The Lasso Segment Is Required for Functional Dimerization of the *Plasmodium* Formin 1 FH2 Domain

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Abstract

Apicomplexan parasites, such as the malaria-causing *Plasmodium* species, utilize a unique way of locomotion and host cell invasion. This substrate-dependent gliding motility requires rapid cycling of actin between the monomeric state and very short, unbranched filaments. Despite the crucial role of actin polymerization for the survival of the malaria parasite, the majority of *Plasmodium* cellular actin is present in the monomeric form. *Plasmodium* lacks most of the canonical actin nucleators, and formins are essentially the only candidates for this function in all Apicomplexa. The malaria parasite has two formins, containing conserved formin homology (FH) 2 and rudimentary FH1 domains. Here, we show that *Plasmodium falciparum* formin 1 associates with and nucleates both mammalian and *Plasmodium* actin filaments. Although *Plasmodium* profilin alone sequesters actin monomers, thus inhibiting polymerization, its monomer-sequestering activity does not compete with the nucleating activity of formin 1 at an equimolar profilin-actin ratio. We have determined solution structures of *P. falciparum* formin 1 FH2 domain both in the presence and absence of the lasso segment and the FH1 domain, and show that the lasso is required for the assembly of functional dimers.

Introduction

Apicomplexan parasites comprise an important group of human and animal pathogens. Best-characterized members of this phylum are the causative agents of malaria (*Plasmodium* spp.) and toxoplasmosis (*Toxoplasma gondii*). These pathogens share a unique mode of actin-dependent motility, characterized by the absence of any specialized organelles or obvious changes in the cell shape [1]. Instead, this so-called gliding motility involves peculiarly short, unstable actin filaments, which work in concert with an unconventional myosin, and a small set of regulatory proteins, governing the rapid cycling of actin monomers back to the growing end of the filament [2]. This miniature machinery lies in a confined space between the plasma membrane and the inner membrane complex of the parasite [3,4] and is linked, through the plasma membrane, to as-yet unidentified host cell receptors and, through the inner membrane complex, to microtubules in the cytoplasmic compartment of the parasite [3].

Despite the importance of actin polymerization for the motility and invasion – and thus survival – of these parasites, most of their cellular actin is present in monomeric form, and short actin filaments are formed only transiently [5]. Actin polymerization in *Apicomplexa* is controlled by a surprisingly small set of regulatory proteins and, for example, most of the conventional actin filament nucleators, such as the Arp2/3 complex and Spire proteins are not present. The core actin regulators in *Plasmodium* include two formins [6], one profilin [7], two actin depolymerization factors [8,9], two capping protein subunits [10], coronin [11], and a C-terminal cyclase-associated protein homologue [12].

Formins are a family of large multidomain proteins, important in many biological processes involving actin polymerization and, as recently discovered, also microtubule dynamics [reviewed in [13–15]]. They are characterized by the presence of two formin homology (FH) domains: the filamentous (F)-actin-binding FH2 domain [16,17] and the proline-rich FH1 domain, which in turn interacts with actin monomers indirectly via other regulatory proteins, such as profilins [18–20]. Formins work, on the one hand, as actin nucleators and, on the other, as processive cappers, which, together with profilins, also accelerate filament growth via recruiting a large pool of monomeric actin close to the barbed (growing) end of the filament [16,21,22]. Previous studies, mainly on mammalian and yeast formins belonging to the mDia family, have shown that the core FH2 domain alone inhibits actin polymerization, most likely via binding to the barbed end and preventing addition of new actin monomers rather than via monomer sequestering [21,23]. The inclusion of an additional region N terminal of the core FH2 domain dramatically changes the properties of formins, turning them into efficient polymerization catalysts [23]. This so-called lasso region, linking the FH1 and FH2 domains, has been shown to form almost the sole contact...
upon the formation of a flexible dimer of FH2 domains [24]. Although the dimeric lasso-containing FH2 domain is sufficient for both nucleation and elongation of actin filaments [16,21], for most formins, profilin has been shown to act as a further accelerator, speeding up polymerization by as much as nearly 20-fold [19]. Formins differ greatly in the number of proline-rich repeats in their FH1 domains and, thus, in the way they recruit profilin-actin. Different formins also utilize different profilin isoforms [25]. Apicomplexan genomes encode two to three FH2-domain-containing formins and a single profilin isoform. *Plasmodium falciparum* profilin (PfPrf1) binds to proline-rich sequences [7], indicating that the profilin-formin-mediated regulation of actin polymerization may be conserved. However, the two *Plasmodium* formin isoforms contain only rudimentary FH1 domains, and it is not clear whether they really participate in the regulation of actin dynamics in a profilin-dependent manner. In addition, *Plasmodium* has a nuclear formin-like protein, which also has a conserved FH2 but no FH1 domain [26]. What is more, none of the regulatory domains conserved in animal and yeast formins are present in the apicomplexan counterparts.

*P. falciparum* formin 1 (PfFrm1) and *T. gondii* formins induce actin filament formation *in vitro* using mammalian actin [6,27]. However, until now, it has not been known if the parasitic formins display the same activity also towards parasite actins, which have dramatically different polymerization properties. Here, we show that PfFrm1 nucleates actin filaments and works on both mammalian skeletal muscle and *Plasmodium* actins *in vitro*. PfPrf1 alone sequesters actin monomers, inhibiting polymerization, and has little or no effect on polymerization in the presence of different PfFrm1 variants. PfFrm1 dimerizes in solution, and the lasso domain, preceding the core FH2 domain, is required for the assembly of functional dimers, and thus, the nucleation activity of PfFrm1.

**Materials and Methods**

Cloning and recombinant protein expression and purification

The gene encoding *P. falciparum* formin 1 FH1 and FH2 domains (PfFrm1-FH1FH2; amino acids 2203–2675) was amplified from genomic DNA 3D7 clone of *P. falciparum* and inserted into the pETM-14 (EMBL) vector using the Nol and XhoI restriction sites. Two shorter constructs, lacking the FH1 domain or the FH1 domain and the so-called lasso segment (PfFrm1-FH2; amino acids 2242–2672 and PfFrm1-FH2Δlasso; amino acids 2272–2672, respectively) were amplified from this vector, and inserts were assembled using multi-enzyme method and ligation independent cloning (SLIC) as described before [28] into the pETM-14 (EMBL) vector followed by annealing. The plasmids were transformed into *Escherichia coli* Rosetta(DE3) (Novagen), the recombinant proteins were expressed using 1 mM isopropyl-β-D-1-thiogalactopyranoside induction at 18°C overnight in lysogeny broth (LB). The hexa-histidine (6×His)-tagged formins were purified on a Ni-NTA column (GE Healthcare) under native conditions in a buffer containing 50 mM sodium phosphate (pH 7.5), 300 mM NaCl, and 5 mM β-mercaptoethanol. A gradient of 10 to 500 mM imidazole in the same buffer was used for eluting the proteins. The 6×His-tags were cleaved during overnight dialysis against 40 mM tris(hydroxymethyl)amino-methane (Tris)-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 1 mM dithiothreitol (DTT) in the presence of either tobacco etch virus (PfFrm1-FH1FH2) or human rhinovirus 3C (PfFrm1-FH2 and PfFrm1-FH2Δlasso) protease. Uncleaved proteins and 6×His-tagged proteases were removed by binding to a Ni-NTA column, and the flow-through fractions were concentrated and applied to a Superdex 200 16/60 column (GE Healthcare), equilibrated with 40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10% glycerol, and 1 mM DTT. Purified proteins were concentrated and stored at −70°C until use in assays.

The gene encoding PfPrf1 was cloned into the pGAT-2 vector [29], which contains a thrombin cleavage site after the 6×His-glutathione-S-transferase (GST) tag, using the Nol and XhoI restriction sites. The protein was expressed in *E. coli* BL21 (DE3), and the 6×His-GST-PfPrf1 was purified essentially as described before [7], using a HisTrap column (GE Healthcare). After dialysis into 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 5% glycerol, PfPrf1 was released from the 6×His-GST tag using thrombin (GE Healthcare). The cleaved PfPrf1 was then passed through a HisTrap column, and the flow-through was subjected to size-exclusion chromatography (SEC) over a Superdex 200 16/60 column (GE Healthcare) in 10 mM Tris-HCl (pH 7.5) and 100 mM NaCl. Peak fractions were collected, concentrated, and stored on ice for subsequent experiments.

Genes encoding *P. falciparum* actin 1 (PfAct1) and *P. berghei* actin 2 (PfAct2) were synthesized and codon-optimized for insect cell expression by Mr. Gene (now part of Invitrogen). The PfAct1 gene was cloned into the pFastBac-HTA vector (Invitrogen) using the Nol and XhoI sites. The cloned plasmid was transformed into DH10Bac (Invitrogen) cells for bacmid generation. Sf9 (Invitrogen) cells were transfected with the PfAct1 bacmid using the FuGene 6 transfection reagent (Roche), and virus particles were harvested after 3 days of infection and used to generate a high-titer viral stock. Typically, 400 µl of the high-titer virus was used to infect approximately 2×10⁶ cells. The cells were harvested three days after infection, and the cell pellets were stored at −20°C. PfAct2 and a 6×His-tag from the pETM-14 vector (EMBL) were amplified, and the inserts were assembled using multi-enzyme method and ligation independent cloning (SLIC) as described before [28] into the pFastBac-Dual vector (Invitrogen), digested with EcoRI and XhoI. PfAct2 was produced in Sf21 (Invitrogen) cells essentially as described before [30]. Briefly, Sf21 cells were transfected with a bacmid generated in and isolated from the DH10MultiBac strain, using the FuGene 6 transfection reagent (Roche). The released virus particles were collected and used to generate a high-titer viral stock. Typically, 400 µl of the high-titer virus was used to infect approximately 2×10⁶ cells. 60–70 h after the growth arrest, cells were harvested and stored at −20°C.

For purification of recombinant PfAct1 and PfAct2, the cells were resuspended in lysis buffer [20 mM N-cyclohexyl-2-ami-noethanesulfonic acid pH 9.5, 250 mM NaCl, 1 mM adenosine-5′-triphosphate (ATP), 2 mM β-mercaptoethanol, 20 mM imidazole, 1× cOmplete Mini EDTA free (Roche)] and sonicated for 15 s. The supernatant was clarified by centrifugation at 18000 g and applied to Ni-NTA agarose (Qiagen). After extensive washing with the lysis buffer, the final washes were performed in a modified G-buffer [5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂, 0.5 mM ATP, and 2 mM β-mercaptoethanol], supplemented with 20 mM imidazole, and the bound proteins were eluted with the modified G-buffer, containing 300 mM imidazole. Subsequently, the proteins were dialyzed against G-buffer [5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂, 0.5 mM ATP, and 5 mM DTT] overnight at 4°C, concentrated, stored on ice, and used within a week.

**Skeletal muscle actin preparation**

Pig loin muscle was obtained from pigs from the University of Oulu Laboratory Animal Centre. The study plan was reviewed and accepted by the local ethics committee of the University of...
Oulu Laboratory Animal Centre (decision number 096/11). According to the Finnish legislation, no official license for animal experiments was needed because the tissues were collected from animals euthanized according to law. Because of ethical reasons, and to fulfill the principle of reduction, the tissues were collected from animals, which were used in other animal experiments, for which there was a separate license from the Finnish National Animal Experiment Board. Essentially, muscle acetone powder preparation and actin purification were performed as described previously [31]. Prior to use in experiments, SEC was carried out in G-buffer over a Superdex 200 16/60 column (GE Healthcare). Pure monomeric actin was stored on ice and used within a week.

Synchrotron radiation circular dichroism spectroscopy

For synchrotron radiation circular dichroism (SRCD) measurements, the formin samples were dialyzed into 10 mM potassium phosphate buffer, pH 7.0, and the concentrations were adjusted to 1.1–1.5 mg/ml. SRCD data were measured on beamline CD1 at the ASTRID synchrotron storage ring, ISA, University of Aarhus, Denmark. Each sample was scanned from 280 to 170 nm in a 100-μm pathlength circular quartz cuvette. Three consecutive scans of each sample were performed, and the corresponding buffer spectrum was subtracted. SRCD data were processed using CDtool [32], and secondary structure deconvolution was carried out at the Dichroweb server [33], using the CDSSTR algorithm [34] and the SP175 reference database [35].

Molecular weight determination by multi-angle static light scattering

Analytical SEC was carried out by injecting 200 μl of each of the three formin samples at 1 mg/ml into a Superdex 200 10/300 GL column (GE Healthcare) coupled to an Akta Purifier (GE Healthcare). The running buffer was 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, and 1 mM DTT, and the flow rate was 0.5 ml/min. The system was attached to a mini-DAWN TREOS (pH 7.0), 50 mM NaCl, and 1 mM DTT, and the flow rate was 0.5 ml/min. Each sample was scanned from 280 to 170 nm in a 100-μm pathlength circular quartz cuvette. Three consecutive scans of each sample were performed, and the corresponding buffer spectrum was subtracted. SRCD data were processed using CDtool [32], and secondary structure deconvolution was carried out at the Dichroweb server [33], using the CDSSTR algorithm [34] and the SP175 reference database [35].

Small-angle scattering

Synchrotron small-angle X-ray scattering (SAXS) measurements were carried out on beamlines X33 (DORIS storage ring) and P12 (PETRA III) at EMBL/DESY, Hamburg, and on beamlines I1711 and I911-4 at MAX-Lab, Lund. Protein concentrations in the measurements varied between 3–20 mg/ml in a buffer containing either 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT (Pf-Frm1-FH1FH2) or 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.5), 150 mM NaCl, and 1 mM DTT (Pf-Frm1-FH1FH2 and Pf-Frm1-FH2Δlasso). Programs of the ATSAS package [36] were used for data processing and analysis. 3D models were built using DAMMIN [37], DAMMIF [38], GASBOR [39], SASREF [40], and BUNCH [40]. SUPCOMB [41] and DAMAVER [42] were used for superpositions and the calculation of averaged models, respectively. A homology model of the Pf-Frm1 FH2 domain, consisting of 435 residues, including the core FH2 domain, the linker, and the lasso, was built based on PDB entry 3O4X [43] as a template, using the Phyre2 server [44]. The sequence alignment gives 23% sequence identity, and the Phyre confidence score for the model is 100%. For BUNCH modeling, the linker and lasso were removed from the homology model and rebuilt as a chain-like model. Rigid body modeling with SASREF was done using two approaches: First, the two protein chains from the homology model were used for rigid body refinement per se. Second, to mimic flexibility of the linker, while keeping the lasso-knob interaction in place, the dimer homology model was cut in half at each of the linker domains, between residues 104 and 105. Hence, rigid body refinement was carried out with two halves of the dimer, each of which contained, in addition to the FH2 domain, the lasso domain from the other subunit instead of the one from the same chain.

For small-angle neutron scattering (SANS), Pf-Frm1-FH1FH2 was dialyzed against 4 mM HEPES (pH 7.5), 20 mM NaCl, in 98% D2O. SANS data were collected on beamline SANS1 at GKSS (Geesthacht, Germany), and were processed on absolute scale.

Biochemical assays

For co-sedimentation assays, 5 mM ATP-actin - either pig skeletal muscle actin or recombinant Pf-Act1/Pf-Act2 - was incubated in G- [5 mM Tris-HCl (pH 7.5), 0.2 mM CaCl2, 0.2 mM ATP, and 0.5 mM DTT] or F-buffer (G-buffer including 2 mM MgCl2, 50 mM KCl, and 2 mM ATP), alone and with 250 mM formins, at room temperature for 1–2 h. After ultracentrifugation at 435000 g for 60 min at 20°C, equal amounts of the supernatants and pellets were analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie Brilliant Blue staining.

Actin polymerization was investigated at 22°C by fluorescence spectroscopy in a 200-μl quartz cuvette by measuring the change in fluorescence intensity (excitation 365 nm, emission 407 nm, bandpass 2 nm for both excitation and emission) with a Horiba Scientific FluoroMax-4 spectrofluorometer. Unlabeled pig muscle actin (5 μM) containing 5% pyrene-labeled rabbit actin (Cyto-skeleton Inc.) was in G-buffer. Actin polymerization was initiated by adding 1/10 of the volume of 10x F-buffer (to a final concentration of 50 mM KCl, 2 mM MgCl2, and 2 mM ATP), in the presence of 0, 1, 5, 10, or 50 mM of the different Pf-Frm1 variants. The change in fluorescence intensity was followed for 1000 s at 10-s intervals. To assess the effect of Pf-Phn on the polymerization kinetics, the assay was also performed with 5 and 10 μM Pf-Phn in both presence and absence of 10 mM formins. The measurements were performed in duplicate, and averages of the individual measurements were compared.

Results

*Plasmodium falciparum* formin 1 forms stable dimers mediated by the lasso segment in solution

We expressed and purified three different variants of Pf-Frm1 (Figure 1A): (i) the FH1-FH2 domains together (Pf-Frm1-FH1FH2), (ii) a longer version of the FH2 domain, which contains the so-called lasso region (Pf-Frm1-FH2), and (iii) a shorter version of the FH2 domain (Pf-Frm1-FH2Δlasso). SEC indicated an apparent dimer size for the two longer Pf-Frm1 FH2 domains (Pf-Frm1-FH2Δlasso). For small-angle neutron scattering (SANS), Pf-Frm1-FH1FH2 was dialyzed against 4 mM HEPES (pH 7.5), 20 mM NaCl, in 98% D2O. SANS data were collected on beamline SANS1 at GKSS (Geesthacht, Germany), and were processed on absolute scale.

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**Note:** The provided text appears to be a continuation of a scientific study on *Plasmodium falciparum* formin 1 and its role in actin polymerization and dimerization. The text includes methods and results pertaining to biochemistry, molecular biology, and structural analysis, along with references to previous studies. The study was published in the journal *PLoS ONE* in March 2012. The text continues with various experiments and analyses aimed at understanding the interactions and properties of the formin proteins. The results indicate that Pf-Frm1 forms stable dimers mediated by the lasso segment, with implications for their role in actin polymerization and potential roles in parasitic infection.
In order to confirm the above findings and to determine the 3D shapes of the different Pf-Frm1 variants, we studied their solution structures using synchrotron SAXS (Figure 2A,B). The basic data extracted from the SAXS scattering curves (Table 1) indicate that the construct with the lasso, but without the rudimentary FH1 domain (Pf-Frm1-FH2), is the most compact of the three constructs. While this was expected in comparison to Pf-Frm1-FH1FH2, it is surprising that the shorter construct, Pf-Frm1-FH2lasso, is more extended. The Pf-Frm1-FH1FH2 construct was also studied with SANS (Figure 2C). The neutron scattering curve had a similar shape to the SAXS curve, indicating the sample suffered no significant damage from X-rays during the SANS measurement.

*Ab initio* model building further confirmed the presence of a compact dimer in the Pf-Frm1-FH2 construct. The longer Pf-Frm1-FH1FH2 is similar, with slightly longer dimensions, in line with the presence of 30 additional N-terminal amino acids, predicted to be unfolded (Figure 2D,E). However, the shortest construct, Pf-Frm1-FH2lasso, is significantly different, being less compact (Figure 2F). While it appeared to be largely dimeric at the concentration used in the SAXS experiments, no compact dimer, such as that seen in the other constructs, was observed. The *ab initio* model built based on the data is elongated, S-shaped, and thin. Furthermore, analysis of the SAXS data indicates this construct to most likely be present as a mixture of monomeric and dimeric forms, which is in line with the SEC/MALS results. Taken together, the data clearly show that the absence of the lasso segment interferes with the dimerization of the formin FH2 domain in solution, resulting also in loss of function of the protein, as presented below.

We also used SRCD spectroscopy to assess the folding state and secondary structure composition of the different formin variants (Figure 2G). Pf-Frm1-FH1FH2 contains 501 amino acid residues, and according to the deconvolution, out of these, 46% (230 residues) are in an α-helical conformation, 9% are in β strands, 13% in turns, and 31% (155 residues) do not form any secondary structure. As FH2 domains are mainly helical, this result is in agreement with known FH2 domain structures and secondary structure predictions. Pf-Frm1-FH2 consists of 450 residues, of which approximately 234 are in α helices and 113 are unordered. The result clearly proves that the FH1 domain is unfolded. Pf-Frm1-FH2lasso has 420 residues, of which approximately 223 are in α helices and 113 unordered. In addition to the predicted presence of a short helical stretch in the lasso, it is likely that the lack of the lasso segment and proper dimerization in the shortest construct leads to some degree of unfolding. This is also in line with the SAXS data, showing a more elongated structure than for the lasso-containing construct.

![Image](https://example.com/image.png)

**Figure 1.** Purification and characterization of the recombinant proteins. **A**, SDS-PAGE analysis showing the purity of the proteins used in this study. The gel was stained using Coomassie Brilliant Blue. The samples are 1. Pf-Frm1-FH1FH2, 2. Pf-Frm1-FH2, 3. Pf-Frm1-FH2lasso, 4. pig muscle actin, 5. Pf-Act1, 6. Pb-Act2, and 7. Pf-Pfn. The molecular weights of the standard proteins in kDa are indicated on the right. The differences in the molecular weights of the *Plasmodium* and pig actins are accounted for by the 6×His-tags present in the recombinant *Plasmodium* actins. **B**, Size-exclusion chromatograms showing a single peak for Pf-Frm1-FH1FH2 (red) and Pf-Frm1-FH2 (blue) and two peaks for Pf-Frm1-FH2lasso (green). The molecular weights of the peaks of Pf-Frm1-FH1FH2 and Pf-Frm1-FH2 correspond to the size of dimers, and the two peaks of Pf-Frm1-FH2lasso to a dimer and a monomer, as determined by MALS (vertical lines with colors corresponding to those of the chromatograms).

doi:10.1371/journal.pone.0033586.g001

54.9 kDa (±6%). The molecular weight of the small peak eluting earlier is 99.0 kDa (±6%), indicating that Pf-Frm1-FH2lasso also has the tendency to form dimers, which are not as tight and are less compact than the dimers of Pf-Frm1-FH1FH2 and Pf-Frm1-FH2.

Table 1. Properties of the three formin versions derived from SEC/MALS and SAXS.

| Sample | Monomer MW* (kDa) | MW from SEC/MALS (kDa) | *Rg** (nm) | *Dmax*** (nm) | Volume (nm³) | Chi, model vs. data (DAMMIN, DAMMIF, GASBOR, BUNCH) |
|--------|------------------|------------------------|-----------|--------------|-------------|-----------------------------------------------|
| Pf-Frm1-FH1FH2 | 57.7 | 105.0 (±1%) | 6.4 | 25 | 352 | 0.9, 1.1, 1.1, 1.4 |
| Pf-Frm1-FH2 | 52.3 | 98.2 (±3%) | 5.2 | 19 | 335 | 1.2, 1.2, 1.7, - |
| Pf-Frm1-FH2lasso | 48.8 | major peak: 54.9 (±6%), minor peak: 99.0 (±6%) | 6.7 | 23 | 299 | 1.3, 2.2, 2.1, - |

The given volume is that of the respective averaged *ab initio* dummy bead model. The Chi-value describes the fit between the experimental scattering data and the model, with values close to unity reflecting a good fit.

*MW = molecular weight.

**Rg = radius of gyration.

***Dmax = maximum particle dimension.

doi:10.1371/journal.pone.0033586.t001
Plasmodium falciparum formin 1 nucleates actin filaments, and the lasso-mediated dimerization is required for this activity

It has previously been shown that the FH1-FH2 and FH2 domains of Pf-Frm1 nucleate filaments of chicken actin [6]. To evaluate the activities of the three Pf-Frm1 variants on Plasmodium actins, we performed co-sedimentation assays by ultracentrifugation, using recombinant Pf-Act1 and Pb-Act2. In low-salt conditions (G-buffer), we see that all three Pf-Frm1 versions induce the Plasmodium actins to polymerize to some extent (Figure 3A). All three formins appear to alone partially pellet in the low-salt condition, indicating that they are not very stable in

Figure 2. Solution structures of the Pf-Frm1 domains. A, Raw synchrotron SAXS data for Pf-Frm1-FH1FH2 (red), Pf-Frm1-FH2 (blue), and Pf-Frm1-FH2lasso (green). B, Distance distribution functions derived from the SAXS data; coloring as in panel A. C, SANS data for Pf-Frm1-FH1FH2. A radius of gyration ($R_g$) of 5.7 nm and a maximum particle dimension ($D_{max}$) of 18 nm can be estimated from the data. D, Averaged ab initio dummy atom models for Pf-Frm1-FH1FH2 (red) and Pf-Frm1-FH2 (blue) created by DAMMIN. The extensions at the extremities most likely correspond to the FH1 domain. E, Averaged ab initio models for Pf-Frm1-FH2 created by DAMMIN (blue) and GASBOR (cyan). The two figures are related by a 90° rotation about the X-axis. Both methods produce very similar models that fit the data. F, Averaged ab initio model for Pf-Frm1-FH2lasso (green) created by DAMMIN, superimposed on the structure of Pf-Frm1-FH1FH2 (red). Note that Pf-Frm1-FH2lasso is highly elongated, lacking a compact domain in the middle. G, SRCD spectra for the Pf-Frm1 variants. Coloring as in panel A. Pf-Frm1-FH2 has the highest relative helical content; see text for details on spectral deconvolution.

doi:10.1371/journal.pone.0033586.g002
the Tris-HCl buffer in the absence of salt. However, a significant part of the proteins is soluble also in G-buffer and upon addition of actin completely moves to the pellet fraction, taking along approximately half of Pf\textsuperscript{-}Act1 and Pb\textsuperscript{-}Act2, which both alone in G-buffer are completely soluble.

In typical polymerization conditions with a high salt concentration (F-buffer), both \textit{Plasmodium} actins polymerize completely under the conditions used in the assay. All three Pf\textsuperscript{-}Frm versions nearly quantitatively co-sediment with \textit{Plasmodium} actin filaments. The formins were allowed to polymerize in the presence or absence of the Pf\textsuperscript{-}Frm versions and separated on SDS-PAGE, as in panel A. The presence or absence of the Pf\textsuperscript{-}Frm versions is indicated above the figure. The molecular weight standards of 70, 55, and 40 kDa are indicated. The same standards were used in all gels shown. The arrowheads indicate the positions of actin, Pf\textsuperscript{-}Frm1-FH1FH2, Pf\textsuperscript{-}Frm1-FH2, and Pf\textsuperscript{-}Frm1-FH2\textsuperscript{lasso} in the gel, from left to right.

doi:10.1371/journal.pone.0033586.g003

To further characterize the activities of the different length formins, we performed polymerization assays using pig muscle actin and monitoring the increase in fluorescence upon co-polymerization of 5% pyrene-labeled actin (Figure 4). Already 5 nM Pf\textsuperscript{-}Frm1-FH1FH2 and Pf\textsuperscript{-}Frm1-FH2 significantly accelerated the initial rate of polymerization (Figures 4A–D), indicating participation in the nucleation of actin filaments. However, Pf\textsuperscript{-}Frm1-FH2\textsuperscript{lasso} had no significant effect on the polymerization rate at the concentrations used (Figure 4E,F). Thus, the FH2 domain dimer, mediated by the lasso region, is both necessary and sufficient for the nucleation activity of Pf\textsuperscript{-}Frm1.
**Figure 4. Effect of **Pf-Frm1** on actin polymerization kinetics.** The effect of Pf-Frm1 on the kinetics of actin polymerization was tested by measuring the change in fluorescence upon incorporation of 5% pyrene-actin into growing actin polymers. The actin concentration used in all experiments was 5 μM. The initial rates (ΔF/s) were calculated as the slope of the linear part (120 s) of the fluorescence curves. In order to facilitate comparison, the samples containing only actin were set to the value 1. A–B, Different concentrations of Pf-Frm1-FH1FH2. C–D, Different concentrations of Pf-Frm1-FH2. E–F, Different concentrations of Pf-Frm1-FH2Δlasso. doi:10.1371/journal.pone.0033586.g004

*Plasmodium falciparum* profilin sequesters actin monomers alone but has little effect on actin polymerization in the presence of formin 1

In order to assess whether *Plasmodium* profilin works together with *Pf*-Frm1 to accelerate actin polymerization, we performed pyrene-actin fluorescence polymerization assays also in the presence of *Pf*-Pfn. As expected, based on the function of profilins from other species, *Pf*-Pfn alone inhibits actin polymerization (Figure 5). However, together with the different *Pf*-Frm1 domains used, *Pf*-Pfn at an equimolar ratio to actin had little or no effect either on the initial rate or the total amount of actin polymerization (Figure 5). At a 2:1 profilin-actin ratio, profilin...
seems to slightly inhibit actin polymerization also in the presence of formins. Thus, we can conclude that Pf-Pfn either binds to actin with a lower affinity than Pf-Frm1 or that Pf-Frm1 nucleates and elongates profilin-actin even in the absence of a canonical profilin-binding FH1 domain.

Discussion

Our current view of the actin-based motility of apicomplexan parasites is somewhat controversial; despite the importance of rapid formation of actin filaments, most of apicomplexan actin is present in the monomeric state [5]. The system also seems rather vulnerable due to the very small number of actin-binding proteins taking part in the rapid treadmilling of actin in the parasites. Especially perplexing is the lack of most of the conventional actin nucleators - while fast nucleation would seem more important than elongation in a system, where only very short, transient actin filaments are required. In essence, the only candidates for actin nucleation in Plasmodium and other Apicomplexa are the formins. Formins in most organisms work as nucleators and processive cappers, protecting the barbed end from capping proteins and accelerating the rate of polymerization by recruiting profilin-actin complexes via their proline-rich FH1 domains [19]. We show here that Pf-Frm1 promotes actin filament assembly on both mammalian skeletal muscle and recombinant Plasmodium actins, suggesting a major role in actin filament nucleation in Plasmodium.

Figure 5. Effect of Pf-Pfn on actin polymerization kinetics in the presence of Pf-Frm1. The effect of Pf-Pfn added at a 1:1 or 2:1 molar ratio to actin on the kinetics of actin polymerization in the presence of the different Pf-Frm1 domains was tested by measuring the change in fluorescence upon incorporation of 5% pyrene-actin into growing actin polymers. The actin concentration used in all experiments was 5 μM. A, Pf-Pfn together with 10 nM Pf-Frm1-FH1FH2. B, Pf-Pfn together with 10 nM Pf-Frm1-FH2. C, Pf-Pfn together with 10 nM Pf-Frm1-FH2Δlasso.

doi:10.1371/journal.pone.0033586.g005
Apicomplexan actins have been reported to polymerize weakly compared to conventional actins [5,45]. Yet, we see both Plasmodium actins almost completely polymerizing in the pelleting assay, in the absence of any nucleators or filament-stabilizing agents. All three Pf-Frm1 constructs studied here associate with the actin filaments and, interestingly, nearly completely co-sediment with the filaments, indicating that, in addition to the growing ends, they may also bind to the sides of the filaments, as has been suggested earlier for e.g. the Drosophila melanogaster formin DAAM [46]. Although bundling has not been shown to happen in apicomplexan actins, this raises the question whether Pf-Frm1 could play a role in bundling and cross-linking of the short parasite actin filaments.

We show that dimerization of the Pf-Frm1 FH2 domain is mediated by the lasso region N terminal to the core FH2 domain, and the formation of a compact FH2 dimer is important for Pf-Frm1 activity. Furthermore, the Pf-Frm1 FH2 domain also weakly induces actin polymerization in low-salt conditions, which indicates a strong interaction and nucleating activity with actin. In our assays, approximately 5 nM Pf-Frm1-FH1FH2 and Pf-Frm1-FH2 were sufficient for an increase in the initial actin polymerization rate. This is in agreement with what has been reported before for both Plasmodium and Toxoplasma profilins, where low nanomolar formin concentrations were enough for a significant increase in the polymerization rates [6,27].

Pf-Frm1 lacks a well-defined FH1 domain and has, instead, only few proline residues scattered in the region preceding the FH2 domain. Consistent with the lack of proline-rich repeats in the Pf-Frm1 sequence, we have not detected an interaction between Pf-Frm1 and Pf-Pfn, using purified recombinant proteins in GST pull-down assays and SEC (unpublished data). Therefore, it is not unexpected that Pf-Pfn had very little, if any, effect on actin polymerization kinetics in the presence of Pf-Frm1. It seems likely that the role of Pf-Pfn is, indeed, to efficiently bind actin monomers in a form that facilitates their incorporation to the barbed ends of the growing actin filaments.

Previously, we have shown that Pf-Pfn binds to proline-rich sequences [7]. Interestingly, the Plasmodium formin 2 isoform contains in its putative FH1 domain two potential profilin-binding regions. It is remarkable that, although T. gondii profilin is highly conserved with Pf-Pfn, it has been reported to be unable to bind proline-rich sequences [27,47]. The apicomplexan profilins are very distantly related to profilins from other phyla but the P. falciparum and T. gondii profilins are 42% identical. In Pf-Pfn, the aromatic nature of the proline-rich-peptide binding site is conserved but many of the exact amino acid positions are not [7]. The same is true for T. gondii profilin [47], and furthermore, there are differences in the aromatic residues of the peptide-binding surface of these two apicomplexan profilins, as well. Therefore, it is possible that they have somewhat divergent functions and mechanisms. Despite the similarities in the actin structures and their regulation in these two parasites, there are also significant differences, which at least partly might be due to the very different life cycles of Plasmodium and Toxoplasma. Whereas Toxoplasma only uses mammalian hosts and displays virtually no cell specificity, Plasmodium needs both a mammalian and an arthropod host and is highly specific with regard to its host cells, depending on the life cycle stage. Furthermore, Plasmodium has two actin isoforms and Toxoplasma only one [48–50]. Therefore, the differences in the actin regulatory proteins may reflect adaptations to both differential actin isoforms and also different mechanisms needed to enter and survive within different host cells.

Our data support the role of a G-actin sequester for Plasmodium profilin but do not exclude the possibility that it works together with Plasmodium formin 2 to accelerate elongation, like in most other organisms. This implies that Plasmodium could have two diverged formins; one functioning rather as a sole nucleator and the other one also as an elongation factor together with profilin. Apparently, in Toxoplasma, such a difference does not exist [27]. For Plasmodium, this remains to be seen, once Plasmodium formin 2 has been properly characterized.

From the structural point of view, our data indicate that the dimeric structure of Pf-Frm1-FH2 in solution is similar to that seen e.g. in the crystal structure of the mouse mDia1 (PDB code 3O4X) [43] (Figure 6). The mouse mDia1 and Pf-Frm1 FH2 domains have 23% sequence identity (Figure 6A). The flexible linker region between the lasso segment and the core FH2 domain provides adaptability that is required for interactions with actin; in the actin-bound state, two actin monomers are bound between the core FH2 domains. Modeling of Pf-Frm1-FH1FH2 using the core FH2 domain and chain-like fragments, with the BUNCH software, provides also a dimeric model, which fits the scattering data very well, while preserving the expected intermolecular interactions (Figure 6B). From other formin FH2 domain structures, it is predicted that the lasso segment wraps around the so-called post region of the opposing FH2 monomer [24,43,51–53] (Figure 6C). From rigid body modeling using a homology model of the dimer (Figure S1), it is seen that rather minor rearrangements of the mDia1 dimer, which can be obtained from some flexibility in the linker region, result in a model that fits the SAXS data very well. The head-to-tail 2-fold symmetrical arrangement of the FH2 domain dimer, combined with the expected flexibility of the linker region between the FH2 and the lasso domains, provides a versatile framework for the binding of actin dimers by the dimerized FH2 domain. In the co-sedimentation assays (Figure 3) we see that all three formin versions associate with actin filaments and also induce formation of filaments in low-salt conditions. However, in the pyrene-actin fluorescence polymerization assay (Figure 4), only the two longer, dimeric versions increase the polymerization rate. It is possible that the FH2 domain in the absence of the lasso and, thus, proper dimerization still binds to actin filaments, as seen before for mouse mDia1 and mDia3 core FH2 domains [23]. Our results indicate that Pf-Frm1-FH2Δlasso is also somehow capable of bringing together actin monomers as short filament nuclei or aggregates, which are large enough to sediment at 435000 g, but cannot support further elongation of the filaments.

Our SAXS and SRCD data also prove that, as predicted based on the sequence, but to our knowledge not visualized for any formin before, the FH1-like domain of Pf-Frm1 is unstructured, and presents itself as two protrusions at opposite ends of the head-to-tail FH2 domain dimer.

Concluding remarks

We have shown that Pf-Frm1 is capable of initiating actin filament formation on both skeletal muscle as well as Plasmodium actins. This is the first time that such a polymerization promoting effect has been shown for purified, recombinant Plasmodium actins. This confirms Pf-Frm1 to be the prime candidate responsible for actin filament nucleation in these parasites. Our data also show that, while Pf-Pfn efficiently sequesters monomers, its monomer sequestering activity does not compete with the nucleation/elongation activities of Pf-Frm1. This, indeed, emphasizes the role of profilin in providing polymerization competent actin monomers to be added to the growing end of an actin filament – probably even in the absence of a direct interaction between profilin and a formin FH1 domain.
In addition, we provide the first structural insight into the dimerization of an apicomplexan formin and prove its relevance for the actin polymerizing activity. The solution structures presented here serve as a first step towards understanding the structure-function relationships in these multidomain proteins. In order to understand the complex interplay between the malaria parasite formins, actin, and other regulatory proteins interacting with them, more structural work at different levels of resolution is of crucial importance.

Supporting Information

Figure S1 Comparison of different models of Pf-Frm1 FH2 domain to the SAXS data. A, Three models are shown: orange, the homology model based on the mDia1 structure (PDB entry 3O4X [43]) – also shown in Fig. 6B superimposed on the BUNCH model; green, a model of the dimer obtained by rigid body refinement of the two FH2 domain chains from the homology model; blue, a rigid body model obtained from two halves of the dimer, such that there is a cut in the linker between residues 104 and 105 (blue arrows). Hence, in the refinement, the lasso belongs to the same rigid body as the corresponding knob. The locations of the lasso segments are indicated by circles. The two monomers in each model are colored slightly differently for clarity. B, Fit (red) of the BUNCH model shown in Fig. 6B to the scattering data of Pf-Frm1-FH1FH2 (black dots). C, Fits of the 3 models shown in A to the scattering data of Pf-Frm1-FH2, corresponding to the model. Coloring of the fits corresponds to the coloring of the respective models in A. The small changes in the dimer conformation in the rigid body models improve the fit significantly. (TIF)
Acknowledgments

We thank Prof. Kai Matuschewski for providing P. falciparum and P. berghei genomic DNA. We are also grateful to Dr. Setsuko Fujita-Becker and Prof. Rasmus Schröder for valuable suggestions regarding skeletal muscle actin purification. User support at synchrotron SAXS (EMBL/DESY, MAX-Lab) and SRCD (ISA) beamlines is gratefully acknowledged, and Dr. Vasyl Haramus and Matti Mälkyssö are specifically thanked for collecting and processing the SAXS data.

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Author Contributions

Conceived and designed the experiments: AI SPB JV IK. Performed the experiments: AI SPB JV PK IK. Analyzed the data: AI PK IK. Contributed reagents/materials/analysis tools: AI SPB JV. Wrote the paper: AI PK IK.
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