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Minireview

Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells

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Abstract ***Plasmodium falciparum*** **causes the most lethal form of malaria in humans and is responsible for over two million deaths per year. The development of a vaccine against this parasite is an urgent priority and potential protein targets include those on the surface of the asexual merozoite stage, the form that invades the host erythrocyte. The development of methods to transfect *P. falciparum* has enabled the construction of gain-of-function and loss-of-function mutants and provided new strategies to analyse the role of parasite proteins. In this review, we describe the use of this technology to examine the role of merozoite antigens in erythrocyte invasion and to address their potential as vaccine candidates.**

Key words: Malaria; Vaccine antigen; Targeted gene disruption; *Plasmodium falciparum*

1. Introduction

Malaria has been a significant burden on humans throughout recorded history and this continues in modern times. The ability of the parasite to develop resistance to antimalarial drugs and the absence of a suitable vaccine to control the disease ensures that malaria remains a major global problem. *Plasmodium falciparum* causes the most severe form of malaria in humans and is responsible for 200–300 million infections per year. Over two million infected people die as a result of the disease annually. The development of a vaccine is a priority, and potential vaccines are being targeted to various stages of the parasite life cycle. The asexual merozoite has been a particular focus in recent times. The merozoite form of the asexual life cycle in the blood stage attaches to the surface of the red blood cell (RBC) thus initiating the invasion process of this host cell. Inside the RBC the parasite replicates and matures into a schizont form which eventually ruptures to release new merozoites and complete the blood stage cycle. Merozoite antigens are exposed to the immune system and consequently these proteins are potential vaccine candidates. Many of these proteins are thought to play a role in merozoite invasion of RBCs but the details of their function remain sketchy at best.

Merozoite invasion takes place following initial interaction with the RBC surface followed by re-orientation to allow the apical end to interact with the membrane of the host cell (Fig. 1). The contents of the apical organelles, the rhoptries and micronemes, are expelled and a tight junction is formed between the merozoite surface and the RBC membrane [1]. The tight junction moves along the surface of the merozoite, possibly via force generated by an actin myosin motor, until the membrane fuses at the posterior end of the parasite. This results in the formation of a parasitophorous vacuole containing the newly invaded merozoite. Many proteins appear to be involved in this complex invasion process, although very little is known about the particular role of any individual protein. One of these proteins, merozoite surface protein 1 (MSP1), has been hypothesised to be involved in the initial interaction of the merozoite with the RBC surface [2,3]. As its name implies, this GPI-anchored protein is found on the surface of the merozoite. This localisation is shared with a number of other GPI-anchored proteins including MSP2 [4], MSP4 [5] and MSP5 [6]. Apical membrane antigen 1 (AMA1) is an integral membrane protein that is initially localised to the neck of the rhoptries although after schizont rupture the protein spreads onto the merozoite surface [7–11]. The function of AMA1 is not currently known. Within the rhoptries, a number of proteins have been identified that may be involved in invasion. This includes the low molecular weight complex that consists of the rhoptry-associated proteins 1 and 2 (RAP1 and RAP2) [12,13] as well as a poorly characterised protein RAP3 [14]. This soluble protein complex is expelled from the rhoptries during invasion and is carried through into the parasitophorous vacuole with the merozoite [15]. The erythrocyte-binding antigen 175 (EBA175) is located in the micronemes and has been shown to bind to the RBC surface molecule glycophorin A in a sialic acid-dependent manner [16–18].

The ability of each of these *P. falciparum* antigens to elicit a protective immune response has led to their consideration as vaccine candidates. However, with the possible exception of EBA175, little is known about the role of any of these antigens in the invasion process. To address this issue, we have used transfection approaches in *P. falciparum* and this has not only provided important information on function, but may also address their validity as vaccine candidates [15].

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2. Analysis of merozoite invasion using gene disruption

The development of transfection in *P. falciparum* has created the possibility to construct loss-of-function mutants by gene disruption [19–21]. It may be expected that genes encoding invasion-related proteins would be essential and therefore difficult to disrupt. However, merozoite invasion pathways appear to be degenerate in nature, involving different parasite ligands and RBC receptors [22]. Therefore it may in some cases be possible to disrupt particular invasion pathways without compromising the viability of the parasite. An indication that this inference may be correct lay in the fact that several *P. falciparum* parasite lines are able to invade RBCs via multiple pathways, while others appear to lack some of these invasion routes [22,23].

We have attempted to disrupt several genes that have been linked to the invasion process. Not surprisingly, many of these appear to be lethal or severely deleterious (Table 1). Interestingly, it was possible to disrupt the *RAP1* gene and this resulted in parasites that express severely truncated forms of RAP1 [15]. Truncated RAP1 still traffics to the rhoptries but can no longer complex with RAP2. In these parasites RAP2 is found in a compartment resembling the lumen of the endoplasmic reticulum rather than in the rhoptries. These results suggest that a function of RAP1 is to localise RAP2 to the rhoptries. This also supports the hypothesis that rhoptry biogenesis is dependent, at least in part, on the secretory pathway in the parasite [24].

Table 1
Gene targeting of genes encoding *P. falciparum* merozoite antigens

Gene	Construct design[a]	End point[b]	Reference
MSP1	KO	NHI[c]	[28]
MSP1	3′ replacement	HI[d]	[28]
MSP2	KO	NHI	Cowman et al., unpublished
MSP2	3′ replacement	HI	Wickham et al., unpublished
MSP3	KO	under way	Mills et al., unpublished
MSP3	3′ replacement	HI	Mills et al., unpublished
MSP4	KO	NHI	Cowman et al., unpublished
MSP5	KO	NHI	Cowman et al., unpublished
RAP1	KO	HI	[15]
RAP2	KO	NHI	O'Donnell et al., unpublished
RhopH3	KO	NHI	O'Donnell et al., unpublished
Ag512	KO	NHI	Baldi et al., unpublished
AMA1	KO	NHI	Triglia et al., submitted
AMA1	3′ replacement	HI	Triglia et al., submitted
EBA175	KO	NHI	Baldi et al., unpublished
EBA175[e]	KO	HI	[25]
S-antigen	KO	NHI	Cowman et al., unpublished
ABRA	KO	NHI	Cowman et al., unpublished

[a]Plasmids for gene targeting were based on either the pHC1 [21] or pHH1 [45] plasmids. These were designed to either 'knock out' (KO) the gene of interest or to integrate into the gene in a manner that would retain gene function (3′ replacement). Construct design and integration procedures were essentially as described in [21,28,46].
[b]End point represents result after three to five drug cycles for integration (4–6 months of continuous culture). Reflects bulk parasite population as observed by Southern blot experiments.
[c]NHI: no homologous integration. Usually manifest as episomal forms that remained after extensive culturing, however, occasionally non-homologous integration was observed.
[d]HI: homologous integration.
[e]This transfection was carried out in the W2mef parasite line while all others were performed in D10.

EBA175 is involved in the glycophorin A-dependent invasion pathway and disruption of this gene was performed to address the role of the conserved 3′ cysteine-rich region, the transmembrane and the cytoplasmic domains by deletion of these regions [25]. Such a truncation had no measurable effect on the level of EBA175 protein expression or its subcellular localisation in the micronemes. Similarly, there was no impairment in the ability of soluble EBA175 to be released into the culture supernatant following schizont rupture. These experiments have shown that the 3′ Cys-rich region, transmembrane and cytoplasmic domains of EBA175 are not essential for merozoite invasion. However, analysis of RBC invasion via the EBA175/glycophorin A route suggested that this pathway was disrupted to such a degree that the mutant lines have undergone a stable switch in invasion phenotype to a sialic acid-independent pathway accounting for 85% of normal merozoite invasion. This suggests that truncation of the C-terminus of EBA175 leads to functional inactivation. These data demonstrate that the *P. falciparum* parasite has the ability to utilise alternative pathways for invasion of RBCs. This property would undoubtedly provide a substantial survival advantage to the parasite in terms of overcoming host receptor heterogeneity and/or immune pressure.

It is clear that it is not possible, with current transfection technology in *P. falciparum*, to disrupt many genes believed to be important in merozoite invasion. Attempts to disrupt most of the genes shown in Table 1 have not been successful; however, this could be due to a number of reasons. Firstly, the D10 parasite, which was used in the majority of gene disruption experiments, lacks at least one of the known invasion pathways [22]. It is possible that *P. falciparum* merozoites require a minimum number of ligands and pathways for successful invasion of the RBC. Consequently, it will be important to test gene disruption in other parasite lines with a broader range of invasion pathways. Secondly, the current transfection procedure results in persistence of a circular form of the transfection plasmids. As a result, selection of parasites with integrated copies of the plasmid in the gene of interest involves cycling of transfectants on and off drug [19,20,26]. However, if the gene disruption event results in a parasite capable of invading but at a reduced efficiency, cycling will be ineffective in selecting them from the population carrying the episomal plasmid. To overcome this it will be essential to develop plasmid vectors containing negative selectable markers such as cytidine deaminase and thymidine kinase, thus allowing the removal of the persisting episomal plasmid. Finally, the gene of interest may be essential for RBC invasion. In this case, it will not be possible to create gene disruptions using normal techniques and an inducible promoter system will be required. Such methods, such as the tetracycline repressor/operator system [27], allow activation and repression of gene expression in a controlled manner. Two examples of essential genes appear to be the MSP1 [28] and AMA1 (Triglia et al., submitted). Both of these genes can be targeted by transfection plasmids only if the recombination events result in gene reconstitution and thus wild-type protein expression.

The ability to disrupt genes believed to be involved in merozoite invasion clearly shows that they are not absolutely essential for this process in vitro. For example, RAP1 together

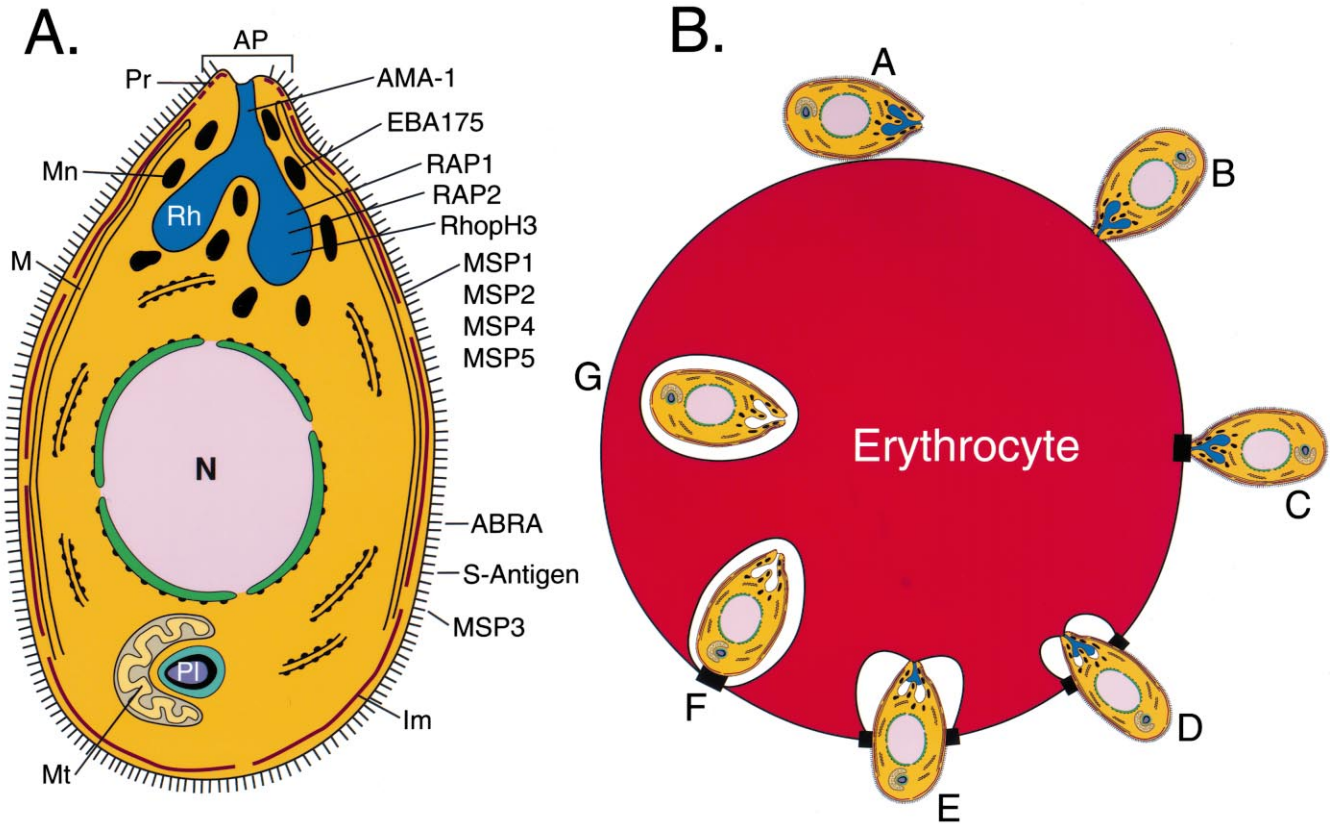

Fig. 1. Invasion of the merozoite form of *P. falciparum* into red blood cells. A: Diagrammatic representation of a malaria merozoite shows some important structural features and the localisation of some *P. falciparum* antigens. Rh, rhoptries; N, nucleus; IM, inner membrane; Mt, mitochondria; Pr, polar rings; Ap, apical end; Pl, apicoplast or plastid; M, microtubules; Mn, micronemes; AMA1, apical membrane antigen 1; MSP1, 2, 3, 4, 5, merozoite surface proteins 1, 2, 3, 4, 5; EBA175, erythrocyte binding antigen 175; RAP1, 2, rhoptry-associated protein 1, 2; RhopH3; ABRA; S-antigen. B: Schematic representation of some of the major morphological events associated with merozoite invasion. (A) Attachment; (B) apical reorientation; (C) junction formation and the beginning of rhoptry discharge; (D and E) penetration of the merozoite past the tight junction into a forming parasitophorous vacuole; (F and G) pinching off of the junction, and resealing of the red blood cell membrane. The surface coat of the merozoite is progressively stripped off as it moves through the tight junction (D to F). The membrane surrounding the fully invaded merozoite is termed the parasitophorous vacuole membrane.

with RAP2 have been viewed as important vaccine candidates as they can partially protect Saimiri monkeys from challenge with *P. falciparum* [13,29]. Furthermore, monoclonal antibodies specific for RAP1 can inhibit invasion into RBCs [30–32]. The ability to disrupt RAP1 and as a consequence the RAP1/RAP2/RAP3 complex demonstrates that it is not essential for invasion and growth of *P. falciparum* in human RBCs. While in vivo studies remain to be carried out, this evidence must temper enthusiasm for RAP antigens as vaccine candidates. It is possible that inhibition of merozoite invasion by RAP1 antibodies occurs via steric hindrance of the invasion process rather than specific inhibition of an essential RAP1 function. This would explain the ability of RAP1 and RAP2 to protect monkeys against *P. falciparum* challenge [13,29]. These findings raise the possibility that breakthrough parasites could arise that can survive in the absence of functional RAP1.

3. What does cross-species complementation of merozoite proteins tell us about function, protective immunity and potential as a vaccine?

Plasmodium species generally show host specificity with respect to RBC invasion. However, some parasites show an ability to invade RBCs from different mammalian hosts. For example *P. falciparum*, which normally invades human RBCs, can also invade mouse RBCs, albeit at a greatly reduced efficiency [33]. Homologues of some merozoite proteins, for instance MSP1 and AMA1, are found across the *Plasmodia* and it is presumed that these molecules are at least in part functionally conserved. It is now possible to perform cross-species complementation experiments in order to explore questions relating to function and protective immunity.

The C-terminal region of *P. falciparum* merozoite surface protein 1 ($MSP1_{19}$) is currently a leading malaria vaccine candidate [34]. The interest in this molecule stems from the finding that antibodies to the epidermal growth factor (EGF)-like domains of $MSP1_{19}$ are associated with clinical immunity to *P. falciparum* [35,36]. Also, active immunisation with $MSP1_{19}$-based vaccines has been shown to provide a degree of protection in various experimental systems [37,38]. These studies, together with the knowledge that EGF-like domains in other molecules possess critical binding functions, suggest an important role for this protein in merozoite invasion of RBCs. Despite extensive molecular epidemiological investigations, it is significant that only limited sequence polymorphism has been identified in *P. falciparum* $MSP1_{19}$ [39,40]. This implies that its sequence is functionally constrained and is used in support of the use of $MSP1_{19}$ as a vaccine.

In light of the sequence conservation of *P. falciparum* $MSP1_{19}$, it was somewhat surprising to find that the $MSP1_{19}$ of the rodent parasite *P. chabaudi* can complement the function of *P. falciparum* $MSP1_{19}$, despite the fact that they are highly divergent in sequence [28]. This implies that the role of $MSP1_{19}$ in RBC invasion is conserved across distantly related *Plasmodium* species and that the sequence of *P. falciparum* $MSP1_{19}$ is not tightly constrained by function as suggested by sequence analysis. This has implications for the use of this molecule in malaria vaccines. Firstly, it is important to consider the possibility that 'escape' mutant parasites, which will presumably acquire point mutations in $MSP1_{19}$, will be selected in vaccinated individuals. Structural analyses of $MSP1_{19}$ have revealed a tight association between the two EGF domains, suggesting that individual point mutations were likely to have consequences for the structural integrity of the domain [41,42]. Therefore, it is possible that such events may require complementary changes elsewhere in the molecule. As a result, point mutations may not accumulate easily in response to immune pressure. However, given our observation that *P. falciparum* can invade efficiently using a divergent $MSP1_{19}$ domain, it is now conceivable that if $MSP1_{19}$ 'escape' mutant parasites do arise in response to vaccination, these may not show a reduced 'fitness' over wild-type parasites. Widespread immunisation with effective $MSP1_{19}$-based vaccines is likely to place much greater selection pressure on the parasite population than is normally evident in infected individuals. This will raise the possibility of such vaccines selecting for *P. falciparum* parasites with antigenically divergent $MSP1_{19}$ domains.

AMA1 is also a leading asexual blood stage protein being considered for inclusion in a malaria vaccine against *P. falciparum*. It was not possible to disrupt the *PfAMA1* gene using PfAMA1 gene 'knockout' plasmids. However, the *AMA1* gene could be targeted by homologous recombination when this recombination results in gene reconstitution. These experiments have suggested that PfAMA1 is critical, perhaps essential, for blood stage growth. Despite this we have complemented the function of the *P. falciparum* AMA1 (PfAMA1) with a divergent *AMA1* transgene from *P. chabaudi* (*PcAMA1*) (Triglia et al., submitted). Importantly, PcAMA1 expression in *P. falciparum* provides trans-species complementation to at least 35% of the function of endogenous PfAMA1 in human RBCs. Furthermore, expression of this transgene in *P. falciparum* leads to more efficient invasion of murine RBCs. These results indicate an important role for AMA1 in the invasion of RBCs across divergent *Plasmodium* species and support the development of this molecule as a malaria vaccine.

4. Concluding remarks

The ability to make loss-of-function and gain-of-function mutants is now assisting dissection of the role of *P. falciparum* proteins in RBC invasion and in providing protective immunity. For instance, disruption of genes encoding proteins that are not essential for invasion in vitro, such as EBA175 and RAP1, has given insights into their function in different invasion pathways. However, this knowledge, together with the many previous studies that employed biochemical methodologies and other biological approaches, has only scratched the surface of our understanding of the molecular basis of *P. falciparum* merozoite invasion. Not only is there still much to be learnt about the molecules with which we are familiar, but with the *P. falciparum* genome project approaching completion many new molecules potentially relevant to this process are being identified [43,44]. For example, the genome possesses several genes homologous to EBA175 that are clear candidates for an involvement in alternative invasion pathways.

The use of microarray, proteomic and potentially other 'whole organism' analytical tools will be essential in utilising the genetic information in the context of understanding merozoite invasion pathways. Such technologies are likely to prove particularly useful in the identification of signalling molecules that regulate processes such as the ontogeny of merozoite antigen expression and perhaps also invasion pathway switching. Improved transfection methodologies are also clearly needed. The availability of inducible promoter systems to derive conditional lethal mutants and of episomal suicide plasmids would greatly assist in the functional analysis of proteins that are essential to *P. falciparum* blood stage growth.

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