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**THE TOXOFILIN-ACTIN-PP2C COMPLEX OF TOXOPLASMA:  
IDENTIFICATION OF INTERACTING DOMAINS.**

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**Abbreviations** : PP2C, type 2C serine threonine phosphatase; GST, Glutathione-S-Transferase; PCR, polymerase chain reaction; IPTG, Isopropylthio- $\beta$ -D-galactoside; PBS, phosphate buffered saline; HPTR, Histidine Patch Thioredoxine; IAEDANS, N-(iodoacetyl)-N'-1-sulfo-5-naphthylethylenamine; TBS, Tris buffered saline; BSA, bovine serum albumin; HRP, horseradish peroxidase; Tf, toxofilin.

## SYNOPSIS

Toxofilin is a 27 kDa protein isolated from the human protozoan parasite *Toxoplasma gondii*, which causes toxoplasmosis. Toxofilin binds to G-actin, and *in vitro* studies have shown it controls elongation of actin filaments by sequestering actin monomers. Toxofilin affinity for G-actin is controlled by the phosphorylation status of its serine 53, which depends on the activities of a casein kinase II and a type 2C phosphatase. To get insights into the functional properties of toxofilin, we undertook a structure-function analysis of the protein using a combination of biochemical techniques. We identified a domain that was sufficient to sequester G-actin and that contains three peptide sequences selectively binding to G-actin. Two of these sequences are similar to sequences present in several G- and F-actin binding proteins, while the third appears to be specific of toxofilin. Additionally, we identified two toxofilin domains that interact with PP2C<sup>†</sup>, one of which contains the serine 53 substrate. In addition to characterizing the interacting domains of toxofilin with its partners, this analysis also provides information for an *in vivo* based approach to selectively and competitively disrupt the protein-protein interactions that are important to parasite motility.

Keywords: actin; toxofilin, serine-threonine phosphatase, protein-protein interaction, peptide spot mapping assay, pyrene actin assay

## INTRODUCTION

The human protozoan parasite *Toxoplasma gondii*, together with other members of the Apicomplexa phylum, invade their host cell by an active process that markedly differs from the passive uptake induced by invasive bacteria [1]. Following host cell entry, the parasite resides within a sub-cellular compartment called the parasitophorous vacuole. The parasite-driven remodelling of this compartment allows molecular exchanges to take place between the host cell and the parasite, thereby promoting subsequent parasite development [2, 3]. The massive multiplication of *T. gondii* tachyzoites within this vacuole ends by host cell lysis and active parasite egress, which lead to parasite dissemination [4, 5]. These events are associated with potentially severe or lethal pathologies, especially when they occur in the brain of immunocompromised humans or during fetal development (for a review see [6, 7]).

The tachyzoite actively enters its host cell by developing a force that relies on actin polymerization [8] and myosin activation [9, 10]. This force is generated upon contact with the host cell, but the signalling underlying parasite actin polymerization remains unclear. A key role has been ascribed to a micronemal protein, MIC2, which is secreted on the parasite surface upon parasite-host cell contact, links the parasite motor to the extracellular cues and is translocated rearwards by the motor as the parasite moves forward [11, 12]. The glycolytic enzyme aldolase has been found to selectively associate with the MIC2 tail and to actin, thereby forming a direct bridge between MIC2 and actin [13].

We previously identified a 27 kDa *T. gondii* protein called toxofilin, which can bind to both mammalian and parasite actin. *In vitro* assays performed with mammalian actin demonstrated that toxofilin sequesters actin monomers and thus controls filament elongation [14]. We also characterized a type 2C serine-threonine phosphatase that copurifies with the actin-toxofilin complex and that selectively dephosphorylates toxofilin on serine 53, thereby increasing toxofilin affinity to G-actin [15].

A central question remains as to whether toxofilin acts on mammalian actin, *Toxoplasma* actin or both. Several results argue in favor of toxofilin targeting host cell actin. We have described a major apical localization of toxofilin in tachyzoites, in particular in intracellular stages of the parasite, an observation consistent with the recent identification of toxofilin as a rhoptry protein following a proteomic analysis of rhoptry contents [16]. Indeed, toxofilin, which carries a N-terminal peptide signal sequence, is likely to be secreted during cell invasion and to act locally on the host cell cortical actin during parasite penetration of the

cell. On the other hand, we cannot exclude also a role of toxofilin on parasite actin dynamics. Our immunolocalization studies showed that toxofilin is also located underneath the plasma membrane and at the posterior end of invading tachyzoites. Recent data obtained with tagged versions of toxofilin expressed via its own regulatory sequences confirmed these observations (data not shown,[16]).

In this study, using a combination of biochemical techniques, we identified the interacting domains of toxofilin with both partners, i.e. PP2C and mammalian actin. Our findings show that besides controlling actin polymerization by sequestering actin monomers *via* its first coil-coiled region, toxofilin also bound to G-actin through its N terminal domain.

## EXPERIMENTAL

**Preparation of recombinant proteins** - We expressed recombinant full and truncated toxofilin in the BL21 strain of *Escherichia coli* using the GST Gene Fusion System (GE Healthcare UK Ltd, Buckinghamshire, England). The full length toxofilin was cloned in pGex6-p3 as described in [14] while CC1, CC1A, CC1Aa, CC1Ab, CC1B, CC1BCC2, NoCC and CC2 domains of toxofilin were prepared as follows (Fig. 1A). CC1 nucleotidic sequence was amplified using the forward primer 5'-GGCCGGATCCCAACAGGAACTAGGGCTGCTC-3' containing a BamHI site (underlined) and the reverse primer 5'-GGCCGTCGACCCTCTGCTCGTTGAGGATTTG-3' containing a Sall site (underlined). For CC1A, the forward primer was the same as for CC1 and the reverse primer was 5'- GGCCGTCGACCTCAGTTGCGAAAGATCCCTC-3'. For CC1B, the forward primer was 5'- GGCCGGATCCCGGAAACGAAGGCTTTGC-3' and the reverse primer was the same as for CC1. For CC1Aa, the forward primer was the same as for CC1 and the reverse primer was 5'-GGCCGTCGACCGTGGCTCTGAGAATTTGCTC-3'. For CC1Ab, the forward primer was 5'-GGCCGGATCCCAAATTTGGACCTCAGGAAGTAC-3' and the reverse primer was the same as for CC1A. For CC1BCC2, the forward primer was the same as for CC1B and the reverse primer was the same as for toxofilin. For NoCC, the forward primer was the same as for toxofilin and the reverse primer was 5'- GGCCGTCGACGCGTGCTGCGACGGAGGG-3'. Finally, CC2 was amplified using the forward primer 5'-GGCCGGATCCGATGCGAGTGGAGCATTAC-3' and the same reverse primer as for toxofilin. The PCR products were then ligated into the BamHI and Sall sites of pGex6-p3 polylinker. Protein expression was induced with either 0.2 mM (CC1, CC1A, CC1Aa, CC1Ab, CC1B and CC1BCC2) or 0.1 mM IPTG (NoCC, CC2 and toxofilin). Recombinant proteins were purified from bacteria lysed in phosphate buffered saline (PBS<sup>-</sup>) supplemented with 0.5% (v/v) Triton X-100 (TX-100) with or without 0.5% (v/v) SB3-14 (myristyl sulfobetaine). After centrifugation at 20,000g for 20 min at 4°C, supernatants were incubated with Glutathione-Sepharose (GE Healthcare) for 3 h at 4°C. The recombinant tagged-polypeptides were eluted in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 10 mM reduced Glutathione while in some cases, tags were cleaved off using Prescission protease (GE Healthcare) for 4 h at 4°C. The HPTR-PP2C (rPP2C) was prepared as described in [15] using Ni-NTA Column Chromatography (QIAGEN S.A, Courtaboeuf, France). Following

purification, polypeptides were dialysed against the relevant buffer for subsequent binding, titrated, supplemented with protease inhibitors and kept on ice until use.

**Native gel assays** - Native gel electrophoresis was carried out as described in [14] with a 7.5% acrylamide gel using G-actin prepared from F-actin labelled with N-(iodoacetyl)-N'-1-sulfo-5-naphthylethylenamine (1-5 IAEDANS, 500  $\mu$ M, Molecular Probes, Eugene, OR). 50  $\mu$ g of IAEDANS labelled G-actin was incubated without or with equimolarity of recombinant GST-toxofilin, GST-CC1, GST-NoCC or GST-CC2 for 1 h at room temperature prior to electrophoresis. F-actin was used as a control.

**G-actin binding assays** - Rabbit muscle actin was purified from acetone powder (Sigma, St Louis, MO) and prepared as described in [17]. It was diluted to 55  $\mu$ M in A buffer (2 mM Tris-HCl pH 8.0, 0.2 mM Na<sub>2</sub>ATP, 0.1 mM CaCl<sub>2</sub>, 0.5 mM  $\beta$ -mercaptoethanol, 0.005% NaN<sub>3</sub>). 4.2  $\mu$ g (2  $\mu$ M) of purified rabbit muscle G-actin was incubated overnight at 4°C with 4  $\mu$ M of GST-fusion proteins prepared in 50  $\mu$ l of B buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 mM CaCl<sub>2</sub>). 30  $\mu$ l of a 50% (v/v) slurry of Glutathione-Sepharose in B buffer were added to the mix which was supplemented with 1 mM DTT, 0.05% (v/v) Tween 20 final concentrations and incubated for 3 h at 4°C. The unbound fraction was collected by centrifugation and the beads were washed with B buffer supplemented with 0.05% (v/v) Tween 20. SDS eluate and fraction samples were analysed by SDS-PAGE [18] and Coomassie staining. Western blotting using the monoclonal anti-C4 actin antibodies (Chemicon International Inc, Temecula, CA) was performed as described in [14] except that the C4 antibodies were used at a 1:1000 dilution.

**Competition assays for binding to G-actin** - 4.2  $\mu\text{g}$  (2  $\mu\text{M}$ ) of purified rabbit muscle actin was incubated or not with 14.5  $\mu\text{g}$  (20  $\mu\text{M}$ ) of clarified CC1, 9  $\mu\text{g}$  (20  $\mu\text{M}$ ) of CC1A, 5.6  $\mu\text{g}$  (20  $\mu\text{M}$ ) of CC1Aa, 3.3  $\mu\text{g}$  (20  $\mu\text{M}$ ) of CC1Ab or a mix of CC1Aa and CC1Ab in 50  $\mu\text{l}$  of B buffer for 1 h at 4°C. CC1-actin and CC1A-actin mixes were added to 10.8  $\mu\text{g}$  (4  $\mu\text{M}$ ) GST-toxofilin while CC1A-actin, CC1Aa-actin and CC1Ab-actin mixes were also added to 8.2  $\mu\text{g}$  (4  $\mu\text{M}$ ) GST-CC1. All reactions were incubated overnight at 4°C. Samples were then treated as described for the G-actin binding assays. Eluate and unbound fractions were analysed by SDS-PAGE and Coomassie staining. Controls were performed with CC1 and CC1A incubated with Glutathione-Sepharose under the experimental conditions.

**Actin pyrene assays and polymerization assays** – Three separate pyrene assays were carried out as in [14] using 2  $\mu\text{M}$  of 20% labelled G-actin. For additional polymerization assays, 12.6  $\mu\text{g}$  (10  $\mu\text{M}$ ) of purified rabbit muscle G-actin was incubated with 12.3  $\mu\text{g}$  (10  $\mu\text{M}$ ) of clarified GST-CC1, 10.3  $\mu\text{g}$  (10  $\mu\text{M}$ ) GST-NoCC, 10.8  $\mu\text{g}$  (10  $\mu\text{M}$ ) GST-CC1A, 9.8  $\mu\text{g}$  (10  $\mu\text{M}$ ) GST-CC1Aa or 9.1  $\mu\text{g}$  (10  $\mu\text{M}$ ) GST-CC1Ab or the toxofilin domains without their GST tags in 30  $\mu\text{l}$  of A buffer. Then, 100 mM KCl, 2 mM  $\text{MgCl}_2$  and 1 mM  $\text{Na}_2\text{ATP}$  were added to induce polymerization of G-actin overnight at 4°C. Unpolymerized and polymerized actins were separated following ultracentrifugation at 200,000g for 30 min at 4°C and both fractions were analysed by SDS-PAGE and Coomassie staining. In some experiments, 12.6  $\mu\text{g}$  (10  $\mu\text{M}$ ) of G-actin was incubated with a mix of 4.4  $\mu\text{g}$  (10  $\mu\text{M}$ ) of CC1 and 14.7  $\mu\text{g}$  (10  $\mu\text{M}$ ) of rPP2C prior to inducing actin polymerization. To examine the potential effect of NoCC on actin in G- conditions, similar assays were conducted without adding the polymerization conditions.

**PP2C binding assays** - 3.9  $\mu\text{g}$  (2  $\mu\text{M}$ ) of HPTR tagged PP2C was immobilized on 10  $\mu\text{l}$  of a 50% (v/v) slurry of Ni-NTA-agarose (QIAGEN) washed in C buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.01% (v/v) TX-100) for 2 h at 4°C. Bound rPP2C was then incubated with the different domains of toxofilin for 1 h at 4°C and the putative complexes were isolated by centrifugation. The unbound fractions were recovered and the beads were washed with 10 times volume of D buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM  $\text{MgCl}_2$ , 0.01% (v/v) TX-100). SDS-SB eluates and fractions were then analysed by SDS-PAGE and Coomassie staining.



**PP2C-CC1-actin complex binding assays** - 3.9  $\mu\text{g}$  (2  $\mu\text{M}$ ) of rPP2C bound to nickel-agarose was incubated either first with the preformed CC1-actin complex in A buffer (1 h at 4°C) or sequentially with 2.3  $\mu\text{g}$  (4  $\mu\text{M}$ ) of CC1 in B buffer (1 h at 4°C) then with 3.4  $\mu\text{g}$  (2  $\mu\text{M}$ ) of clarified G-actin in B buffer (1 h at 4°C). Following incubation, the resin was washed in D buffer and treated as described for the PP2C-binding assay.

**Peptide-spot mapping** - Series of 12 amino acid-long peptides representing toxofilin or CC1A polypeptide which were successively shifted by 2 amino acids were spotted onto a cellulose membrane (Intavis AG, Cologne, Germany). Before use, the membranes were wet in  $\text{dH}_2\text{O}$  then in Tris buffered saline (TBS) supplemented with 0.1% (v/v) Tween 20 (TBST) and saturated in TBST 0.1% containing 3% (w/v) skim milk and 3% (w/v) bovine serum albumin (BSA) for 2 h at room temperature (rt). To detect toxofilin/CC1A-G-actin interacting sequences, membranes were then incubated with 1  $\mu\text{g}/\text{ml}$  of biotinylated G-actin in A buffer supplemented with 1% (w/v) skim milk overnight, at 4°C. After several washes in TBST, the membranes were incubated with streptavidin-HRP at a 1:2000 dilution (Upstate, Charlottesville, VA) for 2 h (rt). To detect toxofilin-PP2C interacting sequences, the toxofilin membranes were incubated overnight at 4°C with 4  $\mu\text{g}/\text{ml}$  of rPP2C in TBST supplemented with 1% (w/v) skim milk. Membranes were washed in TBST, incubated first with affinity purified polyclonal anti-*T. gondii* PP2C antibodies at a 1:4000 dilution for 2 h (rt) and secondly with anti-rabbit-HRP (Jackson ImmunoResearch, West Grove, PA) at a 1:10000 dilution for 2 h (rt). Following extensive washes in TBST and TBST supplemented to 0.2% Tween 20, positive spots were detected using the SuperSignal West Dura Extended Duration Substrate (Pierce biotechnologies, Rockford, IL).

## RESULTS

### **Toxofilin binds to G-actin *via* the CC1A domain and sequesters G-actin.**

To study the structure-function relationships of toxofilin, we performed a series of assays with purified mammalian actin. Sequence analysis of toxofilin predicts that the protein is globular with two coil-coiled regions (contained in the CC1 and CC2 domains respectively, see Figure 1A) and that it carries a signal peptide sequence at the N-terminal end. To map the toxofilin domains involved in actin binding, we first produced several polypeptides encompassing or not the coil-coiled regions (Figure 1A) and tested their binding property to G-actin using pull-down assays and native gel electrophoresis. Since toxofilin was originally isolated by native gel assay with IAEDANS labelled actin [14], we used the same assay conditions to define the toxofilin regions that bind to actin. When fluorescently labelled G-actin (Figure 1B, *lane a*) was incubated with either full length recombinant toxofilin or toxofilin truncated polypeptides CC1 (14.6 kDa), CC2 (5.5 kDa) and NoCC (7.5 kDa) prior to native gel electrophoresis, only toxofilin and CC1 induced a clear shift in actin migration (Figure 1B, *lanes b and c*). In contrast, NoCC and CC2 did not similarly alter actin migration (Figure 1B, *lanes d and e*). Interestingly, NoCC addition to G-actin prevented part of the G-actin pool to enter the 7.5% acrylamide gel as to when F-actin was loaded onto the gel (Figure 1B, *lane f*). This suggested that NoCC could also bind to G-actin and promote actin oligomerization or nucleation. Together these results indicated that toxofilin binding site(s) for G-actin is located in the CC1 polypeptide and is sufficient to capture actin.

To narrow down the putative minimal G-actin binding domain, the 14.5 kDa CC1 domain was cut into a 8.9 kDa CC1A and a 5.5 kDa CC1B polypeptides and we performed chromatography on Glutathione-Sepharose of GST-fusion polypeptides pre-incubated with G-actin. As illustrated in Figure 1C, only GST-toxofilin (*lanes a-c*), GST-CC1 (*lanes d-f*) and GST-CC1A (*lanes g-i*) but not GST-CC1B not GST-CC2 not GST (*lanes j-r*) was able to capture G-actin.

To confirm that the 8.9 kDa CC1A polypeptide could bind G-actin, we tested whether it could compete with either full length toxofilin or CC1 for binding to G-actin. Figure 1D shows a complete inhibition of actin binding to GST-toxofilin in the presence of 5 fold molar excess of CC1 or CC1A (*lanes b-d*). Additionally, actin binding to GST-CC1 was fully prevented by a 5 fold molar excess of CC1A (*lanes f,g*). As controls, actin, CC1 and CC1A did not bind to the glutathione matrix under our conditions (data not shown). Together, these

data demonstrate that CC1A behaved as a competitor of toxofilin for the binding to G-actin suggesting that the toxofilin domain that binds G-actin is located in the CC1A domain.

We then tested whether the G-actin binding property of CC1A was associated to a sequestering activity already described for the full toxofilin. To this end, we performed pyrene actin assays which demonstrated that both CC1 and CC1A polypeptides were sufficient to elicit a sequestering effect on G-actin (Figure 1E). The lower sequestering activity exhibited by CC1A in comparison to CC1 and assessed by the significant differences observed in steady state values using the Student test (22.7 a.u. for CC1 and 62.8 for CC1A, representative of triplicates for three different experiments) is likely to result from a difference in CC1A and CC1 affinity to G-actin. In control, as expected from the pull-down assays, GST-CC2 did not modify the kinetics of actin polymerization (figure1F).

### **Three linear peptidic sequences within CC1A bind to G-actin.**

To identify the important amino acid residues of the CC1A polypeptide that could account for G-actin binding, we performed a peptide spot assay using synthetic overlapping dodecapeptides spotted onto a cellulose membrane. The entire sequence of CC1A was represented, and each peptide was differing from the following by a shift of 2 amino-acids. When probed with biotinylated G-actin as described in the experimental procedures, three series of spots were reproducibly detected (repeated 3 times) (Figure 2A). As seen in Figure 2B, the spots numbered from 6 to 8 sharing the 'AGQAKAAA' sequence bound to G-actin under our conditions. The spots 21-23 sharing the 'DEILRATQ' sequence and the spots 25-27 having in common the 'NLDLRKYE' sequence reacted the most strongly with G-actin. Screening Swissprot Uniprot protein data bases (216,380 sequences May 2006) using the programs "pattern" [19] and "pattinprot" [20] identified a number of putative or well-characterized actin-interacting proteins that displayed high similarity (>70%) with the degenerated 'AGQA[K/R]AAA' (Table 1A) and 'DEIL[R/K]A[T/S]Q' motifs (Table 1B). For example, angiominin, several mammalian myosins and talins were detected by the 'AGQAKAAA' motif. Several mammalian Scinderins (also called Adseverin) and mammalian Actin-Depolymerizing Factors, which are all known to bind to G-actin, were identified by the 'DEILRATQ' motif. For this latter motif, Myosin type II heavy chain, Myosin Vc, skeletal muscle Troponin C, neural Spinophilin (Neurabin-II) and the nuclear anchorage protein Nesprin were also identified (Table 1B). In contrast the third CC1A G-actin binding motif identified only one known actin-binding protein in the data base suggesting that 'NLDL[R/K][R/K]YE' sequence is a more toxofilin specific G-actin binding

motif. It is the microtubule-actin crosslinker MACF1 belonging to the spectraplakins family of cytoskeletal crosslinking proteins [21].

Based on these results, we performed competitive assays for binding to G-actin using two polypeptides from CC1A, named CC1Aa (<sub>69</sub>Q-T<sub>119</sub>) and CC1Ab (<sub>120</sub>Q-S<sub>149</sub>) with GST-CC1A. As observed Figure 2C, addition of CC1A efficiently competed with GST-CC1 for binding to G-actin (*lanes b and c*) but not addition of CC1Aa or CC1Ab (Figure 2C, *lanes d-f*). Interestingly, a mixture of CC1Aa and CC1Ab could not restore the competitive property of CC1A suggesting that the affinities of the two independent peptides for G-actin were lower than the affinity of the CC1A fragment and that the integrity of the sequence was required for binding to G-actin. Using actin polymerization pull down assay, we also tested whether CC1Aa and CC1Ab could prevent actin to polymerize. As seen Figure 2D, the integrity of CC1A was required to fulfil the toxofilin sequestering property.

#### **NoCC binds to G-actin but does not sequester or nucleate G-actin**

As presented above (Figure 1B), the 7.5% polyacrylamide native gel assay suggested that incubation of NoCC with G-actin partially transformed actin monomers into oligomers too large to enter the gel. Additionally, NoCC was found to bind to G-actin in a peptide spot assay (data not shown). Therefore we investigated whether NoCC could capture G-actin in pull down assays ( $n = 6$  separate experiments). As assessed by Western blotting in Figure 3A, under our conditions GST-NoCC captured a small but reproducible amount of G-actin (*lanes c and d*) whereas GST (*lanes a and b*) or GST-CC2 (data not shown) did not. Moreover, in agreement with the native gel data, we found that GST-NoCC did not significantly affect the kinetics of actin polymerization upon addition of salt, magnesium and ATP in a pyrene actin assay (Figure 3B). This result indicated that, at identical concentrations, GST-NoCC did not have the sequestering activity of CC1 (Figure 3B). As expected, steady state values for the sequestering CC1 were statistically different from those obtained with both NoCC and control. However, we reproducibly noticed that the steady state values of fluorescence intensity associated to the amount of F-actin were slightly lower upon addition of GST-NoCC. The samples were subjected to ultracentrifugation and gel electrophoresis: as shown Figure 3C, the amounts of F-actin were similar whatever GST-NoCC was added or not.

Since GST-NoCC weakly captured G-actin, using the same pyrene actin assay, we investigated whether it could induce actin nucleation. While the well known actin binding protein villin displayed a clear nucleating activity at 20 nM in our conditions, GST-NoCC was unable to do so at both 20 and 100 nM (Figure 3D). Finally, we further tested whether

the serine 53 carried by NoCC and known to modulate toxofilin affinity to G-actin through phosphorylation/dephosphorylation had any effect on actin nucleation or on the extent of actin polymerization and found no significant effect (data not shown).

### **The CC1/G-actin complex binds to PP2C through CC1B but not through CC1A.**

We previously showed that PP2C binds the actin-toxofilin complex [15]. To define the interacting domains between the three partners, toxofilin, actin and PP2C, we performed a pull down assay using purified recombinant parasite PP2C, rPP2C. rPP2C was pre-immobilized on nickel agarose through its histidine tag and then incubated with or without toxofilin polypeptides and G-actin. We first tested whether the CC1 domain of toxofilin was sufficient to bind both to G-actin and to PP2C. As shown Figure 4A, the tripartite complex was formed *in vitro* using the CC1 domain. This occurred upon incubation of rPP2C with the preformed CC1-G-actin complex (CC1/G-actin) (Figure 4A, *lane b*) as well as upon sequential incubation of rPP2C with CC1 and then G-actin (CC1+G-actin) (Figure 4A, *lane d*) provided that magnesium was added to the mix in the latter situation. Since G-actin did not bind directly to PP2C under our conditions (Figure 4A, *lane f*), these data strongly suggested that toxofilin CC1 contains a PP2C-binding distinct from the actin-binding sites. Of note, the presence of PP2C did not affect the sequestering effect of CC1 in our actin polymerization assay (data not shown).

To better define the interaction between toxofilin and PP2C, we incubated rPP2C with full length or truncates of toxofilin. While full length toxofilin and CC1 were trapped by rPP2C as expected (Figure 4B, *lanes b and d*), only polypeptides carrying the CC1B sequence (i.e. CC1B and CC1BCC2) interacted with rPP2C (Figure 4B, *lanes h and j*). CC1A or CC2 polypeptides that lack the CC1B sequence did not bind to rPP2C (Figure 4B, *lanes f and l*). Similar results were obtained when we used the vertebrate purified recombinant *Xenopus* PP2C (data not shown).

### **Toxofilin displays two potential binding sites to PP2C within CC1B and NoCC respectively.**

To identify the important amino acid residues of toxofilin that could account for rPP2C binding, we performed a peptide assay on a nitrocellulose membrane spotted with synthetic overlapping dodecapeptides shifted by 2 amino acids and that covered the entire toxofilin sequence. Membranes were probed with 4  $\mu\text{g/ml}$  of purified rPP2C as described in experimental procedures. Two series of spots were reproducibly detected. One containing the

spots 83 to 87 localized within the CC1B domain (dashed lines) consistent with the binding assay. Interestingly, the last three spots (85-87) displayed a stronger signal than the two previous ones (83-84) (Figure 5A) and corresponded to the 'KARKLFQRRHYHVTQ' sequence (Figure 5B). Since no binding was observed for the CC1A-corresponding peptide spots, these data again agree with our binding assay (Figure 4B) that favours an interacting site for PP2C within the CC1B region of CC1. Besides CC1B, PP2C also strongly bound to a 28 amino acid long sequence comprised within the spots 14 to 22 'SVQLSEGMKRLSMRGRSPSPKRGRFESG' (Figure 5B). Interestingly, the signal intensity associated with spots 14 to 18 was stronger than the one observed for spots 19 to 22 (Figure 5A). Since spot 14 (SVQLSEGMKRLS) did not share any amino acid with the spots 21 and 22, it is likely that NoCC carries two independent but connected binding sites for PP2C. In addition, this second binding site within NoCC displays at its C-terminal part the serine 53 that we have previously shown to be phosphorylated by caseine kinase II and a substrate of PP2C.

## DISCUSSION

The 27 kDa toxofilin has been characterized as a G and F-actin binding protein *in vitro* using mammalian muscle actin and in heterologous systems such as mammalian cells. It was also found to tightly bind to parasite G-actin [14]. Phosphorylation of toxofilin on serine 53 by casein kinase II decreases its affinity for G-actin by 14 fold, and a type 2C phosphatase selectively dephosphorylates serine 53, thereby stabilizing the actin-toxofilin complex [15]. It is still unclear whether toxofilin targets parasite or mammalian actin or even both. Recent data from a rhoptry proteome analysis identified toxofilin as a rhoptry protein [16]. As such, toxofilin could be secreted from the secretory rhoptry organelle during host cell invasion, as it has been shown for other rhoptry proteins which can remain in the vacuolar space or eventually inserted into the parasitophorous membrane [22-24]. If toxofilin is released in the host cell cytoplasm, it is likely to act on the host cell actin to facilitate parasite progression through the host cell subcortical actin. In this study, we used mammalian actin to undertake a structure-function analysis of toxofilin. We characterized toxofilin domains and amino-acid sequences that account for the G-actin and PP2C binding properties.

Using native gel electrophoresis and pull down assays, we demonstrated that toxofilin carries a domain that is sufficient for binding to G-actin and to elicit sequestration of actin monomers in actin polymerization assay. This domain we called CC1A is located within a region of 9 kDa that encompasses the first coil-coiled domain. Using a peptide spot mapping assay, we detected three series of overlapping peptides in CC1A that bind to G-actin. Screening of Swissprot Uniprot protein data bases (216,380 sequences May 2006) using “pattinprot” with the common motif of these different overlapping peptides ‘AGQA[K/R]AAA’ provided more than 1000 hits (n= 1054) when a 70% stringency threshold of similarity was applied, among which at least 9 are well known actin binding proteins. This does not mean that such sequence should be considered as a signature motif that accounts for the binding property to actin. It could however provide indication for further and more accurate studies on proteins for which a putative binding property to actin has never been investigated so far or for proteins already reported to bind to actin. Peptide spot assays on peptides carrying systematic mutations were performed and the results prompted us to produce CC1 polypeptides into which we introduced single (L81, K87, L91), double and triple mutations in the ‘ERLIAGQAKAAALQTVHQ’ motif but none of these mutations

prevented actin binding to CC1 in pull down assays suggesting that other sites might be sufficient to capture G-actin (data not shown). By contrast, disrupting the 'DEILRATQNLDLRKYENL' in the middle of the sequence after threonine 119 had a major effect on the actin binding property of CC1A, suggesting that this sequence represents a crucial site for actin binding. When this 'DEIL[R/K]A[T/S]Q' degenerated motif was analyzed *in silico*, 487 sequences were identified among which 14 well-characterized G and/or F-actin binding protein such as members of the ADF/cofilin family, the Gelsolin and Adseverin families and the Myosin superfamily. This analysis supports our experimental data with toxofilin. On the other hand, when the 'NLDDL[R/K][K/R]YENL' motif was examined *in silico*, only one of the 400 proteins detected was indeed described as interacting with actin but also with microtubules [25] and possibly with intermediate filaments as well. It is the microtubule-actin cross linker MACF1. It is tempting to speculate that toxofilin may act in regulating microtubule and actin cytoskeleton interactions to support the development of the vacuole throughout the intracellular life of *T. gondii*. The parasitophorous vacuole is known to rapidly relocate towards the nucleus after invasion in a microtubule-dependent process.

We also found additional regions of toxofilin with actin binding properties, in particular the NoCC domain. In native gel assay, NoCC addition did not induce a similar shift of G-actin as to CC1 but rather prevented actin from running into the gel probably as a result of larger complexes between NoCC and several actin molecules. However, NoCC did not display the sequestering effect elicited by CC1 or any detectable nucleating activity in the pyrene actin assay. These data strongly suggest that apart from CC1A, toxofilin exhibits additional actin binding sites but with different binding features and properties. The complete resolution of the 3D structure and a thermodynamic analysis would provide answers to these questions.

Based on such 3D data, it should be informative to introduce mutations and to assay *in vivo* whether they have an impact on the parasite survival.

Since we previously reported that phosphorylation contributes to toxofilin binding property onto actin, and that PP2C copurified in the actin-toxofilin complex, we also mapped the interactive regions of toxofilin with PP2C. Herein, we show that the toxofilin region encoding the first coil-coiled domain and closely linked to the C-terminal part of CC1A bound to PP2C, as supported by both pull-down and peptide spot mapping assays. An additional sequence in NoCC that bound to PP2C could represent two distinct but connected binding sites according to the peptide spot assay. This sequence in NoCC also contains the amino acid serine 53 previously shown to be substrate of PP2C. When non redundant protein



data bases corresponding to more than 3,000,000 sequences were screened with 'LFQRRHYHVT' motif and imposing a 85% stringency threshold in similarity, only toxofilin was pulled out while 17 hits were found when the stringency threshold for similarity decreased to 70%. Among these positive hits, none corresponded to a protein yet assigned as a PP2C partner. When the same screen was applied with degenerated sequence for basic and alkyl phosphorylatable residues 'LFQ[R/K][R/K]HYHV[T/S]', we obtained only toxofilin at a 90% threshold and 34 hits at a 70% threshold of similarity. Unlike for many kinases or for the PP1 and PP2A serine-threonine phosphatases, there are no clear sequence "signatures" for PP2C dephosphorylation targets although the presence of a basic N-terminal residue at position -3 and no proline adjacent to the C-terminal part of the phosphorylation have been shown to favor dephosphorylation by PP2C [26]. Toxofilin carries an arginine at the residue 50 and the absence of proline after the serine 53 up to residue 60 which is consistent with the theoretical favorable motif for being a PP2C substrate.

Characterizing PP2C substrates is hampered by the lack of specific inhibitors or activators although several major targets are already identified. They are mainly related to cell cycle [27, 28] or to cell apoptosis with the Bcl2 family proapoptotic member BAD [29]. Mammalian PP2Cs are also increasingly analyzed for their role in the homeostasis of the central nervous system as for example PP2C $\alpha$  interacts with Ca<sup>2+</sup> channels and contributes to synaptic transmission in neurons [30]. Therefore the dissection of a define substrate such as toxofilin for the amino-acid requirements that promote both PP2C binding and activity is among the first to be reported and should provide a useful reagent for both PP2C enzymatic characterization and broad modulatory molecule screening. In the future, *in vivo* strategies aiming at competing with endogenous PP2C may rely on non human-related sequences such as the toxofilin identified binding/substrate polypeptide.

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## REFERENCES

- 1 Morisaki, J. H., Heuser, J. E. and Sibley, L. D. (1995) Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell. *J. Cell Sci.* **108** (Pt 6), 2457-2464
- 2 Sinai, A. P., Webster, P. and Joiner, K. A. (1997) Association of host cell endoplasmic reticulum and mitochondria with the *Toxoplasma gondii* parasitophorous vacuole membrane: a high affinity interaction. *J. Cell Sci.* **110** (Pt 17), 2117-2128
- 3 Black, M. W. and Boothroyd, J. C. (2000) Lytic cycle of *Toxoplasma gondii*. *Microbiol. Mol. Biol. Rev.* **64**, 607-623
- 4 Moudy, R., Manning, T. J. and Beckers, C. J. (2001) The loss of cytoplasmic potassium upon host cell breakdown triggers egress of *Toxoplasma gondii*. *J. Biol. Chem.* **276**, 41492-41501
- 5 Hoff, E. F. and Carruthers, V. B. (2002) Is *Toxoplasma* egress the first step in invasion? *Trends Parasitol.* **18**, 251-255
- 6 Hill, D. and Dubey, J. P. (2002) *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect.* **8**, 634-640
- 7 Montoya, J. G. and Liesenfeld, O. (2004) *Toxoplasmosis*. *Lancet* **363**, 1965-1976
- 8 Dobrowolski, J. M. and Sibley, L. D. (1996) *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. *Cell* **84**, 933-939
- 9 Dobrowolski, J. M., Carruthers, V. B. and Sibley, L. D. (1997) Participation of myosin in gliding motility and host cell invasion by *Toxoplasma gondii*. *Mol. Microbiol.* **26**, 163-173
- 10 Meissner, M., Schluter, D. and Soldati, D. (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science* **298**, 837-840
- 11 Carruthers, V. B., Giddings, O. K. and Sibley, L. D. (1999) Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells. *Cell. Microbiol.* **1**, 225-235
- 12 Soldati, D., Dubremetz, J. F. and Lebrun, M. (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int. J. Parasitol.* **31**, 1293-1302
- 13 Jewett, T. J. and Sibley, L. D. (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Mol. Cell* **11**, 885-894
- 14 Poupel, O., Boleti, H., Axisa, S., Couture-Tosi, E. and Tardieux, I. (2000) Toxofilin, a novel actin-binding protein from *Toxoplasma gondii*, sequesters actin monomers and caps actin filaments. *Mol. Biol. Cell* **11**, 355-368
- 15 Delorme, V., Cayla, X., Faure, G., Garcia, A. and Tardieux, I. (2003) Actin dynamics is controlled by a casein kinase II and phosphatase 2C interplay on *Toxoplasma gondii* Toxofilin. *Mol. Biol. Cell* **14**, 1900-1912
- 16 Bradley, P. J., Ward, C., Cheng, S. J., Alexander, D. L., Collier, S., Coombs, G. H., Dunn, J. D., Ferguson, D. J., Sanderson, S. J., Wastling, J. M. and Boothroyd, J. C. (2005) Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interactions in *Toxoplasma gondii*. *J. Biol. Chem.* **280**, 34245-34258
- 17 Pardee, J. D. and Spudich, J. A. (1982) Purification of muscle actin. *Methods Cell Biol.* **24**, 271-289
- 18 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680-685

- 19 Cockwell, K. Y. and Giles, I. G. (1989) Software tools for motif and pattern scanning: program descriptions including a universal sequence reading algorithm. *Comput. Appl. Biosci.* **5**, 227-232
- 20 Combet, C., Blanchet, C., Geourjon, C. and Deleage, G. (2000) NPS@: network protein sequence analysis. *Trends Biochem. Sci.* **25**, 147-150
- 21 Kodama, A., Karakesisoglou, I., Wong, E., Vaezi, A. and Fuchs, E. (2003) ACF7: an essential integrator of microtubule dynamics. *Cell* **115**, 343-354
- 22 Beckers, C. J., Dubremetz, J. F., Mercereau-Puijalon, O. and Joiner, K. A. (1994) The *Toxoplasma gondii* rhoptry protein ROP 2 is inserted into the parasitophorous vacuole membrane, surrounding the intracellular parasite, and is exposed to the host cell cytoplasm. *J. Cell Biol.* **127**, 947-961
- 23 Joiner, K. A., Bermudes, D., Sinai, A., Qi, H., Polotsky, V. and Beckers, C. J. (1996) Structure and function of the *Toxoplasma gondii* vacuole. *Ann. N. Y. Acad. Sci.* **797**, 1-7
- 24 Alexander, D. L., Mital, J., Ward, G. E., Bradley, P. and Boothroyd, J. C. (2005) Identification of the Moving Junction Complex of *Toxoplasma gondii*: A Collaboration between Distinct Secretory Organelles. *PLoS Pathog.* **1**, e17
- 25 Sun, D., Leung, C. L. and Liem, R. K. (2001) Characterization of the microtubule binding domain of microtubule actin crosslinking factor (MACF): identification of a novel group of microtubule associated proteins. *J. Cell Sci.* **114**, 161-172
- 26 Pinna, L. A. and Donella-Deana, A. (1994) Phosphorylated synthetic peptides as tools for studying protein phosphatases. *Biochim. Biophys. Acta* **1222**, 415-431
- 27 De Smedt, V., Poulhe, R., Cayla, X., Dessauge, F., Karaiskou, A., Jesus, C. and Ozon, R. (2002) Thr-161 phosphorylation of monomeric Cdc2. Regulation by protein phosphatase 2C in *Xenopus* oocytes. *J. Biol. Chem.* **277**, 28592-28600
- 28 Cheng, A., Ross, K. E., Kaldis, P. and Solomon, M. J. (1999) Dephosphorylation of cyclin-dependent kinases by type 2C protein phosphatases. *Genes Dev.* **13**, 2946-2957
- 29 Klumpp, S., Selke, D. and Krieglstein, J. (2003) Protein phosphatase type 2C dephosphorylates BAD. *Neurochem. Int.* **42**, 555-560
- 30 Li, D., Wang, F., Lai, M., Chen, Y. and Zhang, J. F. (2005) A protein phosphatase 2 $\alpha$ -Ca<sup>2+</sup> channel complex for dephosphorylation of neuronal Ca<sup>2+</sup> channels phosphorylated by protein kinase C. *J. Neurosci.* **25**, 1914-1923

## FIGURE LEGENDS

**Table 1:** "Pattinprot "screening analysis of Uniprot-Swissprot data base with two toxofilin actin binding motifs 'AGQA[K/R]AAA' (part A) and 'DEIL[R/K]A[T/S]Q' (part B). The proteins identified by the screen and known to interact with actin are listed.

**Figure 1: Toxofilin binds to G-actin via the CC1A domain.** A) Schematic representation of full length toxofilin and toxofilin domains used throughout the study. Amino acids are numbered on top of the schemes. Dashed lines represent the two coil-coiled domains. B) A 7.5% acrylamide native gel assay was performed using 50  $\mu$ g of IAEDANS labelled G-actin incubated without (*lane a*) or with equimolarity of recombinant GST-toxofilin (*lane b*), GST-CC1 (*lane c*), GST-NoCC (*lane d*) or GST-CC2 (*lane e*). F-actin was loaded as a control (*lane f*). C) G-actin binding assays were performed on immobilized GST-toxofilin (*lanes a-c*), GST-CC1 (*lanes d-f*), GST-CC1A (*lanes g-i*), GST-CC1B (*lanes j-l*), GST-CC2 (*lanes m-o*) and GST alone (*lanes p-r*). Following overnight incubation, the resin was washed and bound proteins were eluted in SDS sample buffer prior to SDS-PAGE and Coomassie staining. L indicates the total load, U indicates the total unbound fraction and B the total bound one. Arrowhead indicates the position of G-actin. D) Actin binding competition assays were carried out on immobilized GST-toxofilin (labelled as GST-Tf) using G-actin preincubated without (*lane b*) or with CC1 (*lane c*) or CC1A (*lane d*) and on immobilized GST-CC1 using G-actin preincubated or not with CC1A (*lanes e-g*). Samples were treated as described for C. L indicates the total load and B the total bound fraction. The presence of the competitive fragment is indicated above the gel photograph. E) Pyrene actin polymerization kinetics in presence or not of 500 nM of GST-CC1 ( $\square$ ) and GST-CC1A ( $\times$ ) polypeptides using pyrene actin. Numbers on the right of the curves represent the actin steady state values obtained 16 hours (4°C) after the assay. Stars indicate that the difference with the control values ( $\circ$ ) are statistically significant at  $p = 0.05$  (Student test). F) Pyrene actin polymerization kinetics in presence or not of 500 nM GST-CC1 ( $\square$ ) and GST-CC2 ( $\Delta$ ) polypeptides using pyrene actin. Numbers on the right of the curves represent the actin steady state values obtained 16 hours (4°C) after the assay. Stars indicate that the differences with the control values ( $\circ$ ) are statistically significant at  $p = 0.05$  (Student test) whereas NS indicate that they are not.

**Figure 2: Toxofilin sequesters G-actin via the CC1A domain that contains 3 binding sites.** A) The entire CC1A amino acid sequence was represented onto a cellulose membrane by successive spots of dodecapeptides shifted by 2 amino acids and then probed with biotinylated G-actin and HRP-conjugated streptavidin. B) The sequences corresponding to positive spots using chemiluminescence (A) are presented and framed. The first amino-acid of each lane is numbered on the left. C) Actin binding competition assays were carried out on immobilized GST-CC1 using G-actin preincubated with CC1A (*lanes a-c*) with CC1Aa (*lane d*) or CC1Ab (*lane e*) or with a mix of CC1Aa and Ab (*lane f*). Samples were treated as described for Figure 1. L indicates the total load and B the total bound fraction. The presence of the competitive fragment is indicated above the gel photograph. Arrowheads on the left indicate the position of G-actin and GST-CC1 on the gel. D) Actin polymerization assays were performed by adding 100 mM KCl, 2 mM MgCl<sub>2</sub> and 1 mM Na<sub>2</sub>ATP to G-actin overnight at 4°C and further centrifuged. Supernatants (labelled S) and pellets (labelled P) were subjected to SDS-PAGE followed by Coomassie staining. Actin alone polymerized efficiently (*lanes a-b*) whereas G-actin incubated with GST-CC1 (*lanes c-d*), GST-CC1A (*lanes e-f*) did not. In contrast, GST-CC1Aa (*lanes g-h*) and GST-CC1Ab (*lanes i-j*) failed to prevent actin polymerization.

**Figure 3: NoCC binds to G-actin and does not affect the rate and the amount of polymerization.** A) Actin binding assays were carried out using immobilized GST (*lanes a-b*) or immobilized GST-NoCC (*lanes c-d*) and samples were analyzed by Western blotting using anti-actin antibodies (clone C4). B) Actin polymerization kinetics in presence or not of 500 nM of GST-NoCC (◇) or 500 nM of GST-CC1 (□) polypeptides using pyrene actin. Numbers on the right of the curves represent the actin steady state values obtained 16 hours (4°C) after the assay. Stars indicate that the differences with the control values (○) are statistically significant at  $p = 0.05$  (Student test) whereas NS indicate that they are not. C) Actin polymerization assays were performed as described for Fig. 2. Actin alone under polymerizing conditions (*lanes a-b*) and actin incubated with GST-NoCC in the same conditions (*lanes c-d*). Supernatants (labelled S) and pellets (labelled P) were subjected to SDS-PAGE and further Coomassie staining. For each panel, the positions of actin and GST-NoCC are indicated with arrowheads on the left. D) Actin nucleation assay in presence or not

of 20 nM ( $\diamond$ ) or 100 nM of GST-NoCC (+) polypeptides using pyrene actin. As positive control, villin was used at 20 nM ( $\Delta$ ).

**Figure 4: The CC1/G-actin complex binds to PP2C through CC1B but not through CC1A.** A) Immobilized rPP2C on nickel was incubated with either the preformed CC1-G-actin complex (*lanes a-b*) or sequentially with CC1 then G-actin (*lanes c-d*) or with G-actin alone (*lanes e-f*) for 1 h at 4°C. After extensive washings, Ni-NTA resin-bound proteins were eluted in SDS sample buffer and subjected to SDS-PAGE and Coomassie staining. U indicates the total unbound fraction and B the total bound one. Arrowheads on the left indicate the positions of G-actin, CC1 and rPP2C. B) Immobilized rPP2C was incubated with toxofilin labelled Tf (*lanes a-b*), CC1 (*lanes c-d*), CC1A (*lanes e-f*) CC1B (*lanes g-h*), CC1BCC2 (*lanes i-j*) or CC2 (*lanes k-l*). Samples were treated as described for Figure 4A. Arrowhead on the left indicates the position of rPP2C.

**Figure 5: Two toxofilin amino-acid sequences bind to rPP2C using peptide spot mapping.** A) The entire toxofilin amino acid sequence was represented onto a cellulose membrane by spots of dodecapeptides shifted by 2 amino acids. The CC1B region is represented with dashed lines. The membrane was successively probed with rPP2C, polyclonal anti-PP2C antibodies and HRP-conjugated anti-rabbit antibodies. B) The sequences corresponding to positive spots using chemiluminescence (A) are presented and framed and the first amino-acid of each lane is numbered on the left. In the first series of 28 amino-acid positive sequence, the known PP2C substrate Serine<sup>53</sup> is labelled in bold and underlined.

Table 1

A

Primary accession number	Protein name	Sequence							
CAB72264	Toxofilin	A	G	Q	A	K/R	A	A	A
		A	G	Q	A	K	A	A	A
Q4VCS5	Angiomotin	A	G	Q	i	p	A	A	A
Q21624	Coronin-like	A	G	Q	r	R	A	A	A
Q9QY06	Myosin-9B	A	e	Q	A	R	e	A	A
P05659	Myosin-2 heavy	A	q	e	A	R	A	A	A
O96064	Paramyosin	A	G	l	A	K	A	k	A
P54939	Talin-1 chicken	A	q	Q	A	K	p	A	A
P26039	Talin-1 mouse	A	s	Q	A	K	p	A	A
Q9Y4G6	Talin-2 human	A	k	Q	A	a	A	A	A
Q7ILX4	Talin-2 mouse	A	k	Q	A	a	A	A	A



**B**

Primary accession number	Protein name	Sequence							
		<b>D</b>	<b>E</b>	<b>I</b>	<b>L</b>	<b>R/K</b>	<b>A</b>	<b>T/S</b>	<b>Q</b>
CAB72264	Toxofilin	<b>D</b>	<b>E</b>	<b>I</b>	<b>L</b>	<b>R</b>	<b>A</b>	<b>T</b>	<b>Q</b>
Q28046	Adseverin bovin	y	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>S</b>	<b>Q</b>
Q60604	Adseverin mouse	y	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>S</b>	<b>Q</b>
Q29297	Adseverin pig	y	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>S</b>	<b>Q</b>
Q28372	ADF horse	f	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>T</b>	<b>Q</b>
P06396	ADF human	y	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>T</b>	<b>Q</b>
P13020	ADF mouse	f	<b>E</b>	f	<b>L</b>	<b>K</b>	<b>A</b>	<b>T</b>	<b>Q</b>
P20305	ADF pig	y	<b>E</b>	r	<b>L</b>	<b>K</b>	<b>A</b>	<b>T</b>	<b>Q</b>
Q96SB3	Neurabin II human	<b>D</b>	<b>E</b>	h	<b>L</b>	<b>R</b>	e	<b>T</b>	<b>Q</b>
Q6R891	Neurabin II mouse	<b>D</b>	<b>E</b>	h	<b>L</b>	<b>R</b>	e	<b>T</b>	<b>Q</b>
O35274	Neurabin II rat	<b>D</b>	<b>E</b>	h	<b>L</b>	<b>R</b>	e	<b>T</b>	<b>Q</b>
Q8WXH0	Nesprin 2	<b>D</b>	<b>E</b>	d	<b>L</b>	s	<b>A</b>	<b>T</b>	<b>Q</b>
P02588	Troponin chicken	g	<b>E</b>	<b>I</b>	<b>L</b>	<b>R</b>	<b>A</b>	<b>T</b>	g
P10246	Troponin melga	g	<b>E</b>	<b>I</b>	<b>L</b>	<b>R</b>	<b>A</b>	<b>T</b>	g
Q9NQX4	Myosin 5C	v	<b>E</b>	<b>I</b>	<b>L</b>	<b>R</b>	<b>A</b>	<b>S</b>	k

Figure 1

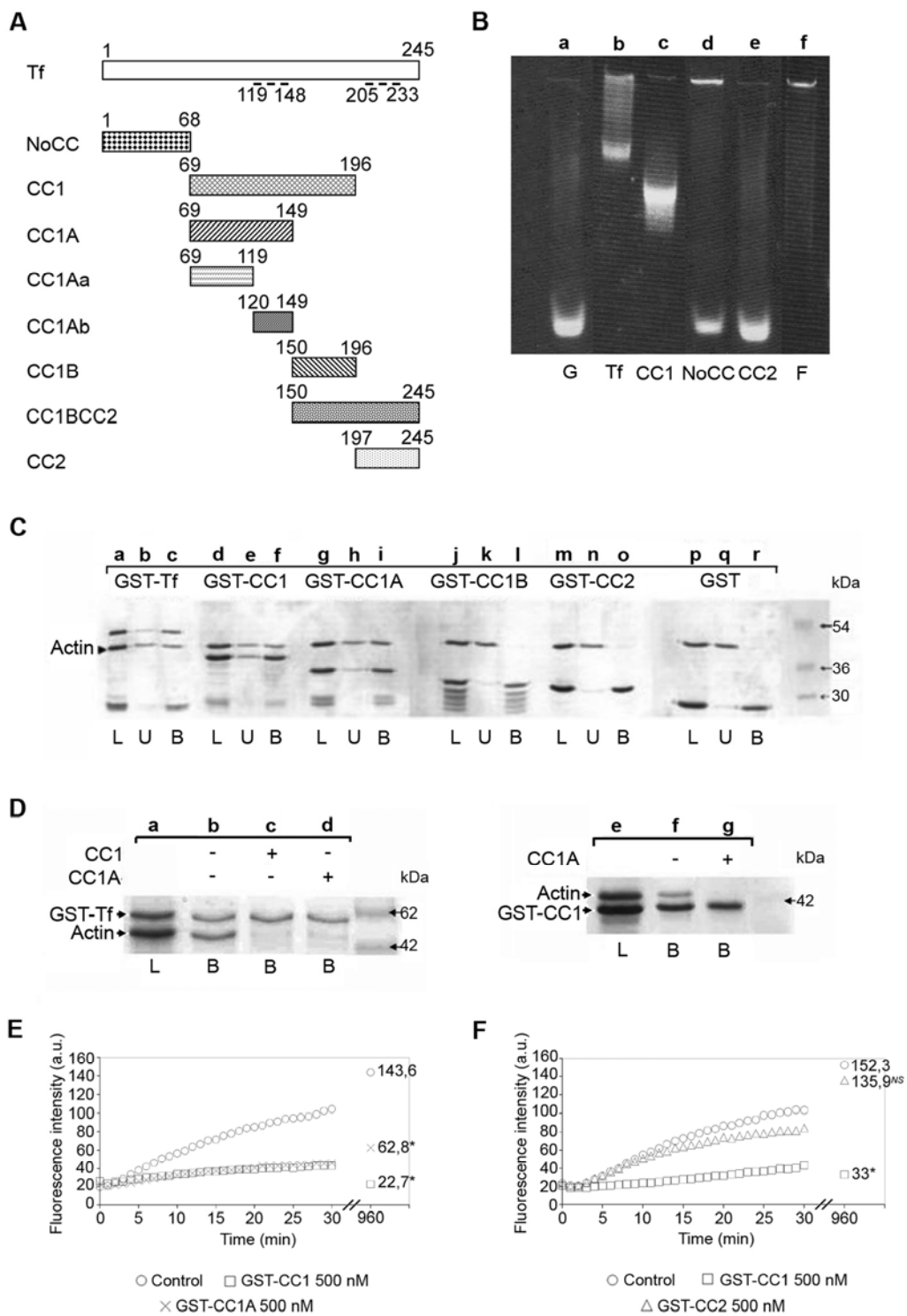


Figure 2

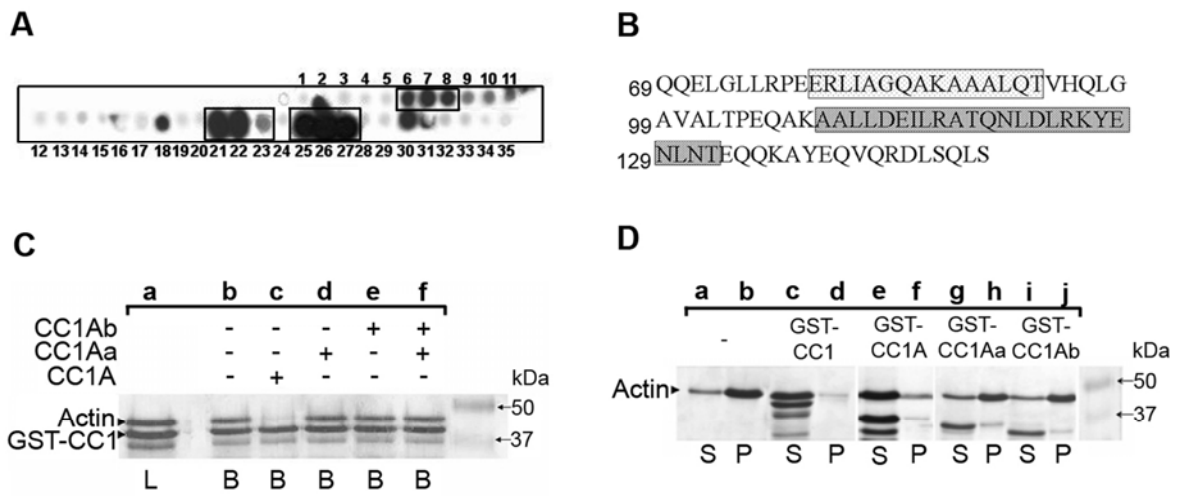
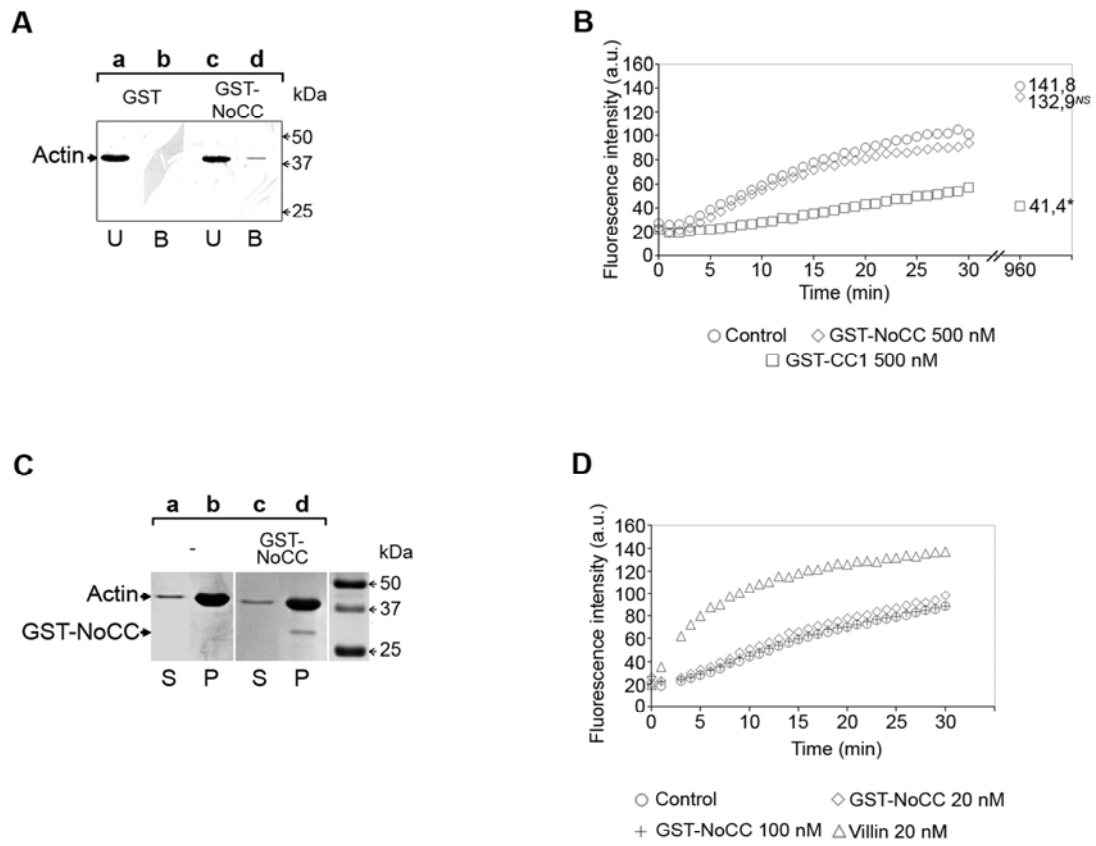


Figure 3



**Figure 4**

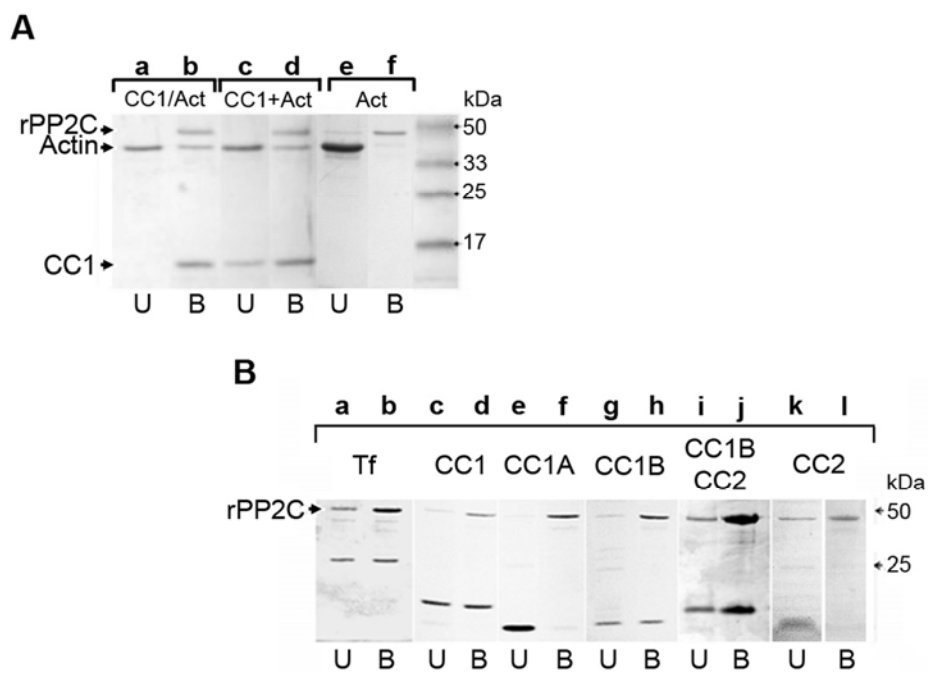


Figure 5

