Minimal requirements for actin filament disassembly revealed by structural analysis of malaria parasite Actin Depolymerizing Factor 1

Wilson Wong¹, Colleen T. Skau³, Danushka S. Marapana¹, Eric Hanssen⁵, Nicole L. Taylor⁶, David T. Riglar¹, Elizabeth S. Zuccala¹, Fiona Angrisano¹, Heather Lewis¹, Bruno Catimel⁷, Oliver B. Clarke², Nadia J. Kershaw², Matthew A. Perugini⁶, David R. Kovar³,⁴, Jacqueline M. Gulbis²,⁸ and Jake Baum¹,⁸

¹ Infection and Immunity Division and ² Structural Biology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia.

Departments of ³ Molecular Genetics and Cell Biology and ⁴ Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637, USA.

⁵ Electron Microscopy Unit and ⁶ Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, Parkville, Victoria 3010, Australia.

⁷ Ludwig Institute for Cancer Research, Melbourne Tumour Biology Branch, Royal Melbourne Hospital, Parkville, Victoria, 3052, Australia.

⁸ Department of Medical Biology, University of Melbourne, Parkville, Victoria 3052, Australia.

Correspondence to:

Jake Baum
The Walter and Eliza Hall Institute of Medical Research
1G Royal Parade, Parkville 3052
Melbourne, Australia
Telephone: 61-3-93452476
Facsimile: 61-3-93470852
Email: jake@wehi.edu.au

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Abstract

Malaria parasite cell motility is a process that is dependent on the dynamic turnover of parasite-derived actin filaments. Despite its central role, actin’s polymerization state is controlled by a markedly reduced set of identifiable regulators compared to other eukaryotic cells. In *Plasmodium falciparum*, the most virulent species that affects humans, this minimal repertoire includes two members of the actin depolymerizing factor/cofilin (AC) family of proteins (PfADF1 and PfADF2). This essential class of actin regulator is involved in the control of filament dynamics at multiple levels, from monomer binding through to filament depolymerization and severing. Previous biochemical analyses have suggested that PfADF1 sequesters monomeric actin but, unlike most eukaryotic counterparts, has limited potential to bind or depolymerize filaments. The molecular basis for these unusual properties and implications for parasite cell motility have not been established. Here we present the first crystal structure of an apicomplexan AC protein, PfADF1. We show that PfADF1 lacks critical residues previously implicated as essential for AC-mediated actin filament binding and disassembly, having a substantially reduced filament-binding (F) loop and C-terminal α4 helix. However, in contrast to previous reports, we demonstrate that PfADF1 is capable of efficient actin filament severing. Furthermore, this occurs in spite of PfADF1’s low binding affinity for filaments. Comparative structural analysis along with biochemical and microscopy evidence establishes that severing is reliant on availability of an exposed basic residue in the F-loop, a conserved minimal requirement that defines AC-mediated filament disassembly across eukaryotic cells.
Introduction

The eukaryotic parasites that cause malaria disease - from the genus *Plasmodium* - require rapid cell movement to complete development, a process dependent on a parasite-derived actomyosin motor (1, 2). Short dynamic actin filaments engage with an internal single-headed myosin, generating force that propels parasites along substrates or into host cells (reviewed in (3)). Drugs that stall actin filament growth or stabilize them from depolymerization each prevent parasite motility when used at high concentrations, demonstrating the importance of dynamic actin (4-8). Despite this central role, however, actin filament turnover across all Apicomplexa, the phylum to which malaria parasites belong, is controlled by only a minimal set of identifiable regulators (3, 9). In *P. falciparum*, the most virulent species causing malaria disease, this minimal set includes two members of the actin depolymerization factor/cofilin (AC) family of proteins, ADF1 and ADF2 (10).

AC proteins function as key regulators of actin turnover in diverse cellular processes (reviewed in (11)). Their function is linked to an ability to interact, to varying degrees, with both monomeric (G) and filamentous (F)-actin. This activity is regulated by phosphorylation (12, 13), phosphoinositide binding (14, 15) and spatial concentration within the cell (16). Structurally, the G-actin sequestering activity has been shown to depend on possession of a conserved binding site involving the AC protein N-terminus, a long α3 helix (following yeast cofilin, ScCOF, nomenclature (17)) and turn connecting the β6 strand and α4 helix (17-19). Equally important is possession of a filament binding site, thought to involve charged residues located at the extended filament binding loop (F-loop) and C-terminal α4 helix as well as the C-terminal tail, which fold together to form a highly conserved F-actin binding motif (19-21).

Whilst ADF2 is thought to function in sexual stages of parasite development (10, 22), studies in the mouse malaria, *P. berghei*, and malaria-related parasite *Toxoplasma gondii* have established that
ADF1 (and orthologue TgADF) is essential for parasite viability (10) and actin filament turnover (23). Biochemical studies of recombinant ADF1 from the most virulent human parasite, *P. falciparum*, have suggested several unique properties. While it can sequester G-actin PfADF1 is unable to disassemble F-actin – a feature attributed to predicted divergence in its structure compared to other better-characterized AC proteins (10). Furthermore, and uniquely, PfADF1 can stimulate nucleotide exchange *in vitro*, a property usually associated with profilin (10). Neither the structural basis for these properties nor *in vivo* functions of ADF1 has been established.

Here we present biochemical and microscopy evidence that, contrary to previous reports, PfADF1 mediates actin filament severing at nanomolar concentration. The crystal structure of *P. falciparum* ADF1 presented here, however, reveals that this occurs despite PfADF1 possessing a markedly reduced F-loop and C-terminal α4 helix. Importantly, conserved charged residues implicated in the F-actin binding and severing motif are absent in the PfADF1 structure with the exception of a single ‘exposed’ basic residue. Using comparative structural analysis of PfADF1 with other AC members and analysis of mutants for this residue, we establish that the exposed basic residue presented by the F-loop defines a minimal motif, conserved across the entire family of AC proteins, required for filament severing.

**Results**

**Spatiotemporal localization of PfADF1 in asexual blood-stage parasites.** Of the two AC proteins expressed in malaria parasites, previous reports have suggested ADF1 is a monomer-sequestering actin regulator with minimal filament-disassembly or binding activity (10). To further dissect the cellular and biochemical properties of AC proteins from *P. falciparum*, we expressed both PfADF1 and PfADF2 in *Escherichia coli* and used these to generate AC-specific antisera (Fig. S1A,B). Immunoblot analyses on whole parasite lysates revealed that PfADF1 is expressed broadly, reaching maximal expression late in the 48 hr asexual blood-stage lifecycle (Fig. 1A), a time when motile merozoite forms are maturing for cell re-infection (1). This peak of expression is similar to that of PfAct1 (Fig. 1A) and other proteins that function in erythrocyte invasion (1). Expression of
PfADF2 was not detected (Fig. S1 A,B) consistent with it having a role in extra-erythrocytic or sexual stages (22). Immunofluorescence assays (IFA) of schizont stages (pre-merozoite release) demonstrated broad labeling across the parasite cytosol with the exception of the nucleus (marked by DAPI) (Fig. 1B). This distribution was consistent with that of PfAct1 (Fig. 1B). Solubility of PfADF1 in cell extracts following hypotonic lysis suggested a free association in the cytoplasm rather than association with detergent soluble membrane fractions (Fig. 1C). In support of this, recombinant PfADF1 displayed low micromolar affinity for phosphatidyl inositol derivatives (Fig. S1C), lipid groups used to recruit AC proteins to the plasma membrane in other eukaryotes (14, 15).

Erythrocyte invasion is a time when actin dynamics are required for parasite movement (5, 6) and, as such, a time when actin regulators are expected to be active (3). We explored PfADF1 localization by IFA in free merozoites captured mid invasion and co-labeled with rhoptry neck protein (RON)4 a marker of the tight junction (24) - the point of close apposition between host and parasite membranes during invasion. This was recently shown to be the site at which a ring of actin forms (24), a feature consistent with engagement of the actomyosin motor at the junction. PfADF1 retained its cytosolic localization during invasion (Fig. 1D) with the exception that labeling was absent at the tight junction or associated actin ring (Fig. 1D). This distribution would support a predominant role for the majority of PfADF1 molecules in actin monomer sequestration, though does not directly address its function with respect to filaments.

**X-ray crystal structure of PfADF1 confirms presence of G-actin binding site.** To assess the biochemical properties of PfADF1 in greater detail we prepared recombinant PfADF1 for crystallographic structure determination. X-ray diffraction from PfADF1 crystals permitted structure determination and refinement to 1.6 Å (Table S1). Defined electron density of the whole protein chain was interpretable except for the disordered C-terminal Lys122, which was omitted. PfADF1 adopts the conserved α/β fold of the AC family - a six-stranded β-sheet in which four anti-parallel β strands (β2-β5) are flanked on either edge by a shorter parallel strand along with four α helices surrounding the β-sheet (Fig. 2A and S2). Structural alignment of PfADF1 with budding
yeast cofilin, ScCOF, showed that the G-actin binding sites (Fig. S2) (18, 25, 26) superimposes closely (RMSD on 41 Cα atoms = 1.6 Å) (Fig. 2B). In PfADF1, β6 is markedly shorter than in ScCOF and, significantly, loops out from the sheet. Since residues from β6 provide contact sites for subdomain 3 of the actin monomer identified in the crystal structure of the mouse-twinfilin-actin complex, it is possible that divergence in this region in PfADF1 may provide the basis for promoting nucleotide exchange (10), inducing a more open configuration in the cleft between subdomain 2 and 4 of the actin monomer. A conserved serine at the N-terminus that is frequently the target for AC-phosphoregulation (12, 13) is well defined in the PfADF1 electron density map (Fig. 2C and D). Given its exposed position and overlap with the G-actin binding site of the N-terminal tail, phosphorylation at this residue would prevent complex formation with actin, suggesting the possibility of phosphoregulation in PfADF1.

**F-actin binding motifs and associated residues are absent in PfADF1.** Comparison of PfADF1 structure with other AC proteins (including PfADF2) revealed significant changes in a key structural motif implicated in actin filament binding and disassembly (17, 19-21) - the F-loop and C-terminal helix (α4), which together form the putative F-actin-binding fold (21, 27). Whilst the basis for AC protein-filament interactions are not known (27) several charged residues in this motif have been implicated in the process (17, 19-21). Chief amongst these are Lys79, Arg80 and Lys82 of ScCOF located on β5 (or Lys95 and Lys96 of HsCOF), and Glu134, Arg135 and Arg138 of ScCOF located on α4 (19, 21) (Fig. S2).

In PfADF1, truncation of α4 has eliminated the latter cluster of charged residues (Fig. 3A, S2 and Fig. S5). Moreover, strands β4 and β5 are substantially shorter than in ScCOF, such that the tips of the connecting F-loops in superimposed Pf and Sc structures are separated by a distance of 16 Å (Fig. 3A, 3C, S2). Importantly, this combination of features alters the molecular geometry so as to preclude a direct interaction between the F-loop and C-terminus. A key difference is that whereas in ScCOF Lys82 is sufficiently close to make an intramolecular hydrogen bond to the α4 main chain
(via Val^{136}, Fig. 3B), the homologous residue in PfADF1 (Lys^{72}) instead makes an intermolecular hydrogen bond to an adjacent molecule in the crystal. Since the distance between Lys^{72} and the C-terminal Ile^{121} is ~13.4 Å, Lys^{72} would be solvent exposed in solution (Fig. 3B). Notably, although the conserved lysine is positioned and oriented similarly in the two structures, in ScCOF Lys^{82} lies midway along β5, whereas in PfADF1 it is the very first residue of a shortened β5. Overall, this leads to a pronounced difference in the morphology and electrostatic appearance of PfADF1. The *Plasmodium* protein has an exposed hydrophobic crevice on an otherwise ‘flattened’ face (Fig. 3C), which in ScCOF is enclosed by α4 and the extended F-loop.

**PfADF1 can mediate filament severing despite substantial divergence in the F-actin binding motif.** To investigate its ability to regulate actin dynamics *in vitro*, we first examined the binding affinity of PfADF1 for rabbit muscle F-actin. A fixed concentration of PfADF1 was incubated with varying concentrations of pre-formed filaments followed by ultracentrifugation to determine the dissociation constant (K_d) between PfADF1 and filaments. SDS-PAGE and densitometry analysis showed that all PfADF1 remained in the recovered supernatant across all filament concentrations tested (Fig. S4), thus precluding determination of the K_d but suggesting that PfADF1 has a very low affinity for actin filaments. Control measurement with PfADF2 (having a more canonical F-loop and C terminal helix (Fig. S5)), whilst still low, showed a K_d of 64.6 µM (Fig 4A and S4).

Despite showing low affinity for actin filaments, sedimentation analysis, where PfADF1 was varied with respect to a fixed concentration of pre-formed filaments, resulted in concentration-dependent increase of actin in the supernatant (p < 0.05, t-test, 0 vs. 16 µM of PfADF1; Fig. 4B,C). This result, in contrast to previous reports (10), unexpectedly demonstrates that PfADF1 can facilitate actin filament severing and/or depolymerization despite possessing a substantially divergent F-actin binding motif. This is in line with recent observations from its homologue in *T. gondii* TgADF1 (28). PfADF2 was similarly able to mediate filament disassembly whilst GST alone had no effect (Fig. 4B,C). To examine if PfADF1 can disassemble filaments composed of native *P. falciparum* Actin1, recombinant PfAct1 was expressed and purified from *E. coli* (Fig. S6) and verified for
structural integrity by circular dichroism spectroscopy (Fig. S6A,B and E), and functionally analyzed by sedimentation under polymerizing conditions (Fig. S6F). As with rabbit actin, PfADF1 was able to induce concentration dependent disassembly of pre-formed PfAct1 filaments (p <0.05, t-test, 0 vs.16 µM of PfADF1, Fig. S6G-H).

To explore the nature of PfADF1-mediated filament disassembly, we visualized pre-formed actin filaments in the presence of PfADF1 by electron microscopy (Fig. 4D). Co-incubation of actin filaments in the presence of 30 or 250 nM of PfADF1 were strongly associated with shorter lengths of pre-formed actin filaments (Fig. 4E, p <0.05, t-test, control vs. 30 or 250 nM PfADF1), suggesting severing. To verify this, direct observation of actin filament kinetics was undertaken with total internal reflection fluorescence (TIRF) microscopy (29). Whilst no increased rate of depolymerization was seen, PfADF1 efficiently severed filaments at a comparable rate to that of Schizosaccharomyces pombe SpCOF and human COF1 (Fig. 4F-H; Movie S1) (16) demonstrating maximal severing rates at between 10 and 100 nM. Combined, these results conclusively demonstrate filament severing by PfADF1. They establish that severing can occur despite a substantial reduction in the extended F-loop and C-terminal α4 helix. Furthermore, they demonstrate that severing does not require a high affinity interaction between an AC protein and an actin filament, consistent with previous findings where optimal severing concentration is significantly lower than the K_d between an AC protein and an actin filament (16).

**A minimal of one solvent exposed basic residue in the F-loop is essential for AC-mediated filament severing.** To investigate a universal mechanism of AC-mediate filament severing, we explored whether Lys^{72}, the only conserved solvent exposed basic residue retained in the F-loop of PfADF1 in comparison to other AC proteins (Fig. 3B, S2 and S5), is the minimal requirement for severing of actin filaments. A mutant PfADF1 was generated, PfADF1.K72A, replacing the exposed basic residue for a non-polar alanine. Circular dichroism spectroscopy confirmed the folding of PfADF1.K72A to be identical to that of the wild type protein (Fig. S6C, D and E). Electron and TIRF microscopy combined with actin sedimentation assays conclusively shown that
the PfADF1.K72A mutant is not able to mediated filament disassembly or severing (Fig. 4C-E, H). This establishes that Lys$^{72}$, the only solvent exposed basic residue in the F-loop, is the essential requirement for PfADF1-dependent filament severing activity. We explored the universality of this mechanism by generating an HsCOF mutant in which the native F-loop was replaced by that of PfADF1, along with comparable elimination of its $\alpha_4$ helix. This HsCOF-Pf mutant was able to mediate severing (Fig. 4H, $p <0.05$, t-test, HsCOF-Pf vs. buffer control). As a control, a reciprocal PfADF1 with the F-loop and $\alpha_4$ helix of HsCOF was generated (PfADF1-Hs), which retained a similar degree of severing (Fig. 4H, $p <0.05$, t-test, PfADF1-Hs vs. buffer control). The reduction of activity compared to native ADFs for both mutants was likely the result of their chimeric nature. Collectively, these data establish that a single solvent exposed basic residue in the F-loop is the minimal requirement for AC-protein mediated actin filament severing.

**Discussion**

Cell motility and host cell invasion in malaria parasites is critically dependent on the regulated assembly and disassembly of actin filaments (5, 6). However, despite its centrality to parasite development malaria and related parasites from the phylum Apicomplexa possess a markedly reduced repertoire of known eukaryotic actin regulators (3). The core repertoire retained includes two formins, profilin, a CAP/Srv2 homolog, capping protein subunits, coronin and two members of the AC family of proteins (reviewed in (3, 9). Of these, CAP/Srv2 and one of the capping protein subunits have been shown to be non-essential for asexual development (9). Given the importance of actin dynamics on asexual development, and in invasion in particular (5), this focuses attention on likely critical roles for the remaining actin regulators. Towards their detailed characterization we present here the first crystal structure of an apicomplexan AC protein, PfADF1, which reveals a substantial divergence in motifs traditionally associated with F-actin binding. Despite these differences in structure and apparent low binding affinity for filaments compared to other AC proteins, PfADF1 is, contrary to previous reports (10), capable of mediating filament disassembly via severing. This is in line with observations from its homologue in *T. gondii* TgADF1 (28).
Importantly PfADF1 is capable of disassembly of PfAct1 filaments, which is markedly divergent from that of other eukaryotes (3).

The crystal structure of PfADF1 completes the triad of the core actin-monomer binding proteins in apicomplexan parasites, with structures recently published for PfProfilin (30) and CAP/Srv2 from the malaria-related parasite Cryptosporidium parvum (31), with each, like PfADF1, possessing features unique to the phylum. Our structural analysis of PfADF1 reveals that it retains the conserved G-actin binding motif, comprised of the N-terminus, long α3 helix and turn connecting the β6 strand and α4 helix. However, structural characteristics associated with filament binding are more divergent. Present understanding has linked F-actin binding and disassembly/severing to charged residues located at the extended F-loop and C-terminal α4 helix as well as the C-terminal tail, which fold together to form a highly conserved F-actin binding motif (19-21). A marked reduction in size of the F-loop and near absence of the α4 helix in PfADF1 based on present understanding of the process would suggest filament disassembly is not a feature of apicomplexan AC proteins. However, we clearly demonstrate that PfADF1 is capable of F-actin severing. This observation is strongly supported by recent biochemical data from the malaria-related parasite Toxoplasma gondii, in which the direct orthologue of PfADF1, TgADF, also mediated actin filament disassembly and severing (28). The ability to mediate disassembly independent of the implicated filament-binding motifs (19, 21) is inconsistent with expectations. Previous characterizations of AC proteins suggested these properties were dependent on charged residues located at the extended F-loop (Arg80 and Lys82 of ScCOF; Lys95,96 of HsCOF; Fig. S2 and 3B) and α4 helix (Glu134, Arg135 and Arg138 of ScCOF; Fig. S2 and 3A) (19, 21), leading to a model in which molecular interactions between AC proteins and the actin filament could be partly driven by salt-bridge or polar interactions. The structure of PfADF1 shows that these charged residues, with the exception of Lys72 (homologous to Lys82 of ScCOF), are absent. This suggests either PfADF1 and its apicomplexan orthologues employ a unique mechanism that facilitates actin severing in the
absence of conserved motifs or that our understanding of AC-mediated disassembly, in general, requires refinement.

Towards the latter possibility, comparative structural analysis of representative AC proteins and their varying capacity to bind and sever F-actin (Table 1) leads us to suggest a revised model in which filament severing does not require high affinity binding to filaments but instead depends on the availability of a free basic residue in the F-loop exposed for interaction with F-actin (Fig. S7). Filament binding is observed with several structurally defined AC proteins, including budding yeast ScCOF and ScABP1, human HsCOF and Acanthamoeba Actophorin (Table 1). Each has a stabilized F-actin binding fold that is ‘anchored’ by polar interactions between the F-loop and α4 helix (Lys$^{82}$ of β5 with main chain amide of Val$^{136}$ of α4 in ScCOF (19)) (Fig. 3B). In ScABP1, which does not cause filament disassembly (32), anchoring is achieved via the homologous Lys$^{80}$ interacting with main chains of Ile$^{135}$, Ser$^{136}$ and Ala$^{138}$. Indeed, when Lys$^{80}$ is substituted for an alanine residue this significantly reduces the binding of the protein to filaments (32) presumably via disruption of the F-binding fold. Similar phenotypes are observed when this anchoring residue is mutated in ScCOF (Lys$^{82}$ to alanine in ScCOF) (19). In HsCOF, anchoring is inverted with interactions mediated between Lys$^{152}$ of α4 and Asp$^{98}$ of the F-loop (Fig. 3B). Mouse twinfilin retains an extended F-loop and α4 helix (26) and superficially resembles a canonical AC protein. However, its F-loop contains significantly divergent residues, (Fig. S2 and 3B) potentially explaining twinfilin’s inability to bind or disassemble actin filaments (33). In this context, the reduction of the F-loop and α4 helix in PfADF1 and absence of necessary polar interactions that facilitate an anchored fold are consistent with PfADF1’s apparent low affinity for filaments. This suggests that the anchored conformation is essential for high affinity binding to actin filaments. Supporting this, PfADF2, which retains a more classical F-loop and C-terminal α4 helix in its predicted structure showed improved affinity for actin filaments relative to PfADF1.

Each of the structurally characterized AC proteins able to sever actin filaments retains a basic residue in the F-loop that is exposed to the solvent (Table 1). The side chain of this basic residue
(Arg$^{80}$ in ScCOF, Lys$^{96}$ in HsCOF and Arg$^{76}$ in AcActophorin, Fig. 3B), which positions toward the α4 helix (following ScCOF), always faces the same direction as the anchoring residue (Lys$^{82}$ of ScCOF) or the exposed residue (Asp$^{98}$ in HsCOF and Lys$^{78}$ of AcActophorin) present in the F-loop, and is predicted to be orientated to interact with subdomain 2 of an actin monomer in the filament (Fig 3B) (21, 27). This ‘exposed’ basic residue is notably absent in ScABP1, where the sole basic Lys$^{80}$ of the F-loop is occupied in polar interactions with the α4 helix. However, it is present in PfADF1, despite reduction of the α4 helix and extended F-loop. This exposed Lys$^{72}$ is the only structurally conserved motif, already implicated in filament binding, which can singularly explain the potential for filament severing in AC proteins. Importantly, we show that substitution of Lys$^{72}$ to alanine disrupts the ability of PfADF1 to mediate filament disassembly and severing. Furthermore, chimeras where the native F-loop and α4 helices of PfADF1 and HsCOF are interchanged can both still mediate filament severing. Collectively, these data demonstrate that AC-mediated filament severing requires a minimal of one exposed basic residue in the F-loop.

In the recent characterization of T. gondii ADF1 (also presumed to lack the anchored fold), addition of the C terminal α4 helix of SpCOF reduced the ability of this AC protein to mediate filament disassembly with a reported, though not shown, increase in F-actin binding potential (28). We predict that addition of an α4 helix structurally altered this protein making it more akin to ScABP1, in which the previously exposed Lys$^{68}$ is drawn into a polar interaction with the C terminal helix. Presence of an exposed basic residue in the F-loop would therefore be expected, irrespective of whether an anchored F-loop/α4 helix fold exists, to enable filament severing. In HsCOF, Lys$^{95}$ was reported to be required for filament severing with an HsCOF.K95QK96Q mutant unable to sever filaments (21) (Table 1). However, the ScCOF.R80AK82A mutant that still possessed Lys$^{79}$ (homologous to Lys$^{95}$ of HsCOF) lost severing activity (19)(Table 1). This rules out Lys$^{95}$ of HsCOF as a requirement for severing, with the loss of activity found in HsCOF.K95QK96Q solely due to the K96Q substitution according to our model here. Consistent with this hypothesis, Lys$^{95}$ of HsCOF and Lys$^{79}$ of ScCOF both face away from the α4 helix (Fig. 3B). Collectively these
observations indicate that for AC-mediated severing to occur the ‘exposed’ basic residue must also orientate in the direction of the $\alpha 4$ helix.

High-resolution details of the interface formed between AC-proteins and the actin filament will be needed to determine the molecular mechanism through which such an exposed residue could mediate severing. Cryoelectron microscopy of AC-decorated actin filaments suggests that the two sites on opposing ends of the AC protein (F-actin and G-actin binding motifs) mediate binding to subdomains 1-2 of the lower and subdomains 1-3 of the upper actin subunit, respectively (21, 27). It has also been suggested that a local twist in the filament structure induced by AC-binding is associated with filament severing (27). Our data suggest the ability to sever must reside in a balance between the presence of functional G-actin binding surfaces and whether an ‘exposed’ basic residue in the F-loop is available (Fig. S7).

The reduced repertoire of actin regulators in malaria and other apicomplexan parasites highlights the power of these remarkable ancient eukaryotes for investigation of universal mechanisms of actin regulation. Furthermore, the reliance on a minimal set of actin regulators in the malaria parasite may constitute a hitherto unexplored target for therapeutic intervention against this increasingly drug resistant pathogen of global significance.

**Materials and Methods**

**Parasite Cultures, Imaging and Immunoblotting.** Wild type (D10) asexual parasites were maintained in standard culture conditions and synchronized as described (24). Free merozoites were isolated and processed for microscopy following (24). Antibodies and image processing are described in SI Materials and Methods.

**Expression and Purification of Recombinant AC proteins and PfAct1.** Full length *PfADF1* and *PfADF2* were PCR amplified from *P. falciparum* genomic DNA and expressed as GST-fusion proteins using the pGEX4T vector (GE Healthcare). PfAct1 was expressed as hexa His-fusion
protein using the pET28 vector (Novagen). Full details on amplification, expression and purification of each AC protein and mutants are described in SI Materials and Methods.

**Crystallization and X-ray Data Collection.** Purified PfADF1 (minus the GST tag) in storage buffer (20 mM Tris, pH8, 10 mM NaCl, 5 mM 2-Mercaptoethanol and 0.02 % NaN₃) was concentrated to 6.6 mg/ml and crystals were grown at 22 °C by hanging drop vapor diffusion mixing 1 µl protein solution with 1 µl of reservoir buffer consisting of 1.8 M (NH₄)₂SO₄, 0.2 M KNa tartrate, 0.1 M NaOAc, pH 4.6. Crystals were equilibrated into cryoprotectant consisting of 0.2 M KNa tartrate, 3 M Na Malonate, pH 4.6. X-ray diffraction data were collected at the Australian Synchrotron. Data were integrated and scaled with HKL2000 (34). Structure determination and refinement is described in SI Materials and Methods. Data collection and refinement statistics for PfADF1 are presented in Table S1. Coordinate and structure factors are available from the Protein Data Bank (3Q2B).

**Actin Biochemical Assays.** Filament disassembly was assayed by analytical ultracentrifugation at 100,000 g (TLA100 rotor, Beckman Coulter Optima TL Ultracentrifuge) with rabbit actin (4 µM; Cytoskeleton, Inc.) or PfAct1 (4 µM) essentially as described (28) with varying concentrations of recombinant PfADF1, PfADF2 or control GST added to pre-formed actin filaments prepared under polymerizing condition for 1 hr in a predominately ADP + Pᵢ rich form 1 hr before sedimentation. Quantification of protein in pellet and supernatant fractions was performed by densitometry analysis (GS-800 calibrated densitometer, Bio-Rad). PfADF1 and 2 (fixed at 5 µM) were added to varying concentrations of pre-formed actin filaments (polymerized for 1hr) for 30 min at room temp. A pre-centrifugation aliquot was removed, the remainder was centrifuged 100,000g for 1hr. An equal amount of pre- and post-centrifugation was analysed by SDS-PAGE and densitometry. The amount of ADF bound to pre-formed actin filaments was determined as [Total ADF (5µM)] – [Free ADF, post-centrifugation]. Free actin concentration was determined as [Total Actin] – [Bound ADF]. Using a fixed concentration of PfADF1 (5 µM), the equilibrium dissociation constant (Kd) was calculated as, Bound/Sites (5 µM) = [Free Actin]/(Kd – [Free Actin]) (35). The data were fit to
this equation using nonlinear regression (Prism). Electron and TIRF microscopy followed established protocols (21, 29) detailed further in SI Materials and Methods.

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References


**Figure Legends**

**Figure 1. Expression and localization of PfADF1 and PfAct1 in *P. falciparum***. (A) Immunoblot of whole parasite lysates across the 48 hr lifecycle (0 hr indicates re-invasion, red arrow) probed with antiserum against PfADF1, PfAct1 (24) and PfAldolase (loading) and PfAMA1 (timing) (1). (B) IFA of mature *P. falciparum* schizont stages labeled with PfAct1, PfADF1 and PfRON4 antisera (r = rabbit, m = mouse). Scale bar = 2µm. (C) Solubility of schizont and merozoite lysate following hypotonic lysis. Control immunolabeling with MSP1-19 (a GPI anchored protein) and Hsp70 as a loading control. P = pellet fraction; S = supernatant fraction. (D) IFA of merozoite invasion probed with anti-PfADF1, PfAct1 and PfRON4 during merozoite invasion. Scale bar = 2µm.

**Figure 2. Crystal structure of PfADF1 confirms the conserved G-actin binding fold**. (A) Schematic representations of the overall fold of PfADF1 related by 90° rotations. (B) Overlay of crystal structures of PfADF1 (purple; 3Q2B) and ScCOF (yellow; 1COF) orientated to show G-actin binding face conserved between two proteins. (C) Cartoon representation of PfADF1 structure showing Ser3 at the N-terminal tail. (D) 2|Fo|-|Fc| electron density map depicting the N-terminal tail of PfADF1. Residue Ser3 is shown as green stick. The map is contoured at 1.2 σ. Water molecules have been removed for clarity.

**Figure 3. F-loop and C-terminal α4 helix implicated in filament disassembly are substantially reduced in PfADF1**. (A) Overlay of the crystal structures of PfADF1 (3Q2B) with AC proteins (MmTwin, 3DAW; SpCOF, 2I2Q and ScCOF, 1COF) shown from two different viewpoints related by 130°. PfADF1 and AC proteins are shown as blue and yellow respectively. (B) Schematic representations of the F-loops from AC proteins. Basic residues in the ‘exposed’ state present in the
F-loops are highlighted in red text. Residues involved in polar interactions between the F-loop and α4 helix are highlighted in purple text. Hydrogen bonds are indicated by dash lines. (C) Transparent surface representation of PfADF1 and ScCOF structures.

**Figure 4. Filament disassembly by PfADF1 and its binding affinity for filaments.** (A) Graph of Bound/Sites versus free protein for PfADF1 and PfADF2. Curve is the best fit to the data, with a $K_d$ of 64.6 $\mu$M, n = 3 for PfADF2. A $K_d$ for PfADF1 was not determinable. (B) Ultracentrifugation of PfADFs with pre-formed actin filaments. S = Supernatant, P = Pellet. (C) Quantification of proportion of actin in respective fractions (shown below gels) represents mean ± SEM for n=3; p = 0.023, t-test, 0 vs 16 $\mu$M PfADF2; p = 0.035 0 vs 16 $\mu$M PfADF1. (D) EM of rabbit actin filaments + or – PfADF1 and PfADF1.K72A mutant. Scale bars are indicated. (E) Quantification of actin filament length in presence of PfADF1 and PfADF1.K72A. Data presented = mean ± SEM. (F-H) TIRF microscopy observation of real-time filament severing by PfADF1. (F) Representative time-lapse micrographs of the indicated reactions. Scale bar = 5 $\mu$m with addition of buffer alone, 250 nM PfADF1 or 250 nM SpCOF at time zero. See also Movie S1. Numbers indicate time in sec. (G) Plot of the severing rate (breaks $\mu$m$^{-1}$ sec$^{-1}$) in the presence of different concentrations of PfADF1. (H) Plot of the severing rate (breaks $\mu$m$^{-1}$ sec$^{-1}$) in the presence of indicated proteins (n=10 filaments per condition) at 100 nM. Values are mean ± SD.

**Table Legends**

**Table 1.** Summary of AC protein actin filament binding and severing properties
**Table 1. Summary of AC protein actin filament binding and severing properties**

<table>
<thead>
<tr>
<th>AC protein</th>
<th>PDB</th>
<th>F-loop</th>
<th>α4 helix</th>
<th>F-binding</th>
<th>Severing</th>
<th>K79</th>
<th>K80</th>
<th>K82</th>
<th>Ref.</th>
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<td>K</td>
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<td>K Anchored</td>
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<td>K exposed</td>
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<td>-</td>
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<td>++</td>
<td>A</td>
<td>V</td>
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<td>G</td>
<td>K ~Anchored</td>
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</tbody>
</table>

* Structurally defined AC proteins with demonstrated severing activity. † HsCOF mutant with HsCOF F-loop replaced by PfADF1 F-loop. ‡ A mutant form of TgADF with an α4 helix of SpCOF fused to the C-terminus of the protein. § ‘~’ Predicted structure in the absence of experimental proof. ¶ No binding to filaments. || Strong severing activity. ** Inability to sever.