The role of proteases in the differentiation of *Acanthamoeba castellanii*

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**Abstract**

Proteases are significant determinants of protozoan pathogenicity and cytolysis of host cells. However, there is now growing evidence of their involvement in cellular differentiation. *Acanthamoeba castellanii* of the T4 genotype elaborates a number of proteases, which are inhibited by the serine protease inhibitor phenylmethylsulphonyl fluoride. Using this and other selective protease inhibitors, in tandem with siRNA primers, specific to the catalytic site of *Acanthamoeba* serine proteases, we demonstrate that serine protease activity is crucial for the differentiation of *A. castellanii*. Furthermore, both encystment and excystment of *A. castellanii* was found to be dependent on serine protease function.

**Introduction**

The protozoan pathogen *Acanthamoeba castellanii* of the T4 genotype can cause the fatal brain disease, granulomatous amoebic encephalitis (GAE), and also a sight-threatening keratitis. The pathogenic genotypes are often seen to display characteristics such as osmotic tolerance, which may be part of the pathogenic process. Under adverse conditions, notably extremes in temperature and pH, desiccation, crowding and nutrient depletion, the infective *Acanthamoeba* trophozoite differentiates into a double-walled cyst. Cysts are highly resistant to harsh conditions, disinfestants such as chlorine, and many chemotherapeutic agents (reviewed in Khan, 2006), presenting a problem in the treatment of *Acanthamoeba* infections. Further, cysts can remain viable for decades without loss of pathogenicity (Mazur et al., 1995), and their capacity to be airborne may indicate a participatory role in disease transmission. However, the molecular mechanisms associated with *Acanthamoeba* encystment are poorly understood. Previous studies have shown that *Acanthamoeba* cysts are partially composed of cellulose (Neff & Neff, 1969; Potter et al., 1972; Weisman, 1976), and that targeting cellulose synthesis in combination with an antiamoebic drug may have the potential to improve treatment of *Acanthamoeba* infections (Dudley et al., 2007). One of the key factors attributed to *Acanthamoeba* is the copious secretion of extracellular proteases (reviewed in Khan, 2006). Predominantly, *Acanthamoeba* secrete serine proteases (Mitro et al., 1994; Leher et al., 1998; Khan et al., 2000; Na et al., 2001), of varying molecular weight: 12, 40, 42, 55, 70, 85, 97, 107, 130, 133 and 230 kDa (reviewed in Khan, 2006), all sensitive to the serine protease inhibitor, phenylmethylsulphonyl fluoride (PMSF). However, both cysteine (Hadas & Mazur, 1993) and metalloproteases (Alsam et al., 2005; Sissons et al., 2006) are known to be secreted by other *Acanthamoeba* genotypes. The genus *Acanthamoeba* elaborates a number of serine proteases. Although several studies have implicated proteases with *Acanthamoeba* pathogenicity (Mitro et al., 1994; Leher et al., 1998; Khan et al., 2000), they may also be salient to *Acanthamoeba* survival, multiplication and cellular transformation. The aim of the present study was to investigate whether protease expression is vital to *Acanthamoeba* differentiation.

**Materials and methods**

All chemicals were purchased from Sigma Laboratories (Dorset, UK), unless otherwise stated. The siRNA duplex oligoribonucleotides [Genbank Accession no. AF221523](https://academic.oup.com/femsle/article-abstract/286/1/9/593034).
(Hong et al., 2000; Lorenzo-Morales et al., 2005) were purchased from Invitrogen Life Technologies with the following 5' sense and antisense nucleotide sequences: CACCGCAGCAGU and GUGCCGUGAGUCC, respectively. The 20 nM pellet was resuspended in 1 mL diethyl pyrocarbonate (DEPC) sterile H2O to yield a 20 μM working stock. For controls, a scrambled siRNA with the following 5' sense and antisense nucleotide sequences CAACUGACCUAGAGUC and GUUCGACUGGGA-CUCAAG was used, as described previously (Lorenzo-Morales et al., 2005).

Culture of Acanthamoeba

Acanthamoeba castellanii belonging to the T4 genotype was obtained from the American Type Culture Collection (ATCC 50492), sourced from a keratitis patient. Amoebae were routinely grown in PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v), and glucose 1.5% (w/v)] and maintained at 30°C in T-75 tissue culture flasks, without shaking, as described previously (Sissons et al., 2004), and the media refreshed 17–20 h before experiments. This resulted in more than 99% of the amoebae remaining in the trophozoite form.

Encystment assays

Encystment assays were performed as described previously (Cordingley et al., 1996; Dudley et al., 2005). Briefly, Acanthamoeba trophozoites (5 x 10⁵ amoebae per well) were incubated with 8% glucose prepared in RPMI 1640 (final volume 500 μL) to induce encystment. The initial number of amoebae was quantified microscopically using a haemocytometer, before the addition of glucose. Plates were left at 30°C, or until 100% encystment was observed. To collect cysts, plates were washed with phosphate-buffered saline containing 0.1 mM CaCl₂ (PBS-Ca) and left to shake for 30 min. Following shaking, cysts were collected by gentle scraping and pelleted by centrifugation at 1000 g for 10 min. The cysts were subsequently stored at 4°C until required.

To quantify encystment, the percentage of Acanthamoeba transforming into cysts was determined as follows: no. of amoebae post-SDS treatment/total number of amoebae pre-SDS × 100 = % encystment. Data are presented as the mean ± SE.

To determine if protease activity is important to Acanthamoeba encystment, amoebae were treated with varying concentrations of the protease inhibitors PMSF, aprotinin, 1,trans-epoxysuccinyl-L-lysylamido(4-guanidine)butane (E64), ethylene glycol-bis(2-aminoethyl ether)-N,N',N'-tetra-acetic acid (EGTA) and EDTA, for 1 h, before the addition of 8% glucose. The inhibitors were also included in the encystment assays. For controls, amoebae were treated with solvents only, or incubated with PYG alone.

siRNA silencing assays

To study the effects of siRNA on Acanthamoeba encystment, encystment assays were performed using short, interfering RNA primers, previously determined to target mRNA of A. castellanii serine proteases (Lorenzo-Morales et al., 2005). Briefly, Acanthamoeba (5 x 10⁵ amoebae per well) were treated with siRNA primers in concentrations ranging from 50 to 200 nM, and left to incubate for 1 h in fresh PYG. Scrambled siRNA was used as a control. Subsequent to incubation, the medium was removed and 8% glucose in RPMI, in the presence, or absence, of primers was added as above. The plates were then maintained at 30°C for 48 h, followed by microscopic counts both before and postaddition of SDS. It is important to indicate that these primers were used previously and they are known to specifically target Acanthamoeba serine proteases (Lorenzo-Morales et al., 2005).

Excystment assays

To investigate whether inhibition of protease activity was equally detrimental to Acanthamoeba excystment, assays were performed using cysts prepared under axenic conditions. Briefly, Acanthamoeba were inoculated onto non-nutrient agar plates and left to incubate for several days at 30°C, or until 100% encystment was observed. To collect cysts, plates were washed with phosphate-buffered saline containing 0.1 mM CaCl₂ (PBS-Ca) and left to shake for 30 min. Following shaking, cysts were collected by gentle scraping and pelleted by centrifugation at 1000 g for 10 min. The cysts were subsequently stored at 4°C until required. For excystment assays, Acanthamoeba cysts (10⁵ cyst per well) were suspended in PYG to encourage transposition, in the presence or absence of various concentrations of the protease inhibitors and left for 48 h at 30°C. After this, excystment, relative to the percentage of amoebae re-emerging from 100% encysted cells, was determined by haemocytometer counting, both before, and subsequent to SDS treatment.

To evaluate whether posttranscriptional RNA repression of serine protease activity would also prove inhibitory to cyst conversion, excystment assays were performed in the presence of siRNA. To quantify excystment, the percentage of Acanthamoeba cysts converting into trophozoites was determined as follows: no. of amoebae pre-SDS treatment/total number of amoebae pre-SDS × 100 = % excystment (e.g. % amoebae multiplication). Data are presented as the mean ± SE.

Zymography

The zymographic assays were performed as described previously (Khan et al., 2000). Briefly, encystment assays were performed as described previously (Lorenzo-Morales et al., 2005). Briefly, excystment assays were performed as described previously (Lorenzo-Morales et al., 2005).
Serine proteases and *Acanthamoeba* differentiation

Serine protease inhibitor PMSF blocks encystment in *A. castellanii*

*Acanthamoeba* of the T4 genotype only elaborate serine proteases, previously shown to be instrumental in host cell cytolysis (Mitro *et al.*, 1994; Leher *et al.*, 1998; Khan *et al.*, 2000) and membrane permeability (Alsam *et al.*, 2005). To determine whether serine proteases are equally crucial to *Acanthamoeba* phenotypic transformation, encystment assays were performed using PMSF, an inhibitor of serine proteases. The results revealed that PMSF exhibited a dose-dependent inhibition of *Acanthamoeba* encystment (Fig. 1) \((P < 0.05)\) using paired \(t\)-test, one-tailed distribution), suggesting that serine proteases play a role in trophozoite differentiation into cysts. However, as PMSF can also inhibit chymotrypsin and papain proteases, we additionally studied the effectiveness of the chymotrypsin, kallikrein inhibitor, aprotinin, and the cysteine inhibitor, E64, on *Acanthamoeba* encystment. Although a 12-kDa chymotrypsin-like serine protease has been characterised in *A. castellanii* isolates (Na *et al.*, 2001), aprotinin did not demonstrably attenuate *Acanthamoeba* encystment (Fig. 1) \((P > 0.05)\) using paired \(t\)-test, one-tailed distribution). In contrast, E64 did appear to initially decelerate encystment, as borne out by the presence of numerous immature cysts in the medium before the addition of SDS (data not shown), though, this inhibitory effect was somewhat negated when the inoculum was increased.

The requirement of calcium and other divalent ions, notably Mg\(^{2+}\) and Zn\(^{2+}\), in diverse cellular functions is well documented. To determine whether metal ion concentration is relevant during encystment, amoebae were treated with the chelating agents EDTA and EGTA (data not shown). In the presence of these chelators the ability of *Acanthamoeba* to transform into cysts was markedly reduced at both tested concentrations (Fig. 1) \((P < 0.05)\).

Genetic interference by siRNAs targeting serine protease genes attenuated encystment in *Acanthamoeba*

siRNA are chemically synthesized, sequence specific, short interfering duplex primers that bind to recognized transcriptional sequences, and hence impede protein translation and initiate silencing of the gene product. A recent study by Lorenzo-Morales *et al.* (2005) has established that siRNA specific to the catalytic domain sequences of serine proteases in *Acanthamoeba* block their ability to produce host cell death. Here, we evaluated the potential of the same duplex primers to suppress encystment in the T4 genotype, and act as a confirmatory tool with respect to our earlier results with PMSF. Using an adapted soaking technique (Bracha *et al.*, 2003; Tabara & Grishok, 2003), we studied the effects of RNA interference during encystment assays. The findings revealed that siRNA interference arrested differentiation in a dose-dependent fashion, reducing overall encystment from an average of 73.1–9.6% (Fig. 2) \((P < 0.05)\). However, siRNA controls were not inhibitory and exhibited the same level of encystment as 8% glucose (Figs 2 and 3). While in growth media (PYG) it was found that proteolytic gene silencing failed to prohibit trophozoite multiplication, as determined by haemocytometer counting before addition of SDS (data not shown), implying that encystment inhibition was not consequential to toxicity (data not shown). To
further confirm the observations made by Lorenzo–Morales et al. (2005), that these primers targeted *Acanthamoeba* serine proteases, and to establish the deleterious effects of the various inhibitors on protease expression, *Acanthamoeba*-conditioned medium was collected in the presence of siRNA and the various inhibitors, and used for zymographic assays. Zymography profiles confirmed that siRNA decreased proteolytic activity in tandem with increased siRNA concentration, but also revealed that amoebae treated with siRNA controls, or incubated in encystment media only, continue to elaborate proteases, and that at the genetic level translation is not suppressed (Fig. 3b). Although, the siRNA duplex did not completely abolish protease expression (lane 3), elevated concentrations of the serine protease inhibitor, PMSF (5 mM), proved deleterious to proteolytic activity (Fig. 3a), as did the chymotrypsin inhibitor, aprotinin; whereas, there was no loss of protease elaboration when cultures were exposed to the cysteine inhibitor (E64). Because protease expression/activation is probably Ca²⁺ dependent, increased inoculums of EGTA, and EDTA, equally suppressed protease expression. Taken together, the foregoing findings indicate that it is specifically serine proteases that are required for *Acanthamoeba* trophozoite transformation into cysts.

**Acanthamoeba** encystment requires serine protease mediation

Previous studies have established that cysteine proteases mediate encystment in *Giardia* and *Entamoeba* (Ward et al., 1997; Makioka et al., 2005). Therefore, to determine whether proteases were similarly imperative to *Acanthamoeba* encystment, we performed encystment assays in the presence of the various inhibitors, as described in Materials and methods. Commensurate with the results for encystment experiments, we observed that PMSF and EDTA significantly arrested *Acanthamoeba* encystment in the T4 genotype (Fig. 4), while aprotinin and E64 (data not shown) had no inhibitory effect on the encystment of *A. castellanii*. In corroboration, when subjected to incubation with siRNA, *Acanthamoeba* encystment was similarly attenuated, notably at concentrations of more than 50 nM (*P* < 0.05), further suggesting serine protease mediation during *Acanthamoeba* differentiation (Fig. 5).

Cellular differentiation in *Acanthamoeba* is a highly controlled and complex process, involving a diverse array of external stimuli together with myriad intracellular processes. An understanding of molecular events during *Acanthamoeba* differentiation is crucial in order to identify potential targets for therapeutic intervention, and/or to intervene at the point of amoebae transmission. A key event following *Acanthamoeba* encystment is the substantial decline in intracellular levels of RNA, protein, triacylglycerides and glycogen, allied with a decrease in cytoplasmic mass (Bowers & Korn, 1969; Neff & Neff, 1969; Weisman et al., 1970; Weisman, 1976), suggesting the involvement of several degradative processes.

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**Fig. 2.** siRNA suppresses *Acanthamoeba* encystment. Duplex or single primers were added to amoebae cultures and encystment was induced using 8% glucose. The siRNA retarded amoebic transformation in a dose-dependent fashion, while scrambled siRNA and single primers had no inhibitory effect on encystment and were comparative with cultures treated with glucose alone. Experiments were performed in duplicate and repeated three times. Error bars represent the mean ± SE.

**Fig. 3.** PMSF and siRNA inhibit extracellular protease activities of *Acanthamoeba*. *Acanthamoeba* were treated with various protease inhibitors (a), or siRNA (b) for 1 h. Next, glucose was added (final concentration 8%) to induce encystment. The inhibitors or siRNA were also included in the encystment assays. Plates were incubated at 30 °C for 48 h. Following this incubation, the cell-free conditioned media were collected and supernatants containing equal amounts of proteins were assayed for zymography on a SDS-PAGE gel using 40 mg mL⁻¹ of gelatin. Note that in the presence of siRNA, protease activity is reduced in a concentration-dependent manner. In contrast, scrambled siRNA (control) showed no inhibitory effect. Results are representative of three independent experiments.
Serine proteases and *Acanthamoeba* differentiation

Using specific inhibitors, in tandem with genetic silencing, we have demonstrated here that serine proteases are material to *A. castellanii* differentiation. We have chemically synthesized siRNA duplex primers that have recently emerged as a novel method for re-evaluating *Acanthamoeba* pathogenicity (Lorenzo-Morales, 2005). These primers have already been shown to be complimentary to the mRNA of *Acanthamoeba* serine proteases (Hong et al., 2000; Lorenzo-Morales et al., 2005), it seemed feasible that posttranscriptional interference would be comparatively deleterious to *Acanthamoeba* differentiation. Indeed, this study demonstrated that proteolytic gene silencing is inhibitory to encystment and excystment in the T4 genotype. The RNA interference significantly inhibited *Acanthamoeba* excystment when administered at concentrations above 50 nM suggesting that trophozoite emergence relies on cell-wall permeability and degradation. Although Persengiev et al. (2004) have reported that short-interfering RNAs can give rise to a number of secondary effects, in this study the employment of siRNA has served as a useful corroborative tool.

*Acanthamoeba* elaborates a number of serine proteases of varying molecular weight (reviewed in Khan, 2006). Their mass diversity is currently unexplained, but may be analogous to the *Entamoeba histolytica* cysteine proteases, in that some are simply precursors to mature enzymes (Que et al., 2002). Alternatively, it is possible that *Acanthamoeba* proteases associatively bind to a multisubunit proteosome-like complex, from which they become disassociated, depending on the cell cycle and/or intensification of the extracellular signal; hence they are subsequently observed as monomeric. As proteasomes are classic cell-sorting and regulatory systems in eukaryotic cells, the latter hypothesis is particularly attractive, not least because protease aggregation is clearly salient to the life cycle of other protozoan organisms (Gonzales et al., 1996, 1999). In corroboration, Gonzales et al. (1996, 1999) have demonstrated that proteasome inhibition has a consequential effect on *Trypanosoma cruzi* amastigote/trypomastigote interchange and *Entamoeba invadens* encystment. Further studies are in progress to address these issues.

Although a chymotrypsin-like serine protease has been identified in *A. castellanii* (Na et al., 2001), the chymotrypsin/kallikrein inhibitor aprotinin had only a moderately reductive effect on encystment (50 μM). Zymography profiling did reveal a loss of proteolytic activity for cultures treated with aprotinin; nevertheless, the inhibitor also failed to downregulate excystment, adding weight to the hypothesis that different proteases may be called into play during alternative morphological events, and are subject to localized trafficking. For instance, the protozoan parasite *Giardia* localizes a cysteine protease to a peripheral cell-wall vesicle before trophozoite expulsion (Ward et al., 1997), where it may well be instrumental in degrading the cyst wall.

Of interest, was the inhibitory potency of the chelating agents EDTA and EGTA. Although *A. castellanii* T1 genotype is known to express a 150 kDa metalloprotease (Alsam et al., 2005; Sissons et al., 2006), to date, none have been purified in the T4 isolate. Certainly, electrochemical gradients and numerous signalling cascades depend on, or are sensitive to, divalent-ion mediation and concentration, but there is conflicting evidence as to the role they play during excystment. The latter stage of encystment involves the

**Fig. 4.** *Acanthamoeba* excystment requires serine proteases. Cysts of *Acanthamoeba* were suspended in PYG (10^5 cysts per well) in the presence of specific protease inhibitors, and left for 48 h. For controls, cysts were inoculated into PYG and RPMI alone in the absence of inhibitors. Excystment was evaluated microscopically as well as by counting, as previously outlined, and data expressed as percent of the original inoculum. Assays were performed in duplicate and repeated five times. Error bars represent the mean ± SE.

**Fig. 5.** siRNA block *Acanthamoeba* excystment. Nonnutrient agar-cultured cysts were suspended in PYG medium (10^5 cysts per well) in the presence of increasing concentrations of the duplex siRNA primers, then maintained at 30 °C for 48 h. At concentrations of > 50 nM, *Acanthamoeba* excystment was completely prohibited (P < 0.05), while scrambled siRNA had no significant effect on cyst transformation and amoebae multiplication in growth media. Data are expressed as percent of the original inoculum. Assays were performed in duplicate and repeated several times. Error bars represent the mean ± SE.
synthesis of the inner cyst wall or endocyst, which is composed of a cross-linked matrix of cellulose (Neff & Neff, 1969; Potter et al., 1972; Weissem, 1976). Synthesis of cellulose is both Mg²⁺-dependent and Ca²⁺-sensitive (Ross et al., 1991; Tal et al., 1998), whereas host–parasite adhesion and postinteraction incites elevated [Ca²⁺] in host cells (Mattana et al., 1997; Meza, 2000).

In summary, our research shows that Acanthamoeba serine proteases are not simply involved in the process of host invasion and cellular cytology, but are equally important for trophozoite differentiation and re-emergence during excystment. One reason that Acanthamoeba infections are notoriously difficult to treat is the rapid propensity of the trophozoite to transform into a highly resistant cyst. Consequently, the inhibition of protease expression, in tandem with genetic silencing, may prove a novel and useful tool to prevent differentiation into the resistant cyst, and so facilitate resolution of the infection.

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References


