Bloodstream form *Trypanosoma brucei* depend upon multiple metacaspases associated with RAB11-positive endosomes

Matthew J. Helms1,*, Audrey Ambit1,*, Paul Appleton2, Laurence Tetley2, Graham H. Coombs2 and Jeremy C. Mottram1,‡

1Wellcome Centre for Molecular Parasitology, The Anderson College, University of Glasgow, Glasgow G11 6NU, UK
2Division of Infection and Immunity, Institute of Biomedical and Life Sciences, Joseph Black Building, University of Glasgow, Glasgow G12 8QQ, UK
*These authors contributed equally to this work
†Author for correspondence (e-mail: j.mottram@udcf.gla.ac.uk)

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Summary
*Trypanosoma brucei* possesses five metacaspase genes. Of these, *MCA2* and *MCA3* are expressed only in the mammalian bloodstream form of the parasite, whereas *MCA5* is expressed also in the insect procyclic form. Triple RNAi analysis showed *MCA2*, *MCA3* and *MCA5* to be essential in the bloodstream form, with parasites accumulating pre-cytokinesis. Nevertheless, triple null mutants (Δmca2/3Δmca5) could be isolated after sequential gene deletion. Thereafter, Δmca2/3Δmca5 mutants were found to grow well both in vitro and in culture and in vivo in mice. We hypothesise that metacaspases are essential for bloodstream form parasites, but that they have overlapping functions and their progressive loss can be compensated for by activation of alternative biochemical pathways. Analysis of Δmca2/3Δmca5 revealed no greater or lesser susceptibility to stresses reported to initiate programmed cell death, such as treatment with prostaglandin D2. The metacaspases were found to colocalise with RAB11, a marker for recycling endosomes. However, variant surface glycoprotein (VSG) recycling processes and the degradation of internalised anti-VSG antibody were found to occur similarly in wild type, Δmca2/3Δmca5 and triple RNAi induced parasites. Thus, the data provide no support for the direct involvement of *T. brucei* metacaspases in programmed cell death and suggest that the proteins have a function associated with RAB11 vesicles that is independent of known recycling processes of RAB11-positive endosomes.

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Key words: Metacaspase, Endosomes, Recycling, *Trypanosoma*, Programmed cell death, RNAi

Introduction
*Trypanosoma brucei* is a unicellular parasitic protozoon responsible for sleeping sickness in humans and nagana in cattle in sub-Saharan Africa. This parasite has a digenetic life cycle, alternating between a mammalian host and the insect vector, the tsetse fly. In the mammalian bloodstream the parasite lives extracellularly, with the surface of the parasite being covered with a dense coat of variant surface glycoprotein (VSG) which is attached to the plasma membrane with a glycosylphosphatidylinositol (GPI) anchor (Ferguson, 1999). The differential expression of VSG genes allows the parasite to undergo antigenic variation and thus evade the host’s immune response (Barry and McCulloch, 2001). The surface VSG undergoes rapid internalisation and recycling (Seyfang et al., 1990) via the flagellar pocket, which is the only site of endocytosis and exocytosis for the parasite. This recycling may function as a secondary defence against immune challenge, aiding in the removal of host anti-VSG antibodies (O’Beirne et al., 1998). The antibodies are internalised, degraded and then secreted (Jeffries et al., 2001; Pal et al., 2003), although the enzymes responsible for degradation are yet to be elucidated. Cysteine peptidases belong to several clans that have separate evolutionary origins (Rawlings et al., 2004). Members of clan CA, containing lysosomal cysteine peptidases, are abundant in trypanosomes and other parasitic protozoa and are important virulence factors and drug targets (Klemba and Goldberg, 2002; Mottram et al., 2004). Metacaspases are clan CD cysteine peptidases that have been identified in plants, fungi and protozoa, but not in worms, flies or mammals (Üren et al., 2000). Metacaspases from plants have been classified into type-I and type-II (Uren et al., 2000) and have been proposed to be part of the developmental cell death machinery. They have been implicated in maintenance of the balance between death and proliferation required for plant embryogenesis (Suarez et al., 2004; Bozhkov et al., 2005). Evidence is also accumulating for a role in cell death of the *Saccharomyces cerevisiae* metacaspase YCA1 (Madeo et al., 2002; Herker et al., 2004; Bettiga et al., 2004; Wadskog et al., 2004; Madeo et al., 2004). Overexpression of YCA1 resulted in both fragmented DNA and chromatin condensation, and these features were abolished by co-incubation with the caspase inhibitor Z-VAD-fluoromethylketone (Madeo et al.,...
Moreover, deletion of \textit{YCA1} appeared to hinder apoptosis. Thus, whereas yeast deleted for \textit{SRO7} (the homologue of a \textit{Drosophila} tumour suppressor gene) showed an increased sensitivity to NaCl stress (Wadskog et al., 2004) with a rapid loss in viability accompanied by markers of apoptosis, the additional deletion of \textit{YCA1} reduced this fast initial drop in viability and the level of DNA fragmentation of the salt-stressed \textit{SRO7}-deficient line. Similarly, the deletion of the yeast deubiquitylating enzyme UbP10 resulted in a complex phenotype characterised by a sub-population of cells displaying markers of apoptosis (Bettiga et al., 2004). The further deletion of \textit{YCA1} suppressed this phenotype, whereas the overexpression of \textit{YCA1} resulted in an increased number of cells displaying apoptotic markers. Furthermore, metacaspase involvement in apoptotic-like cell death has also been reported for yeast with regard to chronological aging (Herker et al., 2004), mRNA stability (Mazzoni et al., 2005), defects in initiation of DNA replication (Weinberger et al., 2005) and in response to viral infection (Ivanovska and Hardwick, 2005).

It has been reported that the heterologous expression of a \textit{T. brucei} metacaspase (T5MCA4) in \textit{S. cerevisiae} resulted in growth inhibition, mitochondrial dysfunction and cell death (Szallies et al., 2002). It has also been proposed that in trypanosomatids metacaspases function as caspase-like enzymes and are involved in apoptosis-like cell death, often called 'programmed cell death' (PCD) (Debrabant et al., 2003; Nguewa et al., 2004). PCD has been reported for a number of trypanosomatids, with there being evidence for cytoplasmic blebbing, DNA fragmentation, phosphoserine exposure, release of cytochrome \textit{C} and changes in mitochondrial membrane potential (Ameisen, 1996; Ameisen et al., 1996; Welburn et al., 1996; Das et al., 2001; Zangger et al., 2002; Lee et al., 2002; Holzmuller et al., 2002; Arnoult et al., 2002a; Arnoult et al., 2002b; Sen et al., 2004; Figarella et al., 2005). However, to date there has been only one report of apoptosis-like cell death in bloodstream form (BSF) \textit{T. brucei} (induced by prostaglandin \textit{D2} (Figarella et al., 2005)), whereas PCD apparently resulted from treatment of procyclic form (PCF) \textit{T. brucei} with the lectin concanavalin A (Welburn et al., 1996; Welburn and Murphy, 1998; Pearson et al., 2000) or reactive oxygen species (Ridgley et al., 1999). Nevertheless, definitive biochemical evidence to support PCD in trypanosomes is lacking and the trypanosome genome appears to lack vital components of two of the central effector pathways of mammalian apoptosis; caspases and their activation, and mitochondrial membrane permeabilization by BCL-2.

We tested the hypothesis that metacaspases in trypanosomatids are involved in PCD by investigating the expression, localisation and function of three \textit{T. brucei} metacaspases: MCA2, MCA3 and MCA5. These three metacaspases have predicted active-site cysteine and histidine residues essential for cysteine peptidase activity, whereas the other two \textit{T. brucei} metacaspases, MCA1 and MCA4, have substitutions in these residues and are predicted to lack cysteine peptidase activity (Mottram et al., 2003). The finding that MCA2 and MCA3 are stage-regulated, that the metacaspases do not undergo any caspase-like processing under normal growth conditions or after prostaglandin \textit{D2} treatment to induce cell death, and that they are colocalised in part in RAB11-positive vesicles argues against a caspase-like involvement in PCD. Rather, the proteins would appear to have a function that is independent of RAB11-dependent recycling of VSG or transferrin receptor, and the degradation and secretion of anti-VSG IgG and transferrin. Loss of this function, which may involve cell-cycle control in \textit{Δmca2/3Δmca5} mutants, has severe, although temporary, effects because adaptation - potentially through changes in cell signalling, allowing survival - had already been initiated during the sequential gene deletion and prolonged selection. However, simultaneous RNA silencing of MCA2, MCA3 and MCA5 leads to a very severe phenotype with an immediate growth arrest, indicating that these three metacaspases play crucial roles for the cell.

\section*{Results}

\subsection*{Expression profile of MCA2, MCA3 and MCA5 in \textit{T. brucei}}

The MCA2 and MCA3 genes are located in a tandem repeat on chromosome 6 of \textit{T. brucei} and the encoded proteins share 89\% amino acid identity, diverging only in the N-terminal region where MCA3 has a small extension relative to MCA2 (Szallies et al., 2002; Mottram et al., 2003). MCA5 is unique among the \textit{T. brucei} metacaspases in that it possesses a C-terminal extension relative to the others (Mottram et al., 2003). An alignment of MCA2, MCA3 and MCA5 (supplementary material Fig. S1) shows the positions of the predicted active site histidine and cysteine residues, together with putative class III WW binding domain motifs. WW binding domains act as ligands for WW domains, which are modules mediating protein-protein interactions in a diverse range of cellular processes (Sudol and Hunter, 2000). Class III WW binding domains are characterised by poly-proline motifs flanked by arginine or lysine residues (Macias et al., 2002). Motifs fulfilling these requirements were identified in the N-terminal regions of MCA2 and MCA3 and the C-terminal extension of MCA5 (supplementary material Fig. S1).

A rabbit polyclonal antibody was raised against a long peptide that, in MCA2 and MCA3, spans the central domain containing both the predicted catalytic cysteine and histidine residues (Fig. S1). Western blot analysis using this antibody on whole-cell lysates of \textit{T. brucei} detected the 37 kDa MCA2 and the 39 kDa MCA3 in the BSF but not the PCF (Fig. 1A). The antibody also detected a major crossreacting protein of 48 kDa and a minor one of 37 kDa in both life-cycle stages, which act as internal controls for protein loading. A sheep polyclonal antibody raised against full-length recombinant MCA5 detected similar levels of expression of the 55 kDa MCA5 in PCF and BSF cell lysates (Fig. 1B). The finding that MCA2, MCA3 and MCA5 were each detected at a similar molecular mass to that predicted from the gene sequences indicates that in wild-type \textit{T. brucei} no major processing of the metacaspases occurs under standard in vitro growth conditions.

\subsection*{Metacaspase function analysed by RNAi}

A gene fragment, identical in MCA2 and MCA3, was cloned into the pT7<sup>11</sup> RNAi vector and used to transfect a BSF RNAi cell line. Western blot analysis confirmed downregulation of MCA2 and MCA3 to undetectable levels in two independent clones (one shown in Fig. 2A) after induction with tetracycline for 24 hours (Fig. 2A, upper left panel), whereas the crossreacting protein of 48 kDa (marked *) and MCA5 levels
Cells appeared to be unable to escape this pre-cytokinesis block before the start of furrow ingression at cytokinesis. An accumulation of 2N2K cells, suggesting a post-mitotic configuration in induced cells was performed to analyse the progression of the parasites through the cell cycle in PCF parasites. In two independent clones (one shown in Fig. 2B), a rapid growth arrest was observed following RNAi induction (Fig. 2B, upper panel). Western blot analysis confirmed almost total downregulation of MCA2, MCA3 and MCA5 after induction with tetracycline (data not shown), but no growth phenotype was observed. This suggests that expression of MCA2, MCA3 and MCA5 is not required for PCF parasites in vitro.

A further BSF cell line was generated for triple RNAi of MCA2, MCA3 and MCA5. In two independent clones (one shown in Fig. 2A, right panel), this was due to downregulation of MCA2 and MCA5 after induction with tetracycline for 24 hours (Fig. 2B, lower panels), whereas the level of the crossreacting protein of 48 kDa (marked * ) remained constant. Indeed MCA2 and MCA3 were not detectable by 4 hours (data not shown). Analysis of kinetoplast and nuclei configuration in induced cells was performed to analyse the progression of the parasites through the cell cycle in comparison to wild type (Fig. 2C). At 4 hours post-induction, a significant increase was observed in the number of cells with multiple nuclei and kinetoplasts was almost four times higher than in the non-induced control, showing that DNA replication and mitosis still occurred but cell division invariably failed. These data suggest that MCA2, MCA3 and MCA5 expression is required for multiplication of BSF T. brucei, but that the proteins can individually compensate for the loss of each other.

MCA null mutants are viable in vitro and in vivo

Since the RNAi studies indicated that T. brucei BSF are viable when MCA5 or MCA2-MCA3 are expressed, BSF MCA-deficient cell lines were generated by sequential rounds of targeted gene replacement (Fig. 3A). The simultaneous deletion of MCA2 and MCA3 was achieved by selection of unique sequences flanking the locus. BSD and PAC resistance markers were used to replace the two alleles from BSF parasites, and two independent clonal lines (Δmca2/3 ) were established. Correct integration of the drug-resistance cassettes and the deletion of MCA2 and MCA3 were confirmed by Southern blotting (Fig. 3B). Genomic DNA was digested with KpnI and the Southern blot probed with the 3’ flanking region. Loss of the 8.8 kb DNA fragment containing the MCA2-MCA3 locus in the null mutants correlated with the appearance of 1.9 and 1.7 kb DNA fragments representing the PAC and BSD cassettes (lanes 4 and 5). The loss of MCA2 and MCA3 protein was confirmed by western blotting (Fig. 3C). The two Δmca2/3 BSF clones were found to be viable in culture and showed no significant differences with regard to growth rate or gross morphology when compared with wild-type parasites. In addition, the Δmca2/3 clones were as virulent to mice as wild-type parasites and achieved a mean parasitaemia of 1×10⁹ ml⁻¹, 4 days after inoculation of 1×10³ cells.

To investigate in more detail the needs for individual MCAs and the possibility of redundancy within the MCA family, MCA5 was deleted from both PCF and BSF parasites. Sequential rounds of targeted gene disruption were carried out with the antibiotic resistance genes for hygromycin and neomycin (HYG and NEO, respectively) as selectable markers. Δmca5 mutants were generated in both life-cycle stages and confirmed by PCR and western blotting (Fig. 3D and data not shown). Both BSF and PCF Δmca5 parasites displayed no discernible difference in either morphology or growth rate compared with wild-type parasites. The same MCA5 deletion constructs were used in an attempt to generate BSF Δmca2/3Δmca5 triple null mutants by sequential rounds of transfection into Δmca2/3 clone 1.
Fig. 2. RNAi of metacaspases in BSF T. brucei. (A). Western blot of cell extracts from an MCA2-MCA3 RNAi clone (left panel) or an MCA5 RNAi clone (right panel), incubated with (+) or without (−) tetracycline (tet) and probed with anti-MCA2-MCA3 or anti-MCA5 antibodies. Asterisk indicates crossreacting 48 kDa protein. (B) Upper panel, growth curve of an MCA2-MCA3 and MCA5 triple RNAi clone with or without induction with tetracycline. Lower panels, western blot of cell extracts from an MCA2-MCA3 and MCA5 triple RNAi clone, with or without induction with tetracycline, probed with anti-MCA2-MCA3 (left) or anti-MCA5 antibodies (right). (C) Upper panel, normal cell-cycle progression in wild-type BSF T. brucei. The kinetoplast division occurs before nuclear division, ultimately followed by cytokinesis. Images illustrate various cell stages with DAPI-stained nuclei (N) and kinetoplasts (K) (white); insets show kinetoplast at higher magnification (blue). Lower panels show nucleus and kinetoplast configurations with or without triple RNAi induction with tetracycline. Others, cells with an abnormal configuration but excluding 2N1K cells. For each time point, 200 cells were analysed. (D) Upper panel, analysis of kinetoplast configuration in 1N1K cells after induction with tetracycline. Bottom panels, aberrant cell-cycle progression in MCA2-MCA3-MCA5 RNAi-induced BSF T. brucei. Images of representative triple RNAi-induced BSF T. brucei. Inset shows kinetoplast at higher magnification. Bars, 10 μm.
of the RNAi data, it was predicted that triple null mutants could not be generated. However, independent Δmca2/3Δmca5 clones were isolated. These clones initially displayed a significantly reduced growth rate compared with wild-type parasites, but after several weeks in culture the growth rate had returned to that of wild-type parasites. At early stages of selection the Δmca2/3Δmca5 parasites also showed a higher number of cells at the pre-cytokinesis stage of the cell cycle than wild-type parasites (wild type: 71% 1N1K, 18% 1N2K, 9% 2N2K; Δmca2/3Δmca5: 54% 1N1K, 24% 1N2K, 17% 2N2K) suggesting a pre-cytokinesis cell-cycle block. Western blotting confirmed the absence of MCA2, MCA3 and MCA5 in the triple mutants (Fig. 3C,D). Re-expression of each MCA gene in Δmca2/3Δmca5 parasites (e.g. Δmca2/3Δmca5:MCA5) was performed by targeting into the tubulin array and western blotting confirmed that expression of MCA2, MCA3 and MCA5 was similar to wild-type levels (Fig. 3C,D). After several weeks in culture, Δmca2/3Δmca5 parasites had the same virulence to mice as wild-type and Δmca2/3 trypanosomes. These data show that in these clones, sequential deletion of MCA2, MCA3 and MCA5 did not affect the viability of BSF trypanosomes in culture or to mice. Thus, with BSF T. brucei there was a major difference in the consequences of knocking down MCA2, MCA3 and MCA5 expression in the RNAi cell lines, which was lethal, and the sequential deletion of MCA2, MCA3 and MCA5, which was not lethal.

To test whether the metacaspases are involved in prostaglandin D 2-induced cell death in BSF T. brucei, wild-type BSF parasites and the Δmca2/3Δmca5 triple null mutants were treated with prostaglandin D 2 and their growth characteristics assessed. As previously reported, prostaglandin D 2 caused death (Figarella et al., 2005), but no difference in the kinetics of cell death was noticed between the two lines (not shown), demonstrating that MCA2, MCA3 and MCA5 are not required as effectors of prostaglandin D 2-induced cell death in BSF T. brucei. In addition, there was no processing of the three metacaspases, analysed by western blotting of BSF cell extracts, after treatment with prostaglandin D 2 for 8 and 24 hours (data not shown).

Metacaspases are located with RAB11 in recycling endosomes

The MCA5 antiserum was used for immunofluorescence studies. In BSF T. brucei, MCA5 localised to a well-defined sub-compartment of the cell located predominantly between the nucleus and kinetoplast (Fig. 4A-C). This is an area of the cell that contains the endosomes, lysosome and organelles of the secretory system (McConville et al., 2002; Overath and

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**Fig. 3.** Generation of metacaspase null mutants. (A) Schematic representation of the MCA2-MCA3 and MCA5 loci together with constructs for gene knockout. Open reading frames (ORFs) are shown as arrows, flanking DNA sequences for targeting are shown as boxes. Genomic DNA was digested with KpnI, separated on a 1% agarose gel, blotted onto Hybond-P membrane and hybridised with a 32P-labelled KpnI-digested DNA for both native and modified MCA2-MCA3 locus is shown. BSD, blasticidin-resistance gene; NEO, neomycin-resistance gene. (B) Southern blot analysis of Δmca2/3. Genomic DNA was digested with KpnI, separated on a 1% agarose gel, blotted onto Hybond-P membrane and hybridised with a 32P-labelled DNA probe comprising the 5’ flanking region used in the knockout strategy. Lane 1, wild type; lane 2, PAC heterozygote; lane 3, BSD heterozygote; lane 4, Δmca2/3 clone 1; lane 5, Δmca2/3 clone 2. (C) Total cell lysates were prepared from BSF parasites and 5×10⁶ cell equivalents subjected to SDS-PAGE and transferred to PVDF membrane prior to immunoblotting with rabbit anti-MCA2-MCA3 antibodies. Lane 1, wild type; lane 2, Δmca2/3; lane 3, Δmca2/3Δmca5; lane 4, Δmca2/3Δmca5:MCA3; lane 5, Δmca2/3Δmca5:MCA2. MCA2 and MCA3 are indicated. Asterisk indicates crossreacting 48 kDa protein. (D) Total cell lysates were prepared from BSF parasites and 5×10⁶ cell equivalents subjected to SDS-PAGE and transferred to PVDF membrane prior to immunoblotting with sheep anti-MCA5 antiserum. Lane 1, wild type; lane 2, Δmca5; lane 3, Δmca2/3Δmca5; lane 4, Δmca2/3Δmca5:MCA5. MCA5 is indicated.
Engstler, 2004). Since the antiserum raised against the peptide of MCA2 and MCA3 showed crossreactivity with other proteins, HA-epitope-tagged versions of MCA2 and MCA3 were generated and integrated at the tubulin locus in BSF parasites. Western blot analysis with an anti-HA monoclonal antibody confirmed that both MCA2HA and MCA3HA were expressed (Fig. 1C). Immunofluorescence revealed MCA2HA and MCA3HA to be localised to a compartment located between the nucleus and kinetoplast and had consistent morphology in all cells analysed (Fig. 4A). A high level of colocalisation was found between MCA5 and both MCA2HA as well as MCA3HA in BSF parasites, showing that the three metacaspases are associated with the same compartment.

To define the sub-cellular compartment in which the metacaspases are located, we carried out colocalisation studies with a variety of well-established organelle markers in BSF parasites (supplementary material Fig. S2). The early endosomes were selectively labelled at 4°C with a tomato-lectin FITC-conjugate (Jeffries et al., 2001), and cells were then co-stained with MCA5. There was clearly no colocalisation of the two signals, showing that the metacaspases are not located in early endosomes. Similarly, no colocalisation of MCA5 was found with lysotracker (Magez et al., 1997), or BODIPY-TR ceramide (Field et al., 2000). Although MCA5-positive structures were localised close to these organelles, the results show that metacaspases are not located in the lysosomal compartment or in the Golgi network.

Having ruled out metacaspase association with early endosomes, the lysosomal compartment and the Golgi network, an analysis of recycling endosomes was carried out with an antibody specific for RAB11, a small GTPase that is an established marker for this compartment in T. brucei (Jeffries et al., 2001; Grünfelder et al., 2003) and other eukaryotes (Volpicelli et al., 2002; Moore et al., 2004). Significant colocalisation of RAB11 and MCA5 was observed by using deconvolution fluorescence microscopy in BSF parasites (Fig. 4B), although separate localisation was also observed for both proteins. The colocalisation was further supported with immunogold transmission-electron-microscopy studies, which showed that RAB11 and MCA5 colocalised in distinct areas of the cell (Fig. 5). Colocalisation was found particularly between nucleus and kinetoplast and also surrounding the flagellar pocket (Fig. 5C). Although labile membrane structures were not easily visualised with our mild fixation procedures, an inferred peripheral pattern was in some cases observed for RAB11 labelling (e.g. Fig. 5A,B). Distinct localisation of both proteins, similar to that shown in the fluorescence studies, was evident from the three sites labelled in Fig. 5D. High-pressure freezing techniques and electron microscopy have shown that half of all endosomal cisternae are RAB11-positive (Grünfelder et al., 2003). The RAB11 compartment in T. brucei has been shown to contain recycling receptors and their cargo, such as transferrin receptor and/or transferrin, and VSG and/or anti-VSG antibody (Jeffries et al., 2001). In this study, we found a significant level of colocalisation of internalised anti-VSG 221 antibody and MCA5 (Fig. 4C). These data provide evidence that a proportion of metacaspases of T. brucei are in close association with RAB11-positive recycling endosomes and their cargo.

Metacaspases are not required for efficient recycling of VSG or the degradation of anti-VSG antibodies

To establish whether the metacaspases are involved in the VSG recycling process in trypanosomes, wild-type parasites were compared with Δmca2/3Δmca5 and triple RNAi parasites. Surface VSG was biotinylated at 4°C prior to 5 minutes of

![Fig. 4. Location of metacaspases. (A) BSF T. brucei parasites expressing either MCA2HA or MCA3HA proteins were fixed and co-stained with anti-HA monoclonal antibody (red) and sheep anti-MCA5 antiserum (green). (B) BSF parasites were fixed and stained with rabbit anti-RAB11 (red) and sheep anti-MCA5 antiserum (green). Single-slice images were analysed with volume deconvolution by using nearest-neighbour algorithm. (C) BSFs were incubated with rabbit anti-VSG221 antiserum at 4°C for 30 minutes and then returned to 37°C for 30 minutes. Fixed and permeabilised cells were stained with anti-MCA5 antiserum (green) and anti-rabbit IgG (red). For all images DNA was visualised with DAPI (blue). DIC images of the parasite are shown. Colocalisation is shown in yellow. Bars, 10 μm.](image-url)
endocytosis at 37°C. Non-internalised biotin was cleaved from the parasite surface and cells were resuspended in medium for 0 to 10 minutes to allow time for recycling to occur. Immunofluorescence analysis of parasites showed that in both lines internalised biotin-VSG colocalised with RAB11-positive structures (data not shown) and that, following a 5-minute or 10-minute chase period, wild-type, Δmca2/3 Δmca5 and induced triple RNAi parasites were able to recycle this internalised VSG and return it to the cell surface (Fig. 6). It therefore appears that, the loss of MCA2, MCA3 and MCA5 does not prevent endocytosis of VSG, the trafficking through RAB11-positive vesicles or its subsequent return to the cell surface.

It has been documented previously that IgG-VSG immune complexes are internalised by T. brucei and that, while the VSG is recycled to the surface, the IgG is degraded intracellularly (O’Beirne et al., 1998; Jeffries et al., 2001; Pal et al., 2003). Anti-VSG IgG has been located in RAB11-positive vesicles, but the peptidases involved in the degradation of IgG and their location within the cell have not been identified. The finding that the metacaspases are present within recycling endosomes, which traffic anti-VSG IgG, raised the possibility that they might be involved in the degradation of the internalised IgG. To investigate this hypothesis, parasites were examined by fluorescence microscopy. The ability to internalise and degrade antibody was unimpaired in Δmca2/3Δmca5 and the triple RNAi mutant eight hours after induction (Fig. 7A). Whole-cell lysates from wild type and Δmca2/3Δmca5 (Fig. 7B), and TCA precipitations of the culture medium (Fig. 7C) were then subjected to SDS-PAGE, transferred to PVDF membrane and probed with an horseradish peroxidase (HRP)-conjugated anti-rabbit IgG to determine the fate of the internalised antibodies. Examination of cell-associated anti-VSG221 IgG illustrated that there was no build-up of IgG within Δmca2/3Δmca5 parasites compared with wild-type parasites (Fig. 7B). A single protein, corresponding to rabbit heavy-chain IgG, was detected at equivalent levels in both cell lines. Analysis of the secreted products illustrated that the degradation of the anti-VSG IgG was very similar in Δmca2/3Δmca5 parasites and wild-type parasites (Fig. 7B). These data show that the Δmca2/3Δmca5 parasites are still functional with regard to anti-VSG antibody internalisation, degradation and secretion, and show that the absence of MCA2, MCA3 and MCA5 from recycling endosomes does not affect this process.

The T. brucei transferrin receptor is a low-abundance protein complex, encoded by two homologous genes ESAG6 and ESAG7 (Steverding et al., 1994; Salmon et al., 1994), limited in its distribution to the flagellar pocket (Steverding et al., 1994). Following internalisation of the receptor-transferrin complex, the reduction in pH within the endosomal system is
believed to cause dissociation of transferrin from the receptor (Dautry-Varsat et al., 1983; Maier and Steverding, 1996). The transferrin receptor is then recycled back to the flagellar pocket by Rab11-positive recycling endosomes (Jeffries et al., 2001) and degraded transferrin is secreted (Steverding et al., 1995; Pal et al., 2003). Analysis of wild-type and Δmca2/Δmca5 parasites showed that both parasites were able to internalise and degrade transferrin (data not shown), showing that the proteolysis of transferrin was unaffected by loss of MCA2, MCA3 and MCA5.

Discussion

The T. brucei genome encodes five putative metacaspases (Szallies et al., 2002; Mottram et al., 2003). MCA1-MCA4 are predicted to have molecular masses of 38 to 40 kDa, whereas MCA5 has an additional C-terminal extension of approximately 15 kDa (Mottram et al., 2003). The MCA5 gene of T. brucei and the single MCA of L. major are syntenic, and phylogenetic analysis suggests that the encoded proteins are more closely related to ScYCA1 than are MCA1-4 of T. brucei (data not shown). This suggests that MCA5 is the basal metacaspase and the possible progenitor of the other trypanosome MCA genes. This idea is also supported by the findings that in T. brucei MCA2 and MCA3 are BSF specific, whereas MCA5 is constitutive and expressed in both the mammalian BSF and in the insect PCF. The occurrence of multiple MCA proteins in T. brucei, with MCA2-MCA3 being only expressed in BSF, suggests that these enzymes play a role that relates closely to the parasite’s survival in the bloodstream of a mammalian host. This study aimed to gain insight into what this is.

The detection of the three metacaspases apparently close to their full-length predicted masses in wild-type parasites cultured in vitro suggests that the metacaspases do not undergo any caspase-like large-scale processing events, at least under the growth conditions tested. A variety of stressful conditions, such as mild heat shock and mild osmotic shock, were tested with BSF trypanosomes in attempts to induce processing of the metacaspases, but none was detected. Likewise, no processing was detected in this study after treatment with the cell-death-inducing molecule prostaglandin D2 (Figarella et al., 2005). Overexpression of the single metacaspase (YCA1) in yeast revealed apparent processing, which was absent when the putatively active site cysteine was substituted (Madeo et al., 2002). Arabidopsis thaliana has nine metacaspase genes, three of which were classified as type-I metacaspases, whereas the remaining six are type-II metacaspases (Uren et al., 2000; Vercammen et al., 2004). Expression of N-terminally tagged type-II metacaspases in Escherichia coli showed that the enzymes undergo caspase-like autocatalytic processing, have arginine/lysine substrate specificity, but do not cleave caspase substrates (Vercammen et al., 2004). Thus, the failure to detect any caspase-like processing of the metacaspases of T. brucei in this study suggests that the T. brucei metacaspases are distinct from both that of yeast and the type-II metacaspases of plants. Indeed, the T. brucei metacaspases may well be more similar to the type-I plant metacaspases, which also did not display any processing under normal growth condition (Vercammen et al., 2004).

The genetic manipulation studies revealed an interesting difference between MCA downregulation with RNAi and MCA deletion with gene knockout. The Δmca2/Δmca5 BSF clones grew very poorly during initial selection, yet recovered over several weeks to grow at the same rate as wild-type parasites. It seems that the parasites were able to adapt to the removal of first MCA2 and MCA3 and then MCA5, possibly by upregulating other peptidases or by activating alternative signalling pathways. RNAi, by contrast, led to rapid

![Fig. 6. Recycling of VSG. Wild-type, Δmca2/Δmca5 and triple RNAi lines, with or without induction with tetracycline for 8 hours. Parasites were biotinylated at 4°C prior to incubation at 37°C for 5 minutes in HMI-9 medium. Remaining surface label was then cleaved by incubation with 50 mM glutathione at 4°C. Cells were prepared for fluorescence microscopy both immediately after the glutathione incubation and following a 5- or 10-minute chase period in HMI-9 medium at 37°C. Parasites were stained with Texas-Red-conjugated streptavidin (red). DNA was visualised with DAPI (blue). Insets, DIC images of the parasite. Bars, 10 μm.](image-url)
downregulation of expression of all three genes at once. We postulate that it is the rapid removal of all three genes at once that resulted in the lethal effect, the parasite can not adapt to overcome this. The data suggest that, in BSF *T. brucei*, MCA2 and MCA3 can compensate for the lack of MCA5 and vice-versa, and this is supported by the finding that the three metacaspases are located in the same subcellular compartment.

Immunofluorescence and electron microscopy studies suggest that a significant proportion of MCA2, MCA3 and MCA5 are located in the same compartment as RAB11 (Fig. 4B and Fig. 5). RAB11 has multiple functions in mammalian cells, with a primary role in recycling endosomes (Ullrich et al., 1996) and the trans-Golgi network (Wilecke et al., 2000), although recent work also suggests an essential role for RAB11 in cytokinesis (Wilson et al., 2005). *T. brucei* RAB11 has been shown to be associated with recycling endosomes that traffic both VSG and the transferrin receptor in BSF (Jeffries et al., 2001; Grünfelder et al., 2003; Engstler et al., 2004). RAB11 is the only known stage-regulated RAB protein in *T. brucei*, displaying a much higher level of expression in the BSF compared with the PCF (Jeffries et al., 2001). Recent studies have shown that the total VSG surface coat is recycled very rapidly (within 12 minutes) (Engstler et al., 2004), thus the upregulated expression of RAB11 in the BSF correlates well with the increased requirement of recycling processes (Jeffries et al., 2001). The differential expression profile of MCA2 and MCA3 in *T. brucei*, together with the proteins’ partial location in RAB11-positive vesicles, pointed towards the enzymes also being involved in recycling processes. As metacaspases are cysteine peptidases, our hypothesis was that the function involved essential processing of proteins within a RAB11-positive compartment. Thus, the findings that triple null mutants and triple RNAi mutants have no major defect in recycling of VSG or in degradation of anti-VSG antibodies is surprising. The phenotype of RAB11 RNAi and the triple MCA RNAi differ significantly. Although in both mutants

![Fig. 7. Degradation of anti-VSG IgG. BSF wild-type, Δmca2/3Δmca5 and triple RNAi lines, with or without induction with tetracycline for 8 hours. Parasites were labelled with anti-VSG221 IgG at 4°C in HMI-9 prior to incubation at 37°C for 5 and 10 minutes in HMI-9. (A) Parasites were fixed and stained with anti-rabbit-FITC conjugate (green) and DAPI (blue). Insets, DIC images of the parasite. Bars, 10 μm. (B) Whole-cell lysates were prepared after the 30-minute incubation and 5×10^6 cell equivalents subjected to SDS-PAGE and transferred to PVDF membrane prior to immunoblotting with an HRP-conjugated anti-rabbit IgG. Lane 1, wild-type BSF; lane 2, Δmca2/3Δmca5. EF-1α served as a loading control. (C) TCA precipitations of the culture medium were resuspended, subjected to SDS-PAGE and transferred to PVDF membrane prior to immunoblotting with an HRP-conjugated anti-rabbit IgG. Lane 1, without anti-VSG221 IgG; lane 2, with anti-VSG221 IgG in the absence of parasites; lane 3, wild-type BSF; lane 4, Δmca2/3Δmca5 BSF. Anti-VSG221 IgGs are indicated together with degradation products.](image-url)
induction of RNAi leads to an immediate cessation of BSF growth, RAB11-induced RNAi cells become rounded with an enlarged flagellar pocket but show no specific cell-cycle block (Hall et al., 2005). By contrast, early after induction, triple MCA RNAi mutants display a delay in kinetoplast segregation and ultimately accumulate in a pre-cytokinesis cell-cycle state. At later time points, after MCA RNAi induction, cells accumulate with multiple nuclei and multiple kinetoplasts. This is because BSF parasites appear to lack a cell-cycle checkpoint that prevents additional rounds of S phase and mitosis prior to the completion of cytokinesis (Hammarton et al., 2003a). The precytokinesis block observed for triple MCA RNAi differs from that observed for RNAi of VSG, where a proposed VSG synthesis checkpoint prevents S phase re-initiation and cells accumulate with a 2N2K configuration (Sheader et al., 2005).

Together, these data argue against an involvement of metacaspases in RAB11-positive recycling endosomes but rather towards RAB11-positive endosomes involved in additional functions. In mammalian cells, RAB11-containing endosomes associate transiently with centrosomes to ultimately accumulate near the cleavage furrow, and are required for cytokinesis completion (Wilson et al., 2005). In PCF T. brucei, RAB11 was shown to migrate coordinately with the basal body during kinetoplast division (Jeffries et al., 2001). In this insect form of the parasite, the flagellar attachment zone area (the region that links the kinetoplast to the flagellum and is involved in kinetoplast division and/or segregation) of the new flagellum marks the position of the cleavage furrow during cytokinesis (Robinson et al., 1995; Kohl et al., 2003). Little is known about the process of cytokinesis in BSF trypanosomes, although it appears to differ significantly from mammalian cells (McKeen, 2003; Hammarton et al., 2003b). Nevertheless, it is tempting to speculate that MCA2, MCA3 and MCA5 are involved in regulation of cleavage furrow formation during BSF cytokinesis.

How the trypanosome MCAs are directed to the compartments in which they reside is unclear. MCA3 and MCA5 have predicted signal peptides [SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP)] Sprob values: 0.929 for MCA3 and 0.986 for MCA5], whereas MCA2 does not. MCA3 and MCA5 are therefore likely to be sorted through the classic secretory pathway, whereas MCA2 might either be trafficked in association with MCA5, MCA3 or another ER-directed protein, or through an unconventional secretory pathway.

This study concentrated on MCA2, MCA3 and MCA5 of T. brucei. The genome of the parasite also encodes MCA1 and MCA4, although these proteins lack the predicted active site cysteine and so, presumably, are not active as cysteine peptidases. However, it cannot yet be ruled out that MCA1 or MCA4 might compensate for the lack of MCA2, MCA3 and MCA5, and fulfill their functions in the mutant parasites. Indeed it will be interesting to determine whether MCA1 or MCA4 are upregulated in the Δmca2/3/mca5 line. MCA1 and MCA4 have extensive sequence identity with MCA2, MCA3 and MCA5, including the region within the catalytic centres (supplementary material Fig. S1), even though both proteins have substitutions at the predicted essential catalytic positions (Szallies et al., 2002; Mottram et al., 2003). Recent analysis has shown that inactive enzyme homologues are abundant in a variety of enzyme families, including peptidases (Pils and Schultz, 2004). It has been proposed that, by adapting existing protein modules, some of these ‘dead’ peptidases have evolved new regulatory functions. This could be the case for MCA1 and MCA4 of T. brucei. However, the loss of the key active-site-residues in these proteins, with the same residues being present in MCA2, MCA3 and MCA5, strongly suggest that the two sets of proteins have different functions.

Clearly, further analysis of the metacaspases in T. brucei is necessary to elucidate their roles and to provide insights into the regulatory networks to which both the active and dead MCAs contribute. Whether these might involve PCD-like phenomena is an open question. Although it has been proposed that metacaspases of yeast (Madeo et al., 2002; Herker et al., 2004; Wadskog et al., 2004) and plants (Hoebenberchts et al., 2003; Bozhkov et al., 2004) have caspase-like functions associated with PCD, we have not been able to find any evidence for similar functions in BSF T. brucei. It cannot yet be ruled out that metacaspases are involved in apoptotic-like cell death in BSF T. brucei, however, the data available suggest that metacaspases have PCD-independent functions that could be associated within RAB11-positive endosomes. Metacaspases also occur in vesicles lacking RAB11, the significance of this awaits elucidation.

Materials and Methods

Culturing and transfection of parasites

Procytic form (PCF) EATRO 795 T. brucei were cultured at 27°C in complete SDM79 medium (Brun and Schonenberger, 1979) supplemented with 10% (v/v) heat-inactivated foetal calf serum (FCS). Bloodstream form (BSF) 427 T. brucei were cultured at 37°C with 5% CO2 in RPMI-9 (Hirumi and Hirumi, 1989) supplemented with 10% (v/v) FCS and 10% (v/v) serum plus. Twenty micrograms of linearized DNA was used to transfect 5×105 mid-log-phase BSF parasites in 0.5 ml ZPFMG (132 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 0.5 mM Mg acetate, 0.09 mM Ca acetate, 55 mM glucose, pH 7) in a 0.4-cm pulse-cuvette at 1.2 kV, 25 μF. After overnight recovery, selection of clones was achieved by limiting dilution with appropriate antibiotics (5 μg ml−1 blasticidin, 2.5 μg ml−1 puromycin, 5 μg ml−1 hygromycin, 1.25 μg ml−1 neomycin, 2.5 μg ml−1 phleomycin) in 24-well plates. For the RNAi study, the BSF 427 pLEw13 pLEw90-6 cell line was used (Wirtz et al., 1999). RNAi was induced with 1 μg ml−1 tetracycline when the cells were at a density of 1×107 cells ml−1.

Plasmids

To generate the MCA2-MCA3 RNAi construct, a 668 bp fragment, sharing 100% identity with MCA2 and MCA3, was amplified by PCR from genomic DNA (strain 427) using Pfu polymerase (Stratagene) and the primer pair OL905 and OL906. The PCR product was cloned into the pGL671 with XbaI and XhoI sites of p2T7i (LaCount et al., 2000) to generate pGL671. For the MCA4 RNAi construct, a 416 bp fragment of MCA4 was amplified by PCR from genomic DNA (strain 427) using Pfu polymerase and the primer pair 0L944 and 0L945. The PCR product was cloned into the HindIII and HindIII sites of p2T7i (LaCount et al., 2000) to generate pGL671. For the MCA2-MCA3-MCA5 RNAi construct, the 668bp MCA2-MCA5 fragment was excised from pGL671 with HindIII and cloned into pGL714 pre-digested with HindIII to generate pGL905. pGL671, pGL714 and pGL905 were digested with NorI and gel-purified prior to transfection.

For deletion of the MCA2 and MCA3 locus, the 5′ flank of MCA2 and the 3′ flank of MCA3 were amplified by PCR using primer pairs OL969 and OL970, and OL971 and OL972, respectively, and cloned sequentially into the Xbal and Apal sites flanking the BSD gene of pTBT-BP K0 (Cross et al., 2002) giving pGL802. Exchange of resistance genes was achieved by excising the PAC gene from pGL698 with EcoRI and cloning into the same sites present in pGL802, replacing the BSD gene with PAC and generating pGL808. pGL802 and pGL808 were digested with NorI-Xhol and the PURA/BSD containing fragments purified for transfection. Gene deletion was by homologous recombination, replacing the locus containing MCA2 and MCA3 and utilizing tubulin intergenic regions contained within pGL802 and pGL808 to ensure expression of drug resistance genes.

For deletion of MCA5, the 5′ and 3′ flanks of the gene were amplified by PCR using primer pairs OL1135 and OL1131, and OL1132 and OL1134, respectively, and cloned sequentially into the NorI-Xhol and Apal sites flanking the BSD gene in pGL802. The BSD gene was then excised with EcoRI and replaced with both HYG and NEO resistance genes to generate pGL906 and pGL907. These were then
digested with NolI and EcoRV, and the fragments containing HYO or NEO purified for transfection.

For expression of MCA2, MCA3 and MCA5, the ORFs were excised from pGL636, pGL864 and pGL805, respectively, with NolI and XhoI, blunt-ended and cloned into pRM481 (a gift from Richard McCulloch, WCM, University of Glasgow, UK), cut with EcoRV, to generate pGL687, pGL688 and pGL927. These plasmids were then digested with HindIII and XhoI, and purified for transfection.

To generate HA-tagged MCA2 and MCA3, equal molar concentrations of OL1125 and OL1126 were heated at 95°C for 10 minutes, allowed to cool slowly to room temperature and then ligated into BglII-XhoI digested pGL683 and pGL864 to generate pGL865 and pGL866, respectively. These modified genes were then excised with NolI and XhoI, and inserted into pGL884 as described above, generating pGL787 and pGL879. Maps and sequence of all plasmids can be provided on request.

Antiserum and immunoblotting

Antiserum specific to MCA2 and MCA3 was generated by immunization of a rabbit with a 80-mer peptide [NDVKQM…(69). …MDLPFT] (supplementary material 4). The gelatin-embedded cells were cut into blocks and rinsed in PBS, then infiltrated with 0.1% Triton X-100 overnight. After washing in PBS, the blocks were digested with 1 mg ml–1 TCA solution was added to four volumes of culture medium, gently mixed and incubated on ice for 10 minutes. After centrifugation at 18,000 g for 1 min TCA solution (HMI-9, 10% (v/v) FCS, 50 mM glutathione, pH 9) and a further 30 minutes incubation on ice. Parasites were subsequently washed a further three times in ice-cold TDB before returning them to HMI-9 at 37°C. Samples were taken at 5 and 10 minutes for analysis by western blot or immune fluorescence microscopy and processed as previously described. Biotin was detected using either Texas-Red-conjugated streptavidin (Molecular Probes) at 1:5000 or HRP-conjugated streptavidin (Sigma) at 1:10,000.

Immunoelectro microscopy

Tokayasu cytosegregation was performed on BSF T. brucei that had been chemically fixed in suspension for 5 minutes at room temperature, followed by 2 hours on ice in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS (pH 7.2). After fixation, cells were rinsed in PBS, sedimented and embedded in 10% (w/v) gelatin in PBS. The gelatin-embedded cells were cut into blocks and rinsed in PBS, then infiltrated for 24 hours with 2.3 M sucrose in PBS. Specimen blocks were mounted on metal stubs in liquid nitrogen and sectioned (70-80 nm) with a Leica Ultracut UCT/FCS. Sections were collected onto formvar-coated nickel grids and stored on ice-cold 2% (w/v) gelatin in PBS (Griffith and Pothuma, 2002). Immuno-labeling of cytosegregation was performed at room temperature using standard procedures, with appropriate controls. Briefly, blocking was carried out using 0.1 M glycine for 20 minutes followed by 5% (w/v) BSA in PBS for 1 hour. Incubation with primary and secondary antibodies (gold conjugates) was for 1 hour. The incubation and washing buffer consisted of PBS (pH 7.4) with 0.2% BSA, HRP-conjugated anti-rabbit and anti-mouse IgG (Promega) and HRP-conjugated anti-sheep IgG (Santa Cruz) was used at 1:5000. Chemiluminescent detection was with the SuperSignal system (Pierce).

Immunofluorescence analysis

For immunofluorescence analyses, log-phase BSF parasites were washed in PBS and air-dried onto slides. Parasites were fixed in –20°C methanol for 15 minutes, then permeabilised with 0.1% Triton X-100 in ice-cold TDB before re-suspension in 1/8 Laemmli sample buffer. Rabbit anti-VSG IgG was detected directly using HRP-conjugated anti-rabbit IgG (Promega).

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