FEBS Letters xxx (2009) xxx–xxx

FEBS Letters
journal homepage: www.FEBSLetters.org

The antifungal plant defensin RsAFP2 from radish induces apoptosis in a metacaspase independent way in *Candida albicans*

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A R T I C L E I N F O

Article history:
Received 27 April 2009
Revised 24 June 2009
Accepted 9 July 2009
Available online xxxx

Edited by Vladimir Skulachev

Keywords:
Apoptosis
Plant defensin
Antifungal
Mode of action
Metacaspase
Candida albicans metacaspase 1

A B S T R A C T

We show that the antifungal plant defensin *Raphanus sativus* antifungal protein 2 (RsAFP2) from radish induces apoptosis and concomitantly triggers activation of caspases or caspase-like proteases in the human pathogen *Candida albicans*. Furthermore, we demonstrate that deletion of *C. albicans* metacaspase 1, encoding the only reported (putative) caspase in *C. albicans*, significantly affects caspase activation by the apoptotic stimulus acetic acid, but not by RsAFP2. To our knowledge, this is the first report on the induction of apoptosis with concomitant caspase activation by a defensin in this pathogen. Moreover, our data point to the existence of at least two different types of caspases or caspase-like proteases in *C. albicans*.

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1. Introduction

Plant defensins are small, basic, cysteine-rich peptides that possess antifungal and in some cases also antibacterial activity (reviewed in [1,2]). Plant defensins are not only active against phytopathogenic fungi, but also against baker's yeast and human pathogenic fungi (such as *Candida albicans*). Moreover, they are non-toxic to human and plant cells, highlighting their therapeutic potential as novel antimycotics [3].

We previously demonstrated that *Raphanus sativus* antifungal protein 2 (RsAFP2), a plant defensin isolated from seeds of radish (*R. sativus*) [4], interacts with the sphingolipid glucosylceramide (GlcCer) in the plasma membrane of susceptible fungi and yeast species [5]. This interaction leads to a subsequent permeabilization of the cell and to cell growth arrest [6]. Fungal and yeast species or mutants with membranes either lacking GlcCer due to the absence of a glucosylceramide synthase (*GCS*) gene as in *Saccharomyces cerevisiae* and *Candida glabrata* [7], or composed of GlcCer with a structurally altered ceramide moiety [8] are resistant to RsAFP2-induced membrane permeabilization and cell death [5,9]. Moreover, RsAFP2 induces endogenous reactive oxygen species (ROS) in *C. albicans*, but not in a RsAFP2-resistant *C. albicans* $gcs\Delta/\Delta$ mutant lacking GlcCer [10]. The accumulation of ROS is one of the phenotypic markers of yeast cells undergoing apoptosis [11].

In this study, we show that RsAFP2 induces apoptosis in *C. albicans*. Moreover, RsAFP2 treatment results in the activation of caspases, which is independent of metacaspase *Ca*Mca1p.

2. Materials and methods

2.1. Materials and microorganisms

RsAFP2 was isolated as described previously [4]. Yeast strains used in this study are *Candida albicans* strain CAI4 [12], BWP17 [13], BWP17 *mca1*Δ/Δ (*mca1*Δ::*FRT*/*mca1*Δ::*FRT*) and BWP17 *mca1* Δ/Δ-*MCA1* (*mca1*Δ::*FRT*/*mca1*Δ::*MCA1-SAT1-FLIP*). Media used were YPD and PDB/YPD [5].

Abbreviations: CaMCA1, *Candida albicans* metacaspase 1; CFU, colony forming unit; DHE, dihydroethidium; GlcCer, glucosylceramide; PI, propidium iodide; PS, phosphatidylserine; ROS, reactive oxygen species; RsAFP2, *Raphanus sativus* antifungal protein 2; TUNEL, terminal transferase-mediated dUTP nick end labeling; z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone

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0014-5793/$36.00 © 2009 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2009.07.004

2.2. Statistical analysis

Statistical analysis was performed using paired *T*-test.

2.3. Construction of *C. albicans mca1Δ/Δ* mutant and the CaMCA1 complemented *C. albicans mca1Δ/Δ* mutant

The *C. albicans mca1Δ/Δ* mutant was constructed using the *URA3* flipper cassette [14], see Supplementary data and Supplementary Fig. 1.

2.4. Antifungal activity assay

Exponentially growing *C. albicans* cultures in YPD ($OD_{600} = 2 \times 10^7$ cells/ml) were incubated with 10 μg/mL RsAFP2 or water in the presence or absence of 80 μM carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone (z-VAD-FMK) caspase inhibitor (Promega, Madison, WI, USA) in PDB/YPD growth medium. After 0 h and 2 h 30 min of incubation at 30 °C, 100 μL aliquots were plated on YPD plates and colony forming units (CFUs) were counted after 2 days of incubation at 30 °C. Percentage killing was calculated based on the ratio of the number of CFUs after treatment with RsAFP2 as compared to water-treated controls. Data are the mean of three independent experiments, each consisting of duplicate measurements.

2.5. Yeast apoptosis assays

Exponentially growing *C. albicans* cultures in YPD ($OD_{600} = 2$) were incubated with 10 μg/mL RsAFP2 or water in PDB/YPD for 2 h 30 min at 30 °C. Percentage killing was determined by performing plating assays in which colony formation of 500 cells on YPD agar plates was analyzed. Apoptotic markers, including ROS levels, phosphatidylserine externalization and DNA fragmentation of yeast cultures ($n = 500$ cells per measurement; values presented in the text are mean of triplicate measurements), were visualized via staining with dihydroethidium (DHE), FITC-labelled annexin V in combination with propidium iodide and TUNEL, respectively, as described previously [11]. For image acquisition, a fluorescence microscope (Axioskop 2; Zeiss), a digital camera (Spot RT-SE; Diagnostic Instruments Inc.), and MetaMorph software (version 6.2r4; Universal Imaging Corp.) were used. The percentage positive cells for the different treatments was calculated relative to the total number of cells (i.e. 500) and is the mean of three independent experiments.

2.6. Caspase activity

Exponentially growing *C. albicans* cultures in YPD ($OD_{600} = 0.5$) were incubated with 120 mM acetic acid or water in YPD pH3.0 supplemented with 80 μg/mL uridine, 80 μg/mL arginine and 40 μg/mL histidine, for 2 h at 30 °C; or *C. albicans* cultures in YPD ($OD_{600} = 2.0$) were incubated with 10 μg/mL RsAFP2 or water in PDB/YPD for 2 h 30 min at 30 °C. Caspase activity was measured using the CaspACE FITC-VAD-FMK In Situ Marker (Promega) [15] using a fluorescence microscope (model Axio Imager Z1), a digital camera (model Axio Cam Mrm), and Axio Vision Rel. 4.6 software. The percentage caspase-active cells for the different treatments was calculated relative to the total number of cells (i.e. 500) and is the mean of three independent experiments.

3. Results

3.1. RsAFP2 induces apoptosis in *C. albicans*

Apoptosis in yeast is best studied at rather high survival rates, since severe killing results in a high necrotic yeast population [16]. We previously demonstrated that treatment of susceptible fungi with 50–100 μg/ml RsAFP2 results in aspecific membrane permeabilization, whereas treatment with lower RsAFP2 doses, i.e. 5–20 μg/ml RsAFP2, results in GlcCer-mediated membrane permeabilization [6]. Therefore, in this study, we used rather low RsAFP2 concentrations, i.e. 1–30 μg/ml. We firstly determined percentage killing and ROS accumulation in *C. albicans* CAI4 cultures after treatment with different concentrations of RsAFP2 (ranging from 1 μg/mL to 30 μg/mL) in PDB/YPD growth medium for different incubation periods (ranging from 1 h to 16 h). Treatment of exponentially growing *C. albicans* CAI4 cultures with 10 μg/mL RsAFP2 in PDB/YPD for 2 h 30 min resulted in about 30% killing, as revealed by plating assays (Fig. 1A), and in endogenous ROS accumulation (visualized by DHE, Fig. 1B). Increasing the RsAFP2 incubation time did not significantly affect percentage killing of *C. albicans* or ROS accumulation by RsAFP2 (Supplementary Fig. 2). In order to clarify if the RsAFP2-induced cell death is of apoptotic nature, the same cultures were assessed for other apoptotic markers, including DNA fragmentation (visualized via the deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay) and phosphatidylserine (PS) translocation from the inner leaflet to the extracellular side of the plasma membrane. PS translocation and loss of membrane integrity were simultaneously analyzed in order to discriminate between apoptotic and necrotic death (visualized via co-staining with FITC-labeled annexin V and propidium iodide (PI)) [11,15]. RsAFP2-treated cultures were characterized by increased ROS and DNA fragmentation levels, and by an excess of annexin V positive/PI negative cells as compared to water-treated cultures (Fig. 1B). Apoptosis in yeast automatically leads to secondary necrosis after time [16,17]. The amount of annexin V negative/PI positive ('PI only') cells that represent necrotic cells (i.e. 1.4 ± 0.3%) was approx. 5-fold lower than the amount of annexin V positive/PI negative cells after RsAFP2 treatment (i.e. 6.1 ± 0.8%), indicating a predominant apoptotic cell death of the *C. albicans* CAI4 culture induced by RsAFP2. Note that

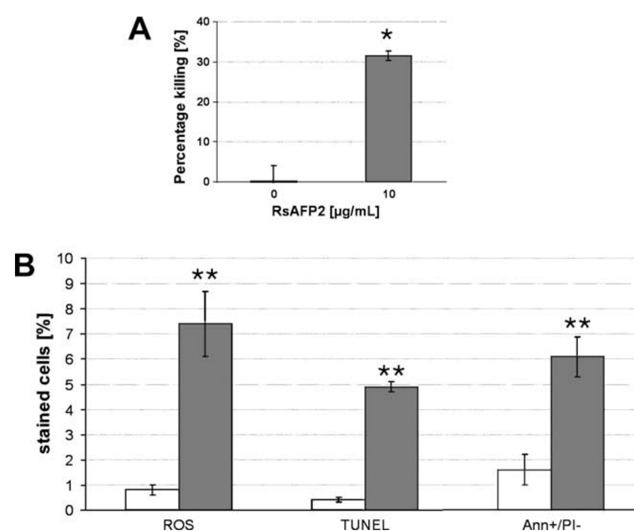

Fig. 1. RsAFP2 induces apoptosis in *C. albicans*. *C. albicans* CAI4 cultures were treated with 10 μg/mL RsAFP2 or water in PDB/YPD growth medium for 2 h 30 min. (A) Percentage killing (colony formation; 100% represents the number of plated cells) of RsAFP2-treated cells and water-treated cells. Data represent means ± S.E.M. $^{*}P < 0.05$. (B) Apoptotic features of RsAFP2-treated (grey bars) and water-treated *C. albicans* cultures (white bars) were assessed by determining the endogenous ROS levels via DHE staining, DNA fragmentation via TUNEL staining and phosphatidylserine externalization and membrane integrity via annexinV/propidium iodide co-staining. In each experiment, 500 cells were evaluated using fluorescence microscopy (100% represents the number of cells, i.e. 500). Values are the mean of triplicate measurements. Data represent means ± S.E.M. $^{**}P < 0.01$.

the amount of *C. albicans* cells displaying apoptotic markers upon RsAFP2 treatment (approx. 5–10%) is typically below the number of dead cells induced by RsAFP2 (approx. 30%), as was reported previously for the apoptotic stimuli acetate, peroxide and farnesol in *C. albicans* [16–18]. However, it cannot be excluded that RsAFP2 induces cell death in *C. albicans* via additional apoptosis-independent mechanism(s).

3.2. RsAFP2-induced apoptotic cell death involves caspase activation

In *S. cerevisiae*, yeast caspase Yca1p is a key player of apoptosis. In *C. albicans*, a putative caspase is encoded by metacaspase 1 (*CaMCA1*) (orf19.5995), which has been recently demonstrated to be involved in oxidative stress-induced cell death by hydrogen peroxide [19]. In order to clarify a possible caspase activation in *C. albicans* upon RsAFP2 treatment, exponentially growing *C. albicans* CAI4 cultures were treated with 10 μg/mL RsAFP2 for 2 h 30 min and afterwards incubated with the FITC-labeled analog of the pancaspase inhibitor z-VAD-FMK, which binds to the active site of caspases in yeast [15]. RsAFP2 treatment led to an increase in the number of fluorescent cells (5.1 ± 0.8%) as compared to water-treated control cultures (1.3 ± 0.1%), indicating that RsAFP2-induced caspase activation.

To examine the involvement of active caspases in RsAFP2 antifungal activity, exponentially growing *C. albicans* CAI4 cultures were treated with 10 μg/mL RsAFP2 in PDB/YPD for 2 h 30 min in the presence or absence of 80 μM of the pancaspase inhibitor z-VAD-FMK [15]. Co-incubation of RsAFP2 with the caspase inhibitor completely blocked RsAFP2-induced killing of *C. albicans*: survival of *C. albicans* culture treated with both RsAFP2 and the caspase inhibitor was 99 ± 7%, whereas survival of the culture treated with RsAFP2 alone was 71 ± 4%. All these data point to an RsAFP2 mode of antifungal mechanism that is dependent on caspase activity.

3.3. *C. albicans* metacaspase 1 (*Ca*Mca1p) is not involved in RsAFP2-induced cell death

Having detected caspase activity during RsAFP2-induced cell death, we next investigated a potential role in the apoptotic process of *Ca*Mca1p, the only reported (putative) caspase in *C. albicans*. For this purpose, we constructed a *C. albicans CaMCA1* homozygous deletion mutant (*mca1*Δ/Δ mutant) in strain BWP17 using the *URA3* flipper cassette (Supplementary data). As a means to control deletion of *CaMCA1* on a biochemical level, we verified the absence of caspase activity induced by acetic acid in the *C. albicans mca1*Δ/Δ mutant. Acetic acid has previously been shown to induce apoptosis in *C. albicans* [16,17] and caspase-dependent apoptosis in *S. cerevisiae* [20]. Treatment of the *C. albicans* wild-type strain BWP17 with 120 mM acetic acid for 2 h resulted in caspase activation (Fig. 2), whereas treatment of *C. albicans mca1*Δ/Δ mutant cultures with 120 mM acetic acid resulted in significantly lower caspase activation. These data point to the significant contribution of *Ca*Mca1p in the overall caspase activation triggered by the apoptotic stimulus acetic acid in *C. albicans*.

In order to investigate whether *Ca*Mca1p is also involved in RsAFP2-induced apoptosis in *C. albicans*, we next evaluated percentage killing upon RsAFP2 treatment and RsAFP2-induced caspase activation of the *C. albicans mca1*Δ/Δ mutant culture and compared it with the RsAFP2-sensitivity and RsAFP2-induced caspase activation of the isogenic *C. albicans* BWP17 and the *CaMCA1* complemented *C. albicans mca1*Δ/Δ mutants. Treatment of either *C. albicans mca1*Δ/Δ mutant, BWP17 or the *CaMCA1* complemented *C. albicans mca1*Δ/Δ mutant cultures for 2 h 30 min with 10 μg/mL RsAFP2 resulted in equal killing of all three strains by RsAFP2 (between 30% and 40% killing for all three strains). Moreover, the percentage of cells with activated caspases upon RsAFP2 treatment did not significantly differ between these 3 strains (Fig. 2). These data demonstrate that the *C. albicans mca1*Δ/Δ mutant (i) is equally sensitive to RsAFP2 and (ii) shows a similar RsAFP2-induced caspase activation as compared to its isogenic parent strain BWP17 and the *CaMCA1* complemented *C. albicans mca1*Δ/Δ mutant.

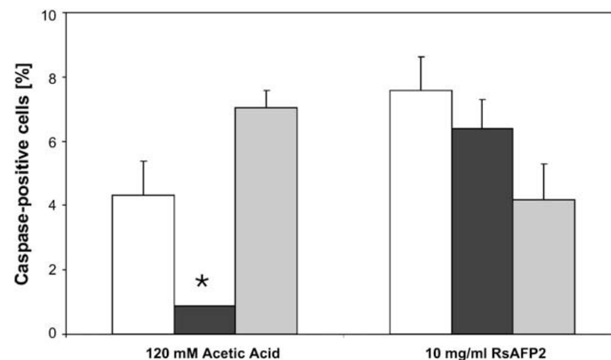

Fig. 2. *C. albicans* metacaspase 1 is not involved in RsAFP2-induced cell death. The percentage of cells with active caspases of the isogenic strains *C. albicans* BWP17 (white bar), the corresponding *C. albicans mca1*Δ/Δ mutant (black bar) and the *CaMCA1* complemented *C. albicans mca1*Δ/Δ mutant (grey bar) upon treatment with 120 mM acetic acid for 2 h, or with 10 μg/mL RsAFP2 for 2 h 30 min (100% represents the number of cells, i.e. 500). Values are the mean of triplicate measurements. Data represent means ± S.E.M. $^{*}P < 0.05$.

4. Discussion

We previously demonstrated that the antifungal activity of RsAFP2 on *C. albicans* involves the induction of ROS generation [10], which is one of the phenotypical markers of apoptosis in yeast [11,17]. In this study, we demonstrated that RsAFP2 indeed induces apoptotic cell death in *C. albicans,* and concomitantly triggers caspase activation. Moreover, the caspase inhibitor z-VAD-FMK alleviated RsAFP2-induced killing of *C. albicans*, demonstrating the important role for active caspases or caspase-like proteases in the antifungal mode of action of RsAFP2. To our knowledge, this is the first study of a defensin causing apoptotic cell death in this pathogen, thereby providing novel mechanistic insights in the mode of action of defensins against the human pathogen *C. albicans*. Note that it cannot be excluded that RsAFP2 induces cell death in *C. albicans* via additional apoptosis-independent mechanism(s).

Compounds or stimuli that have previously been shown to induce apoptosis in *C. albicans* are acetic acid [17], hydrogen peroxide [17], the polyene amphotericin B [17], 1,10-phenanthroline metal complexes [21], silver–coumarin complexes [22], diallyl disulfide [23], disruption of γ-glutamylcysteine synthase [24] or farnesol [18]. Recently, Andrés and coworkers demonstrated apoptotic cell death in *C. albicans* by lactoferrin, a protein present in mammalian mucosal secretions with antifungal and antibacterial activity [25]. The involvement of *C. albicans* metacaspase 1 (*Ca*Mca1p) in apoptosis-induction by these stimuli has only recently been described for hydrogen peroxide [19].

To get more insight in the molecular mechanisms underlying the caspase-linked apoptotic cell death induced by RsAFP2 in *C. albicans*, we assessed the involvement of metacaspase 1 (*Ca*Mca1p) in this process. We showed that *C. albicans* metacaspase 1 is important for the caspase activation induced by acetic acid. In contrast, RsAFP2-induced apoptotic cell death in *C. albicans* is dependent on active caspases but independent on the *C. albicans* metacaspase 1, indicating the existence of (an)other as yet unidentified caspase(s) or caspase-like protease in *C. albicans*. Consequently, this

Please cite this article in press as: Aerts, A.M., et al. The antifungal plant defensin RsAFP2 from radish induces apoptosis in a metacaspase independent way in *Candida albicans*. FEBS Lett. (2009), doi:10.1016/j.febslet.2009.07.004

could imply that different apoptotic pathways (involving different caspases) can be induced in *C. albicans*, depending on the apoptotic stimulus. Indeed, while the gene encoding the metacaspase *Ca*Mca1p is important for caspase activation induced by hydrogen peroxide [19] and acetic acid (this study), it is not affecting the activation of caspases by RsAFP2. In *S. cerevisiae*, various caspase-like proteases have been identified that participate in a Yca1p-independent apoptotic cell death, namely Kex1 protease [26] and Esp1 protease [27]. Whether these caspase-like proteases are implicated in RsAFP2-induced Mca1p-independent apoptosis in *C. albicans* needs to be investigated further.

In conclusion, this study presents the first evidence for an antifungal defensin inducing apoptosis in *C. albicans*. Thus, it sheds light on possible medical applications involving plant defensins in general and RsAFP2 in particular for the treatment of *C. albicans* infections. In this regard, RsAFP2 has recently proven to be prophylactically effective against murine candidiasis, and this at least to the same extent as the antimycotic fluconazole [28].

Acknowledgements

This work was supported by FWO-Vlaanderen (Research Project G.0440.07 to B.P.A.C.). We are grateful for the postdoctoral fellowships to A.M.A. (Research Council) and to K.T. (Industrial Research Found), both from KU Leuven, and for the doctoral fellowship to G.G. from IWT-Vlaanderen. We thank J. Morschhäuser (Universität Würzburg, Germany) for providing us the plasmids used for *CaMCA1* disruption.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.004.

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