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Polyamine biosynthesis in *Xenopus laevis*: the gene xlAZIN2/xlODC2 encodes a lysine decarboxylase

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Abstract

Ornithine decarboxylase (ODC) is a key enzyme in the biosynthesis of polyamines, organic cations that are implicated in many cellular processes. The enzyme is regulated at the post-translational level by an unusual system that includes antizymes (AZs) and antizyme inhibitors (AZINs). Most studies on this complex regulatory mechanism have been focused on human and rodent cells, showing that AZINs (AZIN1 and AZIN2) are homologues of ODC but devoid of enzymatic activity. Little is known about *Xenopus* ODC and its paralogues, in spite of the relevance of *Xenopus* as a model organism for biomedical research. We have used the information existing in different genomic databases to compare the functional properties of the amphibian ODC1, AZIN1 and AZIN2/ODC2, by means of transient transfection experiments of HEK293T cells. Whereas the properties of xlODC1 and xlAZIN1 were similar to those reported for their mammalian orthologues, xlAZIN2/xlODC2 showed important differences with respect to human and mouse AZIN2. xlAZIN2 did not behave as an antizyme inhibitor, but it rather acts as an authentic decarboxylase forming cadaverine, due to its affinity for L-lysine as substrate; so, in accordance with this, it should be named as lysine decarboxylase (LDC). In addition, AZ1 stimulated the degradation of xlAZIN2 by the proteasome, but the removal of the 21 amino acid C-terminal tail, with a sequence quite different to that of mouse or human ODC, made the protein resistant to degradation. Collectively, our results indicate that in *Xenopus* there is only one antizyme inhibitor (xlAZIN1) and two decarboxylases, xlODC1 and xlLDC, with clear preferences for L-ornithine and L-lysine, respectively.

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Introduction

Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the polyamine biosynthetic pathway that catalyzes the formation of putrescine from L-ornithine [1]. The polyamines spermidine and spermine, and their precursor putrescine, are organic cations that interact with different macromolecules, such as nucleic acids and proteins, affecting numerous cellular mechanisms related to cell growth and differentiation, signal transduction, apoptosis and autophagy [2–8]. In mammalian cells, ODC is highly regulated by a series of transcriptional, translational and post-translational mechanisms [1, 9–11]. Interestingly, ODC is a short-lived protein, with a half-live of less than 60 min in most mammalian tissues, and one of the few proteins that are degraded by the proteasome without ubiquitination [12, 13]. In addition, in the degradation of mammalian ODC, the antizyme 1 (AZ1) plays an important role [9, 14–16]. This regulatory protein is induced by increased levels of polyamines through an unusual ribosomal frame-shifting mechanism in the translation of AZ1 mRNA [17, 18]. AZ1 binds to the ODC monomer preventing the formation of the active ODC homodimer, and accelerates the proteasomal degradation of ODC, presumably by inducing the exposure of a cryptic proteasome-interacting surface of ODC [19]. The effects of antizymes on ODC are neutralized by antizyme inhibitors (AZINs), protein homologues of ODC but lacking decarboxylase activity [20–22]. In mammals, two AZINs have been identified (AZIN1 and AZIN2) that differ in their tissue expression profile [22–25]. In contrast to ODC, the degradation of these proteins is ubiquitin-dependent and is decreased by binding to AZ1 [26, 27].

Most studies on the structure, function and expression of ODC, AZs and AZINs have been carried out with the human and rodent versions of these proteins, and in less extension with the yeast and protozoan orthologues [28–31]. *Xenopus laevis* and *Xenopus tropicalis* are clawed frogs that have been used as model organisms in developmental biology. However, little is known about polyamine metabolism in these two species, and most of these studies have been

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68   focused on the changes in ODC activity and polyamine levels during *Xenopus laevis* oogenesis

69   [32–34]. By screening a cDNA library from *Xenopus laevis* eggs, a cDNA corresponding to

70   ODC (XLODC1) was isolated and sequenced [35]. Later, a new paralogue of ODC from

71   *Xenopus laevis* (named xODC2) was identified, and the study of its temporal and spatial

72   expression pattern during early embryogenesis showed that this is quite different from that of

73   xlODC1 [36]. In addition, whereas transfection studies of ODC-deficient mutant C55.7 CHO

74   cells with XLODC1 showed that the *Xenopus* enzyme was functional in this heterologous

75   cellular model [33], to our knowledge, no data on the activity and properties of xODC2 are

76   available. In the Ensembl and Xenbase genome databases three *Xenopus* ODC paralogues are

77   annotated: ODC1, AZIN1 and AZIN2. xAZIN2 gene is also named as xODC2, but it is unclear

78   whether the corresponding protein functions as an antizyme inhibitor or alternatively it is an

79   authentic ornithine decarboxylase. Due to the remarkable properties of mouse AZIN2 found in

80   our previous studies [37–42], it appears relevant to analyze the characteristics of its amphibian

81   orthologue to determine whether this protein functions as an enzyme or as an antizyme

82   inhibitor. In the present work, we have transfected HEK293T cells with expression vectors

83   containing the ORF corresponding to xAZIN2, xODC1, and xAZIN1, and the enzymatic

84   activities and polyamine levels of these transfected cells were compared with those transfected

85   with their murine counterparts. We also analyzed the degradation of the *Xenopus* ODC

86   homologues and the effect of AZ1 on this process. Our results indicate that in *Xenopus laevis,*

87   in contrast to mammalian cells, there are two different decarboxylases of ornithine and lysine,

88   and only one protein acting as an antizyme inhibitor.

89   # Materials and methods

90   ## Materials

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91   L-[1-^{14}C] ornithine and L-[1-^{14}C] lysine were purchased from American Radiolabeled

92   Chemicals Inc. (St. Louis, MO, USA). Anti-Flag M2 monoclonal antibody peroxidase

93   conjugate (A8592), goat Anti-Rabbit IgG antibody peroxidase conjugated (AP132P), EDTA,

94   Igepal CA-630, cycloheximide, L-lysine, putrescine dihydrochloride, cadaverine

95   dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, 1,6-

96   hexanodiamine, 1,7-diaminoheptane, dansyl chloride, proteasome inhibitor MG-132 and

97   protease inhibitor cocktail (containing 4-(2-aminoethyl)benzenesulfonyl fluoride, EDTA,

98   bestatin, E-64, leupeptin, aprotinin) were obtained from Sigma Aldrich (St. Louis, MO).

99   Lipofectamine 2000 transfection reagent, Dulbecco's Modified Eagle Medium (DMEM

100   GlutaMAX), foetal bovine serum (FBS) and penicillin/streptomycin were purchased from

101   Invitrogen (Carlsbad, CA). Pierce ECL PlusWestern Blotting Substrate was from

102   ThermoScientific (IL, USA). Rabbit anti-ERK2 antibody (SC-154) was purchased from Santa

103   Cruz Biotechnology (Texas, USA). The Anti-DYKDDDDK G1 Affinity Resin and the

104   DYKDDDDK peptide were obtained from GenScript. D,L-alpha-difluoromethylornithine

105   (DFMO) was provided by Dr. Patrick Woster (Medical University of South Carolina,

106   Charleston, SC). Gene and protein sequences were obtained from Xenbase

107   (http://www.xenbase.org/, RRID:SCR_003280) and Ensembl (www.ensembl.org) genome

108   databases.

Cell culture and transient transfections

110   Human embryonic kidney cells (HEK293T), obtained from ATCC, were cultured in DMEM

111   (Dulbecco's modified Eagle's medium), supplemented with 10% (v/v) fetal bovine serum, 100

112   units/ml penicillin, and 100 µg/ml streptomycin, in a humidified incubator containing 5% CO_2

113   at 37°C. Cells were grown to ~80% confluence and then were transiently transfected with

114   Lipofectamine 2000 using 1.5 µl of reagent and 0.3 µg of plasmid per well (12-well plates). In

115   co-transfection experiments, the mixtures contained equimolecular amounts of each construct.

116 The plasmid pcDNA3 without gene insertion was used as negative control. After 6 h of

117 incubation, the transfection medium was removed, fresh complete medium was added, and

118 cells were grown for additional 16 hours. Cells were collected and homogenized as described

119 below, whereas the culture media was used for polyamine analysis. In some cases, xlAZIN2

120 and xlODC1 were purified from the cell extracts by affinity chromatography using an anti-Flag

121 resin (GenScript) in accordance with the instructions of the supplier. All the constructs used in

122 transient transfections were cloned into the expression vector pcDNA3.1. The Flag epitope

123 DYKDDDDK was added to the N terminus of xlODC1, xlAZIN1, xlAZIN2, xlAZIN2ΔC,

124 xlAZIN2-mAZIN2, mODC and mAZIN2 and to the C terminus of functional isoforms of

125 murineAZ1, AZ2 and AZ3. All the clones were generated and purchased from GenScript, and

126 sequenced before use.

Western blot analysis

128 Transfected HEK293T cells were collected in phosphate buffered saline (PBS), pelleted, and

129 lysed in solubilization buffer (50 mM Tris–HCl pH 8, 1% Igepal and 1 mM EDTA) with

130 protease inhibitor cocktail (Sigma Aldrich). The cell lysate was centrifuged at 14,000×g for 20

131 min. Equal amounts of protein were separated in 10% SDS-PAGE. The resolved proteins were

132 electroblotted to PVDF membranes, and the blots were blocked with 5% non-fat dry milk in

133 PBS-T (Tween 0.1%) and incubated overnight at 4 °C with the anti-Flag antibody conjugated

134 to peroxidase (1:10000). Immunoreactive bands were detected by using ECL Plus Western

135 Blotting Substrate. ERK2, detected by a rabbit anti-ERK2 antibody (Santa Cruz, USA), was

136 used as loading control. Densitometric analysis was achieved with ImageJ software.

Enzymatic measurements

138 Transfected HEK293T cells were collected in phosphate buffered saline (PBS), pelleted and

139 lysed in solubilization buffer (50mMTris-HCl, 1% Igepal and 1mM EDTA). After

140   centrifugation at 14,000 ×g for 20 min, 5µl of the supernatant were taken to a final volume of

141   50µl with buffer containing 10 mM Tris-HCl, 0.25M sucrose, 0.1 mM pyridoxal phosphate,

142   0.2 mM EDTA and 1 mM dithiothreitol. Decarboxylating activity was assayed in the

143   supernatant by measuring $^{14}CO_2$ released from L-[1-^{14}C] ornithine or L-[1-^{14}C] lysine. The

144   reaction was performed in glass tubes with tightly closed rubber stopper, hanging from the

145   stoppers two disks of filter paper wetted in 0.5 M benzethonium hydroxide, dissolved in

146   methanol. The samples were incubated at 37°C from 15 to 120 minutes, and the reaction was

147   stopped by adding 0.5 ml of 2 M citric acid. The filter paper disks were transferred to

148   scintillation vials, and counted for radioactivity in liquid-scintillation fluid. In some cases, the

149   enzyme activity was calculated by measuring by HPLC the rate of diamine formation

150   (putrescine or cadaverine), after incubation of the cell extracts with different concentration of

151   L-ornithine or L-lysine.

Polyamine analysis

153   Both intracellular polyamines and polyamines generated in the culture media of the transfected

154   cells were measured by HPLC. Transfected HEK293T cells were collected in phosphate

155   buffered saline (PBS), pelleted, and the polyamines were extracted from the cells by treatment

156   with 0.4M perchloric acid. The supernatant obtained after centrifugation at 10,000xg for 10

157   min was used for polyamine determination. For extracellular polyamine analysis, a fraction of

158   the cell culture media was concentrated with a Speedvac Concentrator (Savant Instruments Inc.

159   Farmingdale, NY, USA), and the resulting residue was resuspended in 0.4 M perchloric acid

160   and processed as described above. Polyamines from the acid supernatant were dansylated

161   according to a standard method [43]. Dansylated polyamines were separated by HPLC using a

162   BondaPak C18 column (4.6 x 300 mm; Waters) and acetonitrile/water mixtures (running from

163   70:30 to 96:4 during 30 min of analysis) as mobile phase and at a flow rate of 1 ml/min. 1,6-

164   Hexanediamine and 1,7-heptanediamine were used as internal standards. Detection of the

165 derivatives was achieved using a Waters 420-AC fluorescence detector, with a 340 nm

166 excitation filter and a 435 nm emission filter.

Confocal microscopy

168 Cells grown on coverslips were transfected with xlAzin2, xlOdc1, xlAzin1, mAzin2 or mOdc

169 constructs. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde

170 in PBS and permeabilized with 0.5% Igepal in PBS. For detection of Flag-labelled proteins,

171 cells were incubated with an anti-Flag M2 monoclonal antibody (1:7.000), followed by an

172 Alexa 488-conjugated secondary antibody (1:400). For the staining of nucleus, cells were

173 loaded with DAPI (1:10000) for 5 minutes. Finally, samples were mounted by standard

174 procedures, using a mounting medium from Dako (Carpinteria) and examined with a Leica

175 True Confocal Scanner TCS-SP2 microscope.

Statistical analysis

177 The data were analyzed by Student's t-test for differences between means. $P < 0.05$ was

178 considered as statistically significant.

179

Results

Comparative study of gene and protein structure of *Xenopus* AZIN2 with its paralogues and mammalian orthologues

183 According to the Xenbase genome browser, the gene structures of Azin2 described for *Xenopus*

184 *tropicalis* (XB-GENE-6454420) and *Xenopus laevis* (XB-GENE-6493979) are similar. The

185 comparison of protein sequences between *Xenopus tropicalis* *A*ZIN2 (xtAZIN2)

186 (NP_001015993.2) and *Xenopus laevis*AZIN2 (xlAZIN2) (NP_001079692.1), by using the

8

187   Clustal omega sequence alignment program, revealed a high homology (93.64%) (S1 Fig).

188   Since our preliminary experiments showed that both proteins behave similarly, we selected

189   *Xenopus laevis* for most experiments.

190   Next, we compared the gene structure of *Xenopus laevis* Odc paralogues with their respective

191   murine and human orthologues. Fig 1 shows that the xlAzin2 gene, like mouse Azin2 (mAzin2)

192   and human AZIN2 (hAZIN2), is formed by 11 exons (9 of them are coding exons), whereas

193   xlOdc and xlAzin1 contain 12 exons (10 coding exons), similarly to their murine and human

194   orthologues. The protein homology between the different orthologues of *Xenopus laevis* and

195   mice was analyzed by using the Align Sequences Protein BLAST (NCBI), and the results are

196   shown in Table 1. The sequence homology of xlAZIN2 with respect to xlODC1 or mODC was

197   higher (65% and 63%, respectively) than that ofmAZIN2 (59%). In addition, sequence

198   similarity of mODC to xlODC1 was higher (82%) than that of xlAZIN2 (65%). The lowest

199   identity of xlAZIN2 was with xlAZIN1 (43%). These results indicate that although the genetic

200   structure of xlAzin2 is close to its mammalian orthologues, its protein sequence is closer to that

201   of *Xenopus* or mouse ODC proteins.

202   **Fig 1. Genetic structure of mouse and human ODC paralogues, and their comparison**

203   **with their *Xenopus* orthologues.** Note that exons 7 and 8 in ODC and AZIN1 are fused in

204   only one exon in AZIN2 (blue boxes). Data obtained from Ensembl (www.ensembl.org).

205   **Table 1. Sequence identity between mouse (m) and *Xenopus laevis* (xl) homologue**

206   **proteins.**

207

	xlAZIN2	xlODC1	xlAZIN1	mAZIN2	mODC	mAZIN1
xlAZIN2	100	65	43	59	63	49
xlODC1	65	100	47	52	82	51
xlAZIN1	43	47	100	42	46	67

208   Fig 2 shows the sequence alignment of the proteins corresponding to the three ODC paralogues

209   of *Xenopus laevis* (xlODC1, xlAZIN1 and xlAZIN2) and mODC. The amphibian xlAZIN2, as

210   xlODC1, shares with mODC the 22 residues that are required for the decarboxylating activity

211   [40, 44–48] whereas xlAZIN1, as reported for mammalian AZIN1 and AZIN2, lacks some

212   essential residues such as K69 and C360. These results indicate that, according to these putative

213   catalytic residues, xlAZIN2 appears to be closer to ODCs than to AZINs. Fig 2 also shows that

214   lower homologies were found in the ~70 amino acids residues of the C-terminal region. The

215   identity values of mODC with respect to xlODC1, xlAZIN2 and xlAZIN1 were 63%, 31% and

216   17%, respectively (S1 Table). Since two adjacent segments in the C-terminal region of ODC

217   (segments S1 and S2 in S2 Fig), have been proposed as having different roles in the

218   proteasomal degradation of ODC induced by AZ1 [19], we also calculated the sequence

219   homology in these segments among the different ODC homologues (S2 Fig). S1 Table also

220   shows that the identity values among S1 segments from mODC and its amphibian homologues

221   (77%, 44% and 26%) were higher than those corresponding among the S2 segments (66%,

222   14% and 9%).

223   **Fig 2. Comparison of the amino acid sequences of mouse ODC, xlODC1, xlAZIN1 and**

224   **xlAZIN2 using ClustalW program for multiple sequence alignment.** Asterisks represent

225   amino acid identity; colon and dots represent amino acid similarity between the proteins. Grey

226   background indicates amino acid residues associated with the catalytic activity of mODC that

227   are conserved in the *Xenopus laevis* homologues. In red: substitutions in these critical residues

228   in xlAZIN1.

229

230   **Functional analysis of xlAZIN2 in a heterologous cell system.**

231   To test the potential ornithine decarboxylase activity of xlAZIN2, HEK293T cells were

232   transiently transfected with xlAZIN2, and the decarboxylating activity was measured in

233   homogenates from the transfected cells. In parallel, cells were also transfected with the empty

234   vector and with plasmids containing the coding sequences of xlODC1 and xlAZIN1, in the

235   same vector as xlAZIN2. As displayed in Fig 3A, the homogenates from cells transfected with

236   xlODC1 showed, as expected, a high ODC activity in comparison to those from mock

237   transfected cells. In the case of xlAZIN2, the ODC activity was about 22% of the values found

238   for xlODC1, and much higher than that of xlAZIN1. Western blot analysis revealed that these

239   differences in ODC activity were not due to significant differences in protein expression levels.

240   Both xlODC1 and xlAZIN2 were inhibited by treatment of the cells with 1 mM DFMO (Fig

241   3B). These results suggested that either xlAZIN2 is an antizyme inhibitor more potent than

242   xlAZIN1 for increasing the endogenous ODC activity, or that it may possess intrinsic catalytic

243   activity.

244   **Fig 3. Expression of xlODC1, xlAZIN1 and xlAZIN2 in HEK293T transfected cells.**

245   HEK293T cells were transfected with the corresponding constructs of Flag-xlODC1, Flag-

246   xlAZIN1, Flag-xlAZIN2 or empty vector, as indicated in the Experimental Procedures. (A)

247   Top: ODC activity measured in the cell lysates. Bottom: Western blot analysis of the proteins

248   detected using anti-Flag or anti-ERK2 antibodies. Results are expressed as mean±SE, and are

249   representative of three experiments. (**) $P<0.01$ *vs* pcDNA3 or X-xlODC1. (B) Influence of

250   1mM alfa-difluoromethylornithine (DFMO) on the ornithine decarboxylase activity of

251   xlODC1 and xlAZIN2 cell lysates. (*) $P<0.05$.

252

253   To corroborate the latter possibility, we next analyzed the influence of xlAZIN2 on polyamine

254   levels. For that purpose, we studied the influence of xlAZIN2 transfection on the polyamine

255   content of the transfected cells and on that of the culture media, after 16 h of the transfection.

256   Fig 4A shows the chromatogram traces of the dansylated polyamines obtained by HPLC

257   analysis of HEK293T homogenates from cells transfected with xlAZIN2 or with the empty

258   vector. A dramatic increase in putrescine levels was evident after transfection with xlAZIN2.

259   Unexpectedly, the major increment was observed for cadaverine, the diamine that is produced

260   by decarboxylation of L-lysine, with values about 3-fold higher than those of putrescine. The

261   analysis of the polyamine content of the culture media of the xlAZIN2-transfected cells also

262   showed that cadaverine was the most abundant polyamine, with values about 10-fold higher

263   than those of putrescine (Fig 4B). The finding that the cadaverine to putrescine ratio in the cell

264   cultures was about 3-fold higher than the diamine ratio in the cell extracts revealed that

265   cadaverine is excreted more efficiently than putrescine in this type of cells.

266   **Fig 4. Analysis of the products formed by HEK293T cells transfected with different**

267   **constructs.** After 16 h of transfection, the culture media was aspirated and the cells collected.

268   An aliquot of the media was concentrated and resuspended in perchloric acid 0.4 M, whereas

269   the cells were homogenized in the same acid (200 μl per well). After centrifugation at 12.000

270   ×g for 15 min, the supernatants were dansylated and analyzed by HPLC as described in the

271   Experimental section. (A) Overlapped HPLC chromatogram traces of the dansylated extracts

272   from cells transfected with xlAZIN2 (red line) or with the empty vector pcDNA 3.1 (blue line).

273   Hexanediamine (Hxd) and heptanediamine (Hpd) were used as internal standards. Put:

274   putrescine; Cad: cadaverine; Spd: spermidine; Spm: spermine. (B) Overlapped HPLC

275   chromatogram traces corresponding to the dansylated polyamines present in the culture media

276   of cells transfected with xlAZIN2 (red line) or empty vector (blue line). (C) Comparison of the

277   polyamines found in the culture media of cells transfected with xlAZIN2 (red line) with those

278   of xlODC1, mODC and mAZIN2 (blue line).

279

280 Since it is known that mouse and rat ODCs are able to decarboxylate L-lysine, but less

281 efficiently than L-ornithine [49], we compared the levels of putrescine and cadaverine in cells

282 transfected with xlAZIN2 with those of the cells transfected with xlODC1, mODC or mAZIN2.

283 Figs 4C and 4D show that the ratio cadaverine:putrescine in the cells transfected with any of

284 the two ODCs were lower than one, whereas in the case of xlAZIN2 this ratio was higher than

285 7. These results indicate that xlAZIN2 is more efficient to synthesize cadaverine than

286 putrescine under the cell culture conditions employed in the assays. In addition, in the cells

287 transfected with mAZIN2, only vestigial levels of cadaverine were detected, whereas

288 putrescine levels were similar to those of xlAZIN2 transfected cells (Fig 4E). All these results

289 clearly indicated that xlAZIN2 behaves as an enzyme that can decarboxylate both amino acids

290 L-ornithine and L-lysine to produce putrescine and cadaverine, respectively.

Kinetic analysis of the decarboxylase activity of xlAZIN2

292 The enzyme kinetic parameters were analyzed using cell homogenates from xlAZIN2- or

293 xlODC1-transfected HEK293T cells and different substrate concentrations. Table 2 shows that

294 in the case of xlAZIN2 the Km for L-lysine (1.06±0.25 mM) was lower than the Km for L-

295 ornithine (6.57±1.75 mM), suggesting that the affinity of xlAZIN2 to L-lysine is higher than

296 the one to L-ornithine. The opposite was found for xlODC1, although here the affinity of

297 xlODC1 for L-ornithine was much higher than for L-lysine (Km^{Orn}=0.023±0.008mM and

298 Km^{Lys}=30.1±7.8 mM). Taking the ratio Vm/Km as an indicator of the catalytic efficiency of

299 each enzyme, the results presented in Table 2 indicate that xlAZIN2 was much more efficient

300 to decarboxylate L-lysine than xlODC1, whereas the opposite was found when L-ornithine was

301 the substrate. In parallel experiments, using enzymes purified by affinity chromatography with

302 anti-Flag beads, the Km values found were essentially similar to those presented in Table 2.

303 **Table 2. Comparison of the kinetic parameters of xlAZIN2 and xlODC1.**

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Substrate	L-ornithine			L-lysine		
	Km (mM)	Vm	Vm/Km	Km (mM)	Vm	Vm/Km
xlAZIN2	6.57±1.75	119±23	18.1	1.06±0.25	24.7±2.3	23.3
xlODC1	0.023±0.01	2.85±0.29	124	30.1±7.8	4.67±0.84	0.15

306

307 Vm is expressed as nmol of product formed per h and 10^6 cells. The ratio Vm/Km is

308 expressed in arbitrary units.

309

Study of a possible antizyme inhibitory action of xlAZIN2.

311 Although all above results clearly supported that xlAZIN2 has decarboxylating activity, it

312 could be likely that xlAZIN2 may also act as an antizyme inhibitor. To test this possibility, we

313 analyzed the ability of xlAZIN2 to rescue xlODC1from the predictable degradation induced by

314 AZ1, as early reported for mouse AZIN2 [37]. To this purpose, we carried out different co-

315 transfection experiments using several constructs. The results shown in Fig 5A corroborated

316 that, as expected, AZ1 stimulated the degradation of xlODC1, and that none of the two

317 xlAZIN2 constructs used (either with Flag for western blot detection or without Flag) were

318 able to protect xlODC1 from degradation. In addition, the results shown in this figure also

319 suggested that xlAZIN2 was induced to degradation by AZ1. To confirm this possibility,

320 xlAZIN2 was co-transfected with AZ1, and the cell homogenates were analyzed for

321 decarboxylase activity and xlAZIN2 protein content. Fig 5B clearly shows that AZ1 induced

322 the degradation of xlAZIN2. Taking into consideration that earlier studies showed that mouse

323 AZIN2 protected mouse ODC from degradation, whereas it was not degraded by AZ1 [37], the

324 results shown here do not support a role of xlAZIN2 as an antizyme inhibitor. On the contrary,

14

325   similar experiments using xlAZIN1 showed that AZ1, as it is known for mAZIN1 [26],

326   protected the amphibian protein from degradation (S3 Fig).

327   **Fig 5. Influence of AZ1 on protein levels of xlODC1 and xlAZIN2.** (A) Western blot of

328   lysates of HEK293T cells co-transfected with xlODC1 and different combinations of AZ1 and

329   xlAZIN2. (B) Western blot and ODC activity of lysates of cells co-transfected with Flag-

330   xlAZIN2 and pcDNA3 or AZ1. (***) P<0.001 *vs* pcDNA3 or F-xlAZIN2+AZ1.

331

332   Furthermore, the subcellular distribution of xlAZIN2 in the transfected cells was found to be

333   mainly cytosolic, similar to that of xlODC1 or mODC, and different from that of mAZIN2 (Fig

334   6). All these results demonstrate that the gene annotated as xlAZIN2 in the different gene

335   databanks does not code for a *bona fide* antizyme inhibitor, but instead it encodes for an

336   authentic amino acid decarboxylase with preference for L-lysine as substrate. Therefore, we

337   propose to change its name to lysine decarboxylase (LDC).

338   **Fig 6. Subcellular location of xlODC1 and xlAZIN2 in transfected cells.** Laser scanning

339   confocal micrographs of HEK293T cells transfected with xlODC1, xlAZIN2, mODC or

340   mAZIN2 fused to the Flag epitope. After transfections, cells were fixed, permeabilized and

341   stained with anti-Flag antibody and ALEXA anti-mouse and nuclear DAPI staining, and then

342   examined in a confocal microscope. Flag-proteins are shown in green and nuclei in blue.

343

Degradation of xlAZIN2 in HEK293T cells

345   The half live of xlAZIN2 was calculated by measuring the decay in both enzymatic activity

346   and protein content (estimated by western-blotting), after inhibition of protein synthesis by

347   cycloheximide treatment. Fig 7A shows that xlAZIN2 is a short-lived protein ($t_{1/2} \sim$ 34 min)

348   with a metabolic turn-over higher than that of xlODC1 ($t_{1/2} \sim$ 136 min), under the same

349   analytical conditions (Fig 7B). In addition, the great reduction in the degradative rate elicited

350   by treatment with MG132, a potent inhibitor of proteasomal degradation, shown by Fig 7C,

351   suggests that xlAZIN2 can be degraded by the mammalian proteasome in a similar way to that

352   of their mammalian orthologues.

353   **Fig 7. Protein stability of xlAZIN2 and xlODC1 in transfected cells**. After 16 h of

354   transfection, either with xlAzin2 or xlOdc1, cells were incubated with 200 μM cycloheximide

355   (CHX), harvested at the indicated times, and lysed in buffer containing a protease inhibitor

356   cocktail. (A) Left: Western blot analysis of xlAZIN2 protein using the anti-Flag antibody; right:

357   decay of ODC activity. (B) Similar experiments with xlODC1. Half-lives of xlAZIN2 and

358   xlODC1 in the transfected cells were calculated by linear regression analysis (GraphPad

359   software). (C) HEK293T cells transfected with xlAZIN2 or xlAZIN2+AZ1were incubated for

360   5 h with or without the proteasomal inhibitor MG132 (50 μM). xlAZIN2 protein was

361   determined as in (A). ERK2 was used as a loading control.

362

363   Since it is very well known that the last 37 amino acid residues of the carboxyl terminus of

364   mammalian ODC play a relevant role in its rapid intracellular degradation [50, 51], we decided

365   to analyze the relevance of this C-terminal region in the amphibian protein on the degradation

366   of xlAZIN2. For that purpose, we generated two mutated versions of xlAZIN2 and studied the

367   influence of the different antizymes (AZ1, AZ2, and AZ3) in the degradation of wild type

368   xlAZIN2, and in that of its C-terminal mutant forms, in the HEK293T-transfected cells. The

369   first xlAZIN2 mutant was a truncated form in which the last 21 amino acid residues of the C-

370   term of xlAZIN2 were deleted (xlAZIN2-ΔC). This deleted sequence

371   (CGWEISDSLCFTRTFAATSII) has a poor homology (14%) with the corresponding one in

372   mODC (CAQESGMDRHPAACASARINV). The second mutant was a quimeric protein

373   (xlAZIN2-mAZIN2) in which the mentioned C-terminal sequence in xlAZIN2 was substituted

374   by the corresponding C-terminal region of mAZIN2 (CGWEITDTLCVGPVFTPASIM). Fig

375   8A shows that, whereas AZ1, as earlier shown, increased the degradation of xlAZIN2, AZ2

376   and AZ3 did not stimulate the degradation of this protein. On the opposite, the truncation of

377   the C-terminal region of the protein prevented its AZ1-dependent degradation, indicating that

378   the 21 amino acid residues of the C-terminal region of xlAZIN2, as in the case of mODC, play

379   a relevant role in the degradative process. Again, as in the case of xlAZIN2, the stability of the

380   truncated protein was not significantly affected by any of the other two antizymes (Fig 8B).

381   Moreover, as shown in Fig 8C, the quimeric protein xlAZIN2-mAZIN2 showed against

382   antizymes a behavior similar to that found for xlAZIN2. This indicated that the substitution of

383   the C-terminal of xlAZIN2 by the corresponding region from mAZIN2, did not protect this

384   quimeric protein from the antizyme-induced degradation. Figs 9A and 9B also show that the

385   deletion of the C-terminal region of xlAZIN2 prevented its rapid degradation. The fact that

386   degradation of the quimeric protein xlAZIN2-mAZIN2 was decreased by MG132 (Fig 9C), as

387   already shown by the wild type protein (Fig 7C), suggested that the proteasome participates in

388   the degradation of both proteins.

389   **Fig 8. Influence of the C-terminal region of xlAZIN2 in the degradative process induced**

390   **by AZs.** HEK293T cells were transfected with: (A) xlAZIN2, (B) xlAZIN2 lacking the 21 C-

391   terminal residues (xlAZIN2-ΔC) or (C) with a construct coding for a quimeric protein with the

392   substitution of the 21 C-terminal residues of xlAZIN2 by the C-terminal segment of mouse

393   AZIN2 (xlAZIN2-mAZIN2). In parallel, each one of the constructs was co-transfected with

394   members of the AZ family (AZ1, AZ2, and AZ3). Western-blots were probed with anti-Flag

395   antibody. On the right side, schematic representations of xlAZIN2 and the two mutated

396   proteins.

397   **Fig 9. Protein stability of the mutated forms of xlAZIN2.** (A) After 16 h of transfection with

398   xlAZIN2-ΔC, cells were incubated with 200 μM cycloheximide (CHX), harvested at the

399 indicated times, and lysed in buffer containing a protease inhibitor cocktail. Top: western blot

400 analysis of xlAZIN2-ΔC at different times after CHX addition; bottom: changes in ODC

401 activity after CHX treatment. (B) Influence of the proteasomal inhibitor MG132 (50 μM) on

402 the effect of AZ1 on xlAZIN2-ΔC protein in HEK293T transfected cells. (C) Influence of the

403 proteasomal inhibitor MG132 (50 μM) on the effect of AZ1 on xlAZIN2-mAZIN2 protein in

404 HEK293T transfected cells.

Discussion

406 Our results clearly indicate that xlAZIN2 is devoid of antizyme inhibitory capacity, since it

407 was unable to rescue ODC from the negative effect of AZ1 (Fig 5A). In addition, AZ1 did not

408 protect xlAZIN2 from degradation (Figs 5B and 7C), contrary to what was reported for

409 mAZIN1 and mAZIN2 [26, 37].Unexpectedly, AZ1 accelerated the degradation of xlAZIN2

410 by the proteasome (Fig 7C), as it was also observed for xlODC1 (Fig 5A), and as early

411 described for mammalian ODCs [14, 52].

412 On the contrary, our findings unambiguously demonstrated that xlAZIN2 was able to

413 decarboxylate not only L-ornithine but also L-lysine, producing the diamines putrescine and

414 cadaverine, respectively (Figs 4A and 4B). It was also clear that in the cultured cells transfected

415 with either xlODC1 or mODC, cadaverine was also produced but in a lesser amount than

416 putrescine (Figs 4C and 4D). These results are in agreement with early reports that showed that

417 ODC from rodent tissues was able to decarboxylate both amino acids, although L-lysine less

418 efficiently than L-ornithine [49]. The comparison of the kinetic parameters of xlAZIN2 with

419 those of xlODC1 showed that the affinity of xlAZIN2 for lysine is about 30-fold higher than

420 that of xlODC1, whereas the opposite was evident for ornithine. All these results reveal than

421 in *Xenopus laevis* there are two related genes (xlODC1 and xlAZIN2) coding for enzymes able

422 to decarboxylate both amino acids ornithine and lysine. Whereas the function of xlODC1

18

423   appears to be related with the formation of putrescine, and therefore in consonance with that of

424   mammalian ODCs, our data suggest that it is very likely that the main role of xlAZIN2could

425   be concerned with the synthesis of cadaverine. Although some studies revealed the presence of

426   cadaverine in several amphibian tissues [53, 54], including those adult *Xenopus laevis* during

427   limb regeneration [55], the physiological function of this diamine is mostly unknown. Taking

428   into consideration that the protein sequence of xlAZIN2 is identical to that reported for xlODC2

429   [36], it can be assumed that xlODC2 may have lysine decarboxylase activity, although it should

430   be noted that no enzymatic activity for xlODC2 was measured in the mentioned report.

431   Interestingly, it was also reported that xlODC1 and xlODC2 showed different expression

432   patterns during *Xenopus laevis* embryo development [36]. The specific regional and temporal

433   expression of xlODC2 during specific stages of *Xenopus* embryo development [36], associated

434   to the mentioned lysine decarboxylase activity of xlODC2, suggest that cadaverine may have

435   some role during *Xenopus* embryogenesis, different to that of putrescine. This possibility could

436   also explain the reason for the existence of two apparently similar ODC decarboxylases in

437   *Xenopus*.

438   According to our results, the two *Xenopus* enzymes xlODC1 and xlODC2/xlAZIN2 expressed

439   in mammalian cells share several properties with mouse ODC, such as their cytosolic

440   localization, short half-lives, and AZ1-stimulated degradation by the proteasome. On the other

441   hand, xlODC2/xlAZIN2 differs from mAZIN2 in that the murine protein lacks decarboxylase

442   activity and is located in vesicular-like structures, and that AZ1 protects mAZIN2 from

443   degradation [37, 56, 57]. The mechanisms by which AZs exert opposite effects on the protein

444   stability of ODC and AZINs are not completely understood. Different studies have

445   demonstrated that in ODC there are two regions participating in its rapid turn-over [revised in

446   58]. The first region encompasses amino acid residues 117-140 needed for AZ binding (AZBE

447   region) [52]. The second is the C-terminal region in mammalian ODC [59–61] or the N-

448 terminal region of yeast ODC [31]. Interestingly, our results showed that the deletion of the 21

449 amino acid residues of the C-terminal region of xlODC2/xlAZIN2 made the truncated protein

450 more stable and resistant to AZ1-induced degradation by the proteasome. This result is in

451 agreement with early reports showing that the truncation of the carboxyl-terminal segment of

452 mouse ODC prevented its rapid intracellular degradation [50, 59]. However, the substitution

453 of this C-terminal region in xlAZIN2 by the corresponding one of mAZIN2 did not protect it

454 from AZ1-induced degradation, despite being known that the degradation of mAZIN2 is not

455 stimulated by binding to AZ1 [27]. Taking into consideration the low sequence homology

456 between the 21 amino acid C-terminal tail of xlAZIN2 or that of the quimeric protein with that

457 of mODC (14% and 9%, respectively, as shown in S1 Table), our results support the contention

458 that different C-terminal amino acid sequences may lead to the interaction of these ODC

459 homologous proteins with the proteasome. According to current views, an unstructured

460 terminal domain can be absolutely essential as the initiation site for protein degradation [62,

461 63]. As shown here, in the case of xODC homologues, different C-tail sequences can

462 accomplish this requirement. Apart from the implication of this terminal protein segment (S2)

463 in the initial infiltration of the protein in the proteolytic chamber of the proteasome, recent

464 studies based on structural analyses have proposed that the interaction of ODC-AZ complex

465 with the proteasome requires the exposure of the ODC residues 391-420 [19]. However, the

466 specific role of the different amino acid residues within this pre-terminal sequence (S1) on the

467 interaction with the proteasome is still unknown. As shown in S1 Table, it is clear that the

468 homology of the S1 segment of xlODC1 and xlAZIN2/ODC2, the two amphibian proteins

469 induced to be degraded by AZ1, with that of mODC is higher than those calculated for xlAZIN1

470 or mouse AZINs, proteins whose degradation is not stimulated by AZ1. This finding supports

471 early conclusions based on structural studies that claimed for the relevance of the 391-420 ODC

472 region for interacting with the proteasome [19]. The existence of the invariable sequence

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473   FNGFQ in the S1 segments of mODC, xlODC1 and xlAZIN2/ODC2 (S2 Fig), that according

474   to the above-mentioned structural study forms a short helical turn, suggests that this part of the

475   S1 terminal region may be critical for recognition by the proteasome. If this is the case, the

476   alteration of this sequence in the AZINs could make these proteins resistant to the degradative

477   stimulatory action of AZs.

478   Collectively, our study demonstrates, firstly, that xlAZIN2, although having a gene structure

479   similar to those of mammalian AZIN2s, is not really an antizyme inhibitor, but an authentic

480   decarboxylase with preference for L-lysine as substrate. According to this, the name of xlLDC

481   (or xlODC2), instead of xlAZIN2, should be used. Secondly, our results also extend the

482   previous knowledge on the influence of AZs on degradative aspects, from mammalian ODCs

483   to non-mammalian ODCs different from yeast or trypanosomal ODCs. Our findings support

484   the hypothesis that in the C-terminal region of *Xenopus* ODCs the last 21 amino acid tail is

485   required for antizyme-stimulated degradation of the enzyme, and suggest that the sequence

486   FNGFQ encompassing residues 396~400 may be relevant for the interaction of mammalian

487   and amphibian ODCs with the proteasome.

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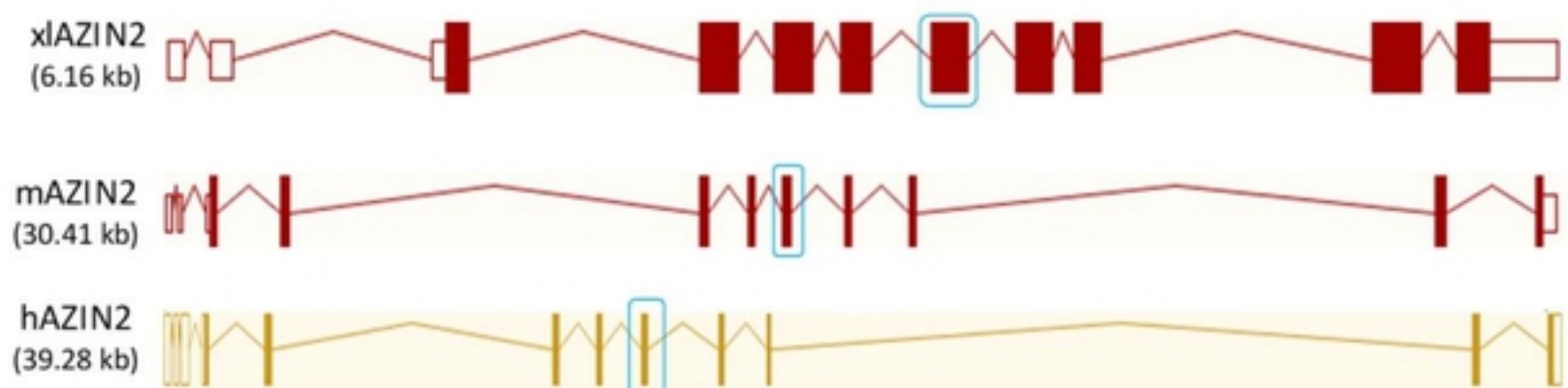667   10.1016/j.molcel.2012.08.029.

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Supporting information

S1 Fig. Comparison of the amino acid sequences of *Xenopus laevis* AZIN2 (xlAZIN2) and *Xenopus tropicalis* AZIN2 (xtAZIN2) using ClustalW program for multiple sequence alignment. Asterisks represent amino acid identity; colon and dots represent amino acid similarity between the proteins. Grey background indicates amino acid residues associated with the catalytic activity of mODC that are conserved in the *Xenopus* homologues.

S2 Fig. Sequences of the C-terminal region of mODC and its paralogues and *Xenopus laevis* orthologue proteins. (A) Scheme of the C-terminal region of mODC, where C represent the ~70 amino acid residues, and S1 and S2 the two subregions that may be important for proteasomal degradation of ODC induced by AZ1. (B) Detailed sequence of the C-terminal region of mODC and its different paralogues and orthologues. Sequences corresponding to S1 (residues 391-420) and S2 (residues 441-461) are underlined.

S3 Fig. Influence of AZ1 on protein levels of xlAZIN1. HEK293T cells were transfected with xlAZIN1-Flag alone or in combination with AZ1. Western-blots were probed with anti-Flag and anti-ERK2 antibodies.

S1 Table. Sequence identity between the C-terminal region of mODC and those of its *Xenopus laevis* homologues. C: terminal region from residues 391 to 461 in mODC; S1 and S2 are two subregions that may be important for ODC proteasomal degradation induced by AZ1; S1: residues 391-423; S2: residues 441-461. (See S2 Fig).

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Figure 1

```
mODC     MSSFTKD-EFDCHILDEGFTAKDILDQKINEVSSSDDKDAFYVADLGDILKKHLRWLKAL    59
xlODC1   MNGFSND-DFDFSFLEEGFCARDIVEQKINEVSLSDDKDAFYVADLGDIVKKHLRWFKAL    59
xlAZIN1  MKGFIEDTNYSIGLLDDSATPRDVVDNYIYEHTL-MGKNAFFVADLGKIVKKHFKWKNIM    59
xlAZIN2  MQGYI-Q-ESDFNLVEEGFLARDLMEEIINEVSQTEDRDAFFVADLGDVVRKHLRFLKAL    58
         *..:   : : .   ::::.     :*:::: * * :   .::**:*****.:::**::: : :

mODC     PRVTPFYAVKCNDSRAIVSTLAAIGTGFDCASKTEIQLVQGLGVPAERVIYANPCKQVSQ   119
xlODC1   PRVAPFYAVKCNDSKAVVKTLSILGAGFDCASKTEIQLVQSIGVSPERIIYANPCKQVSQ   119
xlAZIN1  GHIKPFYTVRCNSSPAVLEILAALGMGFACANKNEMSLVYDLGISMENVVYTNPCKQASQ   119
xlAZIN2  PRVKPFYAVKCNSSKGVVKILAELGAGFDCASKTEIELVQDVGVAPERIIYANPCKQISQ   118
         :: ***:*:**.*  .:. *: :* ** **.*.*:.** .:*:  *.::*:***** **

mODC     IKYAASNGVQMMTFDSEIELMKVARAHPKAKLVLRIATDDSKAVCRLSVKFGATLKTSRL   179
xlODC1   IKYAASNGVQMMTFDSEVELMKVARAHPKAKLVLRIATDDSKAVCRLSVKFGATLKTSRV   179
xlAZIN1  LKYAASVGVHMMTCDNVVELHKVAREAKFNTRLLLRIATDDSKARVRNHLNAKLLLH YATEGISGEEEMNMTFGTTLKNCRH   179
xlAZIN2  IKYAASNGVQMMTFDNEVELSKVSRSHPNARMVLRIATDDSKSSARLSVKFGAPLKSCRR   178
         **:**. **: ** :.* ** *:  * * :*:::*:***:. ..   .:::.**: **..*

mODC     LLERAKELNIDVIGVSFHVGSGCTDPETFVQAVSDARCVFDMATEVGFSMHLLDIGGGFP   239
xlODC1   LLERAKELNVDIIGVSFHVGSGCTDPQTYVQAVSDARCVFDMGAELGFNMYLLDIGGGFP   239
xlAZIN1  LLDCAKELSVEVVGVKFHVSSSSNNPQTYIHALSDARCVFDMAKELGFKMNILDIGGIS-   238
xlAZIN2  LLEMAKNLSVDVIGVSFHVGSGCTDSKAYTQAISDARLVFEMASEFGYKMWLLDIGGGFP   238
         **: **:*.:::*:**.***.*...: ::: :*:**** **:*. *.*:.* :*****

mODC     GSEDTKLKFEEITSVINPALDKYFPSDSGVRIIAEPGRYYVASAFTLAVNIIAKKTVWKE   299
xlODC1   GSEDVKLKFEEITSVINPALDKYFPTDSAVKIIAEPGRYYVASAFTLAVNIIAKKVMVNE   299
xlAZIN1  ---ENEAQLEEVYQAVSPLLDVYFPEGSGTRIIAEPGSFYVSSAFTLAVNVIAKEATEHD   295
xlAZIN2  GTEDSKIRFEEIAGVINPALDMYFPESSDVQIIAEPGRYYVASAFSLAVNIIAKKEVEHS   298
         : : ::**:  ..* ** ***  .*  .:****** :**:***:****:***:    :.

mODC     QPGSDDE-DESNEQTFMYYVNDGVYGSFNCILYDHAHVKALLQKRPKPDEKYYSSSIWGP   358
xlODC1   QSGSDDEEDAANDKTLMYYVNDGVYGSFNCILFDHAHVKPVLTKKPKPDEKFYSSSIWGP   359
xlAZIN1  QHLSSAGKPNSNKPAFIYCMKEGVYGSFARKLSEKLNTAPEVHKKYKDNEPLFASSLLGP   355
xlAZIN2  V--SD-DEENESSKSIMYYVNDGVYGSFNCLVFDHAHPKPILHKKPSPDQPLYTSSLWGP   355
           *.       .. ::.* :::****** :  :: :   : *: . :: ::**: **

mODC     TCDGLDRIVERCNLPEMHVGDWMLFENMGAYTVAAASTFNGFQRPNIYYVMSRPMWQLMK   418
xlODC1   TCDGLDRIVERFELPELQVGDWMLYENMGAYTVAASSTFNGFQRPTLHYVMSRPHWQLMQ   419
xlAZIN1  SYDELDVIVEHCLLPELEVGDWIVFDNMGCGSVNETSPFTDFDKPSLYNFMTFSDWYEIQ   415
xlAZIN2  TCDGLDQIAERVQLPELHVGDWLLFENMGAYTIAASSNFNGFQQSPVHYAMPRAAWKAVQ   415
         : * ** *.*:   ***:.****:::::***. ::   :* *..*::   ::   *    *   ::

mODC     QIQSHGFPPEVEEQDDGTLPMSCAQESGMDRHPAACASARINV----            461
xlODC1   DIKEHGILPEVPEV--SALHVSCARESGMELTPTVCTAASINV----            460
xlAZIN1  DTGNTSES---L-MKSLCVP--CFPLG----EEQLCTD---------            443
xlAZIN2  LLQRGL--QQTEEKENVCTPMSCGWEIS----DSLCFTRTFAATSII           456
                      *.               *
```

A)

ODC activity
(nmol CO_2/ h mg protein)

40
30
20
10
0

**

pcDNA3   F-xlAZIN1   F-xlODC1   F-xlAZIN2

Anti-Flag

Anti-ERK2

B)

ODC activity (%)

150
100
50
0

F-xlODC1   F-xlODC1+DFMO

*

ODC activity (%)

150
100
50
0

F-xlAZIN2   F-xlAZIN2+DFMO

*

Figure 3

Figure 4

A)

F-xlODC1	+	+	+	+	−
AZ1	−	+	+	+	−
xlAZIN2	−	−	+	−	−
F-xlAZIN2	−	−	−	+	+

Anti-Flag

Anti-ERK2

B)

Anti-Flag

Anti-ERK2

***

ODC activity (%)

100

50

0

pcDNA3   F-xlAZIN2   F-xlAZIN2+AZ1

Figure 5

Figure 6

A)

Fig. 7

B)

C)

Figure 7

A)

xlAZIN2	+	+	+	+
AZ1	-	+	-	-
AZ2	-	-	+	-
AZ3	-	-	-	+

Anti-Flag

Anti-ERK2

xlAZIN2

B)

xlAZIN2-ΔC				
AZ1	-	+	-	-
AZ2	-	-	+	-
AZ3	-	-	-	+

Anti-Flag

Anti-ERK2

xlAZIN2-ΔC

C)

xlAZIN2-mAZIN2	+	+	+	+
AZ1	-	+	-	-
AZ2	-	-	+	-
AZ3	-	-	-	+

Anti-Flag

Anti-ERK2

xlAZIN2-mAZIN2

Figure 8

A)

xlAZIN2-ΔC

B)

xlAZIN2-ΔC	+	+	+	+
AZ1	-	+	-	+
MG132	-	-	+	+

xlAZIN2-ΔC

ERK2

C)

xlAZIN2-mAZIN2	+	+	+	+
AZ1	-	+	-	+
MG132	-	-	+	+

xlAZIN2-mAZIN2

ERK2

Figure 9