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Leishmania major metacaspase can replace yeast metacaspase in programmed cell death and has arginine-specific cysteine peptidase activity

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Abstract

The human protozoan parasite *Leishmania major* has been shown to exhibit several morphological and biochemical features characteristic of a cell death program when differentiating into infectious stages and under a variety of stress conditions. Although some caspase-like peptidase activity has been reported in dying parasites, no caspase gene is present in the genome. However, a single metacaspase gene is present in *L. major* whose encoded protein harbors the predicted secondary structure and the catalytic dyad histidine/cysteine described for caspases and other metacaspases identified in plants and yeast. The *Saccharomyces cerevisiae* metacaspase YCA1 has been implicated in the death of aging cells, cells defective in some biological functions, and cells exposed to different environmental stresses. In this study, we describe the functional heterologous complementation of a *S. cerevisiae yca1* null mutant with the *L. major* metacaspase (LmjMCA) in cell death induced by oxidative stress. We show that LmjMCA is involved in yeast cell death, similar to YCA1, and that this function depends on its catalytic activity. LmjMCA was found to be auto-processed as occurs for caspases, however LmjMCA did not exhibit any activity with caspase substrates. In contrast and similarly to *Arabidopsis thaliana* metacaspases, LmjMCA was active towards substrates with arginine in the P1 position, with the activity being abolished following H147A and C202A catalytic site mutations. These results suggest that metacaspases are members of a family of peptidases with a role in cell death conserved in evolution notwithstanding possible differences in their catalytic activity.

Keywords: Metacaspase; Cysteine peptidase; PCD; *Leishmania major*; Trypanosomes; Yeast

1. Introduction

Metacaspases are members of the clan CD, family C14, cysteine peptidases that have been identified in plants, fungi, protozoa and some bacteria (Uren et al., 2000). Functional analyses in several organisms suggest that they could play a role similar to caspases in programmed cell death (PCD). In *Saccharomyces cerevisiae*, the metacaspase YCA1 was found to have caspase-like activity and to be auto-processed. YCA1 was shown to be implicated in yeast PCD of aging cells (Madeo et al., 2002; Herker et al., 2004) and cells defective in some biological functions such as deubiquitination (Bettiga et al., 2004) and mRNA turnover (Mazzoni et al., 2005). Additionally, YCA1 has been associated with PCD of yeast cells exposed to different environmental stresses such as hydrogen peroxide, acetic acid (Madeo et al., 2002; Khan et al., 2005), sodium chloride (Wadskog et al., 2004), heat shock (Flower et al., 2005), hyperosmosis (Silva et al., 2005) and viral toxins (Ivanovska and Hardwick, 2005; Reiter et al., 2005). In plants, metacaspases have been associated with Norway spruce

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PCD during embryogenesis (Suarez et al., 2004; Bozhkov et al., 2005) and tomato plant PCD induced by fungal infection (Hoeberichts et al., 2003). Using yeast as a heterologous system for PCD evaluation, the metacaspases AtMCP1b and AtMCP2b from the plant *Arabidopsis thaliana* were also found to be involved in PCD induced by hydrogen peroxide (Watanabe and Lam, 2005).

Metacaspases harbor the predicted secondary structure and the catalytic dyad histidine/cysteine as described for caspases (Uren et al., 2000). Several of these metacaspases have shown caspase-like auto-processing (Madeo et al., 2002; Vercammen et al., 2004) but specific activity with caspase substrates is still controversial. Yeast cells undergoing PCD have been positively stained with the fluorescein isothiocyanate (FITC)-labeled broad caspase inhibitor z-VAD-fmk (Madeo et al., 2002; Qi et al., 2003), however, unspecific binding and accumulation of this substrate in dead cells has been demonstrated (Wysocki and Kron, 2004). Protein extracts of yeast cells overexpressing YCA1 and exposed to oxidative stress were shown to efficiently cleave the caspase substrates VEID and IETD (Madeo et al., 2002), however, a subsequent study was unable to reproduce these results (Watanabe and Lam, 2005). Similarly, recent publications have shown the involvement of caspase-like proteases during the activation of various types of PCD in plants (Lincoln et al., 2002; Bozhkov et al., 2004; Suarez et al., 2004), however, the AtMCP1b, AtMCP2d and AtMCP2f metacaspases from *A. thaliana* were unable to cleave several caspase substrates (Vercammen et al., 2004; Watanabe and Lam, 2005). In contrast, these *Arabidopsis* metacaspases were found to be auto-processed at arginine and lysine residues and to be active against specific substrates with these amino acids in their P1 positions, thus suggesting that metacaspases could have substrate specificities different to caspases.

Metacaspase genes have been identified in the genomes of the kinetoplastids *Trypanosoma brucei* and *Trypanosoma cruzi*. In *T. brucei* parasites, three of its five metacaspases, namely TbMCA2, TbMCA3 and TbMCA5, were found to be partially associated with RAB11-positive recycling endosomes and no involvement with PCD was observed (Helms et al., 2006). However, the overexpression in yeast of the metacaspase TbMCA4, which is hypothetically inactive due to a cysteine to serine change in the catalytic dyad, led to growth inhibition, mitochondrial dysfunction and cell death (Szallies et al., 2002). On the other hand, the two metacaspases identified in *T. cruzi*, TcMCA3 and TcMCA5, were found to be potentially involved in the PCD of parasites as both proteins migrated to the nucleus in dying epimastigotes after fresh human serum (FHS) exposure and epimastigotes overexpressing TcMCA5 were more sensitive to FHS-induced PCD (Kosec et al., 2006).

Characteristic features of apoptosis have also been described in the human protozoan parasite *Leishmania major*. Cell shrinkage, phosphatidylserine exposure, loss of mitochondrial transmembrane potential, chromatin condensation, DNA fragmentation and endonuclease activity have been found in stationary phase promastigotes and amastigotes in normal cultures and under a variety of stress conditions such as staurosporine treatment, serum deprivation, heat shock and nitric oxide exposure (Arnoult et al., 2002; Zangger et al., 2002). Although caspase activity has been associated with PCD in *L. major*, no caspase gene is present in its genome; and small-molecule cell permeable caspase inhibitors have not protected this parasite against cell death. Apoptotic or pro-apoptotic molecules have, to date, not been described in *L. major* parasites, however a single metacaspase gene (GeneDB name: *LmjF35.1580*) is present.

The *LmjF35.1580* gene codes for a putative type-I metacaspase (LmjMCA) that shares 54% sequence identity with the *T. brucei* TbMCA5 metacaspase, 56.9% identity with the *T. cruzi* TcMCA5 and 36.7% identity with the *S. cerevisiae* YCA1 metacaspase. In order to analyze the proteolytic activity and possible role of LmjMCA in PCD, we have expressed the *LmjF35.1580* gene in yeast cells disrupted for the endogenous YCA1 metacaspase. The survival of cells overexpressing LmjMCA when exposed to hydrogen peroxide, together with its enzymatic activity in cell lysates and as a purified recombinant protein, were evaluated. Point mutations in the catalytic dyad of LmjMCA and a truncated form of LmjMCA lacking its N- and C-terminal domains were also tested to confirm the direct role of LmjMCA in PCD and its activity towards specific substrates.

2. Materials and methods

2.1. Chemicals

Restriction enzymes were purchased from New England Biolabs Inc. (Ipswich, MA) and reagents, unless otherwise stated, were purchased from Sigma–Aldrich Inc. (St. Louis, MO).

2.2. Culture of L. major promastigotes

Leishmania major MRHO/IR/75 promastigotes were grown until stationary phase at 26 °C in M199 medium (Invitrogen AG, Basel, Switzerland) supplemented with 10% heat-inactivated FCS (Seromed GmbH, Wien, Switzerland), 40 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Expression of L. major metacaspase in yeast and generation of point mutants

The coding sequence of the *L. major* metacaspase (GeneDB name: *LmjF35.1580*) was amplified by PCR with primers MCA1fw (5′-CACT**GAATTC**ATGGCAGACC TTTTTGATATTTGGGG-3′) and MCA1rv (5′-CACT**GC GGCCGC**GCCAGGCGGGAGTGGGCT-3′). The coding sequence of the putative catalytic domain of LmjMCA

without the N- and C-termini was amplified with primers CD2fw (5′-CCG**GAATTC**ATGCACCACCACCACCAC CACAAACGGGTCGACAT-3′) and CD2rv (5′-TAA TATATT**GCGGCCGC**CTGCGGCACGCAATG-3′). The PCR products were digested with *Eco*RI and *Not*I restriction enzymes and cloned in to the pESC-His vector (Stratagene, La Jolla, CA) to generate constructs pESC-LCA and pESC-DNC which code for a C-terminally FLAG-tagged LmjMCA (complete sequence) protein and a N-terminally 6× His-tagged and C-terminally FLAG-tagged cd-LmjMCA (catalytic domain) protein, respectively. Point mutations in the putative catalytic dyad were induced in both constructs with a QuikChange® multi site-directed mutagenesis kit (Stratagene) and primers: H147A (5′-TTCCACTTCTCCGGCGCCGGCGGACAAGCCAAG-3′) (His147 to Ala), C201A (5′-ACGTGCGTCTTT GACGCCTGTCACTCAGCCAGC-3′) (Cys201 to Ala) and C202A (5′-TGCGTCTTTGACTGCGCTCACT CAGCCAGCATG-3′) (Cys202 to Ala). All constructs were verified by sequencing (Microsynth AG, Balgach, Switzerland). The pFM21 construct with the coding sequence of a C-terminally FLAG-tagged *S. cerevisiae* metacaspase (YCA1) in the pESC-His vector was provided by Dr. Frank Madeo, University of Tübingen, Germany (Madeo et al., 2002). The Euroscarf YCA1 disrupted strain (Δ*yca1* cells) [Accession No. Y02453 (BY4741; MAT a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; YOR197w::kanMX4)] was transfected with the individual pESC-His vector (vector control) and the constructs pFM21 (YCA1-FLAG protein), pESC-LCA (LmjMCA-FLAG protein), pESC-DNC (cd-LmjMCA protein), and the respective point mutants. Transfected yeast cells were selected and grown in synthetic/dropout (SD/DO) culture medium consisting of 0.67% yeast nitrogen base (Becton, Dickinson and Company, Sparks, MD), an amino acid solution without histidine (20 mg/L adenine, arginine, methionine, tryptophan and uracil; 30 mg/L isoleucine, lysine and tyrosine; 50 mg/L phenylalanine; 100 mg/L leucine; 150 mg/L valine and 200 mg/L threonine) and 2% glucose as a carbon source. Clones were cultured freshly or from −70 °C frozen stocks on SD/DO plates with 2% agar (Becton, Dickinson and Company) and incubated at 30 °C for 4 days. Ten milliliters of SD/DO medium were inoculated with one colony and cultured overnight at 30 °C with continuous shaking. Cultures were diluted to an OD_{600} of 0.1 in 10-ml SD/DO medium and kept in culture until an OD_{600} of approximately 0.5 was reached. Cells were centrifuged and diluted in 1 vol. of SD/DO medium containing 2% galactose instead of glucose for induction of protein expression and maintained at 30 °C with shaking for at least 16 h until harvested.

2.4. Cell lysis and immunobloting

Stationary *L. major* promastigotes were lysed in a solution containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, 0.5% Nonidet P40, 2 mM EDTA, 40 ng/ml leupeptin, 10 μg/ml pepstatin, 0.8 mM *o*-phenanthroline and 160 μM E64. Yeast cells in liquid culture were washed in a 10 mM Tris–HCl/1 mM EDTA solution, lysed in a buffer containing 1.85 M NaOH, 7% β-mercaptoethanol, 1 mM phenylmethylsulphonylfluoride and 5 mM EDTA, precipitated with trichloroacetic acid and diluted in 1× PBS. Protein concentration was quantified using a BCA protein assay reagent (Pierce Biotechnology, Inc., Rockford, IL) with BSA as standard. Twelve percent SDS–PAGEs were used to separate 5 μg of total protein from parasite lysates and 20 μg of total protein from yeast lysates. Proteins were transferred to a nitrocellulose membrane by electroblotting and probed with rabbit polyclonal antibodies produced against a synthetic 76-amino acid peptide (E53) from the caspase-like domain and around the putative His/Cys catalytic dyad of the *T. brucei* TbMCA2/3 metacaspase (NH2-DVLFFHYSG**H**GTQCKSRGDSDEKYDQCIAPVDFQ KSGCIVDDDIHKLLFSRLPEKVRLTAVFDC**C**HSGS IMDLPFT-COOH) sharing 60.5% identity with LmjMCA (α-LmjMCA antibodies). Prior to use, the specificity of the α-LmjMCA antibodies were evaluated in competition assays by mixing 10 μg/ml of the E53 peptide for 30 min at room temperature with α-LmjMCA. Alternatively, murine monoclonal antibodies against the FLAG-epitope (α-FLAG antibodies) (Stratagene) or murine monoclonal antibodies against the Penta-His-epitope (α-5His antibodies) (Qiagen AG, Hombrechtikon, Switzerland) were used. Equivalent protein loading was controlled with a murine monoclonal antibody against the yeast alkaline phosphatase (α-AP). Blots were exposed to the respective horseradish peroxidase-conjugated secondary antibodies (Promega Corp., Madison, WI) and developed by enhanced chemiluminescent staining using ECL™Western blotting system (Amersham Biosciences, Piscataway, NJ). Low range molecular weight standards were used as reference proteins (Bio-Rad Laboratories, Hercules, CA).

2.5. Purification of the catalytic domain of the LmjMCA protein (cd-LmjMCA) expressed in yeast

Yeast cells transfected with the pESC-DNC construct were cultured and induced for protein expression for 18 h. The cell pellet was diluted with 1 vol. of lysis buffer containing 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 10 mM imidazole and 1% Triton X-100 and then mixed with 1 vol. glass beads (Carl Roth GmbH, Karlsruhe, Germany). Cells were lysed by vortexing for 1 min, 10 times. Supernatant was collected after centrifugation and incubated for 1 h at 4 °C with Ni–NTA agarose resin (Qiagen AG) with continuous agitation. The resin was washed twice with 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, 20 mM imidazole and the cd-LmjMCA protein was then eluted with 50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl and 250 mM imidazole. Eluted protein was concentrated in 1× PBS with an Amicon Ultra-4 centrifugal filter device (Millipore, Bedford, MA) prior to protein concentration determination.

2.6. Survival test

Transfected yeast cells were exposed to 0.8 mM H_2O_2 simultaneously with the induction of protein expression. Cells were incubated at 30 °C for 24 h with shaking and diluted to be tested for survival by plating 250 cells on YPD plates consisting of 2% proteose peptone (Merck KgaA, Darmstadt, Germany), 1% yeast extract (Becton, Dickinson and Company), 2% agar (Becton, Dickinson and Company) and 2% glucose. Plates were incubated at 30 °C for 48 h and colonies were counted to calculate survival as the percentage of colonies over plated cells.

2.7. Test for apoptotic marker

Annexin V staining was performed as described previously (Madeo et al., 1997; Balzan et al., 2004) with some modifications. Briefly, 1×10^7 transfected Δ*yca1* cells either exposed or not to 0.8 mM H_2O_2 for 16 h were washed in sorbitol buffer (1.2 M sorbitol, 0.5 mM $MgCl_2$, 35 mM KH_2PO_4, pH 6.8) and digested with 1 mg/ml Zymolyase 20 T (Seikagaku Corp., Tokyo, Japan) in sorbitol buffer for 2 h at 30 °C. Then cells were washed and resuspended in Annexin V binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$ and 1.2 M sorbitol) and incubated for 20 min at room temperature with 10 μl Annexin V-FITC (Invitrogen AG) and 10 μl 500 ng/ml propidium iodide (PI). Ten thousand events were accumulated with a BD FACScan™ apparatus and data was analyzed using the CellQuest™ (Becton–Dickinson Biosciences, San Jose, CA) and Flowjo™ (Tree Star Inc., Ashland, OR) software.

2.8. Enzymatic activity

Transfected yeast cells were harvested following 24 h induction for protein expression. Total protein extracts for caspase activity were prepared with glass beads in 50 μl lysis buffer containing 0.5% Nonidet P40, 20 mM Hepes, 84 mM KCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM phenylmethylsulphonylfluoride. Total protein extracts tested for enzymatic activity with other peptidyl substrates were prepared similarly but in a lysis buffer containing 50 mM KH_2PO_4, pH 7.5, 500 mM NaCl, 1 mM EDTA, 5 mM DTT and 1% CHAPS. Caspase activity was evaluated as described previously (Lauber et al., 2001). Briefly, 40 μg of total protein were diluted in 200 μl of activity buffer (50 mM Hepes, 100 mM NaCl, 10% sucrose, 0.1% CHAPS and 10 mM DTT) and incubated with 50 μM (final concentration) of the fluorogenic substrates *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC), *N*-acetyl-Val-Glu-Ile-Asp-7-amino-4-methylcoumarin (Ac-VEID-AMC) and *N*-acetyl-Ile-Glu-Thr-Asp-7-amino-4-methylcoumarin (Ac-IETD-AMC) (Biomol International LP, Plymouth Meeting, PA). Human caspase-6 cloned in the pESC-His vector (unpublished data) and expressed in Δ*yca1* cells, was used as a positive control for the caspase activity assays. Enzymatic activity with other peptidyl substrates was evaluated with 40 μg of total protein or 1 μg of purified protein diluted in 200 μl of activity buffer (25 mM Hepes, 150 mM NaCl, 10 mM $CaCl_2$, 10% glycerol, 0.1% CHAPS and 10 mM DTT) and incubated with 100 μM of the fluorogenic substrates Boc-Gly-Arg-Arg-7-amino-4-methylcoumarin (Boc-GRR-AMC) (Bachem AG, Switzerland), Z-Arg-Arg-7-amino-4-methylcoumarin (Z-RR-AMC), Z-L-Arg-7-amino-4-methylcoumarin (Z-R-AMC), Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (Z-GGR-AMC) and Boc-Val-Leu-Lys-7-amino-4-methylcoumarin (Boc-VLK-AMC). Activity of 10 ng of trypsin (Boehringer Manheim GmbH, Germany) was tested as a control of the assays. In all cases, the release of AMC was measured each 10 min for 2 h by spectrofluorometry using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. Enzymatic activity was determined as the slope of the resulting linear regression and expressed in arbitrary milli-fluorescence units per minute per μg of protein (mFU/min/μg) or as the fold increase relative to the activity of the vector control.

2.9. Statistical analysis

All the experiments were performed at least three times independently and means and S.D. were calculated. The *t* test was used in statistical analysis and significance was considered when $P < 0.05$.

3. Results

3.1. Metacaspase expression in L. major parasites

The single metacaspase gene (*LmjF35.1580*) identified in chromosome 35 of *L. major* codes for a polypeptide (LmjMCA) of 435 amino acids with an expected molecular mass of 47.2 kDa and a p*I* of 8.2. Based on amino-acid sequence analysis, the LmjMCA polypeptide has a hypothetical secondary structure composed of an N-terminal signal peptide (amino-acid residues 1 to 23), a caspase-like catalytic domain (amino-acid residues 63 to 314) with the catalytic dyad His147/Cys202 and a C-terminal proline-rich domain (amino-acid residues 315 to 435). A hypothetical cleavage site after the N-terminal signal peptide (between amino-acid residues 28 and 29) and several potential glycosylation sites can also be predicted (Fig. 1A).

As an initial approach to study LmjMCA, its expression in stationary phase *L. major* promastigotes was evaluated by immunoblot using rabbit polyclonal α-LmjMCA antibodies. A group of five proteins around the expected molecular mass (47.2 kDa) and two proteins below 31 kDa were recognized in parasite lysates (Fig. 1B, lane a). The specificity of recognition of these proteins by the

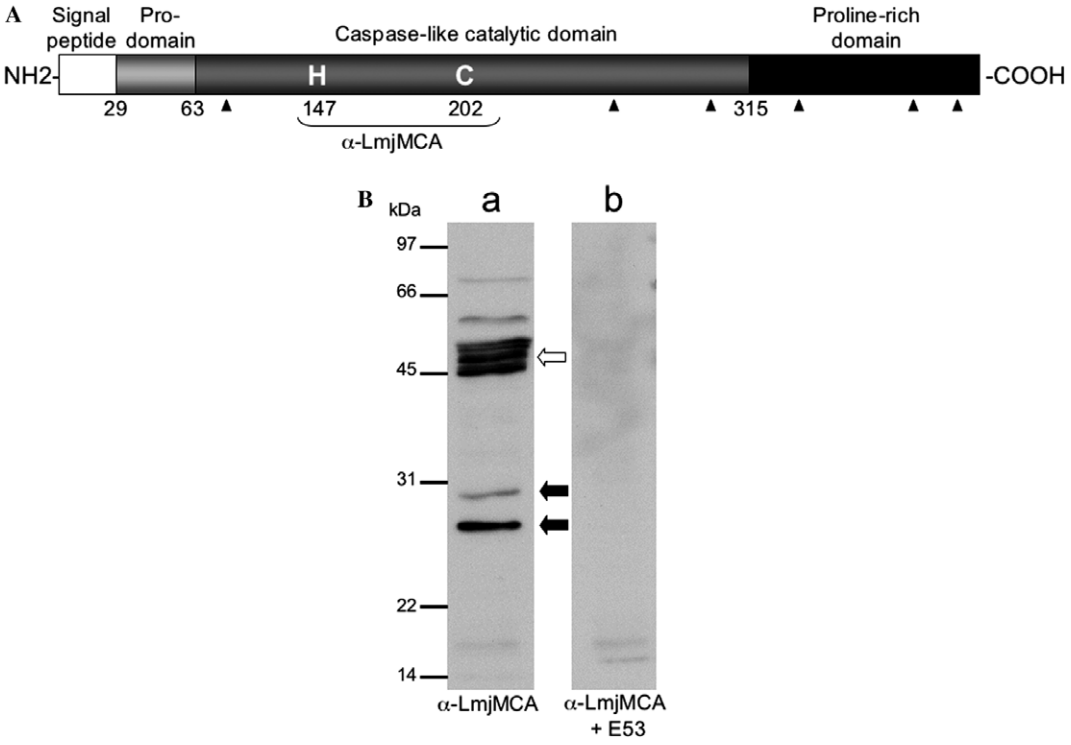

Fig. 1. The *Leishmania major* metacaspase (LmjMCA). (A) Schematic representation of the LmjMCA structure showing a predicted signal peptide in the N-terminus, the caspase-like catalytic domain with the catalytic dyad (His147 and Cys202) and a C-terminal proline-rich domain. Black triangles denote hypothetical glycosylation sites. Recognition site of α-LmjMCA is shown. (B) Immunoblot of stationary phase *L. major* parasite lysates with α-LmjMCA (a) or with α-LmjMCA pre-incubated with the E53 peptide (b). White arrow indicates the predicted full-length LmjMCA (47.2 kDa) and black arrows indicate possible lower molecular weight cleavage products.

α-LmjMCA antibodies was demonstrated by a competition experiment with the E53 peptide (Fig. 1B, lane b). Although this immunoblot confirms the expression of LmjMCA in *L. major* promastigotes, the presence of more than one protein was unexpected. These proteins could correspond to products of post-translational glycosylation and, for the smaller molecular weight proteins, to processed polypeptides of the primary translation product.

3.2. Expression of recombinant LmjMCA in yeast cells

LmjMCA fused to a DNA segment encoding a C-terminally FLAG tag epitope (pESC-LCA construct) was expressed under the control of the GAL10 promoter in *S. cerevisiae* cells disrupted for the endogenous yeast metacaspase (Δ*yca1* cells). The expression of LmjMCA in whole-yeast cell lysates was verified by immunoblot using the α-LmjMCA and α-FLAG antibodies (Fig. 2). As controls, lysates of Δ*yca1* cells transfected with the pESC-His vector alone (vector control) or with the pFM21 construct were also evaluated. Either α-LmjMCA or α-FLAG antibodies did not detect any protein in vector control cells induced for protein expression (Fig. 2, lane a). Cells transfected with the pFM21 construct encoding the FLAG-tagged YCA1 had a single protein detected in the non-induced culture with the α-FLAG at the expected size (51.3 kDa) likely due to a leaky promoter (Fig. 2B, lane b) with several prominent proteins recognized in the induced culture around the expected sizes as previously described (Madeo et al., 2002) (Fig. 2B, lane c). The α-LmjMCA antibodies weakly recognized YCA1 in induced cultures (Fig. 2A, lane c) likely due to some homology in amino-acid sequence between the *Leishmania* and yeast metacaspases.

Three major proteins were detected with the α-LmjMCA antibodies in cells induced for the expression of LmjMCA (Fig. 2A, lane e), which were not present in non-induced cultures (Fig. 2A, lane d); a faint band could be seen at the expected size of full-length LmjMCA (48.5 kDa, white arrow) and two stronger bands at around 40 and 30 kDa (gray and black arrows, respectively). In contrast, the α-FLAG antibodies specifically recognized a protein at the expected size (48.5 kDa, white arrow) in cells expressing LmjMCA (Fig. 2B, lane e). These results suggest that LmjMCA can be detected in transfected Δ*yca1* cells at the expected molecular mass, however the presence of additional bands that are recognized by the α-LmjMCA and not by the α-FLAG antibodies suggests a processing of this protein with cleavage of its C-terminal domain. The 30-kDa protein could correspond to a processing product similar to the one observed around the same molecular mass in parasite lysates (Fig. 1B).

3.3. Auto-processing of LmjMCA in yeast

The auto-processing of some *A. thaliana* metacaspases and YCA1 has been previously demonstrated (Madeo

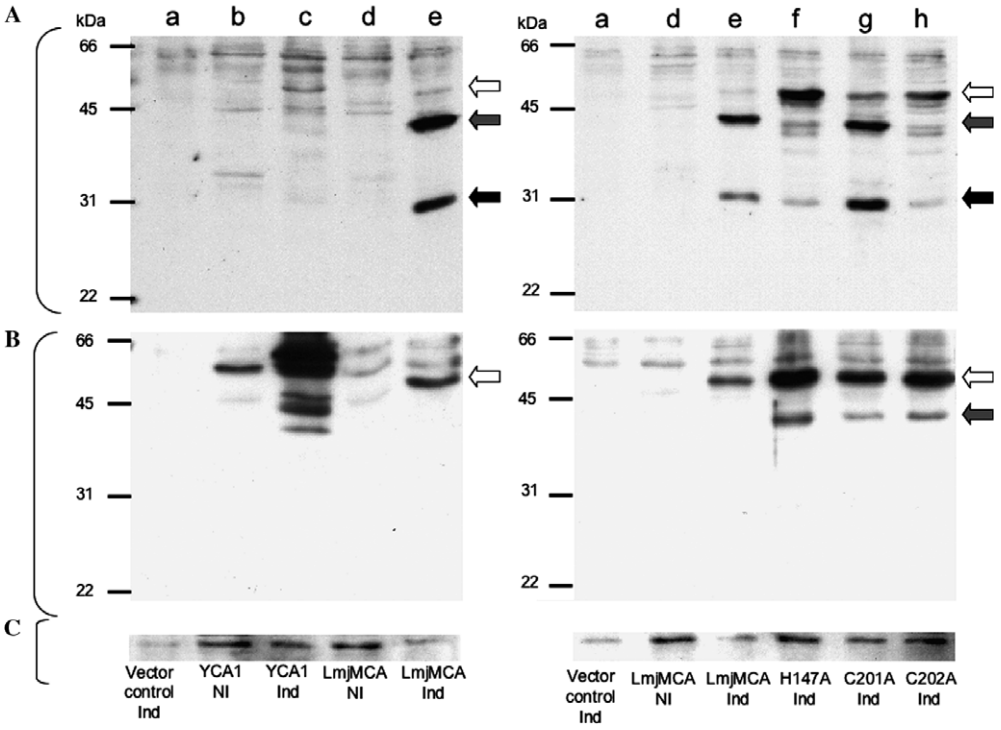

Fig. 2. Expression and auto-processing of LmjMCA in yeast. Δ*yca1* cells transfected with the pESC-His vector alone (a), with the constructs pFM21 (YCA1-FLAG protein) (b and c) and pESC-LCA (LmjMCA-FLAG protein) (d and e) and with the mutants H147A, C201A and C202A (f, g and h, respectively), not induced (NI) or induced (Ind) for protein expression with galactose for 24 h, were lysed and analyzed by immunoblot with α-LmjMCA (A) and α-FLAG (B). α-AP (yeast alkaline phosphatase) immunoblot is shown as loading control (C). White arrow indicates full-length LmjMCA-FLAG (48.5 kDa) and gray and black arrows indicate possible lower molecular weight cleavage products.

et al., 2002; Vercammen et al., 2004; Watanabe and Lam, 2005). The histidine–cysteine catalytic dyad conserved in clan CD cysteine proteases is predicted in positions 147 and 202 of LmjMCA. Another cysteine residue is present at position 201, which could also be involved in LmjMCA activity even though it was predicted not to be part of the catalytic site (Mottram et al., 2003). The three amino acids in positions 147, 201 and 202 were changed in the present study to alanine residues by site directed mutagenesis to evaluate their role in the possible auto-processing of LmjMCA. Expression of these mutants in Δ*yca1* cells was analyzed by immunoblot following 24 h induction (Fig. 2).

The α-LmjMCA antibodies strongly detected the full-length LmjMCA (48.5 kDa – white arrow) in both H147A (Fig. 2A, lane f) and C202A (Fig. 2A, lane h) mutants, while the 40-kDa (gray arrow) and 30-kDa (black arrow) proteins present in the wild-type LmjMCA (Fig. 2A, lane e) were only very weakly recognized in these mutants. Similarly, the α-FLAG antibodies detected, and with more intensity, the full-length protein in all three mutants (Fig. 2B, lanes f, g and h – white arrow) when compared with the wild-type LmjMCA (Fig. 2B, lane e). Interestingly, the 40-kDa protein that is detected only by the α-LmjMCA antibodies in the wild-type LmjMCA, is also detected in all mutants with the α-FLAG antibodies (Fig. 2B, lanes f, g and h – gray arrow). The C201A point mutant (Fig. 2, lane g) presented an intermediate banding pattern with α-LmjMCA and α-FLAG antibodies when compared with the H147A and C202A point mutants and the wild-type LmjMCA, with greater similarity to the wild-type LmjMCA. These results suggest that LmjMCA is auto-processed with cleavage of its C-terminal region and that the amino acids involved in this activity are effectively those predicted to be part of the catalytic dyad, namely histidine 147 and cysteine 202. The cysteine in position 201 does not seem to be essential for LmjMCA auto-processing however; it seems to somehow influence the role of cysteine 202. The detection of a 40-kDa protein in the point mutants by the α-FLAG antibodies suggests that LmjMCA is N-terminal cleaved independently of its auto-processing activity.

3.4. Cell death in yeast cells expressing LmjMCA following exposure to H_2O_2

Previous studies have demonstrated that *S. cerevisiae* cells disrupted for the yeast metacaspase (Δ*yca1*) were able to survive following treatment with hydrogen peroxide. The complementation of these cells with plasmids encoding yeast (YCA1) or plant (AtMCP1b and AtMCP2b) metacaspases rescued the normal susceptibility to oxidative stress, demonstrating a functional role of metacaspases in PCD (Madeo et al., 2002; Watanabe and Lam, 2005). To examine whether LmjMCA is functionally related to YCA1, the survival of Δ*yca1* cells expressing LmjMCA

was evaluated following treatment with H_2O_2. Different concentrations ranging from 0.2 to 1.2 mM H_2O_2 were tested, however, since a similar effect was detected with concentrations over 0.4 mM; results of cells treated with 0.8 mM H_2O_2 for 24 h are shown (Fig. 3). Δ*yca1* cells transfected with the pESC-his vector (vector control) and Δ*yca1* cells complemented with the YCA1 were used as controls. Similar to previous reports (Madeo et al., 2002; Watanabe and Lam, 2005), the vector control cells had a survival rate of 88% following H_2O_2 exposure and Δ*yca1* cells over-expressing the YCA1 had a survival rate of 48.7% under the same conditions. Cells expressing the LmjMCA and exposed to H_2O_2 had a survival rate of 60.6%, thus resembling the survival of YCA1-complemented cells and suggesting that LmjMCA has a similar role to YCA1 in yeast PCD. To evaluate if the catalytic activity of LmjMCA is required for its role in yeast PCD, survival of Δ*yca1* cells expressing the H147A, C201A and C202A LmjMCA point mutants treated with H_2O_2 was also evaluated. An increase in survival rate from 60.6% in the wild-type to 70.3% in the H147A mutant, 77.9% in the C201A and 94.7% in the C202A, was observed (Fig. 3). These results suggest that the involvement of LmjMCA in PCD depends on its catalytic activity.

To confirm that Δ*yca1* cells expressing LmjMCA and exposed to H_2O_2 were dying by PCD, we measured the exposure of phosphatidylserine (PS) at the cytoplasmic membrane of yeast cells with FITC-labeled Annexin V. Yeast cells with a loss of integrity in their membrane (necrotic cells) were detected with propidium iodide (PI) (Fig. 4). Δ*yca1* cells transfected with the pESC-His vector (vector control) (Fig. 4, panel A), complemented with YCA1 (Fig. 4, panel B), and expressing LmjMCA (Fig. 4, panel C), were treated with 0.8 mM H_2O_2 for 16 h prior to staining. In the absence of H_2O_2 no difference in cell staining was observed among the cells tested. However, Δ*yca1* cells complemented with YCA1 (Fig. 4, panel B) and cells expressing LmjMCA (Fig. 4, panel C) had more than double (18.8% and 21.1%, respectively) the percentage of apoptotic cells (Annexin V positive, PI negative – black arrow) present in the vector control (9.28%) following H_2O_2 treatment. There was no difference in the level of cells dying by necrosis (PI positive, Annexin V negative) following H_2O_2 treatment. These results confirm that yeast cells expressing LmjMCA upon exposure to H_2O_2 die by an apoptotic-like process similar to yeast cells expressing YCA1.

3.5. Enzymatic activity of LmjMCA

The auto-processing of LmjMCA and its role in yeast programmed cell death imply that this cysteine protease may have caspase-like enzymatic activity. To assess this hypothesis, total protein extracts of Δ*yca1* cells expressing LmjMCA were evaluated for their activity with the caspase substrates Ac-DEVD-AMC, Ac-VEID-AMC and Ac-IETD-AMC. Total protein extracts of Δ*yca1* cells expressing human caspase-6 were tested with these three substrates as a positive control. The levels of activity obtained with human caspase-6 were: 96.7 mFU/min/μg with Ac-DEVD-AMC, 285 mFU/min/μg with Ac-VEID-AMC and 207.7 mFU/min/μg with Ac-IETD-AMC. These caspase substrates were not significantly cleaved by LmjMCA protein extracts when compared with the vector control, even following H_2O_2 treatment (Fig. 5). Similarly, caspase activity was not observed with protein extracts of Δ*yca1* cells expressing the putative caspase-like catalytic domain of LmjMCA (cd-LmjMCA). In our hands, YCA1 did not have caspase activity as previously reported by Watanabe and Lam (2005).

Considering that *A. thaliana* metacaspases have been demonstrated to cleave a series of substrates with arginine residue in P1 (Vercammen et al., 2004; Watanabe and Lam, 2005) and that LmjMCA could have similar catalytic activ-

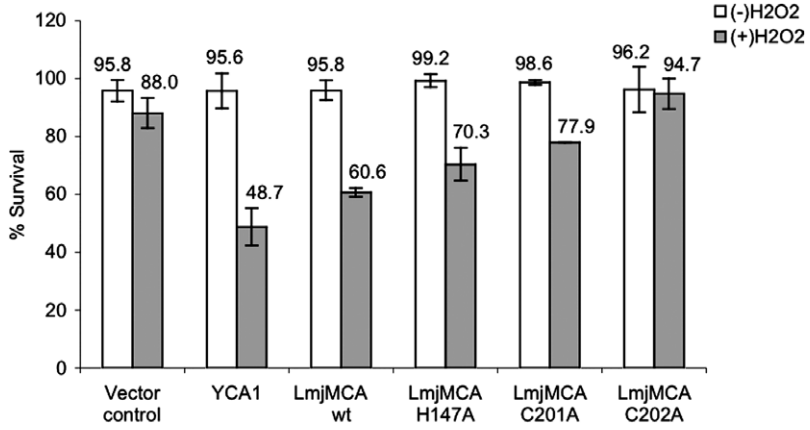

Fig. 3. Survival of Δ*yca1* yeast cells expressing LmjMCA following H_2O_2 exposure. Δ*yca1* cells transfected with the pESC-His vector alone (vector control) and with the constructs pFM21 (YCA1-FLAG protein), pESC-LCA (LmjMCA-FLAG protein) and the H147A, C201A and C202A mutants, were treated with 0.8 mM H_2O_2 for 24 h and tested for survival by incubating 250 cells at 30 °C for 48 h on YPD agar plates. Cell survival was calculated as the percentage of colonies over the total number of plated cells. Survival of transfected Δ*yca1* cells not treated with H_2O_2 are shown as controls (white bars). Means and S.D. are indicated.

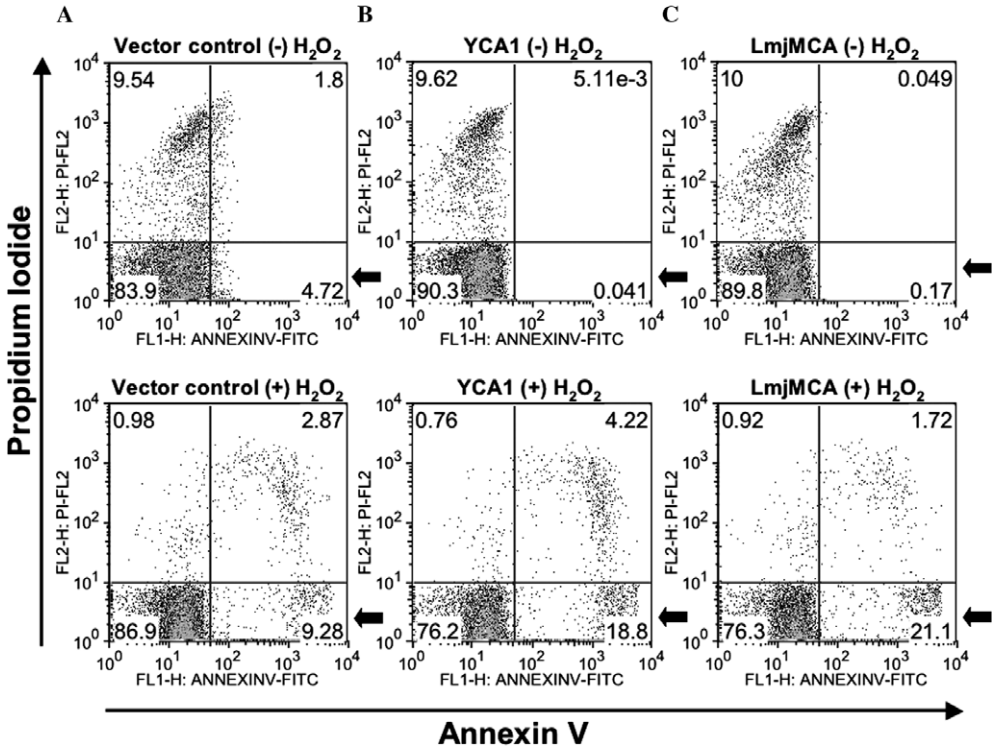

Fig. 4. Phosphatidylserine (PS) exposure at the membrane of yeast cells treated with H_2O_2. Δ*yca1* cells transfected with the pESC-His alone (column A – vector control) and with the constructs pFM21 (column B – YCA1-FLAG protein) and pESC-LCA (column C – LmjMCA-FLAG protein) were not treated (−) or treated (+) with 0.8 mM H_2O_2 for 16 h. Cells were stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and PI to distinguish between apoptotic (Annexin V-positive, PI-negative – black arrows) and necrotic (PI-positive, Annexin V-negative) cells. Data shown is representative of three independent experiments.

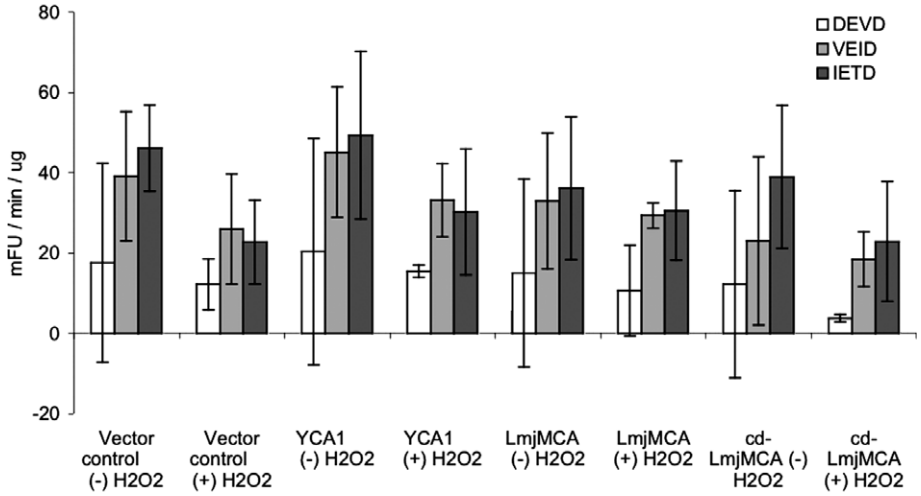

Fig. 5. Caspase activity of LmjMCA. Δ*yca1* cells transfected with the vector alone (vector control) and with the constructs pFM21 (YCA1-FLAG protein), pESC-LCA (LmjMCA-FLAG protein) and pESC-DNC (cd-LmjMCA) were not treated (−) or treated (+) with 0.8 mM H_2O_2 for 24 h. Caspase activity was tested with protein extracts from the transfected cells by measuring AMC release every 10 min for 2 h following incubation with the caspase-specific substrates Ac-DEVD-AMC, Ac-VEID-AMC and Ac-IETD-AMC. Enzymatic activity was determined as the slope of the resulting linear regression and expressed in arbitrary milli-fluorescence units per minute per μg of protein (mFU/min/μg). Means and S.D. are indicated.

ity, total protein extracts of Δ*yca1* cells expressing LmjMCA and cd-LmjMCA were tested with the fluorogenic substrates Boc-GRR-AMC, Z-RR-AMC, Z-R-AMC, Z-GGR-AMC and Boc-VLK-AMC (Fig. 6A). As a control for the assay, 10 ng of trypsin was tested with the Z-GGR-AMC substrate giving 1.4×10^6 mFU/min/μg of

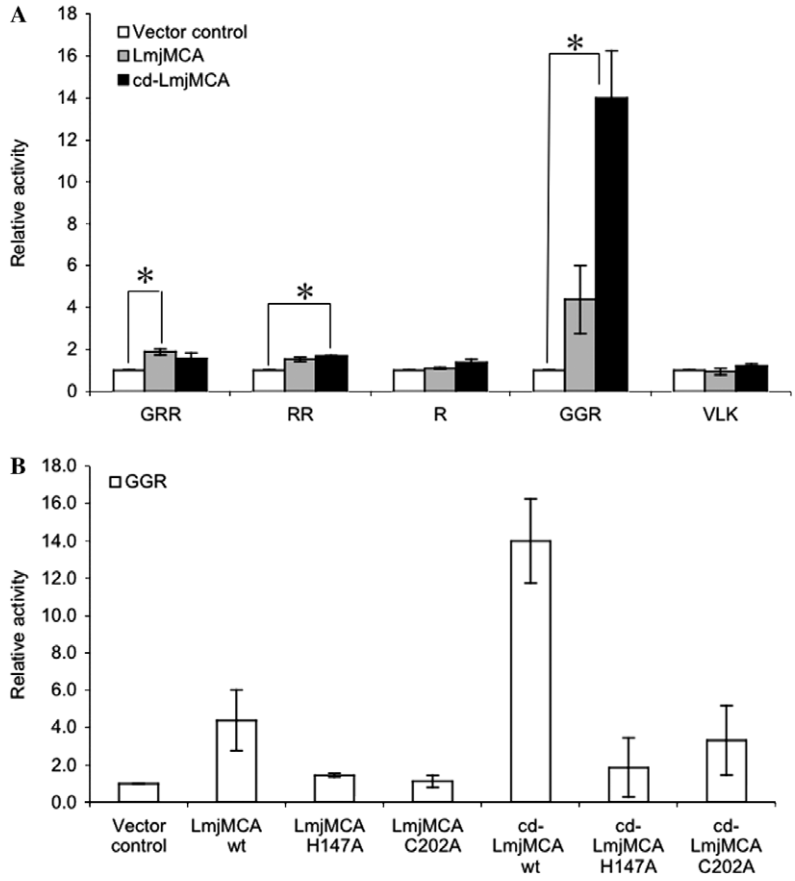

Fig. 6. Enzymatic activity of LmjMCA with peptidyl substrates. (A) Protein extracts form Δ*yca1* cells transfected with the vector alone (vector control) and with the constructs pESC-LCA (LmjMCA-FLAG protein) and pESC-DNC (cd-LmjMCA), were tested for enzymatic activity with the Boc-GRR-AMC, Z-RR-AMC, Z-R-AMC, Z-GGR-AMC and Boc-VLK-AMC substrates. (B) Protein extracts from vector control, LmjMCA-FLAG, cd-LmjMCA and their respective H147A and C202A mutants, were evaluated for its specific activity with the Z-GGR-AMC substrate. The AMC release was measured every 10 min for 2 h to determine the activity as the slope of the resulting linear regression. Relative activity is expressed as the fold-increase relative to the activity of the vector control. Means and S.D. are indicated. $*P < 0.05$.

activity. Significant activity for LmjMCA with the Boc-GRR-AMC substrate ($P = 0.008$) and for cd-LmjMCA with the Z-RR-AMC ($P = 0.016$) and Z-GGR-AMC ($P = 0.0009$) substrates were obtained when compared with the vector control. Different experimental conditions were also tested, with increased calcium concentration (from 10 to 100 mM), changes in temperature (30 and 37 °C) and different pH (5.0, 9.0); however neither the enzymatic activity nor the substrate specificity were improved (data not shown). Activity with the Z-GGR-AMC substrate was significantly higher with the cd-LmjMCA protein, suggesting that the processing of LmjMCA enhances its activity with this substrate. Interestingly, this activity was abrogated when the catalytic dyad (His147/Cys202) was mutated in both LmjMCA and cd-LmjMCA proteins (Fig. 6B), confirming its specificity towards the Z-GGR-AMC substrate.

To exclude the possibility that the observed enzymatic activity is due to any contaminating enzymes present in the total protein extracts, the N-terminally 6× His-tagged and C-terminally FLAG-tagged cd-LmjMCA protein was purified with an Ni–NTA resin, concentrated, immunoblotted and probed with the α-5-His, α-FLAG and α-LmjMCA antibodies. An expected 36-kDa protein was detected with all antibodies (Fig. 7A, black arrow). The enzymatic activity of the purified cd-LmjMCA was then tested with the Z-GGR-AMC substrate (Fig. 7B). A significant increase in activity, around 300 times the activity of the non-purified cd-LmjMCA, was obtained.

4. Discussion

The heterologous expression of mammalian apoptotic molecules in yeast has been shown to induce some features of apoptosis, suggesting the presence of a cell death program in this unicellular organism (reviewed in Frohlich and Madeo, 2000). In *S. cerevisiae*, such a program was first demonstrated by the description of endogenous stimulated cell death following mutation of the ATPase AAA gene *cdc48* (*cdc48*^{S565G}) involved in vesicular fusion (Madeo et al., 1997). Exposure to low doses of hydrogen peroxide also induces PCD in yeast and the disruption of the metacaspase gene (Δ*yca1*) has been found to confer resistance to this kind of oxidative stress (Madeo et al., 2002). The discovery of the YCA1 caspase-related metacaspase and the description of its involvement in PCD has motivated

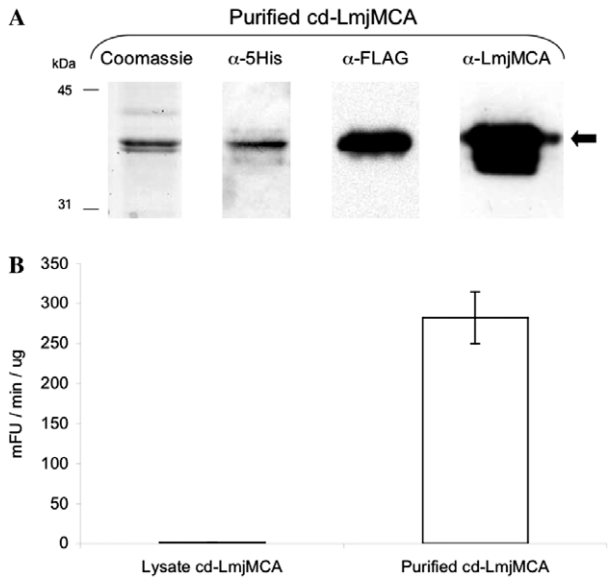

Fig. 7. Activity of the purified cd-LmjMCA with the Z-GGR-AMC substrate. (A) cd-LmjMCA was purified from yeast cells with an Ni–NTA resin, electrophoresed in a 12% SDS–PAGE gel for Coomassie staining and subsequently immunoblotted with the α-5His, α-FLAG and α-LmjMCA antibodies. A 36-kDa protein (black arrow) was detected in all cases. (B) Purified cd-LmjMCA was tested for its activity with the Z-GGR-AMC substrate simultaneously with the non-purified cd-LmjMCA. The AMC release was measured every 10 min for 2 h to determine the activity as the slope of the resulting linear regression. Enzymatic activity is expressed in mFU/min/μg.

the use of yeast as an alternative model to evaluate the function of metacaspases of other organisms. For instance, two *A. thaliana* metacaspases (AtMCP1b and AtMCP2b) expressed in Δ*yca1* cells were demonstrated to be involved in its PCD following oxidative stress and senescence (Watanabe and Lam, 2005). Metacaspase genes have also been found in the genomes of protozoan parasites belonging to *Leishmania*, *Trypanosoma* and *Plasmodium* spp. (Uren et al., 2000; Wu et al., 2003). In contrast to the presence of several metacaspase genes in *Trypanosoma* species (five genes in *T. brucei* and two genes in *T. cruzi*) and *Plasmodium* species (likely two genes in *Plasmodium falciparum*), a single metacaspase gene has been described in the genome of *L. major*, which makes this organism a valuable tool to define the role and effector molecules of cell death pathways in protozoa. In the present study, we describe the effect of the *L. major* metacaspase (LmjMCA) when expressed in YCA1 null mutant *S. cerevisiae* cells. We demonstrated that LmjMCA was able to recover the sensitive phenotype of yeast cells to oxidative stress with survival levels and apoptotic features similar to cells complemented with YCA1, suggesting a similar functional role of both metacaspases in yeast. This function was shown to depend on the enzymatic activity of LmjMCA following analysis of alanine mutations in the catalytic dyad His147/Cys202. Yeast cells expressing these point mutants were more resistant to oxidative stress than cells expressing the wild-type LmjMCA. These results confirmed that LmjMCA could replace a functional YCA1 in the yeast cell death program, suggesting that the role of this family of peptidases in cell death is likely to be conserved in evolution.

In higher eukaryotes, the activation of caspases depends on the cleavage of the p20 and p10 subunits, either by themselves (initiator caspases) or by other caspases (effector caspases). In this study, we demonstrate the presence of cleavage products of LmjMCA and the dependence of this cleavage on its catalytic dyad, suggesting that LmjMCA is auto-processed similar to the auto-processing of initiator caspases in higher eukaryotes. However, LmjMCA did not exhibit any kind of activity towards specific caspase substrates even following cell death induction, thus the LmjMCA endogenous cleavage sites are likely to be different to the cleavage sites described for initiator caspases.

To further study the substrate specificity of LmjMCA, total protein extracts from yeast cells expressing LmjMCA and the catalytic domain of LmjMCA (cd-LmjMCA) were tested for their arginine/lysine activity as was previously described for *A. thaliana* metacaspases (Vercammen et al., 2004; Watanabe and Lam, 2005). The GGR-AMC substrate gave the highest level of activity with cd-LmjMCA. This activity was abrogated when active-site mutants of these constructs were tested and the activity of cd-LmjMCA increased when a purified form of the protein was used in our in vitro assay. These results suggest that LmjMCA is an arginine cysteine protease, which needs to be processed to reach higher levels of activity. We believe this is also the first report for specific enzymatic activity of a purified form of metacaspase. Preliminary results of experiments done with protein extracts from *L. major* parasites show significant enzymatic activity towards the GGR-AMC substrate, although it cannot be excluded that other enzymes present in the extracts could also cleave this substrate.

Considering the activity observed towards the GGR-AMC substrate with LmjMCA, hypothetical auto-cleavage sites in its own sequence may be inferred. For example, three GR sites are present in LmjMCA at amino acids 39, 61 and 298. Immunoblots with α-LmjMCA recognized a 30-kDa protein in lysates of yeast cells expressing LmjMCA. This product may correspond to a cleaved polypeptide at amino acids 39 and 298. This could represent a form of the LmjMCA protein without the signal peptide and without the proline-rich domain (hypothetical molecular mass of 28.4 kDa). The additional molecular weight species at around 40 kDa, which is recognized in the LmjMCA point mutants by the α-FLAG antibodies, likely corresponds to a product of cleavage of the signal peptide and N-terminal processing independent of LmjMCA autocleavage. These results suggest that other specific cleavage sites are present in LmjMCA, which could correlate with the presence of other arginines in its amino acid sequence. Characterization of these cleavage sites is currently in progress.

In conclusion, our results provide strong evidence that LmjMCA is a cysteine protease active towards defined sub-

strates containing arginine in the P1 position, is auto-processed, and that this processing is likely to be essential for its full activity. LmjMCA is potentially the first apoptotic protozoan molecule to be described as being involved in PCD. Although yeast cells constitute a useful model to characterize the LmjMCA, it is still important to evaluate the function of this enzyme in its native environment, the *Leishmania* parasite. The presence of metacaspases in *Leishmania* spp. and other protozoan parasites such as *Trypanosoma* and *Plasmodium* spp. and their absence in mammals, make these proteins and their activating pathways worthy of further characterization with the prospect of identification of novel drug targets and the definition of a cell death program in lower eukaryotes.

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