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Plasmodium falciparum aldolase and the C-terminal cytoplasmic domain of certain apical organellar proteins promote actin polymerization

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ABSTRACT

The current model of Apicomplexan motility and host cell invasion is that both processes are driven by an actomyosin motor located beneath the plasma membrane, with the force transduced to the outside of the cell *via* coupling through aldolase and the cytoplasmic tail domains (CTDs) of certain type 1 membrane proteins. In *Plasmodium falciparum* (Pf), aldolase is thought to bind to the CTD of members of the thrombospondin-related anonymous protein (TRAP) family, which are micronemal proteins and represented by MTRAP in merozoites. Other type 1 membrane proteins including members of the erythrocyte binding antigen (EBA) and reticulocyte binding protein homologue (RH) protein families, which are also apical organellar proteins, have also been implicated in host cell binding in erythrocyte invasion. However, recent studies with *Toxoplasma gondii* have questioned the importance of aldolase in these processes. Using biolayer interferometry we show that Pf aldolase binds with high affinity to both rabbit and Pf actin, with a similar affinity for filamentous (F-) actin and globular (G-) actin. The interaction between Pf aldolase and merozoite actin was confirmed by co-sedimentation assays. Aldolase binding was shown to promote rabbit actin polymerization indicating that the interaction is more complicated than binding alone. The CTDs of some but not all type 1 membrane proteins also promoted actin polymerization in the absence of aldolase; MTRAP and RH1 CTDs promoted actin polymerization but EBA175 CTD did not. Direct actin polymerization mediated by membrane protein CTDs may contribute to actin recruitment, filament formation and stability during motor assembly, and actin-mediated movement, independent of aldolase.

1. Introduction

Like other Apicomplexan parasites, *Plasmodium falciparum* (Pf), a causative agent of malaria has motile forms and can invade host cells. For example, sporozoites are motile and invade hepatocytes, whereas merozoites are not motile but can invade erythrocytes. The merozoite is a polarized cell with specialized subcellular membrane-bound organelles, rhoptries and micronemes, located at the apical end. As found in the other extracellular forms, there is an actomyosin motor located around the periphery of the cell in the space between the surface plasma membrane and flattened alveolar sacs called the inner membrane complex (IMC) that together form the parasite's pellicle. Erythrocyte invasion comprises a series of complex events including low affinity reversible interactions between the surfaces of the two cells, parasite reorientation to position its apical end closely apposed to the host cell surface, ordered discharge of components from the apical organelles, and then entry of the parasite into a parasitophorous vacuole [1]. Invasion is marked by an annulus or moving junction that travels back over the surface of the parasite as it enters the host cell [2].

In the current model of both invasion and motility [3], largely based on initial studies in another Apicomplexan parasite, *Toxoplasma gondii*, the force required is provided by an actomyosin motor: the myosin is tethered to the IMC as part of the glideosome complex [4] and moves actin filaments to the rear of the cell; these filaments are bound through the tetrameric aldolase molecule to

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the cytoplasmic tail domains (CTDs) of certain type 1 membrane proteins that transduce the force to the outside of the cell, binding to cell surface receptors or the substratum and pushing the parasite forwards [5]. Although elegant, the correctness of this model for cell invasion has been questioned recently, based on studies in *Toxoplasma gondii* in which the gene for the myosin and other components of the glideosome were deleted [6], and aldolase was shown to be not essential for cell invasion [7].

The merozoite apical organelles contain many proteins, including a number of type 1 membrane proteins such as apical membrane antigen 1 (AMA1) that is proposed to be important in the formation of the moving junction [8,9], the erythrocyte binding antigen (EBA) and reticulocyte binding protein homologue (RH) protein families that bind to specific receptors on the erythrocyte surface [10–16], and a member of the thrombospondin repeat anonymous protein (TRAP) family found in merozoites (MTRAP) [17]. In sporozoite stage parasites, TRAP has been identified as the adhesin that couples the movement of actin filaments to the outside of the cell [18]; the TRAP CTD binds to aldolase [4,17,19,20]. In merozoites MTRAP is a micronemal protein that shares key features with TRAP, including a thrombospondin repeat domain, a putative rhomboid-protease cleavage site, and a CTD with a conserved subterminal Trp residue [4,21]. Some evidence has been provided that MTRAP can interact *in vitro* with aldolase, suggesting that it is the probable merozoite-specific functional homologue of TRAP. However, although MTRAP resembles TRAP structurally and binds to a red cell surface protein [22], its precise role in merozoite invasion of erythrocytes has not been studied in detail. In addition, the CTDs of RH and EBA family proteins have also been shown to bind to aldolase [23]. Although in one previous study Pf aldolase was co-precipitated with F-actin from parasite lysates [24]; the interaction between Pf aldolase and actin has been largely explored using rabbit actin as a surrogate [25–27]. However, Pf actin is unusual in structure [28,29] and the experimental use of rabbit actin in its place needs to be validated.

In this study we have investigated the interactions between actin, several CTDs and aldolase. We show that Pf actin and aldolase display high affinity interactions. Furthermore, aldolase promotes the polymerization of rabbit actin, a property it shares with some of the CTDs. The fact that some CTDs alone can stimulate actin polymerization suggests that these CTDs could interact directly with actin in the parasite cytoplasm during motor complex assembly. Overall, these interactions may have a role in the recruitment, polymerization and stabilization of actin, and assembly of the motor complex machinery.

2. Materials and methods

2.1. Proteins and peptides

2.1.1. Pf aldolase production and validation

The production of GST-tagged Pf aldolase from sequence inserted into pGEX-5X and transformed into BL21 competent cells (Stratagene) has been described previously [25]. Pf aldolase, with the GST tag removed, was purified and its correct folding confirmed by activity measurements in which fructose 1,6-bisphosphate (F1,6P) cleavage was coupled to the triosephosphateisomerase/α-glycerophosphate dehydrogenase reaction with continuous measurement of NADH consumption monitored at 340 nm (JASCO V550 UV/VIS Spectrophotometer), using an established method [30]. Kinetic analysis was performed using the Lineweaver–Burk plot to calculate the Michaelis–Menten constant (K_m) and the maximum reaction velocity (V_{max}) for the reaction [31].

2.1.2. Purification of actin from P. falciparum merozoites

P. falciparum 3D7 asexual blood stages were cultured in human red blood cells, and merozoites were purified as described previously [24]. Actin was purified from the cells by extracting globular (G)-actin with 25 mM Tris–HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT and clarification by centrifugation at 500,000 × *g* for 20 min as described elsewhere [24]. Filamentous (F)-actin was prepared in actin polymerization buffer (50 mM Tris–HCl (pH 8.0), 500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP), and harvested by centrifugation for 20 min at 500,000 *g*. Protein purity was evaluated by SDS-PAGE and Coomassie blue staining.

2.1.3. CTD peptides and rabbit actin

Synthetic peptides based on the sequence of CTDs were purchased from Biomatik (Wilmington, USA): PfMTRAP (PF3D7_1028700, residues 452–498), YFLRKEKTEKVVQEETKEENFEVMFNDDALKGKDNKAMDEEEFWALE; RH1 (PF3D7_0402300, residues 2942–2971), DNNKMDDKSTQKYGRNQEEVMEIFFDNDYI; and EBA175 (PF3D7_0731500, residues 1445–1502), KYQSSEGVMNENNENNFLFEVTDNLDKLSNMFNQQVQETNINDFSEYHEDINDINFKK. Purified rabbit muscle F- and G-actin were purchased from Sigma–Aldrich. Pyrene-labelled rabbit muscle G-actin, with the fluorescent probe covalently attached to a cysteine side chain [32], was purchased from Cytoskeleton Inc. (Denver, USA).

2.2. Protein biotinylation

G- and F-actin from both rabbit and *P. falciparum* were biotinylated using EZ-Link photoactivatable biotin (Pierce, Thermo Scientific) according to the manufacturer's instructions. The nitrophenyl azide biotin compound, which forms a covalent bond with molecules, was incubated with the protein for 5 min at room temperature during exposure to ultraviolet light (350–370 nm). The non-bound biotin was removed by dialysis into 25 mM Tris–HCl (pH 8.0), 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.5 mM DTT. Biotinylation was confirmed by biolayer interferometry with streptavidin in the Octet system (see below).

2.3. Protein binding assays using biolayer interferometry

The interaction between Pf aldolase and biotinylated G- or F-actin from rabbit and *P. falciparum* was analysed by biolayer interferometry using the Octet Red system (ForteBio) at 25 °C in 96-well microplates. The assays were performed in 100 mM Tris–HCl pH 8.0; all the proteins involved were solubilized in the same buffer using 200 μl per well at the required concentration (0.1–1 μM Pf aldolase) and proceeding as indicated in the manufacturer's instructions. The baseline was established for 20 min and the biotinylated samples were loaded separately onto the streptavidin sensors for 10 min. The sensors were further washed for 5 min and then exposed to Pf aldolase at different concentrations for 20 min, then the dissociation phase was performed for 10 min. The experiments were performed in duplicate or triplicate depending on the experiment. The data were analysed using non-linear least-squares fitting to a 1:1 binding model with in-house software.

2.4. Actin co-sedimentation assay

F-actin purified from merozoites was incubated with 6 μg of recombinant Pf aldolase or GST. Following incubation for 20 min at room temperature, the samples were centrifuged for 20 min at 500,000 × *g* to pellet actin filaments. Protein co-sedimentation was evaluated by resolving the proteins in the supernatant and pellet fractions by SDS-PAGE and staining with colloidal Coomassie blue.