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Molecular analyses of *Toxoplasma gondii* calmodulin-like domain protein kinase isoform 3

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ABSTRACT

Ca^{2+} signaling is thought to play an important role in *Toxoplasma gondii* motility, including invasion of and egress from host cells. Recently, it has been reported that phosphorylation of the glideosome apparatus components of *T. gondii* occurs during invasion. To elucidate the role of *T. gondii* calmodulin-like domain protein kinase in the signaling pathway that bridges Ca^{2+} stimulation and motility, we characterized *T. gondii* calmodulin-like domain protein kinase isoform 3 (TgCDPKif3). TgCDPKif3 is homologous to *Plasmodium falciparum* calcium-dependent protein kinase 1, which has been reported to phosphorylate *P. falciparum* glideosome components. TgCDPKif3 was purified as a fusion protein that was labeled with [γ-^{32}P]ATP, and the label was subsequently removed by phosphatase treatment. Phosphorylation was eliminated when the putative catalytic lysine residue of TgCDPKif3 was replaced with alanine. TgCDPKif3 phosphorylated Histone II_{AS} as a representative substrate in a Ca^{2+}-dependent manner at a high Ca^{2+} concentration. TgCDPKif3 was localized to the apical ends of tachyzoites. TgCDPKif3 showed the translocation between intra- and extracellular tachyzoites. TgCDPKif3 could phosphorylate *T. gondii* aldolase 1 (TgALD1) *in vitro*. The interaction between TgCDPKif3 and TgALD1 was confirmed by the co-immunoprecipitation assay in mammal cells. We suggested that TgCDPKif3 could participate in the motility of *T. gondii* through the phosphorylation of glideosome complex member.

1. Introduction

Toxoplasma gondii is an obligate intracellular parasite of the phylum Apicomplexa, which includes the causative pathogens of toxoplasmosis, malaria, and cryptosporidiosis. Although infection with this parasite is typically asymptomatic, the infection rate is very high; acute toxoplasmosis can be fatal in immunocompromised individuals and can cause severe birth defects or abortion during the first trimester of pregnancy [1].

During the lytic cycle of the parasite, especially in the tachyzoites (the causative stage of acute toxoplasmosis), *T. gondii* needs to be motile, to enter into and escape from host cells. The actin–myosin-based motor complex, which is called the glideosome [2], ensures parasite motility and is conserved among the Apicomplexa [3].

The motility of *T. gondii* tachyzoites is activated by an increase in the cytosolic Ca^{2+} concentration [4], which occurs as the parasites exit the host cells [5,6]. This increase in Ca^{2+} concentration induces the parasites to secrete adhesion molecules from the microneme [7]. The adhesion molecules, which include *T. gondii* micronemal protein 2 (TgMIC2), become anchored to the host surface in association with *T. gondii* aldolase 1 (TgALD1), which connects the *T. gondii* actin 1 (TgACT1) in the glideosome [8,9].

Inhibitor studies have suggested that kinases are crucial controllers of the invasion steps [7,10–12]. So far, the phosphorylation of toxofilin, which is not a member of the glideosome but is the only protein reported to be phosphorylated during the invasion process, has been reported [13]. Recently, the phosphorylation of glideosome members has been reported in *T. gondii* [14] and in *Plasmodium falciparum* by *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) [15]. The increased Ca^{2+} may activate the calcium-dependent protein kinase (CDPK) family (synonym: calmodulin-like domain protein kinase) [16]. The previously characterized TgCDPK1 [17] is thought to be a candidate for the inhibition of invasion by kinase inhibitor KT5976, although TgCDPK1 is neither the only CDPK expressed in tachyzoites nor the only member of the family that is susceptible to kinase inhibitors. With respect to regulation through the phosphorylation of glideosome members, a comparative CDPK family might be active in *T. gondii*. Analyses of the mRNA expression levels and proteomics of ToxoDB (http://www.toxodb.org/) have confirmed that some members of the *Toxoplasma* CDPK family are expressed during the tachyzoite stage, when repeated invasion and egress occur.

Abbreviations: TgCDPKif3, *Toxoplasma gondii* calmodulin-like domain protein kinase isoform 3; TgALD1, *Toxoplasma gondii* aldolase 1; MBP, maltose binding protein; GST, glutathione S-transferase.

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In the present study, we focused on *T. gondii* calmodulin-like domain protein kinase isoform 3 (TgCDPKif3) (GenBank accession no. DQ205646), (ToxoDB gene ID: TGME49_105860), which is homologous to PfCDPK1 (56% similarity) and expressed only in tachyzoite form in mRNA level. TgCDPKif3 expressed in *Escherichia coli* lacks Ca^{2+}-dependency [11], and appears not to be associated with Ca^{2+} signaling under conditions of low Ca^{2+} concentration. However, in the present study, TgCDPKif3 showed Ca^{2+}-dependency in high concentrations of Ca^{2+} and phosphorylated TgALD1 *in vitro*. We also detected differences in the localization of TgCDPKif3 between intra- and extra-cellular tachyzoites. Among the glideosome members, TgALD1 has been reported to translocate from the cytosol of intracellular parasites to the periphery of extracellular parasites [18] or to the apical end of extracellular parasites [8,19]. We found that TgCDPKif3 was bound to TgALD1 when expressed in mammalian cells. The present study provides evidence for the involvement of TgCDPKif3 in invasion, and suggests that it is one of the targets of the kinase inhibitors that inhibit invasion.

2. Materials and methods

2.1. Cells and parasites

Tachyzoites of *T. gondii* RH strain (kindly provided by Dr. Y. Nishikawa) were used in this study. The parasites were maintained in monolayers of Vero cells cultured in Dulbecco's modified Eagle's medium (DMEM) that contained 7.5% fetal calf serum (FCS), 2 mM L-glutamine, 20 mM HEPES (pH 7.5), streptomycin, and penicillin. The host Vero cells were passaged in the same medium. For the expression of flag-TgCDPKif3 and myc-TgALD1, 293 T cells cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 20 mM HEPES (pH 7.5), streptomycin, and penicillin were used.

2.2. Plasmids

T. gondii RH strain mRNA was isolated from infected Vero cells using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The entire TgCDPKif3 open reading frame (ORF) was amplified by RT-PCR using the parasite mRNA as template and the following primers: forward, 5′-GCGAATTCGGGTGCGTCCACTCCAAGAA-3′ and reverse, 5′-GCGGATCCTCAGTGCTTCACTTTGACGT-3′. The amplified fragment was digested with EcoRI/BamHI and cloned into the EcoRI and BamHI sites of pBluescript II KS+ (Stratagene, La Jolla, CA, USA). The resultant plasmid was designated pBS-TgCDPKif3-stop. The pAcGHLT-TgCDPKif3 construct (Fig. 1B) was generated by inserting the EcoRI–NotI fragment of pBS-TgCDPKif3-stop into pAcGHLT-A (BD bioscience, San Jose, CA, USA) to express a glutathione S-transferase (GST) fusion protein. The transfer plasmid with the kinase activity-negative mutant, pAcGHLT-TgCDPKif3KA, was constructed from pAcGHLT-TgCDPKif3 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions (Fig. 1A). For expression in 293 T cells, pFlag-TgCDPKif3 was generated by inserting the HindIII/BamHI fragment of pBS-TgCDPKif3-stop into pFlag-CMV2 (Sigma, St. Louis, MO, USA). The entire TgALD1 ORF was amplified from parasite mRNA by RT-PCR using the primers: 5′-GCGAATTCCTTTGCATTGTAGAGCGAAT-3′ and 5′-GCGGATCCTTAGTACACGTAGCGTTTCT-3′. The amplified fragments were digested with EcoRI/BamHI and cloned into the EcoRI and BamHI sites of pBluescript II KS+. The resultant plasmid was designated as pBS-TgALD-Stop. The EcoRI/XbaI fragment of pBS-TgALD-Stop was inserted into pMal-c (New England BioLabs, Beverly, MA, USA) in frame with maltose binding protein (MBP), to generate pMal-TgALD. For the expression of c-myc-tagged TgALD1 in 293 T cells, pMyc-TgALD1 was generated as follows. The TgALD1 ORF attached to the NdeI/PstI restriction site was amplified from pBS-TgALD-Stop using the primers: 5′-GGAATTCCATATGCTTTGCATTGTAGAGCGAAT-3′ and 5′-AAAACTGCAGTTAGTACACGTAGCGTTTCT-3′. The amplicon was digested with NdeI and PstI and the fragments were inserted into pME18Smyc [20], with the resulting plasmid being designated as pME-TgALD1. Finally, the SspI/XhoI fragment of pCAGGS [21] (used to replace the promoter with the chicken β-actin promoter) was inserted into pME-TgALD1, to generate pMyc-TgALD1 (Fig. 1B).

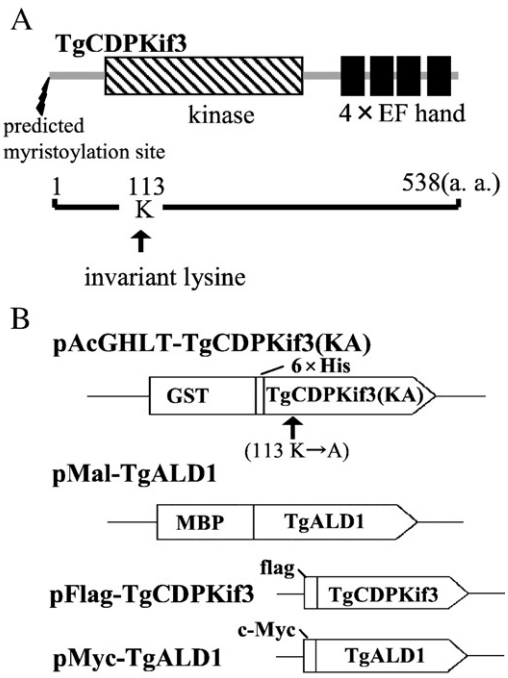

Fig. 1. Schematic diagram of the predicted amino acid sequence of TgCDPKif3 and the construction of the expression plasmids. (A) The sequence around the invariant catalytic lysine is shown. (B) The pAcGHLT-TgCDPKif3(KA), pMal-TgALD1, pFlag-TgCDPKif3, and pMyc-TgALD1 were used to express GST-TgCDPKif3 and GST-TgCDPKif3KA in the baculovirus expression system, MBP-TgALD in the *E. coli* expression system, and Flag-TgCDPKif3 and c-Myc-TgALD in the mammalian expression system, respectively.

2.3. Generation of recombinant baculoviruses

pAcGHLT-TgCDPKif3(KA) or pAcGHLT-A was co-transfected with linearized baculovirus DNA BaculoGold (BD bioscience) into Sf9 cells using Cellfectin (Invitrogen), as described previously [22], to generate recombinant baculoviruses that were designated as Bac-GST-TgCDPKif3(KA) or Bac-GST, respectively. The recombinant viruses were subsequently amplified in Sf9 cells.

2.4. Purification of recombinant proteins

The purification of recombinant proteins expressed in Sf9 cells infected with Bac-GST-TgCDPKif3(KA) or Bac-GST was performed as described elsewhere [22]. Briefly, Sf9 cells (1.0×10^6) infected with each baculovirus (Bac-GST-TgCDPKif3(KA) or Bac-GST) in 0.5 ml of ice-cold buffer C (50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol and 1 mM PMSF) were lysed by sonication. After insoluble material was removed by centrifugation, the supernatants were mixed with 150 µl of a 50% slurry of glutathione-Sepharose beads (BD bioscience) for 2 h. The beads were extensively washed with buffer C and eluted with elution buffer G (10 mM glutathione and 500 mM Tris–HCl, pH 8.0). Next, the eluted supernatants were reacted with Ni^{2+}-NTA agarose beads (Qiagen, Hilden, Germany) for 1 h. The beads were then washed three times with buffer C. Purified proteins captured on the beads were separated by 10% SDS-PAGE and either silver-stained (Fig. 2A) or immunoblotted (Fig. 2B) with rabbit antiserum that contained anti-GST-