Actin Dynamics Is Controlled by a Casein Kinase II and Phosphatase 2C Interplay on *Toxoplasma gondii* Toxofilin

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Submitted August 5, 2002; Revised January 10, 2003; Accepted January 16, 2003
Monitoring Editor: David Drubin

Actin polymerization in Apicomplexa protozoa is central to parasite motility and host cell invasion. Toxofilin has been characterized as a protein that sequesters actin monomers and caps actin filaments in *Toxoplasma gondii*. Herein, we show that Toxofilin properties in vivo as in vitro depend on its phosphorylation. We identify a novel parasitic type 2C phosphatase that binds the Toxofilin/G-actin complex and a casein kinase II-like activity in the cytosol, both of which modulate the phosphorylation status of Toxofilin serine⁵³. The interplay of these two molecules controls Toxofilin binding of G-actin as well as actin dynamics in vivo. Such functional interactions should play a major role in actin sequestration, a central feature of actin dynamics in Apicomplexa that underlies the spectacular speed and nature of parasite gliding motility.

INTRODUCTION

The apicomplexan phylum of protozoan parasites contains important human pathogens, including *Plasmodium falciparum*, the agent of malaria and *Toxoplasma gondii*, which causes serious encephalitis in immunocompromised humans (Dubey, 1994). To date, no vaccine or sterilizing drugs are available against these microorganisms.

Apicomplexan parasites display different life cycle forms and alternate between extra- and intracellular locations in their hosts. Extracellular stages are usually polarized cells called zoites, which not only invade host cells but also are highly motile. Zoite motility is a substrate-dependent, but unique type of locomotion (Russell and Sinden, 1981; King, 1988). Most eukaryotic cells that move on a solid substrate crawl by extending protrusions at their leading edge that adhere to substrate and by retracting the protrusions the cells move forward (Heidemann and Buxbaum, 1998; Small *et al.*, 2002). In stark contrast, apicomplexan zoites glide on the substrate, without changing their shape. Forward gliding is thought to result from secretion of substrate-binding factors at the parasite anterior pole, followed by their redis-

tribution to the posterior pole. Gliding is exceptionally rapid, ~1 to 20 $\mu\text{m/s}$ in vitro (Preston and King, 1996). This is 1 to several order(s) of magnitude faster than the speed of the most rapid crawling cells, such as keratocytes (Small *et al.*, 1999), amoebae (Van Duijn and Inouye, 1991), and polymorphonuclear cells (Mitchinson and Cramer, 1996).

Host cell invasion by apicomplexan zoites is a highly dynamic process, lasting only a few seconds (Morisaki *et al.*, 1995). The apical tip of the parasite attaches to host cell receptors and the host cell-parasite junction is then translocated from the anterior to the posterior pole of the zoite, making the parasite move into the nascent parasitophorous vacuole formed by invagination of the host cell plasma membrane (Aikawa *et al.*, 1978).

Numerous parasite surface molecules have been described in apicomplexan zoites (Boothroyd *et al.*, 1998), and some have been proposed to act as parasite adhesins (Soldati *et al.*, 2001). Recently, a family of transmembrane proteins (MICs), stored in the secretory apical organelle called micronemes and conserved in the Apicomplexa phylum has emerged as central to both gliding and invasive capacities (Sultan *et al.*, 1997; Kappe *et al.*, 1999). These proteins are thought to link the extracellular ligands that are used for parasite traction to a motor system in the parasite (Ménard, 2001).

Although what provides the force for zoite motility and cell invasion remains unknown, actin polymerization in the

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02-08-0462. Article and publication date are at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02-08-0462.

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parasite plays a central role. The use of drugs that interfere with actin polymerization has provided evidence for the importance of actin dynamics in the parasite during both gliding and cell invasion in Apicomplexa (Miller *et al.*, 1979; Poupel and Tardieux, 1999; Shaw and Tilney, 1999). In *T. gondii* tachyzoites, selection of mutants that are resistant to cytochalasins demonstrated that parasite and not the host cell actin polymerization is important for parasite entry into the host cell (Dobrowolski and Sibley, 1996). A role for myosin motor(s) has been suggested by Dobrowolski *et al.* (1997), and recent characterization of TgMyoA provided evidence that it contributes to the zoite motile force (Herm-Götz *et al.*, 2002; Meissner *et al.*, 2002).

Besides myosins (Heintzelman and Schwartzman, 1997), a few actin-binding factors have been identified in *T. gondii*. A homolog of the actin depolymerizing factor/cofilin has been cloned (Allen *et al.*, 1997). In tachyzoites, we identified Toxofilin as an actin-binding protein. Toxofilin was purified in a complex with actin monomers at a 1:1 stoichiometry and shown to regulate association of actin monomers as well as elongation of actin polymers (Poupel *et al.*, 2000). Herein, we report that Toxofilin controls most of the parasite G-actin that is known to form >95% of the actin pool, demonstrating that Toxofilin regulation is key to actin dynamics. We identify and characterize two counteracting Toxofilin regulatory molecules and provide evidence that these contribute to Toxofilin properties on actin dynamics both in vitro and in vivo.

MATERIALS AND METHODS

T. gondii Tachyzoite Recovery, Parasite Gliding, and Host Cell Invasion Assays

The RH *T. gondii* parasites were prepared as described in Poupel and Tardieux (1999). Fresh tachyzoites (5×10^7 in 1 ml) were exposed to the cell-permeant kinase inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB; 62.5, 125, and 250 μ M) or to the solvent (ethanol) (90 min, 37°C) and then incubated with subconfluent HeLa cells. In one control experiment, DRB was added only during the invasion assay (30 min). Intracellular and extracellular parasites were labeled as described previously (Poupel and Tardieux, 1999). For the gliding assay, glass chamber slides were coated by incubation in 50% fetal bovine serum diluted in phosphate-buffered saline (PBS) (1 h, 37°C) followed by rinsing in PBS. Freshly harvested untreated or DRB-treated (250 μ M, 90 min, 37°C) tachyzoites were resuspended in PBS (10^7 in 1 ml) and observed by time-lapse videomicroscopy by using an Axiovert equipped with a temperature-controlled stage (Carl Zeiss, Jena, Germany). Images were collected under low-light illumination by using an intensified charge-coupled device camera (Cool snap HQ; Princeton Scientific Instruments, Monmouth Junction, NJ) at 63 $\times$ magnification.

Exposure of Tachyzoite to [32 P]Orthophosphate and Toxofilin Immunoprecipitation

Parasites (5×10^8) were incubated in phosphate free buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 1.6 mM CaCl₂, 0.5% glucose, 0.1% bovine serum albumin) with 250 μ Ci/ml of orthophosphoric acid (specific activity of 8.8×10^9 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) (120 min, 37°C). Tachyzoites were lysed in 0.4 ml of (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.5% Triton X-100, 0.5% Nonidet P-40, 25 mM NaF, 150 μ M orthovanadate, 3 mM NaPO₄, 1 mM dithiothreitol, 0.5% [vol/vol] protease inhibitor stocks). The supernatant (20 min, 20,000 $\times$ g, 4°C) was precleared on protein A-Sepharose (Phar-

macia AB, Uppsala, Sweden) (1 h, 4°C). This supernatant does not contain F-actin because we have previously shown that extraction of the very few tachyzoite actin filaments required special conditions including jasplakinolide-containing buffer (Poupel and Tardieux, 1999). The unbound fraction was successively incubated with Toxofilin antibodies (overnight, 4°C) and with protein A-Sepharose (2 h, 23°C). Eluates in SDS-PAGE sample buffer were subjected to 12.5% acrylamide gel electrophoresis. Gels were dried and scanned for their radioactivity.

Western Blot Analysis

Western blots were performed as described in Poupel and Tardieux (1999). The antibodies used were affinity-purified rabbit anti-Toxofilin, rat anti-Toxofilin (1:5000), and rabbit anti-*T. gondii* actin antibodies (1:4000). The horseradish peroxidase-conjugated second antibody was used at 1:10000 (Jackson Immunoresearch Laboratories, West Grove, PA) (1 h, 23°C). Cross-reactive proteins were visualized using the ECL system (Pierce Chemical, Rockford, IL).

Production of rToxofilin for Use as a Substrate of Kinases/Phosphatases

We used the vector pGEX6-P3 (Pharmacia AB) containing the Toxofilin encoding cDNA to prepare rToxofilin as described in Poupel *et al.* (2000), but we replaced sarcosyl with *N*-tetradecyl-*N,N*-dimethyl-3 ammonio-1-propanesulfonate (0.5% wt/vol) (Sigma-Aldrich, St. Louis, MO) in the lysate buffer. Soluble rToxofilin was immunoprecipitated with anti-Toxofilin antibodies (overnight, 4°C) and recovered on protein A dynabeads (2 h, 23°C) (Dynal Biotech, Oslo, Norway) before the kinase/phosphatase assay.

Identification and Cloning of *T. gondii* Type 2C Phosphatase (PP2C)

Native Gel and Peptide Microsequencing. After native electrophoresis (Poupel *et al.*, 2000), the gel slice containing the ~36-kDa actin-binding protein was subjected to tryptic digestion (30°C, 18 h, 0.3 mg of trypsin in 0.1 M Tris-HCl, pH 8.6, 0.01% [vol/vol] Tween 20), and the peptides were recovered by high-performance liquid chromatography (HPLC). The sequencing of two peptides gave, respectively, VFDGTVGDFA(Q)ENV and NQSADNITAMTVFFK. The latter was found in one clone from the *T. gondii* database of expressed sequence tags (TgESTzy48A06.r1, November 1999) (WashU-Merk Toxoplasma EST project; Ajioka *et al.*, 1998).

cDNA library screening and DNA sequencing: the oligonucleotide with the sequence: 5'-AGTGCAGACAACATTACTGC-GATG-3' corresponding to part of one peptide microsequence (SADNITAM) was used as the up stream primer, whereas 5'-AGACACACCAAGAATCTCGTC-3' was chosen as the downstream primer in the TgESTzy48A06.r1clone. After polymerase chain reaction (PCR) amplification, the 207-base pair fragment was 32 P labeled (Megaprime kit; Amersham Biosciences, Amersham, United Kingdom) and used to screen a *T. gondii* tachyzoite cDNA library (kindly provided by J.W. Ajioka, University of Cambridge, Cambridge, United Kingdom). The entire nucleotide coding sequence has been deposited at the European Molecular Biology Laboratory nucleotide sequence database (AJ315476).

Production of a Thioredoxin-Hispatch Tg PP2C and Biochemical Characterization

Recombinant TgPP2C was prepared by PCR amplification of a full-length TgPP2C encoding cDNA, by using primers introducing an *Eco*RI restriction site in 5' and a *Xba*I restriction site in 3' (upper strand, 5'-GCCGAATTCCTATGAAGTCTCTGCTGAAATTAG-3'; lower strand, 5'-GCCTCTAGACTAATCAGTCTTCTTGAA-GAAGACTG-3'). The amplified fragment was cloned into the

pThioHis B vector (Invitrogen, Carlsbad, CA). After induction of protein expression (0.1 mM isopropyl β -D-thiogalactoside, 2 h, 37°C), the fusion protein was purified on a nickel column (Probond; Invitrogen) followed by a phenylarsineoxide-agarose column (ThioBond; Invitrogen). Eluates were dialyzed against buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) and stored aliquoted at -80°C. The phosphatase activity of the rPP2C was verified using 32 P-labeled casein in presence of PP1/PP2A-sensitive inhibitor okadaic acid (OA, 1 μ M) or 10 mM EDTA as well as in presence of MgCl₂.

Tachyzoite Cytosol Preparation and Heparin Chromatography

Cytosol. Cytosol was prepared from 10⁹ frozen tachyzoites as described previously (Poupel *et al.*, 2000) but in kinase buffer (buffer A supplemented with 10 mM MgCl₂, 1 mM dithiothreitol, 0.2% [vol/vol] protease inhibitor stocks). In some experiments, cytosols were depleted in PP2C by incubation with 2 μ g of the affinity-purified polyclonal anti-*T. gondii* PP2C antibodies and then with protein A-Sepharose. Control cytosol precleared on protein A-Sepharose and PP2C-depleted cytosol were then subjected to kinase assay.

Heparin Chromatography. A cytosol from 10⁹ parasites was precleared on Sepharose CL-4B and chromatography performed on heparin Sepharose CL-4B (Pharmacia AB) (1 h, 4°C). After several washes, the heparin-bound proteins were eluted in 10 mM Tris-HCl, pH 7.5, 0.5 M NaCl. The eluate was dialyzed against kinase buffer before the kinase assay.

Kinase and Phosphatase Assays

Kinase Reaction on rToxofilin. Two micrograms of immobilized rToxofilin was incubated with a tachyzoite cytosol prepared from 2×10^8 (100 μ l) and precleared on protein A dynabeads. The reaction was started by adding 100 μ M Na₂NTP and 10 μ Ci of the same [γ - 32 P]NTP (ATP, 3000 Ci/mmol; GTP, 6000 Ci/mmol; PerkinElmer Life Sciences) (15 min, 30°C). After washes, rToxofilin and bound proteins were eluted in SDS-PAGE sample buffer before electrophoresis and radioactivity scan (PhosphorImager; Amersham Biosciences, Sunnyvale, CA). To characterize the rToxofilin kinase(s) five types of reagents were used: 1) [32 P]GTP as a unique phosphate source; 2) pharmacological inhibitors such as heparin (20 μ g/ml; Sigma-Aldrich), DRB (100 μ M), chrysin (100 μ M; Sigma-Aldrich), emodin (100 μ M; Sigma-Aldrich), or staurosporine (10 μ M; Calbiochem, San Diego, CA); 3) heparin chromatography eluate and the corresponding unbound fraction; 4) purified human recombinant CKII (α dimer or $\alpha\beta$ tetramer, 250 ng) (gift from C. Cochet, INSERM 4244, Grenoble, France); and 5) a standard CKII peptide substrate (Upstate Biotechnology, Lake Placid, NY).

Kinase Reaction on Tachyzoite Cytosol. Control and PP2C-immunodepleted cytosols were incubated with 100 μ M Na₂ ATP and 10 μ Ci of [γ - 32 P]ATP (15 min, 30°C). Kinase reactions were stopped by addition of SDS-PAGE sample buffer and analyzed by SDS-PAGE followed by radioactivity scan.

Phosphatase Reaction on rToxofilin. The purified rPP2C was added (5 or 20 μ g) during or after the kinase assay. In some control experiments, 1 unit of a recombinant fragment of rabbit catalytic type 1 phosphatase (Upstate Biotechnology) was replacing rPP2C. In experiments where the phosphatase assay was performed on Ser⁵³phosphorylated rToxofilin, the latter was incubated with cytosol supplemented with 10 mM MgCl₂, 2 mM EDTA or a combination of 10 mM MgCl₂ and 2 μ M OA. Eluates containing rToxofilin were treated as described for the kinase assay.

Enzymatic Digestion of 32 P-labeled rToxofilin, HPLC, and Covalent Sequencing

rToxofilin (10 μ g) was phosphorylated using either a tachyzoite cytosol, an eluate after heparin chromatography, or a purified human casein kinase II. After SDS-PAGE, phosphorylated rToxofilin was excised as a band from the gel and incubated with 0.4 μ g of endolysine-C in 50 mM Tris-HCl, pH 8.6, 0.01% (vol/vol) Tween 20 (1 h, 35°C). The peptides were separated by HPLC and eluted with a 20–70% acetonitrile, 0.1% trifluoroacetic acid gradient. The radioactivity contained in each collected fraction was measured using a Cerenkov counter. Radioactive peptides were covalently fixed to Sequelon AA filter (Millipore, Bedford, MA) and sequenced in a 494 sequencer (Applied Biosystems, Foster City, CA).

Cloning, Expression, and Purification of Recombinant *T. gondii* Casein Kinase II Catalytic Subunit

Using the EST (GenBank accession no. BM 189807) and the contigs TGG_3802 and TGG_2216, we reconstituted an open reading frame consistent with the CKII α protein sequence. Indeed, alignment of the translated sequence with known CKII α proteins from related organisms, including *Theileria parva* (GenBank accession no. M92084) and higher organisms confirmed that the reading frame was encoding a CKII α protein. The reverse primer 5'-GAAGTCCG-CAAGACCCAGTCG-3' was used in a reverse transcription reaction containing 30 U of avian myeloblastosis virus reverse transcriptase (Finnzymes) under conditions specified by the supplier except that the reaction was carried out on 3 μ g of RNeasy kit (QIAGEN, Valenica, CA)-purified parasite mRNA. Reverse transcriptase product was amplified using PCR with the same reverse primer and the forward primer 5'-CGAGTACTGGGACTACGAGAAC-3'. The PCR product was cloned into pCRT7/CT-TOPO vector (Invitrogen) and verified by nucleotide sequencing. After induction of protein expression (1 mM isopropyl β -D-thiogalactoside, 2 h, 37°C), the fusion protein was purified on a nickel column (Probond; Invitrogen) and the imidazole eluate was dialyzed against 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂ (2 h, 4°C). It was readily tested in kinase assay with a synthetic Toxofilin peptide encompassing Ser⁵³ by using the CKII assay kit (Upstate Biotechnology). Bacteria that did not carry plasmid were treated similarly to get a control of bacterial kinase activity on the Toxofilin peptide.

Site-directed Mutagenesis on rToxofilin

Using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA), we introduced a single amino acid mutation in rToxofilin replacing serine at position 53 by alanine by using the following primers: 5'-GGAAGATTCGAGGCTGGAGACGAAG-3' and 5'-CTTCGTCTCCAGCCTCGAATCTTCC-3' or by glutamic acid by using 5'-GGAAGATTCGAGGAGGGAGACGAAG-3' and 5'-CTTCGTCTCCCTCTCGAATCTTCC. Positive bacterial clones were verified by nucleic sequencing. rToxofilin mutants (S53A and S53E) were prepared as for wild-type rToxofilin (WT) and subjected to kinase assay.

Ectopic Expression of Green Fluorescent Protein (GFP)-WT and Mutant Toxofilin Fusion Proteins in Fibroblastic Cells

Fibroblastic cell line derived from African green monkey kidney (CV1 line) was transfected with the plasmids encoding GFP-WT, GFP-S53A, or S53E Toxofilin. F-actin was visualized as described in Poupel *et al.* (2000). The samples were examined under an Axiovert (Carl Zeiss). Images were acquired and analyzed using a charge-coupled device coolsnap HQ camera and the MetaMorph imaging system.

Figure 1. Toxofilin is the major G-actin-binding protein. Toxofilin immunoprecipitation from tachyzoite extract. Proteins from the extract (lane a), the immunoprecipitate (lane b), and the flow through (lane c) were separated by SDS-PAGE. Toxofilin and actin were probed by Western blot, by using anti-Toxofilin and anti-actin antibodies.

Surface Plasmon Resonance Experiments

They were performed at 25°C using a Biacore 2000 system (Biacore AB, Uppsala, Sweden). rToxofilin S53E and WT were covalently coupled *via* primary amino groups on CM5 sensor chips surface (Faure *et al.*, 2000). The surface plasmon resonance signals for immobilized Toxofilin on two different flow cells were found to be 1400 pg · mm⁻² for S53E and 1960 pg · mm⁻² for WT. The interaction between G-actin and the chip-immobilized Toxofilin was studied by injecting dilutions of G-actin (25–400 µg/ml, 2 min, 10 µl/min flow rate). The kinetic constants k_{on} and k_{off} for the interaction of G-actin with immobilized Toxofilin were calculated using Biacore BIAEVALUATION 3.1 software (Schuck, 1997).

RESULTS

An Active Type 2C Phosphatase Copurifies with Toxofilin-Actin Complex

Toxofilin was initially characterized as a G-actin-sequestering protein in tachyzoites by forming a 1:1 complex (Poupel *et al.*, 2000). To further evaluate whether Toxofilin is a major player in actin dynamics in *T. gondii*, we first asked whether Toxofilin bound most of the parasite G-actin. For this, we immunoprecipitated Toxofilin from the precleared tachyzoite cytosol by using anti-Toxofilin antibodies and probed the nonprecipitated material with anti-Toxofilin and anti-actin antibodies. As shown in Figure 1, immunoprecipitation of Toxofilin (lane b) efficiently depleted Toxofilin as well as most of G-actin (lane c) from the tachyzoite cytosol (lane a). Similar results were obtained when DNase 1 chromatography was performed (Poupel *et al.*, 2000), whereas preimmune sera did not precipitate Toxofilin or actin (our unpublished data). This showed that most G-actin is complexed to Toxofilin in the tachyzoite cytosol, and thus suggested that Toxofilin may play a key role in parasite actin dynamics.

We previously reported that an additional and less abundant ~36-kDa polypeptide copurified with the actin-Toxofilin complex isolated in native gels (Poupel *et al.*, 2000). Large amounts of the complex were prepared and the ~36-kDa polypeptide was subjected to tryptic digestion and the peptide sequence was derived. Based on these amino acids, a DNA probe was synthesized and a *T. gondii* cDNA library screened and 12 positive clones corresponding to the same gene selected. This gene encodes a 331 amino acid polypep-

tide, with a theoretical isoelectric point of 5.44 and expected molecular mass of 36.81 kDa. It is homologous to a variety of eukaryotic type 2C serine-threonine phosphatases (PP2C), including those from closely related protozoans (Figure 2). PP2Cs form a distinct group among the serine/threonine protein phosphatases that are known to display 11 conserved motifs scattered along the catalytic domain, with eight perfectly conserved amino acid residues that serve as a PP2C signature in both eukaryotes and prokaryotes (Shi *et al.*, 1998) (Figure 2). Among these, it has been proposed that Asp residues (45, 67, 273, and 318), together with Glu-44 and Gly-68, coordinate the active site metal ions (Das *et al.*, 1996). Indeed, PP2Cs are defined by their Mn²⁺ or Mg²⁺ ion dependence and their insensitivity to pharmacological agents blocking the other serine/threonine phosphatase groups (Cohen, 1989). Of note, as described for bacteria (Obuchowski *et al.*, 2000), but not for *P. falciparum*, *T. gondii* PP2C sequence lacks His69 within the conserved motif DGH (motif II), a residue that has been shown to be critical for mouse PP2C activity *in vitro* (Kusuda *et al.*, 1998).

Next, the functional activity of the Toxofilin-associated PP2C was assessed: when ³²P-labeled casein was incubated with the Toxofilin immunoprecipitate, 3.14 ± 0.16 pmol of phosphocasein were dephosphorylated after a 90-min incubation at 30°C (three separate experiments). To check whether the phosphatase activity found in the Toxofilin immunoprecipitate was selective for type 2C the ³²P-casein dephosphorylation assay was performed in the presence of OA, a potent inhibitor of type 1 and type 2A phosphatases. Incubation with OA up to 1 µM did not affect dephosphorylation of phosphocasein, whereas the presence of magnesium was required (our unpublished data). The PP2C activity associated with the Toxofilin immunoprecipitate was estimated as 0.3% of the total PP2C activity measured in cytosol.

Toxofilin Is Phosphorylated *In Vivo* and *In Vitro*

To analyze the relevance of the association of PP2C with Toxofilin, we investigated whether Toxofilin is phosphorylated both *in vivo* and *in vitro*. Therefore, tachyzoites were incubated with [³²P]orthophosphate and Toxofilin was immunoprecipitated using rabbit anti-Toxofilin antibodies. As shown in Figure 3A, the immunoprecipitate contained a ³²P-polypeptide of the apparent size of Toxofilin that was recognized by rat polyclonal anti-Toxofilin antibodies (bottom). Furthermore, we incubated immobilized rToxofilin with the tachyzoite cytosol in presence of [³²P]NTP. As shown in Figure 3B (lane a), rToxofilin was readily detected after SDS-PAGE and Coomassie staining. Gel autoradiography indicated that a major ³²P signal comigrated with rToxofilin (lanes b and c). No ³²P signal was detected comigrating with rToxofilin when the assay was performed in the absence of rToxofilin (lane d). This indicated that rToxofilin is phosphorylated under these *in vitro* conditions.

Toxofilin Is Phosphorylated by a CKII-like Parasite Kinase Activity and by Human CKII

We then attempted to identify the type of kinase activity that phosphorylates Toxofilin by using a variety of kinase inhibitors. Six putative phosphorylation sites were recognized in Toxofilin sequence by using the PROSITE software, among

Figure 2. Toxofilin-associated 36.8-kDa protein is a type 2C phosphatase. Multiple alignment of *Toxoplasma* type 2C phosphatase and four distinct related sequences from eukaryotic members. The accession numbers are the following: T, *Toxoplasma gondii* PP2C, AJ315476; Ha, PP2C alpha human, P35813; Hb, PP2C beta human, O75688; Ld, *Leishmania donovani*, AAB88452; and Pf2, *Plasmodium falciparum* PP2C, CAB62878. Alignments were performed with the ClustalW program. The highly conserved amino acid residues are boldfaced. Note that we only used the repeat 2 of *P. falciparum* PP2C sequence (from amino acid 481–920), because the repeat 1 introduced too many gaps. The peptides that were microsequenced from the purified PP2C are underlined.

which three were consensus sites for CKII (²³TASE, ⁵³SGDE, and ¹⁶⁵TLLE). We found that staurosporine, one of the most potent specific inhibitors of serine/threonine protein kinases that remains poorly effective on CKII (Meggio *et al.*, 1995), did not significantly inhibit rToxofilin phosphorylation by the parasite cytosol (Figure 3B, lane j), compared with the control (lane f). Various inhibitors of CKII have been described, such as glycosaminoglycan heparin (O' Farrell *et al.*, 1999), the nucleotide analog DRB, chrysin, and emodin. Chrysin and DRB are thought to specifically inhibit CKII activity (Shugar, 1994; Critchfield *et al.*, 1997), whereas emodin inhibits CKII, but is poorly effective on other serine/threonine protein kinases (Battistutta *et al.*, 2000). When the tachyzoite cytosol was incubated with any of the CKII inhibitors before the phosphorylation assay, the level of rToxofilin phosphorylation was markedly decreased (Figure 3B, lanes e and g–i).

CKII can use both GTP and ATP as phosphate donor (Allende and Allende, 1995). In agreement with the involvement of a CKII-like activity in Toxofilin phosphorylation, rToxofilin was efficiently phosphorylated in our assay performed with GTP (Figure 3B, lane c). CKII activity is also defined by its lack of dependence on second messengers such as cyclic nucleotides or calcium, which are important for the activity of calcium-calmodulin-dependent protein

kinases or cAMP/cGMP-dependent kinases (Guerra *et al.*, 1999). We found that rToxofilin phosphorylation did not depend on cyclic nucleotides or calcium (our unpublished data). Heparin chromatography has been used extensively for CKII purification in a variety of systems (Litchfield *et al.*, 1990a). Therefore, we subjected the tachyzoite cytosol to heparin Sepharose chromatography and tested whether the eluate could phosphorylate rToxofilin. As shown in Figure 3C, it contained most of the rToxofilin kinase activity (lane b), whereas the flow-through material was markedly depleted in rToxofilin kinase activity (lane c). In addition, when heparin was added to the eluate before the assay, rToxofilin phosphorylation was significantly diminished (lane d). As a control, when the assay was performed with the eluate, but in the absence of rToxofilin no ³²P signal was detected at the size of rToxofilin (lane e). We next asked whether purified human CKII could phosphorylate rToxofilin. CKII is a heterotetramer composed of two catalytic subunits (α or α') associated with two regulatory subunits (β and β'). We observed in vitro that the catalytic α subunit (lane f), as well as the tetramer $\alpha_2\beta_2$ (lane g) of human CKII phosphorylated rToxofilin. At equimolar concentration, the $\alpha_2\beta_2$ tetramer induced higher levels of phospho-transfer onto rToxofilin than the α_2 dimer, which is in agreement with the known enhancing/stabilizing role of β subunit on

Figure 3. Toxofilin is phosphorylated by a CKII-like parasite kinase activity. (A) Toxofilin immunoprecipitation from ^{32}P -labeled tachyzoites. Top, radioactive scan; bottom, Western blot with anti-Toxofilin antibodies (lane a, load; lane b, eluate). (B) rToxofilin was analyzed by SDS-PAGE and Coomassie stain (lane a). Assays were performed by incubation of rToxofilin with cytosol containing either ^{32}P [GTP] (lane c) or ^{32}P [ATP] (lane b, lanes d–j) and in presence of 100 μM DRB (lane e), chrysin (lane g), emodin (lane h), 20 $\mu\text{g}/\text{ml}$ heparin (lane i), or 10 μM staurosporine (lane j). A mock kinase assay was carried out by incubating the cytosol with protein A beads (lane d). (C) Source of kinase: the cytosol (lane a), the heparin chromatography eluate (lane b) supplemented with soluble heparin (lane d), or the corresponding flow through (lane c), a), the

the α subunit kinase activity (Meggio *et al.*, 1992). Acidic synthetic phosphopeptides have proven to be useful tools for isolating CKII, or CKII-like activities (Litchfield *et al.*, 1990b). Therefore, we tested whether the CKII consensus substrate RRRDDDSDDD could be a target for the parasite cytosol and for the parasite fraction eluted of heparin Sepharose. By using similar concentrations of total proteins, the parasite cytosol exhibited a moderate kinase activity on the peptide, whereas the heparin eluate was 5–10 times more active in catalyzing ^{32}P transfer to the substrate (Figure 3D). Addition of a protein kinase A cocktail inhibitor (Upstate Biotechnology) to the kinase-containing fraction did not significantly change the amount of ^{32}P transfer to the CKII peptide (our unpublished data), indicating that a potential parasite protein kinase A activity is not responsible for the phosphorylation of the CKII peptide substrate. These results demonstrate that the kinase activity enriched in the heparin eluate can catalyze phosphate transfer to a consensus CKII peptide. Together, these data strongly suggest that a tachyzoite CKII-like activity is the major source of Toxofilin phosphorylation.

CKII-like Kinase and PP2C Control Ser⁵³ rToxofilin Phosphorylation

To identify the Toxofilin phosphorylation site(s), we incubated rToxofilin with ^{32}P [ATP] in the presence of either the Toxofilin-kinase-enriched eluate, or purified human CKII. From all the rToxofilin peptides generated after endolysine digestion, only one contained detectable amounts of radioactivity. This peptide was then subjected to covalent sequencing, which allowed the detection of significant radioactive counts only associated to Ser⁵³ (Table 1).

Therefore, these data strongly suggest that Ser⁵³ of rToxofilin is the target for both a *T. gondii* CKII-like kinase and a human CKII.

First, to confirm that Toxofilin Ser⁵³ is a substrate for *T. gondii* CKII, we attempted to clone and express recombinant *T. gondii* CKII α before performing kinase assays. Using both an EST (TgEST BM189807) and contigs (TGG_3802 and TGG_2216), we cloned and sequenced the CKII catalytic site, which is shown aligned with other known CKIIs (Figure 4A). Of note, *T. gondii* CKII α displays 29% identity to human CKII α and only 28% identity with CKII α of the related parasite *Theileria parva* (Figure 4A). After expression and semipurification of the CKII α recombinant polypeptide, CKII kinase assays were performed using a synthetic pep-

Figure 3 (cont). heparin chromatography eluate (lane b) supplemented with soluble heparin (lane d), or the corresponding flow through (lane c), 250 ng of α_2 (lane f), $\alpha_2\beta_2$ (lane g) recombinant human CKII. A mock kinase assay was carried by incubating the heparin eluate with protein A beads (lane e). (D) Kinase assay on a synthetic consensus substrate peptide for eukaryotic CKII α . Enzymatic reactions used as kinase the human CKII α (150 ng), or the same protein concentration from either the cytosol or the heparin chromatography eluate. Reactions were absorbed onto phosphocellulose filters and measured using a scintillation counter. After subtraction of the radioactive counts originating from autophosphorylation to the fraction tested, the amount of phosphate transfer catalyzed by the human CKII α in our conditions was arbitrary determined as 100%.

Table 1. Covalent sequencing of the ^{32}P -associated Toxofilin peptide

Amino acid	Heparin eluate	Human rCKII
	CPM	CPM
R	85	66
G	23	67
R	22	0
F	23	68
E	30	66
S	462*	760*
G	65	66
D	36	67
E	27	20
G	18	67
T	15	13
S	19	0
T	16	20
M	19	20
S	22	0
P	17	17
S	24	13
V	14	13
A	23	0
A	17	20

tide derived from the Toxofilin sequence that contains Ser⁵³ (RFESGDEG). As shown in Figure 4B, recombinant T.gCKII α catalyzes ^{32}P transfer onto the Toxofilin peptide, as does human recombinant CKII, whereas, in contrast, no transfer was observed with the control bacterial lysate.

Second, we checked whether Toxofilin Ser⁵³ is important for rToxofilin activity. To this end, we constructed rToxofilin mutants in which Ser⁵³ was replaced by either an alanine residue (S53A) or by a glutamic acid residue (S53E). As shown in Figure 5, [γ - ^{32}P] incorporation into S53A rToxofilin was greatly decreased after incubation with either the purified recombinant human CKII (lane b) or the Toxofilin kinase-enriched fraction (lane e), compared with the wild-type rToxofilin. As expected, γ - ^{32}P incorporation into S53E rToxofilin was equally reduced by both source of kinases (lanes c and f). Indeed, although glutamic acid mimics a constitutively active phosphorylated state, it is not a phosphate acceptor. These data confirm that Ser⁵³ is the major site of Toxofilin phosphorylation by a CKII-like kinase, but because we observed a very faint ^{32}P signal in the mutants, CKII might also weakly phosphorylate other sites.

We then asked whether Toxofilin-bound PP2C dephosphorylates Toxofilin. To this end, a ThioHis-PP2C fusion protein (rPP2C) was produced, and its phosphatase activity was assayed on rToxofilin. We found that rToxofilin phosphorylation was markedly decreased upon addition of purified rPP2C in the kinase assay and the effect was dose dependent (Figure 6A, lanes c and d). In contrast, addition of purified human PP1 did not decrease rToxofilin phosphorylation by the cytosolic fraction (Figure 6A, lane b). We also compared phosphorylation of cytosolic proteins by using either the native cytosol or the PP2C-immunodepleted cytosol. As shown in Figure 6B, several cytosolic proteins displayed higher phosphorylation levels in the absence, compared with the presence of PP2C (compare lanes b and

a). Interestingly, the major hyperphosphorylated protein comigrated with Toxofilin (Figure 6C). These data demonstrate that *T. gondii* endogenous and recombinant PP2C dephosphorylates Toxofilin and rToxofilin, respectively. To test whether native PP2C directly dephosphorylates Toxofilin on Ser⁵³, phosphorylated rToxofilin was produced using human CKII and subsequently incubated with the cytosol. As shown in Figure 7A, Ser⁵³-phosphorylated rToxofilin was dephosphorylated by the cytosol (lane b). In contrast, rToxofilin remained phosphorylated in control buffer (lane a). Furthermore, we found that the phosphatase activity contained in the cytosol required magnesium (lanes b and c) and was insensitive to OA (lane d). These data provide strong evidence that Ser⁵³ dephosphorylation is induced by a selective type 2C phosphatase activity, and not by type 1 or type 2A phosphatases. Finally, to address the question of a potential parasite cofactor for PP2C, we compared the efficacy of rPP2C and endogenous PP2C on dephosphorylation of Ser⁵³. Ser⁵³ phosphorylated rToxofilin was incubated with either buffer, rPP2C or the cytosol, by using the same amount of magnesium-dependent phosphatase activity from both sources. We found that the cytosol contained a specific activity 13 fold higher than the same volume of rPP2C. As illustrated in Figure 7B, under these conditions, rToxofilin remained phosphorylated in presence of buffer (lane a). In contrast, rToxofilin was dephosphorylated by the rPP2C (lane b) and by the cytosol (lane c), but to a much greater extent upon exposure to cytosol. These data raise the question of incomplete folding of the rPP2C, compared with the native molecule. In spite of this possibility, rPP2C was very efficient when added to the cytosol (Figure 6A) and this suggests that a cytosolic cofactor might optimize its phosphatase activity.

Ser⁵³ Is the Major Site of Toxofilin Phosphorylation and Is Important for Toxofilin Function In Vitro as In Vivo

To best visualize the effect of Ser⁵³ substitution on actin dynamics, we used a fibroblastic cell line (CV1) known to display abundant actin stress fibers. As previously described for transfected epithelial HeLa cells (Poupel *et al.*, 2000), ectopic expression in CV1 cells of wild-type GFP-Toxofilin induced a profound disorganization of actin filament with a major breakdown of actin stress fibers (Figure 8, bottom left). CV1 cells expressing either GFP-Toxofilin S53A or GFP-Toxofilin S53E exhibited extremely opposite phenotypes, because the former displayed significant amounts of actin stress fibers (Figure 8, top right, see white arrows), whereas the latter displayed a dramatic loss of actin stress fibers (Figure 8, bottom right). These data showed that Ser⁵³, and presumably its phosphorylation state in vivo, are implicated in the control of Toxofilin properties on actin dynamics. To check whether the effect of Toxofilin Ser⁵³ substitution on actin dynamics in vivo could reflect a modification in Toxofilin-actin binding properties in vitro, we used the Biacore technique. This allowed us to compare the kinetic parameters of G-actin binding to unphosphorylated Ser⁵³ Toxofilin (WT) and to the phosphorylated state of Toxofilin (S53E). As illustrated in Table 2, G-actin binds to WT with an association rate constant (k_{on}) threefold increased compared with Ser53E and a dissociation rate constant (k_{off}) fourfold de-

A

B

Figure 4. A *T. gondii* CKIIα catalytic subunit transfers ^{32}P to a Toxofilin synthetic peptide. (A) Multiple alignment of *Toxoplasma* partial CKIIα catalytic subunit and two distinct related sequences from eukaryotic members. TG, *T. gondii* CKIIα; H, human CKIIα, AJ315847; TP, *T. parva* CKIIα, M92084. Alignments were performed with the MULTALIN program. The highly conserved amino acid residues are in gray. The sequences used to design primers for cloning are underlined. (B) Kinase assay on a synthetic consensus substrate peptide for eukaryotic CKIIα. Enzymatic reactions used as kinase the human CKIIα or the *T. gondii* recombinant CKIIα and as control the eluate from a bacterial lysate chromatographed on a nickel column. Reactions were absorbed onto phosphocellulose filters and measured using a scintillation counter. After subtraction of the radioactive counts originating from auto-phosphorylation to the fraction tested, the amount of phosphate transfer catalyzed by the human CKIIα in our conditions was arbitrary determined as 100%.

creased, leading to a 14-fold increase in K_{dapp} . Therefore, these data suggest a potential role for phosphorylation on Ser⁵³ in the Toxofilin-G-actin interaction.

CKII Inhibitor DRB Decreases Invasion of *T. gondii* Tachyzoites into Mammalian Cells

Because DRB inhibits rToxofilin phosphorylation in vitro and because it has been characterized as a cell-permeant molecule, we tested whether DRB treatment could affect tachyzoite motile properties. We exposed parasites to DRB and assessed their gliding activity as well as their invasive-

ness to host cells. When untreated parasites were added to serum-coated glass and observed by phase contrast video-microscopy, a majority displayed typical circular forward gliding motility (Figure 9A, top), whereas DRB-treated parasites did not move forward, although bending and twirling were still frequently seen (Figure 9A, bottom). Regarding parasite invasiveness, when DRB-treated parasites were incubated with HeLa cells in presence of the drug, their invasive capacities were significantly impaired in a dose-dependent manner (Figure 9B). In contrast, when parasites were exposed to the highest concentration of DRB (250 μM) only during the invasion assay, no loss of parasite invasiveness was detected, which excludes that reduced invasion is due to an effect of DRB on the host cells. In addition, Figure 9C shows that the average number of tachyzoites within an infected cell decreases with increasing concentrations of DRB. As assessed using ethidium bromide and calcein (Molecular Probes), DRB-treated parasites were alive and did not display any plasma membrane damage (our unpublished data). Together, these results suggest that a parasite CKII activity is required for tachyzoite motile properties.

Figure 5. Ser⁵³ is the major site of Toxofilin phosphorylation. Kinase assay was performed on 2 μg of either wild-type, S53A, or S53E rToxofilins by using human α_2 CKII (lanes a–c) or the heparin eluate (lanes d–f).

DISCUSSION

Key events during the *Toxoplasma* life cycle, such as gliding motility, host cell invasion, and egress are critically depen-

Figure 6. Toxofilin-bound PP2C dephosphorylates Toxofilin: in vitro kinase assay. (A) rToxofilin was incubated with cytosol corresponding to 2×10^8 tachyzoites in 100- μ l volume (lane a) supplemented with 1 unit of purified recombinant human PP1 (lane b) or with purified *T. gondii* ThioHis-PP2C at 5 μ g (lane c) and 20 μ g (lane d). (B) Cytosols precleared on protein A-Sepharose (lane a) or PP2C-immunodepleted (lane b) were incubated with [γ - 32 P]ATP. (C) Localization of Toxofilin in the phosphorylated profiles from both control (lane a) and PP2C-immunodepleted (lane b) cytosols by using Western blotting with anti-Toxofilin antibodies.

dent on actin dynamics in the parasite. Little is known about the factors that control actin polymerization in *T. gondii*, or in any other apicomplexan parasite. A striking feature is that most actin is present as monomers in Apicomplexa and that actin filaments have been unusually difficult to visualize in vivo. However, actin filaments in *T. gondii* were recently identified using electron microscopy of jasplakinolide-treated parasites (Shaw and Tilney, 1999), whereas F-actin

Figure 7. Native and recombinant *T. gondii* PP2Cs dephosphorylate Toxofilin on Ser⁵³. (A) rToxofilin was phosphorylated on Ser⁵³ by using 250 ng of purified human CKII and then incubated with kinase buffer (lane a) or with cytosol adjusted to 10 mM MgCl₂ (lane b), to 2 mM EDTA (lane c), or to 10 mM MgCl₂ plus 2 μ M OA (lane d). (B) CKII-phosphorylated rToxofilin was incubated with buffer (lane a) or with the same amount of phosphatase activity from rPP2C (lane b) or parasite cytosol (lane c).

could be detected biochemically under special conditions (Poupel and Tardieux, 1999). The only molecule that has been found to bind actin in Apicomplexa, apart from myosins, is Toxofilin, a protein with no homolog in the database. Toxofilin binds G-actin both in vivo and in vitro and caps barbed-end actin filaments (Poupel *et al.*, 2000). This study describes how Toxofilin properties are regulated.

Toxofilin Is Phosphorylated by a CKII Kinase

CKII is a highly conserved serine/threonine protein kinase found in all eukaryotes examined so far that catalyzes phosphate transfer onto a large number of proteins (Allende and Allende, 1995). CKII activity has already been involved in processes that require actin recruitment, such as the formation of cell-cell adherens junctions (Lickert *et al.*, 2000). More directly, muscle actin has been shown to selectively inhibit CKII kinase in vitro in a dose-dependent manner (Karino *et al.*, 1996). Several lines of evidence indicate that a CKII-like activity phosphorylates Toxofilin in *T. gondii*. First, the phosphate source for Toxofilin phosphorylation by the parasite cytosolic kinase may be either ATP or GTP nucleotides. Second, the parasite kinase activity is inhibited in the presence of CKII inhibitors such as soluble heparin, DRB, chrysin, and emodin at doses usually active in vitro. Third, Toxofilin phosphorylation is insensitive to staurosporine, a broad-spectrum serine/threonine kinase inhibitor, or to second messengers, such as calcium and cyclic nucleotides, features that characterize a CKII-like kinase activity. Finally, as expected for a CKII activity, the Toxofilin kinase activity could be recovered by heparin chromatography. The purified activity catalyzed 32 P incorporation into both recombinant Toxofilin and a synthetic peptide acting as a consensus

Figure 8. Ser⁵³ is important for Toxofilin properties on actin cytoskeleton. Fluorescence microscopy on CV1 cells expressing GFP (top right), GFP-Toxofilin (bottom right), or GFP-S53A Toxofilin (top left), GFP-S53E Toxofilin (bottom left). F-actin was visualized with Alexa 560-phalloidin.

substrate for mammalian CKII α . In agreement with these data, we cloned in *Toxoplasma* a cDNA sequence displaying significant similarities to CKII α from both apicomplexan parasites and higher eukaryotes, confirming the sequence as a bona fide CKII α . In *P. falciparum*, at the time of this study, eight contigs in the genome database (chr2_11953, chr3_3P8, chr7_000012, chr7_000106, chr8_000118, chr11_1, chr12_1, and chr13_1000007; Plasmo database, June 2002) display a high degree of sequence similarity with CKII α from other eukaryotes. In *T. parva* and *T. annulata*, CKII α has been already cloned and characterized (Ole-MoiYoi *et al.*, 1992). Importantly, the recombinant *T. gondii* CKII α polypeptide successfully catalyzed ³²P transfer onto a Toxofilin synthetic peptide encompassing the serine residue that we characterized as phosphate acceptor.

Toxofilin Is Dephosphorylated by a PP2C-like Phosphatase

PP2Cs have been mostly implicated in a variety of cell types, in the control of cyclin dependent-kinase Cdk2 (Chen *et al.*, 1999), as well as a number of mitogen-activated protein kinases (Hanada *et al.*, 2001). PP2C has also been connected to actin dynamics. For example, in *Arabidopsis thaliana* and *Commelina communis*, the ABI1 and ABI2 PP2Cs contribute to the abscisic acid-induced actin filament reorganization observed during stomatal closure (Merlot *et al.*, 2001). In addition,

human platelet PP2C dephosphorylates moesin in vitro, affecting its binding to F-actin (Huang *et al.*, 1999). In *P. falciparum*, an unusual PP2C has been cloned that carries two successive catalytic domains and requires dimerization for optimal activity (Mamoun *et al.*, 1998). One potential substrate of the *P. falciparum* PP2C is the elongation factor 1 β (Mamoun and Goldberg, 2001).

We have shown herein that the 36.8-kDa component of the Toxofilin-actin complex is a PP2C type phosphatase: the corresponding amino acid sequence displays the eight critical amino acid residues conserved in all members of the PP2C family. In addition, we have shown that Toxofilin is selectively dephosphorylated by endogenous or recombinant PP2C, but not by human PP1 that has been shown to be active on several *T. gondii* molecules (Delorme *et al.*, 2002).

Table 2. Biacore analysis of Toxofilin-actin interaction

	k_{off} (10^{-2} s^{-1})	k_{on} ($10^3 \text{ M}^{-1} \text{ s}^{-1}$)	K_d^{APP} (mM)
Toxofilin WT-G-actin	3 ± 0.5	12.8 ± 2	2.3 ± 1
Toxofilin S53E-G-actin	13 ± 0.9	4.0 ± 0.3	32.5 ± 5

Figure 9. CKII inhibitor DRB decreases tachyzoites invasiveness into HeLa cells. (A) Time-lapse videomicroscopy of tachyzoites in contact with serum-coated glass: untreated parasite displayed typical circular gliding within 10 s (top), whereas DRB-treated parasites did not (bottom). The time elapsed between each frame is indicated in seconds. (B and C) Tachyzoites were exposed to DRB before contact with HeLa cells at a 100–1 multiplicity of infection. (B) The number of HeLa cells containing parasites out of 100 cells represents the mean of triplicates for three independent experiments. (C) The number of intracellular tachyzoites was counted on 30 infected cells. Values represent mean of triplicates for three independent experiments and the error bars represent the SD associated to the mean.

Toxofilin Ser⁵³ Is the Site for Regulation and Control of Actin Dynamics by PP2C and CKII In Vivo

The phosphorylation site on Toxofilin was mapped at Ser⁵³ by using a kinase assay with heparin eluate, or human CKII as source of kinase. This residue lies within a CKII phosphorylation consensus site, ES⁵³GDEG, which contains the canonical D at +2 and E at +3 (Songyang *et al.*, 1996). Furthermore, in vitro ³²P incorporation in S53A, or S53E Toxofilin mutants was markedly inhibited using either the parasite heparin-bound eluate, or the human CKII. Interestingly, a PP2C activity present in the parasite cytosolic fraction dephosphorylates Ser⁵³-phosphorylated Toxofilin more efficiently than recombinant PP2C. This may suggest that other isoforms of PP2C might be expressed and efficient at dephosphorylating Toxofilin. It might also be that a cytosolic molecule modulates PP2C activity on Toxofilin and no such regulatory molecule have been yet described for PP2Cs in other eukaryotic systems. If selectivity to Toxofilin and potentially to other parasite PP2C substrates is conferred by partner(s), these might become potential drug targets.

In vivo, the Ser⁵³ residue seems key to the function of Toxofilin on actin assembly/disassembly. Indeed, upon expression of WT Toxofilin in fibroblasts, we observed that most of the actin stress fibers had disassembled, an observation similar to what we previously reported upon expression in epithelial cells. In these cells, the GFP-WT Toxofilin, but not the GFP alone, is found phosphorylated (our unpublished data) but presumably, not all the WT Toxofilin is. In contrast, the whole population of S53E Toxofilin behaves

like phosphorylated and in terms of phenotype, its expression induced an even more striking disorganization of actin stress fibers. However, the Toxofilin-Ser53E mutant protein may not necessarily behave in exactly the same way in vitro or in cells. In contrast, expression of the S53A Toxofilin-GFP fusion did not significantly affect actin stress fiber organization. In addition, our in vitro study using the Biacore system confirmed these results by showing that the stability of the actin-Toxofilin complex depends on the phosphorylation status of Ser⁵³. The phosphorylation of Ser⁵³ on Toxofilin decreased the affinity for G-actin and if we extrapolate this to the in vivo situation observed in fibroblasts, we may speculate that the massive disorganization of actin stress fibers triggered by S53E Toxofilin results more from an effect on F-actin. We have shown that Toxofilin caps actin filaments (Poupel *et al.*, 2000), and we have data characterizing a Toxofilin severing activity on F-actin (our unpublished data). Finally, using time-lapse videomicroscopy, we bring evidence that the parasite CKII activity is required for both gliding and host cell invasion, because DRB treatment that strongly inhibited rToxofilin phosphorylation in vitro also impaired tachyzoite gliding.

A connection between CKII and PP2C has been recently suggested in the microplasmidia *Physarum polycephalum*. First, De Corte *et al.* (1996) reported that fragmin could be phosphorylated in vitro by a CKII-type enzyme without modifying fragmin properties. Second, a large 46-kDa type 2C phosphatase that dephosphorylates CKII phosphorylated-fragmin has been described previously (Waelkens *et al.*,

2000). Nonetheless, neither CKII nor PP2C regulators were detected associated with the actin-fragmin complex.

Our study shows that Toxofilin's properties on actin are mediated by phosphorylation of Ser⁵³ residue through a balance between the counteracting *T. gondii* CKII α kinase and PP2C phosphatase activities. Although the intrinsic properties of actin seem well conserved among "lower and higher" eukaryotes and are central to both cell crawling and gliding motility, it is noteworthy that the phylum of apicomplexan has selected unique mechanisms underlying the speed and the nature of their gliding motion. Such unique mechanisms could integrate transient interactions between Toxofilin, actin, and signaling molecules. Future dissection of the phosphate flux onto Toxofilin within parasites will provide clues on the spectacular dynamic character of actin in these cells.

ACKNOWLEDGMENTS

We thank C. Cochet (Institut National de la Santé et de la Recherche Médicale-Commissariat à l'Energie Atomique, Grenoble, France) for kindly providing with the recombinant CKII enzyme and D. Sibley (Washington University, St. Louis, MO) for the anti-*T. gondii* actin antibody. We are grateful to R. Ménard (Institut Pasteur, Paris, France) for support and help with the manuscript and to G. Milon and G. Langsley (Institut Pasteur) for a critical reading of the manuscript. This project was financed by the Centre National de la Recherche Scientifique (ATiPE Microbiology award to I.T.) and the Foundation for Medical Research (France). V.D. was supported by a Centre National de la Recherche Scientifique-DGA fellowship, whereas X.C. and A.G. were financially supported by the Association for Research against Cancer 4437.

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