti-ODC antibody revealed an important immunoreactivity in the acrosome [Qian et al., 1985]. Since ODC is mainly located in non-haploid testicular cells [Lopez-Contreras et al., 2009], and ODC and AZIN2 share a high grade of sequence homology, the acrosomal immunoreactivity could be the result of cross-reactivity with AZIN2. Third, during spermiogenesis there are fluxes of polyamines in the differentiated cells that led to the accumulation of these polycations in residual bodies [Shin et al., 2007] or in the acrosome. These fluxes, in line with the role of AZIN2 on polyamine uptake [Lopez-Contreras et al., 2008], could be regulated by AZIN2. In fact, spermine has been implicated in the acrosome reaction [Rubinstein and Breitbart, 1991]. On the other hand, taking into consideration that protein processing and degradation are widely considered to be important events for acrosome reaction, the function of AZIN2 in the testis could also be related to the regulation of the turnover of certain proteins. Since AZ3 is able to interact with proteins, such as cyclin D1 or gametogenetin, and proteasomes have been found in acrosome vesicles [Ziemba et al., 2002], one possibility is that AZIN2, by negating the action of AZ3, could regulate protein degradation in this organelle by a ubiquitin-independent process, as reported in other tissues for AZIN1 [Mangold et al., 2008].

Moreover, the recent findings showing that AZ1 may promote the degradation of the oncogenic protein Aurora-A [Lim and Gopalan, 2007], and that the prion [PSI(+)] enhances the expression of antizyme [Namy et al., 2008], associated to the presence of AZIN2 in brain, underlines the possible relevance of this protein in different biological processes.

In summary, our results indicate that AZIN2, in contrast to their homologues ODC and AZIN1, is mainly located in the ERGIC system of mammalian cells, and that translocations between ERGIC clusters and the cytosol appear to be influenced by both the existence of intrinsic AZIN2 sequences and the presence of antizymes. These characteristics of AZIN2 may be of great interest regarding its physiological function in testes and brain, where this gene is mainly expressed.

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