Functional analysis of *Plasmodium falciparum* merozoite antigens: implications for erythrocyte invasion and vaccine development

Alan F. Cowman*, Deborah L. Baldi, Manoj Duraisingh, Julie Healer, Kerry E. Mills, Rebecca A. O'Donnell, Jennifer Thompson, Tony Triglia, Mark E. Wickham and Brendan S. Crabb

The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia

Malaria is a major human health problem and is responsible for over 2 million deaths per year. It is caused by a number of species of the genus *Plasmodium*, and *Plasmodium falciparum* is the causative agent of the most lethal form. Consequently, the development of a vaccine against this parasite is a priority. There are a number of stages of the parasite life cycle that are being targeted for the development of vaccines. Important candidate antigens include proteins on the surface of the asexual merozoite stage, the form that invades the host erythrocyte. The development of methods to manipulate the genome of *Plasmodium* species has enabled the construction of gain-of-function and loss-of-function mutants and provided new strategies to analyse the role of parasite proteins. This has provided new information on the role of merozoite antigens in erythrocyte invasion and also allows new approaches to address their potential as vaccine candidates.

Keywords: malaria; vaccine antigens; targeted gene disruption; *Plasmodium falciparum*; proteins

1. INTRODUCTION

Malaria has been a significant burden on humans throughout recorded history and the ability of the parasite to develop resistance to antimalarial drugs and the absence of a suitable vaccine to control the disease ensures that it remains a major problem. This disease is caused by a number of *Plasmodia* species but *Plasmodium falciparum* causes the most severe form and is responsible for 200–300 million infections per year and of these over 2 million people die as a result of infection with this parasite. The development of a vaccine is a global priority.

Several stages of the complex life cycle of the malaria parasite are potential targets of vaccines. Infection is initiated when the mosquito vector injects sporozoite forms during a blood meal on the human host. The sporozoites rapidly infect liver cells where they develop into merozoites, that upon rupture and release into the circulation are free to invade erythrocytes. Once inside the erythrocyte the parasite replicates and matures into a schizont form which eventually ruptures to release new merozoites to complete the blood-stage cycle. During this asexual developmental cycle some of the parasites can switch to gametocytes. The male and female sexual gametocytes can fuse in the mosquito gut after being taken up during mosquito feeding. These penetrate the midgut and oocysts are formed that eventually develop into sporozoites. The sporozoites are released into the body cavity of the mosquito by mature oocysts and are carried by the

haemolymph to the mosquito salivary glands. They invade the cells of the salivary gland and can be injected into the human host during subsequent mosquito blood meals. Efforts to develop a vaccine to this parasite are being directed at a number of stages of this complex life cycle including transmission of sexual forms to the mosquito, delivery of sporozoites into the human host, invasion and development within liver cells and invasion and development of the asexual stages in the host's blood circulation.

The merozoite form of the asexual life cycle in the blood stage attaches to the surface of the erythrocyte thus initiating the invasion process (Gratzer & Dluzewski 1993). Proteins on the surface of merozoites and those released from the apical organelles such as the rhoptries and micronemes during the invasion process are exposed to the immune system and consequently these proteins are potential vaccine candidates (Anders 1997). Many of these proteins are thought to play a role in merozoite invasion of erythrocytes but the details of their function remain sketchy at best. The asexual merozoite has been a particular focus of attention in recent times for the development of a vaccine.

2. THE MEROZOITE SURFACE

Merozoite invasion is initiated with an initial interaction of this parasite cell with the erythrocyte surface. A subsequent re-orientation step allows the apical end to interact with the membrane of the host cell. The contents of the apical organelles, the rhoptries and micronemes, are expelled and a tight junction is formed between the merozoite surface and the erythrocyte membrane (Gratzer & Dluzewski 1993). The tight junction moves along the sur-

*Author for correspondence (cowman@wehi.edu.au)

face 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.

The initial interaction of the merozoite surface with the erythrocyte membrane must involve proteins on the surface of the parasite and a number of proteins have been characterized. One of these proteins, MSP-1 has been hypothesized to be involved in the initial interaction of the merozoite with the erythrocytes surface (Holder & Freeman 1984; Holder *et al.* 1985). As its name implies, this GPI-anchored protein is found on the surface of the merozoite. This localization is shared with a number of other GPI-anchored proteins including MSP-2 (Smythe *et al.* 1988), MSP-4 (Marshall *et al.* 1997) and MSP-5 (Marshall *et al.* 1998). Other proteins are extrinsically associated with the merozoite surface and are initially located in the parasitophorous vacuole of the schizont stage. These proteins are free to interact with the merozoites before erythrocyte rupture and they probably associate with other proteins on the surface at this time. These proteins include MSP-3 (McColl *et al.* 1994; McColl & Anders 1997), MSP-6 (Trucco *et al.* 2001), acid and basic repeat antigen (ABRA) (Stahl *et al.* 1986), S-Ag (Cowman *et al.* 1985) and SERA (Bzik *et al.* 1988).

The surface coat on the surface of the merozoite has been extensively studied at the morphological level in *Plasmodium knowlesi* and consists of fibrils that are closely spaced (Bannister *et al.* 1986). Each cluster consists of two regions, the outer 40 nM fibril that is 2–3 nM wide and the inner 18–22 nM fibrils, and between the fibrillar coat there appears to be an amorphous proteinaceous covering. The proteins that constitute each of these structures is unknown but probably includes some of the proteins discussed above that have already been localized to the merozoite surface.

3. RHOPTRY AND MICRONEME ORGANELLES

Ultrastructural and biochemical studies have suggested that rhoptries and micronemes play an essential role in the invasion process. These organelles are part of the apical complex and the electron dense rhoptries are connected to the surface of the apical end of the merozoite by a duct-like structure and their contents are expelled during erythrocyte invasion (Aikawa *et al.* 1978). The micronemes are also electron dense and are tubular-shaped vesicles that surround the common duct of the paired rhoptries. A number of proteins located in the rhoptries and micronemes have been identified and characterized, some of which have been shown to have a defined role in erythrocyte invasion.

4. ANALYSIS OF MEROZOITE INVASION USING GENE DISRUPTIONS

The development of transfection in *P. falciparum* has created the possibility to construct loss-of-function mutants by gene disruption (Wu *et al.* 1996; Crabb *et al.* 1997a,b). It may be expected that genes encoding

Table 1. Targeted disruption of genes encoding *P. falciparum* merozoite proteins.

gene	construct design ^a	result ^b	reference
<i>MSP1</i>	KO	NHI ^c	O'Donnell <i>et al.</i> 2000
<i>MSP1</i>	3'-replacement	HI	O'Donnell <i>et al.</i> 2000
<i>MSP2</i>	KO	NHI	M. E. Wickham and A. F. Cowman, unpublished data
<i>MSP2</i>	3'-replacement	HI	M. E. Wickham and A. F. Cowman, unpublished data
<i>MSP3</i>	KO	HI	Mills <i>et al.</i> 2002
<i>MSP3</i>	3'-replacement	HI	Mills <i>et al.</i> 2002
<i>MSP4</i>	KO	NHI	A. F. Cowman and B. S. Crabb, unpublished data
<i>MSP5</i>	KO	NHI	A. F. Cowman and B. S. Crabb, unpublished data
<i>RAP1</i>	KO	HI	Baldi <i>et al.</i> 2000
<i>RAP2</i>	KO	NHI	R. A. O'Donnell, A. F. Cowman and B. S. Crabb, unpublished data
<i>AMA1</i>	KO	NHI	Triglia <i>et al.</i> 2000
<i>AMA1</i>	3'-replacement	HI	Triglia <i>et al.</i> 2000
<i>EBA175</i>	KO	NHI	D. L. Baldi, B. S. Crabb and A. F. Cowman, unpublished data
<i>EBA175</i> ^d	KO	HI	Reed <i>et al.</i> 2000a

^a Plasmids for gene targeting were based on either the pHCI1 (Crabb *et al.* 1997b) or pHH1 (Reed *et al.* 2000b) 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 Crabb *et al.* (1997b) and O'Donnell *et al.* (2000).

^b Result represents analysis of parasites after three to five drug cycles for integration (4–6 months of continuous culture) as observed by Southern blot experiments.

^c Persistent episomal plasmids that remained after extensive culturing, however, occasionally non-HI was observed.

^d This transfection was carried out in the W2mef parasite line while all others were performed in D10.

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 erythrocyte receptors (Dolan *et al.* 1990). Therefore in some cases it may 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 erythrocytes via multiple pathways, while others appear to lack some of these invasion routes (Dolan *et al.* 1990; Okoyeh *et al.* 1999).

We have attempted to disrupt several genes that have been linked to the invasion process and, not surprisingly, many of these appear to be lethal or severely deleterious (table 1). Interestingly, it is possible to disrupt a number of genes including some of those for which there is good evidence that they play an important part in the merozoite

invasion process. 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. First, the D10 parasite, that was used in the majority of gene-disruption experiments, lacks at least one of the known invasion pathways (Dolan *et al.* 1990). It is possible that *P. falciparum* merozoites require a minimum number of ligands and pathways for successful invasion of the erythrocyte. Consequently, it will be important to test gene disruption in other parasite lines with a broader range of invasion pathways.

Second, 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 (Crabb & Cowman 1996; Wu *et al.* 1996; Crabb *et al.* 1997a). 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 has become 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. Recently, we have successfully expressed the thymidine kinase and cytidine deaminase genes in *P. falciparum* and shown that they are able to be used to negatively select the transgenic parasites with the appropriate drugs (Duraisingh *et al.* 2002). Additionally, we have shown that the thymidine kinase gene can be used in a *P. falciparum* transfection plasmid to allow selection of parasites that have incorporated the vector by a double homologous recombination. This is an important technical breakthrough allowing the selection of transfected parasites with deleterious gene disruptions.

Third, the gene of interest may be essential for erythrocyte 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 (Gossen *et al.* 1995), allow activation and repression of gene expression in a controlled manner. Two examples of essential genes appear to be the MSP-1 (O'Donnell *et al.* 2000) and AMA-1 (Triglia *et al.* 2001). 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.

5. MEROZOITE SURFACE PROTEIN 1

There has been a lot of interest in MSP-1 because the C-terminal region MSP-1₁₉ is currently a leading malaria vaccine candidate (Holder 1988). This stems from the finding that antibodies to the EGF-like domains of MSP-1₁₉ are associated with clinical immunity to *P. falciparum* (Egan *et al.* 1996, 1999). Also, active immunization with MSP-1₁₉-based vaccines has been shown to provide a degree of protection in various experimental systems (Daly & Long 1993; Perera *et al.* 1998). 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 erythrocytes. Despite extensive molecular epidemiological

investigations, it is significant that only limited sequence polymorphism has been identified in *P. falciparum* MSP-1₁₉ (Miller *et al.* 1993; Qari *et al.* 1998). This sequence conservation has implied that MSP-1₁₉ is functionally constrained and is used in support of the use of MSP-1₁₉ as a vaccine.

In light of the sequence conservation of *P. falciparum* MSP-1₁₉, it was somewhat surprising to find that MSP-1₁₉ of the rodent parasite *Plasmodium chabaudi* can complement the function of *P. falciparum* MSP-1₁₉, despite the fact that they are highly divergent in sequence (O'Donnell *et al.* 2000). This observation infers that the role of MSP-1₁₉ in erythrocyte invasion is conserved across distantly related *Plasmodium* species and that the sequence of *P. falciparum* MSP-1₁₉ is not tightly constrained by function as suggested by sequence analysis. This has implications for the use of this molecule in malaria vaccines. It is important to consider the possibility that 'escape' mutant parasites, which will presumably acquire point mutations in MSP-1₁₉, will be selected in vaccinated individuals. Structural analyses of MSP-1₁₉ 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 (Chitarra *et al.* 1999; Morgan 1999). 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 MSP-1₁₉ domain, it is now conceivable that if MSP-1₁₉ 'escape' mutant parasites do arise in response to vaccination, these may not show a reduced 'fitness' over wild-type parasites. Widespread immunization with effective MSP-1₁₉-based vaccines will probably 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 MSP-1₁₉ domains.

Attempts to disrupt the MSP-1 gene have been unsuccessful although it is possible to target the gene by homologous recombination provided that it is fully reconstituted in the recombination event (O'Donnell *et al.* 2000). This suggests that MSP-1 is essential for the erythrocyte invasion of merozoites. Interestingly, it is clear that the plasmid constructs designed to disrupt the MSP-1 gene can successfully target the gene as these parasites can be detected by PCR experiments that only detect the successful gene disruption event. An intriguing possibility is that parasites with a disrupted MSP-1 gene are viable but severely restricted in their ability to invade erythrocytes. If this is correct it opens the possibility of deriving parasites with the MSP-1 gene disrupted that can be analysed to determine the direct effect of loss of MSP-1 on merozoite invasion using the newly developed thymidine kinase negative selection transfection plasmids.

6. MEROZOITE SURFACE PROTEIN 3 AND HOMOLOGUES

MSP-3 is a soluble protein initially localized to the parasitophorous vacuole but that interacts with the merozoite surface (McColl & Anders 1997). This protein consists of

a heptad-repeat motif found in three blocks and a coiled-coil region that has similarities to a leucine-zipper motif (McColl *et al.* 1994). We have recently constructed a *P. falciparum* parasite line that contains a disrupted *MSP-3* gene and shown that this does not greatly affect the ability of these parasites to invade erythrocytes (Mills *et al.* 2002). Interestingly, the truncated protein lacking the leucine-zipper-like region was no longer located on the surface of the merozoite suggesting that this region of *MSP-3* may be responsible for binding to another protein on the surface of the cell. Truncation of *MSP-3* also affected the trafficking of ABRA that also was no longer associated with the merozoite surface. These results could be explained if *MSP-3* and ABRA bind to each other and the leucine-zipper region of *MSP-3* binds to another protein on the merozoite surface; however, this remains to be explored. It is interesting that this does not greatly affect the ability of these merozoites to invade erythrocytes suggesting that they are not essential for merozoite invasion.

Recently another protein that has significant homology with *MSP-3* has been identified (Trucco *et al.* 2001). This protein, now termed *MSP-6*, has been shown to be located in the parasitophorous vacuole before associating with the *MSP-1* complex on the merozoite surface (Trucco *et al.* 2001). This protein shares sequence homology with *MSP-3* at the C-terminus where a coiled-coil region is located. It is probable that this region is also required for interaction with another protein as has been suggested with results obtained from truncation of *MSP-3*. Preliminary results suggest that the *MSP-6* gene can be disrupted suggesting that this protein is also not essential for merozoite invasion (K. E. Mills & A. F. Cowman, unpublished data).

7. RHOPTRY-ASSOCIATED PROTEINS 1 AND 2

RAP1 and 2 are located in the body of the paired rhoptries and are important vaccine candidates because it has been shown that antibodies to RAP1 are able to block merozoite invasion *in vitro* (Schofield *et al.* 1986; Harnyutanakorn *et al.* 1992; Howard *et al.* 1998). Additionally, monkeys immunized with RAP1 and 2 are partially protected against parasite challenge (Perrin *et al.* 1985; Ridley *et al.* 1990a).

The RAP1 protein (Ridley *et al.* 1990b; Howard & Schmidt 1995) forms a complex with RAP2 (Howard & Reese 1990). Antibodies to RAP1 immunoprecipitate both RAP1 and RAP2 and an as-yet-uncharacterized third protein that has been termed RAP3 (Howard & Reese 1990). The function of the RAP complex, also known as the lower molecular weight rhoptry complex, is unknown, but it has been thought to play a role in invasion and hence in the maintenance of the blood-stage cycle.

Targeted disruption of the *RAP1* gene in *P. falciparum* has been achieved and this event led to the expression of severely truncated forms of RAP1, and the loss of its ability to interact with RAP2 and RAP3. Also, RAP2 was found to be localized to the lumen of the ER in these mutant parasites, showing that interaction with RAP1 is required for rhoptry localization of this protein. This has important implications for the development and subcellular trafficking of proteins to the rhoptries.

The development of organelles such as the rhoptries

could be controlled by two pathways that target proteins and membranes to the developing structure. First, the proteins could be imported post-translationally into preformed organelles where membrane development is a separate event. This process occurs for mitochondria and peroxisomes, and new organelles are generated by membrane fission (Fujiki *et al.* 1984; Hartl & Neupert 1990). Second, proteins can be co-translationally translocated into the ER and transported via membrane vesicles as either soluble or membrane-bound proteins (Walter & Blobel 1981). These proteins move through other subcellular compartments such as the Golgi where protein maturation and sorting can occur. Finally, vesicles fuse to form or enlarge the organelle and examples of this mode of organellar biogenesis includes lysosomes and secretory granules (Farquhar 1985). There is evidence to suggest that development of rhoptries occurs via this second mechanism using the secretory pathway (Howard & Schmidt 1995). The rhoptry proteins that have been identified have structural characteristics of proteins transported within the secretory pathway including short hydrophobic N-terminal sequences that resemble membrane translocation signal sequences (Lustigman *et al.* 1988; Peterson *et al.* 1989; Howard & Reese 1990; Saul *et al.* 1992).

The localization of RAP2 in the ER of RAP1-disrupted parasites suggests that interaction of RAP1 with RAP2 occurs in the ER followed by trafficking of the protein complex to the rhoptries. The retention of RAP2 in the ER of the mutant parasites is probably due to the inability to be directed to rhoptries rather than a specific ER retention signal such as the amino-acid motif KDEL (Andres *et al.* 1991) which is not present in RAP2. There are several possible reasons why RAP2 is not trafficked to the rhoptries in the RAP1 mutant parasites. First, it is possible that the RAP2 protein, accumulating in the ER of the mutant parasites, is not folded correctly in the absence of interacting RAP1. The mis-folded RAP2 would not be trafficked to the rhoptries and would accumulate in the ER as has been described previously for a number of other proteins in mammalian cells (Gething & Sambrook 1992). Thus accumulation of incorrectly folded RAP2, in the absence of RAP1, would result in the degradation of this protein in the ER. In this hypothesis, retention of RAP2 in the ER of RAP1-mutant parasites is due to incorrect folding of RAP2 due to non-association with full-length RAP1. Second, it is possible that RAP1 but not RAP2 contains a rhoptry-targeting signal and hence RAP2 requires association with RAP1 to be trafficked correctly. Finally, it is possible that localization to the rhoptries is dependent on timing of expression and no localization signals are required.

The short truncated RAP1 product in the RAP1-mutant parasites is still localized to the rhoptries showing that this region of the protein is sufficient to specify rhoptry targeting, but insufficient for association with RAP2 or entry into the erythrocyte with the invading merozoite. Additionally, the truncated RAP1 sequence is sufficient for RAP1 localization into rhoptries of the paired organelles in each merozoite. The analysis of the RAP1-mutant parasites shows that a function of RAP1 is to target RAP2 to the rhoptries. They also suggest that the

function of RAP1 and 2 is not essential for normal merozoite invasion *in vitro*.

The ability to disrupt genes encoding proteins believed to be involved in merozoite invasion clearly shows that they are not absolutely essential for this process *in vitro*. RAP1 together with RAP2 have been viewed as important vaccine candidates as they can partially protect Saimiri monkeys from challenge with *P. falciparum* (Perrin *et al.* 1985; Ridley *et al.* 1990a). Furthermore, mAbs specific for RAP1 can inhibit invasion into erythrocytes (Schofield *et al.* 1986; Harnyuttanakorn *et al.* 1992; Howard *et al.* 1998). 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 erythrocytes. 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 (Perrin *et al.* 1985; Ridley *et al.* 1990a). These findings raise the possibility that breakthrough parasites could arise that can survive in the absence of functional RAP1.

8. PROTEINS IN *PLASMODIUM FALCIPARUM* THAT ARE HOMOLOGOUS TO THE RETICULOCYTE BINDING PROTEINS IN *PLASMODIUM VIVAX* AND THE *PLASMODIUM YOELII* PY235 FAMILY

A high molecular weight rhoptry protein from *P. yoelii* (Py235) has been suggested to be important in the ability of this parasite to invade mature erythrocytes (Preiser *et al.* 1999). Passive transfer of mAbs specific to this protein protect mice infected with a virulent strain, by restricting invasion of reticulocytes (Freeman *et al.* 1980). In *P. vivax* proteins (termed *P. vivax* reticulocyte binding proteins) have been shown to bind reticulocyte-enriched erythrocytes (Galinski *et al.* 1992). Two of these proteins form a complex through non-covalent interactions and co-localize to the apical end of the merozoite. The *P. vivax* reticulocyte binding proteins and Py235 family share similar structures with a signal sequence at the N-terminus and a putative transmembrane domain and cytoplasmic tail at the C-terminus (Galinski *et al.* 2000). These proteins also share a 500 amino-acid region that shows significant homology (Keen *et al.* 1994). The Py235 proteins are encoded by a multigene family of up to 50 members with at least 11 distinct genes spread across different chromosomes of the *P. yoelii* genome (Borre *et al.* 1995). At least one member of this protein family has been shown to bind both mature and immature erythrocytes (Ogun & Holder 1996), a finding consistent with the fact that a Py235 mAb can restrict parasite invasion of reticulocytes (Freeman *et al.* 1980).

Homologous proteins to the *P. vivax* reticulocyte binding proteins and the Py235 family have been identified in *P. falciparum* suggesting that these proteins may be involved in specifying different invasion pathways by the merozoite into human erythrocytes (Rayner *et al.* 2000). Immunofluorescence antibody experiments are consistent with a subcellular localization at the apical end of the

merozoite which is similar to that described for the proteins in *P. vivax* and *P. yoelii*. By analogy with the role of the other members of this family, these proteins may be involved in the targeting of particular erythrocyte subpopulations for invasion by *P. falciparum* merozoites.

We have tested a potential role of the *P. falciparum* homologues to the Py235 and *P. vivax* reticulocyte binding proteins in merozoite invasion of erythrocytes by analysing their ability to bind to mature erythrocytes and reticulocytes (Triglia *et al.* 2001). It was not possible to detect binding activity to mature erythrocytes or cell preparations enriched for reticulocytes. Interestingly, we have identified a *P. falciparum* clone that lacks the gene for one of these proteins showing that it is not required for normal *in vitro* growth; however, it is possible that other members of this protein family are able to provide any missing function as a result of the lack of expression of this particular member of the protein family. We speculate that these proteins in *P. falciparum* are involved in binding of the apical end of the merozoite to the erythrocyte and may specify the ability of the merozoite to invade by specific invasion pathways and receptors on the erythrocyte. This function may be analogous to that described for EBA-175, which has been extensively characterized (see § 9).

9. ERYTHROCYTE-BINDING ANTIGENS

The ability of *Plasmodium* merozoites to invade erythrocytes is contingent upon a rapid cascade of specific interactions occurring between parasite molecules and host erythrocyte receptors. Despite a good understanding of the recognition and invasion processes at the ultra-structural level, the nature and timing of these events at the molecular level remain poorly defined. *Plasmodium falciparum* is known to use different receptors for invasion of erythrocytes and commonly invades via NANA residues present on glycophorin A or B (Dolan *et al.* 1994). Mutant erythrocytes with modifications or deficiencies in glycophorins are, in general, less susceptible to invasion by *P. falciparum* than normal erythrocytes (Cartron *et al.* 1983; Hadley *et al.* 1987). Similarly, treatment of erythrocytes by enzymes that modify the structure of glycophorins renders them less susceptible to invasion (Breuer *et al.* 1983; Hadley & Miller 1988). Moreover, *P. falciparum* appears not to be restricted to invasion via a single class of erythrocyte receptor. Indeed, there is mounting evidence of receptor heterogeneity in both laboratory (Mitchell *et al.* 1986; Hadley *et al.* 1987; Dolan *et al.* 1994) and field isolates (Okoyeh *et al.* 1999). The possibility of 'switching' between, or upregulating, novel invasion phenotypes introduces a further element of complexity into the invasion process and was demonstrated through the ability of Dd2 to be selected for invasion into Nm-treated erythrocytes (Dolan *et al.* 1990).

Based on high degrees of structural conservation at both the genetic and protein levels, the *Plasmodium* erythrocyte binding ligands reported to date have been ascribed to an EBP family (Adams *et al.* 1992) that includes the *P. falciparum* NANA-binding protein, EBA-175. EBA-175 exclusively binds NANA residues present on glycophorin A of human erythrocytes (Camus & Hadley 1985; Sim *et al.* 1994). This protein and other members of the EBP family have extracellular N- and C-terminal cysteine-rich

regions in addition to transmembrane and cytoplasmic domains. The two cysteine rich regions (often referred to as regions II and VI) bear numerous conserved cysteine residues, with region II possessing the erythrocyte binding function (Chitnis & Miller 1994; Sim *et al.* 1994; Ranjan & Chitnis 1999). This reinforces the ability of *P. falciparum* to utilize alternate pathways for invasion of erythrocytes; a property that probably provides a substantial survival advantage in terms of overcoming host receptor heterogeneity and immune pressure.

We have addressed the role of the conserved 3'-cys-rich region, the transmembrane and cytoplasmic domains of EBA-175 through targeted gene disruption (Reed *et al.* 2000a). Truncation of EBA-175 had no measurable effect on either the level of EBA-175 protein expression or its subcellular localization. Similarly, there appears to be no impairment in the ability of soluble EBA-175 to be released into the culture supernatant following schizont rupture. Additionally, the 3'-cys-rich region, transmembrane and cytoplasmic domains of EBA-175 are apparently non-essential for merozoite invasion. In contrast, erythrocyte invasion via the EBA-175-glycophorin A route was disrupted to such a degree that the mutant lines have undergone a stable switch in invasion phenotype. As such, EBA-175 appears to have been functionally inactivated within the truncation mutants. The NANA-independent invasion pathway within the mutant parasites accounts for approximately 85% of invasion into normal erythrocytes. This demonstrates the ability of *P. falciparum* to utilize alternate pathways for invasion of erythrocytes; a property that probably provides a substantial survival advantage in terms of overcoming host receptor heterogeneity and immune pressure.

The ability of *P. falciparum* to utilize alternate invasion pathways for merozoite invasion of human erythrocytes has suggested that there may be paralogues of EBA-175 that are required for these receptor-ligand interactions. We have identified a novel *P. falciparum* ligand, EBA-140, that shares structural features and homology with EBA-175 (Thompson *et al.* 2001). Subcellular localization of EBA-140 suggests that it is located in the micronemes, the same localization as EBA-175. EBA-140 binds to a NANA-dependent receptor on the surface of human erythrocytes. Binding of EBA-140 to this erythrocyte receptor is sensitive to Nm and resistant to trypsin, proteinase K and pronase. The protease-resistant properties of the erythrocyte receptor suggests that it is not glycophorin A or C. Additionally, analysis of mutant erythrocytes from humans has shown that EBA-140 does not bind glycophorin B. Interestingly, we have identified a parasite line that lacks the *EBA-140* gene suggesting that this protein is not essential for *in vitro* invasion. These results suggest EBA-140 may be involved in merozoite invasion using a NANA-dependent receptor on human erythrocytes.

10. APICAL MEMBRANE ANTIGEN-1

One of the leading vaccine candidates is AMA-1 (Deans *et al.* 1984; Peterson *et al.* 1989; Cheng & Saul 1994; Dutta *et al.* 1995; Marshall *et al.* 1995) which is expressed in the late schizont stage of the asexual life cycle of this parasite (Narum & Thomas 1994). The AMA-1 protein

has been localized to the neck of the rhoptry organelles and after schizont rupture a processed form of the protein appears to spread circumferentially around the merozoite surface (Narum & Thomas 1994).

The biological function of AMA-1 is currently unknown, however the subcellular localization and stage-specific expression suggests that it is involved in merozoite invasion. The ability to protect against lethal parasitaemias by passive transfer of the relevant species-specific anti-AMA-1 antibodies into *P. chabaudi*-infected mice supports an important role for AMA-1 in invasion (Deans *et al.* 1984; Thomas *et al.* 1984). Additionally, immunization of mice and monkeys with recombinant AMA-1 protein can protect against blood-stage challenge of mouse and simian malaria parasites respectively (Deans *et al.* 1988; Collins *et al.* 1994). Protective immunization of mice has also been achieved using the correctly folded form of the *P. chabaudi* AMA-1 ectodomain (Anders *et al.* 1996).

An AMA-1 homologue is present in all species of *Plasmodium* that have been examined supporting the view that this molecule is required for an essential function (Marshall *et al.* 1989; Peterson *et al.* 1989; Peterson *et al.* 1990; Thomas *et al.* 1990; Cheng & Saul 1994; Dutta *et al.* 1995). AMA-1 homologues have the structural characteristics of a Type I integral membrane protein and the ectodomain contains 16 cysteine residues that are conserved in all known AMA-1 sequences. The protein can be divided into three domains as defined by the intramolecular disulphide linkages (Hodder *et al.* 1996). Despite this structural conservation, PcAMA1 has diverged considerably from PfAMA1 with the two molecules showing only 51% overall amino-acid sequence identity (Marshall *et al.* 1989).

Targeted disruption of the *PfAMA1* gene appears to be deleterious to blood-stage growth as it has not been possible to derive parasites lacking expression of the protein (Triglia *et al.* 2001). Interestingly, it is possible to complement PfAMA1 function by transgenic expression of the divergent AMA-1 homologue from *P. chabaudi*. However, PcAMA1 does not provide a full function and estimates suggest that expression of this protein correctly in *P. falciparum* can provide approximately 40% of the function in merozoite invasion that PfAMA1 provides. Additionally, expression of PcAMA1 in *P. falciparum* allows these parasites to more efficiently invade murine erythrocytes. These results suggest that AMA-1 provides a critical function in merozoite invasion of erythrocytes by *Plasmodia* species.

11. CONCLUDING REMARKS

The availability of parasites with targeted gene disruptions in *P. falciparum* is now assisting in dissection of the role of proteins in erythrocyte invasion and in providing protective immunity. For instance, disruption of genes encoding proteins that are not essential for invasion *in vitro*, such as EBA-175 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 (Gardner *et al.* 1998; Bowman *et al.* 1999). For example, the genome possesses several genes homologous to EBA-175, such as EBA-140, that are clear candidates for involvement in alternate invasion pathways.

The use of microarray, proteomic and potentially other 'whole-organism' analytical tools will be essential in utilizing the genetic information in the context of understanding merozoite-invasion pathways. Such technologies will probably prove to be particularly useful in the identification of the signalling molecules that regulate processes such as the ontogeny of merozoite antigen expression and perhaps 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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REFERENCES

- Adams, J. H., Sim, B. K. L., Dolan, S. A., Fang, X., Kaslow, D. C. & Miller, L. H. 1992 A family of erythrocyte binding proteins of malaria parasites. *Proc. Natl Acad. Sci. USA* **89**, 7085–7089.
- Aikawa, M., Miller, L. H., Johnson, J. & Rabbege, J. 1978 Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. *J. Cell Biol.* **77**, 72–82.
- Anders, R. F. 1997 Vaccines against asexual blood-stages of *Plasmodium falciparum*. In *New generation vaccines* (ed. M. M. Levine, G. Woodrow, G. Cobon, J. Kaper & G. Garrett), pp. 1037–1057. New York: Dekker.
- Andres, D. A., Rhodes, J. D., Meisel, R. L. & Dixon, J. E. 1991 Characterisation of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. *J. Biol. Chem.* **266**, 14 277–14 288.
- Anders, R. F., Crewther, P. E., Edwards, S., Margetts, M., Matthew, M. L. S. M., Pollock, B. & Pye, D. 1996 A malaria subunit vaccine based on the ectodomain of apical membrane antigen 1 expressed in *Escherichia coli* and refolded *in vitro*. *Vaccine* **16**, 240–247.
- Baldi, D. L., Andrews, K. T., Waller, R. F., Roos, D. S., Howard, R. F., Crabb, B. S. & Cowman, A. F. 2000 RAP1 controls rhoptry targeting of RAP2 in the malaria parasite *Plasmodium falciparum*. *EMBO J.* **19**, 2435–2443.
- Bannister, L. H., Mitchell, G. H., Butcher, G. A., Dennis, E. D. & Cohen, S. 1986 Structure and development of the surface coat of erythrocytic merozoites of *Plasmodium knowlesi*. *Cell Tissue Res* **245**, 281–290.
- Borre, M. B., Owen, C. A., Keen, J. K., Sinha, K. A. & Holder, A. A. 1995 Multiple genes code for high-molecular-mass rhoptry proteins of *Plasmodium yoelii*. *Mol. Biochem. Parasitol.* **70**, 149–155.
- Bowman, S. (and 35 others) 1999 The complete nucleotide sequence of chromosome 3 of *Plasmodium falciparum*. *Nature* **400**, 532–538.
- Breuer, W. V., Ginsburg, H. & Cabantchik, Z. I. 1983 An assay of malaria parasite invasion into human erythrocytes. The effects of chemical and enzymatic modification of erythrocyte membrane components. *Biochim. Biophys. Acta* **755**, 263–271.
- Bzik, D. J., Li, W. B., Horii, T. & Inselburg, J. 1988 Amino acid sequence of the serine-repeat antigen (SERA) of *Plasmodium falciparum* determined from cloned cDNA. *Mol. Biochem. Parasitol.* **30**, 279–288.
- Camus, D. & Hadley, T. J. 1985 A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. *Science* **230**, 553–556.
- Cartron, J. P., Prou, O., Luilier, M. & Soulier, J. P. 1983 Susceptibility to invasion by *Plasmodium falciparum* of some human erythrocytes carrying rare blood group antigens. *Br. J. Haematol.* **55**, 639–647.
- Cheng, Q. & Saul, A. 1994 Sequence analysis of the apical membrane antigen 1 (AMA-1) of *Plasmodium vivax*. *Mol. Biochem. Parasitol.* **65**, 183–187.
- Chitarra, V., Holm, I., Bentley, G., Petres, S. & Longacre, S. 1999 The crystal structure of the C-terminal merozoite protein 1 at 1.8 Å resolution, a highly protective malaria vaccine candidate. *Mol. Cell* **3**, 457–464.
- Chitnis, C. E. & Miller, L. H. 1994 Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion. *J. Exp. Med.* **180**, 497–506.
- Collins, W. E. (and 11 others) 1994 Protective immunity induced in squirrel monkeys with recombinant apical membrane antigen-1 of *Plasmodium fragile*. *Am. J. Trop. Med. Hyg.* **51**, 711–719.
- Cowman, A. F., Saint, R. B., Coppel, R. L., Brown, G. V., Anders, R. F. & Kemp, D. J. 1985 Conserved sequences flank variable tandem repeats in two S-antigen genes of *Plasmodium falciparum*. *Cell* **40**, 775–783.
- Crabb, B. S. & Cowman, A. F. 1996 Characterization of promoters and stable transfection by homologous and non-homologous recombination in *Plasmodium falciparum*. *Proc. Natl Acad. Sci. USA* **93**, 7289–7294.
- Crabb, B. S., Cooke, B. M., Reeder, J. C., Waller, R. F., Caruana, S. R., Davern, K. M., Wickham, M. E., Brown, G. V., Coppel, R. L. & Cowman, A. F. 1997a Targeted gene disruption shows that knobs enable malaria-infected red cells to cytoadhere under physiological shear stress. *Cell* **89**, 287–296.
- Crabb, B. S., Triglia, T., Waterkeyn, J. G. & Cowman, A. F. 1997b Stable transgene expression in *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **90**, 131–144.
- Daly, T. M. & Long, C. A. 1993 A recombinant 15-kilodalton carboxyl-terminal fragment of *Plasmodium yoelii yoelii* 17XL merozoite surface protein 1 induces a protective immune response in mice. *Infect. Immun.* **61**, 2462–2467.
- Deans, J. A., Thomas, A. W., Alderson, T. & Cohen, S. 1984 Biosynthesis of a putative protective *Plasmodium knowlesi* merozoite antigen. *Mol. Biochem. Parasitol.* **11**, 189–204.
- Deans, J. A., Knight, A. M., Jean, W. C., Waters, A. P., Cohen, S. & Mitchell, G. H. 1988 Vaccination trials in rhesus monkeys with a minor, invariant, *Plasmodium knowlesi* 66 kD merozoite antigen. *Parasite Immunol.* **10**, 535–552.
- Dolan, S. A., Miller, L. H. & Welles, T. E. 1990 Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. *J. Clin. Invest.* **86**, 618–624.
- Dolan, S. A., Proctor, J. L., Alling, D. W., Okubo, Y., Welles, T. E. & Miller, L. H. 1994 Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes. *Mol. Biochem. Parasitol.* **64**, 55–63.
- Duraisingh, T., Triglia, T. & Cowman, A. 2002 Negative selection of *Plasmodium falciparum* reveals targeted gene deletion by double crossover recombination. *Int. J. Parasitol.* (In the press.)

- Dutta, S., Malhotra, P. & Chauhan, V. S. 1995 Sequence analysis of apical membrane antigen 1 (AMA-1) of *Plasmodium cynomolgi* bastianelli. *Mol. Biochem. Parasitol.* **73**, 267–270.
- Egan, A. F., Morris, J., Barnish, G., Allen, S., Greenwood, B. M., Kaslow, D. C., Holder, A. A. & Riley, E. M. 1996 Clinical immunity to *Plasmodium falciparum* malaria is associated with serum antibodies to the 19 kDa C-terminal fragment of the merozoite surface antigen, PfMSP-1. *J. Infect. Dis.* **173**, 765–769.
- Egan, A. F., Burghaus, P., Druilhe, P., Holder, A. A. & Riley, E. M. 1999 Human antibodies to the 19 kDa C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 inhibit parasite growth *in vitro*. *Parasite Immunol.* **21**, 133–139.
- Farquhar, M. G. 1985 Progress in unravelling pathways of Golgi traffic. *A. Rev. Cell Biol.* **1**, 489–530.
- Freeman, R. R., Trejdosiewicz, A. J. & Cross, G. A. 1980 Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite. *Nature* **284**, 366–368.
- Fujiki, Y., Rachubinski, R. A. & Lazarow, P. B. 1984 Synthesis of a major integral membrane protein polypeptide of rat liver peroxisomes on free polysomes. *Proc. Natl Acad. Sci. USA* **81**, 7127–7131.
- Galinski, M. R., Medina, C. C., Ingravallo, P. & Barnwell, J. W. 1992 A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. *Cell* **69**, 1213–1226.
- Galinski, M. R., Mengyao, X. & Barnwell, J. W. 2000 *Plasmodium vivax* reticulocyte binding protein-2 (PvRBP-2) shares structural features with PvRBP-1 and the *Plasmodium yoelii* 235 kDa rhoptry protein family. *Mol. Biochem. Parasitol.* **108**, 257–262.
- Gardner, M. J. (and 25 others) 1998 Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum*. *Science* **282**, 1126–1132.
- Gething, M.-J. & Sambrook, J. 1992 Protein folding in the cell. *Nature* **355**, 33–44.
- Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. & Bujard, H. 1995 Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**, 1766–1769.
- Gratzer, W. B. & Dluzewski, A. R. 1993 The red blood cell and malaria parasite invasion. *Semin. Hematol.* **30**, 232–247.
- Hadley, T. J. & Miller, L. H. 1988 Invasion of erythrocytes by malaria parasites: erythrocyte ligands and parasite receptors. *Prog. Allergy* **41**, 49–71.
- Hadley, T. J., Klotz, F. W., Pasvol, G., Haynes, J. D. & McGinniss, M. H. 1987 *Falciparum* malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. *J. Clin. Invest.* **80**, 1190–1193.
- Harnyuttanakorn, P., McBride, J. S., Donachie, S., Heidrich, H.-G. & Ridley, R. G. 1992 Inhibitory monoclonal antibodies recognise epitopes adjacent to a proteolytic cleavage site on the RAP-1 protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **55**, 177–186.
- Hartl, F.-U. & Neupert, W. 1990 Protein sorting to mitochondria: evolutionary conservations of folding and assembly. *Science* **247**, 930–938.
- Hodder, A. N., Crewther, P. E., Mattew, M. L. S. M., Reid, G. E., Moritz, R. L., Simpson, R. J. & Anders, R. F. 1996 The disulphide bond structure of *Plasmodium* apical membrane antigen-1. *J. Biol. Chem.* **271**, 29 446–29 452.
- Holder, A. A. 1988 The precursor to major merozoite surface antigens: structure and role in immunity. *Prog. Allergy* **41**, 72–97.
- Holder, A. A. & Freeman, R. R. 1984 The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. *J. Exp. Med.* **160**, 624–629.
- Holder, A. A., Lockyer, M. J., Odink, K. G., Sandhu, J. S., Riveros, M. V., Davey, L. S., Tizard, M. L. V., Schwarz, R. T. & Freeman, R. R. 1985 Primary structure of the precursor to the three major surface antigens of *Plasmodium falciparum* merozoites. *Nature* **317**, 270–273.
- Howard, R. F. & Reese, R. T. 1990 *Plasmodium falciparum*: hetero-oligomeric complexes of rhoptry polypeptides. *Exp. Parasitol.* **71**, 330–342.
- Howard, R. F. & Schmidt, C. M. 1995 The secretory pathway of *Plasmodium falciparum* regulates transport of p82/RAP-1 to the rhoptries. *Mol. Biochem. Parasitol.* **74**, 43–54.
- Howard, R. F., Jacobson, K. C., Rickel, E. & Thurman, J. 1998 Analysis of inhibitory epitopes in the *Plasmodium falciparum* rhoptry protein RAP-1 including identification of a second inhibitory epitope. *Infect. Immun.* **66**, 380–386.
- Keen, J. K., Sinha, K. A., Brown, K. N. & Holder, A. A. 1994 A gene coding for a high molecular mass rhoptry protein of *Plasmodium yoelii*. *Mol. Biochem. Parasitol.* **65**, 171–177.
- Lustigman, S., Anders, R. F., Brown, G. V. & Coppel, R. L. 1988 A component of an antigenic rhoptry complex of *Plasmodium falciparum* is modified after merozoite invasion. *Mol. Biochem. Parasitol.* **30**, 217–224.
- McColl, D. J. & Anders, R. F. 1997 Conservation of structural motifs and antigenic diversity in the *Plasmodium falciparum* merozoite surface protein-3 (MSP-3). *Mol. Biochem. Parasitol.* **90**, 21–31.
- McColl, D. J., Silva, A., Foley, M., Kun, J. F. J., Favaloro, J. M., Thompson, J. K., Marshall, V. M., Coppel, R. L., Kemp, D. J. & Anders, R. F. 1994 Molecular variation in a novel polymorphic antigen associated with *Plasmodium falciparum* merozoites. *Mol. Biochem. Parasitol.* **68**, 53–67.
- Marshall, V. M., Peterson, M. G., Lew, A. M. & Kemp, D. J. 1989 Structure of the apical membrane antigen I (AMA-1) of *Plasmodium chabaudi*. *Mol. Biochem. Parasitol.* **37**, 281–284.
- Marshall, V. M., Zhang, L.-X., Anders, R. F. & Coppel, R. L. 1995 Diversity of the vaccine candidate AMA-1 of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **77**, 109–113.
- Marshall, V. M., Silva, A., Foley, M., Cranmer, S., Wang, L., McColl, D. J., Kemp, D. J. & Coppel, R. L. 1997 A second merozoite surface protein (MSP-4) of *Plasmodium falciparum* that contains an epidermal growth factor-like domain. *Infect. Immun.* **65**, 4460–4467.
- Marshall, V. M., Tieqiao, W. & Coppel, R. L. 1998 Close linkage of three merozoite surface protein genes on chromosome 2 of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **94**, 13–25.
- Miller, L. H., Roberts, T., Shahabuddin, M. & McCutchan, T. F. 1993 Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). *Mol. Biochem. Parasitol.* **59**, 1–14.
- Mills, K. E., Pearce, J. A., Crabb, B. S. & Cowman, A. F. 2002 Truncation of the merozoite surface protein 3 disrupts its trafficking and that of the acidic basic repeat protein to the surface of *Plasmodium falciparum* merozoites. *Mol. Microbiol.* (In the press).
- Mitchell, G. H., Hadley, T. J., McGinniss, M. H., Klotz, F. W. & Miller, L. H. 1986 Invasion of erythrocytes by *Plasmodium falciparum* malaria parasites: evidence for receptor heterogeneity and two receptors. *Blood* **67**, 1519–1521.
- Morgan, W. 1999 Solution structure of the EGF module pair from the *Plasmodium falciparum* merozoite surface protein 1. *J. Mol. Biol.* **289**, 113–122.
- Narum, D. L. & Thomas, A. W. 1994 Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of *Plasmodium falciparum* merozoites. *Mol. Biochem. Parasitol.* **67**, 59–68.

- O'Donnell, R. A., Saul, A., Cowman, A. F. & Crabb, B. S. 2000 Functional conservation of the malaria vaccine antigen MSP-1₁₉ across distantly related *Plasmodium* species. *Nature Med.* **6**, 91–95.
- Ogun, S. A. & Holder, A. A. 1996 A high molecular mass *Plasmodium yoelii* rhoptry protein binds to erythrocytes. *Mol. Biochem. Parasitol.* **76**, 321–324.
- Okoyeh, J. N., Pillai, C. R. & Chitnis, C. E. 1999 *Plasmodium falciparum* field isolates commonly use erythrocyte invasion pathways that are independent of sialic acid residues of glycoporphin A. *Infect. Immun.* **67**, 5784–5791.
- Perera, K. L., Handunnetti, S. M., Holm, I., Longacre, S. & Mendis, K. 1998 Baculovirus merozoite surface protein 1 C-terminal recombinant antigens are highly protective in a natural primate model for human *Plasmodium vivax* malaria. *Infect. Immun.* **66**, 1500–1506.
- Perrin, L. H., Merkle, B., Gabra, M. S., Stocker, J. W., Chizzolini, C. & Richle, R. 1985 Immunization with a *Plasmodium falciparum* merozoite surface antigen induces a partial immunity in monkeys. *J. Clin. Invest.* **75**, 1718–1721.
- Peterson, M. G., Marshall, V. M., Smythe, J. A., Crewther, P. E., Lew, A., Silva, A., Anders, R. F. & Kemp, D. J. 1989 Integral membrane protein located in the apical complex of *Plasmodium falciparum*. *Mol. Cell Biol.* **9**, 3151–3154.
- Peterson, M. G., Nguyen-Dinh, P., Marshall, V. M., Elliott, J. F., Collins, W. E., Anders, R. F. & Kemp, D. J. 1990 Apical membrane antigen of *Plasmodium fragile*. *Mol. Biochem. Parasitol.* **39**, 279–284.
- Preiser, P. R., Jarra, W., Capiod, T. & Snounou, G. 1999 A rhoptry-protein-associated mechanism of clonal phenotypic variation in rodent malaria. *Nature* **398**, 618–622.
- Qari, S. H., Shi, Y. P., Goldman, I. F., Nahlen, B. L., Tibayrenc, M. & Lal, A. A. 1998 Predicted and observed alleles of *Plasmodium falciparum* merozoite surface protein-1 (MSP-1), a potential malaria vaccine antigen. *Mol. Biochem. Parasitol.* **92**, 241–252.
- Ranjan, A. & Chitnis, C. E. 1999 Mapping regions containing binding residues within functional domains of *Plasmodium vivax* and *Plasmodium knowlesi* erythrocyte-binding proteins. *Proc. Natl Acad. Sci. USA* **96**, 14 067–14 072.
- Rayner, J. C., Galinski, M. R., Ingravall, P. & Barnwell, J. W. 2000 Two *Plasmodium falciparum* genes express merozoite proteins that are related to *Plasmodium vivax* and *Plasmodium yoelii* adhesive proteins involved in host cell selection and invasion. *Proc. Natl Acad. Sci. USA* **97**, 9648–9653.
- Reed, M. B., Caruana, S. R., Batchelor, A. H., Thompson, J. K., Crabb, B. S. & Cowman, A. F. 2000a Targeted disruption of an erythrocyte binding antigen in *Plasmodium falciparum* is associated with a switch toward a sialic acid independent pathway of invasion. *Proc. Natl Acad. Sci. USA* **97**, 7509–7514.
- Reed, M. B., Saliba, K. J., Caruana, S. R., Kirk, K. & Cowman, A. F. 2000b Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**, 906–909.
- Ridley, R. G., Takacs, B., Etlinger, H. & Scaife, J. G. 1990a A rhoptry antigen of *Plasmodium falciparum* is protective in *Saimiri* monkeys. *Parasitology* **101**, 187–192.
- Ridley, R. G., Takacs, B., Lahm, H. W., Delves, C. J., Goman, M., Certa, U., Matile, H., Woollett, G. R. & Scaife, J. G. 1990b Characterisation and sequence of a protective rhoptry antigen from *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **41**, 125–134.
- Saul, A., Cooper, J., Hauquitz, D., Irving, D., Cheng, Q., Stowers, A. & Limpiaaboon, T. 1992 The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **50**, 139–150.
- Schofield, L., Bushell, G. R., Cooper, J. A., Saul, A. J., Upcroft, J. A. & Kidson, C. 1986 A rhoptry antigen of *Plasmodium falciparum* contains conserved and variable epitopes recognized by inhibitory monoclonal antibodies. *Mol. Biochem. Parasitol.* **18**, 183–195.
- Sim, B. K. L., Chitnis, C. E., Wasniowska, K., Hadley, T. J. & Miller, L. H. 1994 Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. *Science* **264**, 1941–1944.
- Smythe, J. A., Coppel, R. L., Brown, G. V., Ramasamy, R., Kemp, D. J. & Anders, R. F. 1988 Identification of two integral membrane proteins of *Plasmodium falciparum*. *Proc. Natl Acad. Sci. USA* **85**, 5195–5199.
- Stahl, H. D., Bianco, A. E., Crewther, P. E., Burkot, T., Coppel, R. L., Brown, G. V., Anders, R. F. & Kemp, D. J. 1986 An asparagine-rich protein from blood stages of *Plasmodium falciparum* shares determinants with sporozoites. *Nucleic Acids Res.* **14**, 3089–3102.
- Thomas, A. W., Deans, J. A., Mitchell, G. H., Alderson, T. & Cohen, S. 1984 The Fab fragments of monoclonal IgG to a merozoite surface antigen inhibit *Plasmodium knowlesi* invasion of erythrocytes. *Mol. Biochem. Parasitol.* **13**, 187–199.
- Thomas, A. W., Waters, A. P. & Carr, D. 1990 Analysis of variation in PF83, an erythrocytic merozoite vaccine candidate antigen of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **42**, 285–288.
- Thompson, J. K., Triglia, T., Reed, M. B. & Cowman, A. F. 2001 A novel ligand from *Plasmodium falciparum* that binds to a sialic acid containing receptor on the surface of human erythrocytes. *Mol. Microbiol.* **41**, 47–58.
- Triglia, T., Healer, J., Caruana, S. R., Hodder, A. N., Anders, R. F., Crabb, B. S. & Cowman, A. F. 2000 Apical membrane antigen 1 plays a central role in erythrocyte invasion by *Plasmodium* species. *Mol. Microbiol.* **38**, 706–718.
- Triglia, T., Thompson, J., Caruana, S. R., Delorenzi, M., Speed, T. & Cowman, A. F. 2001 Identification of proteins from *Plasmodium falciparum* that are homologous to the reticulocyte binding proteins in *Plasmodium vivax*. *Infect. Immun.* **69**, 1084–1092.
- Trucco, C., Fernandez-Reyes, D., Howell, S., Stafford, W. H., Scott-Finnigan, T. J., Grainger, M., Ogun, S. A., Taylor, W. R. & Holder, A. A. 2001 The merozoite surface protein 6 gene codes for a 36 kDa protein associated with the *Plasmodium falciparum* merozoite surface protein-1 complex. *Mol. Biochem. Parasitol.* **112**, 91–101.
- Walter, P. & Blobel, G. 1981 Translocation of proteins across the endoplasmic reticulum II. Signal recognition protein (SRP) mediates the selective binding to microsomal membranes of *in vitro*-assembled polysomes synthesising secretory machinery. *J. Cell Biol.* **91**, 551–556.
- Wu, Y., Kirkman, L. A. & Wellem, T. E. 1996 Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *Proc. Natl Acad. Sci. USA* **93**, 1130–1134.