Molecular characterization of an actin depolymerizing factor from *Cryptocaryon irritans*

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SUMMARY

Actin depolymerizing factors regulate actin dynamics involved in cellular processes such as morphogenesis, motility, development and infection. Here, a novel actin depolymerizing factor gene (*CiADF*$_2$) was cloned from the cDNA library of *Cryptocaryon irritans*, a parasitic ciliate causing cryptocaryonosis. The full-length cDNA of *CiADF*$_2$ was 531 bp. Its open reading frame (ORF) was 417 bp, encoding a polypeptide of 138 aa with typical features of the ADF/cofilin family. Reverse transcription-PCR suggested that *CiADF*$_2$ is expressed in all stages of the life cycle. After site-directed mutagenesis of a non-universal genetic code, the ORF was subcloned in *Escherichia coli*. The bacteria were induced with the addition of isopropylthio-*β*-D-galactoside to express a fusion protein of recombinant CiADF$_2$ (rCiADF$_2$) with glutathione *S* transferase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot confirmed the predicted molecular mass of rCiADF$_2$ of 16·2 kDa. A mouse antibody against rCiADF$_2$ recognized native CiADF$_2$, and rCiADF$_2$ reacted with mouse antisera against *C. irritans* trophonts. CiADF$_2$ was abundant in the plasma around cytostomes, suggesting that CiADF$_2$ is involved in ciliate movement. Moreover, rCiADF$_2$ showed F-actin binding and depolymerizing activity. This study will help to clarify the pathogenic biology of the parasite and develop effective control measures for cryptocaryonosis.

Key words: *Cryptocaryon irritans*, actin depolymerizing factor, molecular characterization.

INTRODUCTION

Actin dynamics play a critical role in the maintenance of eukaryotic cell surface structure, cell motility, cytokinesis, development, differentiation, signal transduction, encystation and excystation (Pollard and Borisy, 2003; Bernstein and Bamburg, 2010; Doi *et al*. 2010; Makioka *et al*. 2011). In parasites, actin dynamics can be also involved in invasion to or release from host cells (Sibley, 2004; Mehta and Sibley, 2011). It is regulated by actin-binding proteins, including actin depolymerizing factors (ADFs), which are major regulatory molecules that usually bind to actin filaments (F-actin) to improve their depolymerization into actin monomers (G-actin) for turnover of actin filaments. ADF has been considered a function node in cellular biology (Bernstein and Bamburg, 2010). For example, ADF from *Toxoplasma gondii* increases the re-circulation of F-actin, which is crucial for the development of parasites from tachyzoites to bradyzoites and for their maintenance of cyst structure (Allen *et al*. 1997). *Plasmodium berghei* parasites lacking the *ADF*$_2$ gene developed normally to the ookinete stage and retained their motility, but they exhibited deficiencies in both the ookinete to oocyst and sporozoite to exo-erythrocytic form transformations, suggesting that ADFs play a pivotal role in the morphological regulation of the complex *Plasmodium* life cycle (Doi *et al*. 2010). Over-expression of ADF/cofilin of *Leishmania donovani* impaired flagellum assembly and consequently cell motility by severely impairing the assembly of the paraflagellar rod, without significantly affecting vesicular trafficking or cell growth (Kumar *et al*. 2012). When the *ADF* gene was knocked out, the flagella failed to wave, which resulted in impaired motility, sensation, invasion of host cells and cytokinesis (Tammana *et al*. 2008, 2010). The ADF of *Eimeria tenella* regulates actin-based cell motility, which is critical for protozoa invading their hosts. Compared with the control, ADF-mRNA levels were down regulated by 63·86% in second-generation merozoites of *E. tenella* treated with diclazuril, which implied that the drug impaired parasite invasive ability by affecting its ADF (Xu *et al*. 2008; Zhou *et al*. 2010). Further experiments suggest that ADF/cofilin of *Entamoeba invadens* is involved in the regulation of actin dynamics, which is related to the encystation and excystation of the parasitic protozoa (Makioka *et al*. 2011).

Cryptocaryon irritans is a ciliated parasite that causes cryptocaryonosis in marine fish, which severely affects

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mariculture in Southeast China. As described by Colorni and Burgess (1997), the life cycle of *C. irritans* is divided into 4 stages, i.e. trophont, protomont, tomont and theront stages. Trophonts parasitize in the epidermis of the skins and gills of various marine fishes and feed on interstitial fluid and cell debris through cytostomes, which result in pathological changes on hosts. When trophonts mature or are irritated by the death of their hosts they drop off their hosts into marine water and become mobile protomonts, which develop to tomonts post-encystation. Tomonts are the productive cysts with strong resistance to the environment and with high production rate, at which more than 200 infective therons are hatched from a single tomont. Newly hatched therons penetrate into skin, gills and eyes of fish hosts to develop into parasitic trophonts. Therefore, it would be crucial to prevent fishes from infective theront invasion, and to inhibit parasite development from trophonts to tomonts, the reproductive stage of the parasite, to reduce the ciliate population. As ADFs might play critical roles in both aspects, they were cloned from a cDNA library of *C. irritans* trophonts. Here, we report the molecular characterization and bioactivity of $CiADF_2$ (GenBank accession number: JQ906266), which would be useful for the development of effective measures to control outbreaks of cryptocaryonosis.

MATERIALS AND METHODS

Parasites and experimental animals

C. irritans trophonts were collected from the gills of infected *Pseudosciaena crocea* cultured in net cages during a cryptocaryonosis outbreak in the coastal area of Xiapu county, Fujian province, China, on 2 July 2009. The collection was carried out by soaking gills showing visible white spots in sterile seawater for 2 h. After discarding the gills, trophonts were collected in the sediment and allowed to develop into tomonts. Part of the tomonts was sampled for extraction of genomic DNA while part was incubated at 27 °C to encourage the development of therons. On the one hand, the genomic DNA was used as template DNA to amplify the ribosomal DNA internal transcribed spacer 1 sequence (rDNA-*ITS*–1) for identification of the parasite strain used in this study. Results of the identification have been given by Sun *et al.* (2011) and showed the strain to be the same as strains Chiayi and PYH4·12 based on the rDNA-*ITS*–1 sequences (Diggles and Adlard, 1997; Sun *et al.* 2006). On the other hand, newly hatched therons were used to infect *Sebastiscus marmoratus* maintained in an aquarium to continue the life cycle of the parasite. All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals outlined by Fujian Normal University.

Collection of the parasites at different stages

Therons were harvested by centrifugation at 1000 ***g*** for 5 min at 4 °C. Trophonts, the parasitic stage of the ciliate, were collected from *S. marmoratus* 3 days post-infection by scraping fish body surfaces with a glass slide in a dish of sterile seawater. Motile trophonts and protomonts were harvested individually and immediately using a pipette. Tomonts were collected 3 days post-infection by placing dishes on the bottom of the aquarium for 24 h, to allow mature trophonts to drop from the fish bodies into the dishes and to encyst. Parasites at each stage were washed in sterile seawater and stored immediately at −80 °C for further use.

Gene isolation from the cDNA library

The cDNA library was constructed as described previously (Huang *et al.* 2012). To sequence expression sequence tags (ESTs), recombinant λTripIE × 2 was converted into recombinant plasmid λTripIE × 2 by transduction of the recombinant phage into *E. coli* BM25·8 strain expressing Cre recombinase. EST sequences were aligned with genes deposited in GenBank to detect clones carrying putative *ADF* genes. Two clones carrying a putative *ADF* gene were isolated and designated as $CiADF_2$.

Examination of $CiADF_2$ mRNA expression

For detecting the expression at different stages of life cycle, total RNA was extracted from the parasites at each stage by using a QIAGEN RNeasy Mini kit (QIAGEN) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed using an RNA amplification kit according to the manufacturer's instructions (Takara Bio Inc.) with 500 ng of total RNA as a template for each 10 μl of reaction mixture. The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 30 min, 95 °C for 5 min, and 5 °C for 5 min. The PCR conditions were 25 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 60 s, followed by 10 min of extension at 72 °C. The sequences of the gene-specific primers for amplification of $CiADF_2$ were 5′-ATGGTATCTACTGGAGTC-3′ and 5′-TCACATTAATAATTCCTTT-3′. An *actin* gene isolated from *C. irritans* (*CiActin*, GenBank Accession number: JN399999) was used as a control and amplified using the following primers, 5′-ATGGCCGAAGACTAACAAGCAG-3′ and 5′-TCAGAAGCATTTTCTGTGTACA-3′.

Site-directed mutagenesis of $CiADF_2$ gene by PCR

One TAA codon was found in the open reading frame (ORF) of $CiADF_2$. To allow gene expression in bacteria, the T in the TAA codon was mutated into

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C with a GeneTailor™ Site-Directed Mutagenesis System (Invitrogen) according to the manufacturer's instructions. Briefly, pTripIE × 2/*CiADF*$_2$ was methylated by DNA methylase and then amplified in a mutagenesis reaction with the overlapping primers 5′-TTATCATTGATATCTGTGGACAAAGAG AAGA-3′ and 5′-TCCACAGATATCAATGATAA TGTTATTTCC-3′ (the underlined nucleotide was the target mutation). The mutagenesis reaction was carried out in a thermocycler. The cycling parameters were 20 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 68 °C for 1 min, followed by 10 min of extension at 68 °C. The mutagenesis mixture was then transformed into wild type *E. coli*, in which linear mutated DNA was circulated and the methylated plasmid DNA template was digested by inherent *Mcr*BC endonuclease.

Su cloning of the modified ORF from pTripIE × 2/ CiADF$_2$ *into pGEX-4T-1*

The modified ORF was amplified using a set of oligonucleotide primers, 5′- CGCGGATCCATGG TATCTACTGGAGTC -3′ and 5′-CGGAATTC TCACATTAATAATTCCTT T-3′ (the underlined sections in each primer indicate *Bam*HI and *Eco*RI recognition sites, respectively), with pTripIE × 2/*CiADF*$_2$ as the template DNA. The PCR product was double-digested with *Bam*HI and *Eco*RI (TaKaRa, Otsu, Japan), followed by ligation into the *Bam*HI-*Eco*RI site of pGEX-4T-1 and then transformation into *E. coli* DH5*α*.

Expression, extraction and purification of recombinant CiADF$_2$ *protein*

E. coli colony containing plasmid pGEX-4T/ *CiADF*$_2$ was cultured in LB medium (1% Bacto Tryptone, 0·5% yeast extract, 1% NaCl, and 0·1% 5 M NaOH) with ampicillin sodium (50 *μ*g/ml) at 37 °C. When the optical density at 600 nm reached between 0·3 and 0·5, *E. coli* was induced to express the recombinant CiADF$_2$ fusion protein (G-rCiADF$_2$) by the addition of 0·5 mM isopropyl-*β*-d-thiogalactopyranoside and incubation for another 4 h. G-rCiADF$_2$ was extracted and purified using glutathione sepharose 4B (GE Healthcare Life Sciences, Uppsala, Sweden) according to the manufacturer's instructions. The recombinant CiADF$_2$ protein (rCiADF$_2$) was further purified by thrombin cleavage to remove glutathione-*S*-transferase (GST).

Production of antibodies against rCiADF$_2$ *and against lysate of trophonts*

Fifteen specific pathogen-free Kunming mice (Shanghai Laboratory Animal Co. Ltd, China) were divided into 3 groups (5 per group) and immunized with rCiADF$_2$ (100 *μ*g/mouse), GST (100 *μ*g/mouse) and lysate of trophonts, respectively. Mice were injected intraperitoneally with the corresponding protein emulsified with equal volume (200 *μ*l/mouse) of Freund's complete adjuvant (Sigma-Aldrich, St Louis, USA). Two booster immunizations were performed using the same dose of antigen emulsified with an equal volume of Freund's incomplete adjuvant (Sigma-Aldrich). The intervals between the immunisations were both 14 days. Ten days after the last immunization, blood was obtained by cardiac puncture, sera against rCiADF$_2$, sera against GST and sera against lysate of trophonts were then separated from the blood cells, respectively. They were stored at −30 °C for further use.

Expression of the native CiADF$_2$ *and antigenicity of rCiADF*$_2$ *detected by Western blot analysis*

To detect the expression of native CiADF$_2$ and immunogenicity of rCiADF$_2$, cell lysates of trophonts/protomonts, tomonts and theronts were sampled and denatured at 100 °C for 5 min in a sample buffer (62·5 mM Tris-HCl at pH 6·8, 2% SDS, 5% *β*-mercaptoethanol, 10% glycerol, and 0·02% bromophenol blue) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% acrylamide gels as described previously (Huang *et al*. 2003). Subsequently, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, USA) and analysed with mouse antibody against rCiADF$_2$ or against GST as the primary antibody (1:100) and horseradish peroxidase-conjugated goat anti-mouse IgG antibody (ICN Biochemicals, Aurora, USA) as the secondary antibody (1: 4000). The reactions were visualized with 3,3′-diaminobenzidine (0·5 mg/ml) and 0·03% H_2O_2. On the other hand, to determine the reactigenicity of rCiADF$_2$, purified rCiADF$_2$ and lysate of trophonts (for positive control) were subjected to Western blot analysis with mouse antibody against the lysate of trophonts as the first antibody. The secondary antibody and the substrate were the same as those mentioned above.

Localization of native CiADF$_2$ *by immune fluorescent antibody testing (IFAT)*

Newly hatched theronts were fixed in 0·15% formaldehyde for 30 min, and then spun down at 800 ***g*** for 5 min. After 3 washes with distilled water, the theronts were pipetted onto glass slides and dried on air. They were then incubated for 30 min with mouse antibody against rCiADF$_2$ or against GST (1:100), respectively, followed by incubation with Alexa 488-conjugated goat anti-mouse IgG antibody

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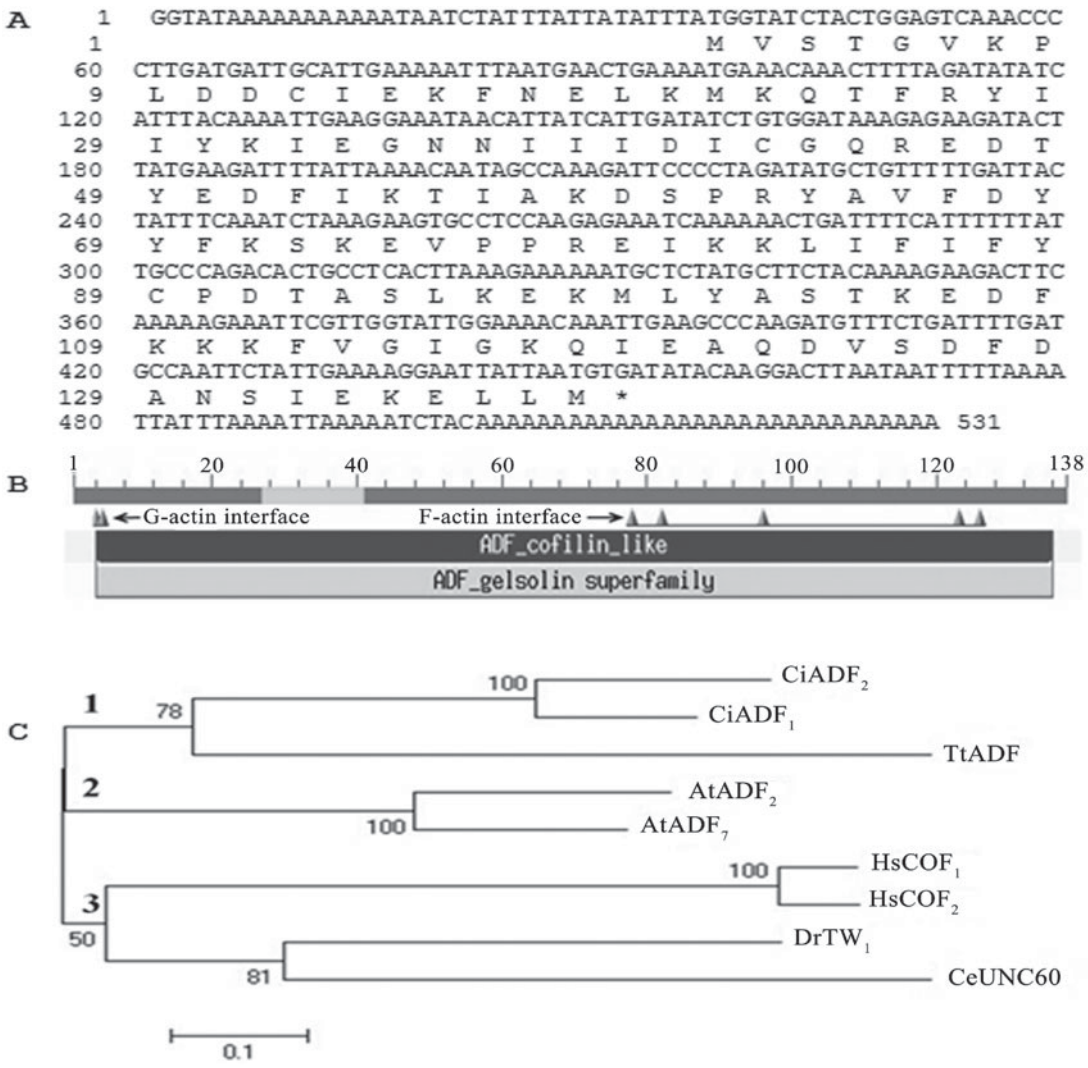

Fig. 1. Characterization of the *CiADF*$_2$ gene and its deduced polypeptide. (A) The full-length cDNA sequence of *CiADF*$_2$ and its deduced amino acid sequence. (B) Schematic structure of CiADF$_2$ showing the ADF/Cofilin-like domain and G-actin and F-actin interfaces. (C) Phylogenetic relationship of CiADF$_2$ with ADFs from several different organisms. AtADF$_2$ (AAB03697) and AtADF$_7$ (AEE85080) are from *Arabidopsis thaliana*; DrTWI (NP_477034) from *Drosophila twinstar*; CeUNC60 (NP_503427) from *Caenorhabditis elegans*; HsCOF$_1$ (NP_005498) and HsCOF$_2$ (NP_068733) from *Homo sapiens*; TtADF (BAH84775) from *Tetrahymena thermophila*.

(1:100 in PBS containing 3% fetal bovine serum) (Sigma) at 37 °C for 30 min. The results were observed under a fluorescence microscopy and photographed.

Detection on bioactivity of CiADF$_2$ *by pull-down assay using analytical ultracentrifugation*

An actin-binding protein assay kit (Cytoskeleton, Denver, USA) was used to detect the F-actin binding and depolymerizing activity of rCiADF$_2$ according to the manufacturer's instruction. Briefly, G-actin was first polymerized to F-actin, which was then mixed with rCiADF$_2$, α-actinin (positive control) and bovine serum albumin (negative control), respectively. The 3 different protein mixtures and F-actin alone, rCiADF$_2$ alone, α-actinin alone and BSA alone were all ultracentrifuged at 150 000 ***g*** for 1·5 h. After the separation of the supernatants from the pellets, each was sampled and subjected to SDS-PAGE analysis.

RESULTS

Characterization of gene sequence

From the cDNA library of *C. irritans* trophonts, 14 clones carrying putative ADF genes were isolated and divided in 2 different ADF genes, *CiADF*$_1$ and *CiADF*$_2$. Reported here is *CiADF*$_2$ with the GenBank accession number JQ906266. As shown in Fig. 1A, the full-length cDNA of *CiADF*$_2$ was 531 bp with an open reading frame (ORF) of 417 bp, which encoded a polypeptide of 138 aa with a predicted molecular weight of 16·2 kDa. There was 1 TAA codon in the ORF, which is a termination

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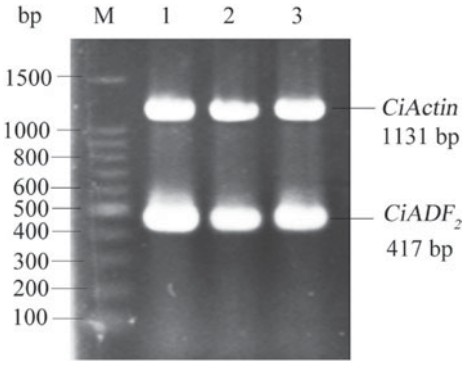

Fig. 2. *CiADF*$_2$ mRNA expression was detected in parasites at different stages using reverse transcription-PCR. M, DNA ladder; lanes 1–3: RT-PCR products amplified from trophonts/protomonts, tomonts and theronts, respectively. Two sets of oligonucleotides were used to amplify *CiADF*$_2$ and *CiActin* (control), respectively.

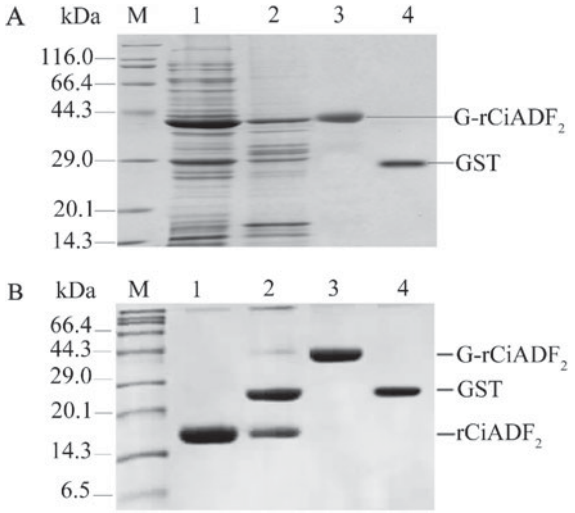

Fig. 3. Expression and purification of the recombinant CiADF$_2$ protein as detected by SDS-PAGE. (A) Expression and purification of the recombinant fusion protein, G-rCiADF$_2$. M, proteins with standard molecular masses; Lanes 1 and 2: soluble and insoluble fractions of the bacterial lysate, respectively, Lane 3: purified G-rCiADF$_2$; Lane 4: GST. (B) Removal of GST from G-rCiADF$_2$ by thrombin cleavage. M, proteins with standard molecular masses; Lane 1: rCiADF$_2$ after cleavage of GST, Lane 2: rCiADF$_2$ and GST, Lane 3: G-rCiADF$_2$ fusion protein, Lane 4: GST.

codon in the universal genetic code, but encodes glutamine in the ciliate genetic code. The ratio of AT to CG in *CiADF*$_2$ ORF was 71 to 29. The deduced polypeptide contained an ADF domain with amino acids typical for interactions with G-actin and F-actin. As shown in Fig. 1B, the 4th and 5th amino acids were a G-actin binding area, and the fragment from the 78th to 126th amino acids was an F-actin binding area. There were 23 acidic amino acids, 23 basic amino acids, 54 hydrophobic amino acids and 38 polar amino acids in the polypeptide, with a pI of 6·36. Ser3 and Tyr68 were found in CiADF$_2$, which are typical for the ADF/cofilin protein family. There was 1 α-helix in a putative F-actin binding area, which is also a conserved trait of the family. The phylogenetic relationship of *CiADF*$_2$ with 9 other ADF/cofilins from different species is shown in Fig. 1C, which indicated that *CiADF*$_2$ is closely related to *CiADF*$_1$ (GenBank accession number: JQ906265) and an *ADF* from *Tetrahymena thermophila* with a similarity of 35%.

mRNA expression profile of CiADF$_2$ *detected by RT-PCR*

As shown in Fig. 2, in all stages of *C. irritans* 2 specific bands were found in the RT-PCR product. The lower band was around 417 bp, corresponding to the predicted size of *CiADF*$_2$ ORF, while that of the upper bands was approximately 1131 bp, consistent with that of *CiActin* ORF.

Recombinant CiADF$_2$ *expressed in E. coli*

The modified *CiADF*$_2$ gene was expressed as the recombinant fusion protein with a GST tag, which was designated as G-rCiADF$_2$. As shown in Fig. 3A, G-rCiADF$_2$ was expressed in both soluble and insoluble fractions with a molecular mass of 42·2 kDa. Glutathione-Sepharose 4B combined with thrombin cleavage to remove the GST tag were used to purify rCiADF$_2$ from the soluble fraction. As shown in Fig. 3B, the molecular mass of rCiADF$_2$ was 16·2 kDa, which corresponded to the predicted result.

Expression of native CiADF$_2$ *and antigenicity of rCiADF*$_2$ *detected by Western blot analysis*

The purified rCiADF$_2$ was used to immunize SPF mice. The resulting polyclonal antibodies against rCiADF$_2$ were applied to detect native CiADF$_2$ protein in *C. irritans*, with a polyclonal antibody against GST as a negative control. As seen in Fig. 4A, a specific positive reaction band presented in each well with lysates of trophonts/protomonts, tomonts and theronts, respectively, which suggested that rCiADF$_2$ was immunogenic to mount an immune reaction in mice. The antibody against rCiADF$_2$ could recognize native CiADF$_2$, the molecular mass of which corresponded to the calculated molecular weight, 16·2 kDa, and the native CiADF$_2$ was expressed at translation level in the entire life cycle of *C. irritans*. On the other hand, as shown in Fig. 4B, polyclonal antibodies against the cell lysate of *C. irritans* trophonts/protomonts reacted not only with native trophont proteins, but also with

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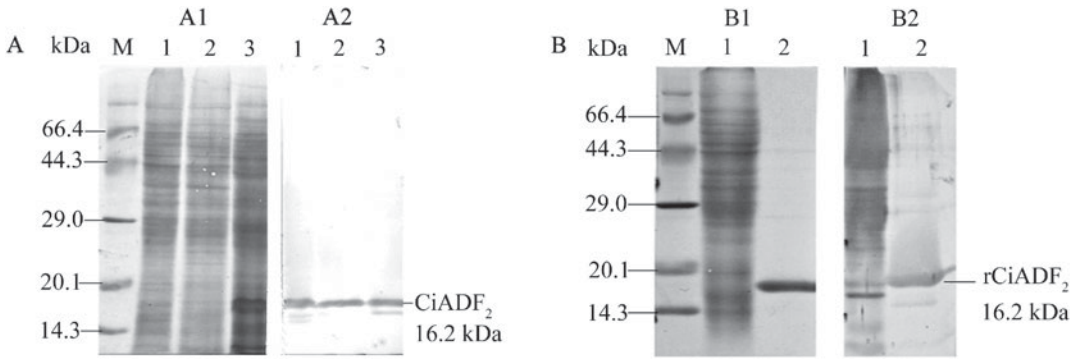

Fig. 4. Expression of native $CiADF_2$ and antigenicity of $rCiADF_2$ as detected by Western blot analysis. (A) Mouse antiserum against $rCiADF_2$ recognized the native $CiADF_2$, which was found in lysates of trophonts/protomonts, tomonts, and theronts. A1, proteins stained with amino black 10B. A2, the same proteins as those shown in A1 were analysed with mouse antiserum against $rCiADF_2$ as the first detection reagent and horseradish peroxidase-conjugated goat anti-mouse IgG antibody as the secondary detection reagent (1: 4000). The reaction was visualized with 3,3′-diaminobenzidine and H_2O_2. M, proteins with standard molecular masses, Lanes 1–3: lysates of trophonts/protomonts, tomonts and theronts, respectively. (B) $rCiADF_2$ reacted with mouse serum against lysate of *Cryptocaryon irritans* trophonts. B1, proteins stained with amino black 10B. B2, the same proteins as those shown in B1 were analysed with serum from mice immunized with native proteins from the lysate of trophonts as the first detection reagent and horseradish peroxidase-conjugated goat anti-mouse IgG antibody as the secondary detection reagent (1: 4000) to detect the reactigenicity of $rCiADF_2$. M, proteins with standard molecular masses. Lane 1, native proteins from the lysate of *C. irritans* trophonts, Lane 2, $rCiADF_2$.

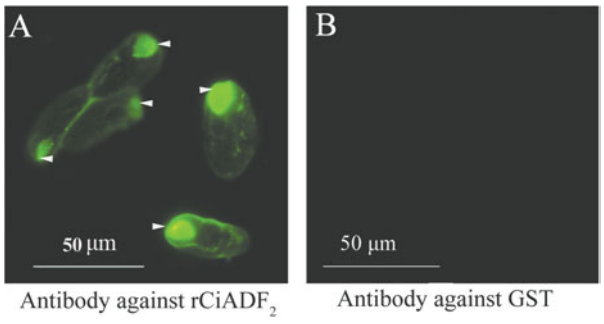

Fig. 5. Localization of native $CiADF_2$ in theronts of *Cryptocaryon irritans* as detected by an immunofluorescent antibody test. Serum from mouse immunized with $rCiADF_2$ (A) or serum from mouse immunized with GST (B) was used as the first antibody. Alexa 488-conjugated antibody against mouse IgG was used as the secondary antibody. The cytostomes of theronts in (A) were pointed out by the white arrows.

$rCiADF_2$, suggesting that the reactigenicity of the recombinant protein.

Localization of native $CiADF_2$ protein

The polyclonal antibody against $rCiADF_2$ was used in an immunofluorescent antibody test (IFAT) to localize the native protein, with a polyclonal antibody against GST as a negative control. The results presented in Fig. 5 demonstrated that $CiADF_2$ was scattered in the cytosol and primarily localized beneath the plasma membrane. However, it was especially enriched in a region about one-third to anterior of the organism, where the cytostome was localized.

F-actin binding and depolymerizing activities of $rCiADF_2$

The activities of actin binding and depolymerization of $rCiADF_2$ were detected by using a pull-down assay combined with analytical ultracentrifugation. BSA and the known actin-binding protein α-actinin were used as the negative and positive controls, respectively. Each of them was incubated with or without F-actin for 30 min and followed by ultracentrifugation. As shown in Fig. 6, α-actinin, BSA, and $rCiADF_2$ were all found in supernatants when they were not co-existing with F-actin, while F-actin alone was found in the sediment. However, after incubation with F-actin, approximately half of the α-actinin and $rCiADF_2$, but none of the BSA, were presented in the sediments post-ultracentrifugation, which implicated F-actin binding activity of $rCiADF_2$. Moreover, in the supernatant from the reaction tube of $rCiADF_2$ plus F-actin, the actin amount was slightly increased when it was compared with those from the tube of F-actin alone and the tube of BSA plus F-actin, which suggested that $rCiADF_2$ partially depolymerized F-actin into G-actin, which presented in the supernatant after ultracentrifugation.

DISCUSSION

In this study, an *ADF* gene was cloned from a *C. irritans* trophont library. The sequence analysis revealed that *$CiADF_2$* and its product had typical characteristics of ADF/cofilin, such as Ser3 and Tyr68, which are G-actin and F-actin binding sites respectively. Therefore, the activity of $CiADF_2$ may

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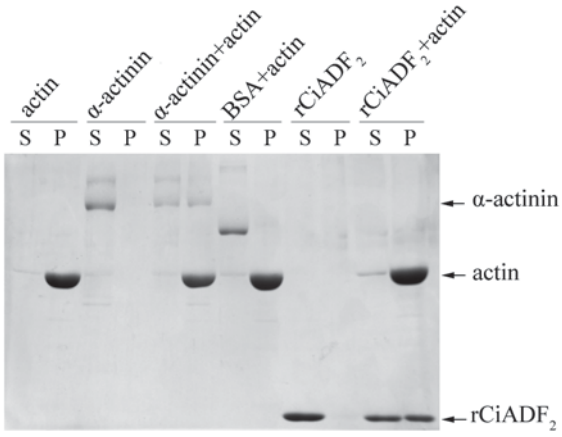

Fig. 6. F-actin binding assay of $rCiADF_2$. Each of the following proteins, α-actinin (positive control), bovine serum albumin (BSA, negative control) and $rCiADF_2$, was incubated with or without F-actin for 30 min, respectively. They were then centrifuged at 150 000 ***g*** for 1·5 h at 24 °C. Subsequently, the supernatants (S) and pellets (P) were collected for each reaction. They were sampled respectively, separated on an SDS-PAGE and stained with 0·1% Coomassie blue. The result showed that approximately half of α-actinin and $rCiADF_2$, but none of the BSA, were co-precipitated with F-actin. Moreover, in the supernatant from the reaction of $rCiADF_2$ plus F-actin, the amount of actin was a little more than those from the reactions of BSA plus F-actin, and F-actin alone. The result suggested that $rCiADF_2$ could bind to and sever F-actin.

be modulated in a way typical of the ADF family, i.e. by phosphorylation of Ser3 with LIM kinase or TES kinase, which would lead to most ADF/cofilin proteins losing their depolymerization activity, and dephosphorylation of Ser3 with SSH, which would restore the depolymerizing activity. Tyr68 is highly conserved in the family and its phosphorylation may enhance protein ubiquitination and degradation, so as to reduce the overall levels of ADF in the cells. *$CiADF_2$* is closely related to an ADF from *Tetrahymena thermophila* (TtADF, Adf73p) with a similarity of 35%, although TtADF has no typical Ser3 and Tyr68, and no genes encoding LIM kinase and SSH were found in the genomic database of *T. thermophila*.

There are non-universal genetic codes in *C. irritans* genes. In *$CiADF_2$*, one TAA codon was found to encode glutamine. However, it would be deciphered as a stop codon by bacterial cells so that the downstream sequence fragment would not be translated. In this study, the TAA codon was mutated successfully into CAA, the universal codon for glutamine. The results showed that the mutated gene was expressed well in bacterial cells. The purified recombinant protein mounted an immune reaction in mice, the antisera of which recognized the native $CiADF_2$. On the other hand, $rCiADF_2$ reacted to antisera against the cell lysate of *C. irritans* trophonts. These results suggested antigenicity of $rCiADF_2$. Judging from the ADF structure, it is a cytosolic protein. However, parasitic trophonts of *C. irritans* have been reported to feed on interstitial fluid and cell debris through cytostomes, which would make uptake of host antibodies against recombinant $CiADF_2$ into the parasitic cytosol possible. Therefore, anti-$rCiADF_2$ might be a candidate for the development of vaccines or diagnostic reagents.

A bioactivity assay showed that $rCiADF_2$ was not only capable of binding to F-actin, but also capable to depolymerize F-actin, which might help to elucidate the actin dynamics during the development of *C. irritans* and its invasion of fish hosts.

Cellular movements are powered by the assembly and disassembly of actin filaments. The assembly of actin filaments at the leading edge of motile cells pushes the plasma membrane forward. $CiADF_2$ was located in the cytoplasm and was especially abundant at one end of the ciliate around the cytostome. It has been reported that microtubules exist around the ciliate cytostome. However, in the case of *C. irritans*, there might also be abundant actin in this area. The turnover of F-actin and G-actin under regulation of $CiADF_2$ and some other actin-binding proteins might play an important role in the movement of the ciliate.

In conclusion, this study improves our understanding of the pathogen biology of *C. irritans* and the control of cryptocaryonosis.

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REFERENCES

Allen, M. L., Dobrowolski, J. M., Muller, H., Sibley, L. D. and Mansour, T. E. (1997). Cloning and characterization of actin depolymerising factor from *Toxoplasma gondii*. *Molecular and Biochemical Parasitology* **88**, 43–52.

Bernstein, B. W. and Bamburg, J. R. (2010). ADF/cofilin: a functional node in cell biology. *Trends in Cell Biology* **20**, 187–195.

Colorni, A. and Burgess, P. (1997). *Cryptocaryon irritans* Brown 1951, the cause of white spot disease in marine fish: an update. *Aquarium Sciences and Conservation* **1**, 217–238.

Diggles, B. K. and Adlard, R. D. (1997). Intraspecific variation in *Cryptocaryon irritans*. *The Journal of Eukaryotic Microbiology* **44**, 25–32.

Doi, Y., Shinzawa, N., Fukumoto, S., Okano, H. and Kanuka, H. (2010). ADF2 is required for transformation of the ookinete and sporozoite in malaria parasite development. *Biochemical and Biophysical Research Communications* **397**, 668–672.

Huang, X., Sun, Z., Guo, G., Zheng, C., Xu, Y., Yuan, L. and Liu, C. (2012). Cloning and characterization of a surface antigen CiSA-32·6 from *Cryptocaryon irritans*. *Experimental Parasitology* **130**, 189–194.

Huang, X., Xuan, X., Yokoyama, N., Xu, L., Suzuki, H., Sugimoto, C., Nagasawa, H., Fujisaki, K. and Igarashi, I. (2003). High-level expression and purification of a truncated merozoite antigen-2 of *Babesia equi* in *Escherichia coli* and its potential for immunodiagnosis. *Journal of Clinical Microbiology* **41**, 1147–1151.

Kumar, G., Srivastava, R., Mitra, K., Sahasrabuddhe, A. A. and Gupta, C. M. (2012). Overexpression of S4D mutant of *Leishmania donovani* ADF/Cofilin impairs flagellum assembly by affecting actin dynamics. *Eukaryotic Cell* **11**, 752–760.

Makioka, A., Kumagai, M., Hiranuka, K., Kobayashi, S. and Takeuchi, T. (2011). *Entamoeba invadens*: identification of ADF/cofilin and their expression analysis in relation to encystation and excystation. *Experimental Parasitology* **127**, 195–201.

Mehta, S., and Sibley, L. D. (2011). Actin depolymerizing factor controls actin turnover and gliding motility in *Toxoplasma gondii*. *Molecular Biology of the Cell* **22**, 1290–1299.

Pollard, T. D., and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453–465.

Sibley, L. D. (2004). Intracellular parasite invasion strategies. *Science* **304**, 248–253.

Sun, H. Y., Zhu, X. Q., Xie, M. Q., Wu, X. Y., Li, A. X., Lin, R. Q. and Song, H. Q. (2006). Characterization of *Cryptocaryon irritans* isolates from marine fishes in Mainland China by ITS ribosomal DNA sequences. *Parasitology Research* **99**, 160–166.

Sun, Z., Zheng, C., Wu, X., Guo, G., Wang, Y. and Huang, X. (2011). The strain and life-cycle of *Cryptocaryon irritans* isolated from *Pseudosciaena crocea* cultured in Xiapu, Fujian. *Journal of Fujian Normal University (Natural Science Edition)* **27**, 101–108.

Tammana, T. V., Sahasrabuddhe, A. A., Bajpai, V. K., Gupta, C. M. (2010). ADF/cofilin-driven actin dynamics in early events of *Leishmania* cell division. *Journal of Cell Science* **123**, 1894–1901.

Tammana, T. V., Sahasrabuddhe, A. A., Mitra, K., Bajpai, V. K. and Gupta, C. M. (2008). Actin-depolymerising factor, ADF/cofilin, is essentially required in assembly of *Leishmania flagellum*. *Molecular Microbiology* **70**, 837–852.

Xu, J. H., Qin, Z. H., Liao, Y. S., Xie, M. Q., Li, A. X. and Cai, J. P. (2008). Characterization and expression of an actin-depolymerising factor from *Eimeria tenella*. *Parasitology Research* **103**, 263–270.

Zhou, B. H., Wang, H. W., Xue, F. Q., Wang, X. Y., Yang, F. K., Ban, M. M., Xin, R. X. and Wang, C. C. (2010). Actin-depolymerising factor of second-generation merozoite in *Eimeria tenella*: clone, prokaryotic expression, and diclazuril-induced mRNA expression. *Parasitology Research* **106**, 571–576.

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