A *Plasmodium* Actin-depolymerizing Factor That Binds Exclusively to Actin Monomers

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ADF/cofilins (AC) are essential F- and G-actin binding proteins that modulate microfilament turnover. The genome of *Plasmodium falciparum*, the parasite causing malaria, contains two members of the AC family. Interestingly, *P. falciparum* ADF1 lacks the F-actin binding residues of the AC consensus. Reverse genetics in the rodent malaria model system suggest that ADF1 performs vital functions during the pathogenic red blood cell stages, whereas ADF2 is not present in these stages. We show that recombinant PfADF1 interacts with monomeric actin but does not bind to actin polymers. Although other AC proteins inhibit nucleotide exchange on monomeric actin, the *Plasmodium* ortholog stimulates nucleotide exchange. Thus, PfADF1 differs in its biochemical properties from previously known AC proteins and seems to promote turnover exclusively by interaction with actin monomers. These findings provide important insights into the low cytosolic abundance and unique turnover characteristics of actin polymers in parasites of the phylum Apicomplexa.

INTRODUCTION

Apicomplexan parasites are important human and animal pathogens. Their gliding motility is actin dependent and involves neither protrusive structures nor any changes in cell shape (Sibley, 2004). Gliding motility and host cell entry are initiated by surface receptor binding to substrate or host cell ligands (Russell and Sinden, 1981). Backward distribution of the receptor–ligand complexes then propel the parasite forward and into the host cell (Kappe *et al.*, 2003; Kappe *et al.*, 2004; Sibley, 2004; Soldati and Meissner, 2004). Thus, rather than inducing endocytosis and hijacking the host cell's force transducing systems, apicomplexans actively cross cell barriers and translocate into the host cytoplasm under simultaneous formation of a replication-competent organelle, the parasitophorous vacuole.

Actin-dependent motility typically depends on the existence of polymers of actin (F-actin), either by interaction of F-actin with the motor protein myosin or through tightly regulated dynamic turnover of F-actin. Apicomplexan gliding motility and host cell invasion are inhibited by cytochalasins, suggesting that actin filaments of the parasite are required for these processes (Dobrowolski and Sibley, 1996). However, microfilaments cannot be visualized within the cytoplasm of parasites using either electron microscopy or fluorescent derivatives of the F-actin binding toxin phalloidin (Dobrowolski *et al.*, 1997; Gantt *et al.*, 2000), presumably

because parasite microfilaments are very short (Schmitz *et al.*, 2005). The cell-permeable drug jasplakinolide induced local filament formation at the apical ends of motile parasites, but it did not give rise to actin polymers within the parasite cytosol (Shaw and Tilney, 1999), suggesting the existence of a highly efficient monomer-sequestering mechanism.

Short microfilaments exist in the space between the parasite plasma membrane and the inner membrane complex, where they are thought to interact with transmembrane receptors on one hand and a myosin motor on the other hand (Kappe *et al.*, 2004). The intrinsic properties of apicomplexan actin may partly explain the restricted turnover of these actin polymers, as recombinant *Plasmodium* actin forms polymers of low stability in vitro (Schüler *et al.*, 2005). Localized and transient microfilament formation may be brought about by the action of actin monomer-binding proteins such as profilin, filament depolymerizing proteins such as ADF/cofilins, and filament nucleating and capping proteins (Pollard and Borisy, 2003). Several such proteins encoded by apicomplexan parasites have been identified and characterized (Allen *et al.*, 1997; Tardieux *et al.*, 1998a,b; Poupel *et al.*, 2000; Matuschewski *et al.*, 2002). Nevertheless, so far the unique turnover characteristics of microfilaments in apicomplexa have not been sufficiently explained.

The actin-depolymerizing factor (ADF)/cofilin family (AC proteins) are ubiquitous eukaryotic proteins that modulate the turnover of the microfilament system in vivo (reviewed in Bamberg, 1999; Carlier *et al.*, 1999; Ono, 2003; Paavilainen *et al.*, 2004). Although unicellular eukaryotes generally express one AC protein, multicellular organisms use several isoforms, some of which may be expressed in a tissue-specific manner. ADF/cofilins bind F-actin in a 1:1 stoichiometry per actin subunit, an interaction that drastically destabilizes the polymers (Carlier *et al.*, 1997; McGough *et al.*, 1997). The ADF/cofilin-induced acceleration of F-actin turnover is largely responsible for the rapid microfilament remodeling observed in vivo (Lappalainen and Drubin, 1997; Rosenblatt *et al.*, 1997). AC proteins also bind monomeric

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Abbreviations used: AC, ADF/cofilin; ADF, actin-depolymerizing factor.

actin (G-actin), thereby inhibiting nucleotide exchange and incorporation into F-actin. These activities are indispensable for the cell, because AC knockouts are not viable (Iida *et al.*, 1993; McKim *et al.*, 1994; Gunsalus *et al.*, 1995) and tissue-specific cofilins are essential for morphogenesis (Gurniak *et al.*, 2005).

The overall structure of ADF/cofilin proteins is a central mixed β -sheet sandwiched in between short α -helices, in most isoforms two on each side (Hatanaka *et al.*, 1996; Fedorov *et al.*, 1997; Leonard *et al.*, 1997; Bowman *et al.*, 2000; Pope *et al.*, 2004). A comparison with other small actin binding proteins showed that a similar architecture is used for twinfilin and gelsolin subdomains (Hatanaka *et al.*, 1996; Lappalainen *et al.*, 1998; Paavilainen *et al.*, 2002). The actin binding site in AC proteins involves a long, kinked α -helix, and the specific F-actin binding residues are located to a central loop protruding from the β -sheet as well as the C-terminal α -helix (Yonezawa *et al.*, 1989; Moriyama *et al.*, 1992; Jiang *et al.*, 1997; Lappalainen *et al.*, 1997; Van Troys *et al.*, 1997; Pope *et al.*, 2000; Guan *et al.*, 2002).

Here, we describe two ADF/cofilin isoforms from the genome of the malaria-causing protozoan *Plasmodium falciparum*. Both display a high degree of homology to known ADF/cofilin sequences. We show that ADF1 is essential for erythrocytic schizogony of the malaria parasite. The coding sequence for ADF1 was expressed in *Escherichia coli*, and the recombinant protein was purified. We describe a biochemical analysis of this protein, in comparison with *Arabidopsis thaliana* ADF1 and *Saccharomyces cerevisiae* cofilin. PfADF1 interacts with monomeric but not with filamentous actin. In addition, PfADF1 promotes nucleotide exchange on actin monomers and sequesters monomers inefficiently.

MATERIALS AND METHODS

Plasmodium ADF Expression Analysis

To study the expression pattern of ADF1 and ADF2 during the *Plasmodium* life cycle, poly(A)⁺ RNA was isolated from gradient-purified late stage schizonts from one infected Sprague-Dawley rat (10⁹ parasites), 8 × 10⁵ infectious salivary gland sporozoites, and 6 × 10⁵ immature oocyst sporozoites using oligo(dT) columns (Invitrogen, Carlsbad, CA). The poly(A)⁺ RNA was used as a template for first-strand cDNA synthesis using oligo(dT) primers (Ambion, Austin, TX) followed by subsequent standard PCR amplification using the following primer sets: ADF1, ADF1for (5' CCGGAATTCGCGATTCGCGTAAATGACAATTGTG 3', EcoRI site is underlined) and ADF1rev1 (5' ATTGCGGCCGCTTATTTAAATCGGCAACATCTAGGGC 3', NotI site is underlined); ADF2, ADF2for (5' GAATTCATCCGACGAATGATTTATGAGTTAAAC 3', EcoRI site is underlined), and ADF2rev (5' TCTAGATTAGCCTTAAGTTCTTCTCAAATTC 3', XbaI site is underlined); and MyoA, MyoAfor (5' CGCGGATCCATGCTGTACAAATGAGGAATTAAC 3', BamHI site is underlined) and MyoArev (5' GGGAATCCATATGATTACCTAATGTAAAAATACCCGCG 3', EcoRI site is underlined). Correct amplification of the ADF1 transcript was confirmed by cloning the PCR product into pBluescript and sequencing from both ends.

PbADF1 Gene Targeting

For disruption of PbADF1, a 540-base pair fragment was amplified using primers ADF1for and ADF1rev1 using *Plasmodium berghei* genomic DNA as template. Cloning of this fragment into the *P. berghei* targeting vector that confers resistance to the antifolate pyrimethamine (Thathy and Menard, 2002) resulted in pINT. For the control integration construct, a 1115-base pair fragment was amplified using primers ADF1for and ADF1rev2 (5' ATTTGCGCGCCGCTTACGATTATCCTATTGTTTATTC 3', NotI site is underlined) and cloned into the targeting vector, resulting in pCONT. Transfection was done as described previously (Thathy and Menard, 2002) with 70 μ g of XbaI-linearized plasmid and gradient-purified schizonts of the pyrimethamine-sensitive *P. berghei* strain NK65. Positive selection for stable integration was by daily injection of 25 mg/kg pyrimethamine. Resistant parasite populations were transferred to naive animals for propagation and genotyping. Genotyping was performed by integration-specific PCRs using primers ADF1test2 (5' TCAAAATGATAAGCGGTATTCGCG 3') and ADF1rev2 for the PbADF1 wild-type signal, and primers Tgfor (5' CCCGACGGACGAATCCAGATGG 3') and ADF1rev2 for the INT disruptant locus. For the CONT control locus

the primers Tgfor and ADF1test1 (5' GTGGAAATCGATATATGGATATAG 3') were used. The PbADF1 wild-type signal was detected through primers ADFtest2 and ADF1test1.

Cloning and Protein Purification

Poly(A)⁺ RNA was isolated from cultured *P. falciparum* mixed blood stages (strain HB3) using oligo(dT) columns (Invitrogen). The poly(A)⁺ RNA was used as a template for first-strand cDNA synthesis (Ambion). PfADF1 was amplified from this cDNA using sequence-specific primers (5' CGTGGATC-CATAAGTGGTATTCGAGTTAATGAT 3' and 5' CCGCTCGAGTTATTTAAGATCAGCAACATCTGT 3'; BamHI and XhoI sites are underlined). The *S. cerevisiae* cofilin expression vector was a gift from Pekka Lappalainen (Institute of Biotechnology, University of Helsinki, Helsinki, Finland).

PfADF1 and ScCof were expressed as glutathione S-transferase (GST)-fusion proteins using pGEX vectors (Amersham Biosciences, Piscataway, NJ). *E. coli* BL-21(DE-3)RPL cells (Stratagene, La Jolla, CA) transformed with the expression constructs were grown to an OD₆₀₀ of 0.7, and expression was induced with 0.1 mM isopropyl β -D-thiogalactoside. Cells were harvested after 3 h of growth at 37°C, suspended in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl containing 0.1 mM phenylmethylsulfonyl fluoride and Complete protease inhibitors (Roche Diagnostics, Indianapolis, IN), and lysed by freeze-thawing and sonication. The lysates were clarified by two 20-min centrifugations at 10,000 and 50,000 × g, respectively, filtered, and applied to GSTrap columns (Amersham Biosciences). The columns were washed with 20 mM Tris-HCl, pH 8.0, 0.5 M NaCl before bound ADF or cofilin proteins were liberated from GST by cleavage with 20 U/ml thrombin overnight at room temperature, or with PreScission protease (Amersham Biosciences) according to the manufacturer's instructions. Contaminants were removed by passage over Q-Sepharose FF (Amersham Biosciences) in 20 mM HEPES, pH 7.1, 1 mM dithiothreitol (DTT). The material passing the Q-Sepharose was concentrated using Centricon devices (Millipore, Billerica, MA), snap-frozen in liquid N₂, and stored at -80°C. The extinction coefficients for the unfolded proteins were calculated (Gill and von Hippel, 1989), and the extinction coefficients in buffer were determined as A₂₈₀ = 0.68 mg⁻¹ml⁻¹ for PfADF1 and 0.97 mg⁻¹ml⁻¹ for ScCof.

A. thaliana ADF1 was expressed in *E. coli* BL-21(DE-3) and purified essentially as described previously (Carlier *et al.*, 1997). Cytoplasmic β -actin from bovine thymus was purified as described previously (Lindberg *et al.*, 1988). Recombinant expression and purification of *P. falciparum* actin I was as described previously (Schöler *et al.*, 2005).

Actin Binding of AC Proteins

The interaction of actin with the ADF/cofilin proteins was characterized at 25°C in 5 mM Tris-HCl, pH 7.6, 0.5 mM ATP, 0.1 mM CaCl₂, 0.5 mM DTT (G-buffer), with the addition of 0.2 mM EGTA and 50 μ M MgCl₂ in the case of Mg²⁺-actin. Monomeric ADP-actin was made by changing to ATP-free G-buffer on PD-10 columns (Amersham Biosciences) and adding ADP to 0.5 mM. Polymerizing conditions were induced by addition of 1 mM MgCl₂ + 0.1 M KCl to G-buffer.

Nucleotide Exchange

After addition of 1, N⁶-ethenoadenosine 5'-triphosphate (ϵ ATP; Molecular Probes, Eugene, OR) to G-actin (8 μ M; 7 μ M in the case of PfAct1) in the absence of excess ATP, the fluorescence increase at >408 nm (excitation at 360 nm) was monitored. Observed rates were determined by curve fitting of the raw data to a first-order equation using MicroCal Origin (OriginLab, Northampton, MA).

Thermal Unfolding of Actin

Thermal stability of actin was assessed as described previously (Schöler *et al.*, 2000). Briefly, actin solutions were incubated in a water bath, either at 50°C or at increasing temperatures at a heating rate of 40 K/h. At intervals, aliquots were removed from the sample and their content of native actin determined using the DNase I-inhibition assay (Blikstad *et al.*, 1978).

Kinetics and Steady-State Levels of Polymerization

Filament formation was induced by addition of 1 mM MgCl₂ + 0.1 M KCl, and monitored by the increase in fluorescence due to copolymerization of 2% pyrene-labeled bovine β -actin (Kouyama and Mihashi, 1980).

Cosedimentation/Sequestering Assay

Samples of actin (8 μ M) were induced to polymerize by addition of 1 mM MgCl₂ + 0.1 M KCl in the presence of 0–24 μ M ADF/cofilin proteins and incubated at room temperature for 6–8 h. After ultracentrifugation in a Beckman airfuge (30000 psi for 15 min), equal amounts of the supernatants and pellets were analyzed by SDS-PAGE. Coomassie-stained gels were scanned and bands quantified using Bio-Rad MultiImage equipment and QuantityOne software. Buffer conditions for the pH-dependence experiment were 10 mM Tris-HCl, 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0, and 30 mM Tris-HCl, pH 8.5, respectively.

Electrophoresis

Denaturing gel electrophoresis was performed using NuPAGE 4–12% Bis-Tris gels and the MES buffer system (Invitrogen). For nondenaturing gel electrophoresis, actin-ADF mixtures were prepared in Mg-G-buffer containing 15% glycerol and either ADP or ATP and incubated for 15 min at 25°C. Mixtures were applied to gels containing 7.5% acrylamide, 80 mM Tris, pH 8.5, 50 μ M MgCl₂, 1 mM DTT, and 0.2 mM either ADP or ATP. Gels were mounted in Novex mini cells (Invitrogen) and run in 40 mM Tris, 30 mM Bicine, pH 8.5, 50 μ M MgCl₂, 1 mM DTT, and 0.2 mM either ADP or ATP at 5 W for ~2.5 h at 10°C. Incidentally, although these conditions were similar to those used by Chen *et al.* (2004), recombinant yeast cofilin migrated readily into our native gels, in contrast to observations by Chen *et al.* (2004).

RESULTS

Plasmodium Has Two ADF Homologues

Using BLAST searches of the *P. falciparum* genome (Gardner *et al.*, 2002), we identified two sequences with ADF/cofilin homology, hereafter referred to as *PfADF1* (PlasmoDB entry PFE0165w) and *PfADF2* (PF13_0326). Sequence comparisons showed that both *Plasmodium* ADFs share roughly 20–30% identity with yeast, plant, and animal ADF/cofilins (Figure 1A). The *PfADF1* gene encodes one of the shortest AC proteins

Figure 1. Apicomplexan ADF proteins share ~30% sequence identity with ADF from other organisms. (A) Structure-based sequence alignment of ADF/cofilins. Sequences were aligned using ClustalW and adjusted manually (Bowman *et al.*, 2000). Positions important for actin binding in *S. cerevisiae* cofilin, as indicated by mutagenesis (Lappalainen *et al.*, 1997) and synchrotron protein footprinting (Guan *et al.*, 2002), are marked by asterisks and circles, respectively. Residue numbers are indicated for *P. falciparum* ADF1 above and for *S. cerevisiae* cofilin below the alignment. The secondary structural elements from the crystal structure of *S. cerevisiae* cofilin (PDB entry 1COF) are indicated below the alignment (arrows for β -strands and spirals for α -helices). Strictly conserved and homologous residues are shown in red and blue, respectively. Sequences shown are *A. thaliana* ADF1 (GenBank sequence ID no. AAC72407), *Toxoplasma gondii* ADF (AAC47717; Allen *et al.*, 1997), *P. falciparum* ADF1 (NP703379), *P. falciparum* ADF2 (NP705497), *Drosophila melanogaster* twinstar (A57569), *Caenorhabditis elegans* unc60A (Q07750), *Homo sapiens* cofilin-1 (NP005498), and *S. cerevisiae* cofilin (Q03048). (B) Structural model of yeast cofilin (PDB entry 1COF) with the secondary structural elements numbered as in A. The F-actin binding site (shaded gray) locates to the F-loop and the adjacent C-terminal helix. Residues which, according to A, are lacking in the primary sequence of *PfADF1* are marked in red. (C) Sequence comparison illustrating that apicomplexan ADF proteins and the actin monomer binding protein twinfilin both diverge from the ADF homology consensus in the central F-actin interaction site. Positions important for interaction of yeast cofilin with actin polymers are indicated by arrows. Sequences shown are *S. cerevisiae* cofilin, *P. falciparum* ADF1, *T. gondii* ADF as well as the N-terminal and C-terminal ADF homology domains of mouse twinfilin (GenBank ID no. AAH15081).

Figure 2. *ADF1* is the major ADF isoform in motile stages of *Plasmodium*. Presence of *ADF1*, *ADF2*, and the unconventional class XIV myosin *MyoA* (as a control for invasive stages) was tested for in cDNAs from three invasive stages of the *Plasmodium* life cycle: oocyst sporozoites (oo) that invade the salivary glands of the *Anopheles* mosquito, salivary gland sporozoites (sg) infectious to the mammalian liver, and schizonts/merozoites (sch) that invade erythrocytes. All genes contain an intron distinguishing amplification from gDNA and cDNA, respectively. No amplification is observed in control reactions without reverse transcriptase (–RT). Note that *PbADF1* transcripts, like *MyoA*, are present in all invasive stages tested.

known, a polypeptide of 122 residues, and the *PfADF2* gene encodes a 143-amino acid protein.

A sequence alignment based on crystal structures of AC family members (Figure 1A) suggests that the *PfADF1* protein features shorter transitions between putative β -sheets 2

and 3 and between putative β -sheets 4 and 5. In addition, the short C terminus may possibly not fold into an α -helix, nor pack against the β 4/ β 5-loop (the F-loop) as in other ADF/cofilins. Yeast cofilin residues involved in actin binding have been identified by site-specific mutagenesis (Lappalainen *et al.*, 1997) and synchrotron protein footprinting (Guan *et al.*, 2002). Interestingly, the *PfADF1* sequence diverges in positions shown to be specific for binding to filamentous actin (ScCof residues R80 in the F-loop and E134/R135/R138 in the C terminus), whereas retaining the general actin binding sequences (Figure 1B). In this respect, *PfADF1* resembles the ADF isoform expressed by *Toxoplasma* (Allen *et al.*, 1997) and the ADF homology domains of the G-actin binding protein twinfilin (Figure 1C). The second isoform, *PfADF2*, resembles conventional ADFs in placement and composition of key sequences.

The *PfADF1* coding sequence was amplified from merozoite-derived cDNA. Similarly, the *P. berghei* ortholog (DQ000975) could be detected in several invasive stages of the *Plasmodium* life cycle (Figure 2). In contrast, we were unable to amplify either the *P. berghei* *ADF2* (DQ000974; Figure 2) or *P. falciparum* *ADF2* sequences (Schöler, unpublished data) from cDNA made from poly(A)⁺ RNA isolated from any one of several *Plasmodium* life cycle stages. These findings suggest that *ADF1* is the major AC protein expressed throughout the life cycle, whereas *ADF2* may serve a more specialized function.

Plasmodium ADF1 Plays an Essential Role during Erythrocytic Schizogony

To test whether *Plasmodium ADF1* is an essential gene, we used reverse genetics in the rodent malaria model parasite *P. berghei* (Thathy and Menard, 2002). Using an integration strategy, we constructed a targeting vector (pINT) that, upon a single crossover event during homologous recombination, would disrupt the endogenous *PbADF1* locus (Figure 3A). To control for gene targeting at the desired locus, we included an integration control plasmid (pCONT). After transfection the control plasmid results in a pseudodiploid allele with one functional *PbADF1* copy (Figure 3A). As expected, the latter resulted in viable recombinant parasites after a single transfection attempt (Figure 3B). In marked

Figure 3. *PbADF1* is essential for survival of erythrocytic stages. (A) Insertion strategy to generate the disruption of the *ADF1* locus. The wild-type (WT) *ADF1* genomic locus is targeted with *Xba*I (X)-linearized targeting plasmids containing the positive selectable marker (*dhfr/ts*), 5' truncations of the *ADF1* open reading frame, and a 3' truncation of *ADF1* in the case of the insertion plasmid (pINT) or a full-length 3' untranslated region in the case of the integration control plasmid (pCONT). On a single crossover event the region of homology is duplicated resulting in a disrupted insertion locus (*INT*) with two nonfunctional gene copies or a control locus (*CONT*) with a functional 5' copy. Black and gray arrows indicate primers that hybridize to regions in the plasmid backbone and outside the *ADF1* targeting fragment, respectively. (B) Genotyping of the recombinant parasite populations after *ADF1* gene targeting.

ing. Integration-specific PCR analysis confirms that gene targeting was successful for both populations tested in the case of the control insertion (*CONT*). In contrast, insertion of the disruption plasmid (pINT) was not detectable in any of the four resistant populations (*INT*). The wild-type-specific PCR reactions (WT) confirm the original genetic background in the *INT* populations. As expected, residual WT signals also are detectable in the nonclonal *CONT* populations. Specificity of the primer combinations was tested by amplification from wild-type DNA (WT) and the targeting plasmids (pl).

contrast, disruption of the *PbADF1* locus was not successful in four independent transfection attempts (Figure 3B). Together, these results indicate that *ADF1* performs vital functions during asexual development of the malaria parasite.

Recombinant PfADF1 Binding to G-Actin

We expressed *PfADF1* in *E. coli* and purified the protein using GST-affinity chromatography (Figure 4A). Most ADF/cofilins bind preferentially to ADP-bound monomeric actin (summarized in Chen *et al.*, 2004) and raise the thermal stability of this otherwise unstable form of actin (Schüler, unpublished data). Therefore, as a test for actin binding activity of the recombinant *PfADF1* protein, its effect on G-ADP-actin during heat denaturation was assessed. As illustrated in Figure 4, B and C, *Arabidopsis* ADF1 substantially stabilized monomeric ADP-actin against thermal unfolding. This was manifested by a shift of the midpoint of the thermal transitions (T_m) from 50.5 to 57°C for CaADP-actin and from 46.5 to 56°C for MgADP-actin. The *Plasmodium* ADF also stabilized monomeric actin, with a shift in T_m to 52°C for CaADP-actin and to 48.5°C for MgADP-actin (Figure 4, B and C). This *PfADF1* effect was moderate but significant (Schüler *et al.*, 2000). Thus, *PfADF1* clearly interacted with monomeric actin.

We recently succeeded to purify yeast-expressed *Plasmodium* actin (Schüler *et al.*, 2005). This allowed us to reconstitute homologous binding partners in the form of recombinant proteins. Here, the effects of *Plasmodium* ADF and yeast cofilin on the unfolding rates of MgADP-actins, incubated at a constant temperature of 50°C, were determined (Figure 5). A threefold molar excess of *Plasmodium* ADF slowed down denaturation of bovine β -actin by a factor of two (unfolding rates of 0.23 and 0.12 min⁻¹, respectively; Figure 5A), whereas yeast cofilin resulted in a fourfold slower unfolding (0.054 min⁻¹). Recombinant *Plasmodium* MgADP-actin unfolded with the same kinetics as the bovine cytoplasmic isoform (unfolding rates of 0.25 and 0.23 min⁻¹, respectively; Figure 5B and Schüler *et al.*, 2005). Surprisingly, threefold molar excess of *Plasmodium* ADF1 accelerated the unfolding of *Plasmodium* actin, whereas yeast cofilin had no effect.

PfADF1 Accelerates Nucleotide Exchange on Monomeric Actin

ADF/cofilins are known to inhibit nucleotide exchange on G-actin (Nishida, 1985), thereby accumulating pools of unpolymerized monomeric actin at sites of rapid filament disassembly. We used the fluorescence increase of ϵ ATP upon binding to actin to measure the effect of *PfADF* and *ScCof* on nucleotide exchange in β -actin (Figure 6). Yeast cofilin inhibited nucleotide exchange on CaATP-actin as expected. *PfADF1*, however, accelerated the rate of nucleotide exchange on β -actin in a concentration-dependent manner. This makes *PfADF* the first known member of ADF/cofilin proteins to accelerate rather than delay nucleotide exchange on actin monomers.

The *Plasmodium* ADF stimulated nucleotide exchange also on recombinant *Plasmodium* actin: a fourfold molar excess of *PfADF1* over 7 μ M *PfAct1* resulted in a 2.5-fold increase in the observed exchange rates (Figure 6B, inset). *PfAct1* alone exchanged nucleotide faster than bovine β -actin (observed exchange rates of 1.88×10^{-3} and 1.18×10^{-3} s⁻¹, respectively).

Using the AC protein-dependent effect on nucleotide exchange, we estimated the affinity of yeast cofilin for monomeric β -actin to be 0.67 ± 0.07 μ M, in good agreement with

Figure 4. Purification of recombinant *PfADF1*. (A) Coomassie-stained SDS-PAGE gel demonstrating the purity of recombinant *PfADF1*. Extracts of *E. coli* cells transformed with pGEX6P1-*PfADF1* were passed over immobilized glutathione. Proteins eluting from that column after protease cleavage were passed over Q-Sepharose. *PfADF1* passed the column (lane f), whereas impurities eluted at higher ionic strength (lanes 1–3). The position of *PfADF1* is marked by an asterisk. (B and C) ADF/cofilins affect the thermal unfolding of monomeric ADP-actin. Samples of 3 μ M CaADP-actin (B) and MgADP-actin (C), alone (open circles) or in presence of a twofold molar excess of AtADF1 (solid circles) or *PfADF1* (triangles), were subjected to heating at a rate of 40 K/h. Aliquots were removed at intervals, and their ability to bind and inhibit DNase was determined as a measure of their content of native actin. AtADF1 and *PfADF1* both shifted the midpoint of the transitions, yet *PfADF1* had a smaller stabilizing effect on actin than AtADF1. Shown are representative measurements from at least two independent experiments. The respective T_m values varied by $<0.5^\circ\text{C}$.

values determined with other isoforms (Hawkins *et al.*, 1993; Hayden *et al.*, 1993). *Plasmodium* ADF1 bound β -actin with an apparent affinity of 10.7 ± 3.9 μ M, in the range of the weakest actin affinities determined for AC proteins (Chen *et al.*, 2004).

Figure 5. *Plasmodium* ADF interacts differently with mammalian nonmuscle actin and recombinant *Plasmodium* actin. Samples of 3 μM bovine nonmuscle (A) and recombinant *Plasmodium* (B) MgADP-actin, alone (open symbols) or in presence of a threefold molar excess of *Plasmodium* ADF1 (filled symbols) or yeast cofilin (dotted symbols), were incubated at 50°C, and their loss of native actin was monitored at intervals as described above (Figure 4, B and C). PfADF1 and ScCof both slowed down the unfolding of cytoplasmic β -actin. Thermal denaturation of *Plasmodium* actin, on the other hand, was promoted by PfADF1 and not affected by ScCof.

PfADF1 Binds Preferentially to Monomeric ADP-Actin

We studied the interaction of PfADF1 with monomeric β -actin with either MgADP or MgATP bound. A gel shift assay (Figure 7) showed that although PfADF1 formed complexes with ATP-actin, the majority of actin monomers did not migrate as a complex (Figure 7A). In contrast, already at low concentrations PfADF shifted the migration of a considerable portion of ADP-actin monomers to the complex (Figure 7B). Yeast cofilin had a similar preference for ADP-bound actin monomers, although complex formation was efficient at lower ScCof concentrations compared with PfADF1, indicating that PfADF1 had a lower affinity for actin monomers than ScCof also under these conditions.

PfADF1 Does Not Modulate Actin Filament Formation In Vitro

AC proteins have several effects on actin polymers, all resulting in a faster turnover of microfilaments in the cell. ADF/cofilins can bind to actin filaments and promote their disassembly. ADF/cofilins probably also sever existing filaments, thereby promoting polymerization onto free filament ends. In addition, although increasing filament numbers, ADF/cofilins decrease the concentration of actin filaments by binding monomeric ADP-actin (Bamburg, 1999; Carlier *et al.*, 1999). Alignments of *Plasmodium* ADF1 with sequences

Figure 6. ADF/cofilins affect the exchange of actin-bound nucleotide. Bovine cytoplasmic β -actin with bound CaATP was freed of excess nucleotide by gel filtration and the fluorescence increase upon binding of ϵ ATP to actin was recorded. In A and B, the black traces show binding of ϵ ATP to 8 μM actin alone, whereas colored traces indicate presence of the AC protein (red, 4 μM ; green, 8 μM ; dark blue, 12 μM ; light blue, 16 μM ; and magenta, 24 μM). Increasing concentrations of ScCof (A) delayed nucleotide exchange, whereas PfADF1 (B) had the opposite effect of stimulating nucleotide exchange on monomeric actin. (B, inset) Recombinant *Plasmodium* actin (7 μM ; black trace) displayed a slightly faster apparent rate of nucleotide exchange as β -actin, and PfADF1 stimulated nucleotide exchange also on the parasite actin (magenta; 21 μM PfADF1). (C) Plot of the observed exchange rates at various AC concentrations in A and B (ScCof, circles; PfADF1, triangles).

of other ADF/cofilins suggested that PfADF1 lacks the typical conserved F-actin binding sites (Figure 1). We studied the interaction of PfADF1 with actin under physiological salt

Figure 7. *Plasmodium* ADF interacts preferentially with ADP-actin monomers. Native gel electrophoresis of monomeric Mg-ATP-actin (A) and Mg-ADP-actin (B) with increasing amounts of either PfADF1 or ScCof. The concentration of monomeric actin was 4 μ M throughout; concentrations of ADF and cofilins are indicated. In the presence of ATP, both PfADF and ScCof form moderate amounts of complexes with actin, whereas in the presence of ADP, both AC proteins efficiently shift the actin band to the position of the complex.

conditions using either spectrofluorimetry of pyrene-labeled actin or sedimentation assays.

When $MgCl_2$ and KCl at physiological concentrations were added to 8 μ M monomeric β -actin, polymer formation proceeded to completion within 10 min (Figure 8A). A two-fold molar excess of PfADF1 had no effect on these kinetics. Under the same conditions, AtADF1 accelerated filament formation dramatically, an effect which is often attributed to severing of existing polymers thus creating new polymerization nuclei (Du and Frieden, 1998). Additionally AtADF1 limited the level of steady-state fluorescence, indicating a lower steady-state concentration of F-actin by sequestering actin monomers.

We were recently able to demonstrate that *Plasmodium* actin has a low intrinsic ability to form polymers and that polymer formation could be stimulated by the nonphysiological factors gelsolin and phalloidin (Schüler *et al.*, 2005). Here, it was interesting to see whether also *Plasmodium* ADF could stimulate polymerization of *Plasmodium* actin. However, a twofold molar excess of PfADF1 did not affect the pyrenyl signal in 4 μ M *Plasmodium* actin, and actin polymer formation was not detected (Schüler, unpublished data). Thus, this corroborates our results established with bovine nonmuscle actin and suggests that the inability of PfADF1 to interfere with actin polymerization is not an artifact caused by combining heterologous binding partners.

For a more stringent measure of the ability of these ADF/cofilins to sequester monomeric actin under polymerizing conditions, serial dilutions of pyrene-labeled F-actin in the presence of either PfADF1 or AtADF1 were made and their fluorescence was determined (Figure 8B). This experiment showed that *Plasmodium* ADF1 (at either 4 or 8 μ M) left both

Figure 8. *Plasmodium* ADF affects neither the kinetics of actin polymerization nor the steady-state concentration of actin polymers. (A) Fluorescence increase due to polymerization of 2% pyrene-labeled β -actin at 8 μ M (open circles). Both the speed of initial filament assembly and the steady-state level of polymers were affected by a twofold molar excess of *Arabidopsis* ADF1 (solid circles) but not by *Plasmodium* ADF1 (triangles). (B) Serial dilutions of filamentous pyrene- β -actin alone (open circles) or in the presence of 2 μ M *Arabidopsis* ADF1 (dotted circles) or 4 and 8 μ M PfADF1 (upward and downward triangles, respectively). In contrast to the plant ADF, PfADF1 did not affect the critical concentration for actin filament formation. Solid circles represent the fluorescence of monomeric actin.

the steady-state pyrene fluorescence of F-actin and the critical concentration of actin polymerization virtually unaffected. On the other hand, *Arabidopsis* ADF1 (at either 2 or 3 μ M) induced a considerable shift in the critical concentration of filament formation. We determined the dissociation constant for AtADF1 and actin to 0.34 ± 0.03 μ M ($n = 3$), in good agreement with published values for AC proteins and skeletal muscle actin measured using the same method (Hawkins *et al.*, 1993; Hayden *et al.*, 1993; Carlier *et al.*, 1997).

PfADF1 Does Not Bind to F-Actin

As a further means to assess the interactions between PfADF1 or yeast cofilin and actin, cosedimentation assays were used. Samples of actin at 8 μ M were incubated under polymerizing conditions with increasing concentrations of either ScCof or PfADF1. High-speed supernatants and pellets were analyzed by SDS-PAGE. The amount of actin in the supernatants was only slightly affected by PfADF1 at a two- to threefold molar excess, suggesting a weak sequestering activity (Figure 9A). In contrast, ScCof efficiently seques-

Figure 9. *Plasmodium* ADF is an inefficient actin sequesterer and does not cosediment with actin polymers. Filamentous β -actin ($8 \mu\text{M}$) was coincubated with increasing concentrations of *Plasmodium* ADF1 (triangles) or yeast cofilin (circles). (A) Whereas yeast cofilin had a profound effect on the concentrations of sedimentable actin polymers, *Plasmodium* ADF1 sequestered actin monomers only weakly at a two- to threefold molar excess. The diagram at right shows a quantitation of the Coomassie-stained actin bands at left. (B) Whereas ScCof cosedimented efficiently with actin polymers, PfADF1 was not detected in F-actin pellets, even at high concentrations. The diagram at right shows a quantitation of the Coomassie-stained PfADF1 and ScCof bands at left. (C) Recombinant *Plasmodium* actin (PfAct1) at $7 \mu\text{M}$ polymerized inefficiently and was found almost exclusively in high-speed supernatants. Fourfold molar excess of PfADF1 did not influence the distribution of PfAct1. (D) PfADF1-activity is independent of pH. Filamentous β -actin ($8 \mu\text{M}$) was incubated alone or with either 6 or $16 \mu\text{M}$ ScCof or PfADF at either pH 6 or pH 8.5 for 4 h, before the samples were subjected to ultracentrifugation and supernatants and pellets were analyzed by SDS-PAGE. ScCof was a more efficient monomer sequesterer at higher pH. In contrast, independent of pH, PfADF did not cosediment with F-actin, and its monomer sequestering activity was low.

tered actin already at an equimolar ratio. PfADF1 did not cosediment with actin polymers at any molar ratio (Figure 9B). ScCof cosedimented efficiently at low ratios, and when

cofilin was added at a 3 M ratio, all F-actin in the pellet was cofilin decorated (Figure 9B). The ability of PfADF1 to sequester actin monomers was not affected by the state of the

actin-bound nucleotide: when polymers were formed in the presence of ADP instead of ATP, PfADF1 did not significantly raise the concentration of actin in the supernatants (Schüler, unpublished data). In combination with its homologous binding partner, PfADF1 did not affect the low polymerizability of recombinant *Plasmodium* actin, and both PfAct1 and PfADF1 remained in the supernatants (Figure 9C).

The monomer sequestering and polymer cosedimentation activities of many AC proteins (especially from vertebrates) are known to vary with pH (Hawkins *et al.*, 1993; Hayden *et al.*, 1993). We tested whether PfADF1 would display the typical AC protein activities at either lower or higher pH compared with the conditions used above. As shown in Figure 9D, PfADF1 cosedimented neither at pH 6 nor at pH 8.5, and its sequestering activity was equally weak over this pH range. As expected (Du and Frieden, 1998), yeast cofilin was more efficient at sequestering actin monomers at the higher pH, whereas we did not observe a pH dependence in its F-actin binding activity.

A prominent effect of AC proteins on actin filaments is the ~25-fold increase in off-rates at the minus end (Carlier *et al.*, 1997). Therefore, it was important to study the turnover kinetics of actin filaments in the presence of *Plasmodium* ADF1. Yeast cofilin strongly accelerated the loss of ϵ ATP-actin subunits from the ends of filaments, whereas PfADF1 had no effect (Schüler, unpublished data).

In conclusion, our experimental results show that actin-depolymerizing factor-1 is an essential protein in pathogenic red blood cell stages of *Plasmodium*. Recombinant PfADF1 binds to actin monomers under low salt conditions but has only a slight sequestering effect under physiological salt conditions. Most importantly, PfADF1 does not interact with actin polymers.

DISCUSSION

The most important physiological function of AC proteins is to accelerate actin dynamics by severing microfilaments and depolymerizing them from their pointed ends (Carlier *et al.*, 1997; Lappalainen and Drubin, 1997; Rosenblatt *et al.*, 1997). However, our biochemical analysis revealed that the essential *in vivo* function for *Plasmodium* ADF1 is not filament depolymerization. Instead, PfADF1 interacts exclusively with monomeric actin. Sequence alignments of the apicomplexan ADFs and other AC family members provide a plausible explanation for this finding. The conserved general actin binding residues of AC proteins are retained in the PfADF1 sequence, whereas the actin polymer binding positions clearly diverge (Figure 1). Thus, we propose that the main function of PfADF1 is not the acceleration of microfilament turnover through a direct interaction. This conclusion can likely be extended to ADF orthologues in *Toxoplasma* and other apicomplexan parasites and functionally separates these proteins from previously known ADF/cofilins.

Apicomplexan ADF sequences resemble twinfilins in that both show similar deviations from the ADF homology consensus, which can explain their inability to bind actin polymers. However, PfADF1 and twinfilins do not share other prominent features in their sequences, such as the C-terminal extension that mediates twinfilin binding to capping protein (Falck *et al.*, 2004). Thus, it seems unlikely that PfADF1 and twinfilin share their functions and regulatory mechanisms. An attractive hypothesis is that apicomplexan ADFs and twinfilins may share a common ancestor, namely, an AC protein with lost F-actin binding capacity. Twinfilin

may have arisen by a gene duplication event from this ancestor protein. Indeed, the apicomplexan ADFs have been placed near the evolutionary split of twinfilin from ADF/cofilins (Lappalainen *et al.*, 1998).

We found some striking similarities between PfADF1 and profilins. Both proteins influence microfilament turnover without interacting with polymeric actin. Unexpectedly, PfADF1 seems to catalyze the conversion of ADP-actin monomers into readily polymerizable ATP-actin monomers, another feature shared with profilin. Possibly apicomplexan ADF is used as an additional, differentially regulated monomer sequesterer besides profilin, a function that may be needed for the unusual dynamics of actin filaments in the parasites (Sibley, 2004). However, we found PfADF1 to be an inefficient sequesterer. AC proteins, although inhibiting the regeneration of actin-bound nucleotide, increase the pool of polymerizable actin because they bind monomers with high off-rates (Carlier *et al.*, 1999). Thus, provided that the kinetics of the interaction between PfADF1 and actin is fast, the major physiological effect of PfADF1 will most likely be the generation of rapidly polymerizable ATP-actin monomers.

The properties of PfADF1 are also reminiscent of CAP/Srv2 (cyclase-associated protein; Moriyama and Yahara, 2002a). Interestingly, *Plasmodium* contains a CAP-like protein (PlasmoDB entry PFA0260c) that is homologous to the C-terminal, actin binding domain of conventional CAP/Srv2 proteins, whereas lacking the N-terminal domain that mediates interaction with both adenylate cyclase and cofilin-actin complexes. The characterization of this protein and its relation to PfADF1 will be an important future goal.

F-actin severing activity of cofilin is essential for cell survival in yeast (Moriyama and Yahara, 2002b). However, the intrinsic instability of *Plasmodium* actin polymers (Schüler *et al.*, 2005) may eliminate the need for both the F-actin severing and the depolymerizing activities of AC proteins. We propose that apicomplexan ADFs are promoters of actin dynamics like typical AC proteins, albeit acting only on monomeric actin.

Because interactions of physiological binding partners may differ somewhat from the interactions of heterologous partners, it seems likely that PfADF1 binds *Plasmodium* and bovine β -actin differently. Yet, our results show that PfADF1 is unable to sever either β -actin or *Plasmodium* actin polymers. We noted that PfADF1 stabilized β -actin against thermal unfolding but destabilized recombinant *Plasmodium* actin. *Plasmodium* and bovine β -actin may differ in their binding of nucleotide and divalent cation. This is indicated by the fact that *Plasmodium* actin exchanged nucleotide faster than bovine β -actin (Figure 6). Because PfADF1 also stimulated nucleotide exchange, our results probably reflect differences in the PfADF1 effects on the actin-bound nucleotide, a strong determinant of the solution structure of actin (Schüler *et al.*, 2000).

Many ADF/cofilins bind to actin in a pH-dependent manner (Hawkins *et al.*, 1993; Hayden *et al.*, 1993). The structural basis for this regulation may be shifts in the position of the F-site (Pope *et al.*, 2004) and/or a pH-dependent conformational change in the actin molecule (Zimmerle and Frieden, 1988), which is recognized by AC proteins (Blondin *et al.*, 2002). As shown here PfADF1, which lacks the F-site, is not pH dependent in its actin monomer binding activity. We conclude that pH-dependent changes in actin are either not involved in the regulation of AC binding to actin monomers or that PfADF1 is unable to react to such changes. AC proteins of most species are negatively regulated by phosphorylation (Morgan *et al.*, 1993) on an N-terminal serine (Ser3 in vertebrates, Ser6 in plants). Although ADFs of api-

complexan parasites preserve serines in the corresponding positions their genomes do not encode orthologues of all metazoan genes involved in regulation of this modification: LIM kinases and slingshot phosphatases (reviewed in Soosairajah *et al.*, 2005) are lacking, whereas the phosphocofilin regulator 14-3-3 ζ (Gohla and Bokoch, 2002) and the cofilin phosphatase chronophin (Gohla *et al.*, 2004) seem to be represented in *Plasmodium* genome databases (Gardner *et al.*, 2002). The ADF/cofilin family also is negatively regulated by phosphatidylinositol 4,5-bisphosphate binding (Yonezawa *et al.*, 1990), providing a third means of regulation. The ability to turn off ADF/cofilin activity is essential for cell cycle progression in *Xenopus* oocytes (Abe *et al.*, 1996). However, it is possible that quick down-regulation of ADF function (through local pH changes or phosphorylation) is dispensable in the absence of F-actin binding in apicomplexan ADFs. Clearly, final assignment of the cellular function of these proteins in the parasites will have to await our understanding of their regulation. Nonetheless, our study establishes that a member of the apicomplexan subfamily of actin-depolymerizing factors exerts its vital physiological function through binding to actin monomers.

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