Functional analysis of apoptosis-inducing factor in the human fungal pathogen *Candida albicans*

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KEY MESSAGES

1. We have cloned and expressed the three putative *Candida albicans* apoptosis-inducing factor (AIF) sequences (orf19.1438, orf19.2175, orf19.2671) in *Escherichia coli* BL21. The proteins are designated as CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-orf19.2671, respectively.
2. The three CaAifp have been purified from *E coli* BL21 to homogeneity.
3. Among the three CaAifp, only CaAifp-orf19.2175 and CaAifp-orf19.2671 possess NADH oxidase activity. The former also exhibits DNase activity.
4. CaAifp-orf19.2175 can functionally complement *S cerevisiae Δaif1* mutant and sub-cellular translocation of this protein is evident upon apoptotic challenge.
5. CaAifp-orf19.2175 is a bona fide *C albicans* AIF.

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Introduction

Apoptosis is a highly organised cellular process that leads to cell death in multicellular organisms. Apoptotic features have been induced in *Candida albicans* following exposure to farnesol, acetic acid, H_2O_2, or a low dose of antifungal agent.[1] Mitochondria play a key role in energy production and cell death. Mitochondrial dysfunction has been regarded as the onset of apoptosis, exemplified by depolarisation of mitochondrial membrane potential, elevation of the level of reactive oxygen species, and release of apoptosis-inducing factor (AIF) and cytochrome *c*.[2] AIF is a highly conserved protein, and *C albicans* AIF has yet to be identified.

In our earlier studies of the antifungal activity of purpurin against *Candida* species, we hypothesised the existence of a mitochondrial-mediated cell death pathway in *C albicans*. In light of the phylogenetic relationship between *S cerevisiae* and *C albicans*, we performed a BLAST database search of the *C albicans* genome using the amino acid sequence of *S cerevisiae* AIF (YNR074c) as query and identified three putative AIF sequences (orf19.1438, orf19.2175, orf19.2671).

This project aimed to identify and characterise cell death mediators in *C albicans* by (1) cloning and expressing the three putative *C albicans* AIF sequences, and (2) determining the cellular localisation and functional role of AIF in energy production and cell death.

Methods

This study was conducted from December 2011 to December 2012.

Strains and cultivation

C albicans SC5314 was used for DNA isolation, and *C albicans* BWP17 (*ura3*, *his1*, *arg4*) was used for gene tagging experiments. They were cultivated on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. *Escherichia coli* DH5α (Novagen) was used for plasmid propagation, and *E coli* BL21 (Novagen) was used for protein expression. Bacterial strains were grown on LB medium at 37°C. *S cerevisiae Δaif1* mutant was obtained from EUROSCARF. *S cerevisiae* and its derivatives were cultivated on SC medium (0.67% yeast nitrogen base without amino acids (Difco), 0.077% CSM-ura supplements (BIO101), 2% dextrose) at 30°C. Unless otherwise specified, 2% bactoagar (Difco) was added to prepare the solid media.

Cloning, expression, and purification of *C albicans* apoptosis-inducing factor

The three putative *C albicans* AIF sequences were obtained by PCR using gene-specific primers (Table 1). The nucleotide sequences of the cloned genes were confirmed by automatic DNA sequencing (TechDragon). To circumvent the unusual codon usage in *C albicans*, the CUG codons in orf19.1438 and orf19.2175 were converted to UUG codon by site-directed mutagenesis using mutagenic primers (Table). The three AIF sequences were subcloned into pET28b (Novagen) and expressed in *E coli* BL21 as C-terminal 6×His-tagged fusion proteins (CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-

orf19.2671). Protein expression was induced by IPTG (1 mM) at 18°C for 20 hours. After induction, the cells were harvested by centrifugation (2400 g, 15 min) at 4°C, washed once and resuspended in ice-cold Buffer A (20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 8) supplemented with 1 mM phenylmethylsulphonyl fluoride and disrupted by sonication on ice (seven cycles, 30 s each, with 30 s intervals). Cell-free extract was obtained by centrifugation (13 000 G, 30 min) at 4°C and filtration (0.2 μm nylon membrane; Millipore). The filtrate was applied to a pre-equilibrated HisTrap HP column (GE Healthcare), and CaAifp were eluted with increasing concentrations of imidazole. The purity of the proteins was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels. Protein concentration was determined by the Bradford method using bovine serum albumin as standard.

NADH oxidase activity

NADH oxidase activity of the purified CaAifp was determined in a 3-mL reaction mixture containing 50 mM KH_2PO_4, 0.1 mM FAD, and 0.07 mM NADH, pH 7 at 30°C. The reaction was followed by decreased absorbance at 340 nm.[3] One unit (U) of NADH oxidase activity was defined as the amount of protein that yielded 1 μmol of NAD^+ per min.

DNase activity

DNase activity of the purified CaAifp was determined in a 100-μL reaction mixture at 37°C containing 0.5 μg plasmid DNA or purified *C albicans* nuclei,[4] 20 μg purified CaAifp, 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.9. At the indicated time, DNA was precipitated by phenol/chloroform/isoamyl alcohol extraction and analysed using gel electrophoresis.

Sub-cellular localisation of CaAifp

The three putative AIF sequences were tagged with green fluorescent protein (GFP) at their C-terminal end using a PCR-based approach via homologous recombination.[5] Briefly, tag cassettes were synthesised by gene-specific primers (Table) and plasmid pGFP-URA3, and were transformed into *C albicans* BWP17. Correct integration of the tag-cassette into chromosomal AIF sequences was identified by PCR, using one primer that annealed within the tag-cassette and another that annealed the AIF sequence (Table). Sub-cellular localisation of the GFP-tagged *C albicans* AIF sequences was evaluated by confocal microscopy (excitation: 480 nm; emission: 530 nm). Mitochondria were stained with MitoTracker Red CMXRos (Invitrogen) (excitation: 579 nm; emission: 599 nm). To evaluate sub-cellular translocation of GFP-tagged CaAifp, fungal cells were challenged by H_2O_2 (5 mM) for 4 hours. Digital images were collected using a CCD camera and processed using Adobe Photoshop.

TABLE. Primers for cloning, site-directed mutagenesis, and gene tagging

Primer name	Sequence[a,b,c] (5' to 3')
Cloning*	
1438-F	**CATatg**acgccaaaagtggtaatcattg
1438-R	**GGATCC**ttaattgactgttgcaccaata
2175-F	**CATatg**tctaaagagaaaagtaaacaga
2175-R	**GGATCC**tcacgtaaaatgctccccagct
2671-F	**CATatg**acagatctcgccaaacaaccac
2671-R	**GGATCC**ttacatatctatagtcgctcta
Site-directed mutagenesis†	
1438-67	taaacacattaaaa<u>AGC</u>ttaattaccaaaaa
1438-640	accagagccgatc<u>AGC</u>gatgctttcaaga
2175-718	gattattgaaaca<u>AGC</u>ggccttttaccca
2175-847	agacgtggttgcc<u>AGC</u>ggtgcaaactcaa
Gene tagging‡	
1438-GFP-F	
1438-GFP-F	atcaatggttcaagagtatcaggattaccggttagggacatgtaagaagtttatt ggtgcaacagtcaatGGTGGTGGTTCTAAAGGTGAAGAATTATT
1438-GFP-R	atcgacgtgtaaaagtgagaaaaaaaaaaaaagttaatatttcagtgttgcaaac atatttcaatagttctTCTAGAAGGACCACCTTTGATTG
2175-GFP-F	accaagctttttaatttggtttgctaaggcaagagattttatgatttccaaagctg gggagcattttacgGGTGGTGGTTCTAAAGGTGAAGAATTATT
2175-GFP-R	cgaaattcacaaaaacgatttctcttaaacacatatatattttgctatctaggtat atggattattctatTCTAGAAGGACCACCTTTGATTG
2671-GFP-F	ggataatgagcattataagcaagagtatgaggattattgtataggtaaaattag agcgactatagatatgGGTGGTGGTTCTAAAGGTGAAGAATTATT
2671-GFP-R	cacaacaacactttgaataaccactaaacttctctgtttcctttattgcttacaca aacttccacaccacTCTAGAAGGACCACCTTTGATTG
Verification	
AIF-TagF	CCTATGAATCCACTATTGAACC
1438-TagR	ATCGACGTGTAAAAGTGAGAAA
2175-TagR	CGAAATTCACAAAAACGATTTC
2671-TagR	CACAACAACACTTTGAATAACC

* Lower case sequences are homology regions; restriction sites for cloning are in bold: *Nde*I (CATATG), *Bam*HI (GGATCC)

† Lower case sequences are homology regions; mutagenic sites are underlined

‡ Lower case sequences are homology regions; upper case sequences anneal tag cassettes

Functional characterisation of CaAifp

The three *C albicans* AIF sequences were subcloned into *S cerevisiae* episomal vector pRS426 (Novagen) and transformed into *S cerevisiae Δaif1* mutant. The transformants were challenged by H_2O_2 and cell survival was determined using a drop test assay. DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick

end labelling (TUNEL) assay using the *In Situ* Cell Death Detection Kit (Fluorescein) (Roche). The fluorescence was quantified using a FACSort flow cytometer (Becton Dickinson) at 530 nm. The data were analysed with the WinMDI 2.9 software.

Results

Preparation and characterisation of the purified CaAifp

The three *C albicans* AIF sequences were cloned. Protein expression was achieved in *E coli* BL21 and the CaAifp (CaAifp-orf19.1438, CaAifp-orf19.2175, CaAifp-orf19.2671) were obtained as C-terminal 6×His-tagged fusion proteins to homogeneity. NADH oxidase activity was only detected in CaAifp-orf19.2175 (4.2±0.5 U/mg) and CaAifp-orf19.2671 (3.9±0.4 U/mg). Only CaAifp-orf19.2175 was able to degrade purified *C albicans* nuclei and plasmid DNA in a time-dependent manner (Fig 1).

Sub-cellular localisation of CaAifp

The sub-cellular localisation of the three CaAifp could be visualised in *C albicans* using confocal microscopy. CaAifp-orf19.1438 and CaAifp-orf19.2671 were cytosolic proteins, whereas CaAifp-orf19.2175 was a mitochondrial protein. Upon apoptotic challenge by H_2O_2, CaAifp-orf19.2175 translocated from the mitochondria to the cytosol (Fig 2).

Complementation study

In the presence of an apoptotic level of H_2O_2, the *S cerevisiae* strain harbouring CaAifp-orf19.2175 exhibited normal apoptotic responses as demonstrated by the drop test assay. In addition, TUNEL assay showed that 54.87%±5.39% of the cell population was TUNEL-positive.

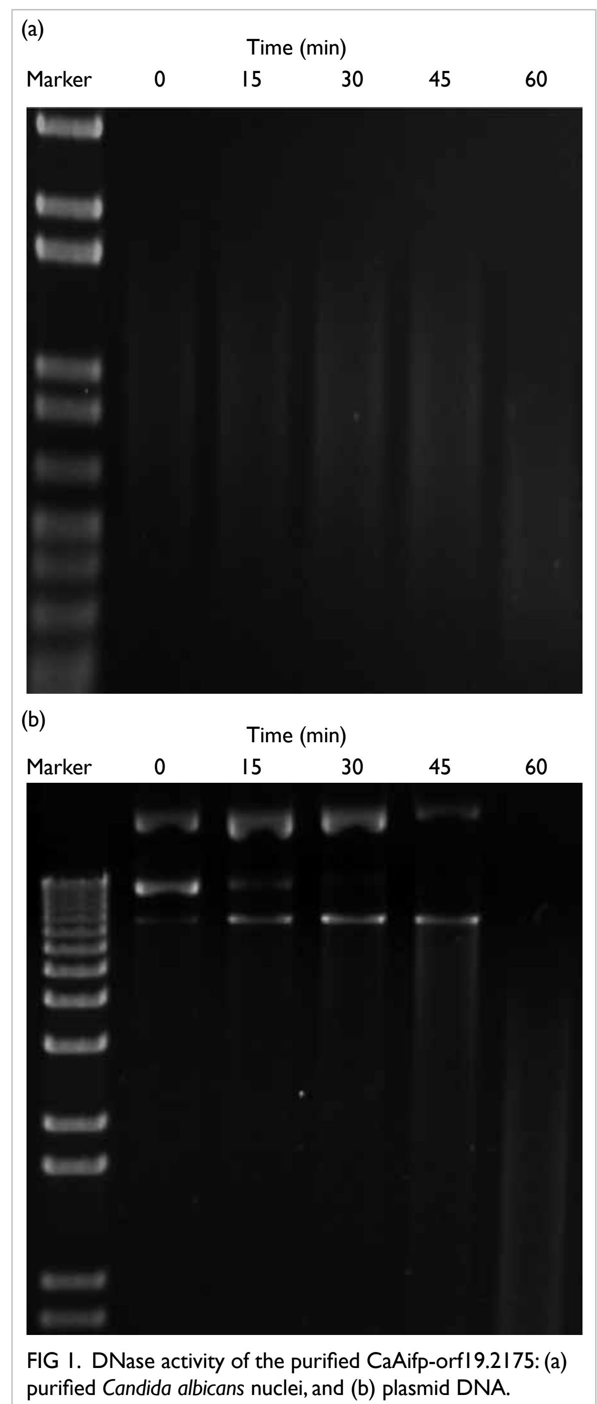

FIG 1. DNase activity of the purified CaAifp-orf19.2175: (a) purified *Candida albicans* nuclei, and (b) plasmid DNA.

Discussion

Three putative AIF sequences (orf19.1438, orf19.2175, orf19.2671) were identified in the *C albicans* genome. In *C albicans*, the CUG codon is translated into serine instead of leucine. Therefore, we performed site-directed mutagenesis to convert the CUG codon to UUG codon in orf19.1438 and orf19.2175 for proper (over)expression in *E coli*. At a lower temperature (18°C) and for a longer duration (20 hours) of IPTG induction to avoid the formation of inclusion bodies, we were able to express the three CaAifp in soluble form. In general, ~1 mg/mL protein was routinely obtained in the crude extract. Addition of a 6×His-tag allowed one-step purification using affinity chromatography.

AIF is a bifunctional enzyme that plays a pivotal role in energy production (NADH oxidase activity) and cell demise (DNase activity). CaAifp-orf19.2175 and CaAifp-orf19.2671 possess NADH oxidase activity, and the former also degrades DNA. More experiments are warranted to understand the nature of its NADH oxidase and DNase activities in terms of enzyme inhibition and co-factor requirements.

The sub-cellular localisation of the *C albicans* CaAifp was readily visualised by confocal microscopy using GFP tagging. Both CaAifp-orf19.1438 and CaAifp-orf19.2671 are cytosolic proteins. CaAifp-orf19.2175 is a mitochondrial protein, as the green and red fluorescence were shown superimposed. The apoptotic features of

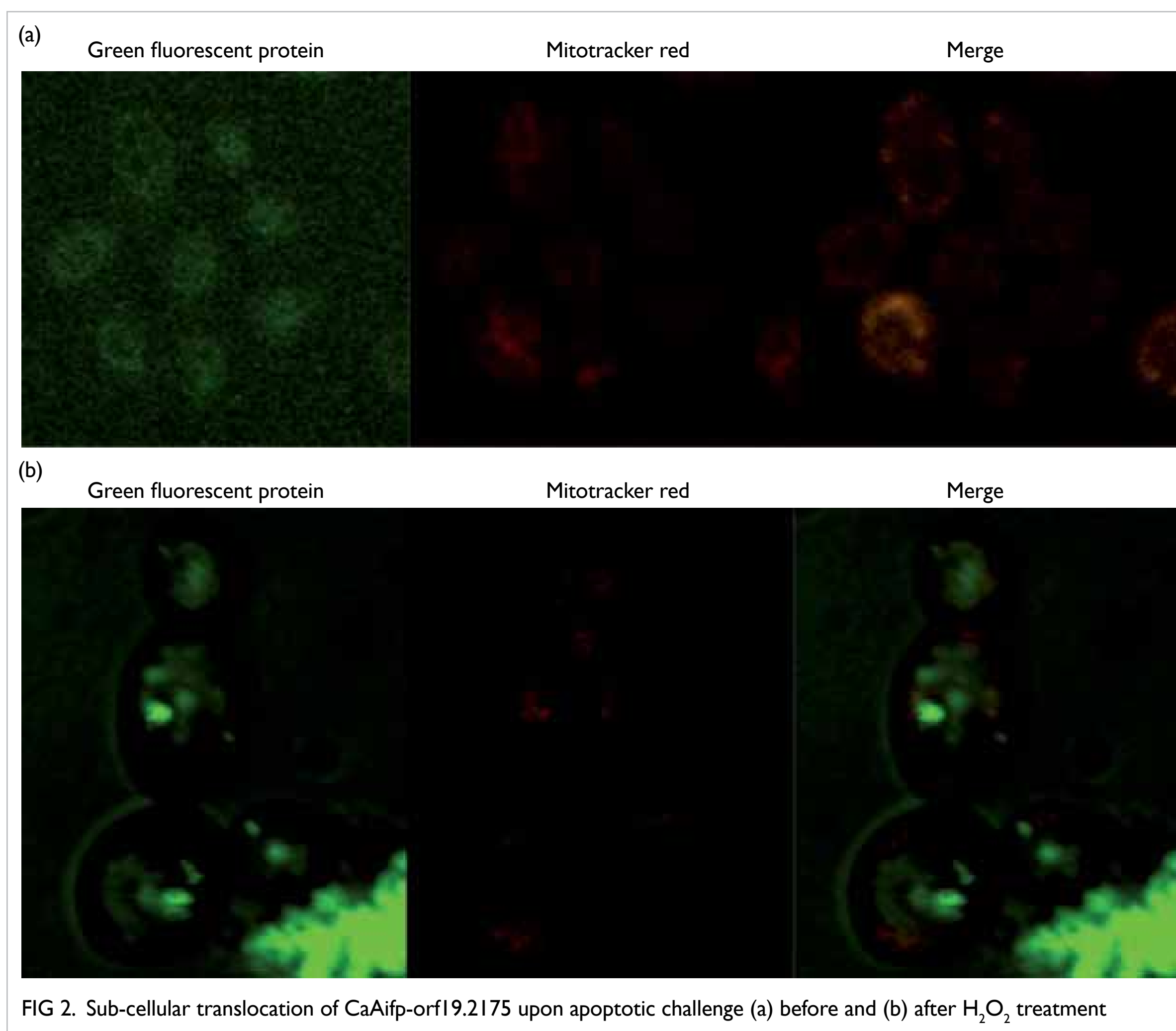

FIG 2. Sub-cellular translocation of CaAifp-orf19.2175 upon apoptotic challenge (a) before and (b) after H_2O_2 treatment

CaAifp-orf19.2175 were demonstrated by its sub-cellular translocation upon oxidative stress, the ability to functionally complement *S cerevisiae Δaif1* mutant, and the presence of TUNEL-positive cells in CaAifp-orf19.2175 complemented the *S cerevisiae* strain in the presence of an apoptotic level of H_2O_2. Our data also suggest phylogenetic conservation of a mitochondrial-mediated cell death mechanism in *S cerevisiae* and *C albicans*. Further studies are needed to establish linkage between the apoptotic functions of CaAifp, caspase, and other effectors in *C albicans* such as cyclophilin, Ras-cAMP-PKA pathway, and antifungal agents.

Conclusion

CaAifp-orf19.2175 is a bona fide *C albicans* AIF. It provides evidence of the phylogenetic conservation of the cell death mechanism in unicellular microorganisms, and opens up a new research area in *C albicans* that focuses on mitochondrial-mediated cell demise.

Acknowledgements

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