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A complex of three related membrane proteins is conserved on malarial merozoites

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ABSTRACT

Invasion of human red blood cells by the malaria parasite *Plasmodium falciparum* is a coordinated, multi-step process. Here, we describe three novel integral membrane proteins that colocalize on the inner membrane complex immediately beneath the merozoite plasma membrane. Each has six predicted transmembrane domains and is conserved in diverse apicomplexan parasites. Immunoprecipitation studies using specific antibodies reveal that these proteins assemble into a heteromeric complex. Each protein was also expressed on insect cells using the baculovirus vector system with a truncated SUMO tag that facilitates maximal expression and protein purification while permitting cleavage with SUMO protease to release unmodified parasite protein. The expressed proteins were successfully reconstituted into artificial liposomes, but were not recognized by human immune sera. Because all three genes are highly conserved in apicomplexan parasites, the complex formed by their encoded proteins likely serves an essential role for invasive merozoites.

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1. Introduction

The phylum Apicomplexa contains numerous unicellular parasites that are important pathogens of humans and animals, causing malaria, toxoplasmosis, and other diseases. Each member is an obligate intracellular parasite that has evolved into a specialized niche, frequently using one or more distinct host cell types. Nevertheless, the process of invading their host cells appears to be highly conserved: both the ultrastructure and many of the implicated proteins are conserved amongst all species studied to date.

Invasion of erythrocytes by *Plasmodium falciparum*, the cause of the most virulent form of human malaria, is a complex process involving multiple receptors on the erythrocyte and a redundant collection of cognate ligands on merozoites, many of which have been identified [1,2]. After receptor–ligand interaction, a coordinated cascade of events – timed release of organelles, formation of a moving junction, de novo generation of a parasitophorous vacuole, rearward transport of surface ligands by an actively recycled actin–myosin motor complex, release of these ligands by proteases, and eventual membrane fusion – are needed to complete invasion. The actomyosin motor is sandwiched between the parasite plasma membrane and an underlying flattened vesicle known as the inner membrane complex (IMC). Beneath these layers, collectively known as the pellicle, is a specialized cytoskeleton that contains microtubule-dynein machinery and various organelles secreted at defined times during the invasion process [3–5].

While certain aspects of the invasion process are now well understood, others remain largely unexplored. For example, little is known about how the above cascade of events is coordinated or how critical information is transduced from the merozoite surface to various locations within the pellicle. Even less is known about what roles the conserved IMC may play for invasive zoites.

To address these questions and to develop a better understanding of the molecular mechanisms of invasion, it will be necessary to identify and characterize the involved proteins. The availability of genome sequence databases for various Apicomplexa, microarray data that reveals genes expressed during the merozoite stage [6], and evolving informatic algorithms for predicting targeting to the invasion machinery [7,8] has created a growing list of candidate proteins for biological and physiological studies. A number of merozoite surface proteins have been identified and implicated as ligands for binding to host erythrocyte receptors. Many of these proteins are either single-transmembrane domain containing

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proteins or are anchored to the membrane only via glycosyl phosphatidylinositol (GPI) at their C-terminus [1,9]. GPI anchors confer segregation to detergent-resistant lipid rafts [10], may permit more rapid lateral migration in the membrane [11], allow release via the action of phospholipases [12], and permit formation of large complexes. Some of these features are well-suited for merozoite surface ligands, which must migrate to the posterior end of the merozoite with the moving junction and are often shed during invasion.

Surprisingly, only one polytopic membrane protein expressed primarily on merozoite forms has been described to date [13]. Here, we identified a highly conserved family of three genes encoding proteins with six predicted transmembrane domains. Our studies indicate that these three proteins are simultaneously expressed and that they form a heteromeric complex on the IMC. Conservation of all three proteins in distantly related apicomplexan parasites suggests expansion in a common ancestor and a critical role for invasive zoite forms.

2. Materials and methods

2.1. Informatics and phylogenetic tree construction

A family of three paralogous genes, here named the *pfm6t* genes, was identified by searching the *P. falciparum* genome database for conserved genes encoding one or more transmembrane domains. The encoded PfM6T paralogs were aligned by the Clustal W algorithm using Vector NTI advance 10.1 (Invitrogen, Carlsbad, CA). Transmembrane domain predictions were carried out with TMHMM 2.0 and membrane topology depictions were constructed with TOPO 2.0 (http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl).

The 37 orthologous sequences were input into MUSCLE [14] to generate a multiple sequence alignment. The two M6Tβ fragments for *Plasmodium chabaudi* (PC000327.01.0 and PC000695.00.0) were combined and assigned to PC000327.01.0, the N-terminal fragment. PY06642 from *Plasmodium yoelli* appears to be a fragment of PY04760 and was excluded; PB300653.00.0 from *Plasmodium berghei* was excluded as a fragment of PB000435.02.0. The alignment was improved manually in MacVector 9.5 (MacVector, Cary, NC) and exported as a Nexus formatted file, which was used for a Bayesian phylogenetic analysis in the program MrBayes 3.1 [15]. The Bayesian phylogeny was the consensus of 7502 post-burn-in samples of two Markov chains each of which ran for 500,000 generations. The tree figure was created using FigTree (http://tree.bio.ed.ac.uk/software/figtree).

2.2. Antibodies

Peptide fragments were selected based on immunogenicity, synthesized, and KLH-conjugated for antibody production by Spring Valley Labs (Sykesville, MD). Polyclonal antibodies producing specific responses to individual PfM6T proteins were successfully raised in mice (identified with the prefix mp) and rabbits (prefix rp): KFSRYTPYPQDTNQNA-c (rp65α) for PfM6Tα; NVEMGVTENNYIKTAQY-c (rp70β and mp21β) and ARYQQTKSDWTLLHFG-c (rp68β) for PfM6Tβ; ELDIEASTENIAACKQC-c (rp64γ and mp7γ) for PfM6Tγ. A mouse monoclonal antibody for PfM6Tα was also generated using the same synthetic peptide as rp65α.

2.3. SDS-PAGE and immunoblots

In vitro parasite cultures (Indo 1 or W2 isolates) were maintained under standard conditions, enriched by the percoll–sorbitol method [16], washed, and used for biochemical studies. Membrane and cytosolic fractions were separated by hypotonic lysis (10 mM Na_2HPO_4, pH 8.0 with 100 μg/mL PMSF, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 2 μM EDTA) and ultracentrifugation at 100,000 × *g* for 1 h at 4 °C. Peripheral proteins were extracted from the membrane pellet in some experiments with 100 mM sodium carbonate, pH 11 [17]. Protein fractions were separated on 4–12% NuPAGE gels (Invitrogen) under reducing conditions, transferred to nitrocellulose, blocked with 5% powdered milk, and probed with specific antibodies at 1:5000–1:1000 dilutions. A rabbit polyclonal antibody against the SUMO tag of expressed proteins was used at a 1:500 dilution (Life Sensors, Malvern, PA). For detection of immune responses against PfM6T proteins, Ni-NTA purified proteins expressed in insect cells were probed with pooled human serum (1:2000 dilution) from 10 healthy adults living in endemic sites in Mali or Cambodia. These sera were provided by Dr. Rick Fairhurst and were collected with written informed consent under an NIAID IRB-approved protocol. After extensive washing, blots were incubated with HRP-conjugated goat anti-mouse, anti-rabbit, or anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:10,000 for 1 h, washed, and visualized using SuperSignal West Pico substrate (Pierce, Rockford, IL).

For stage specificity analysis of PfM6T expression, *in vitro* cultures were synchronized with two consecutive incubations in 5% sorbitol and cultured for indicated durations before harvesting for immunoblotting of matched samples.

2.4. Immunoprecipitation

Antibodies (rp65α, rp70β and mp7γ) were separately cross-linked to Dynabeads protein A (Invitrogen) according to the manufacturer's protocol. Infected cell lysate in 0.1 M Na_2HPO_4, pH 8.0 with 1% CHAPS, 100 μg/mL PMSF, 10 μg/mL leupeptin, 2 μg/mL aprotinin was incubated with these beads or with control beads without cross-linking for 30 min before washing extensively and elution by boiling in loading buffer for immunoblot analysis.

2.5. Indirect immunofluorescence confocal microscopy

Indirect immunofluorescence assays (IFA) were performed as described [18]. Briefly, synchronous cultures of infected erythrocytes were washed to remove serum before making thin smears on glass slides. The cells were air dried, fixed in 1% freshly prepared paraformaldehyde in PBS, washed, and blocked with 5 mg/ml goat serum and 0.1% triton X-100 in PBS. Primary and secondary antibodies were applied in the same buffer at a dilution of 1:100 and incubated at 37 °C for 1 h with extensive washing between antibodies. Colocalization studies used two primary antibodies applied simultaneously. Antibodies against known merozoite surface proteins were mouse monoclonal antibodies against a 42 kDa fragment of MSP-1 (R9256/EcMSP-1_{42}) and AMA-1 (4H9/19), both kindly provided by Dr. Sanjay Singh. Species-specific secondary antibodies coupled to fluoropores were used to detect the distribution of these antigens. Goat anti-mouse or anti-rabbit IgGs conjugated to either Alexa Fluor-488 (green) or Alexa Fluor-594 (red) were obtained from Invitrogen. Where shown, nuclear staining was with 300 nM 4,6-diamidino-2-phenylindole (DAPI). Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and visualized on a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Exton, PA) under a 68× oil immersion objective. Images were processed in Imaris 6.0 (Bitplane AG, Zurich, Switzerland) and uniformly deconvolved using Huygens Essential 3.1 (Scientific Volume Imaging BV, Hilversum, The Netherlands). *x–y* plots showing positional fluorescence intensity along the parasite's apical-posterior axis were also created in Huygens Essential 3.1 and exported to SigmaPlot 10.0 (Systat, San Jose, CA).