Identification of an interaction between calcium-dependent protein kinase 4 (EtCDPK4) and serine protease inhibitor (EtSerpin) in Eimeria tenella

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Abstract

Background: Eimeria tenella is an obligate intracellular apicomplexan protozoan parasite that has a complex life-cycle. Calcium ions, through various calcium-dependent protein kinases (CDPKs), regulate key events in parasite growth and development, including protein secretion, movement, differentiation, and invasion of and escape from host cells. In this study, we identified proteins that interact with EtCDPK4 to lay a foundation for clarifying the role of CDPKs in calcium channels.

Methods: Eimeria tenella merozoites were collected to construct a yeast two-hybrid (Y2H) cDNA library. The Y2H system was used to identify proteins that interact with EtCDPK4. One of interacting proteins was confirmed using bimolecular fluorescence complementation and co-immunoprecipitation in vivo. Co-localization of proteins was performed using immunofluorescence assays.

Results: Eight proteins that interact with EtCDPK4 were identified using the Y2H system. One of the proteins, E. tenella serine protease inhibitor 1 (EtSerpin), was further confirmed.

Conclusion: In this study, we screened for proteins that interact with EtCDPK4. An interaction between EtSerpin and EtCDPK4 was identified that may contribute to the invasion and development of E. tenella in host cells.

Keywords: Eimeria tenella, Calcium-dependent protein kinases, Serpin, Yeast two-hybrid, Bimolecular fluorescence complementation, Co-immunoprecipitation

Background

Eimeria tenella is an obligate intracellular apicomplexan protozoan parasite that causes huge economic losses in the poultry industry. Protozoans have complex life-cycles and need to invade host cells to grow, develop and reproduce. Invasion is a multi-step process that involves the formation, in most cases, of parasitophorous vacuoles within the host cells in which the parasites replicate [1]. Successful invasion of and subsequent escape from host cells, as well as spreading within the host, are important events in the establishment of parasite infections.

Calcium (Ca^{2+}) plays an important role in regulating parasite protein secretion, movement, differentiation, invasion and escape from host cells [2]. In eukaryotic cells, Ca^{2+} is an important signaling molecule, acting as a second messenger and regulating many physiological processes in the body [3]. When cells are stimulated by hormones or electricity, cytoplasmic Ca^{2+} concentrations increase, causing a series of intracellular physiological responses [4]. Calcium-dependent protein kinases (CDPKs) are effectors of Ca^{2+} signaling that play important roles in cells. Recently, CDPKs have been found in plants, green algae and apicomplexan protozoans but have not been reported in bacteria, nematodes, fungi or vertebrates [5]. In cells, CDPKs...
phosphorylate substrate proteins to produce an amplification cascade reaction that transmits the Ca\(^{2+}\) signal. CDPKs have four domains: a variable region, a catalytic region, a link region, and a regulatory region. The catalytic zone can bind to ATP and serine or threonine residues of phosphorylated substrates. In the absence of Ca\(^{2+}\) ions, the linker region binds to the catalytic zone of the substrate and inhibits kinase activity. The regulatory region is a Ca\(^{2+}\) binding zone with EF chiral structure, which allows for CDPKs to be highly compatible with Ca\(^{2+}\) and not dependent on calmodulin [6].

In apicomplexan protozoans, CDPKs have been identified as part of the mechanistic link between Ca\(^{2+}\) signaling and differentiation, motility, invasion and escape from the host cell [2, 7]. Different CDPKs have specific expression patterns at different developmental stages and they regulate many Ca\(^{2+}\)-dependent physiological processes. For example, in *Plasmodium falciparum*, PfCDPK1 can phosphorylate Myosin A tail domain-interacting protein (MTIP) and glideosome-associated protein 45 (GAP45), which are the components of the motor complex that providing a driving force for parasites to invade the host [8]. The peptide P3, a part of the PfCDPK1 junction domain, inhibits the activity of CDPK1 and the secretion of microneme proteins during the invasion of erythrocytes by *P. falciparum* merozoites, indicating that CDPK1 is a key regulatory molecule during movement and invasion of host cells by asexual blood stage *P. falciparum* parasites [9]. Similar findings have been reported in *Toxoplasma gondii*. Conditional suppression of TgCDPK1 results in a block of essential phenotypes, including parasite motility and host cell invasion and escape, indicating that TgCDPK1 controls Ca\(^{2+}\)-dependent secretion of microneme proteins [10]. TgCDPK1 exploits ATP-binding pockets to recognize its substrates, which include the dynamin-related protein DrpB [11]. TgCDPK7 knockdown parasites show significant growth defects and do not progress through cell division. Additionally, TgCDPK7 affects the partitioning and number of centrosomes during parasite division and the polarity of budding, which illustrates that TgCDPK7 is necessary to maintain the distribution and localization of centrosomes in *T. gondii*, an essential process for survival during the breeding stage [12].

Recent studies on *E. tenella* CDPK members have suggested that CDPKs regulate biological functions in *E. tenella* [13–15]. In a preliminary study of its function, it was found that EtCDPK3 was localized to the apical end of sporozoites during the initial invasion stage. Specific antibodies blocking EtCDPK3 inhibit host invasion, indicating that EtCDPK3 participates in host cell invasion and development within the host [16]. In our previous report, another *E. tenella* CDPK, EtCDPK4, was found to be highly expressed during the merozoite stage, although transcriptome levels of EtCDPK4 were highest during the sporozoite stage. Inhibiting the activity of EtCDPK4 reduced sporozoite invasion, indicating that EtCDPK4 participates in host cell invasion [15]. However, the EtCDPK4 regulatory mechanisms and targets in *E. tenella* remain unclear.

Analyses of protein-protein interactions (PPIs) are crucial for the study of various cellular processes and protein function [17]. To further understand the function of EtCDPK4, we conducted yeast two-hybrid (Y2H) screening and identified an interaction between EtCDPK4 and *E. tenella* serine protease inhibitor 1 (*EtSerpin*). Moreover, we confirmed the interaction between EtCDPK4 and *EtSerpin* in DF-1 cells by co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC).

### Methods

**Antiseras and recombinant plasmids**
The following antibodies used for immunoblotting and immunofluorescence assays (IFAs) were prepared and stored at -20 °C in the laboratory: anti-*E. tenella* rabbit antiserum [15], anti-*EtSerpin* rabbit antiserum is described elsewhere [18]. The recombinant plasmid pCAGGS-*EtSerpin* was a gift from Dr. Ye Wang, stored at the laboratory.

The sequences of primers used for PCR are provided in Table 1. To express fusion proteins, EtCDPK4 (ETH_00010685) was cloned into pCDNA3.1-flag (Biovector, Cambridge, MA, USA) and pBIFC-VC155 vectors and the recombinant plasmids were designated pCDNA3.1-flag-*EtCDPK4* and pBIFC-VC155-*EtCDPK4*. *EtSerpin* was cloned into the *SalI* and *XhoI* sites of pBIFC-VN155 using a ClonExpress kit (Vazyme, Nanjing, China).

### Parasite

*Eimeria tenella* (CAAS211116-11) was obtained from the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences. The parasite was...
obtained as previously described [19] by inoculation 2-week-old chickens which were free of infection before experimental inoculation.

Unsporulated oocysts were obtained from theecal contents of chickens infected with $1 \times 10^8$ *E. tenella* sporulated oocysts at 8 days post-infection (p.i.). Sporulated oocysts were harvested from the unsporulated oocysts which underwent sporulation with 2.5% potassium dichromate at 28 °C for 72–120 h in the presence of oxygen. Sporozoites were excysted with trypsin and chicken bile in vitro and purified from cleaned, sporulated oocysts by chromatography over columns of nylon wool and DE-52 cellulose as previously described [19]. Second-generation merozoites (merozoites II) were isolated from theecum and theecal contents of chickens 115 h after infection *E. tenella* and then purified with Percoll [20].

Construction of a Y2H cDNA library of *E. tenella* merozoites II

Yeast strains Y187 and Y2H Gold (Takara, Tokyo, Japan) used for the Y2H screen were prepared according to Yeastmaker™ Yeast Transformation System 2 User Manual (Clontech, Mountain View, CA, USA).

Total RNA was isolated from merozoites II of *E. tenella* with Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. PolyA+ was purified with a PolyA+ Tract mRNA Isolation System kit (Promega, Madison, WI, USA) after the quality of total RNA was assessed. The Y2H cDNA library of *E. tenella* merozoites was constructed using a Make Your Own “Mate & Plate” Library System kit (Clontech, Mountain View, CA, USA). First-strand cDNA was synthesized and then amplified into double-stranded cDNAs (dscDNAs) using long distance PCR. dscDNAs shorter than 200 bp were removed using a Chroma Spin column (Takara, Tokyo, Japan). dscDNAs were cloned into the pGADT7-Rec vector (Clontech, Mountain View, CA, USA). The resulting plasmids were transformed into the Y187 yeast strain according to the instructions. To determine the transformation efficiency, 100 µl of 1:10,000, 1:1000, 1:100 and 1:10 dilutions were spread on 100-mm plates containing synthetic dropout (SD)/-Leu media and incubated at 30 °C for 3–5 days. The remainder was spread on another SD/-Leu plate and the library solution was collected. To calculate the library size, 100 µl of 1:10,000, 1:1000, 1:100 and 1:10 dilutions of the library solution were spread onto media and incubated at 30 °C for 3–5 days. Fifty-one colonies were randomly selected for PCR identification and analysis of library insert size and recombination efficiency.

Y2H library screening

*EtCDPK4* cDNA was inserted in-frame with the GAL4 DNA-binding domain into the vector pGBKTK7-GAL4 to construct the recombinant plasmid pGBKTK7- *EtCDPK4*. A non-autoactivating and non-toxic pGBKTK7- *EtCDPK4* were acquired and bait proteins were expressed [21]. For interaction mating, the bait protein and the library were co-cultured at 30 °C with shaking for 20 h then spreading onto SD/-Leu/-Trp/-His/-Ade (QDO) selection media. After mating, clones were transferred to QDO media. Blue clones were confirmed by culturing on SD/-Leu/-Trp/-His/-Ade supplemented with X-α-gal (QDO/X) media and then cultured on SD/-Leu/-Trp/-His/-Ade supplemented with X-α-gal and aureobasidin A (QDO/X/A) media. Only those clones growing on QDO/X/A media were further characterized. Confirmation of interacting clones was performed by sequencing and non-target plasmids were eliminated. Positive clones were further confirmed by prey plasmid rescue and retransformation into Y2H Gold with pBDGAL4-*EtCDPK4* or with the negative control plasmid (empty pBD-GAL4).

Immunolocalization

Purified, freshly excysted sporozoites and merozoites II were incubated in phosphate-buffered saline (PBS), transferred to a glass slide, and air-dried as previously described [22]. The chicken embryo fibroblast (DF-1) cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. 2.0 × 10^5 cells per well with slices were used for parasite invasion [18]. Purified sporozoites were washed three times with sterile PBS, infected into DF-1 cells then cultured at 41 °C for 2 h. The cells cultured with slices were collected and washed with PBS. The slices were air-dried and fixed in 4% paraformaldehyde for 20 min, then permeabilized with 0.1% Triton X-100 in PBS for 20 min and incubated with 2% bovine serum albumin (BSA) in PBS overnight at 4 °C. At dilutions of *EtCDPK4* and *EiSerpin* antisera for 1 h, a 1:50 dilution of goat anti-rabbit IgG fluorescein isothiocyanate (FITC)-conjugated antibody (Sigma, St Louis, MO, USA) and goat anti-mouse IgG cyanine (Cy3)-conjugated antibody (Sigma, St Louis, MO, USA) were added and incubated for 1 h at 37 °C. 4,6-diamidino-2-phenylindole (10 µg/ml, Beyotime, Haimen, China) was used to stain nuclei for 30 min. After each step, slides were washed three times with PBS. 50 µl Fluoromount Aqueous Mounting Medium (Sigma, St Louis, MO, USA) was added before observation under a fluorescence microscope (Olympus, Tokyo, Japan).

BiFC assay

For BiFC assays, cells must take up the expression vector. Therefore, IFAs were performed to confirm expression of the fusion proteins in the DF-1 cells. The
recombinant plasmids VC155EtCDPK4 and VN155EtSerpin were transfected into 6.0 × 10⁵ DF-1 cells and cultured in six-well plates for 24 h. Briefly, 4 μg plasmid DNA and 10 μl lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were mixed, incubated at room temperature for 20 min, and gently added to the cells. After 6 h, the DNA transfection reagent was replaced with DMEM containing 2% FBS and 200 U/ml penicillin/streptomycin. For IFAs, DF-1 cells transfected with recombinant plasmids were fixed in 2% paraformaldehyde for 20 min. The samples were permeabilized with 0.1% Triton X-100 in PBS pH7.4 for 20 min and then blocked with 2% BSA in PBS at 4 °C overnight. The relevant antisera and the goat anti-rabbit secondary antibodies were used for incubation. Finally, the samples were visualized using a fluorescence microscope.

After confirming that the DF-1 cells could take up the two constructs, the recombinant plasmids VC155EtCDPK4 and VN155EtSerpin were co-transfected into DF-1 cells. 30 h later, the cells were observed under an inverted fluorescence microscope. Two non-fluorescent fragments, pBiFC-bfosVC155 and pBiFC-bjunVN155, form a fluorescent complex that can be detected using a fluorescence microscope were used as positive controls. Two non-fluorescent fragments, pBiFC-bfosVC155 (delta ZIP) and pBiFC-bjunVN155, were used as negative controls.

Co-IP

To confirm their expression in DF-1 cells, the recombinant plasmids pCDNA3.1-flagEtCDPK4 and pCAGGS-EtSerpin were transfected into 2.0 × 10⁶ DF-1 cells and analyzed by western blot. After confirming that the DF-1 cells could take up the two constructs, the recombinant plasmids were co-transfected into cells as described above. pCDNA3.1-flag and flag-EtCDPK4 were used as controls. After 48 h, the transfected cells were washed twice with PBS and lysed with RAPI buffer, cell debris was removed by centrifugation at 12,000× rpm for 10 min. The Co-IP assay was performed using the Pierce Co-Immunoprecipitation kit (Thermo, Waltham, MA, USA) following the manufacturer's instructions using antiserum to EtCDPK4 for coupling to the resin. Samples were analyzed by SDS-PAGE followed by Western blotting then detection with EtSerpin antiserum or anti-flag antibody. The control was incubated with anti-Flag antibody only. The nitrocellulose membranes were incubated with anti-mouse fluorescent secondary antibody for anti-Flag and anti-rabbit fluorescent secondary antibody for EtSerpin antibodies.

Results

Eimeria tenella merozoite II Y2H cDNA library construction

Merozoite II cDNA from E. tenella was cloned into the pGADT7-rec vector. The resulting plasmids were transformed into the Y187 strain and spread onto SD/-Leu plates. Random clones were selected for PCR analysis (Fig. 1). Results showed that 93.2% of recombinants carried DNA sequences with an average length of 500 bp from E. tenella merozoites. The size of the library was 9.6 × 10⁹ CFU and the transformation efficiency of the library was 4.1 × 10⁵ cfu/μg pGADT7-Rec, which was sufficient for subsequent Y2H screening.

Y2H screening for proteins interacting with EtCDPK4

Y2H screening of the E. tenella merozoite II cDNA library with EtCDPK4 as bait resulted in 69 blue colonies formed (Fig. 2a). Comparison of the DNA sequence of the positive plasmids with the genome of E. tenella (http://www.genedb.org) showed that 30 different E. tenella proteins were represented. To test whether these proteins interacted with EtCDPK4 in yeast, the positive plasmids were transformed into Y2H Gold using pGADT7-EtCDPK4 and

![Fig. 1](image-url) Partial PCR products of randomly selected colonies from the Y2H cDNA library analyzed with electrophoresis on a 1% agarose gel. Lanes 1—44: individual recombinant colonies; Lane M: DNA size marker.
spread onto QDO/X/A plates. In total, eight blue colonies (Table 2) formed that contained proteins that interacted with EtCDPK4 in the Y2H system (Fig. 2b).

Co-localization of EtCDPK4 and EtSerpin
IFAs were performed to determine the location of EtCDPK4 and EtSerpin. Sporozoites and merozoites II treated with anti-EtCDPK4 mouse antisera and anti-EtSerpin rabbit antisera showed EtCDPK4 and EtSerpin distributed throughout the cytoplasm (Fig. 3a, c). At 2 h p.i. of DF-1 cells by sporozoites both proteins of EtCDPK4 and EtSerpin were at the apical end (Fig. 3b). The co-localization indicated that the proteins of EtCDPK4 and EtSerpin function in the same location.

Interaction between EtCDPK4 and EtSerpin assessed by BiFC
Expression of the plasmids VC155-EtCDPK4 and VN155-EtSerpin in DF-1 cells was confirmed using IFA

| Gene ID     | Annotation                                               | MW (kDa) |
|-------------|----------------------------------------------------------|----------|
| ETH_00011330| SERPIN1 protein                                          | 45.5     |
| ETH_00024500| Hypothetical protein                                     | 92.3     |
| ETH_00018145| Hypothetical protein                                     | 17.9     |
| ETH_00002065| Hypothetical protein                                     | 38.4     |
| ETH_00009380| DNA-directed RNA polymerases I and III subunit RPAC1     | 36.4     |
| EMH_00033980| Hypothetical protein                                     | 44.1     |
| ETH_00021190| Sec63 domain-containing DEAD/DEAH box helicase          | 246.7    |
| ETH_00007745| apical membrane antigen-1                                | 58.0     |
(data not shown). The recombinant plasmids VC155-EtCDPK4 and VN155-EtSerpin were then co-transfected into DF-1 cells and observed with an inverted fluorescence microscope. When the positive controls bFos and bJun were transiently co-expressed in DF-1 cells, a positive BiFC signal was detected in the cells. A similar positive BiFC signal was detected when VC155-EtCDPK4 and VN155-EtSerpin were transiently co-expressed in DF-1 cells, indicating that EtCDPK4 and EtSerpin interact in DF-1 cells (Fig. 4). In contrast, the...
negative controls bFos (delta ZIP) and bJun did not produce a detectable fluorescent signal.

**Interaction between EtCDPK4 and EtSerpin assessed by Co-IP**

Western-blotting results showed that the fusion protein flag-EtCDPK4 and EtSerpin were successfully expressed in DF-1 cells (Fig. 5a). Co-IP assays showed that when flag-EtCDPK4 and EtSerpin proteins were incubated with resin that was covalently coupled with EtCDPK4 antisera, EtSerpin was eluted with EtCDPK4 from the resin by elution buffer (Fig. 5b). In contrast, when pCDNA3.1-flag was used instead of EtSerpin in control experiments, EtCDPK4 alone was detected in the eluate (Fig. 5b). Based on these data, we conclude that an interaction exists between EtCDPK4 and EtSerpin.

**Discussion**

Biochemical analysis of protein complexes and identification of their components is fundamental to the understanding of protein function [23, 24]. Currently, several methods for identifying protein interactions exist, including Y2H techniques, Co-IP, BiFC, phage display technology and pull down experiments.

In the present study, we used the Y2H technique to screen for proteins that interact with EtCDPK4. High quality libraries are one of the key elements of Y2H screens. In this study, a high quality Y2H cDNA library was constructed using *E. tenella* merozoites II We achieved a recombination rate of 93.2% and a library size of $9.6 \times 10^{-9}$ CFU, which was sufficient for subsequent Y2H screening. A total of eight interacting proteins were identified (Table 2), one of which was previously reported and described as Serpin1 (ETH_00011330) in our lab [18]. Only eight positive interactions were confirmed on a second round screening. There are maybe several reasons: (i) the incorrect folding and/or instability of an AD fusion protein that could interact with its interacting partner, there are maybe some of these AD fusion proteins in the *E. tenella* merozoites II Y2H cDNA library; (ii) the toxicity of some fusion proteins that could affect the viability of transformed cells [25]; (iii) the quality of the library is a key parameter for the success of a screening, although the quality of *E. tenella* merozoites II Y2H cDNA library that we constructed is good, it cannot include all the cDNAs. Some mRNAs encoding putative interacting proteins are expressed at relatively low levels; these proteins might not be identified. In addition there were several identified plasmids (7/30) failed to rescue so there may well be other proteins which interacted with EtCDPK4. In future study, we will screen the putative interacting proteins using other methods. The Y2H system may have technical or biological false positives like any assay system [26]. Therefore, we used other methods, including Co-IP, BiFC and co-localization, to further verify the interaction between EtCDPK4 and EtSerpin.

Detection of PPIs in living cells is particularly important for understanding biological process [27, 28]. One of effective ways for studying PPIs is BiFC [29]. This assay offers several advantages over other techniques such as Y2H. The method enables real-time observation of PPIs in their natural environment, such as in live cells or animals [30, 31]. In addition, the subcellular localization of the PPI can be observed directly from BiFC [31]. So in this study, we used the BiFC to verify the interaction between EtCDPK4 and EtSerpin.

**Fig. 5** Interaction between EtCDPK4 and EtSerpin assessed with Co-IP. a DF-1 cells were transfected with pCDNA3.1-flag-EtCDPK4 and pCAGGS-EtSerpin and cellular lysates were analyzed with immunoblotting with antisera against EtCDPK4 and EtSerpin. b The Co-IP was performed using immobilised antisera against EtCDPK4. Detection of eluted proteins on immunoblots was by EtSerpin and/or anti-flag for EtCDPK4 antibodies.
CDPKs are present in plants, algae, ciliates and apicomplexan parasites. In plants, CDPKs regulate plant development and biotic and abiotic stress responses. The N-terminal domain of CDPK plays a key role in subcellular localization and function [32]. Most CDPKs have myristoylation sites and cysteine residues that allow for N-terminal palmitoylation and contribute to the localization of CDPKs. Arabidopsis thaliana AtCDPK16 is predicted to be localized to the chloroplast based on multiple prediction methods, whereas N-terminal acylation at N-myristoylation and palmitoylation sites prevents localization to the chloroplast [33]. The N-terminus not only determines subcellular localization, but also interacts with target proteins. For example, Nicotiana tabacum NtCDPK1 could phosphorylate the basic leucine zipper transcription factor RSG (repression of shoot growth) in tobacco. A chimeric CDPK containing NtCDPK1 N-terminus fused to AtCDPK9 can also phosphorylate and interact with RSG, although native AtCDPK9 can neither bind nor phosphorylate RSG [34]. Many Arabidopsis CDPKs are membrane localized or membrane associated, which is mediated by N-terminal acylation [35]. In T. gondii, the substrate of TgCDPK1, DrpB, interacts with CDPK1 at the N-terminal ATP-binding pocket [11]. In P. falciparum, PfCDPK7 interacts with phosphatidylinositol 4,5-bisphosphate via its pleckstrin homology domain, guiding its subcellular localization [36]. Functional structure prediction indicates that EtCDPK4 contains three N-myristoylation sites, an ATP binding domain, and a serine/threonine protein kinase activation site [15]. N-myristoylation sites and ATP binding domain contribute to the subcellular localization and functions of EtCDPK4, which include Ca2+ signaling and interacting with substrate proteins. We hypothesise that EtCDPK4 and EtSerpin interact through the ATP binding domain and play a role in sporozoite invasion. The N-terminal myristoylation site of EtCDPK4 may help the complex of EtCDPK4 and EtSerpin to locate to the apex near the membrane surface of parasites when sporozoites invade host cells.

EtSerpin was one of the putative interacting proteins of EtCDPK4. Serine protease inhibitors (serpins) are a class of proteins composed of 300–500 amino acids with a molecular weight between 40–60 kDa. Although intracellular serpins have been reported, most serpins are present in the extracellular environment [37–39]. Over 500 members of the serpin superfamily have been identified in animals, plants, bacteria, archaea and viruses [40]. In mammals, serpins play crucial roles in processes such as blood coagulation and fibrinolysis [38, 41]. Most serpins consist of three β-folds and 8–9 α-helices. The typical serpin structure includes an exposed reactive center loop conformation above the body of the molecule [42]. Most serpins undergo a significant conformational change from the stressed to the relaxed state that can result in inhibition of target proteases. The activity of some small protease inhibitors can be regulated by specific factors. For example, SERPINC1 is a rare inhibitor that inhibits factor Xa with the cofactor heparin in human [43]. SERPINC1, protease and heparin form a stable ternary complex. Therefore, synergistic interactions between serpins and other molecules can result in different roles for serpin proteins. In the case of protein Z dependent protease inhibitors, protein Z as a vitamin K-dependent co-factors to promote the inhibitory activity of the serpin with FXa on negatively charged phospholipid vesicles and calcium [44]. Vaspin is visceral adipose tissue-derived serine protease inhibitor, promotes the phosphorylation of Akt through PI3K signaling pathway [45]. In parasites, Serpin plays an important role in the inflammatory response, regulating host immunity, development and anticoagulation. For example, Ixodes ricinus salivary serpin has anticoagulant activity, including coagulation and fibrinolysis inhibition and binds to cells/macrophages and inhibits TNF secretion [46]. In T. gondii, SERPIN B3 and B4 act via STAT6 activation to inhibit caspase 3, PARP activation, and DNA fragmentation [47]. In this study, Y2H, Co-IP, and BiFC were used to identify an interaction between EtCDPK4 and EtSerpin. We expect that, EtCDPK4 may interact with EtSerpin as a cofactor, similar to SERPINC1 or protein Z, the interaction between EtSerpin and EtCDPK4 may enhance protease inhibitory activity of EtSerpin during sporozoites invasion into host cells. At the same time, the phosphorylation of EtCDPK4 may be elevated by EtSerpin.

In this study, EtCDPK4 and EtSerpin were located mainly in the cytoplasm of sporozoites and merozoites. Co-localization experiments showed that EtCDPK4 and EtSerpin shared the same apical location during the early invasion of sporozoites into DF-1 cells. These results are consistent with previous reports [15, 18]. Another EtSerpin has been reported to be detected 24 h p.i. in DF-1 cells in vitro [18]. Additionally, EtCDPK4 has been detected in the vacuole 12 h p.i. [15]. In vitro, sporozoite invasion inhibition assays indicated that polyclonal antibodies against these two proteins can also reduce the ability of E. tenella sporozoites to invade host cells [15, 18]. Therefore, we speculate that the interaction between EtCDPK4 and EtSerpin is likely to play an important role in sporozoite invasion. When the sporozoites invade the host cells, the complex could release into the host cells to inhibit host protease activity which may delay host cell apoptosis.

Although we confirmed the interaction between EtSerpin and EtCDPK4 with several methods, we also identified but did not confirm other proteins that interact with EtCDPK4 using the Y2H screen. The interaction
between EtSerpin and EtCDPK4 may contribute to the invasion of *E. tenella* in host cells, the complex could inhibit host protease activity to delay host cell apoptosis during sporozoite development in host cells. However, further research on the function of the interaction between *Et*Serpin and *EtCDPK4 at the time of invasion is needed.

**Conclusions**

In this study, we constructed a Y2H cDNA library to screen for proteins that interact with *EtCDPK4*. *Et*Serpin was demonstrated to co-localize and interact with *EtCDPK4*, which may promote to the invasion and development of *E. tenella* in host cells.

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**Authors’ contributions**

HYH and BH conceived and designed the study. LL, BH and QPZ performed the experiments. HD and SHZ analyzed the data. LL and QPZ collected samples. HYH and BH conceived and designed the study. LL, BH and QPZ performed experiments. HD and SHZ analyzed the data. LL and QPZ collected samples. HYH and BH wrote the manuscript. All authors read and approved the manuscript.

**Ethics approval**

The protocol was approved and authorized by the Animal Care and Use Committee of the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

**Competition of interests**

The authors declare that they have no competing interests.

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**References**

1. Santos JM, Soldati-Favre D. Invasion factors are coupled to key signaling events leading to the establishment of infection in apicomplexan parasites. Cell Microbiol. 2011;13:787–96.
2. Billker O, Lounas S, Sibley LD. Calcium-dependent signaling and kinases in apicomplexan parasites. Cell Host Microbe. 2009;5:612–22.
3. Hetherington AM, Brownlee CE. The generation of Ca++ signals in plants. Annu Rev Plant Biol. 2004;55:401–27.
4. Harper JF, Harmon A. Plants, symbiosis and parasites: a calcium signaling connection. Nat Rev Mol Cell Biol. 2005;6:555–66.
5. Hamel LP, Sheen J, Seguin A. Ancient signals: comparative genomics of green plant CDPKs. Trends Plant Sci. 2014;19:79–89.
6. Wan B, Lin Y, Mou T. Expression of rice Ca++–dependent protein kinases (CDPKs) genes under different environmental stresses. FEBS Lett. 2007;581:1179–89.
7. Moreno SN, Ayong L, Pace DA. Calcium storage and function in apicomplexan parasites. Essays Biochem. 2011;51:197–110.
8. Green J, Rees-Channer R, Howell S, Martin S, Knuepfer E, Taylor H, et al. The motor complex of *Plasmodium falciparum* phosphorylation by a calcium-dependent protein kinase. J Biol Chem. 2008;283:30980–90.
9. Bansal A, Singh S, More KR, Hans D, Nangalia K, Yogavel M, et al. Characterization of *Plasmodium falciparum* calcium-dependent protein kinase 1 (PfCDPK1) and its role in microneme secretion during erythrocyte invasion. J Biol Chem. 2013;288:1590–602.
10. Lourido S, Shuman J, Zhang C, Shokat KM, Hui R, Sibley LD. Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in *Toxoplasma*. Nature. 2010;465:359–62.
11. Lourido S, Jeschke GR, Turk BE, Sibley LD. Exploiting the unique atp-binding pocket of Toxoplasma calcium-dependent protein kinase 1 to identify its substrates. ACS Chem Biol. 2013;8:1153–62.
12. Morlon-Guyot J, Berry L, Chen CT, Gubbels MJ, Lebrun M, Daher W. The Toxoplasma gondii calcium-dependent protein kinase 7 is involved in early steps of parasite division and is crucial for parasite survival. Cell Microbiol. 2014;16:95–114.
13. Dunn PP, Bumstead JM, Tomley FM. Sequence, expression and localization of calmodulin-domain protein kinases in *Eimeria tenella* and *Eimeria maxima*. Parasitolology. 1996;113:439–48.
14. Han HY, Lin JJ, Zhao QP, Hui D, Jiang LL, Xu MQ, et al. Identification of differentially expressed genes in early stages of *Eimeria tenella* by suppression subtractive hybridization and cDNA microarray. J Parasitol. 2010;96:95–102.
15. Wang Z, Bing H, Hui D, Zhao Q, Zuo S, Xia W, et al. Molecular characterization and functional analysis of a novel calcium-dependent protein kinase 4 from *Eimeria tenella*. PLoS One. 2016;11:e0168132.
16. Han HY, Zhu SH, Jiang LL, Li Y, Dong H, Zhao QP, et al. Molecular characterization and analysis of a novel calcium-dependent protein kinase from *Eimeria tenella*. Parasitology. 2013;140:746–55.
17. Weithma D, Tzifara T. Imaging protein-protein interactions in plant cells by bimolecular fluorescence complementation assay. Trends Plant Sci. 2009;14:59–63.
18. Jiang L, Lin J, Han H, Zuo Q, Dong H, Zuo S, et al. Identification and partial characterization of a serine protease inhibitor (serpin) of *Eimeria tenella*. Parasitol Res. 2012;110:865–74.
19. Tomley F. Techniques for isolation and characterization of apical organelles from *Eimeria tenella* sporozoites. Methods. 1997;13:171–6.
20. Geyser J, Aymard J, Vanden BH. Simultaneous purification of merozoites and schizonts of *Eimeria tenella* (Apicomplexa) by Percoll flotation and assessment of cell viability with a double fluorescent dye assay. J Parasitol. 1991;77:989–93.
21. Wang ZW, Dong H, Zhao QP, Xia WL, Zhu SH, Men QF, et al. Detection and construction of *Eimeria tenella* CDPK4 bait plasmid for yeast two-hybrid system. Chin J Anim Infect Dis. 2016;24:652–9.
22. Peroval M, Pery P, Labbe M. The heat shock protein 90 of *Eimeria tenella* is essential for invasion of host cell and schizont growth. Int J Parasitol. 2006;36:1205–15.
23. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, et al. Functional organization of the yeast proteome by systematic analysis of protein complex. Nature. 2002;415:141–7.
24. Ho Y, Gruhler A, Heilbut A, Badger GD, Moore L, Adams SL, et al. Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. Nature. 2002;415:180–3.

25. Ferro E, Baldini E, Trabatini L. Use of the yeast two-hybrid technology to isolate molecular interactions of Ras GTPases. Methods Mol Biol. 2014;1120:97–120.

26. Rajapopala SV. Mapping the protein-protein interactome networks using yeast two-hybrid screens. Adv Exp Med Bio. 2015;883:187–214.

27. Remy I, Michnick SW. Application of protein-fragment complementation assays in cell biology. BioTechniques. 2007;42:137–45.

28. Gandhi TK, Zhong J, Mathivanan S, Karthick L, Chandrika KN, Mohan SS, et al. Analysis of the human protein interactome and comparison with yeast, worm and fly interaction datasets. Nat Genet. 2006;38:285–93.

29. Pham CD. Detection of protein-protein interaction using bimolecular fluorescence complementation assay. Methods Mol Biol. 2015;1278:483–95.

30. Kerppola TK. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. Nat Protoc. 2006;1:1278–86.

31. Michnick SW. Protein fragment complementation strategies for biochemical network mapping. Curr Opin Biotechnol. 2003;14:610–7.

32. Dammann C, Ichida A, Hong B, Romanowsky SM, Hrabak EM, Harmon AC, et al. Subcellular targeting of nine calcium-dependent protein kinase isoforms from *Arabidopsis*. Plant Physiol. 2003;132:1840–8.

33. Stael S, Bayer RG, Mehlner N, Teige M. Protein N-acetylation overides differing targeting signals. FEBS Lett. 2011;585:517–22.

34. Ito T, Nakata M, Fukazawa J, Ishida S, Takahashi Y. Alteration of substrate specificity: the variable N-terminal domain of tobacco Ca2+-dependent protein kinase is important for substrate recognition. Plant Cell. 2010;22:1592–604.

35. Cheng SH, Sheen J. Calcium signaling through protein kinases. The *Arabidopsis* calcium-dependent protein kinase gene family. Plant Physiol. 2002;129:469–85.

36. Kumar P, Tripathi A, Ranjan R, Halbert J, Gilberger T, Doerig C, et al. Regulation of *Plasmodium falciparum* development by calcium-dependent protein kinase 7 (PfCDPK7). J Biol Chem. 2014;289:20386–95.

37. Valdivieso E, Perteguer MJ, Hurtado C, Campioli P, Rodríguez E, Saborido A, et al. ANISERP: a new serpin from the parasite *Anisakis simplex*. Parasit Vectors. 2015;8:399.

38. Silverman GA, Bird PI, Carrell RW, Church FC, Coughlin PB, Gettins PGW, et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins: evolution, mechanism of inhibition, novel functions, and a revised nomenclature. J Biol Chem. 2001;276:33293–6.

39. Gettins PGW. Serpin structure, mechanism, and function. Chem Rev. 2002;102:4751–804.

40. Irving JA, Pike RN, Lesk AM, Whisstock JC. Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. Genome Res. 2000;10:1845–64.

41. Rau JC, Beaulieu LM, Huntington JA, Church FC. Serpins in thrombosis, hemostasis and fibrinolysis. J Thromb Haemost. 2007;5(Suppl. 1):102–15.

42. Song KJ, Ahn HJ, Nam HW. Anti-apoptotic effects of SERPIN B3 and B4 via STAT6 activation in macrophages after infection with *Toxoplasma gondii*. Korean J Parasitol. 2012;50:1–6.