Cysteine Protease Inhibitor (AcStefin) Is Required for Complete Cyst Formation of Acanthamoeba

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The encystation of Acanthamoeba leads to the formation of resilient cysts from vegetative trophozoites. This process is essential for parasite survival under unfavorable conditions, such as those associated with starvation, low temperatures, and biocides. Furthermore, cysteine proteases have been implicated in the massive turnover of intracellular components required for encystation. Thus, strict modulation of the activities of cysteine proteases is required to protect AcStefin from intracellular damage. However, mechanisms underlying the control of protease activity during encystation have not been established in Acanthamoeba. In the present study, we identified and characterized Acanthamoeba cysteine protease inhibitor (AcStefin), which was found to be highly expressed during encystation and to be associated with lysosomes by fluorescence microscopy. Recombinant AcStefin inhibited various cysteine proteases, including human cathepsin B, human cathepsin L, and papain. Transfection with small interfering RNA against AcStefin increased cysteine protease activity during encystation and resulted in incomplete cyst formation, reduced encystation efficiency, and a significant reduction in cytoplasmic area. Taken together, these results indicate that AcStefin is involved in the modulation of cysteine proteases and that it plays an essential role during the encystation of Acanthamoeba.

Acanthamoeba is the causative agent of granulomatous amebic encephalitis (GAE) and amebic keratitis. The life cycle of Acanthamoeba is divided into two stages, that is, the vegetative trophozoite stage and the dormant cystic stage. Under challenging conditions, such as those associated with starvation, low temperatures, and biocide treatment, the trophozoite converts to the resilient cyst form (1–5). This differentiation, which is termed encystation, protects Acanthamoeba against host immune responses and allows it to evade the effect of chemical treatments, and thus, encystation decreases treatment efficacies (3, 6). Therefore, the inhibition of encystation during medical treatment could lead to outcomes that are more favorable in cases of amebic infection. However, this goal is hindered by a lack of information about the encystation mechanism involved.

The cysteine proteases are regarded as major drug targets, and the papain family of cysteine proteases, such as cathepsin B and cathepsin L, are localized in lysosomes for intracellular protein degradation (7, 8). Previous studies on Giardia and Entamoeba have shown that cysteine proteases are implicated in parasite survival under nutrient-limited conditions and during developmental differentiation (9–14). In Acanthamoeba, the cysteine protease inhibitor E64d was found to slow encystment (15), and in a previous study, we detected the upregulations of various proteases, including cysteine proteases, by cDNA microarray analysis and comparative expression analysis of expressed sequence tags (ESTs) during encystation (16, 17). Cysteine proteases in lysosomes cause massive proteolytic degradation of unnecessary cytosolic compartments or organelles during encystation and thus must be strictly controlled to protect essential intracellular components from damage for subsequent excystation of cysts to viable trophozoites.

The cystatin I25 superfamily is a well-established family of cysteine protease inhibitors, which is composed of three distinct subfamilies according to the MEROPS classification (18, 19): I25A (stefins, family 1, type 1, and cystatins A and B), I25B (cystatins, family 2, type 2, and cystatins C, E, and S), and I25C (kininogens, family 3, and type 3). The stefins (subfamily I25A) are cysteine protease inhibitors identified in multicellular organisms such as nematodes, trematodes, or ticks based on evaluations of their abilities to protect parasites from extracellular proteolytic damage or to regulate intracellular proteases or based on their immunomodulatory effects (1, 20–30). In protozoan parasites, chagasin, a specific parasite-derived inhibitor of clan CA (family C1 cysteine peptidases), was identified in Trypanosoma cruzi (31). Chagasin and chagasin-like inhibitors (ICP [inhibitor of cysteine peptidases]) lack significant identity with proteins of the cystatin I25 family and are classified as chagasin (clan XI, family I42), which have been suggested to regulate endogenous and/or host cell proteases (31–42). However, little is known regarding the presence of cystatins of the I25 family in protozoan parasites or of their involvement in differentiation.

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In the present study, we identified AcStefin, a cysteine protease inhibitor of the 125 family in Acanthamoeba, which was highly expressed during encystation. In addition, we examined the biochemical properties and the intracellular localization of AcStefin and investigated its roles during the encystation of Acanthamoeba.

**MATERIALS AND METHODS**

**Cultivation of amoeba and induction of encystation and excystation.** *Acanthamoeba castellanii*, which was originally isolated as a eukaryotic cell culture contaminant, was obtained from the American Type Culture Collection, Manassas, VA (ATCC 30011). Amoebae were cultured axenically in peptone-yeast-glucose (PYG) medium at 25°C. Encystment was induced as previously described, with slight modifications (43). Briefly, cells from post-logarithmic-growth-phase cultures were collected aseptically. Approximately 5 × 10^6 cells were then washed with phosphate-buffered saline (PBS) and incubated in 10 ml of encystment medium (95 mM NaCl, 5 mM KCl, 8 mM MgSO₄, 0.4 mM CaCl₂, 1 mM NaHCO₃, 20 mM Tris-HCl [pH 9.0]) for 72 h. The morphological change of cells to cysts was observed under a light microscope. Encystation ratios were calculated by counting cysts under a light microscope after treating them with 0.05% Sarkosyl and 0.22% trypsin blue, which selectively stains nonviable cells (44, 45). The same numbers of cysts from cells transfected with scrambled small interfering RNA (siRNA) or AcStefin siRNA were cultivated in nonnutrient agar covered with an emulsion of Escherichia coli at 27°C. Excystation ratios were calculated by dividing the observed number of cysts by the sum of the numbers of cells 7 days after encystation induction.

**mRNA sequence of Acanthamoeba AcStefin and real-time PCR.** The full-length cDNA sequences of *A. castellanii* AcStefin (accession cluster ID ACL00003305) was first isolated from the taxonomically broad EST database (TBaseDB) (http://tbestdb.bcm.umontreal.ca) and verified by reverse transcription-PCR (RT-PCR). Total RNA was purified by using TRIzol reagent (Gibco BRL, Rockville, MD), and cDNA synthesis was conducted by using a RevertAid first-strand cDNA synthesis kit (Fermentas, Hanover, IN). Real-time PCR was performed by using a GenAmp 5700 SDS unit (Biosystems, Barcelona, Spain), using the default thermocycler program for all genes, that is, 10 min of preincubation at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. The relative expression levels of AcStefin were calculated with respect to the internal standard, *Acanthamoeba* actin. Individual reactions were carried out in 96-well plates containing the following designed primers: AcStefin sense primer 5′-CTTGGTGATGAGTCAAGCTG-3′ and antisense primer 5′-AGTCATCAATGGCTCAGG-3′ and *Acanthamoeba* actin gene (the reference gene) (GenBank accession no. CAA23399) (46) sense primer 5′-AGTTCTCACCACATGGTAACG-3′ and antisense primer 5′-TCGC ACTTCATGATGTTG-3′. All reactions were performed by using SYBR Premix Ex Tag (Takara, Shiga, Japan).

**Transient transfection.** To determine the intracellular localization of AcStefin, the AcStefin gene was cloned into the pUB vector including the *Acanthamoeba* ubiquitin promoter for constitutive expression and which included the gene for reporter enhanced green fluorescent protein (EGFP) (47). The AcStefin gene was PCR amplified with primers that included sites for SpeI at the 5′ end and XbaI at the 3′ end, and AcStefin was inserted into the pUB vector downstream of the EGFP gene. The amplified DNA fragment was completely sequenced (Macrogen, Seoul, South Korea). *A. castellanii* was then transfected with the cloned plasmid as previously described (47). Briefly, approximately 4 × 10⁶ cells were grown to mid-log phase, washed with PBS, and resuspended in 3 ml of PYG culture medium. Aliquots were seeded into a 6-well culture plate and incubated at 25°C. Plasmid DNA (4 μg) was mixed with 20 μl of Superfect transfection reagent (Qiagen, Valencia, CA) and transferred into the cells. After incubation for 24 h, the transfected cells were placed into encystation medium and incubated for 24, 48, or 72 h. EGFP-AcStefin expression was examined by fluorescence microscopy.

**Production of recombinant AcStefin and antiseraum against AcStefin.** To produce recombinant AcStefin protein, its amplified PCR product (sense primer 5′-CTTGGTGATGAGTCAAGCTG-3′ and antisense primer 5′-CTGAGTGCGTACAGACG-3′) was cloned into the pBAD-TOPO vector (Invitrogen, Carlsbad, CA). The *Escherichia coli* TOP10F strain was then transformed with the constructed plasmid. Recombinant AcStefin protein was purified by using Ni-nitriolotriacetic acid (NTA) agarose according to the manufacturer’s instructions (Qiagen), and rat antiseraum against AcStefin was generated by using purified recombinant AcStefin. For immunoblot assays, cell lysates of *A. castellanii* trophozoites and cysts (24 h, 48 h, or 72 h after encystation induction) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and proteins were transferred from gels onto nitrocellulose membranes. Blots were blocked and incubated with antisera collected from AcStefin-immunized rats (1/1,000 dilution) for 1 h. After washing, membranes were incubated with horseradish peroxidase-conjugated anti-rat IgG antibody (1/1,000 dilution) (Amersham Biosciences, Buckinghamshire, United Kingdom), stripped, and reprobed with mouse monoclonal anti-actin antibody (Santa Cruz Biotechnology, CA) to control for equal loading. The proteins were detected by chemiluminescence using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

**Inhibitory activity assay of AcStefin protein.** Inhibitory effects against the cysteine proteases human liver cathepsin L (Calbiochem, San Diego, CA), human liver cathepsin B (Calbiochem), and papain (Sigma-Aldrich, St. Louis, MO) were determined by using the specific proteolytic activity after incubating each enzyme with recombinant AcStefin. Briefly, each cysteine protease (20 nM) was preincubated with 20 nM recombinant AcStefin protein or bovine serum albumin (BSA) (as a control) in reaction buffer (for papain, 100 mM sodium acetate [pH 6.0], 1 mM EDTA, and 2 mM dithiothreitol; for cathepsins B and L, 100 mM sodium acetate [pH 5.0] and 1 mM EDTA) for 30 min at 37°C (for papain and cathepsin B) or 25°C (for cathepsin L). Colorimetric or fluorogenic peptide substrates were then added, and the mixture was incubated for 1 h. The reaction was terminated by adding monooiodoacetate buffer to the mixture, and the absorbance was then measured by using a SpectraMax Gemini XS spectrophotometer ( Molecular Devices, Sunnyvale, CA). Substrates for the cysteine proteases were benzoyloxycarbonyl-l-arginine–l-arginyl–4-nitroanilide (Z-Arg-Arg-pNA) (Calbiochem) for human cathepsin B, N-benzoyloxycarbonyl–Phe–Ala–7-aminol–4-trifluoromethyl coumarin (Z-Phe-Arg-AFC) (TFA) (Enzo Life Science, Farmingdale, NY) for human cathepsin L, and N-acetylphenylalanine–glycine–4-nitroanilide (Ac-Phe-Gly-pNA) (Calbiochem) for papain. p-trans-3-Cardboxoxirian–2-carbonyl–l-leucylgmatine (E64) was used as a control. To calculate approximate K, values of AcStefin against individual enzymes, different concentrations of AcStefin (0 to 100 nM) were incubated with each enzyme (20 nM), and substrate hydrolysis was monitored, as described above. Cysteine protease activities in cell extracts of scrambled-siRNA- or AcStefin-siRNA-transfected cells were determined by using the specific cathepsin L substrate Z-Phe-Arg-AFC (Enzo Life Science, Farmingdale, NY). Cell extracts (10 μg) containing reaction buffer (100 mM sodium acetate [pH 5.0], 1 mM EDTA) were preincubated for 30 min at 25°C before addition of 10 μM substrate. For controls, cell extracts of scrambled-siRNA or AcStefin-siRNA-transfected cells were incubated with 10 nM recombinant AcStefin protein or 10 nM E64. After incubation for 1 h at 25°C, reactions were monitored as described above.

**Knockdown of AcStefin.** siRNA targeting the AcStefin gene of *A. castellanii* was synthesized by Sigma-Proligo (Boulder, CO), based on the cDNA sequence of the gene. The siRNA oligonucleotide was designed with a fluorescein isothiocyanate (FITC) modification to allow transfection efficiency to be determined by flow cytometry. The sequence of the upper strand was 5′-GUUCCAAUUGCGAGUGAU-3′. *A. castellanii* trophozoites plated at a density of 4 × 10⁵ cells were transfected with siRNA (4 μg) against AcStefin or with scrambled siRNA as a negative control.
control (Ambion, Austin, TX), and transfection efficiencies were deter-

Immunofluorescence assay and transmission electron microscopy. After isolating amoebae expressing EGFP-fused AcStefin by FACSAria flow cytometry (BD Biosciences, San Jose, CA), cells were allowed to ad-
hire to culture dishes and examined by confocal laser scanning micros-
copy using an LSM 5 Exciter apparatus (Carl Zeiss, Hamburg, Germany). EGFP-fused AcStefin- and LysoTracker Red DND 99 (Molecular Probes, Eugene, OR)-mediated fluorescence was measured by using band-pass filters that provided excitation and emission wavelengths of 500 to 530 nm and 570 to 590 nm, respectively. For transmission electron microscopy (TEM), cell suspensions were centrifuged, and sediments were washed three times in cold PBS. The sediments were then prefixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h, rinsed with 0.1 M phosphate buffer, postfixed with 1% osmium tetroxide for 2 h, rinsed twice with 0.1 M phosphate buffer, dehydrated by using an ethyl alcohol gradient (50%, 70%, 80%, 95%, and 100%), treated twice with propylene oxide resin (1:1 dilution) for 20 min, embedded in epoxy resin (Embed-812; Electron Microscopy Sciences), and incubated at 37°C for 12 h, 45°C for 12 h, and 60°C overnight. Ultrathin sections were cut on a Reichert-Jung ultramicrotome and stained with uranyl acetate and lead citrate. Sections were observed by using a model H-7000 TEM instrument (Hitachi, Tokyo, Japan).
chi, Tokyo, Japan). Areas of intercyst space and of cytoplasm of scrambled-siRNA- or AcStefin-siRNA-transfected cells were quantified by image analysis using ImageJ Java software (http://rsb.info.nih.gov/ij/), by calculating numbers of pixels in regions of interest. Changes in the intercyst space or cytoplasm area were determined by dividing the pixel count of scrambled-siRNA-transfected cells by the pixel count of AcStefin-siRNA-transfected cells.

Identification of the Stefin homologue in *A. castellanii*. Previously, we studied the cyst-specific gene expression profile of *Acanthamoeba* and found that cystatin-like proteins are highly upregulated during encystation (17). Because the role of cystatin (a member of the I25 family) in encystation has not been studied in protozoan parasites, we focused on *Acanthamoeba* cystatin-like proteins to determine whether cystatin is involved in the encystation of *Acanthamoeba*. The gene showing homology with cystatin contained a 291-bp open reading frame (ORF) encoding a 96-amino-acid peptide with a calculated molecular mass of 10.5 kDa. When the deduced amino acid sequence was aligned with those of other proteins, the encoded protein exhibited strong sequence similarity with subfamily I25A (stefins) of the cystatin superfamily characterized in other organisms, such as *Fasciola gigantica* stefin-1 (28), *Clonorchis sinensis* CsStefin-1 (29), *Schistosoma mansoni* cystatin (2), and human cystatin A (48) (Fig. 1A). The encoded protein has a highly conserved Gln-Val-Val-Ala-Gly (QVVAG) motif in the β-hairpin loop of I25A subfamily members (stefins), which has been implicated in binding to target enzymes (49). Therefore, this sequence was designated AcStefin (*Acanthamoeba castellanii* stefin).

AcStefin is functional and highly expressed during the encystation of *A. castellanii*. Next, recombinant AcStefin was expressed in *E. coli* to examine its biochemical properties. To ascertain the enzymatic characteristics of AcStefin, recombinant AcStefin was prepared, and its inhibitory effects against C1 family cysteine proteinase, such as human cathepsin L, human cathepsin B, and papain, were determined by using those specific peptide substrates. AcStefin was found to inhibit all enzymes tested (Fig. 1B and C), indicating that recombinant AcStefin protein is functionally active as a cysteine proteinase inhibitor. We then examined the expression levels of AcStefin in *Acanthamoeba* trophozoites and cysts using specific antibodies against the recombinant AcStefin protein. As shown in Fig. 1D, the expression of AcStefin was hardly detected in trophozoites but was significantly enhanced at 24 h postencystation and maintained at 72 h (Fig. 1D, lanes 2 to 4).

AcStefin localizes to lysosomes/autophagolysosomes in *Acanthamoeba* cysts. To determine the intracellular localization of AcStefin protein, *Acanthamoeba* trophozoites were transfected with an EGFP-fused AcStefin plasmid (pUb-EGFP-AcStefin); transferred into encystation medium; stained with LysoTracker Red; which labels acidic compartments such as lysosomes and autophagolysosomes; and examined by fluorescence microscopy (50, 51). As shown in Fig. 2, the distribution of AcStefin in trophozoites showed a dispersed pattern of fluorescence in the cytoplasm, indicating that EGFP-AcStefin is expressed in the cytoplasm of trophozoites. After transfected amoebae were transferred into encystment medium, the expressed EGFP-AcStefin fusion protein was also found to be partially colocalized with these LysoTracker-stained bodies, suggesting that some AcStefin is localized in *Acanthamoeba* cyst lysosomes or autophagolysosomes.

Encystation and excystation of *Acanthamoeba* are not successful in AcStefin knockdown cells. To determine the involvement of AcStefin in the encystation of *Acanthamoeba*, siRNA-mediated gene silencing was used to reduce the expression level of endogenous AcStefin. After transfection with siRNA against AcStefin or scrambled control siRNA, transfected trophozoites were transferred into encystation medium, and the transcriptional expression levels of AcStefin were examined by real-time PCR. As shown in Fig. 3A, AcStefin mRNA expression was markedly decreased in cysts at 24 h postencystation from AcStefin-siRNA-transfected trophozoites, and this was maintained for 72 h after encystation, indicating that siRNA-mediated gene knockdown was successful in these transfected cells. Next, we examined the effect of the siRNA-mediated silencing of AcStefin on cysteine proteinase activity in *Acanthamoeba* cysts by determining the specific activities of cathepsin L in scrambled-siRNA- and AcStefin-siRNA-transfected cells using fluorometric assays. As shown in Fig. 3B, hydrolysis of cathepsin L increased at 24 h postencystation and gradually decreased at 48 h and 72 h in scrambled-siRNA-trans-
Incomplete encystation by AcStefin siRNA. (A) Trophozoites were transfected with scrambled or AcStefin siRNA; transferred into encystment medium; incubated for 24 h, 48 h, or 72 h; and examined for transcriptional changes of AcStefin in scrambled siRNA (light bars) and AcStefin siRNA (dark bars) transfectants by real-time PCR. Transfection efficiencies (80 to 88%) of transfected with scrambled or AcStefin siRNA; transferred into encystment medium; incubated for 24 h, 48 h, or 72 h; and examined for transcriptional changes of AcStefin in scrambled siRNA (light bars) and AcStefin siRNA (dark bars) transfectants by real-time PCR. Transfection efficiencies (80 to 88%) of transfected with scrambled or AcStefin siRNA; transferred into encystment medium for 24 h, 48 h, or 72 h, and numbers of mature cyst-like cells were counted under a microscope. The results show the means ± standard deviations of experiments performed in triplicate. (B) Cysteine protease activities of Acanthamoeba. Cell extracts of scrambled-siRNA- and AcStefin-siRNA-transfected cells were prepared at 0, 24, 48, or 72 h postencystation. The expression of AcStefin was normalized to that of actin. (C) Cysteine protease activities of Acanthamoeba cysts. Cell extracts of scrambled-siRNA- and AcStefin-siRNA-transfected cells were prepared at 0, 24, 48, or 72 h postencystation. The expression of AcStefin was normalized to that of actin. (D) Cysteine protease activities of Acanthamoeba cysts. Cell extracts of scrambled-siRNA- and AcStefin-siRNA-transfected cells were prepared at 0, 24, 48, or 72 h postencystation. The expression of AcStefin was normalized to that of actin.
caused by cytosolic degradation by cysteine proteases. Collectively, our findings support the view that AcStefin is essential for encystation and that AcStefin regulates intracellular cysteine protease activity during encystation.

DISCUSSION

Cysteine proteases in lysosomes are involved in many cellular processes and are key players in the macromolecular turnover associated with the degradation of the cellular components (7, 53). Recent studies of Giardia and Entamoeba have revealed that cysteine proteases are implicated in encystation (9, 11, 54–56). In Giardia, the expressions of encystation-specific cysteine proteases (ESCPs) (a cathepsin C family of enzymes) are developmentally regulated during encystation (57). Entamoeba invadens EiCP-B9, a cyst-specific cysteine protease, is expressed near the cyst wall of immature cysts and throughout mature cysts (11). The inhibition of the activities of cysteine proteases during encystation by cysteine protease inhibitors such as E64 or Z-Phe-Arg-CH2F reduces the cyst-forming ability of E. invadens (13, 14). In the present study, the level of cathepsin L activity of cyst lysates was elevated at 24 h.
postencystation and was inhibited by treatment with recombinant AcStefin or E64, indicating that the activities of cysteine proteases are indeed highly upregulated during the encystation of Acanthamoeba. In the present study, we found that AcStefin is a cysteine protease inhibitor in A. castellanii, and we examined its inhibitory effect on cysteine proteases and on the encystation of Acanthamoeba. Because AcStefin is highly expressed in cysts, we upregulated the activities of cysteine proteases by AcStefin knockdown and examined the resulting effects on Acanthamoeba encystation and excystation. Cysteine protease activities were found to be greater in AcStefin knockdown cysts than in control Acanthamoeba cysts. Interestingly, encystment appeared to be accelerated by AcStefin knockdown, but most AcStefin knockdown cells exhibited incomplete cyst formation, as only a few mature cysts were present, as determined by Sarkosyl treatment. Furthermore, TEM-based ultrastructural analysis revealed defects in exocyst walls and significant cytoplasmic degradation, which favors the view that in Acanthamoeba, cysteine protease regulation is required for successful cyst formation by preventing the proteolytic degradation of intracellular components required for subsequent excystation. Several cystatins have been found to inhibit cysteine proteases and to have immunomodulatory effects on nematodes (20–27), and the regulatory effects of intracellular cysteine proteases have been found to protect against proteolytic damage in tetramyelates (28, 29). In protozoan parasites, endogenous protease inhibitors have been found to regulate protease activity by blocking deleterious effects of host proteases from Plasmodium falciparum (34), Trypanosoma cruzi (31), Leishmania mexicana (32), and Entamoeba (36). In Plasmodium, falstatin, a cysteine protease inhibitor of P. falciparum, is required to facilitate red cell invasion (34), and PlbICP (Plasmodium berghei inhibitor of cysteine proteases) has been reported to play important role in sporozoite invasion and host cell survival (35). In T. cruzi, the overexpression of chaegasin, an inhibitor of cysteine proteases (including cruzain), decreased infectivity in cell culture (31), and in L. mexicana, virulence in mice was markedly abolished by disrupting the activities of cysteine protease inhibitors (32). However, these cysteine protease inhibitors were classified as members of the chagasins (family I25), and thus, no member of the cystatins (family I25) has yet been identified in protozoan parasites. Accordingly, to the best of our knowledge, this is the first report of a cystatin of family I25 in a protozoan parasite. In our previous studies, we determined that autophagy is essential for the encystation of Acanthamoeba (58, 59), and it is likely that the higher expression levels of cysteine proteases during encystation are associated with degradation of intracellular contents in autophagosomes (60). Murine cystatin C (CysC) is known to play a protective role under conditions of neuronal challenge by inducing autophagy via the mTOR pathway, which leads to enhanced proteolytic clearance of autophagy substrates by lysosomes, whereas the inhibitory effects of CysC on cathepsin B are not required for these neuroprotective functions (61). Although in the present study, suppression of AcStefin expression caused incomplete cyst formation and significantly diminished excystation efficiency in Acanthamoeba, we did not determine whether AcStefin induces autophagy via a specific signaling pathway in Acanthamoeba. Recently, we identified a cysteine protease that is significantly upregulated during encystation and found it to be associated with lysosomes and autophagolysosomes (62). However, the identities of the cysteine protease targets of AcStefin were not determined in the present study. Also, our findings do not exclude the possibility that the cysteine proteases of trophozoites, and not those of cysts, are inhibited by AcStefin during encystation. Thus, further studies are required to address these questions and to determine the mechanism responsible for Acanthamoeba encystation at the molecular level.

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