Molecular Characterization and Functional Analysis of a Novel Calcium-Dependent Protein Kinase 4 from *Eimeria tenella*

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**Abstract**

*Eimeria tenella* is an obligate intracellular parasite that actively invades cecal epithelial cells of chickens. The basis of cell invasion is not completely understood, but some key molecules of host cell invasion have been discovered. This paper investigated the characteristics of calcium-dependent protein kinase 4 (EtCDPK4), a critical molecule in *E. tenella* invasion of host cells. A full-length *EtCDPK4* cDNA was identified from *E. tenella* using rapid amplification of cDNA ends. *EtCDPK4* had an open reading frame of 1803 bp encoding a protein of 600 amino acids. Quantitative real-time PCR and western blotting were used to explore differences in *EtCDPK4* transcription and translation in four developmental stages of *E. tenella*. *EtCDPK4* was expressed at higher levels in sporozoites, but translation was higher in second-generation merozoites. In *vitro* invasion inhibition assays explored whether *EtCDPK4* was involved in invasion of DF-1 cells by *E. tenella* sporozoites. Polyclonal antibodies against recombinant *EtCDPK4* (r*EtCDPK4*) inhibited parasite invasion, decreasing it by approximately 52%. Indirect immunofluorescence assays explored *EtCDPK4* distribution during parasite development after *E. tenella* sporozoite invasion of DF-1 cells *in vitro*. The results showed that *EtCDPK4* might be important in sporozoite invasion and development. To analyze *EtCDPK4* functional domains according to the structural characteristics of *EtCDPK4* and study the kinase activity of r*EtCDPK4*, an *in vitro* phosphorylation system was established. We verified that r*EtCDPK4* was a protein kinase that was completely dependent on Ca\(^{2+}\) for enzyme activity. Specific inhibitors of r*EtCDPK4* activity were screened by kinase activity *in vitro*. Some specific inhibitors were applied to assays of DF-1 cell invasion by *E. tenella* sporozoites to confirm that the inhibitors functioned *in vitro*. W-7, H-7, H-89, and myristoylated peptide inhibited DF-1 invasion by *E. tenella* sporozoites. The experimental results showed that *EtCDPK4* may be involved in *E. tenella* invasion of chicken cecal epithelial cells.
Introduction

The protozoan phylum Apicomplexa comprises thousands of obligate intracellular parasites, many of which cause significant human and animal health problems. For example, *Toxoplasma gondii* infects approximately one-third of the global human population and causes severe disease in immunocompromised patients and pregnant women [1]. Other examples are *Plasmodium falciparum*, the causative agent of malaria, which causes more than 1 million deaths per year [2] and *Eimeria* species, the protozoan parasites that cause the severe intestinal disease coccidiosis [3, 4]. Avian coccidiosis is a major disease of poultry caused by parasitic *Eimeria* species including *Eimeria tenella*, *Eimeria necatrix*, *Eimeria acervulina*, *Eimeria maxima*, *Eimeria brunetti*, *Eimeria mitis* and *Eimeria praecox*. Coccidiosis causes severe economic losses in the poultry industry every year [5]. *Eimeria* have complex life cycles and need to invade the intestinal epithelium of chickens to develop and propagate. The invasion of host gut epithelial cells by *Eimeria* species is a complex, multistep process that begins with the apical attachment of the parasite to the host cell. This is followed by rapid internalization to form an intracellular, parasitophorous vacuole (PV) that encloses the newly invaded parasite, enabling its survival within the host [6]. To perpetuate the infection, *Eimeria* need to egress from infected cells and then rein invade uninfected cells. In response to these events, parasites have developed regulatory mechanisms for self-proliferation and invasion. During these processes, specialized secretory organelles known as micronemes, rhoptries and dense granules deliver cargo proteins in a coordinated fashion. Secreted proteins are thought to be central to invasion and the establishment of infection [7, 8]. However, secretion by these organelles is controlled by intracellular calcium as a second messenger, which is important in signal transduction cascades, including for protein secretion, gliding motility, invasion of and egress from host cells, proliferation and differentiation [9].

In Apicomplexan parasites, calcium-dependent protein kinases (CDPKs) are main receptors of Ca^{2+} signals [10, 11]. CDPKs have been identified throughout the plant kingdom and in some protozoans, but not in animals or fungi [12]. CDPKs have two key domains, a Ser/Thr kinase domain and an EF-hand-type calcium-binding domain. They also contain an N-terminal variable domain, an auto-inhibitory junction region and a C-terminus [13]. The N-terminal domain shows the highest sequence divergence among CDPKs and often contains myristoylation or palmitoylation sites that are believed to be associated with subcellular targeting [14]. The C-terminal domain is also variable and differs in lengths and amino acid compositions among CDPKs. The N- and C-terminal variable domains are suggested to determine the specific function of individual CDPKs [15].

Increasing evidence suggests that CDPKs control important physiological events in Apicomplexan parasite life cycles. For example, conditional suppression of *T. gondii* CDPK1 (*TgCDPK1*) weakens microneme protein secretion, parasite gliding motility, host cell invasion and egress ability [16, 17]. *PbCDPK4* from *Plasmodium berghei*, an ortholog of *TgCDPK1*, regulates cell cycle progression in the male gametocyte [18]. Genetic disruption of *TgCDPK3* demonstrates that it has a regulatory function in parasite physiology in addition to the ionophore-induced egress process [19, 20, 21]. Apicomplexa parasites contain multiple CDPK genes; *Plasmodium* species possess seven [22], Billker *et al.* found that *T. gondii* contains 12 [23]. To our knowledge, only three *E. tenella* CDPK members (*EtCDPK1*, *EtCDPK2* and *EtCDPK3*) have been studied [24, 25], and the physiological functions of most *EtCDPKs* remain unclear. Studies suggest that CDPKs regulate biological functions in Apicomplexa parasites. However, CDPKs regulatory mechanisms and targets remain unclear in Apicomplexa parasites. Nonetheless, this family of CDPKs has received attention as potential drug targets in Apicomplexan parasites.
Because the CDPKs have essential functions in Apicomplexan parasites and are absent in mammalian and avian hosts, CDPKs are promising targets for research on drugs against Eimeria species and related Apicomplexans parasites [26]. Some selective inhibitors against their kinase activity have been generated [27, 28].

We studied new members of the E. tenella CDPK family. We carried out a comprehensive analysis including the cloning, sequencing, protein expression and characterization of a novel EtCDPK4 gene and protein. We provided novel insights into E. tenella invasion and development from a detailed study of the expression of EtCDPK4. This study aimed to provide information for further research and discovery of other members of the CDPK family of E. tenella.

Materials and Methods

Ethics Statement

This research with chickens was approved by the Shanghai Administration Committee of Laboratory Animals (GB14925-2010) and performed in accordance with the Chinese Academy of Agricultural Sciences Institutional Animal Care and Use Committee guidelines.

E. tenella Propagation and Purification

The Shanghai strain of E. tenella was isolated from a sample collected from a chicken farm in Shanghai, China in 1985 and was maintained in our laboratory (Resource Number: CAAS 21111601, Shanghai Veterinary Research Institute innovation team of protozoosis preservation, Chinese Academy of Agricultural Sciences). 60 healthy AA chickens were fed with coccidian-free water and feed. E. tenella was propagated as previously described [29] by passage through 2-week-old coccidian-free chickens. Unsporulated oocysts were obtained from the cecal contents of chickens at 8 days post-infection (p.i.). Some unsporulated oocysts was purified and stored in liquid nitrogen. The rest were incubated in 2.5% potassium dichromate to induce sporulation. After sporulation, oocysts were collected and purified. Sporozoites were prepared from cleaned, sporulated oocysts by excystation in vitro. Second-generation merozoites were collected from ceca at 120 h p.i. from chickens inoculated with $8.0 \times 10^4$ sporulated oocysts per bird. Isolation was carried out as previously described [30]. Isolated sporozoites and second-generation merozoites were stored in liquid nitrogen.

The chicken embryo fibroblast cell line DF-1, derived from East Lansing Line (ELL-0) chicken embryos, was used for infection, inhibition assays and immunofluorescence experiments [31].

Molecular cloning of the EtCDPK4 full-length cDNA by RACE

Total RNA was extracted from E. tenella sporozoites using TRIzol reagent (TaKaRa, Tokyo, Japan) according to the manufacturer’s protocol. RNA quality was analyzed by 1% agarose gel electrophoresis and visualization with Ethidium Bromide staining. Total RNA concentration was quantified by UV spectrophotometry (Eppendorf, Hamburg, Germany). Rapid amplification of cDNA ends (RACE) was carried out with GeneRacer kits (Invitrogen, Carlsbad, CA, USA) to obtain the full-length 5’- and 3’-termini. RACE primers were designed based on gene sequences of the CDPK family (ETH_00010685, http://www.genedb.org/Homepage/Etenella). Approximately 10 μg of total RNA was used to synthesize 5’- and 3’-RACE-Ready cDNA. Sequences were amplified by Touchdown PCR with either 5’- RACE or 3’- RACE gene-specific primers (Table 1) and GeneRacer 5’- or 3’- primers. Nested PCRs were performed with nested gene-specific primers and 5’- or 3’- RACENEST primers (Table 1). Amplification products were subjected to electrophoresis using 1% agarose gels. Single bands were extracted, purified,
cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and propagated in *Escherichia coli* TOP10 (TIANGEN, Beijing, China) competent cells. Clones were sequenced by Shanghai Sunny Biotechnology Co., Ltd. Sequences of 5'-RACE and 3'-RACE were compared with the original *EtCDPK4* open reading frame (ORF) sequence using DNASTar software (Promega) to determine overlap. Full-length cDNA sequences were submitted to GenBank (Accession No. KU925778).

Bioinformatics analysis of *EtCDPK4*

The sequence of full-length *EtCDPK4* cDNA was analyzed. Signal peptide sequences, transmembrane and hydrophobic regions, genetically mobile domains and conservative structure predictions were identified using SignalP (http://www.cbs.dtu.dk/), TMPRED (http://www.ch.embnet.org/), Hydrophobic (http://web.expasy.org/), SMART (http://smart.embl-heidelberg.de/) and CDD (http://www.ncbi.nlm.nih.gov/Structure/) computational tools. Secondary structure, antigen index, flexible region and surface probability of *EtCDPK4* were analyzed using DNASTar.

Expression and Purification of Recombinant CDPK4 Proteins

Total RNA was extracted from sporozoites and the first-strand cDNA templates were generated by M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using oligo (dT) as primer. A 1660-bp (from 303 bp to 1962 bp) fragment of *EtCDPK4* encoding 550 amino acids with a calculated molecular weight of 62.6 kDa was amplified using primers *EtCDPK4UP* and *EtCDPK4LOW* according to the ORF sequence. Sequence-specific primers were designed to contain sites for *Bam*HI in the forward primer (*EtCDPK4UP*) and *Sal*I in the reverse primer (*EtCDPK4LOW*) (Table 1). PCR amplification was performed as follows program: 94˚C for 5 min, 38 cycles 94˚C for 90 s, 56˚C for 45 s and 72˚C 90 s, followed by 10 min at 72˚C. After sequencing, fragments were digested with *Bam*HI and *Sal*I, purified from agarose gels by TIANgel Midi Purification Kits (TIANGEN) and ligated into the corresponding sites of the expression vector pCold I and sequenced. The pCold I-*EtCDPK4* plasmid was transformed into *E. coli* (BL21) to produce a recombinant protein (r*EtCDPK4*) with a 6×-His tag at the N-terminus. r*EtCDPK4* protein was induced by 1.0 mM IPTG (Sigma, St. Louis, MO, USA) at 16˚C. Induced bacterial cells were incubated for 24 h and harvested by centrifugation. Cell pellets were lysed by sonication and the insoluble portion of the pellet was suspended in 10 mM imidazole binding buffer and purified by His Bind Resin (Merck, Darmstadt, Germany). Yield of the affinity-purified protein was estimated using a Biophotometer (Eppendorf, Hamburg,

| Primer ID           | Primer Sequences                  |
|---------------------|-----------------------------------|
| 5'-RACE Primer      | 5'-TCGAAATATCTTCCGGCAGGGCGACTCA-3' |
| 5'-RACEN EST Primer | 5'-CGACTCATCCACTGGGGGAAGCTGCAA-3' |
| 3'-RACE Primer      | 5'-CGAAACTGTCTCGGGGCGGAGACTA-3'   |
| 3'-RACEN EST Primer | 5'-GGCGATGGGCGAGATTGCTGGGGAGCA-3' |
| *EICDPK4UP*         | 5'-CGGATCGAGCCAGGTTGATATTGGGAGGAGTT-3' |
| *EICDPK4LOW*        | 5'-GGGATCCGAGAAACTGCTGGTGGTCTGAG-3' |
| *EICDPK4-RT(UP)*    | 5'-GCCATCAGATTGCAAGGA-3'          |
| *EICDPK4-RT(LOW)*   | 5'-CTCAAGACATCCACATC-3'           |
| 18Sr RNA sense      | 5'-TTGATGCGAGTCTGGTGATCT-3'       |
| 18Sr RNA antisense  | 5'-CTTCGCTGCTGCTTCTTATAGT-3'      |

doi:10.1371/journal.pone.0168132.t001
Germany). Purified rEtCDPK4 protein was visualized by 12% SDS-PAGE. Then purified protein was stored in aliquots at – 20 °C.

**Production of Anti-rEtCDPK4 Serum and Identification of rEtCDPK4**

Two 2-month-old rabbits were immunized with 0.2 mg rEtCDPK4 emulsified in Freund Complete Adjuvant (Sigma) by intraperitoneal injection. Rabbits were boosted three times with Freund’s incomplete adjuvant at 2-week intervals. Eight days after final immunization, polyclonal antibody serum was separated from two rabbits blood.

rEtCDPK4 was resolved by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Bellerica, MA, USA). Western blots were performed according to standard procedures by using rabbit anti-merozoite protein sera (1:500) previously obtained in our lab or anti-**His**-tag monoclonal antibody (1:2000). Native rabbit IgG (1:1000) was the negative control. IRDye 800CW goat anti-rabbit IgG (LI-COR, Lincoln, NE, USA) and IRDye 680RD donkey anti-mouse IgG (1:25,000) (LI-COR, Lincoln, NE, USA) were used as the secondary antibody. Indirect Enzyme Linked Immunosorbent Assay (ELISA) was used to determine rabbit anti-rEtCDPK4 serum titers.

**EtCDPK4 Transcription and Translation Analysis in E. tenella Life Stages**

Total RNAs isolated from four life stages of *E. tenella* (unsporulated oocysts, sporulated oocysts, sporozoites and second-generation merozoites) were treated with DNase I (Invitrogen) according to the protocol. Quality and quantity of total RNAs were assessed as described above. The first cDNAs were generated by SuperScript II reverse transcriptase (Invitrogen) using random primers. Quantitative real-time PCR (qRT-PCR) was performed on an Eppendorf Mastercycler ep Realplex (Eppendorf, Hamburg, Germany) using the SYBR1 green I dye method. Negative (no template) controls were included. A fragment encoding the *E. tenella* 18S ribosomal RNA was used as a control. Reactions were carried out in triplicate and experiments were performed six times. Primers for real-time PCR are in Table 1. Primers for *EtCDPK4* (EtCDPK4-RT[UP] and EtCDPK4-RT[LOW]) and 18S rRNA were designed by the Beacon Designer program (Corbett Robotics, USA). Relative mRNA expression level was determined as the ratio of *EtCDPK4* to 18S rRNA.

To investigate expression of *EtCDPK4* in developmental stages, lysates from *E. tenella* unsporulated oocysts, sporulated oocysts, sporozoites and second-merozoites were prepared using cell lysis buffer for western Blot and IP (Beyotime, Haimen, China). Protein concentrations were determined using BCA Protein Assay kits (Beyotime) and separated on 12% SDS-PAGE. Western blots were performed according to standard procedures [32]. Anti-rEtCDPK4 antibodies were used at 1:100 and mouse monoclonal anti-α-tubulin antibodies (Sigma) at 1:1000 were controls. Secondary antibodies were used as above. IRDyes were detected using an ODYSSEY Infrared Imaging System (LI-COR).

**Assays for rEtCDPK4 protein kinase activity**

To calculate rEtCDPK4 activity units in catalytic reactions, we defined one unit rEtCDPK4 activity as a nanomole of phosphate group transferred to a substrate per minute per milliliter. *In vitro* phosphorylation reactions were performed using Non-radioactive PepTag assays (Promega). Assays (25 μL) were carried out in 20 mM HEPES-KOH, pH 7.4, 1.3 mM CaCl₂, 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 5 μg sonicated phosphatidylserine, 1 mM phenylmethylsulfonyl fluoride, 5 ng leupeptin, 5 ng aprotinin, 2 mM mercaptoethanol, 0.05% Triton X-100, 47.5 μM PepTag®-C1-Peptide, 2 μL peptide protective solution, with an aliquot of purified
rEtCDPK4 (10 μg, initial concentration about 1.0 mg/mL). Phosphorylation reactions were performed for 30 min at 30˚C and stopped by heating at 95˚C for 15 min. Phosphorylated C1 peptides were separated from non-phosphorylated peptides by electrophoresis on 0.8% agarose gels at 140 V for 30 min. To estimate the amount of phosphorylated peptide, bands were excised from gels under UV light, melted at 95˚C and mixed (325 μL) with gel solubilization solution (75 μL, Promega) and glacial acetic acid (100 μL). Optical absorbance values of solutions were measured with a NanoDrop2000/2000C spectrophotometer at 570 nm. rEtCDPK4 enzyme activity was measured using Beer’s Law as described above.

**Effect of Ca^{2+} Concentrations on rEtCDPK4 Activity**

Based on rEtCDPK4 kinase activity assays, the initial reaction system was improved. The kinase activity assay had two portions: qualitative and quantitative detection. Assays (30 μL) were carried out in 20 mM HEPES-KOH, pH 7.4, 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 5 μg sonicated phosphatidylycerine, 1 mM phenylmethysulfonyl fluoride, 5 ng leupeptin, 5 ng aprotinin, 2 mM mercaptoethanol, 0.05% Triton X-100 (v/v), 47.5 μM PepTagC1-Peptide, 2 μL peptide protective solution, and an aliquot of purified rEtCDPK4 (10 μg, initial concentration about 1 mg/mL), and five concentrations of CaCl₂: 0 μM, 10 μM, 50 μM, 100 μM, or 1000 μM. Negative controls and rEtCDPK4 sample controls were used. Phosphorylation reactions were for times and temperatures as above. Phosphorylated-C1-peptides were separated and amounts of phosphorylated peptide estimated as above. rEtCDPK4 kinase activity was measured by spectrophotometer and experiments were performed twice.

**Screening and Analysis of rEtCDPK4 Specific Inhibitors**

By bioinformatics analysis of EtCDPK4 functional domains, we chose seven inhibitors: W-7 (Sigma), H-7 (Sigma), H-89 (Beyotime), staurosporine (Beyotime), D-sphingosine (Sigma), Ro-31-8220 (Sigma) and myristoylated peptide (Promega). These inhibitors belonged to three categories: ATP competitive inhibitor, hypothetical-substrate inhibitor and Ca^{2+}-binding-domain inhibitor. Staurosporine, H-7, H-89, W-7 and Ro-31-8220 belong to the category of ATP competitive inhibitor; Myristoylated peptide belongs to the hypothetical-substrate inhibitor; D-sphingosine belongs to the Ca^{2+}-binding-domain inhibitor. Inhibitory concentrations were as recommended by the supplier instructions. The inhibitors’ final concentrations were adjusted to 100 μM in the reaction system. Assays (30 μL) were carried out in 20 mM HEPES-KOH, pH 7.4, 1.3 mM CaCl₂, 1 mM DTT, 10 mM MgCl₂, 1 mM ATP, 5 μg sonicated phosphatidylycerine, 1 mM phenylmethysulfonyl fluoride, 5 ng leupeptin, 5 ng aprotinin, 2 mM mercaptoethanol, 0.05% Triton X-100, 47.5 μM PepTag C1-Peptide, 2 μL peptide protective solution, and purified rEtCDPK4 as above and specific inhibitor. Negative controls and rEtCDPK4 samples without inhibitors were included. Phosphorylation reactions, separation of phosphorylated-C1-peptides and estimation of amounts were as above. Experiments were performed three times. Data differences among groups were analyzed by one-way analysis of variance (ANOVA) Duncan test.

**Inhibition of DF-1 Invasion by Anti-rEtCDPK4 Polyclonal Antibody or rEtCDPK4 Specific Inhibitors**

The chicken embryo fibroblast cell line DF-1 was used for inhibition assays [33]. Antibodies were purified with Protein A+G Agarose (Beyotime). E. tenella sporozoites were labeled using carboxyfluorescein diacetate, succinimidyl ester (CFDA SE, Beyotime) according to the manufacturer’s instructions. Labeled sporozoites (1.0 × 10⁸) were resuspended in 1 mL CM (Gibco, Grand Island, NY, USA), purified anti-rEtCDPK4 polyclonal antibody, IgG from native rabbit
serum (negative control), or an equivalent volume of PBS (normal control) was added to labeled sporozoites to final concentrations of 50, 100, 200, 300 or 400 μg/mL respectively. Sporozoites were incubated at 37°C for 2 h, washed twice in sterile phosphate buffered saline, then infected 2.0 × 10⁶ DF-1 cells in 24-well plates (Corning, NY, USA). After cultured 16 h at 41°C, cells were collected and analyzed by flow cytometry (Beckman Coulter, USA). Uninfected DF-1 cells were the control. Infected cells, uninfected cells, and free sporozoites were gated using RXP software (Beckman Coulter, USA) to count infected (labeled sporozoites) and uninfected (fluorescence-free) cells. All of invasion-inhibition assays were performed in triplicate. Percentages of cells infected in the presence or absence of anti-rEtCDPK4 polyclonal antibody were used to calculate inhibition rate, as previously described: inhibition = 100% × (1− [% (infected cells Antibody treatment) /% (infected cells negative control)]) [34].

E. tenella sporozoites were labeled as above. Labeled sporozoites (4.0 × 10⁸) were resuspended in 1 mL of DMEM and incubated with specific inhibitors of rEtCDPK4 (100 μM) or dimethyl sulfoxide (DMSO; negative control) (100 μM) for 2 h at 37°C. All assays were performed in triplicate. Uninfected DF-1 cells were controls. Infected cells, uninfected cells, and free sporozoites were gated as above to count infected (labeled sporozoites) and uninfected (fluorescence-free) cells. Percentages of infected cells in the presence or absence of rEtCDPK4 specific inhibitors were used to calculate inhibition rates as described.

Indirect Immunofluorescence Assays of rEtCDPK4 Expression During First Schizogony

DF-1 cells were infected with sporozoites at 41°C, 5% CO₂ at one sporozoite per cell in DMEM (Gibco) supplemented with 5% FBS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine. Purified freshly excysted sporozoites were incubated in PBS or complete medium for 2 h at 41°C and washed before transferring to a glass slide and air-dried as previously described [35, 36]. Sporozoites incubated in CM for 2 h at 41°C were used to infect cells. After infection 12, 24, 36, 48, 60 and 72 h, cells were collected and washed before transferred to glass slides and air-dried. Slides were fixed in 2% paraformaldehyde in PBS for 30 min and permeabilized using 1% Triton X-100 in PBS for 30 min. Slides were blocked with PBS containing 2% (w/v) bovine serum albumin at room temperature for 2.5 h. A 1:500 dilution of anti-rEtCDPK4 polyclonal antibody was added and incubated for 2 h at 37°C. Then a 1:500 dilution of a goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma-Aldrich, St. Louis, State of Missouri, USA) was added and incubated for 2.5 h at 37°C. Nuclei were stained with 15 μg/mL 4, 6-diamidino-2-phenylindole (DAPI) (Beyotime) at room temperature for 10 min. After each step, slides were washed six times with PBS containing 0.5% (v/v) Tween-20. Finally, slides were mounted using 100 μL Fluoromount Aqueous Mounting Medium (Sigma-Aldrich). Before observation under a fluorescence microscope (Olympus, Tokyo, Japan), 50 μL 1, 4—diazabicyclo [2. 2. 2] octane (DABCO; Sigma) was added.

Statistical Analysis

Statistical analysis was performed using Microsoft Office Excel for Windows version 2013 (Redmond, Washington, USA) and GraphPad Prism 5.0 (GraphPad, La Jolla, CA, USA). All data, including EtCDPK4 Transcription and Translation Analysis in E. tenella Life Stages, Inhibition of DF-1 Invasion by Anti-rEtCDPK4 Polyclonal Antibody or rEtCDPK4 Specific Inhibitors, Effect of Ca²⁺ Concentrations on rEtCDPK4 Activity, Screening and Analysis of EtCDPK4 Specific Inhibitors, were analyzed. Differences among groups were tested by one-way analysis of variance (ANOVA) Duncan test. The data are presented as mean ± standard deviation (SD). P < 0.05 was considered significant and P < 0.01 highly significant.
Results

Characteristics of EtCDPK4 Sequence by Bioinformatics

The full-length EtCDPK4 cDNA was 2499 bp with a single ORF of 1803 bp (positions 186 bp–1988 bp) encoding a polypeptide of 600 amino acid residues with a calculated molecular mass of approximately 68.3 kDa. Fig 1 shows the complete nucleotide sequence of EtCDPK4 and the deduced amino acids. The cDNA contained a 5’- untranslated region (UTR) of 185 bp and a 3’- UTR of 511 bp. The 3’- UTR contained a characteristic poly A tail (AAAAAA) but without a classic polyadenylation signal (AATAAA).

TMPRED analysis of the transmembrane regions of the EtCDPK4 amino acid sequence found a transmembrane region in the Val302—Ile324 position. SignalP analysis revealed that the protein most likely does not contain a signal peptide. Structural module and conservative structure predictions indicated that the protein contained distinct domains characteristic of a calcium-dependent protein kinase, including an amino-terminal kinase domain with a typical serine/threonine-kinase active site, a carboxy-terminal calmodulin-like domain with four EF-hand motifs and an amino-terminal calmodulin-like domain with two EF-hand motifs for calcium-binding (Fig 2). Also predicted were three N-myristoylation sites and two N-glycosylation sites. The gene was designated EtCDPK4 (GenBank Accession No. KU925778). DNAstar analysis of the secondary structure, antigen index, flexible regions and surface probability of EtCDPK4 showed a polypeptide rich in α-helix, β-sheet, β-turn, random coil and flexible regions that might contribute to polypeptide chain folding to tertiary structures. The polypeptide antigen-index score was relatively high at 1.7. Therefore, the EtCDPK4 polypeptide had antigenic sites and was predicted to be immunogenic. Surface probability analysis of the EtCDPK4 polypeptide had high scores of about 6.0, suggesting that the function of the EtCDPK4 was involved in the E. tenella membrane surface.

Expression and Purification of Recombinant CDPK4

The 1660-bp fragment (location: 118 bp—1777 bp) of the EtCDPK4 ORF was amplified by RT-PCR according to the structural and functional domains, because it is difficult to amplify the full-length of ORF of EtCDPK4. The protein encoded by this fragment contained all distinct domains, including serine/threonine-kinase active site, EF-hand motifs for calcium-binding. Then the fragment was ligated with expression vector pColdI to create an expression plasmid pCold-EtCDPK4. After sequencing, pCold-EtCDPK4 was transformed into E. coli BL21 to induce expression of recombinant protein. Electrophoresis results showed that rEtCDPK4 was a soluble protein. Lysates of E. coli included a band on SDS-PAGE gels with a molecular weight (MW) close to the theoretical 62.6 kDa MW of EtCDPK4. rEtCDPK4 was purified using column affinity chromatography and identified by SDS-PAGE (Fig 3A).

Production of Anti-rEtCDPK4 and Identification of rEtCDPK4

Serum was generated using rabbits. Western blots showed that rEtCDPK4 was recognized by rabbit anti-merozoites serum or anti-His tag monoclonal antibody (Fig 3B, 3C and 3D). The rabbit anti-rEtCDPK4 serum titer was 1:12, 800 by indirect ELISA.

EtCDPK4 Transcripts and Protein in E. tenella Developmental Stages

To determine the mRNA level of EtCDPK4 in unsporulated oocysts, sporulated oocysts, sporozoites, and second-generation merozoites of E. tenella, total RNA was subjected to qPCR analysis. Among the four development stages, EtCDPK4 mRNA was highest in sporozoites; transcripts were nearly undetectable in unsporulated oocysts and merozoites (Fig 4).
A Novel Calcium-Dependent Protein Kinase 4 from *Eimeria tenella*
The presence of EtCDPK4 protein in the four developmental stages was determined by immunoblotting using antibodies from rabbits immunized with rEtCDPK4. Anti-α-tubulin monoclonal antibodies were used as internal reference controls. Anti-rEtCDPK4 labeled the same 68.3-kDa band in second-generation merozoites, unsporulated oocysts and sporozoites with weak reactivity in sporulated oocysts (Fig 5A). Thus, the EtCDPK4 had a high expression level in stage of the second-generation merozoites, while the EtCDPK4 in other three developmental stages of the sporozoites, unsporulated oocysts and sporulated oocysts were approximately at the same level (Fig 5B).

Protein Kinase Activity of rEtCDPK4

rEtCDPK4 was purified by column affinity chromatography (Fig 3A) and protein kinase activity was measured based on nonradioactive detection of in vitro phosphorylation of a PKC-specific fluorescent synthetic peptide substrate. Phosphopeptides were detected under UV light and quantified spectrophotometrically (Fig 6A, 6B and 6C). rEtCDPK4 had protein kinase activity with a reaction rate of about 0.87 nmol•min⁻¹•mL⁻¹ protein or 0.87 units/mL.

Effect of Ca²⁺ Concentration on rEtCDPK4 Activity

Enzyme activity was strongly dependent on Ca²⁺ concentrations with a calculated Kᵦ of 10–1000 μM based on incubating rEtCDPK4 with various Ca²⁺ concentrations for in vitro phosphorylation assays (Fig 7A, 7B and 7C). With no Ca²⁺, rEtCDPK4 was inactivated and no substrate was phosphorylated. With increasing Ca²⁺ concentrations, the activity of rEtCDPK4 was enhanced in spite of the band relatively weak.

Analysis of rEtCDPK4 Specific Inhibitors

Seven inhibitors were applied to rEtCDPK4 activity assays with quantitative detection (Fig 8B). The activity of rEtCDPK4 in the reaction system with different inhibitors was detected by measuring the absorbance value of the cutting gel band at the 570 nm. The results showed that the absorbance values of phosphorylation reaction products treated by W-7, H-7, H-89, staurosporine, Ro-31-8220 and myristoylated peptide were lower than the sample control, but the absorbance value of phosphorylation reaction products treated by D-sphingosine was very close to the sample control. The calculation formula of kinase activity was showed in Fig 8A. Calculations for rEtCDPK4 activity showed that W-7, H-7, H-89, staurosporine, Ro-31-8220 and myristoylated peptide significantly decreased enzyme activity, while D-sphingosine had no effect.
EtCDPK4 Localization During In Vitro Infection by Immunofluorescence

Anti-rEtCDPK4 was used to localize EtCDPK4 in sporozoites and during first schizogony. EtCDPK4 exhibited a homogenous distribution pattern throughout the cytoplasm of sporozoites except for the refractive body and second-generation merozoites incubated in PBS for 2 h (Fig 9A and 9C). In contrast, when sporozoites were incubated in culture medium, EtCDPK4 expression did not change significantly (Fig 9B). After sporozoites invaded host cells, EtCDPK4 was mainly localized to the cytoplasm of parasites, except for the refractive body. Green fluorescence intensity was enhanced at this phase. Foci of intense EtCDPK4 staining were closely associated with the parasitophorous vacuole membrane (Fig 9D, 9H and 9J). Observations at 36 h p.i. showed that EtCDPK4 protein increased and was distributed in trophozoites (Fig 9E). Labeled EtCDPK4 was eventually uniformly dispersed in immature and mature schizonts and decreased in immature schizonts (Fig 9F, 9G, 9H, 9I and 9J). After formation of first-generation merozoites from mature schizonts in DF-1 cells, labeling increased (Fig 9K).

Anti-rEtCDPK4 and rEtCDPK4 Specific Inhibitors Inhibited DF-1 Cell Invasion

To evaluate EtCDPK4 effects on invasion of DF-1 cells by E. tenella sporozoites, invasion inhibition assays were performed. Protein function was blocked by pre-incubation of sporozoites with purified antibody against rEtCDPK4 (Fig 10A) before DF-1 cell infection. AbEtCDPK4
inhibited invasion to 52% at an antibody concentration of 400 μg/mL, compared to infection with non-treated sporozoites. Inhibition was dose dependent. By comparison, native rabbit-sera IgG did not have a significant effect on invasion by *E. tenella* sporozoites (Fig 10B).

Flow cytometry used to determine the effect of specific inhibitors on labeled sporozoites is in Fig 11. W-7, H-7, H-89 and myristoylated peptide significantly decreased invasion, while staurosporine and Ro-31-8220 had no effect.

**Discussion**

In this work, the calcium-dependent protein kinase 4 of *E. tenella* was cloned and identified. The full-length cDNA of *EtCDPK4* was 2499 bp, with a 1803 bp ORF encoding a protein of 600 amino acids. The 5'-UTR was 185 bp. The 3'-UTR of 511 nucleotides ended with a poly (A) tail. Sequence analysis indicated that the protein contained domains characteristic of CDPKs: an N-terminal protein kinase domain, a C-terminal calmodulin-like domain with four calcium-binding EF-hand motifs, an N-terminal calmodulin-like domain of two calcium-binding EF-hand motifs, a junction domain and a very short N-terminal variable region.

Searching the *E. tenella* genome database, the deduced amino acid sequence had 100% identity with a gene (ETH_00010685) encoding a putative CDPK. These results suggested that *EtCDPK4* was a member of the *E. tenella* CDPK family.

CDPKs, encoded by multigene families, are widespread in plants and some Apicomplexan parasites. However, only four genes encoding for putative CDPKs of *E. tenella* are in the *E.
According to the number of calcium-binding EF-hand motifs, Apicomplexan parasite CDPKs are classified into four major categories [23]. The first category contains proteins with canonical CDPK structures containing four C-terminal EF-hand motifs. The second category contains proteins with three C-terminal EF hands. The other two groups of CDPKs have one or more N-terminal EF hands followed by a Ser/Thr kinase domain and three or four C-terminal EF-hand motifs. Motif scan results classified EtCDPK4 in the other group of canonical Apicomplexan parasite CDPKs. The EF-hand motif structure of Cryptosporidium parvum CDPK6 was also similar to EtCDPK4 [37]. The arrangement of the N-terminal EF domains is unusual and their role in the regulation of kinase activity has not been examined. Bioinformatic analysis on EtCDPK4 showed that it has three N-myristoylation sites. Several canonical CDPKs in parasites contain consensus motifs for N-myristoylation or palmitoylation, a feature also seen in plant CDPKs, many of which show membrane localization [38, 39]. This result suggests that association with membranes may be important in localization, as has been shown for PfCDPK1, which is modified by both [38].

**Fig 5. Analysis of western blots of EtCDPK4 at different of *E. tenella* stages probed with anti-rEtCDPK4.**

A, qualitative analysis of EtCDPK4 at different stages of *E. tenella* by western blot; α-tubulin, internal reference protein. B, relative quantitative analysis of EtCDPK4 expression difference at different stages of *E. tenella*. Lanes: UO, unsporulated oocysts; SO, sporulated oocysts; Spz, sporozoites; Mz or Mrz, second-generation merozoites. Bars with different letters were significantly different (P < 0.05) and the error bars indicate standard deviations. WB, western blot. M, protein weight standard (from top to bottom: 170 kDa, 130 kDa, 100 kDa, 70 kDa, 55 kDa, 40 kDa, 35 kDa, 25 kDa, 15 kDa).

doi:10.1371/journal.pone.0168132.g005

tenella genome database [24], including EtCDPK4. According to the number of calcium-binding EF-hand motifs, Apicomplexan parasite CDPKs are classified into four major categories [23]. The first category contains proteins with canonical CDPK structures containing four C-terminal EF-hand motifs. The second category contains proteins with three C-terminal EF hands. The other two groups of CDPKs have one or more N-terminal EF hands followed by a Ser/Thr kinase domain and three or four C-terminal EF-hand motifs. Motif scan results classified EtCDPK4 in the other group of canonical Apicomplexan parasite CDPKs. The EF-hand motif structure of Cryptosporidium parvum CDPK6 was also similar to EtCDPK4 [37]. The arrangement of the N-terminal EF domains is unusual and their role in the regulation of kinase activity has not been examined. Bioinformatic analysis on EtCDPK4 showed that it has three N-myristoylation sites. Several canonical CDPKs in parasites contain consensus motifs for N-myristoylation or palmitoylation, a feature also seen in plant CDPKs, many of which show membrane localization [38, 39]. This result suggests that association with membranes may be important in localization, as has been shown for PfCDPK1, which is modified by both
palmitate and myristate [40]. The role of such modifications in regulation of activity has not been explored in parasites.

The mRNA and protein levels of EtCDPK4 were examined in four developmental stages of *E. tenella*. Results from qPCR showed that mRNA for *EtCDPK4* was highest in the sporozoites and the lowest in the unsporulated oocysts and second-generation merozoites. These results showed that the *EtCDPK4* gene was transcribed predominantly at a distinct phase of the *E. tenella* life cycle. However, western blots showed that protein levels were highest for *EtCDPK4* in second-generation merozoites and weakest in sporulated oocysts. This difference may be the result of underlying molecular mechanisms and signaling pathways in the four stages of *E. tenella* and should be investigated in more detail in future studies [41].

Previous studies of Apicomplexans parasites found that each *CDPK* gene is expressed predominantly at a distinct phase of the parasite life cycle. For example, another *CDPK* isoform of *E. tenella*, *EtCDPK1*, is expressed in sporulated oocysts, sporozoites and merozoites, but not in unsporulated oocysts, as determined by western blots [25]. *EtCDPK3* has the highest expression in sporozoites by qPCR or western blots compared with other stages [24]. In *P. falciparum*, *PfCDPK1* is mainly expressed in the asexual blood stages of the parasite, particularly in late-stage schizonts [40]. In contrast, *PfCDPK3* is expressed specifically in the sexual erythrocytic stage [42]. *PfCDPK4* is detected only in *P. falciparum* gametocytes [32]. In the Apicomplexan parasite *T. gondii*, *TgCDPK1* and *TgCDPK3* are produced in the tachyzoite stage, but *TgCDPK2* protein is not detectable in this stage [43, 44].

In our study, mRNA for *EtCDPK4* was highest in sporozoites, but *EtCDPK4* protein was higher in second-generation merozoites than in the other three stages. A high number of transcripts does not necessarily indicate corresponding amounts of translated protein, which is
related to gene functions in the various the parasite stages [37]. Nonetheless, we propose a biological significance for CDPK4 in the *E. tenella* life cycle. Sporulated oocysts in the environment require material for metabolism from the storage of oocysts [45]. Without an energy supply, sporulated oocysts maintain a low metabolic rate until they can infect fresh host cells [46]. However, during this period, sporozoites are present in sporulated oocysts, and the energy required by sporozoites is provided by oocysts, so metabolism is moderate [47, 48]. To adapt to this mechanism, physiological activity of sporozoites is regulated at the gene level [49]. Protein translation consumes more energy in sporozoites, therefore mRNA for *EtCDPK4* was significantly higher in the sporozoites stage. When the merozoite stage invades the host cell and obtains nutrients, merozoites are in schizogony. In this period, the merozoites have higher metabolism and translation is activated [50], so merozoites can invade cells for schizogony and gametogony functions. At this time, *EtCDPK4* has physiological and biochemical functions, so parasites generate a large amount of *EtCDPK4* protein. *EtCDPK4* protein

**Fig 7. Kinase activity dependence on Ca**$^{2+}$**concentration by qualitative assays.** SC, *rEtCDPK4* sample control; NC, negative control. Five Ca$^{2+}$ concentrations were: 0, 10, 50, 100, and 1000 μM. A, Qualitative detection assay; B, A$_{570nm}$ value of phosphorylated short peptides at indicated calcium concentrations; C, *rEtCDPK4* enzyme activity under indicated Ca$^{2+}$ concentrations. Phosphorylation, the gel band that the PepTag®C1-Peptide was phosphorylated by *rEtCDPK4*. Non-phosphorylation, the gel band that the remainder PepTag®C1-Peptide was not phosphorylated by *rEtCDPK4*.

doi:10.1371/journal.pone.0168132.g007
expression was increased sufficiently to be detected by western blot. Therefore, we propose that although EtCDPK4 transcripts were moderately expressed in merozoites, EtCDPK4 protein could have a high expression in the same stage.

Protein phosphorylation on Ser-/Thr- residues is a key post-translational modification required for signal transduction in eukaryotes [51]. CDPKs have a Ser/Thr kinase domain that phosphorylates downstream substrates. CDPK activity can be detected by either radioactive or non-radioactive methods [52, 53]. In this experiment, the rEtCDPK4 activity was detected by non-radioactive methods. rEtCDPK4 catalyzed the phosphorylation of the C1-peptide PKC substrate in vitro and rEtCDPK4 kinase activity could be maintained in a stable range. The kinase activity of rEtCDPK4 depended on Ca$^{2+}$ concentration. In the absence of calcium, rEtCDPK4 did not have activity. This result was in agreement with results of CDPK kinase activity assays in Digitaria sanguinalis mesophyll cells [54]. Therefore, we propose that EtCDPK4 activity is completely dependent on calcium in the cytoplasm of E. tenella.

From the pharmacological perspective, EtCDPK4 has unique structural features that could be the target of specific inhibitors in parasites because these targets do not exist in the host. Crystal structures of TgCDPK1 and CpCDPK1 show an enlarged ATP-binding pocket due to glycine at the "gatekeeper" position adjacent to an adenine recognition site [55, 56]. Therefore, the activity of CDPK4 could be blocked by inhibitors. In this study, using a functional module of EtCDPK4, we selected seven specific inhibitors: W-7, H-7, H-89, staurosporine, Ro-31-8220, myristoylated peptide and D-sphingosine. The effect of Ca$^{2+}$-concentrations on rEtCDPK4 kinase activity and specific inhibitors was determined. The organic compounds W-7, H-7, H-89, staurosporine, Ro-31-8220 and myristoylated peptide significantly decreased enzyme activity, while D-sphingosine had no effect on rEtCDPK4 kinase activity. Previous studies reported that activity of D. sanguinalis CDPKs and the rice CDPK OsCDPK14 can be effectively inhibited by W-7 [54, 57] and H-7, staurosporine, Ro-31-8220, and myristoylated peptide inhibit CDPK kinase activity in D. sanguinalis mesophyll cells [54]. H-89 blocks CDPK kinase activity of French beans in enzyme activity assays in vitro [58]. D-sphingosine, a well-known physiological inhibitor of PKC is a broad-spectrum PKC inhibitor. However, the EtCDPK4 structure was much more complex than PKC and therefore, the inhibitory effect of
D-sphingosine on rEtCDPK4 was likely blocked by calcium ions in the reaction system. This might be why D-sphingosine did not inhibit rEtCDPK4 activity. This result was similar to findings from previous studies [59] that D-sphingosine does not affect the activity of protein kinase C in porcine theca cells. D-sphingosine did not inhibit enzyme activity but stimulates phospholipase D activity in 7721 human hepatocarcinoma cells [60].

Inhibition of DF-1 cell invasion by *E. tenella* sporozoites using specific inhibitors of rEtCDPK4 showed that W-7, H-7, H-89 and myristoylated peptide significantly decreased sporozoite invasion activity, while staurosporine and Ro-31-8220 had no effect. This result might be because the compounds inhibited the activity of rEtCDPK4 *in vitro* but were
removed by a regulation mechanism [61] in *E. tenella* sporozoites and could not block the kinase activity of *EtCDPK4 in vivo*. This would explain the lack of effect on invasion.

We determined experimental conditions for *in vitro* screening of four specific inhibitors *rEtCDPK4* activity. W-7, H-7, and H-89 are broad-spectrum kinase inhibitors and that affected DF-1 invasion by *E. tenella* sporozoites. Myristoylated peptide derived from the pseudosubstrate sequence of β-PKC is a selective inhibitor of PKC subtypes in human fibroblasts [62]. In our study, inhibition by myristoylated peptide was modest but significant for DF-1 invasion by *E. tenella* sporozoites. These results showed that the inhibitors effectively blocked the activity of *EtCDPK4* kinase and inhibited invasion by *E. tenella* sporozoite of DF-1 cells.

Expression of *EtCDPK4* was detected by indirect immunofluorescence assays. Expression of *EtCDPK4* was high in mature schizonts and forming first-generation merozoites. Therefore, suitable organic compounds that block the *EtCDPK4* activity may inhibit development of *E. tenella* in host cecum epithelial cells. We conclude that *EtCDPK4* is likely to be an excellent molecular target candidate for anti-coccidiosis drug or vaccine research.

Using antibodies raised against *rEtCDPK4*, we showed by indirect immunofluorescence assays that *EtCDPK4* kinase was located on the surface and in the cytoplasm of *E. tenella* sporozoites and merozoites. CDPKs were found in several subcellular localizations including membranes and cytoplasm and some isoforms were in more than one compartment [40]. Indirect immunofluorescence assays also showed that *EtCDPK1* [25] and *EtCDPK3* [24] were near the apical end and on the surface of *E. tenella* sporozoites. *PfCDPK1* is present in the membrane and organelle fractions of blood-stage parasites and the membrane fraction of ring-stage infected erythrocytes [63, 64]. *EtCDPK4* has a transmembrane region and three N-myristoylation sites (positions: 8 to 13, 152 to 157, and 253 to 258). Myristoylation sites are vital for membrane targeting and signal transduction in plants and *P. falciparum* response to environmental stress or the host cell environment [40, 65]. This might be one reason that *EtCDPK4* was found on sporozoite and merozoite membranes. *EtCDPK4* localization in intracellular parasites showed mobilization to the membranes of refractile bodies in the anterior of sporozoites. When *E. tenella* developed in DF-1 cells, specific staining was more intense than in trophozoites, mature schizonts or first-generation merozoites, but decreased in immature schizonts by fluorescence intensity. Later during sporozoite development in DF-1 cells, *EtCDPK4* was found in PV membranes. The PV is a crucial structure that protects the parasite against the
host environment [7]. Invasion and the formation of PVs is mediated by different parasite organelles, including micronemes and rhoptries [66]. PV composition evolves during infection according to metabolite exchange between the parasite and host cell [67]. When merozoites escape from mature schizonts to invade new host cells, they must pass through the PV membrane. Thus, we postulated that \( \text{EtCDPK4} \) was important in merozoite maturity and release. Western blots showed that \( \text{EtCDPK4} \) protein increased significantly in merozoite stages. This result was similar to localization of \( \text{EtCDPK1} \) at the apical end of sporozoites after addition to Mardin-Darby bovine kidney cells. \( \text{EtCDPK1} \) protein was higher in mature schizonts than in immature schizonts. \( \text{EtCDPK1} \) appeared to be specifically involved in sporozoite invasion of host cells and in release of merozoites from mature \( \text{Eimeria tenella} \) schizonts [25].

Fig 11. Inhibition of DF-1 invasion by \( \text{E. tenella} \) sporozoites using specific inhibitors of \( \text{EtCDPK4} \). The symbol \(*\) representability \( P < 0.05 \), significant difference; \(**\) representability \( P < 0.01 \), very significant difference. And the error bars indicate standard deviations.

doi:10.1371/journal.pone.0168132.g011
In *P. falciparum*, *PfCDPK1* has a key role in schizont development, microneme protein secretion, invasion of host erythrocytes and regulating mRNA expression to assure timely and stage-specific protein expression [68, 69, 70]. *TgCDPK7* is crucial for *T. gondii* differentiation, growth and proper maintenance of centrosomes [71]. Knock out of *PbCDPK3* leads to a pronounced defect in ookinete transmission to the mosquito midgut epithelium and terminates oocysts production [72, 73]. *PfCDPK5* is essential for regulating parasite egress from erythrocytes [74], whereas *PbCDPK6* is critical for controlling the sporozoite switch from a migratory to an invasion phenotype [75]. Finally, Sharma *et al.* demonstrated that *PfCDPK7* binds to PI (4, 5) P2 and controls *P. falciparum* development in host erythrocytes [76]. These results suggest that *EtCDPK4* is related to the invasion and survival of *E. tenella* intracellular stages.

Previous *in vitro* invasion inhibition assays showed reduced sporozoite invasion in the presence of monoclonal or specific polyclonal antibodies [34, 36, 77]. In our study, *in vitro* invasion inhibition assays using specific antibody against *rEtCDPK4* showed partial blockage of the invasion of sporozoites into cells. Inhibition of sporozoites was modest at 52%. Therefore, *EtCDPK4* might be a key factor in host cell invasion by *E. tenella* sporozoites. While further studies are needed to determine the exact function of *EtCDPK4*, because this kinase family is absent from the parasite’s hosts, it represents a target that might be exploited for chemotherapy against Apicomplexans parasites.

In conclusion, we cloned, expressed and characterized a CDPK4 from *E. tenella*, adding substantially to the current understanding of its function in the *E. tenella* invasion process. Given the importance of *EtCDPK4* in invasion, host cell adhesion and *E. tenella* development in cecum epithelial cells, the results of this study have implications for both novel chemotherapeutic and immunotherapeutic approaches to interfering with *EtCDPK4* function in *E. tenella*.

**Acknowledgments**

This work was supported by the National Natural Science Foundation of China (Grant No. 31572266, No. 31272557) and Central Public Welfare Research Institutions and Basic Scientific Research Business Expenses (No. 2016JB10). We would like to thank Prof. Chan Ding (CAAS, Beijing, China) for generously providing the DF-1 cell line used in this study.

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