Identification and biochemical characterisation of *Acanthamoeba castellanii* cysteine protease 3

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Research

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Abstract

Background: *Acanthamoeba* spp. are free-living amoeba that are ubiquitously distributed in the environment. This study examines pathogenic *Acanthamoeba* cysteine proteases (*Ac*CPs) belonging to the cathepsin L-family and explores the mechanism of *Ac*CP3 interaction with host cells.

Methods: Six *Ac*CP genes were amplified by PCR. Quantitative real-time polymerase chain reaction was used to analyse the relative mRNA expression of *Ac*CPs during the encystation process, and between pre- and post-reactivated trophozoites. To further verify the role of *Ac*CP3 in these processes, *AcCP3* recombinant proteins were expressed in *Escherichia coli*, and the hydrolytic activity of *AcCP3* was determined. The influence of the *Ac*CP3 on the hydrolytic activity of trophozoites and the toxicity of trophozoites to human corneal epithelial cells (HCECs) was examined by inhibiting *Ac*CP3 expression using siRNA. Further, the levels of p-Raf and p-Erk were examined in HCECs following coculture with *Ac*CP3 gene knockdown trophozoites by western blotting.

Results: During encystment, five out of six *Ac*CPs exhibited decreased expression, and only *AcCP6* was substantially up-regulated at the mRNA level, indicating that most *Ac*CPs were not directly correlated to encystation. Further, six *Ac*CPs exhibited increased expression level following trophozoite reactivation with HEp2 cells, particularly *Ac*CP3, indicating that these *Ac*CPs might be virulent factors. After refolding the *Ac*CP3 recombinant protein, the 27 kDa mature protein from the 34 kDa pro-protein hydrolysed host haemoglobin, collagen and albumin, and showed high activity in an acidic environment. After *Ac*CP3 knockdown, the hydrolysis activity of trophozoite crude protein against gelatin was decreased, suggesting that these trophozoite had decreased toxicity. Compared with untreated trophozoites or negative control siRNA-treated trophozoites, *Ac*CP3-knockdown trophozoites were less able to penetrate and damage monolayers of HCECs. Western blot analysis showed that after inhibiting the expression of trophozoite *Ac*CP3, the activation levels of the Ras/Raf/Erk/p53 signalling pathways in HCECs were decreased.

Conclusions: *AcCP6* was correlated to encystation. Further, *Ac*CP3 is a virulent factor in trophozoites, and participated in the activation of the Ras/Raf/Erk/p53 signalling pathways of host cells.

Background

Acanthamoeba castellanii are free-living amoeba that can cause Acanthamoeba keratitis in humans after infection, as well as granulomatous amoebic encephalitis and cutaneous acanthamoebiasis in immunocompromised individuals [1, 2]. In recent years, serious *Acanthamoeba* infections have been associated with an increase in the number of contact lens wearers and immunocompromised patients. This parasite has a life cycle with two phases, the trophozoite and the cyst, which differ morphologically. When trophozoites are exposed to starvation conditions or a change in osmotic pressure, the trophozites transform into cysts to better withstand harsh environmental conditions. This parasite readily encysts in response to nutrition source deprivation, osmotic shock or a combination of both [3]. Thus, the growth

and encystation of *Acanthamoeba* trophozites, which are regulated by a number of biological factors, can lead to persistent infections and influence the pathogenicity of this parasite.

The first stage of *Acanthamoeba* infection is the interaction between mannose binding protein (MBP) on the surface of the parasite and the mannose receptor on the surface of host cells [4]. Next, a variety of proteolytic enzymes are secreted into the host, hydrolysing the host's tissue and resulting in tissue oedema, inflammation and necrosis [5]. Among these enzymes, serine proteases [6] and cysteine proteases (CPs) [7] are the main proteolytic proteins. Studies have shown that many serine proteases with different molecular weights are involved in the degradation of host cells and extracellular matrix [8] during pathogenic *Acanthamoeba* infection, and are inhibited by proteasome inhibitors in a concentration dependent manner [6].

Many protozoa utilise CPs for nutrient uptake, host infection, tissue invasion and environmental adaptation (encystation). It was well established that pathogenicity-related CPs, including cysteine protease 1 (CP1), CP2 and CP5 are expressed during the pathogenic process of *Entamoeba histolytica* [9]. In *Giardia lamblia*, CPs and UDP-Nacetylglucosamine pyrophosphorylase are required during encystation. *Acanthamoeba* express many CPs with different molecular weights, including 38.5, 43, 50, 59, 70, 100 and 130 kDa proteases [10–13]. Following sequencing and analysis, it was found that both the 990 bp *A. castellanii* CP gene (*Ac*CP) [11] and the 1359 bp *A. culbertsoni* CP gene (*Ac*CP2) [14] belong to the L cathepsin CP family. *Ac*CP2 contains an Ex3Rx3Wx2N motif in the proregion and a proline/threonine-rich C-terminus. The amino acid sequence of *Ac*CP contains a catalytic site with five residues, as well as ERFNIN and GNFD motifs. Moreover, recombinant *Ac*CP protein was capable of hydrolysing host proteins, including haemoglobin, albumin, IgG, IgA and adhesion proteins, suggesting that *Ac*CP may be an important pathogenic protease in *Acanthamoeba* [11].

The pathogenic process of *Acanthamoeba* mainly depends on the hydrolysis activity of the pathogenic proteases secreted by trophozites, which activate a series of cell signalling pathways in host cells. Several studies have revealed that various downstream molecules are involved, including G-protein-coupled receptors, beta adrenalin receptors [15], Toll-like receptor-4 (TLR4), TLR4-Myeloid differentiation primary response gene 88 (MyD88), Nuclear Factor-κB (NF-κB), Extracellular signal-regulated kinase (Erk) [16], PI3K [17]and cytosolic phospholipase (A2αcPLA2α) [18]. Due to the complex pathogenic mechanisms of *Acanthamoeba* infection, cytopathogenic effects in host cells are not solely mediated by these signalling molecules. Instead, changes in the expression levels of other signalling molecules in the host may also be involved. The Ras/Raf/Erk signalling pathway is well known as a key intracellular signal transduction pathway that regulates cell differentiation, proliferation and apoptosis, and which is itself regulated by various growth factors. When cells are stimulated, Ras protein kinase expression is up-regulated, thereby activating downstream Erk1/2 protein and inducing apoptosis. Further, the activation of Ras/Raf/ERK signalling can lead to the activation of the tumour suppressor p53. Increased phosphorylation of p53 can up-regulate apoptotic proteins and inhibit bcl-2 proteins, eventually inducing apoptosis [19]. However, whether the Ras/Raf/Erk/p53 signalling pathways of host cells are involved in the pathogenesis of *Acanthamoeba* has not yet been verified. In addition, it has not been reported

whether the CPs secreted by trophozoites are involved in the activation of the Ras/Raf/Erk/p53 signalling pathways of host cells.

Here, we amplified the CP genes *of A. castellanii* and determined their roles in the pathogenesis and encystation of trophozoites. We confirmed that AcCP3 is specifically required for the pathogenicity and virulence of trophozoites. By potentially activating the Ras/Raf/Erk/p53 signalling pathway of host cells, *AcCP3* is important in the pathogenesis of *Acanthamoeba* and may be involved in the pathophysiology of infection.

Methods

Amoeba and cell culture

Acanthamoeba castellanii (strain ATCC30011) was obtained from the American Type Culture Collection (ATCC). Trophozoites were cultured axenically in peptone-yeast-glucose (PYG) medium. Cultures were incubated at 26 °C and trophozoites were harvested in the late log phase after subculture for 72 h. HEp-2 cells purchased from ATCC were cultured in EMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Thermo), 100 U/ml penicillin and 100 μg/ml streptomycin. Human corneal epithelial cells (HCECs) purchased from Bioleaf (Shanghai, China) were grown in DMEM (Gibco) with 10% FBS. Both cells were cultured in a 37 °C incubator with 5% CO2.

Encystation Assays

Encystation assays were performed as described previously [20] with slight modifications. Briefly, trophozoites from post-logarithmic growth phase cultures were treated with phosphate buffered saline (PBS; containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4) with 50 mM MgCl2 and 10% glucose in culture plates at 30 °C (5 × 105 trophozoites per ml of medium) for 72 h. The treated materials were collected at 24 h, 48 h and 72 h for RNA extraction.

Cloning of *Ac*CP genes and expression level analysis

The CP genes of *Acanthamoeba* were cloned from the cDNA of trophozoites using the primers listed in Table S1 by PCR. PCR was performed in a 9902 Veriti 96-well Thermal Cycler (Applied Biosystems) (94 °C for 3 min; 35 cycles of 94 °C for 15 s, 55 °C for 30 s and 72 °C for 1 min; followed by 72 °C for 7 min). The amplified PCR products were purified and ligated into a pMD19-T vector (Takara), and the nucleotide sequences were obtained by automated sequencing.

A.castellanii mRNA was extracted from trophozoites and cysts using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesised using a PrimeScript® 1st strand cDNA synthesis kit (Takara). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to analyse the expression level of AcCPs. The primers for AcCPs genes and the GAPDH internal reference are listed in Table S2.

Expression, purification and refolding of recombinant *AcCP3* protein

The correct plasmids containing AcCP3 were amplified with primers containing *Hind*III and *BamH*I restriction sites, and the PCR products were ligated into the pQE-30 expression vector (Qiagen). The sequences of all constructs were confirmed on both strands and analysed with Vector NTI software (Invitrogen, Waltham, USA). Plasmids were transformed into M15 (pREP4) cells (Qiagen) for protein expression. The selected clones were cultured in Luria-Bertani broth containing 100 μg/ml ampicillin and r*Ac*CP3 expression was induced using 1 mM isopropyl-β-D-thiogalactoside for 3 h at 37 °C. The recombinant r*Ac*CP3 protein was purified using a QIA Express kit in accordance with the manufacturer's instructions. The purity and mass of protein was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Since the N-terminal peptide sequence in recombinant CPs can inhibit the hydrolytic activity of the protease, it was necessary for the recombinant *Ac*CP3 to be refolded to obtain the mature peptide with hydrolytic activity [21]. Refolding of the purified recombinant protein was performed as described previously [11, 21]. In brief, purified r*Ac*CP3 (2 mg) was slowly added to 100 ml refolding buffer, containing 100 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetracetic acid (EDTA), 250 mM L-arginine, 5 mM reduced glutathione (GST), and 1 mM oxidised glutathione. The protein was gently stirred at 4 °C overnight, and then dialysed against 10 mM Tris-HCl (pH 7.5). The obtained r*Ac*CP3 was further processed as described previously [22]. In brief, sodium acetate buffers with pH 4.0 to 7.0 were used to analyse the optimal pH condition required for obtaining the fully matured r*Ac*CP3 enzyme. The concentration of matured r*Ac*CP3 enzyme was measured using a protein assay (Bio-Rad).

Analysis of biological characteristics of Ac**CP3**

To assess the role of *Ac*CP3 in *Acanthamoeba* pathogenesis, the reactivation of the physiological properties of trophozoites was performed using HEp-2 cell monolayers as described previously [23]. In brief, HEp-2 cells were cultured in 75 cm^2 tissue culture flasks (Corning) at 37 °C under sterile conditions until the monolayer covered the bottom of the flask completely, at which point the supernatant was removed. Trophozoites (10^6) suspended in 25 mL physiological 0.9% NaCl were inoculated onto the monolayer, three times consecutively. Cocultures of amoebae and HEp-2 cells were incubated at 26 °C until the monolayer was completely lysed. Reactivated trophozoites were collected and total mRNA was extracted. qRT-PCR was performed to analyse the expression level of *Ac*CP3. In addition, MBP was used as an example cytotoxicity protein in *Acanthamoeba* [24]. 18S rDNA was used as internal reference [25]. The primers for *Ac*CP3, MBP and 18S rDNA are listed in Table S2.

To determine the effect of AcCP3 on host proteins, haemoglobin (from human blood), collagen (from human placenta) and albumin (from bovine serum) were purchased from Sigma. Each protein (2 mg/ml) was incubated with matured r*Ac*CP3 (100 nM) in 50 mM sodium acetate (pH 4.0 or pH 7.0) with 1 mM GST for 3 h at 37 °C. The reactions were terminated by adding reducing sample buffer and the degradation activity of r*Ac*CP3 was analysed by SDS-PAGE.

The protease activities of trophozoite crude proteins were analysed using Novex™ 10% Zymogram Plus (Gelatin) Protein Gels (Thermo Fisher scientific) in accordance with the manufacturer's instructions. 1.5 μg crude protein extract was added to each lane. Various inhibitors for different proteases were used.

Crude protein extracts treated and untreated with inhibitors (1 h before electrophoresis) were analysed with zymography. The final concentrations of inhibitors were as follows [26]: for serine proteases, 5 mmol/L phenylmethylsulfonyl fluoride (PMSF); for metalloproteases, 2 mmol/L EDTA; for CPs, 40, 60, 80 and 100 mmol/L N-ethylmaleimide (NEM).

Ac CP3 gene silencing

siRNA targeting the catalytic domain of *Ac*CP3 was synthesised by Ribobio (Guangzhou, China) and based on the cDNA sequence. The sequence of the forward strand was 5'-AGUACAUCAUCAACAACAA-3'. Trophozoites were plated at a density of 5×10^4 cells in 48-well plates and cultured overnight, then transfected with siRNA (15 μg/ml) for 12 h using SuperFectin™ In Vitro siRNA Transfection Reagent (Pufei). As a control, a negative siRNA provided by Ribobio was also applied to cultured trophozoites. Untreated trophozoites and transfection reagent-treated trophozoites were also processed. After the transfection, the differentially treated trophozoites were harvested to determine the efficacy of the knockdowns by examining the expression level of *Ac*CP3 with qRT-PCR, and then for the cytopathic tests.

Effect of Ac*CP3*-knockdown in trophozoites on *Acanthamoeba*-mediated cytotoxicity

To determine the effects of reduced *Ac*CP3 expression on *Acanthamoeba*-mediated HCECs death, cytotoxicity assays were performed as previously described [27]. Confluent HCEC monolayers in 12-well culture plates (Corning) were incubated with differentially treated trophozoites (ratio 1:2) at 37 °C in a 5% CO_2 atmosphere for 24 h. Four different experimental groups were included. Group 1, normal cultured confluent HCECs; Group 2, HCECs co-cultured with normal trophozoites; Group 3, HCECs co-cultured with negative control siRNA-transfected trophozoites; Group 4, HCECs co-cultured with *Ac*CP3-knockdown trophozoites. The cytopathic effects for the different groups of trophozoites were observed using light microscopy (Olympus). The trophozoites were detached with cold 2 mM EDTA-PBS buffer, chilled on ice for 20 min and then harvested. In addition, HCECs were cultured in fresh DMEM medium with CCK 8 reagent for 2 h, and the cytotoxicity was determined by measuring dehydrogenase release (Cell Counting Kit-8, DOJINDO). The absorbance of each well was measured at 450 nm using a Model 680 Microplate Reader (Bio-Rad). In order to investigate the signalling pathways in HCECs activated by *Acanthamoeba*, four groups of differentially treated HCECs were harvested after co-culture with amoeba for 24 h. qRT-PCR was performed to analyse the expression level of the *Ras* gene (NM_004985.4). The forward strand was 5'- AGGAAGCAAGTAGTAATTGATGGA-3', the reverse strand was 5'- GCCTGTTTTGTGTCTACTGTTCT-3'. Human GAPDH used as an internal reference, with forward strand 5'- TCACCACCATGGAGAAGGC-3', reverse strand 5'- GCTAAGCAGTTGGTGGTGCA-3'.

Western Blotting Assays

The activation states of Raf, Erk and p53 in differentially treated HCECs were determined using western blotting assays as previously described [28]. Briefly, four groups of differentially treated HCECs were harvested for western blotting after coculture with amoeba for 24 h. The cells were lysed in 100 μL lysis buffer (20 μL Phosphatase inhibitors, 20 μL Protease inhibitor cocktail, 100 μL PBS, 5 μL NP-40, 855 μL

ddH_2O). The cell lysate (20 μg) was resolved by SDS-PAGE and transferred to PVDF membranes (Roche). We used the following primary antibodies purchased from Cell Signalling: rabbit anti-p-Raf (Ser338), rabbit anti-Raf, rabbit anti-p-Erk (Thr202/Tyr204), rabbit anti-Erk, mouse anti-p-p53 (Ser15), and mouse anti-p53. HRP-labelled goat anti-mouse and goat anti-rabbit secondary antibodies (Abcam), and rabbit polyclonal to β-actin (Abcam), were detected with Tanon™ High-sig ECL Western Blotting Substrate (Tanon), observed with an ECL detection system (Tanon), and the scanned images were quantified using Image-Pro Plus 4.5.1 software (Media Cybernetics).

Statistical analysis

The results of qRT-PCR were calculated using the $2^{-\triangle\triangle Ct}$ method. Statistical analyses were performed using GraphPad Prism software (San Diego). Significance was calculated by one-way analysis of variance followed by a Tukey test or Student's t-test. Data are expressed as mean ± SD and at least three independent experiments were performed for each experiment.

Results

Identification of pathogenic *Ac*CPs

The sequences encoding full-length CP genes were amplified using PCR procedures. Five new open reading frames (ORF) corresponding to CP genes were identified in *A.castellanii*. The sequences of *Ac*CP3, *AcCP10*, *AcCP9*, *AcCP5* and *AcCP8* with the accession numbers LC472809, LC472813, LC472812, LC472811, LC472810, respectively, (Table S3) have been submitted to the GenBank database. *AcCP8* does not present the typical characteristics of CP. The sequences of *AcCP7* (XM004358251) and *AcCP6* (XM004341651) from ATCC30011 have also been confirmed in the Neff strain.

The amino acid residues Q, C, H, N and W that act as the catalytic site of cathepsin L-family CPs exist in *Ac*CPs. The modified ERFNIN and GNFD motifs that are conserved in cathepsin L-like CP family members have also been observed in *Ac*CPs [29]. We found that while *AcCP10* had no C active site, the amino acid sequences of the other CPs contained the conserved C site, which is mainly involved in the refolding of the protease. The nucleotide sequence homology of ATCC30011 and Neff 7 CPs was as high as 99%.

Expression pattern of *Ac*CPs during encystation

qRT-PCR was used to analyse the expression levels of the *Ac*CPs during the encystation process. As shown in Fig. 1, the mRNA levels of *Ac*CP3, *AcCP10*, *AcCP8*, *AcCP9* and *AcCP7* were decreased at 24 h, 48 h and 72 h (with the exception of *AcCP7* at 72 h), indicating that these *Ac*CPs may not be involved in encystation. In contrast, *AcCP6* was gradually but substantially up-regulated during encystation, suggesting that while most cathepsin L-family CPs were not involved in encystation, *AcCP6* may play a uniquely important role in the formation of cysts.

The relationship between *Ac*CPs and protozoan virulence

In order to investigate the role of *Ac*CPs in *Acanthamoeba* virulence, trophozoites that were axenically grown *in vitro* for several years were reactivated with HEp2 cells. The results of quantitative PCR analysis of *Ac*CP and MBP mRNA expression in pre- and post-reactivated trophozoites are shown in Fig. 2. The MBP expression level was up-regulated in post-reactivated trophozoites. In addition, the relative mRNA expression levels of six of the CPs were up-regulated following trophozoite reactivation, while the expression level of *Ac*CP3 was significantly increased in trophozoites that invaded HEp2 cells (Fig. 2). These data indicate that *Ac*CPs may play an important role in *Acanthamoeba* pathogenesis by mediating host cell damage during infection. Further, *Ac*CP3 in particular may act as a potential pathogenic factor during human cell invasion.

Refolding of r *Ac* CP3 and degradation of host proteins

The *Ac*CP3 gene contains a 993-bp ORF that encodes a 329-amino acid protein. A portion of the prodomain and the entire mature domain of *Ac*CP3 was amplified and expressed using a pQE-30 expression vector. *Ac*CP3 was expressed in M15 competent cells as an inclusion body protein with an apparent molecular mass of 34 kDa (Fig. 3a lane 1). The recombinant protein was purified by Ni-NTA affinity chromatography and then refolded. The refolded sample was further processed under reducing acidic conditions to allow the refolded protein to be processed into a fully active enzyme. The size of fully activated mature r*Ac*CP3 coincided with the size predicted for the mature protease (27 kDa) (Fig. 3a lane 2). However, despite our extensive efforts to produce enzymatically active recombinant enzymes, we failed to successfully perform zymography with 0.1% gelatin as a substrate (data not shown). However, we were able to carry out an assay of host protein degradation using r*Ac*CP3. SDS-PAGE showed that r*Ac*CP3 possesses proteolytic activity against human haemoglobin, collagen and bovine serum albumin. All protein substrates used in this assay were hydrolysed at acidic pH, but no hydrolysis was observed at neutral pH (Fig. 3b, c, d).

Low proteolytic activity of *Ac*CP3 knockdown trophozoites

In order to investigate the biological role of *Ac*CP3 in *Acanthamoeba* trophozoites, trophozoites were transfected with *Ac*CP3-siRNA and qRT-PCR revealed that the mRNA level was significantly reduced in *Ac*CP3 gene knockdown trophozoites (Fig. 4a). There was no significant difference in the growth and proliferation rate of trophozoites between the knockdown and wild-type strains (data not shown). Further, there were no differences observed between superfectin-treated trophozoites (superfectin) and negative control siRNA-transfected trophozoites (Ne) compared to untreated trophozoites cultured with PYG. Subsequently, the hydrolytic activity of *Ac*CP3 knockdown trophozoites was compared to that of wild-type trophozoites and protease inhibitor-treated trophozoites (Fig. 4b). 1.5 μg of differentially treated crude protein extracts were added to lane 1 to lane 8, and lane 10. The crude protein extracts in lanes 1 and 4, corresponding to wild-type trophozoites, showed high hydrolytic activity. Lane 2 showed that the serine proteinase inhibitor PMSF could inhibit this hydrolytic activity. Lane 3 showed that the metalloprotease inhibitor EDTA had no effect on this hydrolytic activity. Lanes 5 to 8 showed that 40, 60, 80 or 100 mmol/L NEM could inhibit this hydrolytic activity, and the hydrolytic activity decreased with

increasing NEM concentration. The ability of PMSF and NEM to inhibit the hydrolytic activity of crude protein extracts from trophozoites confirmed previous results [26]. In lane 9, 1.5 μg crude protein extract from trophozoites transfected with negative control siRNA showed that the negative control siRNA has little effect on the hydrolytic activity. Lane 10 treated was the same as lane 5. In lane 11 is shown the effect *Ac*CP3 siRNA on the hydrolytic activity of trophozoites. Zymography revealed that the proteolytic activity in trophozoites following *Ac*CP3 knockdown was decreased, suggesting that the virulence of these trophozoites was also decreased.

Effect of *Ac*CP3 knockdown on protozoa-mediated cytotoxicity

Cytotoxicity assays were performed with four differently treated HCECs as described in the methods section. When HCECs were cultured alone, the cells grew into a confluent monolayer and were tightly connected to one another, while a small number of cells lifted off of the dish because of overgrowth (shown as Fig. 5a). The white arrow in Fig. 5b, c and d indicates the cellular voids formed when HCEC monolayer cells were destroyed by trophozoite penetration. Figure 5b shows confluent HCECs co-cultured with wild-type trophozoites; in this case, most adherent HCECs became suspended and the tight connections between the cells were disrupted, resulting in the formation of cellular voids. Figure 5c shows confluent HCEC monolayers were also destroyed by negative control siRNA-transfected trophozoites, and the cytotoxicity did not differ significantly from that of untreated trophozoites. As shown in Fig. 5d, trophozoites transfected with *Ac*CP3 siRNA exhibited a decrease in *Acanthamoeba*-mediated HCEC cytotoxicity, and while the HCECs exhibited a few cellular voids, most of the HCECs remained attached. To test the cytopathic activity, a monolayer of HCECs was co-cultured with differently treated trophozoites. After 24 h, the trophozoites were removed. HCECs were sequentially cultured in fresh DMEM medium with CCK 8 reagent for 2 h and then analysed by photometry to assess the dehydrogenase released from cells. As shown in Fig. 5e, HCECs treated with *Ac*CP3 knockdown trophozoites exhibited the highest dehydrogenase release compared to HCECs in treatment groups 2 and 3. These results indicate that the cytotoxicity induced by *Acanthamoeba* in HCECs was decreased in *Ac*CP3 knockdown trophozoites, further suggesting that *Ac*CP3 is a pathogenic factor for *Acanthamoeba*.

Inhibiting the expression of *Ac*CP3 reduces target cell Ras/Raf/Erk signalling

Four groups of HCECs treated as described in the methods section were harvested after co-cultured with trophozoites, qRT-PCR was used to analyse the mRNA expression levels of Ras genes. The data shows (Fig. 6a) that the expression level of K-Ras was significantly increased in HCECs co-cultured with wide-type trophozoites (group 2). On the contrary, the expression level of K-Ras was significantly decreased in HCECs cocultured with *Ac*CP3 knockdown trophozoites (Group 4). This indicates that *Ac*CP3 siRNA significantly decreased the mRNA expression level of K-Ras in *Acanthamoeba*-treated HCECs. In contrast, the mRNA expression levels of H-Ras and N-Ras showed no differences between groups 2 and 4 (data not shown). Previous studies have demonstrated that Ras recruits Raf kinase into a complex, which mediates Raf activation. Raf then phosphorylates MEK1 and MEK2, which in turn activate Erk1/2 by the tandem phosphorylation of threonine and tyrosine residues [30]. Thus, we further examined the levels of p-Raf

and p-Erk in HCECs exposed to *Ac*CP3 gene knockdown trophozoites by western blotting. That data shows that (Fig. 6, group 2) untreated trophozoites could significantly increase the phosphorylation levels of Raf, Erk, and p53 proteins in HCECs. Compared to untreated cultured confluent HCECs (group 1), HCECs co-cultured with untreated trophozoites (group 2) or HCECs co-cultured with negative control siRNA-transfected trophozoites (group 3), the levels of p-Raf and p-Erk in HCECs were markedly decreased after co-culture for 24 h ($P < 0.01$). Previous studies have suggested that phosphorylation of p53 might be an important mechanism in Ras/Raf/Erk-induced apoptosis. Thus, we also examined the levels of p-p53 in HCECs cocultured with *Ac*CP3 knockdown trophozoites. The data shows that the level of p-p53 was also markedly decreased after treated for 24 h ($P < 0.01$). These results demonstrated that inhibiting the expression of *Ac*CP3 protein could reduce the *Acanthamoeba*-mediated phosphorylation levels of Raf, Erk and p53 proteins in HCECs. This suggests that *Ac*CP3 might participate in the activation of Raf, Erk and p53 proteins in HCECs.

Discussion

CP family proteins are essential to the life cycle of several protozoa by influencing several diverse processes, such as nutrient intake, protein degradation of parasites, immunomodulators, host cell invasion and encystment/excystment, in addition to well-established roles in protein processing and catabolism. They are also an important virulence factor for parasites [31]. It has recently been reported that *A. castellanii* expresses CPs with proteolytic effects [11]. However, little research has been done on the pathogenic and encystment mechanisms of *Acanthamoeba* CPs. Long-term sterile passage culture of *Acanthamoeba* trophozoites *in vitro* may reduce the toxicity of the trophozoites [32]. However, a potential method to reactivate attenuated trophozoite virulence was identified by growing the trophozoites on HEp-2 cell monolayers three times using the *Acanthamoeba* 1BU strain (ATCC no. Pra-105) [23]. Using this method, we found that the expression levels of six *Ac*CPs were higher following reactivation, especially *Ac*CP3, suggesting that *Ac*CPs may play a pathogenic role in *Acanthamoeba*. *In vitro*, *Ac*CP3 could effectively hydrolyse host haemoglobin, collagen and albumin after refolding, and it had strong hydrolytic activity in an acidic environment but almost no hydrolytic activity in a neutral environment. This characteristic is similar to that of vivapain from *Plasmodium vivax* [33] and CsCF-6 from *Clonorchis sinensis* [21].

The pathogenic process of *Acanthamoeba* in the host cell is regulated by various signalling molecules. G protein-coupled receptor and β adrenalin receptor inhibitors block the activity of trophozoite proteases, thereby affecting the growth, encystation, vitality, and pathogenicity of the parasite [15]. The HCECs used in our experiments primarily express the epithelial markers E-cadherin, ZO-1, and β-catenin. It has been reported that Toll-like receptor 4 (TLR4) is an important pathogenic target of *Acanthamoeba* in human corneal epithelial cells (HCECs). After attaching to the surface of host cells, *Acanthamoeba* activates TLR4, which in turn affects intracellular MyD88, NF-κB and Erk [34]. It has been found that thrombinase and trypsin can activate protease-activated receptor (including PAR1 and PAR2) in HCECs, and promote the secretion of pro-inflammatory factors such as IL-6, IL-8 and TNF-α, leading to ocular inflammation [35]. Furthermore, studies have shown that *Acanthamoeba*-induced host cell death is related to PI3K

signalling [17]. *Acanthamoeba*-induced host cell apoptosis is associated with mitochondrial overexpression of pro-apoptotic proteins [36]. It was found that cytosolic phosolipase A2 alpha (cPLA2-α) is involved in *Acanthamoeba*-induced apoptosis of HCECs [18]. To determine whether *Acanthamoeba Ac*CP3 interacted with HCECs via the TLR4 receptor, it will be necessary to determine whether CPs can bind to the TLR4 receptor on the surface of HCECs via pathogen-associated molecular patterns. In this study, zymography revealed that the proteolytic activity of trophozoites was decreased following *Ac*CP3 gene knockdown, and trophozoites after *Ac*CP3 gene silencing exhibited a decrease in *Acanthamoeba*-mediated HCEC cytotoxicity. The mechanism of *Ac*CP3 in *Acanthamoeba*-mediated HCEC cytotoxicity must be confirmed with regards to its role in cell signalling pathways.

The Ras/Raf/Erk signalling pathway is the main signalling pathway regulating the occurrence and development of tumours, and is important in regulating apoptosis [19]. This pathway activates endogenous apoptotic pathways, such as the release of mitochondrial cytochrome c [37], as well as the activation of caspase-9 [38], caspase-8 [39], and p53 [40]. The ability of the amoeba to activate the Ras/Raf/p53 signalling pathways of host cells during infection has not been verified. Ras signalling molecules include H-Ras, K-Ras, and N-Ras [41]. The role of Ras in HCECs has been confirmed, and it has been reported that Ras can regulate the inflammatory response in the cornea of mice [42]. In addition, the Ras/Erk signalling pathway can be activated by hepatocyte growth factor [43] in HCECs. In this study, we found that *Acanthamoeba* infection mainly affected the expression level of the *K-Ras* gene in HCECs, and western blot analysis showed that trophozoites could also increase the phosphorylation levels of Raf, Erk1/2, and p53 in HCECs; taken together, this indicates that *Acanthamoeba* could activate the Ras/Raf/ERK/p53 signalling pathways of host cells. Moreover, studies have found that serine and cysteine protease inhibitors can down-regulate Ras pathway-induced apoptosis. Protease inhibitors can inhibit the activation of Ras, which may be related to the involvement of proteases in tumour cell invasion and metastasis [44]. In summary, proteases are involved in the activation of Ras protein and downstream signalling pathways. In this study, the phosphorylation of Ras/Raf/ERK/p53 signalling pathway components in HCECs was decreased when the expression of *Ac*CP3 was inhibited in trophozoites. This suggests that *Ac*CP3 is involved in the activation of the Ras/Raf/ERK/p53 signalling pathway during the trophozoite pathogenic process.

It is not well understood how CP expression and function changes during encystation in *Acanthamoeba*. We analysed the transcription levels of six genes and found that *Acanthamoeba* CP *AcCP6* was the most highly expressed CP gene during the encystation process. This indicates that while most cathepsin L-family CPs are not involved in encystation, *AcCP6* may participate in encystment. Of these CP genes, *AcCP6* emerges as the most highly expressed and exhibits developmental regulation, with expression increasing dramatically during encystation and cyst stages. In general, the process of encystation involves the coordinated secretion of cyst wall materials to the periphery of a cell. It was suggested that trophozoites produce abundant cyst wall proteins, including *Ac*CP6, which are packaged into encystation-specific materials in response to environmental cues. Indeed, cysteine endopeptidases in *Giardia* were localised to the encystation-specific vesicles during encystation. When fixed cysts were subjected to fluorescence in situ hybridization with *AcCP3* and *AcCP5* DNA probes, *AcCP3* and *AcCP5* were visualized

in both submembranous and nucleus of cysts (Fig. S1). Our data indicated that some CPs could also be regulated by acidification of encystation-specific packages; it has been suggested that encystation vesicles fuse with peripheral vacuoles prior to formation of the cyst wall and that the activity of encystation-specific CP (*Ac*CP6) toward protein substrates is greatly reduced in an acidic compartment.

Conclusions

These results suggested that various *Acanthamoeba* CPs may be required during pathogenic and cyst formation processes. We propose that various CPs play important roles in the regulation of pathogenic and encystment processes, especially *Ac*CP3 and *Ac Ac*CP6. Further study of the pathogenic mechanisms of *Acanthamoeba* trophozoites will provide a platform for the development of new anti-*Acanthamoeba* drugs. Further work will be required to clarify the role of the other CPs in *Acanthamoeba*. The present study preliminarily confirmed that *Acanthamoeba* trophozoites activate the Ras/Raf/Erk/p53 signalling pathways of HCECs and contribute to host cell death. The phosphorylation of the Ras/Raf/Erk/p53 signalling pathway decreased after inhibiting the expression of *Ac*CP3, indicating that *Ac*CP3 may be an important pathogenic factor involved in the pathogenesis of trophozoites.

Abbreviations

AcCPs: *Acanthamoeba* cysteine proteases; HCECs: human corneal epithelial cells; CP: cysteine proteases; TLR4: Toll-like receptor-4; MyD88: TLR4-Myeloid differentiation primary response gene. NF-κB: Nuclear Factor-κB; Erk: Extracellular signal-regulated kinase, PBS: phosphate buffered saline; qRT-PCR: quantitative real-time polymerase chain reaction; SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis; EDTA: ethylenediaminetetracetic acid. GST: glutathione; PMSF: phenylmethylsulfonyl fluoride. ORF: open reading frames.

Declarations

Availability of data and materials

All data generated or analyzed during this study are included in the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XC and MF designed the study. ZW, DW, MF conducted the work. XC acquired funding. ZW and DW wrote the first draft of the manuscript. XC, MF and HT revised the manuscript. All authors read and approved the final manuscript.

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Figures

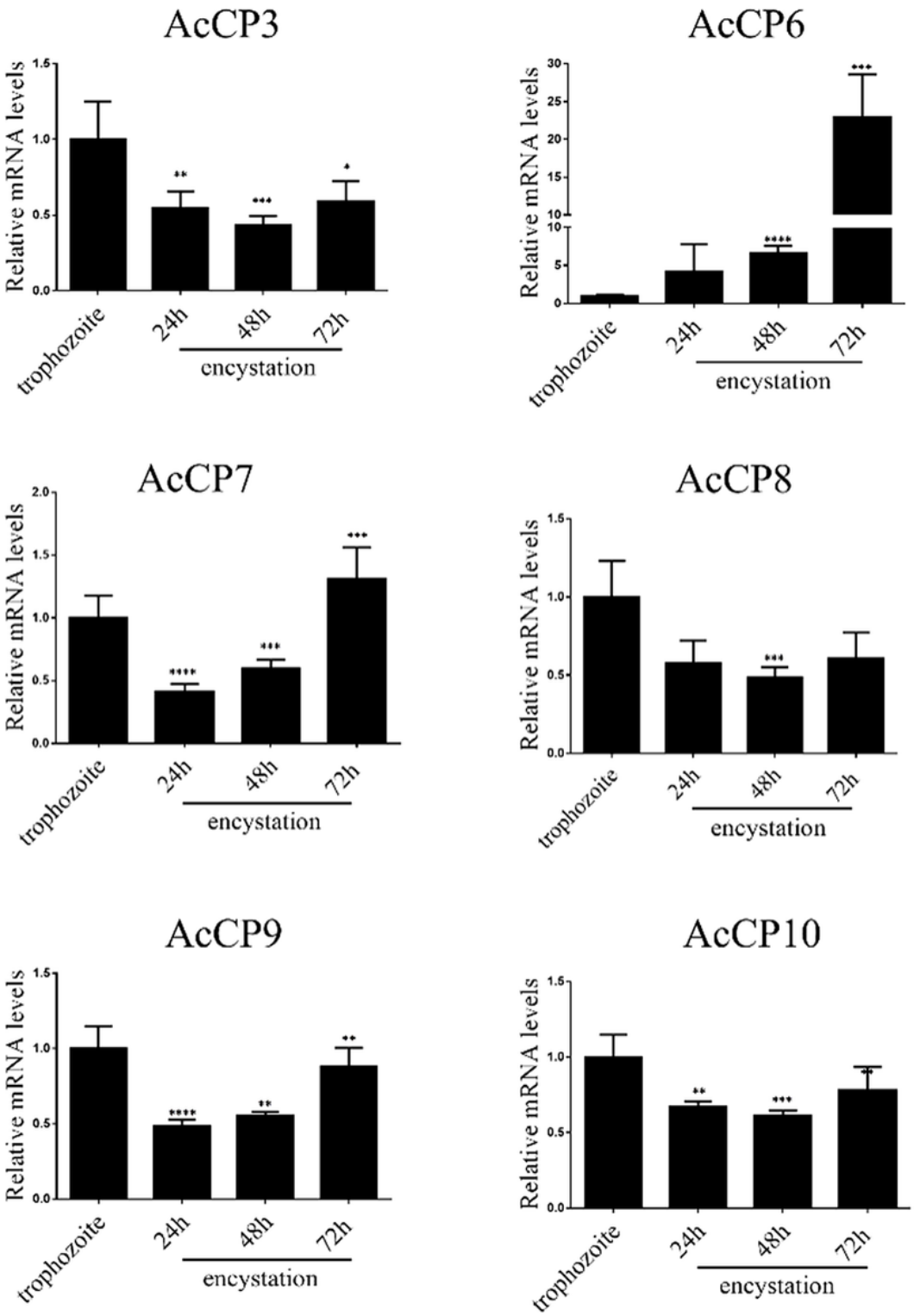

Figure 1

The AcCPs expression levels during encystation using qRT-PCR analysis. The experiments were repeated three times, and the average values were presented with vertical bars representing standard deviations. *means significantly different (P<0.05) by Student's t-test, ** P<0.01, ***P < 0.001, **** P < 0.0001.

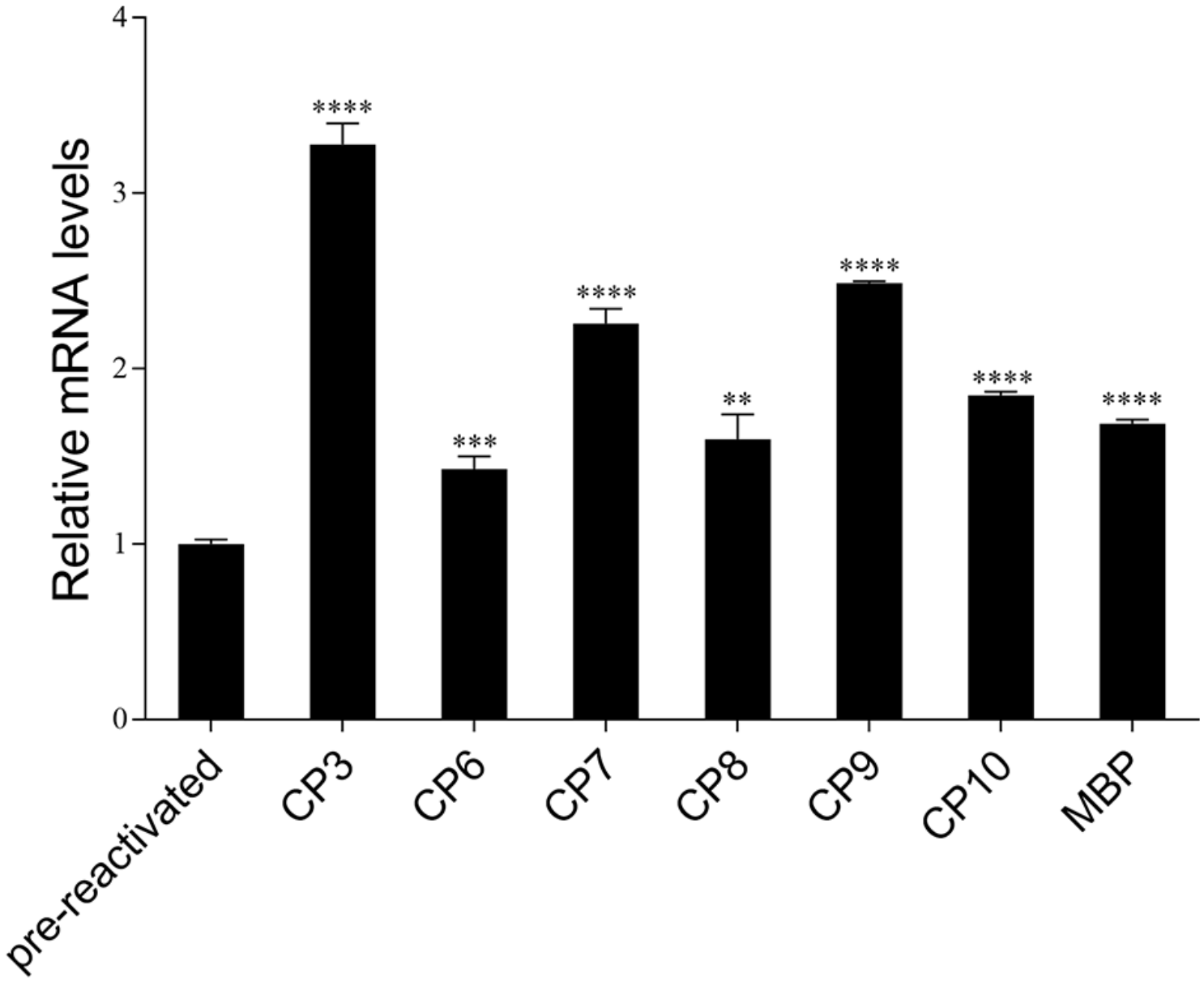

Figure 2

The expression levels of AcCPs and MBP between pre- and post-reactivated trophozoites. AcCPs includes AcCP3, AcCP6, AcCP7 AcCP8, AcCP9 and AcCP10. Values indicate the mean (±SD) of three experiments. Student's t-test was used to analyze the data. *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.

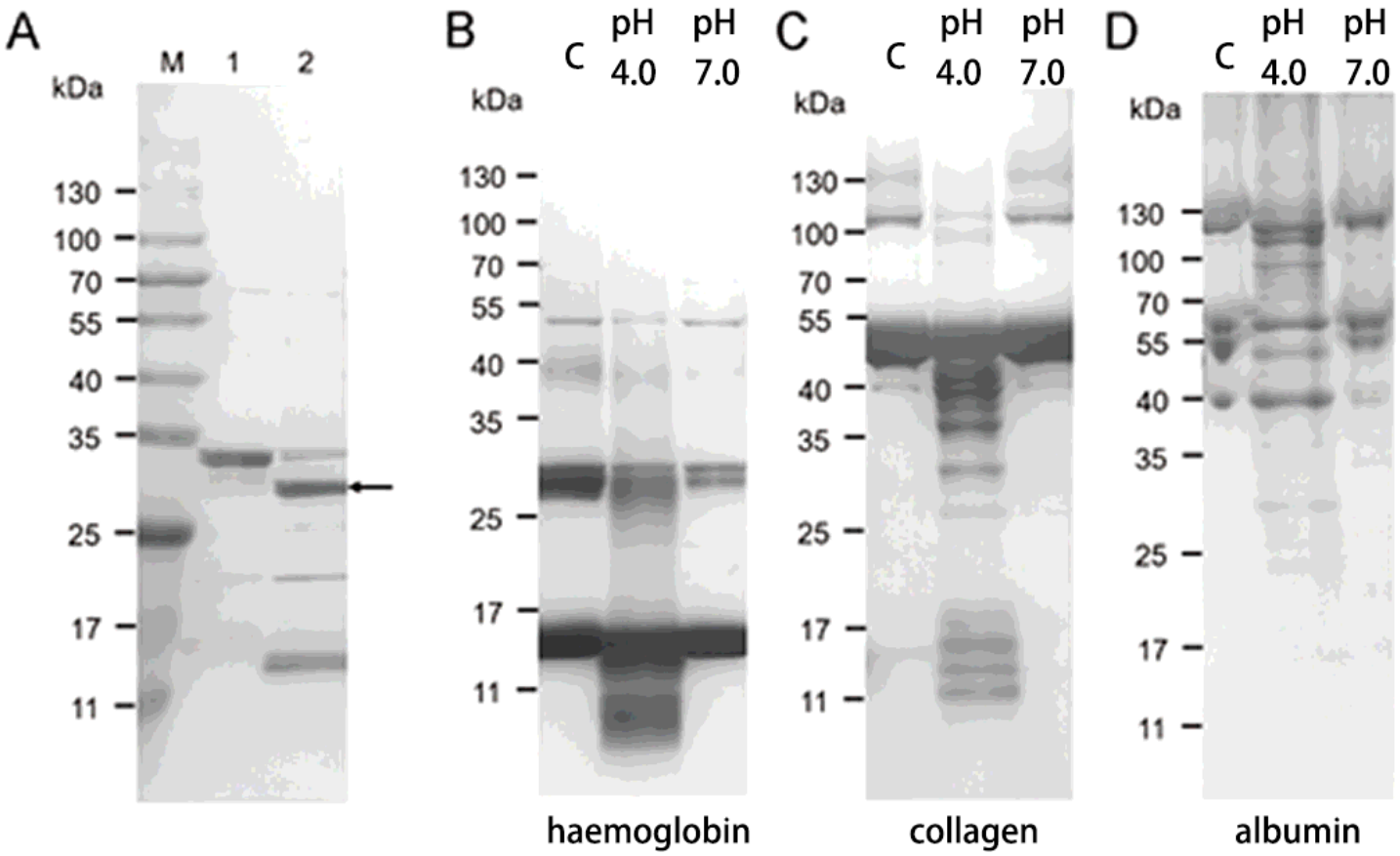

Figure 3

Expression, purification and refolding of rAcCP3 and degradation of host proteins. (A) rAcCP3 were analyzed by SDS-PAGE. Lane 1, purified rAcCP3. Lane 2, refolded rAcCP3. (B, C, D) Degradation of haemoglobin, collagen and albumin by rAcCP3 in reaction system of pH 4.0 and pH 7.0, respectively.

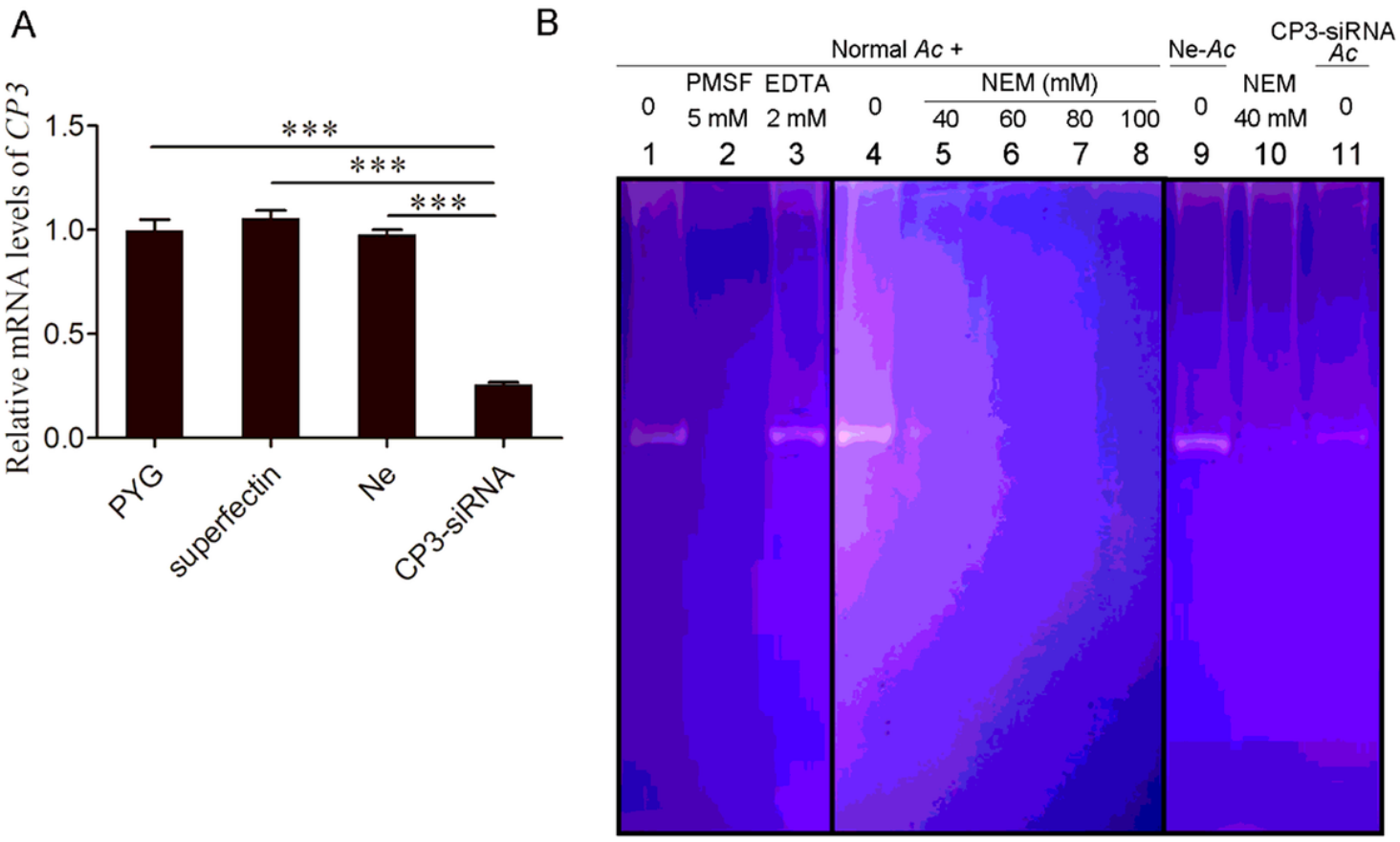

Figure 4

Zymography showed low hydrolytic activity of AcCP3 gene knockdown trophozoites. (A) AcCP3 mRNA expression level of wild-type trophozoites (PYG), only superfect treated trophozoites (superfect), negative siRNA transfected trophozoites, AcCP3-siRNA transfected trophozoites. (B) Zymography test of different trophozoites crude extract proteins. Lane 1 and lane 4 showed normal trophozoites crudes proteins with no treatment. Lane 5 and lane 10 showed normal trophozoites crudes proteins pretreated with 40 mM NEM. Lane 10 added negative siRNA transfected trophozoites crude proteins. Lane 11 added AcCP3 gene knockdown trophozoites crude proteins. Independent experiments were repeated three times. Significance was calculated by one-way analysis of variance (ANOVA) followed by a Tukey test. Vertical bars indicate SD. ***P < 0.001.

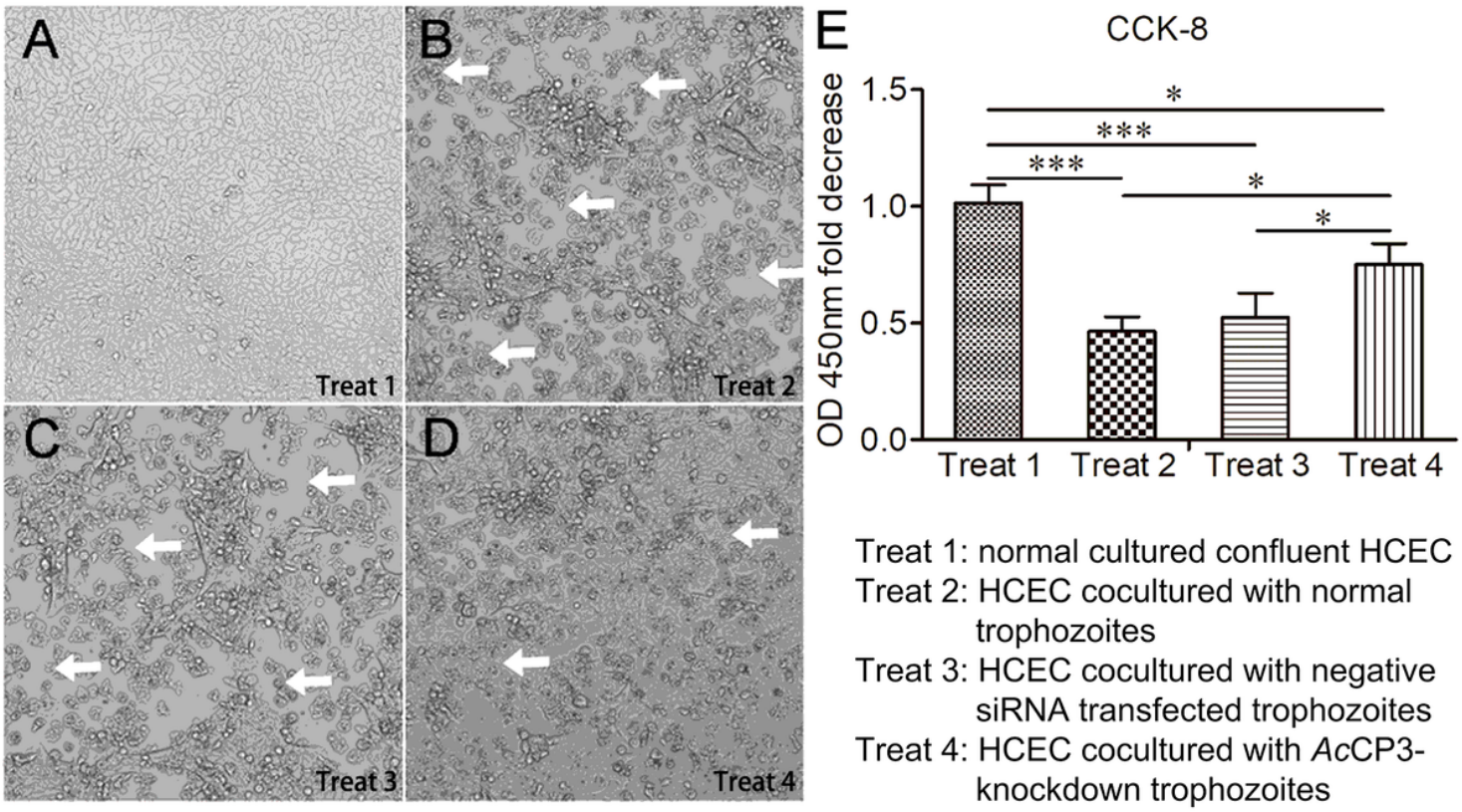

Figure 5

Microscopy and CCK-8 demonstrating that AcCP3 gene knockdown trophozoites exhibited a decrease in Acanthamoeba-mediated HCEC cytotoxicity. (A to D) Pictures took under light microscopy. (A) normal cultured confluent HCEC (treat 1). (B) HCEC cocultured with normal trophozoites (treat 2). (C) HCEC cocultured with negative siRNA transfected trophozoites (treat 3). (D) HCEC cocultured with AcCP3-knockdown trophozoites (treat 4). (E) OD 450 nm fold decrease compared to treat 1 HCEC cells. Independent experiments were repeated three times. Vertical bars indicate SD. *P < 0.05; ***P < 0.001

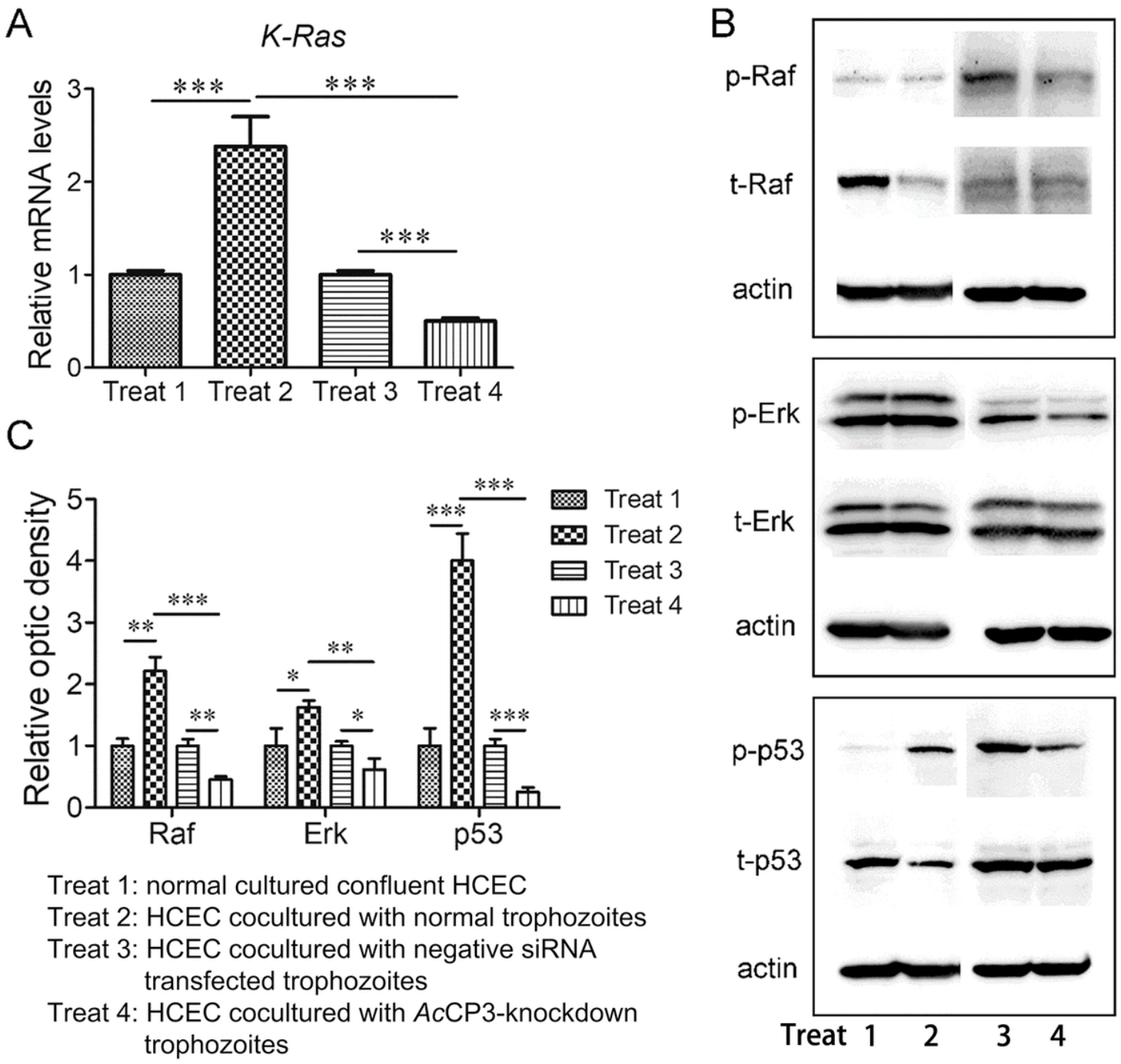

Figure 6

AcCP3-siRNA significantly decreased the phosphorylation levels of Raf, Erk, and p53 in Acanthamoeba-mediated HCEC cells. Four groups of HCEC were treated the same as above. (A) qRT-PCR analyzed the mRNA expression level of K-Ras gene. (B) Western blot analysis the phosphorylation level of Raf, Erk and p53 proteins in HCEC cells. (C) The optic densities of phosphorylation level of Raf, Erk and p53 proteins in HCEC cells. Normal cultured confluent HCEC (treat 1), HCEC cocultured with normal trophozoites (treat 2), HCEC cocultured with negative siRNA transfected trophozoites (treat 3), HCEC cocultured with AcCP3-knockdown trophozoites (treat 4). Independent experiments were repeated three times. Vertical bars indicate SD. *P < 0.05; **P < 0.01; ***P < 0.001.

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