Unusual properties of Plasmodium falciparum actin: new insights into microfilament dynamics of apicomplexan parasites

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Abstract Plasmodium falciparum, the etiologic agent of malaria, is a facultative intracellular parasite of the phylum Apicomplexa. A limited turnover of microfilaments takes place beneath the parasite plasma membrane, but the cytoplasm of apicomplexans is virtually devoid of F-actin. We produced Plasmodium actin in yeast. Purified recombinant Plasmodium actin polymerized inefficiently unless both gelsolin and phalloidin were added. The resulting actin polymers appeared fragmented in the fluorescence microscope. Plasmodium actin bound DNaseI about 200 times weaker than bovine non-muscle actin. Our findings suggest that the unique properties of Plasmodium actin can explain some of the unusual features of apicomplexan parasite microfilaments.

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1. Introduction

The phylum of Apicomplexa contains unicellular eukaryotes that are obligate intracellular parasites in a wide range of invertebrate and vertebrate hosts. Apicomplexan parasites, such as Plasmodium and Toxoplasma, are important human pathogens. Despite vast differences in host range and target cell specificity, these parasites share their mechanism of motility and host cell entry [1]. Apicomplexan gliding locomotion, unlike most forms of eukaryotic cell motility, does not involve cilia, flagella or cytoplasmic extensions, and occurs without apparent changes in cell shape. Evidently, gliding locomotion and host cell entry are mechanistically linked and require transmembrane surface receptors that specifically bind substrate or host cell ligands [2]. One class of receptors needed for apicomplexan motility are transmembrane proteins of the thrombospondin-related anonymous protein (TRAP) family [3]. TRAP is thought to transmit an extracellular recognition event to the parasite cytoplasm by binding to actin filaments via the bridging molecule aldolase [4]. The TRAP-aldolase-actin complex may in turn interact with unconventional class XIV myosins [5] immobilized to the inner membrane complex, an additional membrane layer that is supported by microtubules. According to such a model, translocation of the receptor-ligand complexes to the posterior end of the parasite would thus propel the parasite forward and into the host cell [6].

Toxoplasma invasion of and mobility within cytochalasin D-resistant host cells is inhibited by cytochalasin D, indicating that actin filaments of the parasite are required for these processes [7]. However, actin filaments have not been detected under physiological conditions in any apicomplexan parasite using conventional methods, such as electron microscopy [8,9]. Parasite microfilaments cannot be visualized using fluorescent derivatives of the F-actin binding toxin, phalloidin [10], but it remains unclear whether this is due to the absence of actin polymers or failure of F-actin to bind phalloidin. Comparison of the total and sedimentable parasite actin content suggests the absence of a significant pool of filamentous actin [11]. Thus far, cytoplasmic actin filaments can only be visualized after jasplakonolide treatment of parasites [12]. Under these conditions, actin filament polymerization is induced at the apical end of motile cells. Together, these findings suggest that actin polymerization is the rate-limiting step in apicomplexan gliding motility, and that parasite microfilaments are under unusually tight spatial and temporal regulation.

Actin is exceptionally well conserved throughout evolution. Apicomplexan actins form a distinct subfamily within the conventional actins [13,14]. For instance, Plasmodium falciparum actin I (PfACT1; PF14_0124) shares ~83% sequence identity with actins from yeast and from vertebrate muscle and non-muscle sources [13]. The molecular differences between host and parasite actins are of great interest because they may pinpoint to distinct molecular mechanisms for regulation of the parasite microfilament system. While Toxoplasma gondii possesses a single conventional actin gene [11], P. falciparum has two [13]. PfACT1 is ubiquitously expressed throughout the Plasmodium life cycle and represents the abundant isoform [15,16]. In contrast, PfACT2 (PF14_0124) expression is restricted to sexual stages that form the diploid zygote and, hence, may serve a more restricted function during transmission to the mosquito vector.

Turnover of actin and the microfilament system is regulated by a number of regulatory proteins. Apicomplexa are no
exception, although some families of actin binding proteins are missing or present only as single isoforms [17]. In addition to potential intrinsic actin properties, the observed divergent characteristics of the apicomplexan microfilament system may be due to parasite-specific regulatory protein activities. Members of some families of apicomplexan actin binding proteins have been identified and characterized [18–22], but so far none seem to fulfill the biochemical requirements for a major and unique modulator of actin dynamics.

To study the role of the parasite-encoded actin in this context, we have conducted a biochemical analysis of purified recombinant PfACT1. We show that *Plasmodium* actin alone polymerizes inefficiently and can form polymers only in the combined presence of gelsolin and phalloidin. The actin polymers bind tetramethyl rhodamine isothiocyanate (TRITC)-phalloidin and appear fragmented in the fluorescence microscope. *Plasmodium* actin has a low affinity for DNaseI, suggesting that the molecular basis for polymer instability localizes to the conformation of subdomain 2. Our findings suggest that the unique properties of *Plasmodium* actin are the basis for the observed unusual dynamics of microfilaments in the parasite.

2. Materials and methods

2.1. Cloning and protein purification

To clone the PfACT1 cDNA (Genbank Accession Code NP0701803), we isolated polyA+ RNA 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). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion). The poly A+ RNA was used as a template for first-strand cDNA synthesis (Ambion). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion). The poly A+ RNA was used as a template for first-strand cDNA synthesis (Ambion). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion). The poly A+ RNA was used as a template for first-strand cDNA synthesis (Ambion). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion). The poly A+ RNA was used as a template for first-strand cDNA synthesis (Ambion). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion). The poly A+ RNA was used as a template for first-strand cDNA synthesis (Ambion). *PfACT1* was amplified from this cDNA using sequence-specific primers and subcloned into yeast expression (Ambion).

2.2. Biochemical characterization of PfACT1

Actin was studied at 25 °C in 5 mM Tris–HCl, pH 7.6, 0.5 mM ATP, 0.1 mM CaCl₂ and 0.5 mM DTT (G-buffer), with the addition of 0.2 mM EGTA and 50 μM 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), adding ADP to 0.5 mM, and finally inducing cation exchange by addition of 0.2 mM EGTA and 50 μM MgCl₂.

Actin binding to DNaseI was determined using the DNaseI-inhibition assay [26], using an assay concentration of DNaseI of 2 nM. Dissociation constants were calculated by linear curve fitting to double-reciprocal plots of the binding data using Microlab Origin. Thermal stability of actin was assessed as described [27]. Actin filament formation was monitored through the increase in fluorescence due to copolymerization of 2% pyrene-labeled [28] bovine β-actin after addition of 1 mM MgCl₂ + 0.1 M KCl to monomeric actin. The interaction of filamentous actin with TRITC-phalloidin was monitored by taking advantage of the 20-fold increase in fluorescence at 590 nm (λex = 544 nm) upon TRITC-phalloidin binding to F-actin [29].

3. Results

3.1. Purification of recombinant *P. falciparum* actin 1

Recombinant production of actin is difficult because of the absence of proper chaperones in some expression systems [30] and interference from endogenous actin isoforms in other systems. We have previously used temperature-inducible expression in *S. cerevisiae* to make milligram amounts of vertebrate non-muscle actin ([31] and references therein). The same approach was used here to produce and purify PfACT1.

Yeast-expressed PfACT1 was purified using immobilized DNaseI. The heterologous actin was separated from endogenous yeast actin by hydroxyapatite chromatography. We have previously observed that actins with more acidic N-terminal sequences generally interact tighter with the hydroxyapatite matrix. Consistent with this observation, *Plasmodium* actin eluted at higher ionic strength than the less acidic β-actin purified in previous studies and separated well from the basic yeast actin (data not shown).

To exclude the possibility that recombinant *Plasmodium* actin was unstable or partially unfolded, the stability of MgADP-actin, incubated at a constant temperature of 50 °C, was determined. Importantly, the parasite actin unfolded with the same kinetics as the bovine cytoplasmic isoform, i.e., we measured unfolding rates of 0.25 and 0.23 min⁻¹, respectively.

3.2. DNaseI binding of PfACT1

It has previously been reported that both *Plasmodium* [32] and *Toxoplasma* actins [11] interact with DNaseI. However, these studies did not discriminate between weak and strong binding. Employing a standard DNaseI inhibition assay [26], we found that PfACT1 bound DNaseI with an affinity of 104 ± 9 nM, while the endogenous yeast actin from the same actin preparation displayed a typical dissociation constant of 0.52 ± 0.11 nM (Fig. 1). This profound difference in DNaseI binding compared to actins from higher eukaryotes has important implications for the properties of PfACT1. Previously, Field et al. [32] used DNaseI inhibition to estimate the cytosolic concentration of G-actin in *Plasmodium* merozoites, relying on the DNaseI inhibition activity determined for bovine cytoplasmic β-actin [26]. However, β-actin like yeast actin binds DNaseI with subnanomolar affinity [33]. Thus based on our present results, we propose that the intercellular actin concentration in *Plasmodium* merozoites has been underestimated and may account for considerably more than 0.3% of soluble merozoite proteins.

3.3. F-actin polymerization dynamics of PfACT1

The peculiar polymerization pattern of apicomplexan actins in vivo has been extensively observed [11,12,32]. The recombinant PfACT1 enabled us to study the polymerization reaction in vitro using purified components. *Plasmodium* actin, either alone or in the presence of the filament capping protein gelsolin or the polymer stabilizing peptide phalloidin, did not show any signs of polymer formation (Fig. 2). We performed these experiments at a standard actin concentration of 4 μM, about a factor of 20 above the critical concentration for polymerization of yeast and non-muscle actin. Due to the limited amounts of purified PfACT1, we could not test whether increasing the actin concentration to higher micromolar concentrations would eventually result in spontaneous filament formation.
When we pre-incubated 4 μM PfACT1 with 20 nM gelsolin and added 1 μM phalloidin after 30 min into the polymerization reaction, we measured slow polymerization (Fig. 2B). Under these conditions, the apparent forward rate constant for polymer formation was approximately 10-fold lower than for bovine β-actin. Nevertheless, after 2 h the rate of pyrene-label incorporation reached similar levels when compared to our control actin, suggesting a specific effect in the dynamics of polymerization rather than an intrinsic deficiency to form filaments. Complete incorporation of pyrene-labeled bovine β-actin also confirmed that heteromeric polymers could be formed.

3.4. PfACT1 binds phalloidin

Since neither Plasmodium nor Toxoplasma show cytosolic phalloidin staining, it has been debated whether parasite actin polymers are capable of binding phalloidin. In analogy to ciliate actin [34], apicomplexan actin filaments would be present but simply not bind to phalloidin and, hence, remain elusive to fluorescent imaging techniques. Since we observed that phalloidin facilitated the polymerization of recombinant Plasmodium actin (Fig. 2B), we wanted to test whether these filaments were stainable with TRITC-phalloidin for visualization in the fluorescence microscope. We incubated polymerized PfACT1 filaments, as measured by incorporation of pyrene-labeled bovine β-actin, with TRITC-labeled phalloidin. We could detect abundant PfACT1 filaments that, however, looked short and fragmented in comparison to β-actin polymers (Fig. 3A). These results demonstrate that Plasmodium actin polymers indeed bind phalloidin. This interaction was further studied by titrating TRITC-phalloidin with actin under polymerizing conditions and monitoring the increase in TRITC-fluorescence intensity upon binding of labeled phalloidin to F-actin [29]. Over the actin concentration range tested,
TRITC-fluorescence increased by a factor of 4, clearly indicating TRITC-phalloidin binding to *Plasmodium* actin (Fig. 3B). The slope of the fluorescence increase was lower than in the control actins. This may be caused by a lower concentration of actin filaments, or their lower affinity for TRITC-phalloidin, or both. Thus, we conclude that recombinant *Plasmodium* actin is capable of binding phalloidin. We suggest that the inability to stain apicomplexan microfilaments with phalloidin in situ may be caused by an unusually short filament length. Alternatively, the phalloidin binding site may be masked by filament binding proteins, possibly in combination with a weak affinity for phalloidin or its fluorescent analogs.

4. Discussion

We show here that purified recombinant *Plasmodium* actin polymerizes poorly in vitro and binds DNaseI with low affinity. In an attempt to correlate these characteristics with structural features of the *Plasmodium* actin molecule, we marked those positions in vertebrate β-actin which represent non-homologous substitutions in the *Plasmodium* actin sequence (Fig. 4A). First and most important, all of these substitutions locate to the surface of the actin molecule, suggesting that the architecture of *Plasmodium* actin is similar to those actins of which the structures have been determined by X-ray crystallography. The non-homologous replacements fall into three categories: (i) positions in the DNase binding loop in subdomain 2; (ii) positions running along the outer edge of the large domain (subdomains 3 and 4) of the molecule; (iii) a patch of amino acids on the face of subdomain 1, including the N-terminus.

The divergent residues of *P. falciparum* actin in the DNase binding loop are illustrated in an alignment of the N-terminal parts of several actin sequences (Fig. 4B). Two of the rabbit skeletal muscle actin residues involved in crystal contacts with DNaseI [35] differ in PfACT1: Muscle actin R39, the ε-amino group of which is involved in a salt cluster with residues in DNaseI, is replaced by a lysine in PfACT1. Possibly of greater significance ε-actin Q41, which is involved in backbone interactions with DNaseI, is a proline in PfACT1. *Tetrahymena* actin, which also has a proline in position 41, did not detectably bind DNaseI [34]. Thus, the reason for the weak affinity of these actins for DNaseI may be the smaller number of intermolecular interactions.

The structural basis for the weak DNaseI binding activity of *Plasmodium* actin may also lie in a limited flexibility of its subdomain 2. A large body of structural and biochemical evidence suggests that a greater part of subdomain 2, in particular the DNase binding loop, is flexible and that this flexibility is modulated by the bound nucleotide (see [36,37]). While this part of the actin molecule is disordered in several of the crystal structures, residues H40–G48 adopt an α-helical conformation in the structure of tetramethyl rhodamine (TMR)-ADP-actin [38]. The proline in *Plasmodium* actin position 41 likely affects the flexibility of that part of subdomain 2. If actin activities are regulated by structural transitions in the DNase binding loop, *Plasmodium* actin can be expected to differ in this respect.

The second category of divergent *Plasmodium* actin residues contains residues that may contribute to polymer stability. Replacements M269K and S271A are located in a hydrophobic loop shown to influence the stability of the actin monomer and its ability to polymerize [39]. These replacements, as well as M283K and the DNase loop discussed before, are near interprotomer contacts in the structural model of F-actin [40]. Thus, these replacements may cause the limited ability of *Plasmodium* actin to form polymers. Other replacements running along the edge of subdomains 3 and 4 may influence the regulation of the microfilament through interaction with putative *Plasmodium* actin regulators.

Finally, the non-homologous replacements around the location of the N-terminus may represent adaptations to binding of myosin. The composition of the N-terminal sequence has been shown to modulate myosin binding [41,42]. In addition, crosslinking studies [43] and the analogy with gelsolin segment 1 [44] suggest ADF/cofilin proteins to bind near that site. Inci-

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**Fig. 4.** (A) Illustration of the locations of non-homologous replacements in *Plasmodium* actin. The positions of divergent replacements are shown in CPK colors on the structural model for vertebrate β-actin (pdb accession code 2btf). The nucleotide:cation complex is shown in green. (B) Sequence alignment over the N-terminal regions, including the major DNase binding site, of rabbit skeletal muscle ε-actin (RnActA), *S. cerevisiae* actin (ScAct), and *P. falciparum* actins I and II (PfActI and PfActII).
dentally, ADF/cofilin binding to filamentous actin also involves actin subdomain 2 [45,46]. ADF/cofilins and gelsolin/capping protein are two of the most important protein families regulating actin filament turnover. These divergent replacements, again, corroborate unusual Plasmodium microfilament dynamics on the level of its actin sequence.

The limited intrinsic polymerizability of Plasmodium actin can be overcome by combined action of two non-physiological factors, gelsolin and phalloidin. This suggests that parasite-specific proteins exist to perform similar tasks in a regulated fashion. The Plasmodium genome does not encode gelsolin; however, Plasmodium apparently contains capping protein as an α/β-heterodimer similar to other lower eukaryotes lacking gelsolin [22]. Based on the successful heterologous expression of PfACT1, we can now test the biochemical roles of putative Plasmodium actin binding proteins in a homologous in vitro reconstitution system.

Apicomplexan actins share several of their divergent amino acid replacements with Tetrahymena actins [13]; however, the latter have a higher degree of divergence from the actin consensus. One of the properties not shared by the actins of Plasmodium and Tetrahymena actin is the ability to bind phalloidin. We have shown here that purified recombinant PfACT1 interacts with phalloidin, in contrast to Tetrahymena actin which was found not to bind phalloidin [34]. These differences support structural models which place the tryptophan of phalloidin in the vicinity of W79 and Y198 of actin, since both of these side chains are conserved in Plasmodium but diverge in Tetrahymena actin. All other side chains which have been implicated in phalloidin binding by various methods are conserved in both Tetrahymena and Plasmodium actins. This line of reasoning is supported by the fact that Eimeria actin, which binds phalloidin [47], contains the conserved W79 and Y198. Incidentally, the unique substitution A114L in Tetrahymena actin, near the site which can be crosslinked to phalloidin derivatives [48], introduces a bulky side chain that may have consequences for phalloidin binding. Plasmodium and Eimeria actin have a glycine and alanine, respectively, in position 114.

Given the high degree of sequence conservation across the actin family and the unusual properties of the Plasmodium microfilament system, the non-homologous replacements in actin presumably reflect the modulation of the molecule for Plasmodium-specific actin dynamics. An intriguing possibility emerging from our results is that PfACT1 assembles only into short polymers in vivo, similar to the fragmented filaments we observed in vitro (Fig. 3A). Until recently, it was proposed that a continuous filamentous actin scaffold up to a length of 10 µm would be necessary to power parasite gliding motility. This view was recently challenged through the localization of the myosin motor to the inner membrane complex instead of the plasma membrane [49], and the isolation of the actin-bridging molecule aldolase [4] that links TRAP family invasions directly to actin instead of myosin, as initially assumed. The unanticipated reverse orientation of the motor complex [6] leads to an important prediction for the role of actin. In order for an actin-myosin motor to be still functional in backward distribution of the receptor-ligand complexes, actin polymers must be rather short. Immobilized motor myosins are anchored to the inner membrane complex which now serves as the scaffold [49]. Short actin polymers are pulled backwards by a set of myosin motors and passed on to the next motor unit. Hence, Plasmodium actin no longer needs to form typical microfilaments that determine shape and orientation of a cell but oligomerize into small units that serve as the backbone for a limited number of receptor-ligand complexes. Our in vitro studies of PfACT1 support such a model, since this unusual actin lacks the unifying intrinsic property to form elongated microfilaments.

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