Ammodytoxin, a secretory phospholipase A<sub>2</sub>, inhibits G<sub>2</sub> cell-cycle arrest in the yeast Saccharomyces cerevisiae

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INTRODUCTION

Secretory phospholipases A<sub>2</sub> (sPLA<sub>2</sub>s) belong to the superfamily of enzymes that catalyse the hydrolysis of the ester bond at the sn-2 position of phosphoglycerides. They are involved in several (patho)physiological processes, ranging from inflammation, cell injury and tumour resistance to neurotoxicity [1,2], and are thus important under physiologically normal conditions as well as in disease.

Ammodytoxin (Atx), an sPLA<sub>2</sub> (secretory phospholipase A<sub>2</sub>), binds to γ and ε isoforms of porcine 14-3-3 proteins in vitro. 14-3-3 proteins are evolutionarily conserved eukaryotic regulatory proteins involved in a variety of biological processes, including cell-cycle regulation. We have now shown that Atx binds to yeast 14-3-3 proteins with an affinity similar to that for the mammalian isoforms. Thus yeast Saccharomyces cerevisiae can be used as a model eukaryotic cell, which lacks endogenous phospholipases A<sub>2</sub>, to assess the in vivo relevance of this interaction. Atx was expressed in yeast cells and shown to be biologically active inside the cells. It inhibited G<sub>2</sub> cell-cycle arrest in yeast, which is regulated by 14-3-3 proteins. Interference with the cell cycle indicates a possible mechanism by which sPLA<sub>2</sub>s are able to cause the opposing effects, proliferation and apoptosis, in mammalian cells.

Key words: secretory phospholipase A<sub>2</sub>, G<sub>2</sub> cell-cycle arrest, cell proliferation and death, Saccharomyces cerevisiae, model organism, 14-3-3 protein.

EXPERIMENTAL

Strains and media

The following strains were used: yUP10 (S288c-derived; MATα ura3 Met<sup>+</sup>), pex11Δ (S288c-derived, EUROSCARF accession no. Y17129; MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 YOL147c::kanMX4) and the corresponding wild-type strain BY4742 (S288c-derived, EUROSCARF accession no. Y10000; MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0). BY4742 and pex11Δ strains were obtained from EUROSCARF (European Saccharomyces cerevisiae Archive for Functional Analysis, Frankfurt, Germany).

The gene coding for mature AtxA [19] was cloned in a PRS316-derived plasmid (ARS1 CEN4 URA3) under the control of the GAL1 promoter and transformed into the respective strains

Abbreviations used: Atx, ammodytoxin; GIIA, group IIA; sPLA<sub>2</sub>, secretory phospholipase A<sub>2</sub>; MALDI–TOF–MS, matrix-assisted laser-desorption ionization–time-of-flight MS.

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using the lithium acetate method. Strains transformed with the empty vector were used as the control in all experiments. Cells were grown in the synthetic minimum uracil dropout medium [YNB CSM − URA (where YNB stands for yeast nitrogen base and CSM − URA for complete supplement medium without uracil); 1.7 g of YNB + 0.67 g of CSM − URA + 2% (w/v) glucose or 2% (w/v) galactose in 1 litre of water], either with glucose (2%) as the carbon source, which represses Atx expression, or with galactose (2%) as the carbon source, which induces Atx expression. yUP10 strain was grown in rich [YPD (1%, w/v, yeast extract, 2%, w/v, peptone and 2% glucose)] medium for isolation of 14-3-3 proteins.

**Isolation of Bmh1p and Bmh2p**

Bmh1 and Bmh2 proteins (i.e. yeast 14-3-3 proteins) were isolated by the method of Toker et al. [20]. Frozen yeast (80g), strain yUP10, was thawed and homogenized in 100 ml of buffer A (0.25 M sucrose, 20 mM Tris/HC1, pH 7.5, 2 mM EDTA, 10 mM EGTA and 1 mM dithiothreitol) to which proteinase inhibitors (31 µg/ml benzamidine, 25 µg/ml bacitracin, 2 µg/ml soya-bean trypsin inhibitor, 1.4 µg/ml pepstatin and 1 µg/ml leupeptin) had been added immediately before use. The final preparation was concentrated and dialysed against 50 mM Tris (pH 8.4) and 0.1 mM CaCl2. Western-blot analysis, using rabbit anti-14-3-3 concentrated and dialysed against 50 mM Tris (pH 8.4) and 0.1 mM CaCl2 over the chip and following the changes in refractive index at the sensor surface. The interaction between Bmh1/2 proteins and the chip-immobilized Atx was studied by injecting solutions of Bmh1/2 proteins in 50 mM Tris (pH 8.4) and 0.1 mM CaCl2. Western-blot analysis, using rabbit anti-14-3-3/β polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), was performed to verify the identity of the isolated proteins as 14-3-3 homologues.

**Surface plasmon resonance**

Surface plasmon resonance experiments were performed at 25°C using a Biacore X system. Atx in 10 mM Mes (2-morpholinoethanesulphonic acid) buffer (pH 6.5) and 5 mM CaCl2 was immobilized on the CMS sensor chip as described in [21]. The interaction between Bmh1/2 proteins and the chip-immobilized Atx was studied by injecting solutions of Bmh1/2 proteins in 50 mM Tris (pH 8.4) and 0.1 mM CaCl2 over the chip and following the changes in refractive index at the sensor surface. The sensor chip was regenerated with 5 mM NaOH between consecutive injections.

**Determination of the mutation rate and of the ratio of G1/G2 phase cells**

H2O2 treatment

Atx-expressing and control cells of the strain yUP10 were grown in the medium that induces Atx expression from early exponential phase until they reached late exponential phase. Cells were aliquoted and H2O2 was added to 5 mM of final concentration; the same volume of distilled water was added to mock-treated samples. The cells were rotated at 250 rev./min and 30°C for an additional 3 h to determine the G1/G2 cell ratio, and for 16–48 h to determine the mutation rate. The viability of the cells was determined by plating appropriate dilutions on YPD plates and it was above 50% for wild-type cells after 3 h treatment under our experimental conditions.

Determination of the G1/G2 ratio

Ethanol (1 ml) was added to 0.5 ml of yeast culture and the cells were rotated overnight. Cells were pelleted, washed with PBS. RNase was added to 1 mg/ml concentration and the samples were incubated for 1 h at 37°C, or alternatively overnight at 4°C. Then, 1 µl of 20 mg/ml protease K in water was added and the samples were vortex-mixed and incubated for 1 h at 50–55°C. The cells were pelleted, washed with PBS, resuspended with 200 µl of 100 µg/ml propidium iodide in PBS and incubated overnight in the dark at 4°C. They were then analysed using an FACSCalibur (Becton Dickinson, San Jose, CA, U.S.A.) flow cytometer, and at least 100 000 viable cells were counted for each measurement. Three independent experiments were performed for each strain under each condition and then average values and S.D. were calculated.

**Proteomic analysis**

Atx-expressing and control cells of the strain yUP10 were grown from early to late exponential phase in the medium that induces Atx expression and harvested by centrifugation. Proteins were isolated and 50 µg of protein from each sample was labelled with 100 µg/ml propidium iodide in PBS and incubated overnight in the dark at 4°C. They were then analysed using an FACSCalibur (Becton Dickinson, San Jose, CA, U.S.A.) flow cytometer, and at least 100 000 viable cells were counted for each measurement. Three independent experiments were performed for each strain under each condition and then average values and S.D. were calculated.

**RESULTS**

**Binding of Atx to yeast 14-3-3 homologues**

The two yeast homologues of 14-3-3 proteins, Bmh1p and Bmh2p, were isolated from yeast and their interaction with Atx was assayed using surface plasmon resonance (Figure 1). The apparent
Expression of active Atx in yeast cells

Atx expressed in yeast *S. cerevisiae* under the control of the galactose-inducible GAL1 promoter was biologically active. This was shown by expressing it in the *pex11Δ* strain that is defective in peroxisome biogenesis and inheritance [24,25]. The *pex11Δ* strain harbouring the GAL1-Atx plasmid grew on galactose medium significantly slower than the wild-type BY4742 strain or the respective strains harbouring empty plasmids (Figure 2).

Growth rates of the wild-type strain harbouring the empty vector, the wild-type strain harbouring the GAL1-Atx plasmid and the *pex11Δ* strain harbouring the empty vector did not differ significantly under any of the conditions tested, and expression of an inactive Atx mutant had no effect on the *pex11Δ* strain (results not shown), confirming that the observed inhibition of growth was a consequence of active Atx expression. Subsequent replacement of galactose by glucose in the medium resulted in the *pex11Δ* cells harbouring the GAL1-Atx plasmid starting to grow again at the wild-type growth rate, indicating that the effect of Atx on the *pex11Δ* strain was cytostatic.

Effect of intracellular Atx on H$_2$O$_2$-mediated cell-cycle arrest

Bmh1p and Bmh2p are required for a proper response to DNA damage, in particular by involvement in DNA damage checkpoints [26]. We therefore considered the possible effects of Atx on the cell cycle of the wild-type strain of *S. cerevisiae*. Different growth perturbations were used to compare the responses of Atx-expressing and control yUP10 strains. After treatment of cells in late exponential phase with 5 mM H$_2$O$_2$, we observed an approx. 2-fold lower survival of Atx-expressing yeast cells compared with control cells. Since, in *S. cerevisiae*, H$_2$O$_2$ is known to cause cell-cycle arrest specifically in the G$_2$ phase [27], inhibition of the arrest was considered as a possible cause of decreased survival of the Atx-expressing strain. The ratios of the number of cells in the G$_1$ and G$_2$ phases of Atx-expressing versus control cells were therefore determined after treating the cells with H$_2$O$_2$, using propidium iodide staining and flow cytometry. Under non-perturbed growth conditions, the ratio was the same for the two strains, i.e. 1:1.04 ± 0.13 for the Atx-expressing strain and 1:1.03 ± 0.07 for the control strain. However, when the cells were treated with 5 mM H$_2$O$_2$ for 3 h, the Atx-expressing strain showed no significant change in the G$_1$/G$_2$ ratio (1:1.08 ± 0.04), whereas the control strain showed a decrease (1:1.23 ± 0.06), indicative of G$_2$ cell-cycle arrest, as reported in [27]. These observations suggest that Atx inhibited the G$_2$ cell-cycle arrest in yeast *S. cerevisiae*.

Inhibition of the G$_2$ cell-cycle arrest leads to increased mutation rate [28]. In keeping with this, when the yUP10 cells were exposed to 5 mM H$_2$O$_2$ for periods between 16 and 48 h, the mutation rate, measured by the occurrence of canavanine resistance, was 5–50-fold higher in Atx-expressing cells than in control cells, confirming the effect of Atx on the regulation of the cell cycle, and explaining the observed decreased survival rate of the Atx-expressing strain after H$_2$O$_2$ treatment. No difference in mutation rate was observed between non-treated Atx-expressing and control strains.

### Proteomic analysis

Changes in protein expression in the Atx-expressing wild-type yUP10 strain were determined by two-dimensional electrophoresis. Six polypeptides with >2-fold higher expression in Atx-expressing cells than in the control strain were detected using fluorescent labelling of proteins with Cy2 and Cy5, and identified by MALDI–TOF-MS (Table 1; Figure 3). No underexpressed proteins were found.

Spot 1 corresponded to Tef1p and/or Tef2p. Since they have identical amino acid sequences, it was not possible by this method to determine which of the two proteins was overexpressed. Tdh3p was clearly identified in spot 2, despite its 96% identity with Tdh42p isofrom, as was the Ydl086w protein of unknown function. In the case of the degradation product in spot 5, it was impossible to distinguish between the two isofroms. Adh1p and Rad2p were identified as degradation products, corresponding to partial sequences in the databases with GenBank entries CAA24600 and CAA69080 respectively.

Table 1 Overexpressed proteins in the Atx-expressing yeast strain

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein</th>
<th>Apparent MM/pI</th>
<th>Protein function</th>
<th>Fold overexpression</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tef1p*</td>
<td>50 kDa/9.5</td>
<td>Translation elongation factor EF 1α</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>Tdh3p*</td>
<td>36 kDa/7.0</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>Ydl086w</td>
<td>25 kDa/6.0</td>
<td>Unknown</td>
<td>2.2</td>
</tr>
<tr>
<td>4</td>
<td>Adh1p (N-terminal peptide CAA24600)</td>
<td>14 kDa/6.5</td>
<td>Alcohol dehydrogenase</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>Tdh2/3p* (degradation product)</td>
<td>13 kDa/6.4</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>2.2</td>
</tr>
<tr>
<td>6</td>
<td>Rad2p (N-terminal peptide CAA69080)</td>
<td>6 kDa/7.0</td>
<td>Involved in nucleotide excision repair</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* From the data, it could not be determined which of the two isofroms was overexpressed.

DISCUSSION

Atx was shown to bind *in vitro* to evolutionarily conserved mammalian proteins, γ and ε isofroms of 14-3-3 proteins and calmodulin [6,7]. There are two yeast homologues of 14-3-3 proteins, Bmh1p and Bmh2p, which are 92% identical in amino acid sequence and 72 and 73% identical with the human ε isofrom respectively. We demonstrated in the present study that Atx binds...
in vitro to yeast homologues of 14-3-3 proteins, Bmh1p and Bmh2p, with apparent $K_a$ values similar to those measured for the interaction between Atx and mammalian 14-3-3 proteins (Figure 1). On the other hand, Atx does not bind in vitro to yeast calmodulin, Cmd1p (U. Petrović and I. Križaj, unpublished work). We took advantage of these facts to use yeast S. cerevisiae, which has no endogenous, intracellularly active PLA$_2$s, as a model eukaryotic system to elucidate the effect of binding of Atx to 14-3-3 proteins in vivo.

The mature form of Atx was expressed in yeast cells. Comparison of the phospholipolytic activity of lysates of the Atx-expressing yUP10 strain under the GAL1 promoter with those of the control strain showed that Atx is enzymatically active and that the number of active Atx molecules per cell is approx. $10^7$ [18], which is probably more than the amount of Atx that enters the mammalian cell on envenomation in vivo. Here, the biological activity of Atx inside the yeast cell was confirmed by expressing the phospholipase in peroxisome-deficient strains. The expression of Atx inhibited the growth of pex11A yeast cells (Figure 2), as well as of pex3A and pex5A strains (M. Mattiazzi, U. Petrović and I. Križaj, unpublished work). One possible explanation for the requirement of functional peroxisomes for the viability of Atx-expressing strains is its intracellular phospholipolytic activity generating free fatty acids that are toxic to S. cerevisiae [29,30]. In wild-type yeast, free fatty acids are known to exert oxidative stress by uncoupling the respiratory chain [31], causing a transient arrest in cell multiplication before metabolic adaptation that includes fatty acid $\beta$-oxidation in peroxisomes [30]. Yeast strains with impaired peroxisome biogenesis and inheritance have decreased levels of fatty acid $\beta$-oxidation [24,32]. According to this hypothesis, in peroxisome-deficient strains, the metabolic adaptation to free fatty acids produced by enzymatically active Atx would be hampered; therefore in the Atx-expressing strain the growth arrest would be persistent. The fact that Atx exerts a cytostatic rather than a cytotoxic effect on the pex11A strain is in line with this hypothesis. Our experimental results support the notion that it is the intracellular activity of Atx that causes the observed effects. First, no phospholipolytic activity was detected in the extracellular medium of Atx-expressing yeast cells [18] and secondly, addition of Me-indoxam, a cell-impermeable inhibitor of sPLA$_2$s [16,33] that also inhibits Atx activity, to the medium did not suppress the growth inhibition of Atx-expressing pex11A strain (results not shown).

In S. cerevisiae, Bmh1p and Bmh2p are involved in the induction of G$_2$ cell-cycle arrest [26]. GIIA sPLA$_2$s cause proliferative and proapoptotic effects in mammalian cells and it has been proposed that separate pathways lead to these opposing effects [12–14]. Alternatively, these effects could be caused by a common effect on cell-cycle regulation, and the final outcome of the sPLA$_2$ activity would depend on the cell type and/or environmental factors. In keeping with this latter possibility, we checked whether Atx affects G$_2$ cell-cycle arrest. Unlike in wild-type S. cerevisiae, where H$_2$O$_2$ causes specific G$_2$ cell-cycle arrest [27], addition of H$_2$O$_2$ did not affect the G$_1$/G$_2$ phase ratio in the Atx-expressing wild-type strain. This could either mean that the Atx-expressing strain is resistant to H$_2$O$_2$-mediated DNA damage [34] or that G$_2$ cell-cycle arrest is inhibited in the presence of this sPLA$_2$. The increase in mutation rate and decrease in viability of the Atx-expressing strain demonstrated in the presence of H$_2$O$_2$ strongly favour the second explanation. Moreover, we observed no significant difference in the rate of H$_2$O$_2$ degradation between Atx-expressing and control strains (results not shown), further pointing to inhibition of G$_2$ cell-cycle arrest in the Atx-expressing strain.

The proposal that interaction of GIIA sPLA$_2$ with 14-3-3 proteins affects cell-cycle regulation could also apply to mammals.
14-3-3 proteins are involved in the onset of the G2 cell-cycle arrest in mammalian cells [35,36], and molecules that interfere with their function are potent abrogators of G2 cell-cycle arrest [37,38]. Human GIIA sPLA2s, which induces apoptosis in neurons [14], binds to 14-3-3 proteins in vitro (G. Faure and I. Križaj, unpublished work). In addition, besides the human GIIA sPLA2s and all the Atx isoforms, AtxA, AtxB and AtxC, other GIIA sPLA2s bind in vitro to yeast 14-3-3 proteins: βbungarotoxin, taipoxin, and ammodytin L, a non-neurotoxic paralogue of Atx with their function are potent abrogators of G2 cell-cycle arrest in mammalian cells [35,36], and molecules that interfere with G2 cell-cycle arrest is the mechanism behind the proliferative effect of Atx in yeast. To establish this putative connection in mammalian cells, we can speculate, on the basis of our results from the yeast system, that binding to 14-3-3 proteins and consequent inhibition of the G2 cell-cycle arrest would lead to faster growth and pronounced proliferation, whereas, in other instances, such as cells with potentially lethal mutations, the inability to repair DNA damage would result in non-permissive genomic changes and would result in (apoptotic) cell death.

Proteomic analysis was used for a global-view comparison of Atx-expressing and control strains. The common denominator connecting three of the proteins shown to be overexpressed (Table 1), Tdh3p, Adh1p and Tef1/2p, is that they have been reported as the prime targets of S-thiolation in response to H2O2 [40]. In the case of glyceraldehyde-3-phosphate dehydrogenase, three isoforms are present in yeast, namely Tdh1p, Tdh2p and Tdh3p, of which only Tdh3p is the major target of S-thiolation [41]. Similarly, of the five isoforms of alcohol dehydrogenase in yeast, only Adh1p was identified as a target protein [40]. Translational elongation factors, including Tef1/2p, are the third functional group of the S-thiolation target proteins [40]. According to the ‘guilt-by-association’ paradigm, this suggests that most of the observed changes in the proteome are not the consequence of a specific effect of Atx on the metabolic processes in which the overexpressed proteins are involved, but rather are related to the putative oxidative stress caused by Atx expression. The oxidative stress could be a direct consequence of the free fatty acids generated by Atx, as described above. Another overexpressed protein was identified as Rad2p, a homologue of xeroderma pigmentosum group G protein, involved in DNA nucleotide excision repair [42,43]. In addition, Ydl086w, a protein with undetermined function, TF1IB of the RNA II polymerase holoenzyme Sua7p [44], and a DEAD-box helicase Dbp8p [45], indicating its involvement in nuclear processes, possibly including DNA repair. None of the identified overexpressed proteins is known to be involved in any other process affected by Atx in yeast. It is therefore reasonable to conclude that Atx expression in yeast activates the DNA damage repair machinery, which is in agreement with the effect of Atx on the inhibition of the G2 cell-cycle arrest, since this checkpoint is also important when the cells are growing in the absence of any mutagenic effect, and its impairment in mammalian cells results in increased DNA damage [46].

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