Structure of the MTIP–MyoA complex, a key component of the malaria parasite invasion motor

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The causative agents of malaria have developed a sophisticated machinery for entering multiple cell types in the human and insect hosts. In this machinery, a critical interaction occurs between the unusual myosin motor MyoA and the MyoA-tail Interacting Protein (MTIP). Here we present one crystal structure that shows three different conformations of Plasmodium MTIP, one of these in complex with the MyoA-tail, which reveal major conformational changes in the C-terminal domain of MTIP upon binding the MyoA-tail helix, thereby creating several hydrophobic pockets in MTIP that are the recipients of key hydrophobic side chains of MyoA. Because we also show that the MyoA helix is able to block parasite growth, this provides avenues for designing antimalarials.

Cell invasion machinery | myosin-tail-interacting protein | Plasmodium | gliding motility

The ancient phylum of unicellular Apicomplexa harbors most of the intracellular parasites known, including Plasmodium species, which are the causative agents of malaria in different continents and subcontinents. Malaria is one of the world’s most devastating diseases, caused mainly by Plasmodium falciparum and Plasmodium vivax, with one to two million, mostly young, victims world wide (1).

These eukaryotic parasites have developed a sophisticated multiprotein machinery for entering multiple cell types in the human host and the insect vector (2–6). For malaria parasites the major motor components of this machinery (Fig. 1A) appear to be the same for entering, and leaving, insect cells as well as human host hepatocytes and erythrocytes (7–10). During invasion by malaria parasites, adhesive transmembrane proteins bind to the host cell (6, 10) and are linked to the invasive motor via an interaction between the cytoplasmic tail of the transmembrane proteins and aldolase (11, 12), which in its turn interacts with actin. The parasite invasive motor is composed of actin (13) and an Apicomplexan-specific myosin molecule, MyoA (Fig. 1B) (14). This parasite-encoded actin–myosin A-MTIP motor is present in all Apicomplexans for which sequence information is available (9, 13, 15–17). Studies of MyoA in Toxoplasma have demonstrated its essential role in parasite invasion and gliding motility (4). A protein assembly, of which the MyoA-tail-interacting protein (MTIP) is a key member, tethers the motor to the inner membrane complex (IMC). MTIP (Fig. 1C) shows distant sequence homology to the myosin light chains and calmodulin family even though MTIP misses the canonical calcium binding motif present in many members of this protein family (15, 17, 18). The binding of MTIP from Toxoplasma gondii and from Plasmodium species to Apicomplexan myosin is uniquely mediated via the tail of MyoA (Fig. 1B) rather than via the neck region as is the case with muscle myosin interacting with the myosin light chain (15, 17). Studies using the yeast two-hybrid system and an in vitro protein-binding assay (data not shown) have demonstrated that the C-terminal 15 aa of MyoA are necessary and sufficient for its interaction with MTIP (15). This MyoA-tail displays only a partial “IQ-motif” (19) as seen in partners of non-calcium-dependent calmodulin family members. These regions of MTIP and MyoA are extremely well conserved in the Plasmodium and Toxoplasma parasites (Fig. 1 B and C). Therefore, structural characterization of this critical interaction is of great interest for developing agents that interfere with host cell processes of the parasites. Because of the diverse manners in which calmodulin family members interact with their partners (20–22), a detailed structural analysis is also of intrinsic biological interest.

Results

MTIP Inhibition. The relatively small size and the overall basic nature of the minimal MTIP interacting region of the Plasmodium MyoA prompted us to examine its ability to affect parasite biology. Exogenously added 15-mer C-terminal MyoA-tail peptide inhibited the growth of P. falciparum strain 3D7 cultures in vitro with an IC50 of 84 μM (Fig. 2). The specificity of the peptide’s effect is demonstrated by the failure of a peptide with the R812A/K813A double mutation to inhibit parasite growth in this assay. Mutation of these residues, which are part of a trabisic motif within the C-terminal tail of MyoA, results in the inability of MyoA to interact with MTIP (15). (The corresponding residues within the tail of Toxoplasma MyoA are essential for localizing TgMyoA to the periphery of tachyzoites; ref. 23.) As an additional control, a sequence-randomized peptide fails to inhibit parasite growth. Examination of Giemsa-stained films from treated parasites revealed many extracellular merozoites, supporting the notion that the MyoA-tail peptide inhibits invasion of red blood cells. These in vivo results, like recent in vitro results (24), provide prima facie validation of the MyoA-tail MTIP interaction as a potential drug target.

The Three MTIP Conformations. Obtaining well diffracting MyoA-tail-MTIP crystals required exploring different length variants of MTIP from a variety of Plasmodium species and the use of different peptides representing the tail of MyoA. Eventually, we succeeded in growing crystals of residues 79–205 from P. knowlesi MTIP in complex with the P. yoelii MyoA C-terminal tail. The structure was solved by de novo by selenomethionine SAD procedures (see Table 1, which is published as supporting information on the PNAS web site). Quite remarkably, the asymmetric unit contained three MTIP subunits in three different conformations with one of these subunits in complex with the MyoA-tail (Fig. 3A).

The MTIP subunit appears to consist of two domains with three helices in the N-terminal domain, two of which form a...
so-called “EF hand” (20), and four helices in the C-terminal domain, which form two EF-hands (Fig. 3B). None of the EF hands is observed to bind calcium, in agreement with the absence in MTIP of the calcium-binding fingerprint described for the calmodulin family as mentioned above (15, 17, 18). The C-terminal domain of MTIP also contains two short β-strands, formed by residues 160–161 and 193–194, which form a small anti-parallel β-sheet. The N- and C-terminal domains are connected by a 28-residue long central helical structure, which runs from residue 125 to 152 and incorporates the last helix, α4, of the N-domain and the first helix, α5, of the C-domain. The helix is almost straight in each subunit with bends of 4°, 14°, and 10° in subunits 1, 2, and 3, respectively. This surprising crystal content allows for comparing three independent subunits and, thereby, assessing the flexibility among unliganded MTIP molecules and the alterations occurring upon the crucial interaction with the MyoA-tail. The N-terminal domains, comprising residues 92–134, appear to be very similar in the three subunits as reflected in mutual rms deviations of 0.56 Å for 43 residues. However, the C-terminal domains present an altogether different picture. The C-domains

Fig. 2.  *P. falciparum* growth inhibition by MyoA-tail peptide. Late trophozoite and schizont stages of *P. falciparum* clone 3D7 were incubated with varying concentrations of (i) the *P. yoelii* 15-mer MyoA-tail peptide (filled squares), (ii) a 15-mer peptide with randomized sequence of MyoA-tail (open triangles; the amino acid sequence of this peptide is N-SRQKAVIMLHMRARV-C), or (iii) the MyoA peptide bearing the R812A/K813A double mutation (open circles) for 24 h, at which point [3H]hypoxanthine was added to the cultures for an additional 24 h. Percent growth was determined by comparing [3H]hypoxanthine incorporation in solvent-treated parasites with peptide-treated parasites.

**Fig. 1.** The MyoA-MTIP components of the *Plasmodium* cell invasion motor. (A) Schematic representation of the organization of the components of the *Plasmodium* invasion machinery. MTIP binds the tail of MyoA, which localizes MyoA to the inner membrane complex (IMC). Additionally, MTIP is tethered to the IMC via an interaction with the GAP45–GAP50 complex, with GAP50 being an integral membrane protein of the IMC (43). The head domain of MyoA interacts with short actin filaments that are indirectly linked via an aldolase tetramer to the cytoplasmic domain of a transmembrane protein (11, 12), which binds the cell being invaded via its exocellular adhesive domain(s). Because the position of MyoA is fixed on the IMC, the action of the myosin head domain results in the actin–aldolase–transmembrane protein complex being displaced toward the posterior end of the parasite (black arrow), resulting in forward movement of the parasite. PMT, parasite microtubules; IMC, inner membrane complex; PPM, parasite plasma membrane; cpm, cellular plasma membrane (cell being invaded); MTIP, MyoA-tail interacting protein; MyoA, myosin A. (B) Amino acid sequence alignment of MyoA-tail homologs. Several Apicomplexan sequences are shown. The green bar indicates the cocrystallized *P. yoelii* MyoA-tail, which was N-terminally acetylated. Conserved residues are in light red, completely conserved residues in dark red. “*A. irradians* MHC” is *Aequipecten irradians*, or scallop, myosin heavy chain. The residues in contact with MTIP are indicated with green asterisks. (C) Sequence alignment and secondary structure of MTIP and homologs. Sequence alignment of MTIP from several *Plasmodium* species, *Toxoplasma gondii*, and *A. irradians* (scallop) is shown. Secondary structure elements of the *P. knowlesi* structure subunit 3 are depicted in the top rows of the alignment. The helices are numbered according to the comparison with the scallop muscle essential light chain (ELC) and other related proteins (Fig. 6). The structure based sequence alignment of the closest homolog to *P. knowlesi* MTIP, i.e., subunit 3 of the ELC of *A. irradians* (PDB code 1wdr, chain C), is included on the last row. The central helix runs from residues 125 to 152. The 17 MTIP residues crucial for interaction with the MyoA-tail are indicated with green asterisks above the *P. knowlesi* sequence.
of subunits 1 and 2 exhibit intriguing differences, even though neither of them binds a target helix. Whereas helices α6 and α7 maintain a constant orientation with respect to one another, helices α5 and α8 change their orientation with respect to the rigid α6-loop-α7 unit by ~9° and ~17°, respectively (Fig. 3A). This finding suggests that the conformation of the unliganded C-domain may be flexing all of the time in solution.

Much larger conformational changes are observed when comparing the C-terminal domains of unliganded subunit 1 and the MyoA-tail-binding subunit 3, which differ by an rms deviation of 3.1 Å for 62 Cα atoms. It appears that residues Phe-159 to Phe-188, i.e., the α6-loop-α7 unit, form an essentially rigid framework with an rms deviation of 0.65 Å for 30 equivalent Cα atoms in subunits 1 and 3. With respect to this framework, the rest of the domain changes dramatically upon binding the MyoA-tail helix. Specifically, helix α5 of the first EF hand of this domain moves by ~38° with respect to the framework to avoid a collision with the MyoA helix bound. Helix α8 of the second EF hand moves ~14° with respect to the framework upon binding the MyoA-tail helix. Also, the loops between helices α5 and α6, and between α7 and α8, are very different (Fig. 3B). A major change within the α6-loop-α7 unit concerns Trp-172, the
terminal residue of α6, whose side chain adopts different rotamers in subunits 1 and 3 resulting in an ~8 Å shift of its Cα atom. Amidst all these alterations, the two antiparallel β-strands of MTIP keep forming a two-stranded β-sheet (Fig. 3B). The conformational change upon MyoA-tail helix binding by the C-domain can be described as an opening up of its two EF hands, allowing this domain to present an extensive binding region to the target protein MyoA (Fig. 3B).

The MTIP MyoA-Tail Complex. The MyoA-tail exhibited excellent electron density (see Fig. 7, which is published as supporting information on the PNAS web site) and engages in contacts with three different subunits in the crystals. However, only the interactions with the C-domain of subunit 3 are extensive: burying 1,389 Å² of solvent-accessible surface by engaging 10 residues of the MyoA-tail to contact 17 residues of MTIP (Figs. 1 B and C and 4). In contrast, the other two MyoA-tail subunit contacts bury only 660 and 500 Å² buried surface. A combination of hydrophilic and hydrophobic interactions is responsible for forming the complex between the tail and the tail-binding protein. MyoA residues Gln-808 and His-810 make numerous hydrogen bonds with MTIP, whereas electrostatic interactions mainly involve the first and third residues from the conserved tribranched RKR motif spanning residues 812–814 (Fig. 6). MyoA Arg-812 forms a salt bridge with MTIP Glu-180, whereas MyoA Arg-814 is interacting with the side chain of MTIP Asp-202. Our structure also concurs with interaction studies using the yeast two-hybrid system, which was initially used to isolate MTIP (15) and reported the first MyoA-tail-MTIP interactions. Mutagenesis to Ala of each of the two MyoA-tail Arg residues of the tribranched motif, and each of the three hydrophobic MTIP-facing residues, results in a failure to interact with MTIP (see Fig. 5), which is in complete agreement with our structure of the MyoA–MTIP complex.

Three hydrophobic MyoA residues, Leu-804, Val-807, and Ile-811, all on one side of the tail helix, are pointing toward the MTIP surface and fit into hydrophobic pockets (Fig. 4; see Fig. 8, which is published as supporting information on the PNAS web site). The 17 residues of P. knowlesi MTIP interacting with the MyoA-tail are highly conserved, with several pocket-forming residues, including Phe-149, Leu-169, Leu-176, and Phe-199, totally identical among the sequenced Plasmodium and Toxoplasma MTIPs (Fig. 1C). Again, site-directed mutagenesis of several key residues of MTIP significantly reduce or eliminate interaction with the MyoA-tail (see Fig. 5).

The 10 residues of the P. yoelii MyoA-tail contacting MTIP appear to be highly conserved among Plasmodium species (Fig. 1B and Fig. 9, which is published as supporting information on the PNAS web site). Only the first three conserved sites of the 11-residue IQ motif (“IQxxR/GxxR”) described for non-calmodulin-dependent calmodulin partners (19) are present in the MyoA-tail sequence interacting with MTIP. In contrast, the Gly at the seventh position is in P. yoelii MyoA replaced by Lys-813, and the canonical Arg at the 11th position of the motif is replaced by Ala-817. The P. yoelii Lys-813 is completely conserved among Plasmodium and Toxoplasma species (Fig. 1B). The large side chain of Lys-813 in MyoA, corresponding to position 7 of the IQ fingerprint, has major consequences as described below.

Structural Comparisons. Comparison of MTIP with related structures using DALI (25) shows closest structural MTIP homolog scallop muscle myosin essential light chain (ELC) in complex with the myosin heavy chain (MHC) (26, 27). Both the ELC C-terminal domain and the MTIP C-terminal domain have no calcium bound, the latter being also in agreement with recent studies on the MTIP–Myosin A interactions in P. falciparum (24). The α6–loop–α7 units of the C-terminal domains of ELC and MTIP subunit 3 superimpose within 1.3 Å for 30 aa with only 16.7% sequence identity (Fig. 6). This superposition of the α6–linker–α7 units brings 13 Cα atoms of the bound proteins, MHC and MyoA, within 0.99 Å of each other (Fig. 6). Also, the side chains of the equivalent MHC Arg-790 and MyoA Arg-812 residues, and of the equivalent MHC Tyr-792 and MyoA Arg-814, adopt similar positions. Clearly, the two complexes have several features in common as far as target helix binding by the C-terminal domains is concerned.

Yet, there is also a distinct difference in the scallop ELC–MHC complex compared to that of MTIP and MyoA. The former has the two domains of ELC surrounding the heavy chain on both sides, whereas, in our MTIP–MyoA complex, the MyoA-tail interacts only with the C-domain of MTIP (Fig. 6A). However, this finding is in accord with the above-mentioned deviation of MyoA from the canonical IQ-motif at the seventh position, which is, in contrast, well followed in the MHC helix interacting with the ELC. In the ELC–MHC complex, Gly-791 at the seventh position of the motif in the MHC makes contacts with Asn-43 of the N-terminal domain of the ELC. As the corresponding residue is a lysine (number 813) in P. yoelii MyoA, it is not surprising that the MTIP N-domain is not observed to form a clamp surrounding the MyoA-tail, because a large side chain at this position in the MHC would clash with the loop Arg-38 to Asn-43 of the ELC.

Conclusion

The MTIP Conformations. One single crystal form revealed two structures of the uncomplexed Plasmodium MTIP molecule, and
the 30 MyoA complexes. The superposition operation was obtained by superimposing the N-terminal domains of the ELC and MTIP (see text). Note that the difference is likely a major factor in explaining the different positions of the N-terminal domain of ELC. The corresponding residue in MyoA is Lys-813. This because this residue of the IQ motif in the MHC is interacting with residue 43 of the target helix by MTIP and ELC. The position of the N-terminal domains is clearly visible as is the good correspondence in binding mode of the target helix by MTIP and ELC. Fig. 6. Comparison of Plasmodium the MyoA-MTIP and scallop muscle ELC-MHC structures. (A) Superposition of the scallop muscle MHC-ELC and the MTIP–MyoA complexes. The superposition operation was obtained by superimposing the 30 α6-loop–α7 unit residues of the C-terminal domain of the essential light chain (ELC) of scallop muscle (PDB code 1wdo, chain C) onto the corresponding residues of the C-terminal domain of P. knowlesi MTIP subunit 3. Dark blue, C-terminal domain of MTIP; light blue: N-terminal domain; cyan, C-terminal domain of ELC; gray, N-terminal domain of ELC; red, the MyoA-tail bound to MTIP; yellow, the scallop muscle MHC chain bound to ELC. The difference in position of the N-terminal domains is clearly visible as is the good correspondence in binding mode of the target helix by MTIP and ELC. (B) Stereoview of the C-domains of MTIP and ELC binding target helices MyoA and MHC, respectively. Subunit 3 is in blue, MyoA-tail is in red, ELC is in cyan and MHC is in yellow. The blue spheres indicate the start and end residue of the core region used for the superposition. The Cα atom of MHC Gly-791 is highlighted with a yellow sphere, because this residue of the IQ motif in the MHC is interacting with residue 43 of the N-terminal domain of ELC. The corresponding residue in MyoA is Lys-813. This difference is likely a major factor in explaining the different positions of the N-terminal domains of the ELC and MTIP (see text). Note that the α6-loop–α7 units are very similar, whereas helices α5 and α8 adopt quite different angles with respect to these constant units, i.e., the EF hands of ELC and MTIP rearrange differently upon binding to their respective target helices.

Perspectives for the Design of New Antimalarials. Given the enormous burden of the malaria parasite in the developing world and the increase of drug resistance (28–30), we like to point out that all of the 17 P. knowlesi MTIP residues engaged in critical interactions with MyoA are identical in MTIP from the major malaria parasite P. falciparum as well as in P. vivax, the second most important malaria causing agent (Fig. 1C). Moreover, of these 17 contact residues, 11 residues are different in the closest human homolog, myosin alkali light chain 1. With the observation that the MyoA-tail peptide itself inhibits parasite growth (Fig. 2), we present an exciting platform for designing compounds that prevent essential cell entry processes in hepatocytes and erythrocytes by the two major human malaria parasites, an exciting foundation for the development of novel classes of antimalarials.

Materials and Methods

Mutagenesis and Yeast Two-Hybrid Assays. A Gal4 DNA binding domain vector containing amino acids 803–817 of P. yoelii MyoA (pBD–MyoA) or a Gal4 activation domain vector containing full length P. yoelii MTIP (pAD–MTIP) was subjected to site-directed mutagenesis to alter key residues using the QuikChange Site-Directed Mutagenesis kit (Stratagene). The DNA sequences of the resulting plasmids were confirmed and the plasmids were subsequently transformed into yeast strain PJ69–4a. β-Galactosidase activity was measured essentially as described (31). The values are represented as percentage wild-type activity (pBD-MyoA + pAD-MTIP) and are the average values of duplicate aliquots from two independent yeast colonies.

P. falciparum Growth Inhibition Assay. We used a modified version of the [3H]hypoxanthine incorporation method of Dejardins et al. (32) for assessing growth inhibition by the MyoA-tail peptides or its mutated versions. Briefly, synchronized (74% late trophozoites, 13% schizonts, 12% ring stages) P. falciparum strain 3D7 parasites were seeded at 2% parasitemia in 96-well plates and treated with varying concentration of the peptides as well as the solvent (DMSO) for 24 h. [3H]Hypoxanthine was added to the cultures and incubation was continued for an additional 24 h. Incorporation of [3H]hypoxanthine by the parasites was determined by liquid scintillation. Growth inhibition was assessed as decrease in [3H]hypoxanthine incorporation in treated parasites relative to the control parasites treated with the solvent.

Plasmid Construction. A region encompassing amino acids 79–205 of P. knowlesi MTIP was amplified from genomic DNA (a gift from Carole Long, National Institute of Allergy and Infectious Diseases, Bethesda) using primers containing a 5’-BamHI site and TEV protease cleavage site (underlined) (5’-ATGGATCCGAGACCTGTAATCCAGGAAGATATGTTCAATACTAA-CAATATATCCCTACAGAAC-3’ and a 3’-Xhol site (5’-ATCTCGAGCTAGGACCAAATCTCAGCAGC-3’). After restriction endonuclease digestion, the DNA fragment was cloned into the Escherichia coli expression vector pET28a (Novagen), and the DNA sequence was confirmed.

Expression, Purification, and Crystallization. For large-scale expression of the protein, the vector was transformed into E. coli BL21(DE3) Rosetta strain (Novagen). An overnight culture was grown at 37°C in LB medium with 50 mg/liter kanamycin and 35 mg/liter chloramphenicol. The cells were grown at 37°C until an OD600 of 0.6. After a further 30 min, 1 mM IPTG was added and expression continued overnight. Selenomethionine protein was prepared following the metabolic control procedure (33).

Cells were lysed by sonication in a buffer (buffer A) of 10 mM Tris, 10 mM imidazole (pH 7.3), 200 mM sodium chloride, and 1 mM TCEP, along with one “Complete–EDTA” tablet (Roche) and 1 mg/ml lysozyme. Protein was purified by using a NiNTA (Qiagen) resin column, TEV protease was added to cleave off
the His-tag, the solution was passed again over a NiNTA column, and the protein in the flow-through was concentrated to 0.3 mM. Crystallization of *P. knowlesi* MTIP79–205 with 0.75 mM MyoA-tail (residues 803–817; Sigma) by the sitting-drop vapor diffusion method yielded the best crystals in a mixture of 20% wt/vol PEG200, 0.05 M sodium acetate (pH 5.3), and 1 mM TCEP at room temperature. For cryoprotection, they were transferred to a solution of 30% PEG200 in the same buffer with the addition of 0.75 mM MyoA-tail.

**Structure Determination.** Data were collected at the Advanced Light Source synchrotron beamline 8.2.2 at the Se absorption edge using 1° rotation images in the inverse beam mode. Data reduction was carried out with MOSFILM/SCALa [34] and XDS (35). The crystals belong to group P61 with three subunits per asymmetric unit. Twelve Se sites were located by the program SHELXD (36). Density modification by SHELXE (37) and chain alternation by TEXTAL (38) resulted in a partial model. Density averaging could not be used because of significant differences in all three subunits. Manual tracing and rebuilding using COOT (39) alternated with multiple rounds of composite omit maps with CNS (40) resulted in a model which contains in MTIP subunit 1 residues 88–203; in subunit 2 residues 89–189 and 195–204; in subunit 3 residues 79–204; and in the MyoA-tail residues 803–815. Finally, a TLS refinement with REFMAC3 (41) and the optimal TLS groups from the TLSMD web server (42) resulted in a crystallographic R factor of 22.9% (*R*merge = 28.3%) and good geometry (see Table 1). The final models were analyzed with validation tools in COOT (39) as well as MOLPRO-BITY (42) and SFCHECK (34).

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