Molecular & Biochemical Parasitology 168 (2009) 55–62

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Effects of calcium signaling on *Plasmodium falciparum* erythrocyte invasion and post-translational modification of gliding-associated protein 45 (PfGAP45)

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ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 19 June 2009
Accepted 23 June 2009
Available online 1 July 2009

Keywords:
Erythrocyte invasion
Glideosome
Merozoite
Calcium signaling
Post-translational modification

ABSTRACT

Plasmodium falciparum erythrocyte invasion is powered by an actin/myosin motor complex that is linked both to the tight junction and to the merozoite cytoskeleton through the Inner Membrane Complex (IMC). The IMC association of the myosin motor, PfMyoA, is maintained by its association with three proteins: PfMTIP, a myosin light chain, PfGAP45, an IMC peripheral membrane protein, and PfGAP50, an integral membrane protein of the IMC. This protein complex is referred to as the glideosome, and given its central role in erythrocyte invasion, this complex is likely the target of several specific regulatory effectors that ensure it is properly localized, assembled, and activated as the merozoite prepares to invade its target cell. However, little is known about how erythrocyte invasion as a whole is regulated, or about how or whether that regulation impacts the glideosome. Here we show that *P. falciparum* erythrocyte invasion is regulated by the release of intracellular calcium via the cyclic-ADP Ribose (cADPR) pathway, but that inhibition of cADPR-mediated calcium release does not affect PfGAP45 phosphorylation or glideosome association. By contrast, the serine/threonine kinase inhibitor, staurosporine, affects both PfGAP45 isoform distribution and the integrity of the glideosome complex. This data identifies specific regulatory elements involved in controlling *P. falciparum* erythrocyte invasion and reveals that the assembly status of the merozoite glideosome, which is central to erythrocyte invasion, is surprisingly dynamic.

1. Introduction

Infection by *Plasmodium* parasites results in 200–500 million cases of malaria each year, with between 1 and 3 million of those cases being fatal [1,2]. This illness represents a significant burden on global public health—a burden that is becoming more severe as drug-resistance spreads among both parasite and mosquito populations, and as environmental factors converge to increase the number of at-risk individuals [3]. Malaria can result from infection by any of four species of *Plasmodium* parasite, but almost all malaria mortality is caused by infection with *Plasmodium falciparum*. Like other *Plasmodium* parasites, *P. falciparum* infects through the bite of an infected mosquito and causes disease as a result of its subsequent invasion and occupation of erythrocytes.

As all clinical symptoms of malaria result from the parasite's intraerythrocytic development, a clear understanding of the molecular mechanisms that underpin the process of erythrocyte invasion could allow the development of invasion–inhibitory therapeutics. Erythrocyte invasion begins when a merozoite comes into contact with a circulating red blood cell (RBC) and forms an initial interaction with the RBC surface. This is followed by a reorientation step that allows the merozoite to bring its apical organelles into direct opposition to the RBC plasma membrane and form the tight junction—a close and irreversible interaction between the merozoite and RBC that is then translocated along the merozoite's periphery as invasion proceeds. As the tight junction is passed along the merozoite periphery, the RBC plasma membrane is forced to invaginate to form the parasitophorous vacuole (PV), and invasion is complete once the merozoite has passed the tight junction to its posterior and moved completely into the fully formed PV [4].

This tightly coordinated sequence of events relies on a variety of specific molecular interactions. The initial interactions that allow RBC recognition and tight junction formation depend on ligands on the merozoite surface that interact with specific receptors on the RBC surface. These interactions are subject to immune selection pressure and as a result the merozoite proteins responsible for them are highly variable and are often part of multi-member protein families with overlapping and interchangeable functions

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doi:10.1016/j.molbiopara.2009.06.007

[5]. However, the process of tight junction translocation depends on a multi-member myosin associated protein complex that is conserved across a number of Apicomplexan species [6–8]. This complex functions by anchoring the motor protein, PfMyoA, to the Inner Membrane Complex (IMC), a set of flattened membrane cisternae that are associated with the merozoite cytoskeleton. The anchoring of PfMyoA to the IMC is essential for the generation of directional force, which occurs when PfMyoA binds and releases F-actin filaments that are associated with specific transmembrane proteins in the tight junction [6,9,10]. This myosin associated protein complex was first described in *Toxoplasma gondii*, but is present in *P. falciparum* merozoites and in other invasive/motile *Plasmodium* life cycle stages; it is collectively referred to as the glideosome [6–8]. The glideosome in *P. falciparum* merozoites consists of PfMyoA, an associated light chain, PfMTIP, an IMC-associated peripheral membrane protein, PfGAP45, and an IMC integral membrane protein, PfGAP50, which is thought to function in anchoring this complex to the IMC [6,8].

As outlined above, erythrocyte invasion involves complex interactions between two eukaryotic cells, the merozoite and the erythrocyte, and must rely on the coordinated action of a tightly controlled regulatory network. This regulatory network is likely active at numerous points in the invasive process, and given its central role in invasion, the glideosome is likely an important regulatory target. In order to function properly, the glideosome must be both fully assembled and activated as the tight junction is translocated rearward, and both assembly and activity of this complex could be regulated. Furthermore, while some of the broad regulatory elements responsible for controlling the overall process of RBC invasion have been defined — calcium signaling and protein phosphorylation are known to play an invasion regulatory role, for example — the specific signaling pathways active in controlling invasion have been poorly characterized, and we know nothing of the subsequent impact of these pathways on the glideosome [11–13]. In order to address these major gaps in our understanding of *P. falciparum* erythrocyte invasion, we set out to describe the specific signaling elements involved in the regulation of erythrocyte invasion, and to determine the impact of these pathways on the assembly status of the glideosome and on the post-translational modification of one of its constituents.

2. Materials and methods

2.1. P. falciparum culture and invasion assays

P. falciparum parasite strains were cultured in O^+ human erythrocytes at 5% hematocrit and 10% O^+ human serum as described previously [14]. Synchronization of parasite cultures was by treatment with 5% sorbitol.

For invasion assays, mid-late stage 3D7 schizonts (43–46 h post-invasion at 1–2% parasitemia) were incubated with signaling effector compound or compound solvent alone and allowed to reinvade. After 12–16 h, assays were smeared and Giemsa-stained and fully formed rings were counted. Each experiment was performed at least three times in duplicate or in triplicate. Invasion in the presence of the compound solvent alone was considered to be 100%, and the influence of each signaling effector compound on ring formation was compared to its own solvent control in order to calculate invasion efficiency.

2.2. PfGAP45 immuno-affinity purification column preparation and usage

To generate an immuno-affinity purification column specific for PfGAP45, affinity purified PfGAP45 rabbit antisera (prepared as previously described [8]) was coupled to NHS-Sepharose loaded onto a glass Econo-Column (Bio-Rad, 1 ml column volume). In order to purify PfGAP45 and the associated glideosome, late schizonts (45–48 h post-invasion) were collected and lysed in 1% TX100 IP lysis buffer (1% TX100, 50 mM Tris–Cl pH 8.0, 5 mM EDTA) with complete protease inhibitor cocktail (Sigma) and phosphatase inhibitor cocktail II (Calbiochem) at a concentration of $1–5 \times 10^8$ parasites/ml. After lysis on ice for 30 min, parasite material was spun at 13,000 × *g* for 10 min at 4 °C and lysate supernatants were put over the PfGAP45 immuno-affinity column by gravity flow. After washing with 5–10 column volumes of 1% TX100 IP lysis buffer, PfGAP45 and its binding partners were eluted from the column using 3 column volumes of elution buffer (.1 M glycine, .5 M NaCl, pH 2.5–2.8). Elutions were collected directly into 1/10 volume neutralization buffer (1 M Tris–Cl pH 9.0) and were either mixed with an equal volume 2× SDS sample buffer with 10% β-mercaptoethanol or were chloroform/methanol precipitated and resuspended in 1× SDS sample buffer with 5% β-mercaptoethanol for analysis by SDS-PAGE.

Experiments examining PfGAP45 and its associated complex members under treatment with invasion–inhibitory compounds were performed exactly as described above except as follows. For each signaling compound used, an invasion–inhibitory concentration of either the compound under study or an equal volume of the compound solvent was added to well synchronized, mid-schizont (42–45 h post-invasion) stage parasites and culture was maintained for between 2 and 4 h before lysis of parasites in 1% TX100 IP lysis buffer and application to the PfGAP45 immuno-affinity purification column as described above.

2.3. SDS PAGE, Western blotting, and Pro-Q analysis

PfGAP45 column elutions were loaded onto either 10% or 12% polyacrylamide Tris–glycine gels for SDS-PAGE. For Western blot analysis, SDS-PAGE gels were transferred to either nitrocellulose or PVDF membranes and blocked in 2% milk/PBS solution overnight. Membranes were probed with anti-glideosome sera and then with HRP-conjugated secondary sera and visualized using enhanced chemoluminescence (ECL, Amersham Biosciences). Densitometry was performed using ImageJ analysis, which is freely available at http://rsbweb.nih.gov/ij/. For Pro-Q analysis, SDS-PAGE gels were transferred to PVDF membranes and treated with Pro-Q membrane stain per manufacturer's instruction (Molecular Probes). Following Pro-Q staining, PVDF membranes were also subjected to Western blot analysis as described above.

2.4. PfMTIP immuno-depletion

Immuno-depletion experiments were basically immunoprecipitation (IP) experiments performed repeatedly with the same lysate. Briefly, 600 μl of 1% TX100 IP lysate was pre-adsorbed against Protein G/Sepharose for 1 h at 4 °C and then added to 20 μl of either rabbit pre-immune sera or rabbit anti-MTIP. This initial IP was left overnight at 4° with rotation. The resulting immune-complexes were precipitated by incubation with Protein G/Sepharose for 1 h at 4 °C, after which IP supernatants were again added to 20 μl of rabbit pre-immune or MTIP sera and incubated with rotation at 4 °C for 2 h. Protein G/Sepharose was again added to pull-down the subsequent immune-complexes, and this process was repeated for three rounds of 2 h incubation/1 h immunoprecipitation. Complete protease and phosphatase inhibitor cocktails were added with each addition of antisera. After immuno-depletion, the resulting IP lysate was split into 200 μl aliquots for IP with MTIP, GAP45, or GAP50 rabbit antisera as described above. Subsequent immune-complexes were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting as described above.