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A type-1 metacaspase from *Acanthamoeba castellanii*

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Summary

The complete sequence of a type-1 metacaspase from *Acanthamoeba castellanii* is reported comprising 478 amino acids. The metacaspase was recovered from an expression library using sera specific for membrane components implicated in stimulating encystation. A central domain of 155 amino acid residues contains the Cys/His catalytic dyad and is the most conserved region containing at least 30 amino acid identities in all metacaspases. The *Acanthamoeba castellanii* metacaspase has the most proline-rich N-terminus so far reported in type-1 metacaspases with over 40 prolines in the first 150 residues. Ala–Pro–Pro is present 11 times. Phylogenies constructed using only the conserved proteolytic domains or the complete sequences show identical branching patterns, differing only in the rates of change.
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Introduction

Acanthamoeba castellanii is a small, free-living amoeba although some isolates are opportunistic human pathogens (Marciano-Cabral et al., 2000). It is closely related to the cellular slime mold *Dictyostelium discoideum* as shown by a number of studies e.g. proteasome subunit sequence alignments (Bouzat et al., 2000). *Acanthamoeba castellanii* feeds phagocytically on bacteria and has the ability to encyst, manufacturing a cellulose-containing cyst wall in order to survive periods of "adverse" conditions. When conditions improve, the amoeba emerges from the cyst and recommences growth. The environmental circumstances that trigger encystation and the molecular details of the pathways leading from the stimulus to the mature cyst are incompletely understood. However, various specific conditions have been shown to trigger encystation experimentally in rich media, among which are increasing osmolarity (Cordingley et al., 1996) and various monoclonal antibodies (mAbs) that bind to the surface of the amoebae (Villemez et al., 1985).

The mAbs that bound to the surface of the amoeba and under specific circumstances triggered

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encystation potentially provided ways to identify components of membrane complexes, possibly proteins, involved in triggering encystation. However, upon further analysis many of the mAbs binding to the surface recognized epitopes that were either completely or partly composed of carbohydrates. Since recombinant bacterial proteins are not glycosylated, the mAbs could not be used to directly screen an expression library and therefore an indirect approach was used to identify protein components in these membrane complexes. In this procedure we isolated membrane complexes, solubilized with non-ionic detergent in order to preserve non-covalent associations, using one of the mAbs that recognized a carbohydrate determinant on the surface of the amoebae. Proteins from these immune complexes were used to raise polyclonal sera and using these sera we isolated expression clones from a cDNA library. One of the clones identified in this way encoded the type-1 metacaspase reported here.

Metacaspases are a group of genes encoding cysteine proteases found in higher plants, fungi and protists. They have been classified into two distinct subgroups, designated types 1 and 2 (Uren et al., 2000). Type-1 metacaspases have diverse N-terminal extensions, often proline-rich, which are absent in type-2 molecules and a central proteolytic domain. Type-2 metacaspases have an N-terminal proteolytic domain and C-terminal regions varying in both length and sequence. There is a single type-1 metacaspase gene in the *Saccharomyces cerevisiae* genome but, in contrast, there are multiple metacaspase genes, both types 1 and 2, in multicellular plants such as *Arabidopsis thaliana* in which the multiple gene copies are probably paralogues arising by gene duplication and subsequent divergence of the copies during the evolution of the increasing cellular diversity in higher plants (Vercammen et al., 2004).

D. discoideum has no metacaspase gene, however the *D. discoideum* genome has a paracaspase gene that was used as the starting sequence for the PSI-BLAST searches that led to the identification of the metacaspase gene family (Uren et al., 2000). The human paracaspase identified in the same study by Uren et al. (2000) is related to the caspases, the well-characterized group of cysteine proteases that form an integral part of the apoptotic machinery in metazoa (Cohen, 1997). The distant but significant homologies between caspases, paracaspases and metacaspases have prompted many efforts to establish functional parallels between these groups of proteins. Metacaspases have since been shown to be involved in "apoptotic-like cell death" in *Saccharomyces cerevisiae* (Silva et al., 2005). However, they are also implicated in other processes such as sporulation in *Aspergillus nidulans* (Thrane et al., 2004) and plant embryogenesis (Suarez et al., 2004; Bozhkov et al., 2005) in which specific cells in the plant embryo undergo a form of programmed cell death. These studies suggest that the diverse intracellular roles of metacaspases are still incompletely understood.

We report here the identification of a type-1 metacaspase from *Acanthamoeba castellanii* and report a comparative study with other type I metacaspases and a discussion of possible functional roles.

Materials and methods

Acanthamoeba castellanii cultures

Acanthamoeba castellanii (Neff) cultures were grown axenically as described previously except glucose was included at a final concentration of 1.5% or 3% w/v (Cordingley et al., 1996).

Immunoprecipitation with F1 Mab

Monoclonal antibody F1 (Villemez et al., 1985) was linked to affigel and used for immunoprecipitations from an *Acanthamoeba castellanii* lysate. 1×10^8 *Acanthamoeba castellanii* cells were harvested and lysed in 50 mls 2% Np40/1 × PBS/protease inhibitor cocktail (4 mM pefabloc, 0.7 μg/ml pepstatin A, 5 μg/ml Leupeptin, 50 μg/ml TLCK). F1-affigel or affigel alone was added to lysate cleared by centrifugation at 10,000 rpm for10 min and agitated gently at 4 °C for 2 h. The F1-affigel was spun down, washed 3 × with (0.2% Np40/1 × PBS/protease inhibitor cocktail) followed by 3 × with PBS. The F1-affigel with bound material was boiled in SDS–PAGE sample buffer, beads were removed by centrifugation, and samples electrophoresed on 8–20% gradient gels as described below. Comassiae blue-stained protein bands were excised from gels and used to immunize mice as described below.

SDS–PAGE gels and western blots

The gels used in these experiments were 8–20% gradient gels in the buffer conditions of Leammli (Laemmli, 1970). Protein samples for gels were boiled in sample buffer (10% SDS, 5% 2-mercaptoethanol, 125 mM Tris–Cl pH 6.8, 10% glycerol). For western blots, samples were electrophoresed on

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