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A NOVEL *TOXOPLASMA GONDII* CALCIUM-DEPENDENT PROTEIN KINASE

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Summary:

Toxoplasma gondii is an obligate intracellular parasite that infects all types of cells in humans. A family of calcium-dependent protein kinases (CDPKs), previously identified as important in the development of plants and protists, was recently shown to play a role in the infectivity of apicomplexans, and in motility and host cell invasion in particular. We report here the isolation of a new calcium-dependent protein kinase gene from the human toxoplasmosis parasite, *Toxoplasma gondii*. The gene consists of 12 exons. The encoded protein, TgCDPK4, consists of the four characteristic domains of members of the CDPK family and is most similar to PfCDPK2 from *Plasmodium falciparum*. We measured TgCDPK4 activity, induced by calcium influx, using a kinase assay. A calcium chelator (EGTA) inhibited this activity. These findings provide evidence of signal transduction involving members of the CDPK family in *T. gondii*.

KEY WORDS : *Toxoplasma gondii*, protein kinase, calcium-dependent protein kinase, TgCDPK4, calcium, gene.

Résumé : UNE NOUVELLE PROTÉINE KINASE DÉPENDANTE DU CALCIUM CHEZ *TOXOPLASMA GONDII*

Toxoplasma gondii *est un parasite intracellulaire obligatoire qui infecte tous les types de cellules chez l'homme. Une famille de protéines kinases dépendantes du calcium (CDPK) a été identifiée uniquement chez les plantes et les protistes. Chez les apicomplexes, CDPK a été récemment décrite pour jouer un rôle dans l'infectivité, la mobilité et l'invasion des cellules hôtes. Nous reportons ici le clonage et le séquençage d'une nouvelle protéine kinase dépendante du calcium de* Toxoplasma gondii *TgCDPK4. Le gène est composé de 12 exons. La séquence de TgCDPK4 comporte les domaines caractéristiques des sérine/thréonine kinases et quatre domaines EF liant le calcium. TgCDPK4 présente des similarités avec les CDPK des plantes et celles de* Plasmodium falciparum *(Pf CDPKs). TgCDPK4 possède une activité kinase stimulée par le calcium. Un chélateur du calcium (EGTA) inhibe cette activité. L'ensemble de ces résultats semble indiquer que TgCDPK4 joue un rôle dans la transduction du signal chez* Toxoplasma gondii.

MOTS CLÉS : *Toxoplasma gondii*, protéine kinase, calcium, gène, Tg CDPK4.

INTRODUCTION

The apicomplexan protozoan *Toxoplasma gondii* is an obligate intracellular parasite that typically causes asymptomatic infections, but acute toxoplasmosis is a leading source of congenital neurological defects at birth and may be life-threatening in immuno-suppressed patients. The parasite invades and replicates within almost all nucleated mammalian cells and host cell invasion is critical for the growth and reproduction of *T. gondii* (Joiner *et al.,* 1993; Dobrowolski *et al.,* 1996). The biochemical pathways involved in host cell invasion have not been identified, but several lines of evidence suggest that Ca^{2+} is important for parasite motility (Mondragon & Frixione, 1996; Smith, 1995; Werk, 1985; Pezella *et al.,* 1997) and the attachment of *T. gondii* to its host cell. Ca^{2+} plays a key role in signal transduction in eukaryotic cells, by activating protein kinase cascades (Chao *et al.,* 1992). Mitogen-activated protein (MAP) kinases, also known as extracellular signal-regulated protein kinases (ERKs), represent a crossing point between intracellular signaling through protein tyrosine kinase activation involving G protein-coupled receptors, Ca^{2+} channel-coupled receptors and protein kinase C (Robinson & Cobb, 1997). MAP kinase activation is one of the most rapid cellular responses to growth and differentiation factors, and to various external stimuli (Blenis, 1993). We have recently (Roisin *et al.,* 2000) characterized two ERK/MAPkinases from *Toxoplasma gondii* sharing two epitopes with the eukaryotic MAP kinases (ERK1 and ERK2).

An unusual class of calcium-dependent protein kinases has been described containing calmodulin-like domains (CDPKs) and the kinases can be activated by calcium in the absence of calmodulin, or phospholipids. First described in plants (Harper *et al.,* 1991) such enzymes have also been identified in algae (Yuasa & Muto, 1992) and in apicomplexan parasites such as *Eimeria maxima* (Dunn *et al.,* 1996) and *P. falciparum* (Li *et al.,* 2000;

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Zhao *et al.,* 1993). *T. gondii* contains more than 20 CDPK or CDPK- related kinases (Nagamune & Sibley, 2006). In the present, two related *T. gondii* CDPK isoforms have been recently identified, where TgCDPK1 seems to play a central role in regulating parasite motility and host cell invasion (Kieschnick *et al.,* 2001). Indeed, TgCDPK1 was found to be inhibited by KT5926, a selective inhibitor of calmodulin-dependent and myosin light chain kinase, which is known to block the mobility of *T. gondii* (Dobrowolski *et al.,* 1997). Recently another TgCDPK3 clone was isolated from the DNA library (Donald *et al.*, 2006). In this study, we report the characterization of a new CDPK (TgCDPK4) from *T. gondii*.

MATERIALS AND METHODS

Parasites

Growth and isolation of parasites: *T. gondii* strain RH was maintained by serial passage in the peritoneal cavity of OF11 Swiss mice (Iffa Credo, France), as previously described (Roisin *et al.*, 2000; Creuzet *et al.,* 1998), or in mouse NIH-3T3 fibroblast cell cultures.

Invasion assays and immunocytofluorescence

Mouse NIH-3T3 fibroblast cells were cultured on 22 mm^2 glass coverslips (10^5) and infected with 5×10^5 tachyzoites as previously described (Roisin *et al.,* 2000; Creuzet *et al.,* 1998). Parasites were detected with a mouse monoclonal anti-*Toxoplasma* antibody (generously provided by Dr J.F. Dubremetz, Montpellier France), or with anti-TgCDPK4 antibodies.

Preparation of TgCDPK4 specific antisera

Anti-sera against the 42 kDa band TgCDPK4 trunkated protein were raised in two toxoplasmosis-free rabbits. Each animal was immunized subcutaneously with 100 µg of purified recombinant TgCDPK4 protein under standard conditions (Eurogentec, France) on days 0, 14, 28 and 56. The serum obtained will be referred to hereafter as anti TgK4a and TgK4b serum. The anti-sera were affinity purified with the TgCDPK4 trunkated antigen. Sera samples taken before the first immunization will be referred to as pre-immune sera. These pre-immune anti-sera failed to detect any protein in parasite extracts.

Cell culture and transient transfection

Eukaryote Cos-7 cells (SV40-transformed African green monkey kidney cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum (FCS). Subconfluent Cos-7 cells (about 2×10^6 cells) plated in 35 mm-diameter dishes were transiently transfected with 1 µg of pAS1B-TgCDPK4, using the FuGENE6 reagent (Roche).

Immunoprecipitation

All steps were carried out at 4° C. Frozen parasites or transfected cell lysates (pAS1B-Tg CDPK4 fusion proteins) were rapidly thawed and homogenized at 4° C in 20 µl of lysis buffer containing 1 % NP40, 1 % DOC, 0.1 % SDS, 158 mM NaCl, 10 mM Tris pH 7.8, 1 mM PMSF (phenylmethylsulfonyl fluoride) and 1 mM Na_3VO_4 (RIPA buffer). Lysates were cleared by centrifugation at 15,000 g for 20 minutes, and incubated with protein G-agarose-conjugated antibodies for two to three hours (anti-HA or antibodies raised against CDPK4, anti-TgK4a/b). Immunoprecipitates were washed four times with lysis buffer and subjected to immunoblotting (Benes *et al.,* 1998) and kinase assays.

Kinase assays

Kinase activities were measured using MBP or histone H1 (Life Technologies, Inc) as the substrate. Assays were performed in a standard reaction mixture (20 µl) containing 20 mM Tris-HCl pH 8.0, 20 mM $MgCl^2$, 2 mM $MnCl^2$, 100 µM [γ^{32}P ATP] (10 µCi/assay, Amersham) and 2 µg histone H1 and/or 5 µg of MBP. Reactions were initiated by adding 1 µg of the recombinant protein kinase substrate or 1 µg of *Toxoplasma* extract per reaction. The reaction was allowed to proceed for 45 minutes at 30° C. and stopped by adding Laemmli buffer. The mixture was then boiled for five min, and analyzed by SDS-PAGE in 12 % polyacrylamide gels. The gels were dried and subjected to autoradiography. We assayed the auto-phosphorylation of recombinant protein kinases in the same conditions in the absence of exogenous protein substrate. Autoradiographs were analyzed by densitometry.

Abreviations

The abreviations used are:
- CDPK: calcium-dependent protein kinase;
- ERK: extracellular signal-regulated protein kinase;
- MAPK: mitogen-activated protein kinase,
- GST: glutathione S-transferase;
- MBP: myelin basic protein;
- Tg K4: antibody against TgCDPK4;
- PCR: polymerase chain reaction.

RESULTS AND DISCUSSION

TgCDPK4 was initially isolated from a *T. gondii* expression library with antibodies to mammalian ERK1 and ERK2 (Santa-Cruz Biotechnology). The

deduced amino-acid sequence of the cDNA clone coded for 452 amino acids with a calculated molecular mass of 42 kDa. TgCDPK4 was expressed in bacteria and the purified recombinant protein used to generate two specific sera in rabbits (anti TgK4a and TgK4b). Antisera were subsequently affinity purified. Theses antibodies detected a parasite protein of 67 kDa indicating that the initial cDNA was incomplete (data not shown). The pre-immune sera did not detected protein in parasites extracts (data not shown). Full length TgCDPK4 encoding a 594-amino acid protein with a predicted molecular mass of 67 kDa was obtained by RT-PCR using primers based on sequence information available at ToxoDB database (http://toxodb.org/). A search of the ToxoDB database showed that the 105434 bp TGG994543 gene contained the sequence of the TgCDPK4. The gene sequence predicted by GENSCAN contained 20 introns. TgCDPK4 starts at exon 5 and ends at exon 16 of a 26833 bp sequence of the gene The predicted translation initiation site was located in exon 5. TgCDPK4 displayed conservation of all of the key functional motifs typical of CDPKinases, including a serine/threonine kinase domain similar to that of calcium/calmodulin-dependent protein kinases, followed by a highly conserved junction domain connecting the kinase region to the C-terminal calmodulin-like domain and contains four EF-hand calcium binding site. This calmodulin-like domain is 40 % similar to calmodulin, and corresponds to the calcium-binding domain of the archetype CDPK (Day *et al.,* 2002).

Not surprisingly, sequence comparisons showed that TgCDPK4 was most similar to CDPKs of apicomplexan parasites *P. falciparum* PfCDPK2 (Farper *et al.,* 1997) and *E. maxima* EmCDPK (Dunn *et al.,* 1996) and plants such as *A. thaliana* CDPK/AK1 (Harper *et al.,* 1993) and soybean CDPKg. (Harper *et al.,* 1991. The sequence of TgCDPK4 has been deposited in Genbank under the accession number AJ488146.

Figure 1 shows the CLUSTALW 1.8 multiple alignment of TgCDPK4 with TgCDPKI, TgCDPK2, TgCDPK3 sequences from *T. gondii*, PfCDPK1, PfCDPK2, PfCDPK3 and PfCDPK4 sequences from *Plasmodium falciparum*. Motifs commonly found in protein kinases are present in this enzyme. TgCDPK4 contains a glycine loop motif ($G^{38}XG^{40}XXG^{43}$) and an invariant lysine ($^{59}AIK^{61}$ and Lys^{71}) presumably involved in ATP binding. Tg CDPK4 also has a number of motifs commonly found in serine/threonine protein kinases: $^{79}EVAI^{82}$, $^{246}HRDLKLEN^{252}$, $^{268}IDF^{270}$, $^{295}APE^{297}$, $^{308}DLW^{310}$ and Tyr^{317}. The calmodulin-like C-terminal domains of TgCDPK4, like those of all known CDPKs, contain four EF-hand calcium-binding sites. Pair-wise analyses with the full protein sequence was shown in Table I. While TgCDPK1 and TgCDPK3 showed 63 % identity with each other, TgCDPK4 showed only 25 % identity with others TgCDPK isoforms. Tg CDPK1,2,3 were more closely related to the Pf CDPKs (37 to 73 %) than TgCDPK4 (23 to 28 %). The phylogenetic tree based on the amino-acid sequence also show that the TgCDPK4 is less related to CDPK that to the other Tg CDPKs and PfCDPKs isoforms (Fig. 2).

In a recent publication, Donald *et al.* (2002, 2006) reported that TgCDPK1 and TgPKG (cGMP protein kinase) share a common sensitivity to pyrrole and imidazopyrimidine inhibitors due to a threonine residue in the catalytic binding pocket and substitution of this threonine residue with methionine prevented inhibitor binding. As TgCDPK3, the new TgCDPK4 also insert with an invariant methionine residue in the catalytic binding pocket $^{194}EKGHKRIHLVM^{204}ELCTGKELYD^{214}$. This invariant methionine residue was found also in PfCDPK2, PfCDPK4, but not in TgCDPK1. As decribed recently by Donald *et al.*, 2006, TgCDPK4 would most likely be resistant to both classes of compound.

The distribution of TgCDPK4 in extracellular and intracellular *T. gondii* parasites was analyzed using affinity purified anti-TgK4a and anti-Tg4b antibodies and compared to the anti-*T. gondii*-P30 surface protein (Fig. 3). In conventional fluorescence microscopy, the anti-P30 antibody decorated the surface of *T. gondii*, lining the cell contours (Fig. 3A1 and A4), as expected. In contrast, the anti-TgK4a and anti Tg-K4b antibodies displayed a cytoplasmic distribution in tachyzoites, as well in extracellular (3A2, 3A3) and in intracellular parasites (3A5 and 3A6) with some accumulation in the basal region. The protein was not detected in the cytoplasm of infected host cells (Fig. 3A5 and 3A6) demonstrating the parasite-specific nature of the two anti-TgK4 anti-

	TgCDPK1	TgCDPK2	TgCDPK3	TgCDPK4	PfCDPK1	PfCDPK2	PfCDPK3	PfCDPK4
TgCDPK1	100	42	63	26	57	44	37	73
TgCDPK2		100	38	27	40	43	38	39
TgCDPK3			100	26	62	47	38	61
TgCDPK4				100	23	27	28	25
PfCDPK1					100	43	35	56
PfCDPK2						100	44	45
PfCDPK3							100	36
PfCDPK4								100

Table I. – A multiple sequence alignment was obtained by pair-wise (% identity).

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TgCDPK1   MGQQESTLGG----------AAGEPRSRGHAAGTS  25
TgCDPK2   MPHHQCST----------SQRRHSIS----------T  17
TgCDPK3   MGCVHSKNPHS----------KHAGAAGEKPD-ASLEKGGQSKGSAPSS  38
TgCDPK4   MADPLSFFNSLTHTPLFTSRIKTKVKLEQVYDVSNHVL**GTGISGA**VRIGHHRQSRRQV**AI**  60
PfCDPK1   MGCSQSSN----------VKDFKTRRSKFTN  21
PfCDPK2   MGNHLSVN----------KLKRKKKKKSFLN----------IYGKNTNEN-TSKQS  35
PfCDPK3   MNDLIIKNNKKGSCDVIIKYKCKKSDENIKRRKSSHKYIKNKS-VVLGRSIMTNKKEKLK  59
PfCDPK4   MGQEVSSVNN----------TKNEHHKTNKKSLKG  25

TgCDPK1   GGPGDHLH----------AT----------PGMFVQHSTAIFSDRY-K  52
TgCDPK2   QAADEAAGGG----NRVSFK----------RSAFILANTGPITNYY-T  50
TGCDPK3   GTGDSGKG----------TGSPDTKRD----------SMPMTPGMYITQQKAHLSDRY-Q  77
TgCDPK4   **K**TLCLSAMAPKRTLMLYN**E**VAIYLQVDHPNICKLLEVFVDDGEERKPAAESPRLAGNRER  120
PfCDPK1   GN-NYGK----------SGN-NKNSE----------DLAINPGMYVRKKEGKIGESY-F  57
PfCDPK2   NDYKYDIN----------TSCIS---REGTTTLE----------RKNLILCHSGKLEDKY-I  73
PfCDPK3   GALKYKGSKKEIKICNKKSMIKNDKDENTTLKSMKSDNFKFSRRGFILSFTGNLEDFYNL  119
PfCDPK4   GNERHEMK----------ESSVGISKKIVENSFNNSKLRPGMFIQNSNVVFNEQY-K  71

TgCDPK1   GQRVL**GKGSFGEV**ILCKDKITGQEC**AVK**VISKR----------QVKQKTDKE----SLLREV  100
TgCDPK2   VSKTI**GRGTWGEV**KLVIDNGTGARR**AAK**KIPKC----------YVEDAD----RFRQEI  95
TGCDPK3   RVKKL**GSGAYGEV**LLCKDKLTGAER**AIK**IIKKS----------SVTTTSNSG----ALLDEV  125
TgCDPK4   AESAEAR**G**DSRSLVGSSPVHAVGKT**GDK**LVRTPVSS----EGTPLRAEDLKSEEQDDREE  176
PfCDPK1   KVRKL**GSGAYGEV**LLCREKHGHGEK**AIK**VIKKSQFDKMKYSITNKIECDDKIHEEIYNEI  117
PfCDPK2   IDEKL**GQGTYGCV**YKGIDKVTNQLY**AIK**EEKKD----------RLKNIN----RFFQEI  118
PfCDPK3   SKEPL**GKGTYGCV**YKATDKLLKISR**AVK**VVSKK----------KLKNIP----RFRQEI  164
PfCDPK4   GIKIL**GKGSFGEV**ILSRDKHTGHEY**AIK**VISKK----------HVKRKTDKE----SLLREV  119

TgCDPK1   QLLKQLDHPNIMKLYEFFEDKGYF**YLV**GEVYTGGELFDEIISRKRFSEVDAARIIRQVLS  160
TgCDPK2   EIMKSLDHPNIVRLYETFEDMTDF**YLV**MEYCTGGELFDRLVHQGVFTEALACRIMRQILA  155
TgCDPK3   AVLKQLDHPNIMKLYEFFEDKRNY**YLV**MEVYRGGELFDEIILRQKFSEVDAAVIMKQVLS  185
TgCDPK4   RKERRGSCMSRTSISSDEKGHKRI**HLV**MELCTGKELYDRLARKKRYSEKDAGRVTRQMLS  236
PfCDPK1   SLLKSLDHPNIIKLFDVFEDKKYF**YLV**TEFYEGGELFEQIINRHKFDECDAANIMKQILS  177
PfCDPK2   EIMKKLDHPNIVKLYETYENDNYI**YLI**MELCSGRELFDSIIENGSFTEKNAATIMKQIFS  178
PfCDPK3   DIMKNLDHPNVVKLLETFEDSNQI**YLV**MELCTGGELFDKIVKKGCFVETFASFIMKQIFS  224
PfCDPK4   ELLKMLDHINIMKLYEFFEDNNYY**YLV**SDVYTGGELFDEIISRKRFYEIDAARIIKQILS  179

TgCDPK1   GITYMHKNKIV**HRDLKPEN**LLLESKSK-DAN**IRIIDFG**LSTHFEA-SKKMKDKI**GT**AYYI  218
TgCDPK2   AVAYCHAHRVA**HRDLKPEN**FLFLHDNP-ESP**IKLIDF**GLAARFKPGQPMR-TRAGTPYYV  213
TGCDPK3   GTTYLHKHNIV**HRDLKPEN**LLLESKSR-DAL**IKIVDFG**LSAHFEV-GGKMKERL**GT**AYYI  243
TgCDPK4   AINYCHQRHIC**HRDLKLEN**WVFRDDSD-DA**PLKLIDFG**FSRIFHP-GVRMTAMH**GT**VYYV  294
PfCDPK1   GICYLHKHNIV**HRDIKPEN**ILLENKHS-LLN**IKIVDFG**LSSFFSK-DNKLRDRL**GT**AYYI  235
PfCDPK2   AIFYLHSLNIV**HRDLKPEN**FLFQSENK-DSL**LKIIDFG**LSKNLGTGEFTT-TKA**GT**PYYV  236
PfCDPK3   VLNYLHIRNIC**HRDIKPEN**FLFYDMTP-ESL**IKIIDFG**LASYFTHNNYEMKTKA**GT**PYYV  283
PfCDPK4   GITYMHKNNVV**HRDLKPEN**ILLETKNKEDMII**IKIIDFG**LSTHFEY-SKKMKDKI**GT**AYYI  238

TgCDPK1   **APE**VLHGTYDEK**CDVW**ST**GVILYILLSG**CPPFNGANEYDILKKVEKGKYTFELPQWKKVS  278
TgCDPK2   **SPQ**VLEGRYGPE**CDVW**SA**GVMMYILLCG**YPPFNAPSDRAIMNKVRAGHYTFPDSEWSRVS  275
TgCDPK3   **APE**VLRKKYDEK**CDVW**SC**GVILYILLCG**YPPFGGQTDQEILKRVEKGKFSFDPPDWTQVS  303
TgCDPK4   **APE**VMDGKYNEK**CDLW**SI**GVIVYMLLSG**SPPFTGHGDQEILIKIRRCKYNMDGPRWRGIS  354
PfCDPK1   **APE**VLRKKYNEK**CDVW**SC**GVILYILLCG**YPPFGGQNDQDIIKKVEKGKYYFDFNDWKNIS  295
PfCDPK2   **APQ**VLDGKYDKK**CDIW**SS**GVIMYTLLCG**YPPFYGDTDNEVLKKVKKGEFCFYENDWGSIS  296
PfCDPK3   **APQ**VLTGSYNYK**CDMW**SS**GVLFYILLCG**YPPFFGESDHEILSMVKKGKYQFKGKEWNNIS  343
PfCDPK4   **APD**VLHGTYDEK**CDIW**SC**GVILYILLSG**CPPFNGSNEYDILKKVEAGKYTFDLPQFKKIS  298

TgCDPK1   ESAKDLIRKMLTYVPSMRISARDALDHEWIQTYT-KEQISVDVPSLDNAILNIRQFQGTQ  337
TgCDPK2   LQAKDLISRLLDRHPRTRISAEQALRHAWFAMHA---PGD-HFEPLGLDILSKFRRFQGLS  329
TgCDPK3   DEAKQLVKLMLTYEPSKRISAEEALNHPWIVKFCSQKHTDVGKHALTGALGNMKKFQSSQ  363
TgCDPK4   EQAKHFIASLLRRNPEERPSAEEALKHPWLVAAEKEALADTEIDVS---VLKSMQRFAACS  412
PfCDPK1   EEAKELIKLMLTYDYNKRITAKEALNSKWIKKYANNINKSD-QKTLCGALSNMRKFEGSQ  354
PfCDPK2   SDAKNLITKLLTYNPNERCTIEEALNHPWITQMTKSH-EHVELSST---LLKNLKNFKKEN  353
PfCDPK3   EEAKDLIKRCLTMDADKRICASEALQHPWFKKKKYAFNMDMKMDIH---VLENFKNYGLLL  401
PfCDPK4   DKAKDLIKKMLMYTSAVRISARDALEHEWIKMMTSKDNLNIDIPSLELSIANIRQFQSTQ  358

TgCDPK1   **KLA**QAALLYMGSKLTSQDETKELTAI**FHKMDKNGDG**QLDRAELIEGYKELMRMKGQDASM  397
TgCDPK2   **RLK**KLALTVIAQHLEDS-EIEGLKNL**FTQLDTEGDG**VLTVEEIRKGIERSG-VHLPPDMV  387
TgCDPK3   **KLA**QAAMLFMGSKLTTLEETKELTQI**FRQLDNNGDG**QLDRKELIEGYRKLMQWKGDTVSD  423
TgCDPK4   **AIK**RASLALIAMSMN-AQQLDDLERL**FRKIDIDNSG**CIKMDRMVAVLVTFLDVP----  465
PfCDPK1   **KLA**QAAILFIGSKLTTLEERKELTDI**FKKLDKNGDG**QLDKKELIEGYNILRSFK-NELGE  413
PfCDPK2   **ELK**KIALTIIAKHLCDVEINN-LRNI**FIALDVDNSG**TLSSQEILDG----LKK  401
PfCDPK3   **KFQ**KLAMTIIAQQSNDYDVEK-LKST**FLVLDEDGKG**YITKEQLKKG----LEK  449
PfCDPK4   **KLA**QAALLYMGSKLTTIDETKELTKI**FKKMDKNGDG**QLDRNELIIGYKELLKLKGEDTSD  418

TgCDPK1   LDASAVEHEVDQVLDAV**DFDKNGYIEYSEF**VTVAMDRKTLLSRERLERA**FRMFDSDNSGK**  457
TgCDPK2   LED----VLREV**DTAGTGSIDYTEF**IAACLHQSHYIREEACRAA**FRVLDINGDGL**  439
TgCDPK3   LDSSQIEAEVDHILQSV**DFDRNGYIEYSEF**VTVCMDKQLLLSRERLLAA**FQQFDSDGSGK**  483
TgCDPK4   ----RDEALRIFQRI**DQTRAEEINYSEF**LAATLQTRIALNQQLIREA**FERFDVDNSGH**  519
PfCDPK1   LKN---VEEEVDNILKEV**DFDKNGYIEYSEF**ISVCMDKQILFSEERLRDA**FNLFDTDKSGK**  471
PfCDPK2   IGYQKIPPDIHQVLRDI**DSNASGQIHYTDF**LAATIDKQTYLKKEVCLIP**FKFFDIDGNGK**  461
PfCDPK3   DG-LKLPYNFDLLLDQI**DSDGSGKIDYTEF**IAAALDRKQ-LSKKLIYCA**FRVFDVDNDGE**  507
PfCDPK4   LDNAAIEYEVDQILNSI**DLDQNGYIEYSEF**LTVSIDRKLLLSTERLEKA**FKLFDKDGSGK**  478

TgCDPK1   **ISSTE**LATIFG----VSDVDSETWKSVLSE**VDKNNDGEVDFDEF**QQMLLKLCG----  506
TgCDPK2   **VSAQE**LRQVFH---MAGDLETDAAA----ELLE CGLMRKVPSLALV----  515
TGCDPK3   **ITNEE**LGRLFG----VTEVDDETWHQVLQE**CDKNNDGEVDFEEF**VEMMQKICDVKVK  536
TgCDPK4   **ISLEN**LRYVLG----DSYDSLSVEEILRQ**CDRKQNGVIEFDEF**MLALTGDESGVLE  571
PfCDPK1   **ITKEE**LANLFG----LTSISEQMWNEVLGE**ADKNKDNMIDFDEF**VNMMHKICDNKSS  524
PfCDPK2   **ISVEE**LKRIFGRDDIENPLIDKAID---SLLQE**VDLNGDGEVNKNYN**HEKG----  509
PfCDPK3   **ITTAE**LAHILYNGNKKGNITQRDVNRVKRMIRD**VDKNNDGKIDFHEF**SEMMKLKF----  562
PfCDPK4   **ISANE**LAQLFG----LSDVSSECWKTVLKE**VDQNNDGEIDFKEF**RDMLVKLCNY----  528

TgCDPK1   ----------
TgCDPK3   H---------- 537
TgCDPK4   EWELDGPCCSRRRANSSSESQHE 594
PfCDPK1   ----------
PfCDPK2   ----------
PfCDPK3   ----------
PfCDPK4   ----------
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Fig. 1. – CLUSTAL W: multiple alignment of the predicted amino-acid sequence of the new TgCDPK4 (AJ488146) with the TgCDPK1 (AF333958), Tg CDPK2 (AF333959), TgCDPK3 (DQ205646) sequences from *Toxoplasma gondii*. PfCDPK1 (A45472), PfCDPK2 (X99763), PfCDPK3 (AF106064) and PfCDPK4 (CAD50923) sequences from *Plasmodium falciparum*. The highly conserved amino-acid residues are shown in bold typeface. Sequences were aligned with the CLUSTAL W (1.8) multiple sequence alignment program.

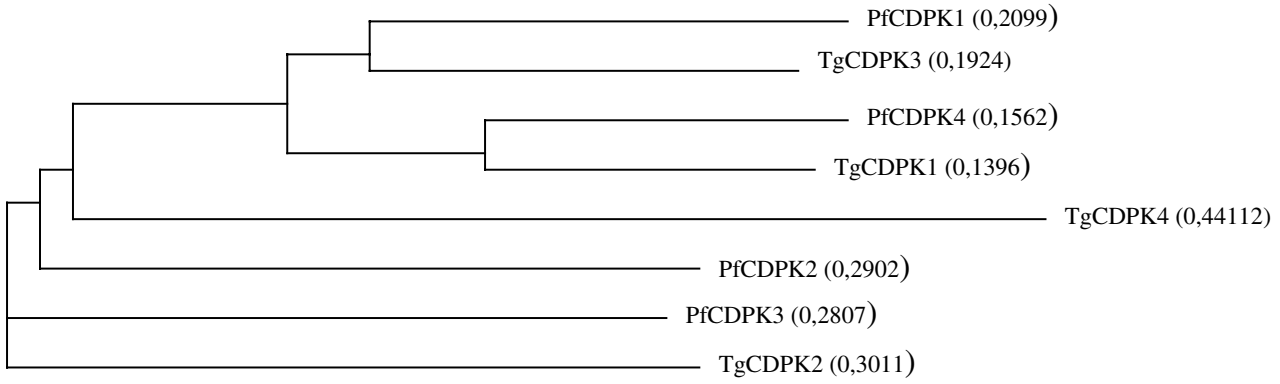

Fig. 2. – Phylogenetic tree showing the relationship between *Toxoplasma gondii* and *Plasmodium faciparum* CDPKs were generated using Vector NTI.

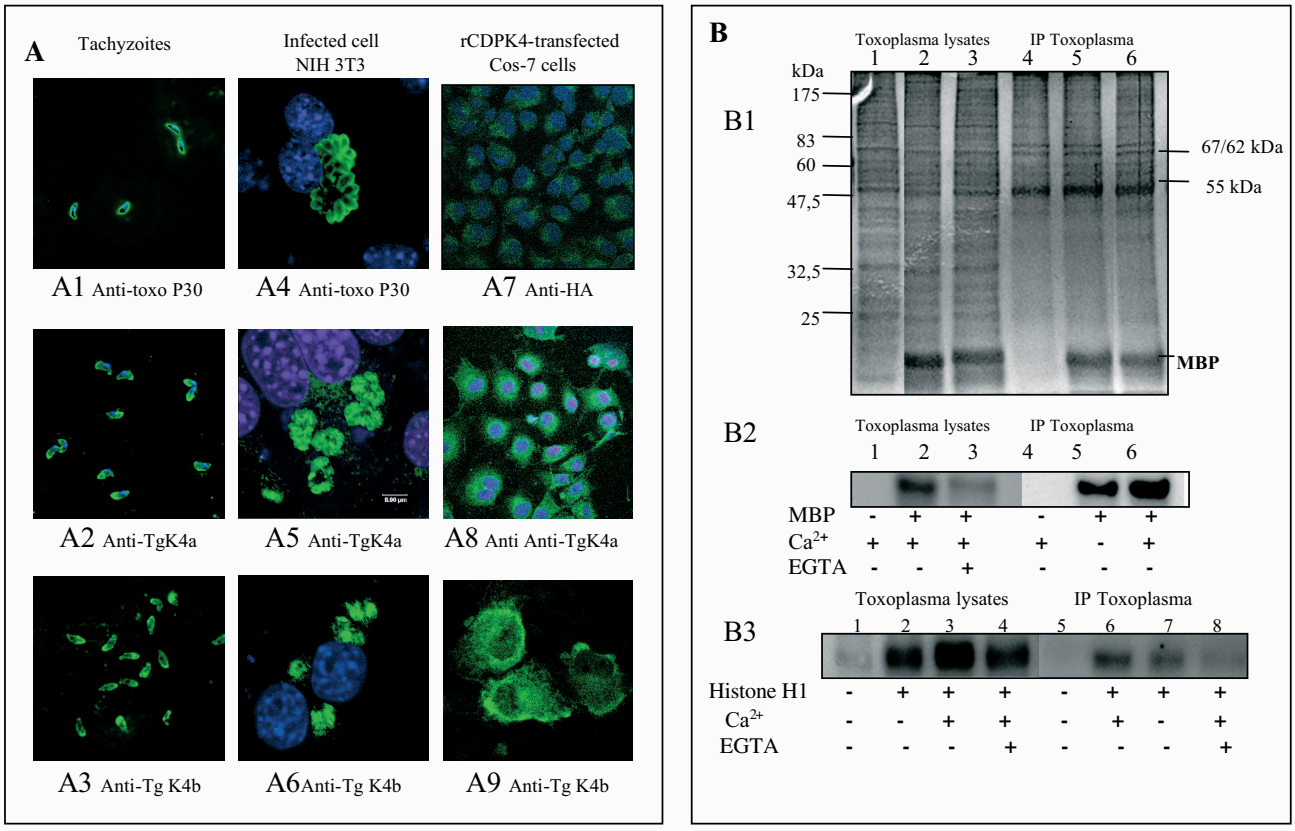

Fig. 3. – A. Immunolocalization of TgCDPK4 in *Toxoplasma gondii* and in transfected Cos-7 cells. Extracellular tachyzoïtes were harvested from the peritoneal fluid of mice infected four days previously with *Toxoplasma gondii* and intracellular tachyzoïtes were obtained from infected NIH 3T3 cells, as described (Roisin *et al.,* 2000; Creuzet *et al.,* 1998). Parasites were detected with a mouse monoclonal anti-*Toxoplasma* antibody (panel A1 and A4) or with specific antiTg-K4 antibodies, stained by incubation with CY3-conjugated anti mouse IgG or CY3-conjugated anti rabbit IgG, and FITC-conjugated anti rabbit IgG, respectively (panels A2, A3, and A5, A6). Cos-7 cells were transfected with the expression vector pAS1B-Tg CDPK4 encoding HA epitope-tagged TgCDPK4. Cells were fixed and permeabilized 24 h after transfection. TgCDPK4 was detected with an anti-HA antibody panel A7 or with specific the anti-TgCDPK4 antibodies anti-Tg K4a (A8) or anti-TgK4b (A9) followed by staining with CY2-conjugated anti-rat IgG (panel A) and FITC-conjugated anti rabbit IgG, respectively (panels B and C). TgCDPK4 was detected in the cytoplasm of Cos-7 cells. No significant background staining was observed with pre- immune sera (data not shown)

B. Kinase phosphorylation assay of TgCDPK4: whole lysates or immunoprecipitates of parasite lysates with anti-TGK4b antibody were assayed for kinase activity. Incubation of the tachyzoite extract (lanes 1, 2 and 3), and the immunoprecipitate (lanes 4, 5, 6) with [γ^{32}P ATP] in the absence of MBP (lane 1, 4) or in the presence of MBP as a substrate (lanes 2, 3, 5, 6). (B1) coomassie blue staining and (B2) autoradiography; (B3) incubation of the tachyzoite extract (lanes 1, 2, 3 and 4), and the immunoprecipitate (lanes 5, 6, 7 8) with [γ^{32}P ATP] in the presence or absence of histone H1 as a substrate.

C. Kinase phosphorylation assay with the recombinant protein rTgCDPK4. Equal amounts of recombinant TgCDPK4 were assayed in the presence or absence of MBP and/or histone H1 as substrates. (A) coomassie blue staining; (B) autoradiography.

bodies used. No significant background staining was observed with pre- immune sera (data not shown)
In pAS1B-CDPK4-transfected Cos-7 cells, recombinant protein stained with anti-HA antibody was found to accumulate in the cytoplasm (Fig. 3A7). The recombinant protein was also recognized by anti-TgCDPK4 (Fig. 3A8 and 3A9) antibodies. The subcellular distribution of TgCDPK4 protein in transfected Cos-7 cells shows that the protein is not nuclear, but is instead diffused throughout the cytoplasm.
The ability of TgCDPK4 to phosphorylate myelin basic protein (MBP), or histone H1 as substrate was assayed following Ca^{2+} and EGTA treatment in parasite lysates and in immunoprecipitates from parasite lysates with anti-TgK4b antibody (Fig. 3B). Two major bands of 67 and 62 kDa (the 55 kDa band on the Coomassie blue-stained gel corresponds to IgG) were immunoprecipitated from parasite lysates by anti-TGK4b antibodies (Fig. 3B1 lanes 4, 5 and 6). The pre-immune sera failed to detect any protein in parasites extracts (data not shown).
In parasite lysates and immunoprecipitates, optimal CDPK4 kinase activity required calcium and maximal activation was obtained with10 µM of Ca^{2+} (Fig. 3B2, lanes 2 and 6). As expected, TgCDPK4-mediated phosphorylation of MBP decreased in presence of 1 mM EGTA (Fig. 3B2, lanes 3 and 5).
We also assayed TgCDPK4 kinase activity using histone H1 as a substrate, again in presence of Ca^{2+}, or EGTA (Fig. 3B3). Quantification by scanning densitometry revealed an increase in histone H1 phosphorylation by both lysates and immunoprecipitates (150 and 140 %), respectively in the presence of 10 µM Ca^{2+} (Fig. 3B3, lanes 3 and 6). In this assay EGTA inhibited CDPK4 kinase activation by 52 % in lysates (Fig. 3B3, lane 4) and by 67 % in immunoprecipitates (Fig. 3B3, lane 8).
Our results demonstrate that as for TgCDPK1 described by Kieschnick *et al.,* 2001, TgCDPK4 can phosphorylate both MBP and histone H1 *in vitro* and that incubation with calcium ions induced an increase in its kinase activity, demonstrating Ca^{2+}-dependence. On the contrary, the TgCDPK3 recently described by Donald *et al.,* 2006 was insensitive to EGTA and not dependent on exogenous calcium. In *P. falciparum*, PfCDPK1 (Zhao et *al.,* 1994) requires concentrations of calcium an order of magnitude higher than those required by the soybean enzyme. The large number of CDPKs in plants may reflect specialization of the various isoforms in calcium binding and activation. Calcium-binding properties have been demonstrated experimentally for only a few CDPKs, and are difficult to predict from sequence data for a given isoform. Cytoplasmic calcium fluctuations play a key role in the regulation of host cell invasion by many apicomplexan parasites, including *P. berghei* (Billker et *al.,* 2004) and *T. gondii*. In *T. gondii*, the release of Ca^{2+} from intracellular stores governs tachyzoite egress, microneme secretion, motility, and host cell invasion (Carruthers & Sibley, 1999; Lovett & Sibley, 2003).
Transfected Cos-7 cell extracts were subjected to immunoprecipitation with an anti-HA antibody. Immunoprecipitates gave two major bands at 67 and 62 kDa on Coomassie blue-stained gels (Fig. 2C1, lanes 1-4).
In vitro kinase assays with various divalent cations as cofactors showed that maximal phosphorylation of MBP and histone H1 by recombinant TgCDPK4 required the presence of 5 µM Ca^{2+} (Fig. 2C2, lanes 1 and 3). The incubation of recombinant TgCDPK4 with EGTA in the kinase assay mixture greatly reduced the activity of this protein (Fig. 2C, lanes 2 and 4). Scanning densitometry showed that EGTA decreased the phosphorylation of MBP and histone by the recombinant protein rTgCDPK4 by a maximum of 80 % ± 15 % (Fig. 2C2). MBP and histone were not phosphorylated (Fig. 2C2, lane 5) by the truncated recombinant TgCDPK4 protein.

CONCLUSION

We isolated and characterized a new calcium-dependent protein kinase (TgCDPK4) from *T. gondii*. The predicted amino-acid sequence of TgCDPK4 shows similarity to CDPKs of plants and apicomplexan parasites.
Among the 20 CDPK or CDPK-related kinases identified in *T. gondii*, only TgCDPK1 (Kieschnick *et al.,* 2001) and TgCDPK3 proteins (Donald *et al.*, 2006) were detected in tachyzoïtes. Because TgCDPK4 shares low identities with other TgCDPKs isoforms, we can assume that anti-TgCDPK4 antibodies are specific to TgCDPK4 and the cross reactivity with other TgCDPKs isoforms seems unlikely. Immunolocalization with TgK4a and TgK4b antibodies showed that TgCDPK4 was cytoplasmic in parasites (extracellular or intracellular infected cells) and transfected Cos7 cells.
This new calcium-dependent protein kinase, TgCDPK4, may play an important role in the invasion of host cells by the parasite. As TgCDPK4 belongs to CDPK family, which does not exist in human cells, it may serve as a new therapeutic target with minimal toxic side effects (Doerig *et al.,* 2002).

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