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Metacaspase 2 of *Trypanosoma brucei* is a calcium-dependent cysteine peptidase active without processing

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Abstract **Metacaspases are cysteine peptidases that are distantly related to the caspases, for which proteolytic processing is central to their activation. Here, we show that recombinant metacaspase 2 (MCA2) from *Trypanosoma brucei* has arginine/lysine-specific, Ca^{2+}-dependent proteolytic activity. Autocatalytic processing of MCA2 occurred after Lys55 and Lys268; however, this was shown not to be required for the enzyme to be proteolytically active. The necessity of Ca^{2+}, but not processing, for MCA2 enzymatic activity clearly distinguishes MCA2 from the caspases and would be consistent with different physiological roles.**

Keywords: Caspase; Metacaspase; Cysteine protease; Calcium

1. Introduction

Metacaspases (MCAs) are Clan CD, family C14 cysteine peptidases that were identified in plants, fungi and protozoa based on homology with a caspase-like domain from *Dictyostelium* [1]. In general, MCAs possess the catalytic dyad of histidine and cysteine that comprises the active site of the caspases, but otherwise display low overall sequence similarity. As caspases have a well-studied role in programmed cell death (PCD), a similar function has been proposed for the MCAs.

There are five MCAs in *Trypanosoma brucei*, just three of which (MCA2, MCA3 and MCA5) have preserved the catalytic cysteine and histidine residues [2,3]. The necessity of these three proteins for the bloodstream form of the parasite has been investigated by RNAi and genetic deletion [4]. Cells recover from sequential knockdown, but more acute simultaneous down-regulation of all three by RNAi led to a defect in cytokinesis and cell death. No association between prostaglandin-induced PCD of *T. brucei* [5] and the presence of MCA2, MCA3 and MCA5 was found [4]. MCA4, however, was found to induce cell death when over-expressed in yeast [2]. Taken together, the current evidence indicates that there are functional MCAs in *T. brucei*, although their precise roles need to be elucidated.

In this work, we show that MCA2 of *T. brucei* has cysteine peptidase activity. This is Ca^{2+}-dependent, with arginine/lysine specificity, and is not dependent upon the autocatalytic processing that the enzyme undergoes.

2. Materials and methods

2.1. Production of recombinant proteins

The *MCA2* coding sequence was amplified from *T. brucei* genomic DNA using oligonucelotides OL910 (GCCATATGTGCTCCTTAATTACACAACTCTGTG) and OL909 (CTCGAGCTATTGGATAGATCTGTCAACAG). The amplified sequence was inserted in to the NdeI and XhoI sites of pET28a+ (Novagen) to generate pBP54, encoding MCA2 with an N-terminal His-tag. The plasmid pBP54 was transformed into BL21:DE3 *Escherichia coli* and bacteria were grown in Overnight Express medium (Novagen) for 16 h and then lysed in B-Per protein extraction solution (Pierce). His-MCA2 was purified using Ni-NTA affinity matrix (Qiagen), followed by anion exchange chromatography on a Poros HQ column (ABI) in 50 mM Tris–HCl, pH 7.0, over a 0–1 M NaCl gradient. MCA2 was collected in the flow-through from this chromatography step. The plasmid pGEXtu-fAwt, encoding GST-tagged EF-Tu from *E. coli*, was kindly provided by Professor Charlotte Knudsen (Aarhus University, Denmark). The protein was expressed in BL21:DE3 at 28 °C for 4 h. GST-EF-Tu was purified from cell lysates using glutathione-Sepharose (Sigma) in 50 mM Tris, pH 8.0, and elution with 20 mM glutathione.

2.2. Site-directed mutagenesis

MCA2 mutants (K55G, K268G and K55,268G, C212G, C213G) were generated using the QuikChange Site-directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. For mutation of the Arg residue in the linker region, the primers were 5′-GCAGCGGCCTGGTGCCGGGCGGCAGCCATATGTGC-3′ and 5′-GCACATATGGCTGCCGCCCGGCACCAGGCCGCTGC-3′. Incorporation of mutations was confirmed by DNA sequencing.

2.3. Protein cleavage assays and $CaCl_2$-induced auto-processing of MCA2

For $CaCl_2$-induced auto-processing, MCA2 was incubated with $CaCl_2$ at 10 mM, unless otherwise indicated, for 30 min at 37 °C. Samples were analysed by reducing SDS–PAGE on 12% gels. For Edman sequencing (University of Dundee Proteomics Facility), proteins were transferred to PVDF membrane (Amersham).

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Abbreviations: MCA, metacaspase; PCD, programmed cell death; AMC, 7-amino-4-methylcoumarin; EF-Tu, elongation factor Tu; GST, glutathione *S*-transferase; TLCK, tosyl-lysyl-chloromethylketone

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2.4. Fluorometric assays for MCA2 activity

MCA2 activity was assessed by measuring hydrolysis of the fluorogenic substrates Z-GGR-AMC and Z-GRR-AMC (Bachem), using excitation and emission wavelengths of 355 nm and 460 nm, respectively. Specific activities were calculated using a 7-amino-4-methylcoumarin standard (AMC). The substrates Abz-VRPRQ-EDDnp, Abz-IKLRQ-EDDnp and Abz-IKLKQ-EDDnp synthesised according to Hirata et al. [6] were used with excitation and emission wavelengths of 320 nm and 420 nm, respectively. Substrates were used at 10 μM and measurements collected on an En Vision plate reader (Perkin Elmer). Assays were done in 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl, 5 mM DTT and 10 mM $CaCl_2$ unless otherwise stated. All experiments were run in triplicate. Statistical analysis was performed using Student's *t*-test. A value of $P < 0.05$ was statistically significant.

3. Results

3.1. MCA2 has arginine/lysine specificity

When expressed in bacteria, the N-terminal His-tag of MCA2 was removed, as an anti-His antibody failed to detect the expressed protein. The N-terminal tag remained intact when active-site mutants of MCA2 were expressed (not shown), indicating that the N-terminal cleavage was autocatalytic. To determine the site of cleavage, MCA2 lacking the His-tag was partially purified using anion exchange chromatography, and the truncated protein subjected to N-terminal sequencing (Fig. 1A). This revealed that the recombinant protein had been cleaved after an Arg residue that derives from the expression vector, thus demonstrating that MCA2 will accept Arg at the P1 position. This specificity was confirmed by our observation that a predominant *E. coli* protein was degraded during MCA2 expression. The degraded protein was identified by mass spectrometry as elongation factor Tu (EF-Tu). To identify the cleaved sites, recombinant GST-EF-Tu was purified and incubated with bacterial expression extracts containing MCA2. This resulted in the production of two major proteins (Fig. 1B), for which N-terminal sequences were obtained. The upper protein yielded the N-terminus of the GST fusion. The lower protein contained a mixture of two sequences (ALEGDA and GSALKA), showing cleavage after a Lys and an Arg, respectively. These data indicate that MCA2 has Arg/Lys specificity. To facilitate purification and further biochemical analysis of MCA2, the Arg in the linker sequence was mutated to Gly. This lead to retention of the His-tag and permitted affinity purification of recombinant MCA2. This recombinant enzyme was used for all analyses except where stated otherwise.

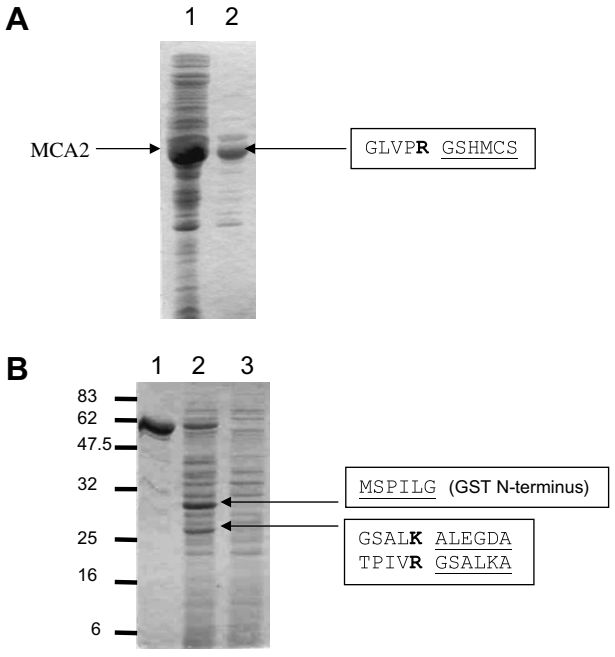

Fig. 1. Cleavage specificity of MCA2. (A) Expression and partial purification of MCA2. Whole bacterial lysates expressing MCA2 (lane 1) and MCA2 after a one step partial purification using anion exchange chromatography (lane 2) were analysed by SDS–PAGE. The N-terminus of processed MCA2 (underlined) was determined by Edman sequencing. The inferred P1 residue is in bold. (B) Cleavage of *Escherichia coli* GST-EF-Tu by MCA2-expressing bacterial cell lysates. Lane 1, GST-EF-Tu; lane 2, GST-EF-Tu + MCA2-lysate; lane 3, MCA2-lysate. Two major GST-EF-Tu degradation products (arrowed) were subjected to Edman sequencing. The resulting N-terminal sequences are underlined. The inferred P1 residues are in bold.

The ability of MCA2 to cleave the substrates Z-GGR-AMC and Z-GRR-AMC was assessed. MCA2 cleaved both substrates well, with the rate of cleavage for Z-GGR-AMC being 1.5-fold greater than that for Z-GRR-AMC. In addition, the tetrapeptide substrates Abz-VRPRQ-EDDnp, Abz-IKLRQ-EDDnp and Abz-IKLKQ-EDDnp [7] were also cleaved well by MCA2 (Abz-VRPRQ-EDDnp > Abz-IKLRQ-EDDnp > Abz-IKLKQ-EDDnp using 10 mM substrate). Further, we found that MCA2 has a pH optimum of 7.0 and was inhibited by the cysteine/serine peptidase inhibitors leupeptin (93% inhibition at 100 μM), antipain (91% inhibition at 100 μM), and tosyl-lysyl-chloromethylketone (TLCK) (70% inhibition at 100 μM). E-64, a potent inhibitor of clan CA cysteine peptidases but which is not effective against other clan CD peptidases, did not inhibit MCA2 at 10 mM.

3.2. Ca^{2+}-dependent activity of MCA2

As type II MCAs from *Arabidopsis thaliana* exhibit a Ca^{2+}-dependent activity against Arg/Lys-specific substrates [8], we tested the Ca^{2+}-dependency of MCA2 activity. MCA2 activity in the presence of 0.5 mM Ca^{2+} was boosted approximately 2-fold by the addition of 10 mM $CaCl_2$ (Fig. 2A). MCA2 activity was completely abolished by the presence of the Ca^{2+}-chelating agent EGTA (Fig. 2A). The effects of Ca and EGTA were significant when compared to buffer control ($P < 0.05$). Other divalent cations Cu^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} were unable to enhance MCA2 activity when used at 10 mM (not shown). MCA2 activity was responsive to Ca^{2+} concentrations in a dose-dependent manner up to 10 mM, where the enhancing effect began to plateau (Fig. 2B).

Most mammalian caspases require proteolytic processing to convert the inactive zymogen into an active peptidase [9]. MCAs from *Leishmania major* [10,11], *A. thaliana* [8,12] and yeast [13] also undergo caspase-like processing. Therefore, we asked whether Ca^{2+}-induced activation of MCA2 was accompanied by proteolytic processing events. Purified recombinant MCA2 was incubated with $CaCl_2$ and analysed by SDS–PAGE. As seen in Fig. 2C, 1 mM $CaCl_2$ was sufficient to induce autolytic processing of MCA2, generating two distinct products of approximately 30 kDa and 6 kDa. These two fragments were sequenced by Edman degradation and found to possess N-termini that were consistent with cleavage

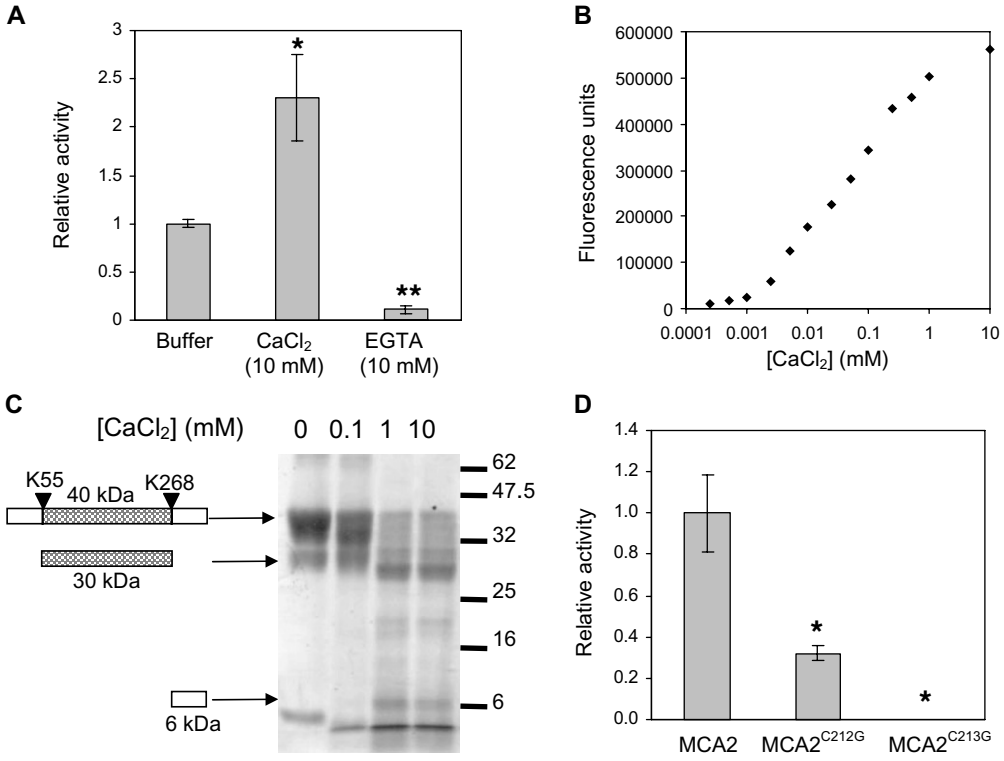

Fig. 2. $CaCl_2$ induces cleavage and activation of MCA2. (A) Cleavage of Z-GGR-AMC by MCA2 was measured in a buffer containing 0.5 mM $CaCl_2$, supplemented with 10 mM $CaCl_2$ or 10 mM EGTA. MCA2 activity is expressed as a relative value and given as means ± S.D. $^{*}P < 0.05$; $^{**}P < 0.001$ when compared to buffer control. (B) Cleavage of Z-GGR-AMC by MCA2 was measured by fluorometric assay in buffer supplemented with the indicated concentration of $CaCl_2$. (C) MCA2 was incubated at 37 °C for 30 min with the indicated concentrations of $CaCl_2$ and analysed by SDS–PAGE. (D) Activities of MCA2 and the mutants $MCA2^{C212G}$ and $MCA2^{C213G}$ were measured in the presence of 10 mM $CaCl_2$ using Z-GGR-AMC. MCA2 specific activities are expressed as relative values and given as means ± S.D. $^{*}P < 0.05$ when compared to MCA2 control.

after Lysine 55 (generating the 30 kDa product) and Lysine 268 (generating the 6 kDa product). A band at ~5 kDa appeared even at 0.1 mM $CaCl_2$, which could be the result of an initial cleavage after Lysine 268, but we failed to confirm this using Edman degradation. When processed MCA2 was analysed by size exclusion chromatography, all proteolytic products were recovered in the same fractions (not shown), indicating that MCA2 auto-processing products remain associated. Similarly, when Ca^{2+}-activated MCA2 was purified by Ni-NTA Sepharose, all processed fragments bound to the resin. In the absence of the three-dimensional structure, we are unable to conjecture how these fragments associate, however non-reducing SDS–PAGE indicated that they are not linked by disulphide bonds (not shown).

A sequence alignment of caspases and trypanosome MCAs around the active site predicts that Cys213 is the active site cysteine in MCA2 [3]. There is an additional Cys at position 212, which is conserved in all five *T. brucei* MCAs. To test whether either Cys212 or Cys213 is required for catalytic activity, each residue was individually mutated to Gly and the activity of the mutants measured. No activity against Z-GGR-AMC could be detected for $MCA2^{C213G}$, indicating that this residue functions in the active site (Fig. 2D). The activity of $MCA2^{C212G}$ was also significantly reduced relative to wild-type MCA2, which is probably due to the proximity of the substitution to the active site. Consistent with this observation, $MCA2^{C213G}$ could not undergo Ca^{2+}-induced auto-processing, while $MCA2^{C212G}$ could, albeit to a lesser extent than wild-type MCA2 (not shown).

3.3. Auto-proteolysis is not essential for MCA2 activity

MCA2 activation requires Ca^{2+} and there is also auto-catalytic processing, posing the question of whether cleavage at Lys55 and/or Lys268 is required for MCA2 activity. The current evidence is that processing is required for the activation of both plant and yeast MCAs [5,13]. To address this, MCA2 was treated with varying amounts of $CaCl_2$, inducing auto-catalytic processing (Fig. 3A). Half of each sample was treated with EDTA to remove any free Ca^{2+} and MCA2 activity was then measured. MCA2 activity increased with the amount of free Ca^{2+} present. In samples in which free Ca^{2+} had been depleted with EDTA, however, MCA2 activity was completely eliminated (Fig. 3B). We conclude from this that processing is not sufficient for MCA2 activity, but that the presence of Ca^{2+} is required. To determine whether processing is required for MCA2 activation, the processing sites were removed by mutagenesis. Both Lys55 and Lys268 were mutated to Gly, generating the double mutant $MCA2^{K55,268G}$. This mutant was resistant to Ca^{2+}-induced auto-processing when compared to the wild-type protein, even at the highest $CaCl_2$ concentration of 10 mM (Fig. 3C). Importantly, despite the lack of processing in $MCA2^{K55,268G}$, it exhibited full proteolytic activity in the presence of Ca^{2+} (Fig. 3D). Altogether, these results show that Ca^{2+} alone is sufficient for MCA2 activity.

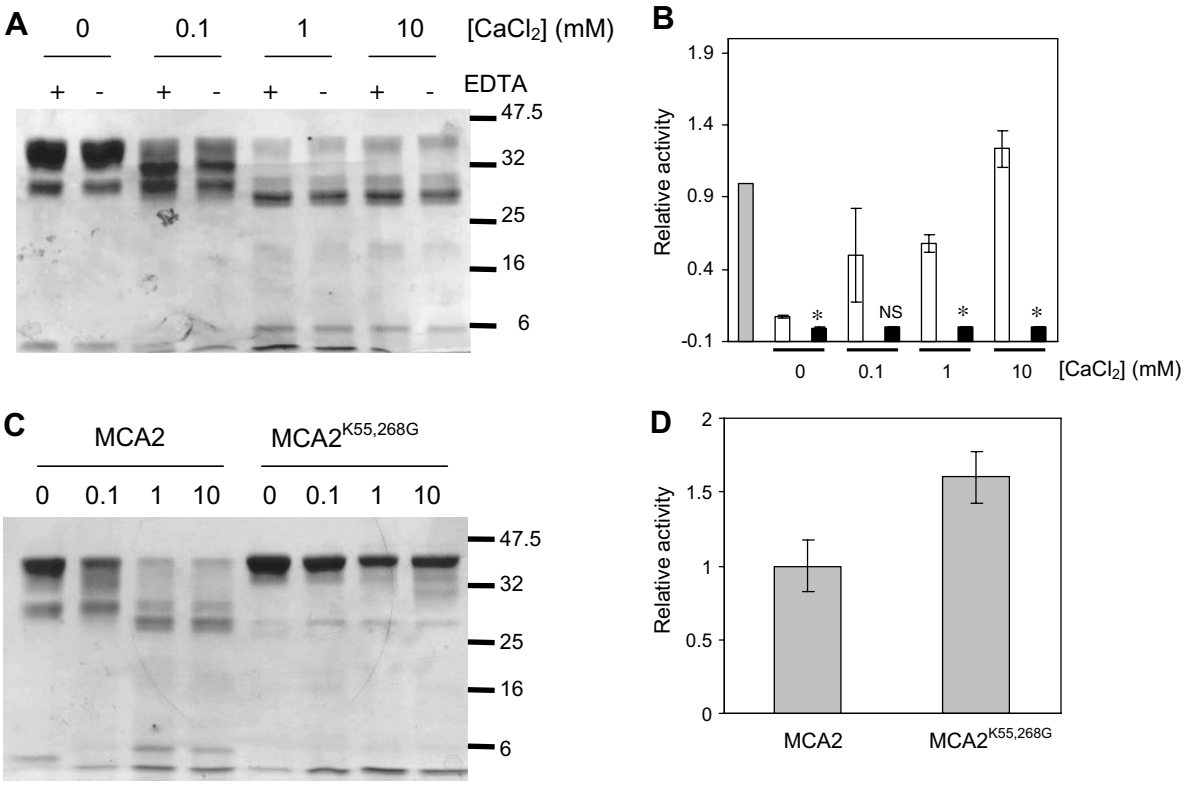

Fig. 3. Cleavage is not required for MCA2 activity. (A) MCA2 was activated with $CaCl_2$ for 30 min at 37 °C. Free calcium was then removed, where indicated, by the addition of excess EDTA. The samples were then analysed by SDS–PAGE. (B) The proteolytic activity of the samples in A was measured by fluorometric assay (without EDTA, white bars; with EDTA, black bars) using the substrate Z-GGR-AMC. As a positive control, MCA2 activity was measured in buffer containing 10 mM $CaCl_2$ (grey bar). The activity (means ± S.D.) relative to the positive control is shown. $^{*}P < 0.005$ when compared to sample not treated with EGTA. NS: not statistically significant. (C) SDS–PAGE of MCA2 and the auto-processing mutant $MCA2^{K55,268G}$ after treatment with $CaCl_2$ for 30 min at 37 °C. (D) Activities of MCA2 and $MCA2^{K55,268G}$ measured using a fluorometric assay as in Fig. 2D.

4. Discussion

This is the first demonstration that a MCA of *T. brucei* is an active cysteine peptidase. MCA2 has an Arg/Lys specificity at the P1 position, being able to cleave after these residues in the context of both a short peptide and a whole protein. The P1-specificity of MCA2 is distinct from the Asp-specificity of the caspases, but in accordance with the data observed for MCAs from *L. major* [10], plants [8,12,14], yeast [12] and filamentous fungi [15]. Other studies have found caspase-like activities in lysates from yeast cells over-expressing YCA1 [13], or embryonic cells from Norway spruce [16]. However, these studies provided no evidence that MCAs were directly responsible for cleaving caspase substrates and it remains possible that Arg/Lys-specific MCAs activate enzymes with some Asp-specificity. More recently, Guaragnella et al. [17] found that yeast YCA1 was involved in acetic acid-induced cell death, but that its effect could not be blocked with a caspase-specific inhibitor, further suggesting that yeast MCA activity is different from caspase activity.

The activity of MCA2 is strictly Ca^{2+}-dependent, requiring about 1 mM $CaCl_2$ for maximum activity. Ca^{2+} concentrations may reach this level in the acidocalcisome, an organelle rich in ions and polyphosphate [18]. However, MCA2 was found to occur predominantly in RAB11-positive endosomes in *T. brucei* with no apparent localisation in acidocalcisomes [4]. The basal cytosolic level of Ca^{2+} reported for bloodstream form *T. brucei* is 100 nM, and is only increased by 40–60 nM with the addition of thapsigargin [19]. The concentrations in different sub-cellular compartments, and under different stimuli, are unknown. The plant type II MCAs, to which *T. brucei* MCAs are most similar in that they also display Ca^{2+}-dependent activities, also require higher than known endogenous concentrations of Ca^{2+} for their activity in vitro [12,14]. The calpains, a large family of Ca^{2+}-dependent cysteine peptidases, also require significantly higher levels of Ca^{2+} for activation in vitro than have been measured intracellularly. It has been postulated that association with cofactors or phospholipids, proteolysis, or a combination of these factors, can lower the Ca^{2+}-activation threshold for calpains [20]. Similar mechanisms might exist for MCA2 activation. For example, in vivo, auto-catalytic processing may lower the Ca^{2+} requirement to within the normal intracellular range.

We have shown that MCA2 is auto-catalytically processed, but that this processing is not necessary or sufficient for peptidase activity, whereas processing is the key step in activation of caspases. Similar processing is also considered to be essential for the activity of plant and yeast MCAs [8], making the trypanosomatid MCA2 unusual. It is possible that, in vivo, processing has a role in MCA targeting and/or protein interactions and thus may have an indirect regulatory function. We have so far been unable to observe MCA2 processing

in vivo but its occurrence may be restricted to specific points of the cell cycle [11] or specific induction conditions, such as stress.

The Ca^{2+}-dependency of MCA2 raises the possibility that intracellular Ca^{2+} levels are used to control the physiological function of MCA2. Ca^{2+} is probably an important regulator of diverse cellular functions in trypanosomes and at least four organellar pools of Ca^{2+} are available to the parasite, namely the ER, nucleus, mitochondrion and acidocalcisome [21]. Interestingly, a Ca^{2+}-dependent cell death pathway has been described in *T. brucei* [22] and further studies will focus on whether intracellular Ca^{2+} fluctuations or translocation of MCA2 into Ca^{2+}-rich compartments correlate with variations in MCA activity and give insights into the protein's in vivo functions.

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