Original article

Cloning of metacaspase gene expressed in the opportunistic human pathogenic fungus, *Aspergillus fumigatus*

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Introduction and objective: Caspases belong to a distinct class of cysteine proteases which also includes hemoglobinases, gingipains, clostripains, and separases, the proteases involved in chromosome segregation in mitosis and meiosis. Two families of predicted Caspase Homoglobinase Fold (CHF)-proteases were identified and shown to be more closely related to the caspases than to other proteases of this class and hence dubbed paracaspases and metacaspases. The aim of present study was to isolate genes particularly caspase-related ones up-regulated in the earliest stages of the stationary phase (up to four hours) and using clones to perform a PCR on cDNA prepared from exponential and stationary phase mRNA.
Materials and methods: After the use of the clorimetric assay to get evidence of caspase activity, both strands of cDNA were used for cloning the PCR products. The whole plasmid and freeze-dried material were sent for automated sequencing. By searching in NCBI homepage, particularly Blast part, all the alignment sequences were shown and then after similar genes were recognized as well.
Results: There was no sign of any cDNA in the midlog and 4h stationary phases which shows no RNA synthesis in those phases. The extended sequence of the gene was found to have a high level of identity (87%) with a metacaspase from *Schizosaccharomyces pombe* through a BLASTX search.
Conclusion: This information may be used to fabricate microarrays which would enable a genome-wide analysis of gene expression of *Aspergillus fumigatus* as it enters and during the stationary phase.

Keywords: Cloning, Metacaspase, Gene, Fungi, *Aspergillus fumigatus*

Introduction

Caspases are the principal proteases that are activated during animal apoptosis and cleave a variety of proteins, ultimately leading to cell death and disintegration [1,2]. Caspases belong to a distinct class of cysteine proteases which also include hemoglobinases, gingipains, clostripains,

and separases, the proteases involved in chromosome segregation in mitosis and meiosis. Recent sequence and structure analyses revealed a much greater diversity of caspase-related proteases than previously suspected [3]. Two families of predicted Caspase Homoglobinase Fold (CHF)-proteases were identified and shown to be more closely related to the caspases than to other proteases of this class and hence dubbed paracaspases and metacaspases [4].

A possible regulatory role for the human paracaspase in certain forms of Programmed Cell Death (PCD) has been demonstrated [4]. More recently, the yeast metacaspase has been shown to mediate programmed cell death upon peroxide treatment and in aged cultures, which not only supports the role of metacaspases in apoptosis but also indicates that PCD occurs even in (at least some) unicellular eukaryotes via mechanisms related to those in multicellular organisms [5]. A major role for metacaspases in plant PCD is also likely, given the proliferation of the genes coding for metacaspases in plant genomes, the absence of other caspase homologs in plants, and the fusion of some of the plant metacaspases with the LSD1 Zn-finger, a regulator of plant PCD [4].

The metacaspases whilst absent in metazoans and animals, are present in plants, fungi and protists, leading to the suggestion that the metacaspases represent an earlier ortholog of the capsase family. This view has recently been strengthened by the demonstration that the yeast metacaspase *YCA1*, undergoes caspase-like processing and activation during apoptotic-like cell death induced by H_2O_2 and the stationary phase. Moreover, it has been shown that metacaspase activity in *A. fumigatus* contributes to the apoptotic-like loss of membrane phospholipid asymmetry at stationary phase [5].

The case of caspases-paracaspases is more complicated because this branch of the superfamily so far has not been detected in eukaryotes other than animals and slime mold. This distribution is compatible with a second, later HGT from α-proteobacteria to eukaryotes or with independent loss of the paracaspase gene in multiple eukaryotic lineages.

The aim of present study was to isolate genes particularly caspase-related ones up-regulated in the earliest stages of the stationary phase (up to four hours) and using clones to perform a PCR on cDNA prepared from exponential and stationary phase mRNA.

Materials and methods

Caspase activity measurement

The colorimetric assay for caspase activity was based on the cleavage of a p-nitroaniline (p-NA) dye from the C-terminal of the peptide substrates, which were Ac-YVAD-pNA for caspase-1, Ac-DEVD-pNA for caspase-3 and Ac-IETD-pNA for caspase-8 (2mM). Ten microliter of the aldehyde (-CHO) inhibitors (2 mM), which are slowly reversible enzyme inhibitors and interact at the enzyme substrate-binding site, were also used. Ac-YVAD-CHO, Ac-DEVD-CHO and Ac-IETD-CHO were used as inhibitors for caspase-1, -3 and -8, respectively. One hundred milliliter of modified Vogel's media was inoculated with 1×10^8 conidia.

One milliliter of *A. fumigatus* spores were incubated on an orbital shaker (225rpm) at 37°C for 16h [4,5]. This yielded 5-10g wet weight mycelium. Mycelia were harvested by filtration onto sterile muslin and ground in liquid nitrogen and the biomass was resuspended in 20ml of ice-cold cell lysis buffer (50mM Hepes; pH 7.4, 1mM DTT, 0.5mM EDTA and 0.1% Chaps) and centrifuged at 1500g for 10min (Mistral 300I, MSE). The

supernatant was collected as the cytoplasmic fraction. Protoplasts were centrifuged at 1500g for 10min and resuspended with 20ml of ice-cold cell lysis buffer [50mM Hepes; pH 7.4, 1mM DTT, 0.5mM EDTA and 0.1% (v/v) Chaps and 0.1% (v/v) Tween 20] and incubated for 5min on ice before being centrifuged at 10,000 g for 10min at 4°C (Sorvall RC 5B, Plus). Samples (10-20µl) were dispensed into a 96-well microtiter-plate with 70-80µl assay buffer [50mM Hepes; pH 7.4, 100mM NaCl, 0.1% (v/v) Chaps, 10mM DTT, 1mM EDTA and 10% (v/v) glycerol], substrate (10-20µl; 2mM final concentration of each Ac-YVAD-pNA for caspase-1, Ac-DEVD-pNA for caspase-3 and Ac-IETD-pNA for caspase-8) and when required inhibitors (10µl of 2mM as final concentration of each Ac-YVAD-CHO, Ac-DEVD-CHO and Ac-IETD-CHO) and incubated at 37°C for 10min.

Absorbance (405nm) was measured in a microplate reader (Lambda E; MWG-Biotech). To calculate the specific activity of the samples, p-nitroaniline calibration standard was diluted to 50µM in assay buffer and 100µl of the p-nitroaniline calibration standard (50µl) was added to 2 wells of the microtiter-plate.

cDNA Synthesis

First strand cDNA synthesis

One microliter of the RNA sample was added to 1µl of 3′ smart CDS primer II A (5′-AAGCAGTGGTATCAACGCAGAGT-$ACT_{(30)}N_{-1}N$-3′; 10µM), 1µl of the smart II A oligonucleotide (5′-AAGCAGTGGTAT-CAACGCAGAGTACGCGGG-3′; 10µM) and 2µl of ddH_2O, mixed and spun briefly in a microcentrifuge and incubated at 70°C in a thermal cycler for 2min. Samples were spun briefly and 2µl of 5x first strand buffer (250mM Tris-HCl; pH 8.3, 375mM KCl and 30mM $MgCl_2$) and 1µl of each DTT (20mM), 50x dNTP (10mM) and powerscript reverse transcriptase added to the reaction tubes. After vortexing gently, samples were spun briefly in a microcentrifuge and then incubated at 42°C for 1h in a thermal cycler. For the last part of the first strand cDNA synthesis, all the samples were diluted to 40µl with TE buffer (10mM Tris; pH 7.6, 1mM EDTA) and heated at 72°C for 7min. Samples were stored at -80°C for not more than one month.

Second Strand cDNA Synthesis

After preheating the PCR thermal cycler to 95°C, the master mix (74µl of deionised H_2O, 10µl advantage 2 PCR buffer [40mM tricine-KOH; pH 8.7 at 25°C, 15mM KOAc, 3.5mM $Mg(OAc)_2$, 3.75µg.ml^{-1} BSA, 0.005 % (v/v) Tween-20 and 0.005 % (v/v) Nonidet-P40] and 2µl of each 50x dNTP, 5′ PCR primer II A (10µM; 5′-AAGCAGTGGTATCAACGCAGAGT-3′) and 50x advantage 2 polymerase mix [1.0 % (v/v) glycerol, 0.3mM Tris-HCl; pH 8.0, 1.5mM KCl and 1.0µM EDTA] were mixed by vortexing and spun briefly in a microcentrifuge. 90µl of the master mix was then aliquoted into each sample tube and placed in the preheated thermal cycler. First strand cDNA was denatured at 95°C for 1min and thermal cycling was as follows: 95°C for 15sec, 65°C for 30sec and 68°C for 6min; for 19 cycles. 2µl of 0.5M EDTA was added to each tube to terminate the reaction and stored at -20°C.

Plasmid

The linearized pT-Adv vector was used for direct T/A cloning of PCR products. This vector includes the lacZα fragment for α-complementation (blue/white screening) in *Escherichia coli*. Other features include ampicillin- and kanamycin-resistance genes for selection in *E. coli*; a col E1 origin of replication for propagation in *E. coli*; an f1

origin for rescue strand for mutagenesis and single-strand sequencing; and a T7 promoter and priming site for *in vitro* transcription of sense RNA.

Cloning into pT-Adv

The PCR products were used immediately after amplification for optimal ligation efficiency due to the potential of the 3′ A-overhangs on the PCR products to degrade over time. One tube of pT-Adv was briefly centrifuged to collect all the liquid in the bottom. To estimate the amount of PCR product for ligation with 50ng (20 fmol) of pT-Adv, the following formula was used:

$$\text{X ng PCR product} = \frac{(\text{y bp PCR product})(50\text{ ng pT} - \text{Adv})}{(\text{size of pT} - \text{Adv: } \sim 3{,}900\text{ bp})}$$

Where x ng is the amount of PCR product of y base pairs to be ligated for a 1:1 (vector:insert) molar ratio. For the best ligation efficiencies, 1.0µl of the PCR sample, 1.0µl of 10x ligation buffer (60mM of Tris-HCl; pH 7.5, 60mM of $MgCl_2$, 50mM of NaCl, 1mg.ml^{-1} of BSA, 70mM of ß-mercaptoethanol, 1mM of ATP, 20mM of dithiothreitol and 10mM spermidine), 2µl of pT-Adv (25ng.µl^{-1}), 5µl of ddH_2O and 1µl of T4 DNA ligase were mixed by pipetting the ligation reaction and was incubated at 14°C O/N.

Sequencing

All cDNA products that required sequencing were cloned using the pT-Adv vector and after purification, cut by EcoR I to linearize the whole plasmid and freeze-dried material was sent to MWG (MWG Biotech-Ltd, Mill court, Featherston Road, Wolverton mill South, Milton Keynes, MK12 5RD) for automated sequencing. Means and standard errors of the mean (SEM) were assessed by the single factor analysis (ANOVA) using the SPSS computer programme. Statistical significance was considered to be achieved only if the *P* value multiplied by the number of comparisons was less than 0.05.

Results

As different genes may be expressed at different times during the stationary phase, two samples from the stationary phase were selected, one sample 2h post-stationary phase and a second 4h post-stationary phase. cDNA was synthesized from mRNA isolated 2h post-stationary, 4h post-stationary and mid log phase. As shown in figure 1, a strong band of around 470bp was present in the cDNA from all three samples. Specific primers for each of these genes were used in a PCR with all cDNA samples from the mid log phase, the 2h and 4h post-stationary samples and the subtracted cDNA.

As shown in figures 1 and 2, for the metacaspae gene a single PCR product of the expected size was found only when cDNA from the 2h post-stationary phase sample was used as a template. There were also no sign of any cDNA in the midlog and 4h stationary phases which shows no RNA synthesis in those phases. The similarity between genes were shown in the figure 2 is more than 80% particularly with *S. pombe*. Although, the second gene (*Saccharomyces cerevisae*) was also similar to the found gene, but the number of nucleotides were lower than the first one (Fig. 2).

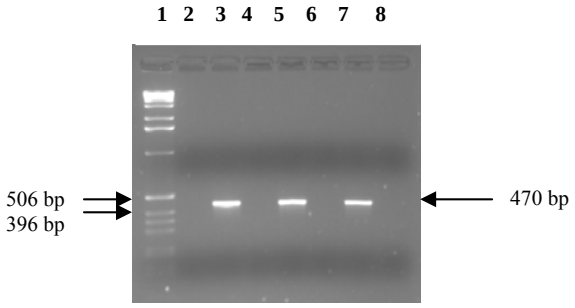
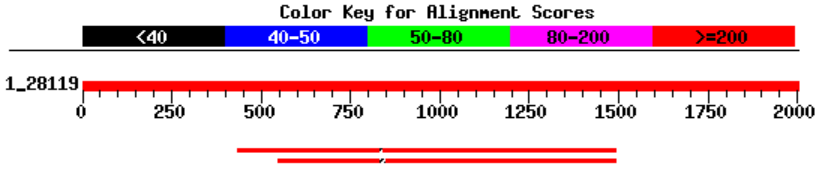


Fig. 1: Expression of the gene in exponential & stationary phase. PCR was performed with primers and cDNA templates. Lane 1: molecular weight markers (1kb ladder); Lane 2: PCR without any template (negative control); Lane 3: genomic DNA (positive control); Lane 4: mid-log phase cDNA; Lane 5: 2h post-stationary phase cDNA; Lane 6: 4h post-stationary phase cDNA; Lane 7: 2h post-stationary phase subtracted cDNA; Lane 8: 4h post-stationary phase subtracted cDNA

i)

CAGTAAGAGTCTCTCAAAGTCTTTCACTGGTGTTGGAAGGTTTCGTTGACTGACCGTTCGAAAGACAAATGCAGAACCATC
ATCACCAACAATCTTCCTACGGCGGCGGTTACCCCGGCCAAGCGTACCGTGAGCAACATCCTCCTCCGAATCCTTACGGGTA
TGGCCAGCCCTCCCCTCAGCCTGGTTACGGCGCGCCTCCTCCACACAACGGTTACGGTGTATGTCCTCATCAGTGAAGATAA
CTGTGAACAAAAAAAAATGCAAGTGTGGCTGACTTTGTATGCAGCAACCGCCGTCAGGATACGGCCAGCCGCCGCCGCCTA
CTGGAAATGCGGTGTATGGTGGAAGACAGCCTGGTATGTGATCGCGTTTGGTGCAGGAAGAACTGCATTCTGCTTTGGAAA
GTGTCAGTATGCGGCCGCTGACATCTGCAACACAGGCATGAACCAGTATCAGAACACCTACTCGCACGGTCATCAGGGTGG
TCCACCACCACCACCCACTGATCCGGTCGCGTTCGGCCACGGAGCTCCACAGGGATACAGTTTCCAGTACTCTCGATGCACT
GGTAAGCGCAAGGCCTTGCTGATTGGAATCAACTATTTCGGGCAGAAGGGCCAGCTGCGTGGTTGTATCAACGATGTCAAG
AACATGTCGACGTATTTGAACCAGAACTTCGGCTACGCGCGCGAGGACATGGTGCTGTTGACGGACGACCAGCAGAACCCC
ATGAGCCAGCCTACGAAGGCTAACATCCTGCGGGCGATGCATTGGTTGGTCAAGGATGCGCAGCCGAATGATTCGCTCTTT
TTCCACTACTCAGGTGAGACCGTACTTTGACGGGTGCTTCCCACAATGAAACTAAAATTTTGTCTGAGACAGGTCATGGTGG
TCAGACTCCGGACTTGGATGGCGACGAGGAAGACGGATATGATGAGGTCATCTACCCGGTCGACTTTCGACAAGCCGGTCA
CATTGTGGACGACGAGATGCACCGGATCATGGTCCGGCCACTACGCCCGGGAGTACGCTTGACCGCAATCTTCGACTCGTG
TCACTCGGGCTCCGCCTTGGACCTCCCCTACATCTACTCGACACAGGGTATCCTCAAGGAGCCCAACCTGGCCAAGGAGGC
CGGACAAGGGCTCCTGGGTGTGGTCTCGGCCTATGCGCGCGGCGACATGAGCGGCATGGTGTCCACCGCGGTTGGGTTCTT
GAAACGCGCCACCAAGGGCGACGAGGCCTACACGCGCAGCAAGCAGACCAAGACCAGCCCGGCGGACGTCATCATGTGGT
CTGGCAGCAAGGACAGCCAGACCAGCCAGGATGCTCAGATCGGTGGCCAGGCCACCGGCGCCATGTCCTGGGCGTTCATCA
CCGCTCTCCGCAAGAACCCCCAGCAGAGCTATGTACAGTTACTGAACAGTATCCGGGATGAGTTGGCAACCAAGTACTCGC
AGAAACCGCAGCTGAGCTGCAGTCATCCATTGGGTAAGCATTCGTCCTTGTGTTTTGGTCTCTGATCTAAAAGGGAAACATC
GCTAACGGAAGTAATAATATATAGACACGAACCTTCTCTACGTCATGTAAATAAGTTACGCATAATGGCCCGCTGTATTCTC
GCTCGGTTTGCATGGGAGGAAAAATGAAAATAGGCGAGTTGCCTGGCTGATTACGGATTCTCGCAATATCTCCTTGTCTATT
GTCTTCATTGCTTGTTTACTTTCGCTTGCGATGGGGTATTTTTTTTCAGTATAAGGAAACCTCGACCTGTAATTTGCTTGTTCC
TTGCATAGTCTGGTTCACTAGCTTGGAGACCCTGCAAGGAGTGGCTTATGCAGGTGCATTTGTATATGATTTCATGGTCGTA
GTATATCATCATCTGTCTATTGTTTCAAAGTTATCATAAAAACTTTTCGTCGTGACGTAACATTGCGGACTTCATGCACAATT
TTCGGCGAGTTCGGGAAGCAGGCTCAGGGAGATCCTTTCGAACAT

ii)



E value = $1e^{-54}$

Sequences producing significant alignments:

gi|19076003|ref|NP_588503.1|
Metacaspase *S. pombe*
E value = $4e^{-56}$
gi|6324771|ref|NP_014840.1|
Metacaspase (Mca1p), *S. cerevisiae*

Fig. 2: Genomic DNA sequence and blastx report. i) Genomic DNA sequence from contig 30 encoding a metacaspase, ii) BLASTX report from NCBI

Sequencing was performed on the double-stranded cDNA templates of the 33 clones obtained from the 2h and 4h post-stationary phase. After removing flanking vector sequence and poly A or T tails, sequences were used to perform a BLASTX search of the *A. fumigatus* genomic database at TIGR (http://www.tigr.org/). This enabled the contig and position of the sequence along the contig to be determined and allowed identical clones to be grouped together and assigned to a single gene. A total of 14 genes were identified amongst the cDNA clones. As the cDNA sequence obtained

represented the 3' end of the gene, the sequence of each putative gene was extended 5' by 1000bp using the TIGR database and used to perform a BLASTX search against the NCBI database (http://www.ncbi.nlm.nih.gov/).

This process was repeated, extending the 5' sequence by a 1000bp each time until the whole predicted ORF and at least 500bp of upstream and downstream flanking sequence was obtained. A nucleotide alignment of the cDNA and corresponding genomic DNA sequence was then performed to determine if the cDNA sequence spanned any introns. The extended sequence of the gene was found to have a high level of identity with a metacaspase from *S. pombe* through a BLASTX search (Figs. 2i & ii). GeneFinder predicted that the ORF was 1389bp containing no introns and encoding a protein 462 amino acids in length (Fig. 2ii).

Discussion

In filamentous fungi, entry into the stationary phase is followed by process of autolysis, whereby the mycelium stops growing and actively secretes lytic enzymes that result in the macromolecular breakdown of the hyphae [6]. In our studies, entry of *A. fumigatus* into the stationary phase was associated with a rapid loss in the viability of the mycelium and of protoplasts derived from stationary phase mycelium. As shown in figure 1 viability decreased by ca. 50 %, 4h after entering stationary phase and by over 85 %, 8h after entering stationary phase. In contrast in the yeast *S. cerevisiae*, although loss of viability occurs progressively through the stationary phase, the loss of viability is considerably slower, occurring over a period of days or weeks depending on the medium used, rather than hours [7]. Our results are consistent with other studies made in filamentous fungi where cell death occurs relatively rapidly on entry to the stationary phase and is followed later by autolysis [8].

In yeast cells, there appear to be no homologues of the many key mammalian apoptosis regulators such as Apaf-1, Bax and Bcl-2 [7]. Paracaspases were detected in animals, slime mold, and one group of bacteria, the *Rhizobiales*, with a notable expansion in *Mesorhizobium loti*, whereas metacaspases are present in plants, fungi, early-branching eukaryotes, and a variety of bacteria. Phylogenetic analysis of the caspase-like protease superfamily shows a clear affinity with the eukaryotic metacaspases, paracaspases, and the classic caspases with the corresponding predicted proteases from the *Rhizobia*, which belong to the α-subdivision of the Proteobacteria, the free-living ancestors of the mitochondria.

Homology searches against these classical metazoan regulators including caspases also failed to detect any homologues in the *A. fumigatus* TIGR database. Recently, with the use of iterative PSI-BLAST searches, a new family of proteins related to the caspases, the paracaspases and the metacaspases have been described [4]. Moreover, the metacaspases whilst absent in metazoans and animals, are present in plants, fungi and protists, leading to the suggestion that the metacaspases represent an earlier ortholog of the capsase family.

This view has recently been strengthened by the demonstration that the yeast metacaspase *YCA1*, undergoes caspase-like processing and activation during apoptotic-like cell death induced by H_2O_2 and the stationary phase. Moreover, the broad spectrum cell-permeant caspase inhibitor Z-VAD-fmk, which has been used to efficiently block caspase activity in intact cells in numerous systems [9] was also found to block both TUNEL staining and translocation of PS in yeast [7]. In our

studies, using specific caspase substrates for caspase-1, -3 and -8, activity against the caspase-1 and caspase-8 substrates were found to increase during the onset of the stationary phase [10,11].

The translated amino acid sequence for *YCA1*, was used to perform a blast search against all six possible translations of the *A. fumigatus* genomic DNA at TIGR to search for a metacaspase homologue. Interestingly, two different regions were identified with a 56 % and a 38 % homology to metacaspase from *S. cerevisiae* and *S. pombe* (Figs. 2i and 2ii) respectively. Thus it appears that the apoptotic machinery may be more complex in *A. fumigatus* than in the yeasts [12-14]. As apoptosis involves active protein synthesis and up-regulation of specific genes, we attempted to isolate genes up-regulated in the earliest stages of the stationary phase (up to four hours) and four randomly selected clones were used to perform a PCR on cDNA prepared from exponential and stationary phase mRNA as shown in figure 1.

The entire genome of *A. fumigatus,* which was shotgun sequenced in a joint project between the Sanger Centre and the TIGR Institute, will shortly be fully assembled following sequencing of a few remaining gaps in the sequence and mapping onto chromosomes [13,14]. It is likely then, that this information may be used to fabricate microarrays which would enable a genome-wide analysis of gene expression of *A. fumigatus* as it enters and during the stationary phase. However in the future, all of the isolated clones should be screened in a similar manner as it is uncommon to be completely successful and to isolate only differentially expressed transcripts. The diversity of the genes identified as up-regulated in the stationary phase suggests that only a fraction of up-regulated genes were isolated. In addition, there may be a temporal array of stationary phase gene expression, with some genes expressed early in the stationary phase and others later. Therefore one future approach may be to perform subtractive hybridisation at a number of points during the stationary phase.

Conclusion

The study presented here give compelling evidence for a primitive apoptosis-like pathway analogous to that now being investigated in the yeast *S. cerevisiae* and provides preliminary data that this process in the filamentous fungi is likely to be more complex than in yeast. The development of microarray technology will be crucial if rapid advances are to be made in understanding how *A. fumigatus* participates in its own death and may ultimately reveal unique novel targets for a new generation of antifungal agents.

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