Identification and characterization of Schizophyllum commune type I metacaspases

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ABSTRACT

The role of programmed cell death in filamentous fungi is not well-understood, but is important due to the role of fungi in opportunistic infections. Plants, fungi and protozoa do not have caspase genes, but instead express the homologous proteins denoted metacaspases. To better understand the role of metacaspases in fungi we present an analysis of the sequences and activities of all five Type I metacaspases from Schizophyllum commune (ScMC), a mushroom-forming basidiomycete that undergoes sexual reproduction. The five Type I metacaspases of S. commune can be divided into two groups based on sequence similarity. Enzymes both with and without the N-terminal prodomain are active, but here we report on the constructs without the prodomains (Δpro). All five ScMCΔpro proteins show the highest enzymatic activity between pH 7 and 8 and require calcium for optimal activity. Optimal Ca2+ concentrations for ScMC1Δpro and ScMC2Δpro are 50 mM, while ScMC3, ScMC4Δpro and ScMC5Δpro activity is optimal around 5 mM calcium. All five S. commune metacaspases have similar substrate specificity. They are most active with Arg in the P1 position and inactive with Asp in the P1 position.

1. Introduction

Processes of programmed cell death (PCD) are of interest throughout all kingdoms of life. While long-studied in metazoans [1], the role of PCD in filamentous fungi has only been investigated more recently and is not well understood (reviewed in Ref. [2]). Caspase proteins are key components of PCD pathways in metazoans, and their complex role has been clearly elucidated in many studies [3]. Plants, fungi and protozoa do not have caspase genes, but do express the homologous proteins denoted metacaspases [4]. Unlike caspases which cleave most efficiently after Asp, metacaspases cleave most efficiently after Arg and Lys [5]. In filamentous fungi PCD plays a role in development, reproduction, responses to stress, and plant invasion [2,6]. Recent studies indicate that the proteolytic activity of metacaspases is involved in PCD, but the details of this role are not yet clear [7].

Metacaspases are members of the MEROPS C14 family of proteases based on the geometry of the active site residues [8], and have been divided into types I-III (reviewed in Ref. [9]). All three types contain a strongly conserved p20 catalytic region with a Cys-His catalytic dyad similar to that found in caspases. The three types of metacaspases also have a smaller p10 region with high sequence similarity but unclear function. The p20 and p10 regions are connected by a region of low sequence similarity that varies in length. Only the Type I metacaspases contain a long N-terminal region in the same position as the prodomain in caspases. The genomes of multicellular organisms typically encode multiple Type I and Type II metacaspases, while Type III metacaspases have only been identified in a few organisms [10].

The structures of two type I metacaspases have been determined [11,12], but no structures of Type II or Type III metacaspases are known. Type I metacaspase structures are comprised of a core caspase-hemoglobinase fold with a central 8-stranded β-sheet. In both structures the catalytic Cys is located in the loop between β-strands 4 and 5, analogous to the location of the active site loop in caspase-7 [4]. Unlike in caspases, β-strand 5 is not adjacent to β-strand 4, and the linker loop between these strands is much shorter in metacaspases. These observations are consistent with the fact that Type I metacaspases are active as intact monomers [5,11] rather than requiring the proteolytic processing typical of caspases [13].

Metacaspases from plants, protozoa and fungi preferentially cleave after Arg in most in vitro studies on fluorogenic peptide substrates [5] and have...
significantly lower activity when presented with Lys-containing peptides. In contrast, when examining self-cleavage sites, both Arg and Lys are found at sites internal to metacaspases [5,14]. Some metacaspases require calcium for activity [12,15], although whether calcium directly increases autocatalysis or causes a general increase in activity is unclear. Recent work has suggested a role for the p10 region in calcium binding in the metacaspase from the alga *Guillardia theta* [10]. A physiological role for calcium in non-self recognition, a part of the mating process in filamentous fungi, has been identified [2]. Calcium also broadens substrate sequence recognition in a *T. brucei* metacaspase [14].

To better understand the role of metacaspases in fungi we present an analysis of the sequences and activities of all five Type I metacaspases from *Schizophyllum commune* (abbreviated ScMC), a mushroom-forming basidiomycete that undergoes sexual reproduction.

2. Materials and methods

2.1. Identification and alignment of metacaspases

Metacaspases were initially identified by searching the genome of *S. commune* at the JGI [16] with the sequence of the *Saccharomyces cerevisiae* metacaspase MCA1 (Q08601, also known as Yca1) as a query using a similarity cutoff of E > 10⁻⁵. Sequences were aligned using ClustalOmega [17].

2.2. Cloning

The original cDNA template for cloning of *S. commune* metacaspase (ScMC) genes was generated from *S. commune* strain T26 (Fungal Genetics Stock Center #9300). Compatible PCR products of the small ubiquitin-related modifier (SUMO)-containing vector [modified by removing the LIC region from pET His6 Sumo TEV LIC cloning vector (2S-T), a gift from Scott Gradia (Addgene plasmid # 29711)] and the metacaspase encoding fragments were amplified (SI Tables 1–3). Metacaspase fragments both with and without (Δpro) the N-terminal prodomain were generated. Assembly reactions were prepared following the manufacturer’s instructions (New England Biolabs) including a 2:1 ratio of metacaspase-encoding fragment to SUMO vector backbone fragment. After a 15 min incubation at 50 °C, NEB Salpa chemically competent E. coli were transformed with the assembly product. Sequences of purified plasmid DNA were confirmed by Sanger sequencing.

Site-directed mutagenesis was performed on sequence-verified plasmids. The plasmid was PCR amplified using primers (SI Tables 1–3) generated by NEBase changer software (New England Biolabs). After amplification of the mutant plasmids, KLD treatment and transformation were performed according to the manufacturer’s instructions. All mutant plasmid sequences were confirmed by Sanger sequencing.

2.3. Expression and purification

Chemically competent BL21(DE3) cells were transformed using heat shock with the SUMO-ScMC plasmids containing a His-Tag N-terminal to SUMO. Transformants were incubated at 37 °C and 250 rpm until OD₆₀₀ = 0.5–0.7 (4–6 h). The starter culture was added to 100–500 mL LB medium and carbenicillin (0.05 mg/mL) and incubated for a minimum of 10 h at 27 °C and 250 rpm. (No protein is expressed if cultures are grown at 37 °C.) Cell pellets were stored at -20 °C or -80 °C.

Cell pellets were resuspended in 1.5 mL 1X CelLytic B (10X from Sigma-Aldrich, diluted with 40 mM Tris, pH 8.0) per gram of cell pellet. Following resuspension, 1.5 μL of 5 mg/mL DNasel was added per gram of cell pellet. The cell suspension was vortexed for 2 min and incubated at 37 °C for 20 min. The supernatant was stored on ice, and the extraction was repeated using 1 mL of 1X CelLytic B and 20 μL of 5 μg/mL DNase I per gram of cell pellet. The supernatants were combined and loaded onto 200 μL of HIS-Select Nickel Affinity Gel beads (Sigma-Aldrich), previously equilibrated with Wash Buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 8.0), for every 1 mL of supernatant. The beads were incubated with rocking at 4 °C for a minimum of 10 h and then washed twice with Wash Buffer. Protein was eluted by incubation at 4 °C for 10 min in Elution Buffer (250 mM imidazole, 50 mM sodium phosphate, 0.3 M NaCl, pH = 8.0). Recovered protein was aliquoted and used immediately or stored in Elution Buffer plus 30% v/v glycerol at -20 °C. Protein does not maintain activity if frozen and thawed.

Purification was confirmed via protein gel electrophoresis on 12% Tris-glycine polyacrylamide gels and western blot with a His-Tag mouse antibody (1:3,000 dilution of Cell Signaling Technology 27E8) as the primary antibody, and goat anti-mouse (1:10,000 dilution of Invitrogen 626520) as the secondary antibody.

2.4. Activity assays

Assays were performed at room temperature in microplate wells with a total volume of 200 μL containing MC Assay Buffer (50 mM Tris @ pH 7.5, 100 mM NaCl, 5–75 mM CaCl₂), 5 mM DTT, 10 μM substrate (Z-GGR-AMC or other fluorogenic peptide (Bachem)), and metacaspase protein at a concentration that gives a rate between 0.1-1.5 RFU/s. Fluorescence at an excitation of 355 nm and an emission of 460 nm was monitored in a Spectramax M3 microplate reader ( Molecular Devices) for 10 min at 25 °C. When optimizing pH from 4-10, the MC Assay buffer contained a mixture of 25 mM glycine, 25 mM acetic acid, 25 mM MES, and 75 mM Tris buffers that was titrated to the appropriate pH, 100 mM NaCl and 5 mM CaCl₂ (ScMC3, ScMC4, ScMC5) or 75 mM CaCl₂ (ScMC1 and ScMC2).

3. Results and discussion

3.1. ScMC sequences

The five metacaspase sequences including the prodomain are between 350-473 amino acids in length, a considerable difference (Fig. 1). The size variation narrows to 254–296 amino acids without the prodomains. Although some metacaspases contain zinc finger motifs in the

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Fig. 1. Visual representation of full-length ScMC1-5 protein domains to scale. The constructs without the prodomain are enclosed by the gray box, and the catalytic Cys and His (red) and the calcium-binding region (purple) are indicated.
promodon, sequence analysis of the S. commune metacaspases did not result in the identification of any commonly observed motifs other than the metacaspase C14 protease fold [18].

The five Type I metacaspases of S. commune can be divided into two groups based on sequence similarity. ScMC1-3 are 50–62% identical to each other and ScMC4 and 5 are 55% identical, but sequences in one group are only 30–45% identical to members of the other group. Four out of the five S. commune metacaspases contain a typical N-terminal prodomain region, with ScMC3 being the only exception. ScMC3 lacks both the prodomain typical of Type I metacaspases and the long linker region typical of Type II metacaspases. As such, it is best described as a Type I metacaspase with a very short prodomain-like region. In addition to the prodomain, each metacaspase contains both the p20 and p10 domains characteristic of caspases and metacaspases. The locations of the ScMC prodomain and p20 and p10 region boundaries were identified by sequence alignment with the AtMC1.

Overall, the p20 sequence is highly conserved (43–70% identical) among the ScMC sequences, and the p10 is less well conserved, but still significantly conserved (32–63% identical) (Fig. 2). The sequence linking the p20 and p10 regions is not conserved and varies in length (Fig. 2). The N-terminal region is poorly conserved among ScMC sequences. In all five ScMC sequences and the metacaspases from S. cerevisiae (Yca1), T. brucei (TbMCA2), and A. thaliana (AtMC1), conserved residues are shaded in gray. The p20-like and p10-like regions are outlined with green and blue, respectively. Important residues are highlighted: active site (red), the putative calcium binding site (purple), the putative S1 binding site (yellow), and the alternative Cys (light red). Image generated with Jalview.

Fig. 2. Sequence alignment of the five Type I metacaspases from S. commune and the metacaspases from S. cerevisiae (Yca1), T. brucei (TbMCA2), and A. thaliana (AtMC1). Conserved residues are shaded in gray. The p20-like and p10-like regions are outlined with green and blue, respectively. Important residues are highlighted: active site (red), the putative calcium binding site (purple), the putative S1 binding site (yellow), and the alternative Cys (light red). Image generated with Jalview.

3.2. Cloning and expression

The cloning template cDNA was S. commune strain T26 leading to some differences when compared with the sequences from S. commune strain H48 in the NCBI database. In ScMC2 the Ser53 codon is AGC instead of TCC, and in ScMC5 there is a 6 nucleotide in-frame insertion after position 501 leading to an additional 2 aa (His, Gly). A larger difference is present in ScMC3 due to a splice prediction error in the database at nucleotide 642 that leads to a 74 base insertion, a frame shift, and the use of a different stop codon.
ScMC3 protein is produced in these systems, it is found in insoluble tag fusion vectors such as pQE (Qiagen) and pET (Novagen). While a codon that extends the protein sequence at the C-terminus of each protein. When linked to the SUMO sequence, the quitin-related modifier (SUMO) sequence is fused via a linker to the N-terminus of strand β1, so the Δpro constructs begin with the amino acid that aligns with the second residue of this β strand [11]. This residue aligns with the cleavage site between the prodomain and the p20-like region in plant metacaspases [19]. Since the prodomain for ScMC3 is less than 10 residues in length, it was only cloned and expressed as the full length protein. For ScMC1, 2, 4, and 5 both forms of the protein had similar enzymatic activity against peptide substrates, but the presence of the prodomain produced more complex banding patterns on SDS gels suggesting that more than one form of the metacaspase may be involved in catalysis. Therefore, it was not possible to know what fragments of the constructs with the prodomains were responsible for cleavage during activity assays, or what regulatory regions these fragments contained. The data presented here is focused on the forms without the prodomain (Δpro) to allow for a more accurate characterization of the active region of the S. commune metacaspase proteins.

3.3. Metacaspase activity

All five ScMCΔpro proteins have the highest enzymatic activity between pH 7–8 (Fig. 3), consistent with other metacaspases [7,12,19,22,23]. The active site His will be at least partially deprotonated in this pH range, allowing it to act as a base to increase the nucleophilicity of the active site Cys in the reaction mechanism. Mutation of both active site residues (His 264 to Gln and Cys 320 to Ala) in ScMC1 produced an enzyme with ~1% of the wild-type activity demonstrating the importance of these residues for catalysis (SI Table 4). All five S. commune metacaspases have similar substrate specificity (Fig. 4). The Z-GGR-AMC substrate is cleaved most rapidly, indicating preference for Arg at the cleavage site (P1) and smaller amino acids in the P2 and P3 sites. For ScMC1-5Δpro, the Z-R-AMC substrate had less than 60% of the activity compared to Z-GGR-AMC, indicating that the presence of residues in the P2 and P3 sites increases activity. A peptide preference for Arg at the cleavage site (P1) and smaller amino acids in the P2 and P3 sites. For ScMC3, the P2 and P3 sites. For ScMC1-5Δpro, the Z-R-AMC substrate had less than 60% of the activity compared to Z-GGR-AMC, indicating that the presence of residues in the P2 and P3 sites increases activity. A peptide preference for Arg at the cleavage site (P1) and smaller amino acids in the P2 and P3 sites. For ScMC1-5Δpro, the Z-R-AMC substrate had less than 60% of the activity compared to Z-GGR-AMC, indicating that the presence of residues in the P2 and P3 sites increases activity. A peptide preference for Arg at the cleavage site (P1) and smaller amino acids in the P2 and P3 sites.
ScMC4Δpro and ScMC5Δpro is optimal around 5 mM calcium and noticeably decreases above 30 mM calcium. Only ScMC1Δpro is completely inactive in the absence of calcium, while ScMC2Δpro, ScMC3, ScMC4Δpro and ScMC5Δpro retain partial activity without calcium (Fig. 5). The optimal calcium concentrations obtained for all five ScMC proteins are similar to results of other characterized metacaspases [10,19,25].

During purification ScMC1Δpro remains intact and produces a single band on an SDS gel (SI Fig. 1) and Western Blot (data not shown). This is consistent with the fact that ScMC1Δpro absolutely requires calcium for activity. The concentration of calcium present in the purification buffers is very low and this prevents ScMC1 from undergoing any autoprocessing. The other four *S. commune* metacaspases (ScMC2-5Δpro) produce more complex banding patterns on SDS gels (SI Fig. 1) and Western blots (data not shown) due to their autoproteolytic activity even in the absence of calcium.

Studies on the enzymes with the prodomains (+pro) were complicated by moderate expression levels and proteins with very low specific activities. For example, ScMC1+pro had only ~5% the specific activity of ScMC1Δpro (SI Table 4). Metacaspases typically have relatively low catalytic efficiencies and undergo autoproteolysis during concentration and storage. As a result, reliable studies of wild-type full-length protein are difficult due to the presence of multiple proteolysis products. Enough ScMC1+pro was purified to perform activity assays. The pH optimum and substrate specificity were the same for ScMC1Δpro and ScMC1+pro (Fig. 6). However, the activity of the enzyme including the

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**Fig. 5.** Activity of all five metacaspases at varying calcium concentrations. The activity of each protein was determined at pH 7.5 with Z-GGR-AMC substrate (ScMC1 (yellow), ScMC2(red), ScMC3(purple), ScMC4(green), ScMC5(blue)). Data are plotted as a percentage of the highest value. Three trials were performed for each protein with at each calcium concentration. Error bars represent standard deviations. (*Denotes missing data at 9mM, 30 mM, and 60 mM calcium for ScMC1-4 and at 25, 50 mM, and 75 mM calcium for ScMC5.)

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**Fig. 6.** Comparison of the activity of ScMC1 at varying pH and [calcium] with the putative prodomain (+pro, brown) and without (Δpro, yellow) the putative prodomain. Assays were done at pH 7.5 and 5 mM calcium with Z-GGR-AMC substrate. Data are plotted as a percentage of the highest value. Three trials were performed for ScMC1Δpro and a single trial for ScMC1+pro. Error bars represent standard deviations.
prodomain is much lower at all calcium concentrations, and adding calcium does not result in a significant increase in activity (Fig. 7). The enzyme without the prodomain is strongly activated by increasing calcium concentrations.

While the active sites seem to function similarly in different forms of the enzyme, enzyme activity is regulated by calcium levels and autophotolysis. This is consistent with recent work in plants showing that metacaspases can be activated in vivo both by autotaxic cleavage of the metacaspase and by increases in calcium levels [26].

4. Conclusions

Five Type I metacaspases in the fungus *Schizophyllum commune* have been identified and characterized. The multicellular basidiohyte *S. commune* is a member of the group of mushroom-forming fungi; has roles in human health, agriculture, and biotechnology; and is the only member of this group of organisms that can be cultured on defined media. This makes it an attractive model for metacaspase function in vivo in basidiohytes. Analysis of the sequences of the ScMC proteins reveals significant similarities between the yeast, plant and *T. brucei* metacaspases at residues involved in reactivity and substrate specificity that are the most well-characterized in the literature. Despite the sequence similarity among the five *S. commune* metacaspases, the difference in response to calcium by ScMC1 when compared to the other ScMC proteins suggests that each enzyme may play a specific physio-
ological role. ScMC1 could play a critical role in cellular pathways where calcium dependence helps regulate enzymatic activity. In yeast, metacaspases have been shown to cleave the DNA-damage inducible protein 1 (Ddi1), and it is known that Ddi1 cleavage is regulated by changes in calcium concentrations [27]. Ca²⁺ signaling is a frequently observed response to cell death inducing compounds in fungi [2]. Intracellularly, free calcium concentration is typically in the nanomolar range and total calcium (free + bound) is in the low millimolar range [28], so only in the presence of a dramatic increase in calcium concentration would a response from ScMC1 be physiologically relevant.

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