Abstract Metacaspases constitute a new group of cysteine proteases homologous to caspases. Heterologous expression of Trypanosoma brucei metacaspase TbMCA1 in the budding yeast Saccharomyces cerevisiae resulted in growth inhibition, mitochondrial dysfunction and clonal death. The metacaspase orthologue of yeast, ScMCA1 (YOR197w), exhibited genetic interaction with WWM1 (YFL010c), which encodes a small WW domain protein. WWM1 overexpression resulted in growth arrest and clonal death, which was suppressed by coconitament overexpression of ScMCA1. GFP-fusion reporters of WWM1, ScMCA1 and TbMCA4 localized to the nucleus. Taken together, we suggest that metacaspases may play a role in nuclear function controlling cellular proliferation coupled to mitochondrial biogenesis. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Metacaspase; Cysteine protease; WW domain; Mitochondrion; Saccharomyces cerevisiae; Trypanosoma brucei

I. Introduction

Cysteiny1 proteases of Clan CD [1] serve diverse functions in various organisms. Legumains are involved in lysosomal degradation, caspases control apoptosis, a form of programmed cell death (PCD), and modulate signal transduction. Gpi8 proteins are a core component of the endoplasmic reticulum glycosylphosphatidylinositol (GPI) transamidase complex, gingipains are virulence factors of pathogenic bacteria [1] and separins trigger anaphase progression [2]. All these proteases share a typical secondary and, where known, tertiary core structure composed of several consecutive β-strands and α-helices around a catalytic dyad of histidine and cysteine constituting the active center [1,3,4]. Metacaspases have been denoted as putative Clan CD peptidases by iterative sequence analyses and seem to be homologous to caspases and paracaspases [5]. They occur in virtually all non-metazoan eukaryotic lineages and might be functional homologues of metazoan caspases, which are primarily involved in PCD [5].

PCD is thought to be a product of the coevolution of the eukaryotic cell and its mitochondrial endosymbiont [6–10]. It is anticipated to occur not only in metazoans, but also in plants and unicellular eukaryotes [10–12]. Conclusive genetic evidence for the existence of PCD in plants and in Dictyostelium was presented [13,14]. Interestingly, plant PCD could be inhibited by expression of various metazoan antiapoptotic genes such as p35 of baculovirus [15], which acts as a subversive substrate upon diverse caspases [16], and Bcl-xL, a protector of mitochondrial function and integrity [13].

On unicellular eukaryotes, such as the yeast Saccharomyces cerevisiae, cellular death can be inflicted by expression of diverse PCD mediating metazoan gene products. Bax for instance caused clonal death of yeast by perturbation of their mitochondria [17,18].

We performed studies on the metacaspases of S. cerevisiae and Trypanosoma brucei in budding yeast to take advantage of the versatility of which gene function can be assessed in this model organism. Recent comprehensive two-hybrid screens of yeast protein interactions [19,20] revealed interactions of the yeast metacaspase (Mca1p, YOR197p) with several proteins, amongst others Wwm1p (YFL010p) [19]. WWM1 (YFL010c) encodes a protein of 211 amino acids and is characterized by a high glycine content (14%) and a high hydrophilicity. It was categorized as a potential hydrolphin, which are often involved in osmotic stress response [21]. Wwm1p contains a 40 amino acid N-terminal domain with two signature tryptophan (W) residues (WW domain), a widespread module mediating protein–protein interactions in a variety of cellular processes [22].

2. Materials and methods

2.1. Cloning of TbMCA1–5

Genes of T. brucei metacaspases TbMCA1–5 were cloned by reverse transcription based polymerase chain reaction (RT-PCR) as described previously [23] using Pwo polymerase (Roche, Mannheim, Germany) into pBluescript KS+ (Stratagene, La Jolla, CA, USA). Primer sequences were TGT GTG GAC TGA TCA CTT CCG ACT GCC C (TbMCA1 sense), GTG TCA ACA CCG ACT TAT GCA CAT (TbMCA1 antisense), TAA TTT TCC AGC CAC ATC CTT CCG ACT GCC C (TbMCA2 sense), GTG GTA GAA GCT CTG CCT GCA ACA ACA (TbMCA3 sense), AAC CCT TCT GCA GCT CCC AGG CAC (TbMCA23 antisense), GCG GAT TCC TGA AAA CAT GAG AGG C (TbMCA4 sense), CAC ATC TTC ATT CCA GCC AAA GAG (TbMCA4 antisense), AGT ATG TAA GAC AAA ATA CAG GAA GC (TbMCA5 sense), GAG GCA ATC CCT GCA GCA.

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CCA GGC TTG (TbMCA5 antisense). Sequencing was carried out by GATC GmbH (Konstanz, Germany) according to the Sanger-dideoxy-nucleotide method. Sequences have been submitted to EMBL (accession numbers AJ437301, AJ437302, AJ437303, AJ437304, AJ437305).

2.2. Metacaspase sequence retrieval and analysis

The sequence of Rhodobacter sphaeroides was obtained from the Rhodobacter genome project by the University of Texas (http://mng.uh.tmc.edu/;sphaeroides), designated putative ORF ‘OR0035’. The sequence of Geobacter sulfurreducens was obtained from the TIGR database (http://www.tigr.org), designated contig 2947. Sequences of fission and budding yeast as well as of Arabidopsis were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov); accession numbers AAG38593, Z777105, AAC24380, AAD11574 and CAB79420). Metacaspase sequences were aligned with DNAMAN software (version 4.15, Lynnon BioSoft). Each one of the primary sequences was calculated for putative secondary structures, the alignment was fit manually for conserved secondary structures.

2.3. Yeast expression constructs

For expression in yeast several vectors were used with the desired genes under control of inducible promoters. Vector pRS416 (cen, URA3, MET17 (formerly MET25) promoter) [24], Yep52 (U, LEU2, GAL10) as well as pYES2 (A, URA3, GAL1 promoter) were used. TbMCA1-5 were cloned into pRS416, Yep52 and pYES2 (only TbMCA1, Yep52 and pYES2). SpMCA1 was cloned from Schizosaccharomyces pombe by RT-PCR using primers CAT GAC AAG CTT CCA GAC MCA1 CTG AAG CTT CTA TAA AAC CAT GGC AAG ATT CAT GTC TGA GCT ACA ACT CCA ATC CTT ATA AC (sense) and GAC LEU2 TbMCA1 antisense). Sequencing was carried out by 1:100 for amplification of full length gene using the primers listed above, carrying the defined nucleotide changes (verified by sequencing).

2.4. Yeast strains, growth and media

S. cerevisiae strains employed were BY4742 (S288C background), designated contig 2947. Sequences of fission and budding yeast as well as of Arabidopsis were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov); accession numbers AAG38593, Z777105, AAC24380, AAD11574 and CAB79420). Metacaspase sequences were aligned with DNAMAN software (version 4.15, Lynnon BioSoft). Each one of the primary sequences was calculated for putative secondary structures, the alignment was fit manually for conserved secondary structures.

2.5. Yeast transformations carried out as described [25].

2.6. TCC overlay assay

Tetrazolium red (2,3,5-triphenyltetrazolium chloride, Sigma, Germany) was solved (0.5%) in 100 mM Tris-HCl pH 7.0 containing 1% agar; 20 ml were poured on culture plates. This procedure has been described previously [26].

2.7. Microscopy

Cells (200 µl of OD600 0.5–1) were incubated in water with an appropriate amount of dye (bisbenzimide, 1 µg in 1 ml stock solution, MitoTracker® W5, W5 stock solution, W5, tetramethyl rhodamine, 1 µM) in 20 µl culture plates. This procedure was run in parallel. All series of experiments were repeated at least three times.

3. Results

3.1. Sequence analysis of metacaspases

Amino acid sequences of several metacaspases were aligned with respect to putative common secondary structures (as shown as supplementary information on the net: http://www.plc.chemie.uni-tuebingen.de/midu/miduhome.html). This alignment includes the single metacaspases of Saccharomyces and Schizosaccharomyces, four Arabidopsis orthologues and orthologues of Rhodobacter sphaeroides and G. sulfurreducens. The two latter α- and δ-proteobacterial sequences are the only bacterial sequences with definite homology to the eukaryotic ones. The genes of the five T. brucei metacaspases TbMCA1–5 were cloned from bloodstream form trypanosomes by RT-PCR, using sequences provided by the TIGR database (www.tigr.org).


GGA TAT GGT GCC CAA ACT; ScMCA4W57A: TTG TTT GAC TCT GCT CAT TCG GGT ACA; TbMCA4ΔIBSA: CAT CAG TGC GCC CAT GAC ATT ATG; TbMCA4ΔIBSA: CAT TAC TCG GGT GCC AGT CCA GGT; and TbMCA4ΔIBSA: CAT GTG TTC GAC GCC TCG CAC TAC GGT.
consecutive secondary structures are predicted to occur in all metacaspases alike. In accordance with Clan CD peptidases the catalytic dyad histidine and cysteine residues are well conserved.

Apart from the high homology region, metacaspases show only a limited similarity to each other in the last third of their sequences (see web site as stated above). This region seems to be analogous to the small subunit of caspases [3]. The region that separates both main blocks of homology is variable in length and becomes particularly large in metacaspases of plants (‘latex-abundant RNA protein’ [27], ‘type II metacaspases’ [5], which are not included in our alignment). Another common feature of the metacaspases presented here is their proline-rich N-terminus (see Fig. 1); the aforementioned metacaspases lack this N-terminus.

Notably, in TbMca1p a tyrosine substitutes for the putative catalytic histidine and serine substitutes for the putative catalytic cysteine. However, a cysteine residue is located immediately adjacent, as is also the case in TbMca4p. As in other Clan CD peptidases some conserved glycine residues can be found which may constitute ‘oxanion holes’, facilitating nucleophilic attack by the catalytic dyad of histidine and cysteine. Residues which may specify the P1 scissile bond amino acid (for which Clan CD members are always highly specific) may be constituted by two conserved aspartate residues. This would imply that metacaspases are specific for a basic P1 amino acid such as arginine or lysine.

An extraordinary feature of metacaspases is the occurrence of a conserved cysteine between the first strand and helix motifs (see web site as stated above). Superposition of these predicted secondary structures upon the known crystal structures of caspase-1 and gingipain [3,4] puts this conserved cysteine in spatial proximity to the catalytic dyad.

3.2. Deletion or overexpression of ScMCA1 is not detrimental to mitochondrial function

Assuming that metacaspases were introduced into eukaryotes by their mitochondrial progenitor (see Section 4), we expected a physiological function of metacaspases in mitochondrial biogenesis. Since the yeast S. cerevisiae is unique in its ability to live with dysfunctional mitochondria, this model eukaryotic organism seems apt to study a putative mitochondrial biogenesis function of metacaspases. However, a yeast strain deleted in its only endogenous metacaspase gene, ScMCA1 (mca1Δ strain), did not show any petite phenotype that is characteristic for compromised essential mitochondrial functions. In addition, no slow growth phenotype, no alteration in clonogenicity rates or impairment of respiratory competence was noted under various regimes tested such as different temperatures, different carbon and nitrogen sources. Likewise, overexpression of ScMCA1 using different plasmids and promoters (cen MET17, 2µ GAL11/10) did not lead to any obvious phenotype (data not shown).

3.3. Expression of T. brucei metacaspase TbMCA4 induces a petite phenotype in yeast

Five metacaspase genes of T. brucei (TbMCA1–5) were heterologously expressed in yeast, as well as the only one of S. pombe (SpMCA1). Of these, only expression of TbMCA4 brought about phenotypic consequences. Strains expressing TbMCA4 were significantly retarded in growth (Fig. 1A). Furthermore, clonogenicity of TbMCA4 expressing cultures diminished gradually but irreversibly over time of expression (Fig. 1C). Clonogenicity loss was preceded by loss of respiratory competence (Fig. 1D,E). Even a short induction of TbMCA4 was sufficient to abolish respiratory competence completely (Fig. 1D).

The phenotypes induced by TbMCA4 expression in yeast were completely lost when the putative catalytic dyad residues histidine-164 and cysteine-218 were both independently mutated to alanine. Yeast expressing the respective alleles of TbMCA4 was able to grow as the control (Fig. 1A). This result clearly demonstrates that the effects of TbMca4p depend on the putative catalytic dyad residues. Therefore, the assumption that Tbc4p is a cysteine proteinase inferred by sequence homology to Clan CD proteases is confirmed.

In addition, exchange of the other conserved cysteine amongst metacaspases, cysteine-98 in TbMca4p (to alanine, the adjacent cysteine was changed to alanine simultaneously), rendered TbMCA4 inactive as well (Fig. 1A).

To monitor abundance and shape of mitochondria in TbMCA4 expressing yeast mitochondrial staining was performed. Staining with MitoTracker Green FM dye revealed that there was no significant difference in mitochondrial abundance or shape in TbMCA4 expressing yeast compared with control cells. However, staining with MitoTracker CMXRos was significantly weaker in TbMCA4 expressing yeast than in the control cells (see web site as stated above). Accumulation of MitoTracker CMXRos within mitochondria is dependent on free thiols and thus indicative of metabolically functional organelles.

3.4. Metacaspase–GFP fusion constructs localize to the nucleus

To determine intracellular localization of ScMca1p and TbMca4p in yeast, the gene for GFP was fused to the respective C′ ends of the respective metacaspase genes and expressed in yeast cells upon induction from pRS416.

The ScMca1–GFP fusion apparently localized to the nucleus, as did the TbMca4–GFP fusion (Fig. 2B,C). Apart from nuclear localization, ScMca1–GFP fluorescence could also be monitored throughout the cell (Fig. 2B), this localization was indistinguishable in wild-type and △mca1 yeast (data not shown).

3.5. Metacaspase ScMCA1 exhibits genetic interactions with WWMI

Protease function generally is expected to be controlled tightly. Thus it was not surprising that ScMCA1 overexpression did not result in a phenotype as seen by heterologous expression of TbMCA4. Considering that ScMca1p function might be controlled by endogenous activator or inhibitor proteins, we focused on a reported physical interaction of ScMca1p with Wwm1p (YFL010p) [19]. Interestingly, overexpression of WWMI in wild-type yeast (pRS416) caused severe growth retardation and loss of clonogenicity, similar to the effects exerted by TbMCA4 (Fig. 1B,C). These effects were independent of ScMCA1, since a △mca1Δ strain was also susceptible to WWMI overexpression (Fig. 1B, data not shown). A △wwm1Δ strain was likewise susceptible to WWMI pRS416-based expression but otherwise did not reveal any phenotype considering growth, clonogenicity rates and respiratory competence as compared to the respective wild-type strain (data not shown).

Simultaneous overexpression of WWMI and ScMCA1 re-
Fig. 1. Ten-fold dilution series of wild-type yeast spotted on CM/glucose agar plates. A: Vector based expression (pRS416) of TbMCA4. Left panel: control (promoter shut off). Right panel: expression of TbMCA4 and mutant alleles. First row: empty vector (pRS416), second row: TbMCA4, third row: TbMCA4C98A, fourth row: TbMCA4H164A, fifth row: TbMCA4C218A. B: Vector based expression (pRS416) of WWM1. Left panel: control (promoter shut off). Right panel: expression of WWM1. First row: empty vector in wild-type strain, second row: WWM1 in wild-type strain, third row: empty vector in mca1Δ strain, fourth row: WWM1 in mca1Δ strain. C: Upper panel: growth of wild-type yeast in liquid CM/glucose medium expressing TbMCA4 (○), WWM1 (▲) and control (●). Lower panel: clonogenicity (colonies counted) of wild-type yeast cultures expressing TbMCA4 (○), WWM1 (▲) and control (●), corresponding to growth shown in upper panel. D: Outgrown colonies of wild-type yeast culture previously having expressed TbMCA4 (same experiment as depicted in Fig. 2C) divided into respiration competent (white parts of bars) and respiration incompetent (black parts of bars) ones as judged by TCC-overlay assay. E: Example of TCC overlay assay with plate of outgrown colonies of wild-type yeast culture previously expressing TbMCA4 for 2 h (independent experiment from that shown in Fig. 2C,D). Bigger red staining colonies are respiration competent, smaller white colonies are respiration incompetent.
resulted in suppression of the phenotypes caused by the former alone. Alleles of \textit{Sc\textsubscript{MCA1}} mutated in the conserved cysteine (cysteine-176 and cysteine-297) or histidine (histidine-241) residues were capable to suppress \textit{WWW1} overexpression as effectively as wild-type \textit{Sc\textsubscript{MCA1}}. A truncated form of \textit{Sc\textsubscript{MCA1}}, however, lacking the 5\textsuperscript{P} region encoding the proline-rich N-terminus was not able to suppress overexpressed \textit{WWW1} (Fig. 3A, data on suppression of loss of clonogenicity not shown). Since WW domains like the one found in Wwm1p are known to interact with proline-rich protein domains [22], suppression of overexpressed \textit{WWW1} by overexpressed \textit{Sc\textsubscript{MCA1}} is most readily explained by titration of Wwm1p by the N-terminal domain of Sc\textsubscript{Mca1p}.

Growth inhibition, loss of clonogenicity and suppression by \textit{Sc\textsubscript{MCA1}} were also achieved with a pRS416-based \textit{WWW1}-GFP fusion construct (data not shown). The resulting Wwm1–GFP fluorescence localized to the nucleus (Fig. 2D), but upon suppression by \textit{Sc\textsubscript{MCA1}} was observed in large aggregates distributed throughout the cell, no longer co-localizing with the nucleus (Fig. 3B).

Fig. 2. Images of wild-type yeast cells expressing GFP fusion constructs (pRS416). Left panels: GFP fluorescence. Middle panels: Nuclear fluorescence (stained with Hoechst). Right panels: Nomarski optics. A: Control cells (solely GFP). B: Sc\textsubscript{Mca1–GFP}. C: Tb\textsubscript{Mca4–GFP}. D: Wwm1–GFP.
4. Discussion

Heterologous expression of \( T b M C A 4 \) was found to induce respiration deficiency in yeast. This is obviously due to the peculiarity of \( S. c e r v i s i a e \) of living without fully functional mitochondria, although the organelle itself remains essential nevertheless [28]. Loss of clonogenicity (clonal lethality) occurred as a much later, downstream event of \( T b M C A 4 \) expression, so that yeast colonies with irreversibly damaged respiratory competence (‘petites’) were obtained at high efficiency. Thus it is apparent that the action of \( T b M C a 4 p \) primarily affects yeast by compromising mitochondrial function. However, no concomitant alteration in mitochondrial abundance, shape or distribution could be observed by MitoTracker Green FM or electron microscopy (data not shown). Only MitoTracker CMXRos, a fluorescent dye specific for free mitochondrial thiols and thus indicative for mitochondrial functions, was not efficiently sequestered by mitochondria of \( T b M C A 4 \) expressing yeast, underscoring that abnormal mitochondrial biogenesis takes place besides the loss of respiratory competence.

The site of \( T b M C a 4p \) action on yeast seems to be the nucleus as suggested by a \( T b M C a 4p-G F P \) fusion reporter. Although this latter construct was non-functional, the nuclear localization of a \( S c M C a 1p-G F P \) reporter supports this idea. Furthermore, yeast growth inhibition by Wwm1p-GFP also coincided with a nuclear localization of this fusion construct, which was suppressed by \( S c M C A 1 \) overexpression. Finally, the effect of \( T b M C a 4p \) upon yeast mitochondrial respiration competence and its presumptive nuclear localization suggests that metacaspases are involved in an aspect of transcriptional control affecting mitochondrial biogenesis.

Development of respiration incompetence is very common for mutations in genes necessary or essential for mitochondrial biogenesis (\( P E T \) genes) [29]. For instance, expression of a truncated cytochrome \( b c 1 \) complex assembly protein of which the gene was disrupted resulted in a temperature-dependent petite phenotype [30]. The most striking parallels to the effects of \( T b M C A 4 \), however, were reported from heterologous expression of mammalian Bax in yeast. Bax is an important proapoptotic effector in mammalian PCD which induces growth retardation, loss of clonogenicity and a permanent loss of respiratory competence in yeast [17,18,31–34]. These effects of Bax could be suppressed by coexpression of another related Bcl family member, Bcl-x\(_L\) [17,32]. A general protection of yeast mitochondria was achieved by expression of another Bcl family member, Bcl-2 [35]. Furthermore, even plant PCD was suppressed by expression of Bcl-x\(_L\) [13]. Therefore it appears notable that we were unable to relieve \( T b M C A 4 \) expressing yeast by coexpression of mouse Bcl-x\(_L\) (data not shown).

Coexpression of antiapoptotic baculovirus p35 also did not affect \( T b M C A 4 \) expressing yeast (data not shown), while this caspase inhibitor was able to suppress PCD in plants [15]. If metacaspases are mediators of PCD in plants and unicellular eukaryotes, which appears to be an attractive speculation [5,10], we expected Bcl-x\(_L\) and p35 to relieve \( T b M C A 4 \) expressing yeast. Our aforementioned experimental results do not support this idea, however.

The physiological relevance of \( W W M 1 \) still remains unsolved. It is intriguing to notice that moderate overexpression of this gene was inhibiting cellular proliferation in such a potent manner, considering the high transcript levels that were reported from growing yeast for this gene [36]. In a recent overexpression screen \( W W M 1 \) was found to be one of the strongest growth inhibitors, which caused cells to arrest in G1 [37]. Suppression of \( W W M 1 \) by the yeast metacaspase \( S c M C A 1 \) appears an interesting fact, although the physiological relevance of this interaction remains elusive. Based on our results, we would like to suggest that Wwm1p plays a specific role in metacaspase function. Since Wwm1p-GFP localization was altered by overexpression of \( S c M C A 1 \), we assume that exclusive nuclear localization of Wwm1p-GFP is due to an increased dosage of Wwm1p.

Sequence analysis shows that metacaspases constitute a new group of cysteinyl proteases. Accordingly, exchange of the
putative catalytic dyad residues of TbMca4p by site-directed mutagenesis resulted in loss of all phenotypes caused by expression of the respective wild-type gene TbMCA4 in yeast. In addition, exchange of the other conserved cysteine residue within metacaspases also rendered TbMca4p inactive. This indicates that metacaspases possess a second catalytic nucleophile, which might be important either for maturation or for the catalytic activity itself. Notably, a conserved cysteine is also found in Gpi8p transamidases at a comparable site (our unpublished observations). Exchange of this residue in the essential yeast ScGpi8p was possible, however [38]. So far, we cannot exclude that metacaspases are not typical proteases, but rather transamidases, which mediate protein lipidation similar to the related Gpi8 proteins.

In our experiments, tagging of TbMca4p (N- or C-terminally with c-myc or FLAG epitopes or with GFP) always resulted in a loss of function (data not shown). These tagged variants might not fold properly and consequently are hindered in their activity or in their (self-) maturation. It appears fortuitous that TbMca4p becomes active in yeast at all, since expression of the four other T. brucei metacaspases, the single metacaspase of S. pombe, or overexpression of ScMCA1 did not lead to any visible phenotype.

In order to demonstrate protease activity of TbMca4p, raw extracts of yeast expressing TbMCA4 or purified recombinant TbMca4p from Escherichia coli were incubated with a set of synthetic quarterpeptides with either N, D, R or K preceding a fluorogenic group (7-amino,4-methylcoumarin). Protease activity could not be monitored, however (data not shown).

Metacaspase-like sequences with limited homology to metacaspases occur in many bacteria. Since the putative secondary structures of these different sequences appear to be very similar to metacaspases, they appear to be distant homologues of metacaspases [5]. However, only the α-proteobacterial RmMca1p of R. sphaeroides (and the α-proteobacterial GoMca1p) shows a high degree of sequence homology with the eukaryotic metacaspases. Thus, in view of the endosymbiont hypothesis [39], it appears likely that metacaspases were acquired by the eukaryotic cell from its protomitochondrial endosymbiont. Interestingly, analysis of the yeast mitochondrial proteome revealed that only a minority of proteins (50 out of 400) are clearly derived from the α-proteobacterial ancestor, reflecting an unexpectedly high degree of coevolutionary development of symbiont and host [40]. If the metacaspases are indeed α-proteobacterial origin, they would be quite unique for their non-mitochondrial localization. The only other, also hypothetical, example known so far are aspartic proteases involved in cell cycle control [41].

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