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Toxofilin, a Novel Actin-binding Protein from *Toxoplasma gondii*, Sequesters Actin Monomers and Caps Actin Filaments

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Toxoplasma gondii relies on its actin cytoskeleton to glide and enter its host cell. However, *T. gondii* tachyzoites are known to display a strikingly low amount of actin filaments, which suggests that sequestration of actin monomers could play a key role in parasite actin dynamics. We isolated a 27-kDa tachyzoite protein on the basis of its ability to bind muscle G-actin and demonstrated that it interacts with parasite G-actin. Cloning and sequence analysis of the gene coding for this protein, which we named Toxofilin, showed that it is a novel actin-binding protein. In in vitro assays, Toxofilin not only bound to G-actin and inhibited actin polymerization as an actin-sequestering protein but also slowed down F-actin disassembly through a filament end capping activity. In addition, when green fluorescent protein-tagged Toxofilin was overexpressed in mammalian nonmuscle cells, the dynamics of actin stress fibers was drastically impaired, whereas green fluorescent protein-Toxofilin copurified with G-actin. Finally, in motile parasites, during gliding or host cell entry, Toxofilin was localized in the entire cytoplasm, including the rear end of the parasite, whereas in intracellular tachyzoites, especially before they exit from the parasitophorous vacuole of their host cell, Toxofilin was found to be restricted to the apical end.

INTRODUCTION

Eukaryotic cells remodel their actin cytoskeleton continuously in response to both intracellular and extracellular signals. This remodeling is crucial in mediating not only cell motility but also many other fundamental cellular functions. Extensive in vitro work has led to the current understanding at the molecular level of the in vivo actin dynamics (Welch *et al.*, 1997; Ayscough, 1998).

Reorganization of filamentous actin varies according to the cell type and the stimulus. It can be an extremely localized, discrete, and transient phenomenon but can also result in sustained reorganization involving extended parts of the cell. However, despite the variation in its manifestation, the reorganization of actin cytoskeleton relies primarily on the intrinsic properties of the actin molecule (G-actin) to assemble and disassemble into filaments (F-actin) (Carlier, 1990, 1991). The organization of the cytoskeleton is controlled by a cohort of actin-associated proteins, which tightly coordinate the intrinsic dynamic properties of actin and support its spatial organization into various dynamic structures within the cells (Stossel, 1993).

Coccidies are protozoan parasites that belong to the phylum of Apicomplexa, members of which are human pathogens of major medical importance. The causative agent of malaria, *Plasmodium falciparum,* causes death of >2 million children every year (Marsh and Snow, 1997), and other Apicomplexa such as *Toxoplasma gondii* and to a lesser extent *Cryptosporidium parvum* are devastating human pathogens when they parasitize immunocompromised hosts (Kasper and Buzoni Gatel, 1998). Certain developmental stages of these parasites including the sporozoites of *Plasmodium, Cryptosporidium,* and *Toxoplasma* as well as the tachyzoites of *Toxoplasma,* move by a gliding motion across either a mucous layer or an extracellular matrix before encountering their host cells. They

In memory of O. Tardieux.

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Abbreviations used: ADF, actin-depolymerizing factor; DMEM, Dulbecco's modified Eagle's medium; EST, expressed sequence tag; GFP, green fluorescent protein; GST, glutathione *S*-transferase; HFF, human foreskin fibroblast; 1,5-IAEDANS, *N*-(iodoacetyl)-*N*′-1-sulfo-5-naphthylethylenamine; Ig, immunoglobulin; r-Toxofilin, recombinant Toxofilin.

subsequently enter these cells by an active process, and once in a suitable intracellular niche, they either multiply and/or differentiate (for reviews, see Silverman and Joiner, 1997; Dubremetz, 1998), two steps required for parasite spreading before transmission to a new host.

The strategies selected by these parasites for either gliding onto a substratum or for invading their host cells depend on the dynamics of their actin cytoskeleton (King, 1988; Dobrowolski and Sibley, 1996; Preston and King, 1996). However, although it has been established that actin dynamics is required for the progression of the parasite's life cycle, studies on the molecular basis of parasite actin dynamics have been hampered by the transient and discrete nature of actin cytoskeleton remodeling. As a consequence, only limited knowledge is available for Apicomplexa compared with what is known for other eukaryotic cells.

Recent data from our and other laboratories demonstrated that *Toxoplasma* has a strikingly low amount of assembled actin compared with the usual F- to G-actin ratio observed in other eukaryotic cells (Dobrowolski *et al.*, 1997b; Poupel and Tardieux, 1999). The estimated amount of extractable F-actin in our study represented <5% of the total actin, and this yield increased slightly when parasites were treated before extraction with the actin-stabilizing drug jasplakinolide. Indeed, tachyzoite actin filaments have not been detected in situ either by phalloidin staining or by classical electron microscopy. However, Heuser and Sibley report that microfilaments can be observed by freeze—etch microscopy beneath the plasma membrane of gliding tachyzoites (in Dobrowolski and Sibley, 1997). Our recent data demonstrate that, although remarkably small, the pool of F-actin in *T. gondii* tachyzoites remains competent for assembly and disassembly, and for coupling to a myosin-type motor activity (Poupel and Tardieux, 1999).

Parasite molecules, capable of controlling actin monomer sequestration and desequestration in association with molecules regulating actin filament turnover, are expected to be among the major effectors responsible for the unusual low F-actin content in *Toxoplasma*. Therefore, stimuli inducing actin polymerization during parasite gliding and host cell entry will have to locally decrease the critical actin concentration required for filament assembly. In the eukaryotic cells analyzed thus far, such changes in the critical actin concentration are known to be elicited by fluctuations in the activity of capping proteins and profilin, whereas they are amplified by other G-actin-binding proteins (Schafer and Cooper, 1995; Perelroizen *et al.*, 1996).

We present here the identification and characterization of a *T. gondii* novel actin-binding protein, which we named Toxofilin. Toxofilin sequesters muscle G-actin and inhibits its polymerization in vitro. Additionally, it associates with muscle F-actin by capping the actin filament end. When Toxofilin was ectopically overexpressed as green fluorescent protein (GFP)-tagged protein in mammalian nonmuscle cells, it clearly disrupted the actin cytoskeleton and caused disassembly of actin stress fibers. In tachyzoites, Toxofilin binds G-actin and copurifies with a parasite F-actin-containing fraction, suggesting that it may control parasite actin dynamics as well. Such a role was further suggested by the highly variable localization pattern of Toxofilin in the moving parasite, i.e., during gliding or host cell entry.

MATERIALS AND METHODS

Parasite Production and Recovery

The RH *T. gondii* strain was propagated in female Swiss mice as described by Poupel and Tardieux (1999). The parasites were pelleted in PBS^- containing 0.1% (vol/vol) protease inhibitor stocks 1 and 2 and were stored at −70°C until use. The protease inhibitor stocks were composed, for stock 1, of 4-[2-aminoethyl]benzenesulfonylfluoride (5 mg/ml), aprotinin (2 mg/ml), leupeptin (2 mg/ml),and benzamidin (16 mg/ml) in H_2O and, for stock 2, of pepstatin A (5 mg/ml in DMSO).

Parasite Handling for Further Protein Extraction and Affinity Chromatography

Frozen tachyzoites (10^9) were thawed on ice and lysed by five liquid nitrogen freezing and defreezing cycles in 2 ml of buffer A (20 mM Tris-Cl, pH 8.0, 50 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA) supplemented with 0.5% (vol/vol) protease inhibitor stocks. The extract was centrifuged (15 min, 1000 × *g*, 4°C) to remove nuclei and unbroken cells. The supernatant was centrifuged (30 min, 100,000 × *g*, 4°C) in a TL100 table-top ultracentrifuge (Beckman Instruments, Palo Alto, CA) using the TLA 100.3 rotor. The final supernatant was applied to either a monomeric actin column prepared as described by Miller and Alberts (1989) with actin purified from a rabbit muscle acetone powder (Pardee and Spudich, 1982) or a DNase1 affinity column as previously described (Fahrni, 1992). Both columns were washed with 20 column volumes of buffer A supplemented with 0.05% Nonidet P40. G-actin-bound proteins were recovered by elution with 3 column volumes of buffer B (buffer A supplemented with 3 mM $MgCl_2$ and 1 mM Na_2ATP) followed by precipitation with trichloroacetic acid containing 0.04% (wt/vol) sodium deoxycholate. DNase1-bound proteins were directly eluted in SDS-PAGE sample buffer. Samples were boiled and kept at −70°C until analysis by SDS-PAGE electrophoresis. To recover parasite F-actin, proteins were extracted in 2 ml of actin-stabilizing buffer C (60 mM 1,4-piperazinediethanesulfonic acid, 25 mM HEPES, 125 mM KCl, 2 mM $MgCl_2$, 5 mM EGTA, 100 μM Na_2ATP, 1 μM phalloidin, pH 7.2) containing 0.1% (wt/vol) saponin (Sigma, St. Louis, MO) and 0.5% (vol/vol) protease inhibitor stocks (Poupel and Tardieux, 1999).

Peptide Microsequencing, cDNA Library Screening, and DNA Sequencing

The gel slice containing the 27-kDa actin-binding protein from the parasite was subjected to tryptic digestion (30°C, 18 h, 0.3 mg of trypsin in 0.1 M Tris-Cl, pH 8.6, 0.01% Tween 20). The tryptic peptides were recovered by HPLC on a DEAE-C18 column. The sequence of each peptide was found in different clones from the *T. gondii* database of expressed sequence tags (ESTs; Washington University–Merk Toxoplasma EST Project, St. Louis, MO). Nondegenerate primers were synthesized for amplification of the target sequence from the clone identified as TgESTzy57g11. The oligonucleotide with the sequence 5′-CG GAG NAG CCC TAG TTC CTG-′, corresponding to the amino acid sequence QELGLLR, was used as the downstream PCR primer, whereas 5′-TCA AGT GAC CAA GGC GGT CGA-3′ was chosen as the upstream primer. The PCR conditions for amplification of the 294-bp DNA product were as follows: a hot start of 2 min at 94°C followed by 35 cycles (1 min, 94°C; 30 s, 61°C; and 45 s, 72°C) and a final elongation step at 72°C for 10 min. The 294-bp fragment recovered was ^{32}P labeled using random priming (Megaprime kit; Amersham, Arlington Heights, IL), purified on a Sephacryl S-400 HR column (Pharmacia, Uppsala, Sweden), and used as a probe to screen a *T. gondii* tachyzoite cDNA library (kindly provided by J.W. Ajioka, Cambridge University, Cambridge, United Kingdom). After two rounds of screening, 10 independent overlapping clones were selected, and their cDNA was prepared for nucleotide sequencing performed by Genset (Paris,

France), using both vector and *T. gondii* Toxofilin-specific primers (Genset).

Native Electrophoresis with IAEDANS-labeled Muscle Actin

Polymerized rabbit muscle actin was prepared from clarified G-actin by adding KCl to 50 mM, $MgCl_2$ to 2 mM, and Na_2ATP to 1 mM. The Cys-373 of F-actin (50 μM) was labeled with *N*-(iodoacetyl)-*N*′-1-sulfo-5-naphthylethylenamine (1,5-IAEDANS, 500 μM; Molecular Probes, Eugene, OR) as described by Dos Remedios and Cooke (1984). A cytosolic fraction from 5×10^8 frozen tachyzoites was prepared as described in the previous paragraph by using 400 μl of buffer D (2 mM Tris-Cl, pH 8.0, 0.2 mM $CaCl_2$, 0.1 mM dithiothreitol, 0.2 mM Na_2ATP) supplemented with 0.5% (vol/vol) protease inhibitor stocks. One hundred twenty-five microliters of cytosol were incubated (15 min, 4°C) with 50 μg of labeled G-actin (in a 25-μl volume) under rotating agitation and analyzed by native gel electrophoresis as described by Safer (1989). In some experiments, the interaction of recombinant Toxofilin with G-actin was also tested by native gel electrophoresis. For this, 30 μg of recombinant toxofilin (in 125 μl buffer A), obtained as described below, were incubated (15 min, 4°C) with 50 μg of labeled G-actin (in a 25-μl volume) before analysis on a native gel. Fluorescent bands were visualized under a UV lamp (λ = 321 nm).

Expression of Glutathione S-Transferase (GST)–Toxofilin Fusion Protein and Recombinant Toxofilin Purification

The fragment for expression of Toxofilin was prepared by PCR amplification of a full-length Toxofilin-encoding cDNA using primers introducing a *Bam*HI restriction site at position 5′ and a *Sal*I restriction site at position 3′. For amplification of the upper strand (5′-GGCCGGATCCATGGCGCAATACAAGTCACGC-3′) and of the lower strand (5′-GGCCGTCGACTTACGACGAGGGCATAGCGCC-3′), the amplified fragment was cloned into the expression vector pGEX6-P3 (Pharmacia) after digestion with *Bam*HI and *Sal*I of both fragment and vector. For expression of the GST-Toxofilin, an *Escherichia coli* clone (BL21 strain) positive for the plasmid was grown up to OD of 1 and induced with isopropylthio-β-D-galactoside (1 mM, 3 h, 37°C). At the end of the induction period, the bacteria were pelleted and subsequently lysed in buffer D (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, Triton X-100 [0.5% vol/vol], *N*-lauryl sarcosyl [1.5% vol/vol; Sigma]) at 4°C. The supernatant recovered after centrifugation (15,000 $\times$ *g*, 15 min, 4°C) was incubated with glutathione-Sepharose (Pharmacia) (4°C, overnight), and the beads were washed with 40 bead volumes of buffer C supplemented with 0.1% Triton X-100. The bound GST-polypeptide was cleaved with prescission protease to recover the recombinant Toxofilin (r-Toxofilin) without GST (Pharmacia). r-Toxofilin was lyophilized as 100-μg aliquots and stored at −70°C until use.

Antibodies

The mouse monoclonal antibody anti-P30 (Biogenex, San Ramon, CA) recognizes a major tachyzoite surface. The mouse monoclonal anti-MIC3 (Tg42F3.2F5) and the anti-GRA1 (Tg 17–43) recognize, respectively, the MIC3 protein from tachyzoite micronemes and the GRA1 protein from tachyzoite-dense granules. These antibodies were kindly provided by M.F. Cesbron (Institut Pasteur, Lille, France). Antibodies against r-Toxofilin were obtained by immunizing two rats and a rabbit with r-Toxofilin, and the positive sera were affinity purified on an r-Toxofilin HiTrap-*N*-hydroxysuccinimide-activated affinity column (Pharmacia).

Electrophoresis and Western Blotting

Proteins were separated by SDS-PAGE (12% gel; Laemmli, 1970) and transferred to Hybond-C nitrocellulose (Amersham) using a semidry blotting apparatus (Hoefer, San Francisco, CA; 45 min, 1.5 mA/cm^2). The membrane was blocked and treated after incubation with the appropriate antibodies as described by Tardieux *et al.* (1998). Membranes were incubated overnight at 4°C either with a rat anti-*T. gondii* Toxofilin serum diluted 1:5000 in Tris-buffered saline and 0.1% (vol/vol) Tween 20 and/or a rabbit anti-*T. gondii* actin antibody diluted to 1:4000. After several washes in Tris-buffered saline and 0.1% (vol/vol) Tween 20, the blots were incubated (1 h, 23°C) with HRP-labeled anti-rat immunoglobulin (Ig) diluted to 1:5000 (Jackson ImmunoResearch, West Grove, PA) or HRP-labeled anti-rabbit Ig diluted to 1:3000 (Amersham). In the actin cosedimentation experiment, rabbit actin was detected using the mouse monoclonal antibody clone C4 (dilution 1:4000) followed by the Western blot kit reagents (ECF, Amersham).

Pyrene-labeled Muscle Actin Polymerization Assay

Actin was prepared as described by Tardieux *et al.* (1998). Pyrene-labeled actin was obtained as described by Kouyama and Mihashi (1981). The final concentration of rabbit actin was 100 μg/ml. The concentration of r-Toxofilin in the assay varried between 1 and 2 μM. To eliminate potential aggregates, r-Toxofilin was ultracentrifuged before its addition to actin (4°C, 30 min, 100,000 $\times$ *g*). Polymerization was carried out at 23°C in buffer P (100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 5 mM Tris-HCl, pH 8.0), and monitored in a Spex fluorimeter (F-2000; Hitachi, Tokyo, Japan) at λ_{ex} = 350 nm and λ_{em} = 390 nm. The steady-state values of F-actin were obtained after 16 h incubation at 4°C and the samples were subsequently centrifuged (4°C, 2 h, 100,000 $\times$ *g*, in a TL100.A rotor; Beckman) and analyzed by SDS-PAGE and Western blot.

Protein Immunoprecipitation

Toxofilin was immunoprecipitated from the final 100,000 $\times$ *g* supernatant of the parasite lysate supplemented with 0.5% (vol/vol) Triton X-100 and 0.5% (wt/vol) BSA (fraction V; Life Technologies, Gaithersburg, MD) using rabbit anti-Toxofilin followed by protein A-Sepharose (Pharmacia). Washes were successively performed in buffer A containing both 0.5% (wt/vol) BSA and 0.5% (vol/vol) Triton X-100 and then in buffer A containing 0.5% (vol/vol) Triton X-100 and buffer A. Immunocomplexes were eluted in SDS-PAGE sample buffer. Samples were boiled and kept at −70°C until analyzed by SDS-PAGE.

Human Cell Culture

Human foreskin fibroblasts (HFFs) were cultured between the 11th and 25th passages in Dulbecco's modified Eagle's medium (DMEM) with Glutamax medium (Life Technologies) supplemented with 10% (vol/vol) FBS (Dutscher, Brunath, Israel) and penicillin-streptomycin (10 μg/ml, Life Technologies). HeLa cells were cultured under the same conditions as a laboratory-established cell line.

Expression and Detection of GFP-Toxofilin Fusion Protein in HeLa Cells

The fragment for expression of Toxofilin was obtained by PCR amplification of a full-length Toxofilin-encoding cDNA using primers introducing 5′-*Sal*I and a 3′-*Bam*HI restriction sites. The primers used were 5′-GGCCGTCGACATGGCGCAATACAAGTCACGC-3′ for the upper strand and 5′-GGCCGGATCCTTACGACGAGGGCATAGCGCC-3′ for the lower strand.

The amplified fragment was cloned into the pEGFP-C1 MCS (Clontech, Cambridge, United Kingdom) expression vector after digestion of both insert and vector with *Bam*HI and *Sal*I enzymes.

The GFP-Toxofilin coding plasmid was prepared from a positive clone of *E. coli* (strain DH5α; Qiagen [Hilden, Germany] method) and used for transfections of 50% confluent HeLa cells. Transfections were performed with the $CaCl_2$ phosphate transfection mix-

ture overnight (37°C, 5% CO_2). Cells after two washes in DMEM containing 10% FBS, were plated on glass coverslips and incubated overnight (37°C, 6% CO_2) for 20 h. Expression of the GFP-Toxofilin was analyzed by fluorescence microscopy, and F-actin was stained with rhodamine-phalloidin. Forty to 60% of the transfected cells expressed GFP-tagged Toxofilin.

Immunofluorescence of Extracellular and Intracellular Parasites

Free *T. gondii* tachyzoites freshly isolated from mice were allowed to glide on glass slides in PBS^-. The parasites were fixed in situ by paraformaldehyde (2% in PBS^-, 15 min, 23°C), washed in PBS^-, treated with NH_4Cl (50 mM in PBS^-, 5 min), and deposited on a glass slide at a density of 10^7/ml to air dry.

HFF cells were plated in 3.5-cm-diam Petri dishes at a density of 2×10^5 cells per dish on acid-washed 12-mm circular glass coverslips. After overnight culture in DMEM and Glutamax (Life Technologies) containing 10% (vol/vol) FBS and antibiotics, tachyzoites were added to the cells (2×10^7 per dish) for 15 min (37°C, 5% CO_2). Some coverslips were then washed in PBS^- and fixed (2% paraformaldehyde, 0.1 M lysine, 0.05 M $NaPO_4$, pH 7.4) for 15 min, whereas others were further incubated in fresh 10% medium for 20 h (37°C, 5% CO_2) before fixation. Cells were labeled with the mouse monoclonal antibody anti-P30 (dilution 1:300 in PBS^- supplemented with 2.5 mg/ml goat serum). After several washes, cells were incubated with the Alexa-488 goat anti-mouse IgG (heavy and light chain, Molecular Probes; 7 μg/ml, 1 h, 23°C). The excess of conjugate was washed away with PBS^-, and cells were permeabilized with Triton X-100 (0.1% (vol/vol) in PBS^- containing 2.5 mg/ml normal goat serum). Free extracellular parasites were permeabilized for 1 min (23°C), and HFFs were infected with parasites for 3 min (23°C). The detergent was removed by several PBS^- washes, and cells were incubated with nonimmune or affinity-purified immune sera. Both the rat and the rabbit anti-Toxofilin affinity-purified sera were diluted at 1:200 in PBS^- containing 2.5 mg/ml goat serum. The staining was subsequenctly revealed by incubation with the Alexa-568 goat anti-rat or anti-rabbit IgG (heavy and light chain, Molecular Probes; 7 μg/ml, 1 h, 23°C). Unbound fluoroconjugate was removed with PBS^- washes, and DAPI (Sigma; 5 μg/ml) was added for 5 min before the last PBS^- wash. When needed, F-actin of HFF or HeLa cells was stained with rhodamine-phalloidin (0.66 mg/ml, 30 min, room temperature; Molecular Probes).

The samples were examined under an epifluorescence microscope (Axiophot; Zeiss, Oberkochen, Germany) attached to a cooled charge-coupled device camera (Photometrics, Tucson, AZ), using a 63× Plan-Apochromat lense. Images were aquired using the IPlab software (Signal Analytics, Vienna, VA) and analyzed by NIH Image (National Institutes of Health, Bethesda, MD) or Adobe (Mountain View, CA) Photoshop software.

For the quantitation of F-actin fluorescence, cells from several fields were analyzed using the NIH Image program. The area around each cell was delineated, and the mean fluorescence intensity was measured in pixels. The results were presented as the mean of the fluorescence intensity for each group of cells ± SD.

Electron Microscopy

Actin filaments at steady state (2 μM) were incubated with purified r-Toxofilin (1 μM) for 15 min (23°C). Affinity-purified anti-Toxofilin antibodies were added (2 μg/ml, 15 min, 23°C), and the r-Toxofilin was revealed by anti-rabbit antibodies conjugated to 5-nm gold particles. The actin filaments were pelleted through a 30% sucrose cushion in F-actin stabilizing buffer (50,000 × *g*, 30 min, 4°C) and were further processed for electron microscopy as described by Tardieux *et al.* (1998). The samples were analyzed using a Phillips (Eindhoven, The Netherlands) CM12 transmission electron microscope.

RESULTS

T. gondii Tachyzoite Cytosolic Extracts Contain a 27-kDa Protein That Binds Mammalian G-actin

To isolate *T. gondii* tachyzoite proteins sequestering actin monomers, we initially searched for tachyzoite cytosolic proteins that could bind to muscle rabbit G-actin. For these studies we used affinity chromatography and native gel electrophoresis. When a 100,000 × *g* cytosolic extract from *T. gondii* tachyzoites (Figure 1A, lane b) was passed through an affinity column of rabbit muscle G-actin, a protein migrating in SDS-PAGE with a molecular mass of ~27 kDa was reproducibly recovered in the eluate and visualized on blots by Ponceau S staining (Figure 1A, lane a, triangle). Moreover, when rabbit G-actin fluorescently labeled with IAEDANS was incubated with a 100,000 × *g* cytosolic extract from *T. gondii* tachyzoites and the mixture was submitted to native gel electrophoresis, several fluorescent bands were detected (Figure 1B, lane a). Only one fluorescent band was observed when the same amount of G-actin was analyzed alone (lane c). When actin polymerization was promoted, a much fainter band of fluorescently labeled G-actin was detected (lane d).

The additional fluorescent bands detected in Figure 1A, lane a, represent complexes of labeled G-actin with parasite polypeptides. To dissociate and separate the complexes composing the two major shifted bands (Figure 1B, lane a, *1 and *2) SDS-PAGE was performed (Figure 1C). Both complexes were shown to contain a comigrating 27-kDa protein (marked with a triangle), which was visualized after transfer to nitrocellulose membrane and Ponceau S staining (Figure 1C, lanes a and b). Two other minor bands of ~40 and 47 kDa were also detected in both complexes. No fluorescent bands were observed in the *T. gondii* cytosolic fraction (Figure 1B, lane b), and the 40- and 47-kDa parasite proteins were not detected from the gel slice that migrated at the same position as the fluorescent actin-containing band marked as *1 (Figure 1C, lane c). This strengthened our conclusion that muscle G-actin interacts with at least three parasite proteins. As expected, the fluorescent band marked as *0, which migrated faster during native gel electrophoresis, contained only G-actin (Figure 1C, lane d). In addition, the 27-kDa protein recovered from either of the two purification procedures (affinity chromatography [Figure 1A, lane a] and native gel electrophoresis [Figure 1B, lane a]) appears to be the same. First, its digestion with trypsin yielded identical peptide profiles. Second, antibodies made against the recombinant Toxofilin recognized the same 27-kDa T.*gondii* actin-binding protein isolated by either of the two above-described procedures (our unpublished results).

Identification of Toxofilin cDNA and Protein Sequence

Peptide microsequencing of two tryptic peptides from the isolated 27-kDa *T. gondii* actin-binding protein gave rise to the following partial sequences: QAALAGQILNEQR and QQELGLLRPEER. Each peptide was encoded by several different *T. gondii* ESTs (www.cbil.upenn.edu/ParaDBs/; Ajioka *et al.*, 1998). Using nondegenerate primers, we obtained by PCR a 294-bp fragment, which was used as a probe to screen a tachyzoite cDNA library. We isolated 10 overlapping clones from which we obtained a single open

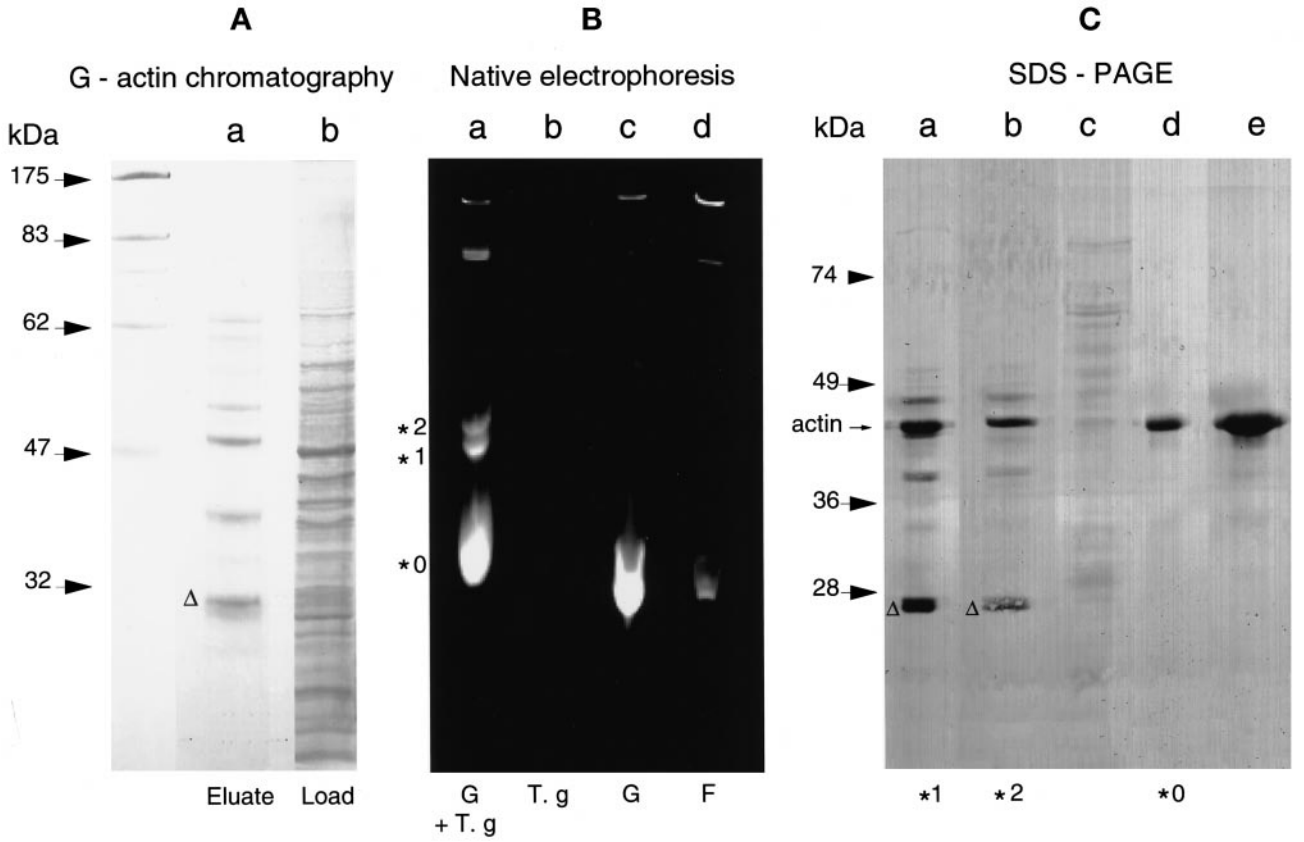

Figure 1. The *T. gondii* 27-kDa protein binds to G-actin. (A) G-actin chromatography. A 100,000 × *g T. gondii* cytosolic extract was subjected to affinity chromatography over a rabbit muscle G-actin column. Proteins bound to G-actin were eluted by 3 mM $MgCl_2$ and 1 mM ATP. These proteins were further separated by SDS-PAGE on a 12% gel, transferred to a nitrocellulose membrane, and visualized with red Ponceau S stain. Lane b, cytosolic fraction corresponding to 5×10^7 *T. gondii* tachyzoites; lane a, eluate. The polypeptide migrating with a molecular mass of ~27 kDa is marked with a triangle. (B) Native gel electrophoresis. A 100,000 × *g* parasite cytosolic extract (T.g) was incubated with IAEDANS-labeled G-actin (G), and the protein complexes were separated by native gel electrophoresis. The bands were visualized under a UV lamp (λ = 312 nm). Lane a, three bands were detected and marked, respectively, *0, *1, and *2; lane b, parasite cytosolic extract without fluorescent actin; lane c, fluorescent G-actin; lane d, fluorescent G-actin incubated under polymerization conditions to reach steady state before native gel electrophoresis. (C) Ponceau S staining on blot. Slices of the native gel (see B) containing either complex *0, *1, or *2 or from the adjacent lanes from positions corresponding to the migration of *0, *1, or *2 were boiled in SDS-PAGE sample buffer and analyzed on a 12% gel. The separated proteins were subsequently transferred onto a nitrocellulose membrane and stained with Ponceau S. Lanes a and b, material from bands *1 and *2, respectively. Apart from actin, three other similar bands were detectable in the complexes *1 and *2, including a band of ~27 kDa (triangle); lane c, sample extracted from a slice cut from lane b on the native gel. The slice corresponded to polypeptides comigrating with *1; lane d, material from the band *0 from the native gel; lane e, material containing G-actin from lane c of the native gel.

reading frame coding for a 27,085-Da protein (GenBank accession number AJ132777). This 246-amino-acid protein is predicted to be basic (pI = 9.63) and to contain two coiled-coil domains (amino acids 120–149 and 206–234). No consensus nucleotide-binding or actin-binding sites were predicted by the sequence. Southern blot analysis was performed on genomic *T. gondii* DNA after digestion with either *Bam*HI, *Eco*RI, or *Hin*dIII restriction enzymes. In all cases, only one signal was obtained, strongly suggesting that this protein is encoded by only one gene copy in the *T. gondii* (our unpublished results). The DNA and protein sequence of this newly identified protein was compared with all the known protein sequences in the nonredundant GenBank, but no significant similarity with other known proteins was revealed. Therefore the 246-amino-acid T. *gondii* acting-binding protein is novel, and we named it Toxofilin.

The Recombinant Toxofilin Binds Directly to G-actin and Controls Actin Polymerization In Vitro

To investigate the activities of Toxofilin in vitro, we expressed it in *E. Coli* as a recombinant protein (r-Toxofilin). To study whether r-Toxofilin binds directly to G-actin and whether it affects actin dynamics, we performed 1) a native gel electrophoresis, 2) a pyrene-labeled actin polymerization assay, and 3) an actin cosedimentation assay.

When r-Toxofilin was added to IAEDANS-labeled muscle G-actin and the mixture was separated on a native gel,

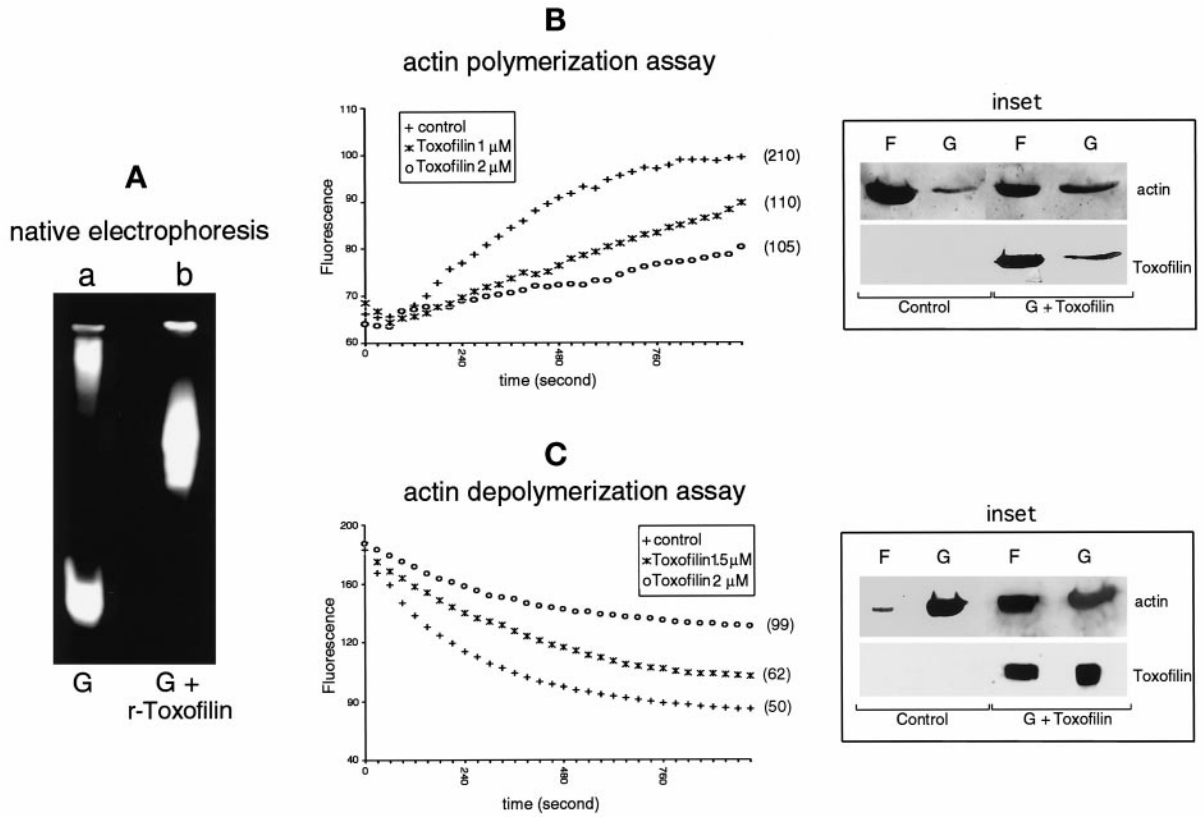

Figure 2. Recombinant Toxofilin controls actin dynamics in vitro: it binds directly to actin monomers and caps actin filaments. (A) Native gel electrophoresis. IAEDANS-labeled G-actin was incubated with or without r-Toxofilin (lanes b and a, respectively) before electrophoresis on a native gel. (B) Pyrene-labeled G-actin polymerization assay. G-actin at 3 μM and 1% pyrene labeled was incubated with or without r-Toxofilin before induction of polymerization. The kinetics of actin polymerization was monitored in a Spex fluorimeter (F-2000; Hitachi) at λ_{ex} = 350 nm and λ_{em} = 390 nm. +, kinetics of actin polymerization in the absence of r-Toxofilin; *, with 1 μM r-Toxofilin; ○, with 2 μM r-Toxofilin. Fluorescence intensity is measured in arbitrary units. Steady-state values are given in parentheses. Inset, upper panel, the amount of G and F-actin pelleted after ultracentrifugation (2 h, 100,000 × *g*, 4°C; as described by Tardieux *et al.*, 1998); lower panel, amount of r-Toxofilin recovered in the supernatant (G) and pellet (F). (C) Pyrene-labeled F-actin depolymerization assay. Ten micromolar steady-state F-actin was induced to spontaneously depolymerize upon dilution to 2 μM. The kinetics of actin depolymerization was illustrated by the curve labeled +. Kinetics of actin depolymerization was also followed in the presence of r-Toxofilin at 1.5 μM (*) and 2 μM (○). Inset, upper panel, amount of G and F-actin; lower panel, amount of r-Toxofilin recovered in the supernatant (G) and pellet (F).

we observed a shift in the migration profile of actin (Fig. 2A, lane b) compared with the migration of actin alone (lane a). In the mixture with r-Toxofilin actin ran slower. Of note, at equimolar concentrations, all the G-actin detected by fluorescence was complexed to r-Toxofilin. This result strongly argues for a direct binding of r-Toxofilin to G-actin.

To further analyze the effect of r-Toxofilin on the kinetics of muscle actin polymerization and depolymerization, we used a pyrene-labeled actin polymerization assay. The effect of r-Toxofilin on the extent of inhibition of actin polymerization was concentration dependent, as demonstrated by the steady-state values given in parentheses (Figure 3B). These steady-state values were obtained 16 h after the assay was begun. The insets in Figure 2B show that, at steady state, there was a significant decrease of sedimented actin in the presence of r-Toxofilin (upper panel), whereas r-Toxofilin copelleted with F-actin (lower panel). For this determination we used an anti-Toxofilin antibody raised against the r-Toxofilin. The copelleting of r-Toxofilin could not be an artifact attributable to aggregation, because we routinely ultracentrifuged r-Toxofilin-containing solutions before using them in the assay.

The Recombinant Toxofilin Has F-actin Capping Activity In Vitro

When r-Toxofilin was added to preassembled actin filaments that were allowed to depolymerize spontaneously, the rate of monomer dissociation was clearly slower compared with the control (Figure 2C). Similarly, the effect was concentration dependent, as illustrated by the steady-state values in parentheses. The insets in Figure 2C illustrate that F-actin was protected from dissociation (upper panel) by the r-Toxofilin found associated with it (lower

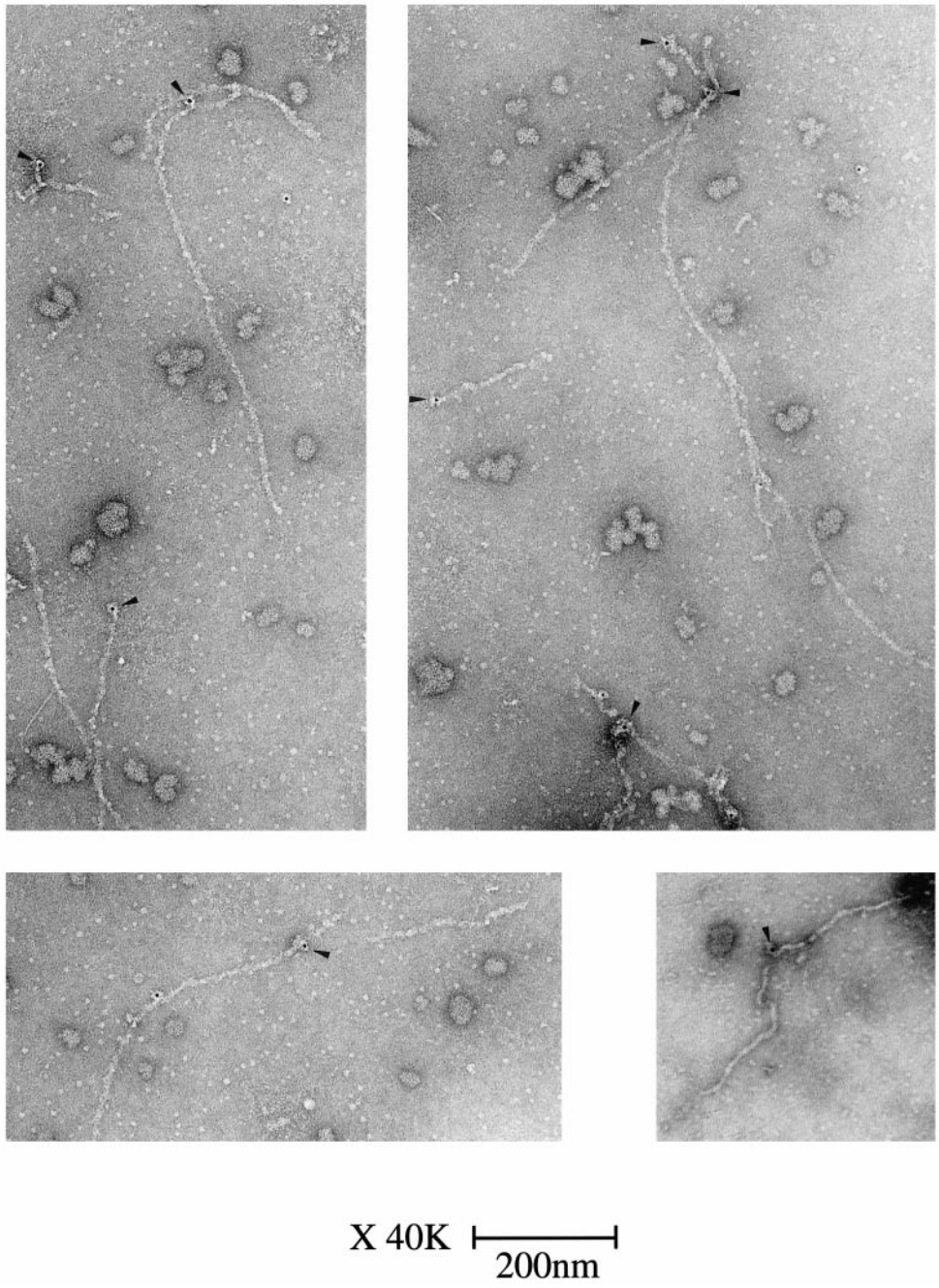

Figure 3. Toxofilin localizes at one end of actin filaments. Actin filaments at steady state (2 μM) were incubated with purified r-Toxofilin (1 μM). Immunolocalization of r-Toxofilin was revealed by anti-rabbit antibodies conjugated to 5-nm gold (see arrows). Actin filaments were negatively stained with 2% uranyl acetate.

panel). These data suggested that Toxofilin could function as a capping protein.

Additionally, electron micrographs displaying localization of Toxofilin on actin filaments strongly suggested that it caps one end of the filaments (Figure 3). Quantitative analysis was performed on 50 randomly selected actin filaments from three separate experiments. By statistical analysis we compared the frequency by which gold particles appeared either at one end or along the side of actin filaments. First, for filaments on which only one gold particle was bound (46 of 50, 45 of 50, and 48 of 50, respectively, for the three replicates), the frequencies for each gold particle to be found in either position were not different among the three replicates ($\chi^2 = 1.40$; df = 2; p = 0.497). Second, when the values from the three replicates were pooled together, the association of gold particles with one end of a filament was found to be highly significant (pooled frequencies compared with half of the particles in each position: $\chi^2 = 8.02$; df = 1; p = 0.0046). And finally, in the few filaments that had several gold particles bound (4 of 50, 5 of 50, and 2 of 50, respectively, for the three replicates), there was always one localized at one filament end.

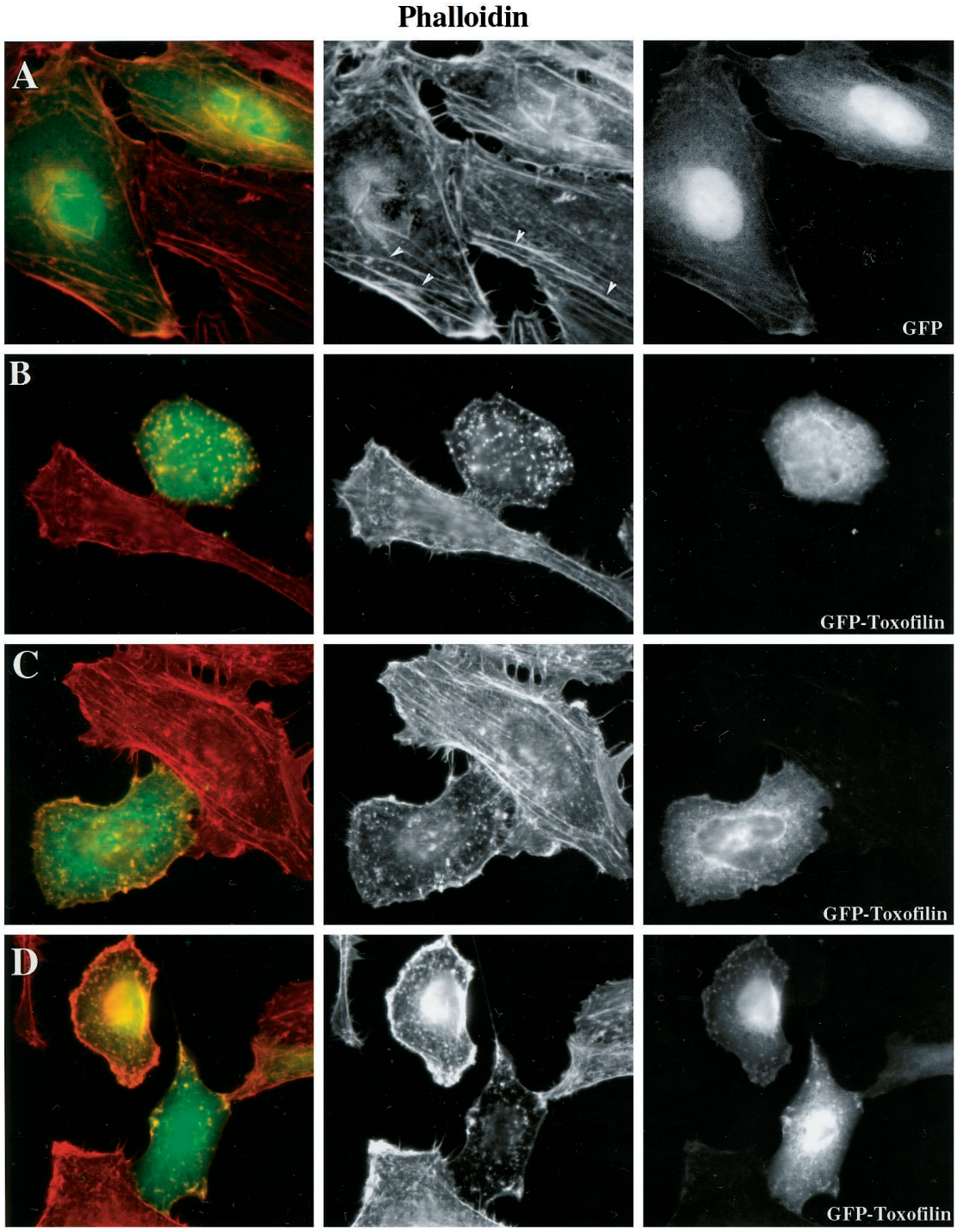

Figure 4.

Ectopically Expressed GFP–Toxofilin Disrupts F-actin Cytoskeleton in Mammalian Nonmuscle Cells

We have presented above biochemical evidence that r-Toxofilin can control muscle actin dynamics in vitro. To examine whether Toxofilin could affect actin dynamics in vivo, we overexpressed GFP-tagged Toxofilin in epithelial cells (HeLa). When GFP was expressed alone (Figure 4A), the F-actin stained with phalloidin was similar to that observed in nontransfected cells, with well-organized stress fibers (see

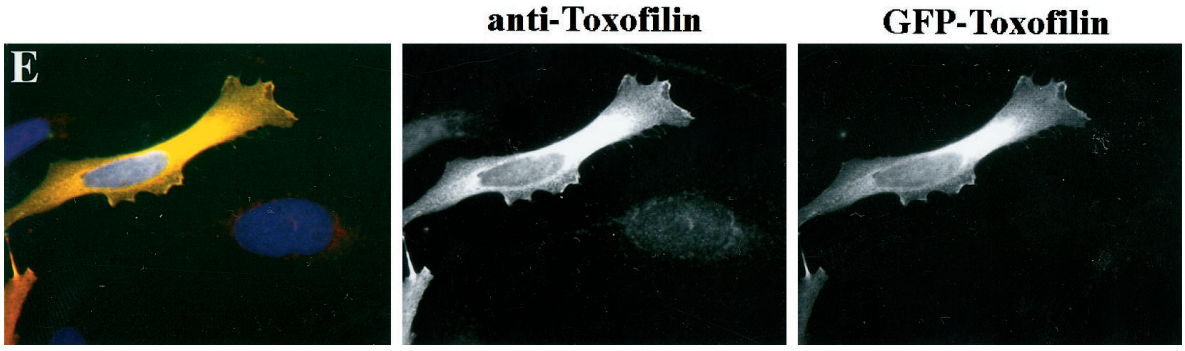

Figure 4 (facing page). Expression of GFP-Toxofilin fusion protein affects actin dynamics in mammalian nonmuscle cells. HeLa cells (50% confluent) were transfected by the $CaCl_2$ phosphate method with plamids encoding either GFP-Toxofilin or GFP (see MATERIALS AND METHODS) and were incubated overnight before replating on glass coverslips. The cells were incubated for 20 h (37°C, 5% CO_2) before processing for immunofluorescence (see MATERIALS AND METHODS). For revealing the F-actin or Toxofilin, the cells were permeabilized after fixation and stained with either rhodamine-phalloidin or with an anti-Toxofilin antibody, respectively. (A) Phalloidin staining of F-actin in HeLa cells expressing GFP. Well-organized actin stress fibers are visible. (B–D) Phalloidin staining of F-actin in cells expressing GFP-Toxofilin. Actin stress fibers are disorganized. (D) Different levels of expression of the GFP-Toxofilin. (E) Detection of Toxofilin with an anti-Toxofilin antibody in HeLa cells transfected with the GFP-Toxofilin-encoding plasmid.

arrows). In contrast, cells expressing GFP-tagged Toxofilin no longer displayed actin stress fibers (Figure 4, B–D). Instead, they had a punctate staining pattern resembling what is usually seen in cytochalasin D-treated cells (in particular, Figure 4B).

We quantified the amount of F-actin in a sample of HeLa cells expressing GFP-Toxofilin fusion protein by measuring the intensity of fluorescent phalloidin in each cell using the NIH Image analysis program. By comparing the fluorescence associated with F-actin in cells that express or do not express GFP-Toxofilin, we found a reduction of ~45% in the fluorescence of the cells expressing GFP-Toxofilin. The same comparison done between the cells expressing or not expressing GFP did not show any significant difference (Table 1).

In addition, it was clear that the extent of the effect of the GFP-tagged Toxofilin on the actin morphology depended on the levels of expression of the fusion protein (Figure 4D). Only ~43% of the transfected cells showed clear defects on the actin cytoskeleton, whereas the rest of the cells, usually expressing lower levels of GFP-Toxofilin, had smaller disappearance of the actin stress fibers. The expression of fluorescent Toxofilin in HeLa cells transfected with the plasmid encoding GFP-Toxofilin was confirmed by immunostaining with the anti-Toxofilin antibody (Figure 4E).

In parallel, we could recover the GFP-Toxofilin protein from transfected HeLa cells by immunoprecipitation of actin (our unpublished results), demonstrating that the phenotype observed in the transfected cells was at least partially due to direct binding of GFP-Toxofilin to G-actin.

Table 1. F-actin quantitation

No. of cells analyzed	Expression of GFP-toxofilin	Expression of GFP	Mean fluorescence intensity (pixels)	% Control fluorescence intensity
29	+	−	1180 ± 250	55.8
42	−	−	2115 ± 582	
33	−	+	1784 ± 311	107.0
39	−	−	1668 ± 416	

The fluorescence associated with HeLa cells from several fields was analyzed using the NIH Image program. The area around each cell was delineated, and the mean fluorescence intensity was measured in pixels. The results are presented as the mean of the fluorescence intensity for each group of cells ± SD. Control, cells not expressing GFP or GFP-toxofilin.

T. gondii Toxofilin Binds to Parasite G-actin and Is Also Associated with a Parasite Fraction Containing F-actin

To characterize Toxofilin in the parasite, we first examined whether the protein was associated with parasite actin that was isolated on the basis of its affinity to DNase 1. Using affinity chromatography, we observed that the recovered 43-kDa band copurified with other products of which the major band migrates as a 27-kDa polypeptide (Ponceau S-stained blot; Figure 5A, lane a, *). We identified by Western blot analysis that the 43-kDa band corresponds to actin (lane b), and the 27-kDa copurifying product corresponds to Toxofilin (lane c). An anti-*T. gondii* actin and the anti-Toxofilin andibodies were respectively used for this determination. In addition, direct immunoprecipitation of parasite actin coprecipitated Toxofilin (our unpublished results), whereas actin (marked with a sphere) coprecipitated with immunoprecipitated Toxofilin (Figure 5B, lanes b and d).

Based on the information obtained from the studies with mammalian actin, we subsequently investigated whether Toxofilin was also present in the detergent-insoluble fraction containing parasite F-actin. As assessed by Western blot (Figure 5C), Toxofilin indeed cofractionated with a parasite F-actin (lanes b and d). Interestingly, Toxofilin migrated as a doublet in the detergent-insoluble fraction (lane d), suggesting the presence of a modified form of Toxofilin.

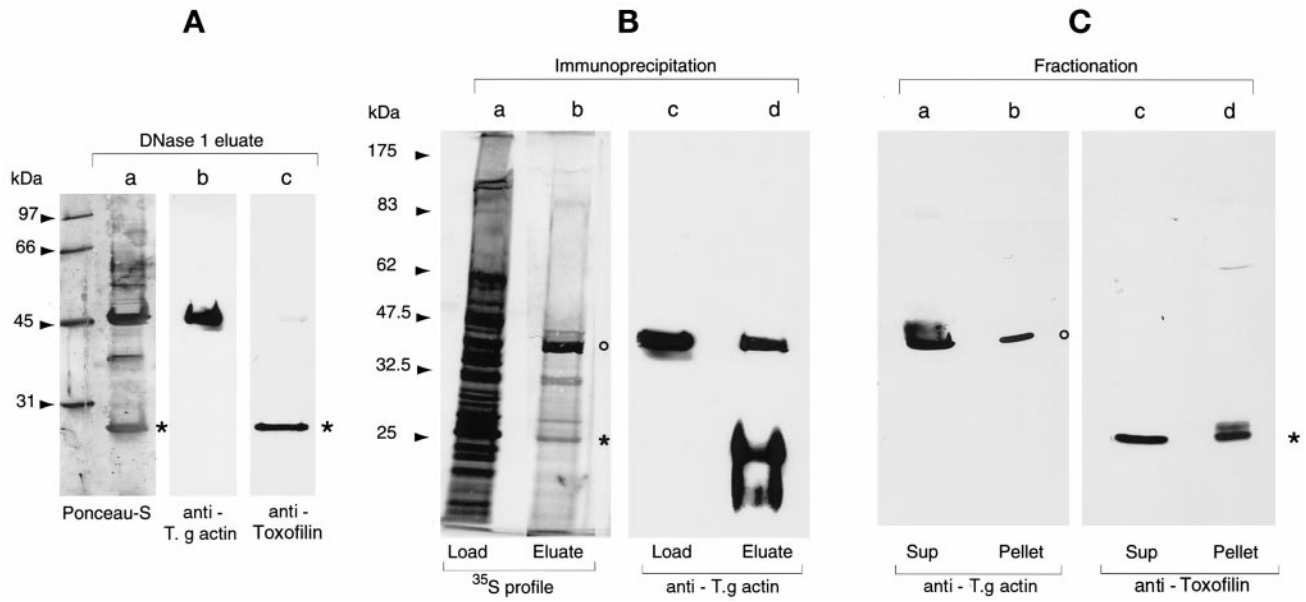

Figure 5. *T. gondii* Toxofilin binds to parasite G-actin and is associated with a parasite fraction containing F-actin. (A) DNase 1 affinity chromatography. DNase 1-bound proteins were eluted in SDS-PAGE sample buffer and separated on a 12% acrylamide gel. After transfer to a nitrocellulose membrane, eluted proteins were visualized with Ponceau S staining (lane a). Lane b, the eluate was probed for actin with an anti-actin antibody specific for *T. gondii* actin; lane c, the eluate was probed for Toxofilin with an anti-Toxofilin antibody. Toxofilin is marked with a star. (B) Toxofilin immunoprecipitation. A 100,000 × *g* parasite cytosolic extract was prepared from [^{35}S]methionine-cysteine-labeled tachyzoites (lane a, load) and immunoprecipitated with anti-Toxofilin antibody (lane b, eluate). Toxofilin is marked with a star. Both the cytosolic extract and the immunoprecipitate contained actin (○) as assessed by Western blot using an anti-*T. gondii* actin-specific antibody (lanes c and d, respectively). (C) Protein fractionation. Parasite proteins were extracted in F-actin-stabilizing conditions to recover fractions containing G-actin (lane a) or F-actin (lane b; ○. Both fractions contained Toxofilin (∗) as assessed by Western blot analysis using an anti-Toxofilin antibody (lanes c and d, respectively).

Redistribution of Toxofilin in Tachyzoites during Gliding and Host Cell Entry

Using immunofluorescence microscopy, we observed that Toxofilin was mostly found apically in the cytoplasm of tachyzoites and excluded from the nucleus (Figure 6). However, in extracellular gliding parasites the staining varied strikingly among individuals. It was observed either as densely organized in the apical cytoplasm (Figure 6A, 1, large arrowhead) or distributed as patches in the apical part of the cytoplasm (Figure 6A, 1 and 3, small arrows). However, it was often found in an apical, arrowlike distibution (Figure 6A, 2, small arrowhead) with occasional additional staining behind the nucleus at the distal part of the parasite (Figure 6A, 2, large arrow). Interestingly,images catching active host cell entry always showed a positive staining for Toxofilin at the distal end of the parasite membrane (Figure 6A, 4 and 5, small arrows). Entering parasites were detected on the basis of the incomplete staining of the parasite (Figure 6A, 4 and 5, small arrowheads). The partial staining of parasite surface was achieved because of the restricted accessibility of an antibody recognizing a major *Toxoplasma* surface molecule (P30), to only the part of the parasite that still remains extracellular, i.e., its posterior end. To further characterize the localization of Toxofilin, we costained with antibodies recognizing secretory dense granules (Figure 6B, 1) or micronemes (Figure 6B, 2). Toxofilin did not colocalize with either of these organelles.

In contrast to motile parasites, Toxofilin presented a strong and uniform staining in all the parasites once they have entered the host cell (our unpublished results). At the end of the parasite intracellular life before exit from the host cell, the newly formed tachyzoites still display a strong uniform pattern for Toxofilin at their apical side (Figure 7 with inset 2×).

DISCUSSION

T. gondii tachyzoites, as other Apicomplexan parasites, rely on the assembly and disassembly of their actin cytoskeleton to invade their host cells. Actin assembly and disassembly (Dobrowolski and Sibley, 1996) and actin–myosin coupling activity (Dobrowolski *et al*., 1997a) underlie the process by which surface molecules are capped during parasite motility and invasion. Both of these mechanisms provide the energy necessary for the capping process and therefore generate the force driving parasite movement on a substratum or into its host cell. The molecules controlling these mechanisms remain to be identified. However, it is clear that the changes in the abundance, location, and organization of actin filaments are expected to be under the control of actin monomer sequestration and desequestration and of filament turnover.

Because tachyzoites display an unusually low amount of F-actin, we hypothesized that sequestration of monomeric actin through the activity of actin-associated proteins may

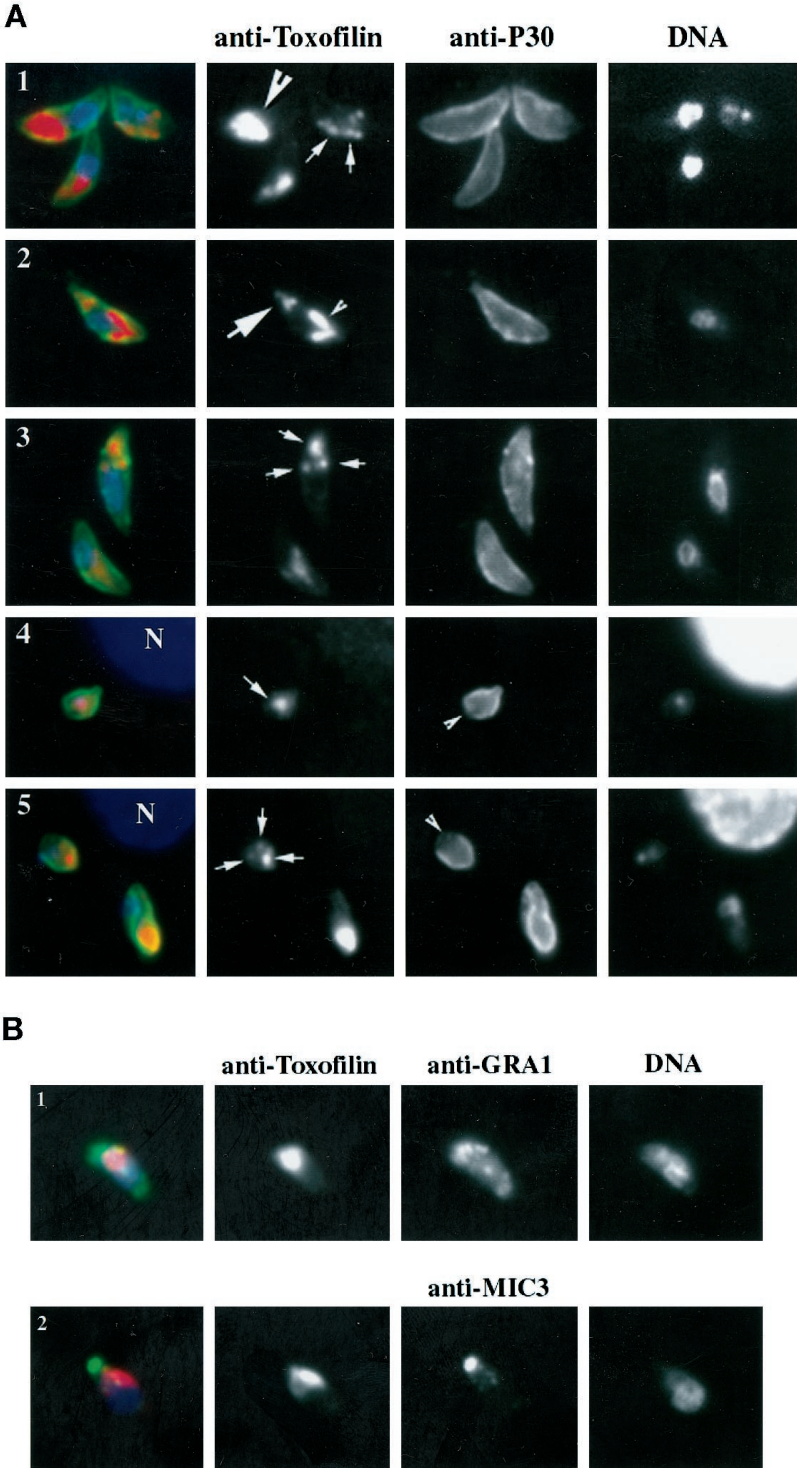

Figure 6. Toxofilin localization during tachyzoite motility and host cell entry is dynamic. (A, 1–3) Tachyzoites were allowed to glide on glass slides before being fixed and stained for the major surface protein P30 with mouse monoclonal anti-P30 followed by a secondary anti-mouse antibody conjugated to Alexa-488. Cells were then permeabilized and stained for Toxofilin using the anti-Toxofilin antibody followed by an anti-rabbit antibody conjugated to Alexa-568. Nuclei were stained by DAPI (see MATERIALS AND METHODS). In green, the surface of the parasite; in red, Toxofilin distribution; in blue, nuclei. (A, 4 and 5) Tachyzoites were incubated for 10 min with HFF cells plated on glass coverslips. Coverslips were washed in PBS and processed for immunofluorescence (see MATERIALS AND METHODS) either immediately or after further incubation for 10 or 20 h (37°C, 5% CO_2). Extracellular tachyzoites were stained with the anti-P30 followed by the anti-mouse antibody conjugated to Alexa-488. The cells were subsequenty permeabilized and stained for Toxofilin followed by an anti-rabbit antibody conjugated to Alexa 568. Nuclei were stained by DAPI. Extracellular parasites were detected by the green fluorescent staining on their surface, whereas tachyzoites in the process of entering the host cell present an incomplete staining of their surface (arrows). (B) Gliding tachyzoites were processed as for A. After fixation they were permeabilized and stained for either their micronemes, using the mouse monoclonal antibody anti-MIC1, or for their dense granules, using the mouse monoclonal antibody anti-GRA3. In both cases, the secondary anti-mouse antibody was conjugated to Alexa-488, and the staining is shown in green. After removal of the unbound conjugated antibody, the cells were stained for Toxofilin and for DNA as described above.

play a key role in parasite actin dynamics. Such actin-sequestering proteins have been well described in systems ranging from yeast to mammals, and they are mostly members of three large families, the profilins, the thymosins, and the actin-depolymerizing factor/cofilins (Maciver, 1998). In *T. gondii,* a 13.5-kDa depolymerizing factor that shares a high degree of similarity with the ubiquitous actin-depolymerizing factor (ADF)/cofilin family has been described (Allen *et al.*, 1997). Proteins of this family are able to increase the rate of depolymerization of ADP-bound F-actin from the

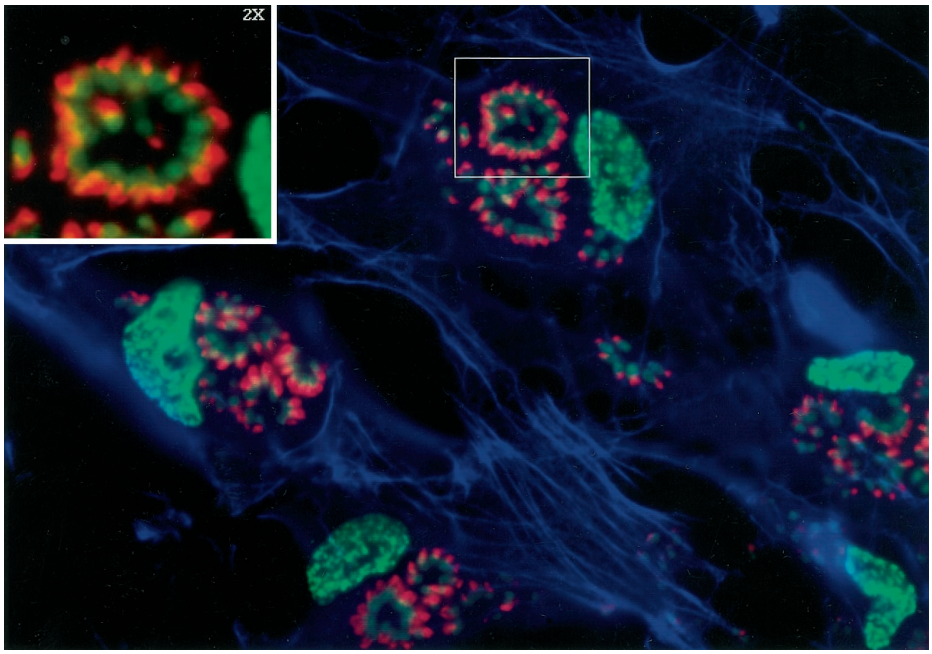

Figure 7. In intracellular tachyzoites, Toxofilin distributes uniformly in the apical cytoplasm. Tachyzoites were incubated for 10 min (37°C, 5% CO2) with HFF cells plated on glass coverslips. The nonbound parasites were removed by changing the medium, and the infected cells were further incubated for 20 h as described in MATERIALS AND METHODS. At the end of the incubation period the cells were fixed, and extracellular tachyzoites were stained with the anti-P30 antibody followed by the anti-mouse antibody conjugated to Alexa-488, seen in red. The cells were subsequently permeabilized and stained for Toxofilin followed by an anti-rabbit conjugated to Alexa-568. Nuclei were stained by DAPI, shown here in green (artificial color). The actin cytoskeleton of the host cell was stained with rhodamine-phalloidin, shown in blue (artificial color). The indicated area was enlarged 2× and is presented as an inset.

pointed filament ends as well as to induce the spontaneous breaking of actin–actin contacts and thus play a major role in controlling dynamics (Carlier, 1998).

On that basis, we were interested in identifying parasite proteins with a sequestering function. Using complementary biochemical approaches, we initially isolated a parasite 27-kDa protein on the basis of its ability to bind rabbit muscle G-actin and demonstrated that this protein formed a complex with parasite G-actin. It was subsequently identified as a novel actin-binding protein. This protein does not display any significant similarity to any other known actin-sequestering proteins such as profilins or thymosins or with any other known actin-binding protein. Additionally, it does not contain any known consensus sequence for actin binding site. We named this protein Toxofilin.

Actin-binding proteins often act in concert with each other or with other proteins, some of which are regulatory molecules (Pantaloni and Carlier, 1993; Mccollum *et al*., 1996; Narumiya *et al*., 1997). Our data, in particular those obtained after native electrophoresis, suggested that Toxofilin participated in a protein complex containing G-actin. By analyzing how r-Toxofilin interacted with actin, both with a native gel assay and an actin polymerization assay, we demonstrated that r-Toxofilin was capable by itself of sequestering G-actin in vitro. The inhibition of actin assembly in presence of r-Toxofilin was also observed using the cosedimentation assay. In addition, r-Toxofilin is likely to cap actin filaments, an activity that was further supported by immunoelectron microscopy. r-Toxofilin was clearly localized at one end of most of the actin filaments analyzed. Nevertheless, it does not act as a heterodimer like the classical actin-capping proteins described in every other eukaryotic cell studied thus far (Schafer and Cooper, 1995), nor does it seem to increase the initial rate of actin polymerization as do other known capping proteins that promote actin nucleation (Cooper and Pollard, 1985).

Actin dynamics was also clearly affected when Toxofilin was expressed in mammalian nonmuscle cells. GFP-Toxofilin ectopic expression in epithelial cells (HeLa) resulted in loss of actin stress fibers, a result that was probably due to inhibition of the renewal of actin stress fibers. The extent of the effect on actin cytoskeleton depended on the level of GFP-Toxofilin expression. We claim that this is a direct effect on the actin cytoskeleton, because immunoprecipitation of actin from GFP-Toxofilin-expressing cells coprecipitated Toxofilin as well. This piece of data further supports the monomer-sequestering activity of Toxofilin suggested by the in vitro kinetics of actin polymerization. Despite its dramatic effect on the actin cytoskeleton, ectopic expression of GFP-Toxofilin did not affect the microtubule network (our unpublished results).

To localize endogeneous Toxofilin in tachyzoites we used the anti-Toxofilin antibodies. As expected from previous reports on actin localization (Dobrowolski *et al*., 1997b) and from our biochemical data, Toxofilin primarily stained the cytoplasm in the anterior part of the *T. gondii* tachyzoite. However, it is noteworthy that a different distribution of Toxofilin was observed in gliding tachyzoites with respect to the intracellular and immobile parasites. Indeed, when tachyzoites were freshly isolated from mice and allowed to glide, Toxofilin staining was heterogeneous from one parasite to the other, sometimes clearly in patches, whereas in other cases it was restricted under the apical membranes with occasionally a patch at the rear side. We examined whether the patchy distribution could be due to an association with organelles, especially with the numerous dense granules, but we did not observe any colocalization.

Actin dynamics in *T. gondii* tachyzoite is not only required during gliding but also during host cell entry. *T. gondii* tachyzoites invade their host cells within few seconds once they have reoriented their apical side to establish contact

with the host cell membrane (Silverstein and Joiner, 1997). Concomitantly, they secrete part of the content of their apical organelles, mainly the rhoptries (Carruthers and Sibley, 1997). This is a regulated secretion event that participates in the formation of the parasitophorous vacuole membrane together with the invagination of the host cell plasma membrane (Suss-Toby *et al.*, 1996; Lingelbach and Joiner, 1998). We detected tachyzoites when they were still "half in–half out" of their host cell. In these parasites, we always observed a redistribution of Toxofilin, including a strong patch at the distal part of the parasite, which had not yet been internalized. It is known that during gliding Apicomplexan parasites cap some of their surface molecules, which move away from the apical side (the advancing part of the cell) toward the distal end. It is accepted that parasite actin filaments associated with cross-linked surface molecules move rearward during the capping process. These filaments are formed by assembly in the front of the cell. This implies that actin monomers have to be locally desequestered under the membranes to be competent for association through the nucleation of new actin filaments or through elongation of actively growing preexisting filaments. In the latter case, preexisting filaments should uncap to lower the critical concentration and generate free ends for monomer assembly. Toxofilin release may be involved during this uncapping step as well, because it could regulate the pool of available monomers for actin assembly. The newly elongated actin filaments formed under the membrane would then rapidly dissociate at the rear side, leaving monomers to be sequestered by Toxofilin.

Once into their host cell and during their entire intracellular growth phase, tachyzoites are more or less immobile. In these parasites, the staining of Toxofilin was significantly stronger and homogeneously localized throughout the apical cytoplasmic part for every parasite. In contrast to the uniform pattern observed in immobile parasites, the broad pattern of Toxofilin distribution observed in gliding tachyzoites may reflect a highly dynamic behavior of Toxofilin, probably coordinated with the dynamic state of actin.

It remains to be clarified how Toxofilin could be involved in the actin polymerization process controlling tachyzoite gliding, invasion into host cells, and further intracellular development. Genetic manipulation of the level of protein expression in live *T. gondii* tachyzoites will help answer these questions (Boothroyd *et al.*, 1995; Donald and Roos, 1995). Finally, taking into account that the gliding motility of Apicomplexan parasites displays some unique features, it is expected that understanding how Toxofilin acts in the parasite in vivo should permit the identification of novel molecules critically involved in long-term survival of Apicomplexan parasites. This could create new possibilities for targeted therapeutic approaches against Apicomplexan-caused diseases.

In conclusion, Toxofilin, besides its great importance for understanding Toxoplasma biology, will become an interesting tool for the dissection of actin dynamics in other systems, as is already the case for proteins from other microorganisms, in particular *Listeria monocytogenes* (Lasa and Cossart, 1996; Carlier and Pantaloni, 1997, Moreau and Way, 1999).

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