Chapter 1

General Introduction

1.1 Malaria is a global health problem.

Malaria has posed a serious threat to the human race from antiquity and around 40% of the world's population live in areas at risk of malaria transmission today (Snow *et al*., 2005; Winzeler, 2008). Caused by the apicomplexan parasite, *Plasmodium*, malaria accounted for at least 216 million clinical cases globally in 2010, with 655,000 to 1.2 million deaths (WHO, 2011; Murray *et al*., 2012). The mortality rates were highest in sub-Saharan Africa and 85- 90% of the malaria-related deaths occurred amongst children under the age of five and pregnant women.

Long neglected as a disease of poverty that primarily affects the developing world, investment for prevention and treatment of malaria has seen a rapid surge within the last decade (Snow *et al*., 2008; Geels *et al*., 2011; Greenwood & Targett, 2011). Funding from a number of agencies including the Global Fund, the World Bank and the US President's Malaria Initiative has enabled the scaling up of control measures for malaria, such as treatment with artemisinin-combination therapy and the use of insecticide-treated bed nets (Snow *et al*., 2008; Geels *et al*., 2011; Greenwood & Targett, 2011). Widespread implementation of these tools has led to a significant decrease in the incidence of malaria in endemic countries, with a 31% reduction in global malaria deaths from 2004 to 2010 (Murray *et al*., 2012). However, the current *status quo* is threatened by the potential spread of artemisinin-resistance parasite strains from south-east Asia and the propagation of insecticide-resistance in the mosquito vector populations (Greenwood & Targett, 2011; Geels *et al*., 2011).

An efficacious vaccine that provides sufficient protective immunity against malaria is clearly imperative for challenging this disease on a global scale (Crompton *et al*., 2010). However, numerous attempts at developing such a vaccine have yielded only limited success so far, mainly

due to inadequate understanding of the complex biology of *Plasmodium* and its mechanisms of immune evasion (Greenwood& Targett, 2011; Geels *et al*., 2011).

1.2 Five different species of *Plasmodium* can cause malaria in humans.

Until very recently only four species of *Plasmodium*, namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae* were believed to infect humans, with *P. vivax* being the most geographically widespread and *P. falciparum* the most lethal (Mendis *et al*., 2001). The discovery of naturally-acquired *P. knowlesi* infections amongst human populations in Southeast Asia has, however, changed this perception (Singh *et al*., 2004; Lee *et al*., 2011). *P. knowlesi*, a simian parasite found primarily in macaques, is thought to be transmitted to humans zoonotically, with no evidence (yet) of the parasite having undergone an adaptive host-switch from monkey to human (Lee *et al*., 2011).

The work described in this thesis was focused mainly on *P. falciparum*, the malaria parasite associated with the most severe clinical symptoms and the majority of malaria-related fatalities. (Snow *et al*., 2005).

1.3 The closest relatives of *P. falciparum* are great ape parasites of the *Laverania* family.

Interestingly, *P. falciparum* is only distantly related to the other human malaria parasites and is generally believed to have undergone a host switch after an ancient zoonotic transfer event from a non-human primate host (Prugnolle *et al*., 2011a; Rayner *et al.*, 2011; Duval and Ariey, 2012). However, the identity of the immediate predecessor of *P. falciparum* is a matter of some contention (Liu *et al*., 2010; Prugnolle *et al.*, 2011b). The chimpanzee parasite, *P. reichenowi*, first identified in the early 1920s, was the only known close relative of *P. falciparum* for many decades and widely believed to be its predecessor (Rayner *et al*., 2011). This paradigm was

challenged in 2009, by the discovery of another, closely-related *Plasmodium* species, *P. gaboni*, in two pet chimpanzees in Gabon (Ollomo *et al*., 2009). This study was followed by four others which reported the identification of *P. falciparum*-like parasites in African apes (Rich *et al*., 2009; Krief *et al.*, 2010; Prugnolle *et al*., 2010; Rayner *et al*., 2011). No definite conclusions, however, could be drawn from these studies with respect to the number and natural host-preferences of the parasite species, due to only a relatively few samples being analysed, most of them derived from captive apes. African apes are highly endangered, so invasive studies of wild-living populations are not a possibility (Rayner *et al*., 2011). In a large scale study conducted by Liu *et al*. (2010), this hurdle was circumvented by collecting and analysing more than 3000 fecal samples from forest-dwelling apes in Africa. Sequence analysis of *Plasmodium* mitochondrial, apicoplast and nuclear DNA recovered from these samples led to the identification of six distinct clades of *P. falciparum*-related parasites, collectively referred to as the *Laverania* family, found at high prevalence rates in great apes. The six clades were observed to be strictly host-specific with *P. reichenowi* (C1), *P. gaboni* (C2) and *P. billcollinsi* (C3) found only in chimpanzees and *P. praefalciparum* (G1), *P. adleri* (G2) and *P. blacklocki* (G3) restricted to gorillas (Figure 1). Furthermore, *P. praefalciparum* was identified as a likely direct predecessor of *P. falciparum*.

This postulated gorilla-origin of *P. falciparum* has since been brought into question by Prugnolle *et al*. (2011b). In their study, Prugnolle and colleagues analysed more than 300 blood samples from ten different species of African monkeys and discovered the presence of *P. falciparum*-like sequences in one sample (out of 29 tested) from one species, the old world monkey, *Cercopithecus nictitans* (greater spot-nosed monkey). Although the possibility of *P. falciparum*

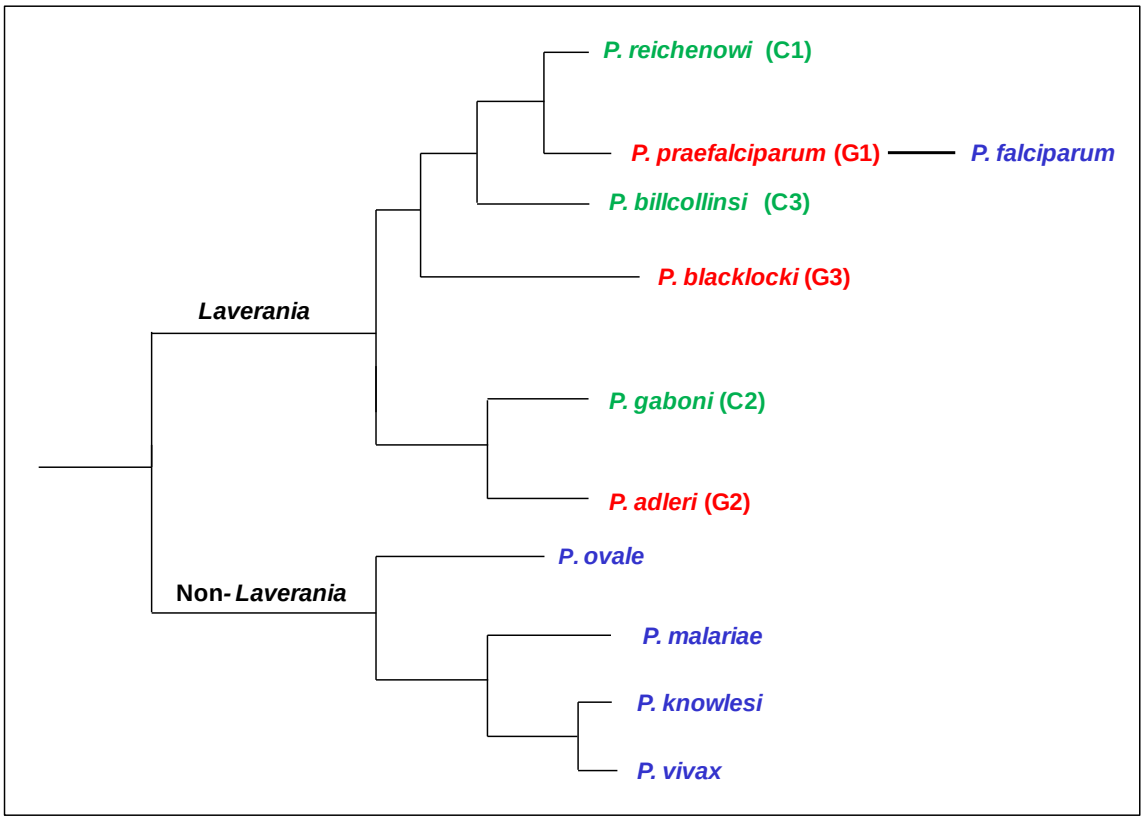

Figure 1. Simplified diagram of the phylogenetic relationships between human and great ape *Plasmodium* species. The two main lineages of *Plasmodium* are indicated with the parasites colour coded according to the primary host infected in the natural environment. Blue-human, red- gorilla, green-chimpanzee.

originating in monkeys rather than in gorillas was raised on the strength of this observation, it is yet to be validated by more substantial evidence (Prugnolle *et al*., 2011b). In fact, a very recent survey of wild-living *C. nictitans*, found no *P. falciparum*-like parasites in any individual (out of 300 tested), leading to the proposal that the original sample detected in a pet monkey may be a rare case of human to primate transmission (Ayouba *et al*., 2012).

1.4 *P. falciparum* has a complex life cycle.

The life cycle of *P. falciparum*, which comprises multiple morphologically distinct states, involves two stages of asexual multiplication in the human host and a period of sexual reproduction in the mosquito vector (Figure 2) (Winzeler, 2008; Kappe *et al*., 2010). Upon being injected into the peripheral circulation of a human host by the infectious bite of a female *Anopheles* mosquito, *P. falciparum* sporozoites migrate to the liver where they divide and differentiate in hepatocytes to form schizonts. After an incubation period of about 10 days, these schizonts rupture to release merozoites which in turn invade erythrocytes and multiply in progressive 48-hour cycles. In this stage, the number of merozoites can increase by 10^3-10^9 fold and some of the parasites leave the cycle to differentiate into male and female gametocytes. Upon ingestion by a mosquito, these gametocytes undergo fertilisation and maturation in the midgut to generate infective ookinetes which migrate through the midgut wall developing into oocysts. The oocysts harbour sporozoites which, when released, accumulate in the salivary glands of the mosquito, ready for transmission to other human hosts.

The erythrocytic stage of the parasite, obligatory for maintaining a sustained infection in the human host, is also responsible for the numerous clinical symptoms associated with malaria (Miller *et al*., 2002; Evans & Wellems, 2002). The release of merozoites into the blood stream following the rupture of host erythrocytes, at the end of each 48-hour development cycle, is

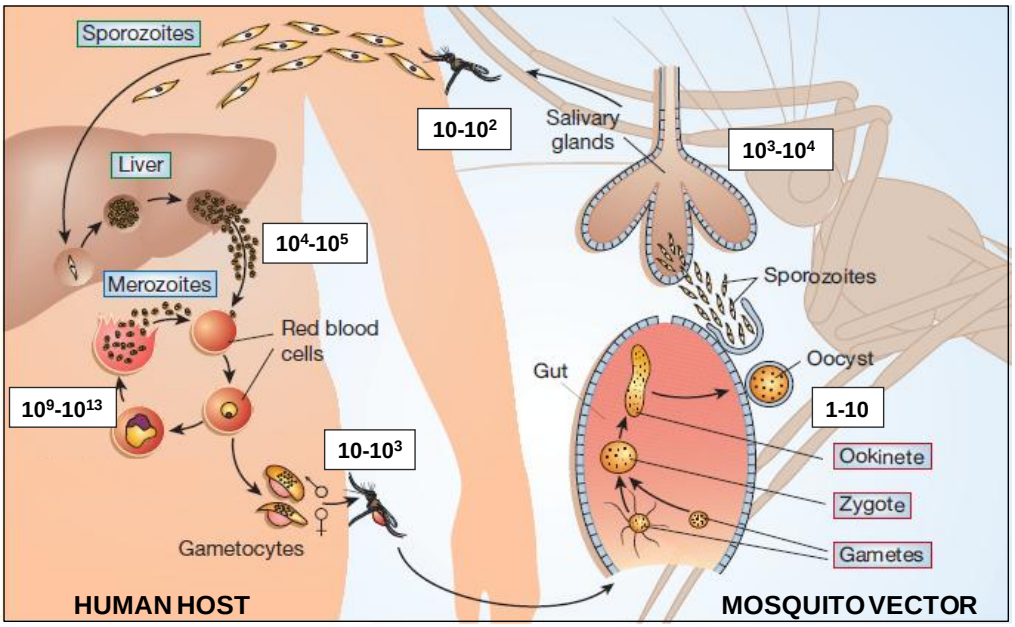

Figure 2. The life cycle of *Plasmodium falciparum* in the human host and the *Anopheles gambiae* mosquito vector. Sporozoites injected into the human host by the *Plasmodium*-infected mosquito first migrate to the liver, where they invade hepatocytes and develop into merozoites. Upon release into the blood stream, these merozoites infect erythrocytes, within which they grow and multiply to generate daughter merozoites that continue onto invade other erythrocytes, once discharged. All the clinical symptoms of malaria are caused by the erythrocytic stage of the parasite. Some intra-erythrocytic stage parasites develop into gametocytes, which are taken up by the mosquito vector during feeding. Gametes fuse to form zygotes which develop into ookinetes that infect the mosquito midgut. The resulting oocysts produce sporozoites which accumulate in the salivary glands of the mosquito ready for transmission to the next human host. The numbers shown indicate parasite population sizes at different stages of the life cycle. (The schematic was adapted from Ménard, 2005).

associated with the periodic fever, paroxysms and sweats characteristic of malaria. The sequestration of *P. falciparum*-infected erythrocytes in microcapillaries perfusing critical organs such as the brain and lung can cause serious life-threatening conditions including severe anemia, coma and pulmonary edema.

Understanding the molecular basis of the machinery that mediates the invasion of erythrocytes by *P. falciparum* merozoites is hence crucial to the development of new anti-malaria therapeutics (Bannister & Mitchell, 2003; Cowman & Crabb, 2006).

1.5 The invasion of human erythrocytes by *P. falciparum* merozoites is a multi-step process mediated by interactions between cell-surface proteins.

P. falciparum merozoites are ~1.2 μm in length and ellipsoidal with a polar structural organisation (Figure 3 A) (Garcia *et al*., 2008). Three sets of secretory organelles, the rhoptries, micronemes and dense granules are located at the apical end together with three cytoskeletal rings (polar rings) that provide structural support. The organelles which carry the genetic information and metabolic machinery necessary for parasite growth and development, the nucleus, mitochondrion and apicoplast, lie at the wider posterior end (Bannister & Mitchell, 2003; Garcia *et al*., 2008). Each merozoite also has a double membrane structure called the inner membrane complex (IMC), located immediately beneath the plasma membrane (except for a small gap at the apex) and connected to it by numerous actin filaments (Farrow *et al*., 2011). The cytosolic side of the IMC is coupled to two or three microtubules, which extend in parallel from the third polar ring to the posterior and play a role in the targeting of apical organelles during merozoite assembly, in addition to their mechanical role (Garcia *et al*., 2008). The outer surface of the merozoite is covered by a 15 nm thick, adhesive coat comprising clumps of narrow, protruding bristles (Garcia *et al.*, 2008).

Invasion of erythrocytes by *P. falciparum* merozoites is driven by a series of specific molecular recognition events between surface proteins on the host and parasite cells (Figure 3 B) (Bannister & Mitchell, 2003; Cowman & Crabb, 2006; Garcia *et al*., 2008; Harvey *et al*., 2012). Although the process of erythrocyte invasion is fairly well understood at the gross ultrastructural level (Aikawa *et al*., 1978), the precise timing and nature of the molecular events are still in the process of being elucidated. The initial tethering of a merozoite to a target erythrocyte is reversible and can occur at any point on the parasite surface. This is presumed to be mediated by proteins which constitute the outer coat of the parasite via relatively long-range (20-30 nm) interactions with their erythrocyte receptors (Cowman & Crabb, 2006; Garcia *et al*., 2008). The merozoite then undergoes re-orientation so that the apical end of the parasite is brought into juxtaposition with the host surface membrane allowing a much closer interaction (Aikawa *et al*., 1978; Gilson & Crabb, 2009). The re-orientation process appears to involve the partial wrapping of the erythrocyte surface around the merozoite and enables proteins secreted from the micronemes and the rhoptries to form an irreversible "tight junction" between the parasite and the host (Aikawa *et al*., 1978; Bannister *et al*., 1975). The movement of the ring-like tight junction from the apical to the posterior end of the merozoite is driven by an acto-myosin motor coupled to the IMC and leads to the internalisation of the merozoite within a parasitophorous vacuole inside the erythrocyte (Keeley & Soldati, 2004; Baum *et al*., 2006). The surface coat of the parasite is removed at the moving junction by the calcium sensitive serine protease, SUB2, a micronemal protein that translocates across the parasite surface (Harris *et al*., 2005; Withers-Martinez *et al*., 2012). Once the posterior end of the parasite is reached, the adhesive proteins that mediate the tight junction are also removed. This is proposed to involve serine proteases of the rhomboid family, ROM1-4, which cleave the proteins from within the phospholipid bilayer,

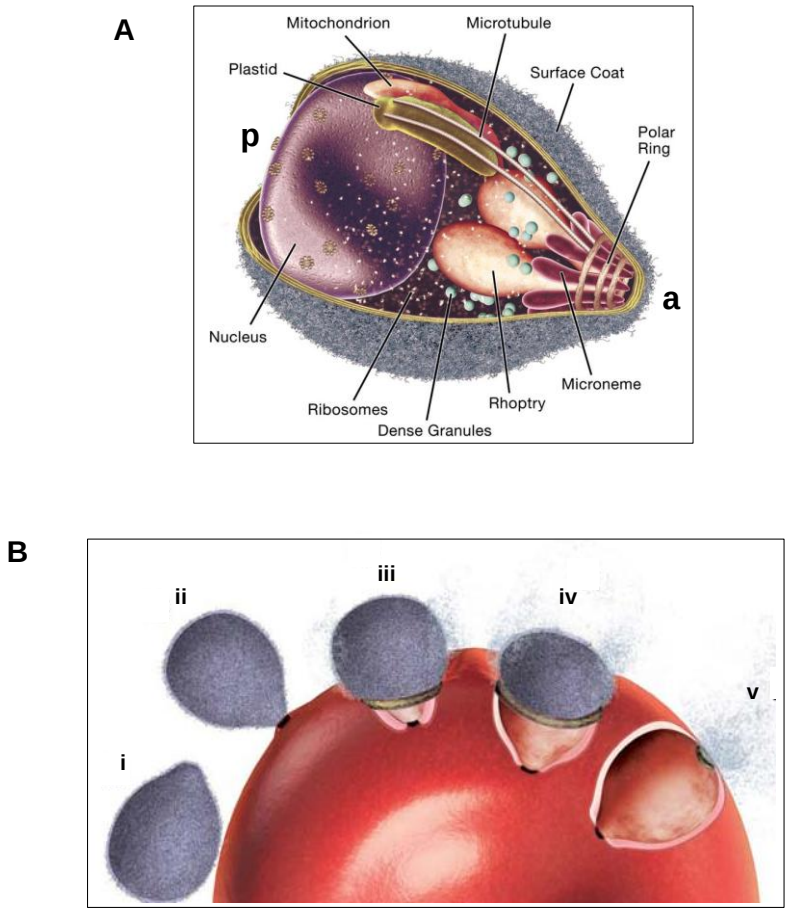

Figure 3. Invasion of erythrocytes by *Plasmodium falciparum* merozoites. A) The merozoite has a polarised structural morphology, a-apical end, p-posterior end. The secretory organelles at the apical end, the rhoptries, micronemes and dense granules, release their contents during erythrocyte invasion, whereas the bristly, adhesive coat which covers the entire outer surface of the cell plays an important role in initiating contact with erythrocytes. **B)** The multiple stages of erythrocyte invasion. i) Initial reversible tethering of the merozoite is mediated by 'long-distance' interactions between proteins constituting the outer coat of the parasite and erythrocyte receptors. ii) Re-orientation of the merozoite brings the apical end in contact with the erythrocyte, allowing proteins secreted from the apical organelles to form a tight junction by interacting with their erythrocyte receptors. iii) and iv) Movement of the tight junction from the apical to the posterior end of the parasite is powered by the actin-myosin motor. The surface coat of the parasite is removed at the moving junction by a serine protease. v) Adhesins forming the tight junction are proteolytically removed upon reaching the posterior end and the parasite is sealed within the parasitophorous vacuole. (The schematics were adapted from Cowman & Crabb, 2006).

facilitating the resealing of the membrane (O'Donnell *et al*., 2006; Garcia *et al*., 2008).

The release of micronemal and rhoptry proteins during invasion is proposed to be a sequential, two-step process, occurring in response to distinct external signals (Singh *et al*., 2010). Exposure of *P. falciparum* merozoites to the low K^+ concentrations found in the blood plasma, leads to a rise in its cytosolic Ca^{2+} levels, which triggers the secretion of micronemal proteins to the apical surface. The subsequent interactions of the micronemal proteins with their erythrocyte receptors restore cytosolic Ca^{2+} to basal levels, triggering the release of rhoptry proteins.

1.6 Primary contact with erythrocytes is proposed to be mediated by proteins that constitute the outer coat of *P. falciparum* merozoites.

More than 30 proteins have been identified by genomic analysis to comprise the outer coat of *P. falciparum* merozoites and these are likely to be important for the initial recognition and reversible binding of erythrocytes by the parasite (Cowman & Crabb, 2006; Garcia *et al*., 2008). The glycosylphosphatidylinositol (GPI)-anchored ligands of the surface coat are in general essential for normal erythrocyte-stage development and are mostly clustered within detergent-resistant membrane domains with their associated peripheral proteins (Sanders *et al*., 2005; Cowman & Crabb, 2006). MSP1, the most abundant of the GPI-anchored ligands is synthesised as a 195 kDa precursor and subsequently processed to generate a complex of four fragments held together non-covalently on the surface of merozoites until erythrocyte invasion (Bentley, 2006; Garcia *et al.*, 2008). The GPI-anchored fragment, $MSP1_{42}$, is again proteolytically cleaved by SUB2 just distally to two tandem EGF (epidermal growth factor) domains, during invasion, to generate a 19 kDa fragment, $MSP1_{19}$, which is held on the merozoite as it enters the erythrocyte (Blackman *et al*., 1991; Harris *et al*., 2005). Prevention of this second processing step has been shown to preclude invasion (Blackman *et al*., 1994; Bentley, 2006). Although a definitive

function for MSP1 in invasion is yet to be identified, there is some evidence to suggest that it may be a ligand for the multi-pass protein band 3, the second most abundant protein on human erythrocytes (Goel *et al*., 2003). The other GPI-anchored proteins on the merozoite surface include Pf12 and Pf38 which carry dual 6-cys domains and are predicted to be putative ligands for erythrocyte receptors, based on structural similarity to SAG proteins of *Toxoplasma gondii* (Gerloff *et al*., 2005; Cowman & Crabb 2006).

Peripheral proteins are secreted into the parasitophorous vacuole and attach to the surface of developing merozoites, via interaction with GPI-anchored ligands (Cowman & Crabb, 2006; Garcia *et al*., 2008). Such proteins identified to date include members of MSP3, MSP7 and SERA families and Pf41, another 6-cys ligand (Sanders *et al*., 2005; Cowman & Crabb, 2006; Garcia *et al*., 2008). MSP6 (MSP3.2) and MSP7 (MSP7.1) are known to form a non-covalent complex with the fragments of MSP1 on the merozoite surface and peptides derived from these have been demonstrated to bind to erythrocytes; however, the biological implications of these interactions are still poorly understood (Burgess *et al.*, 2005; Kadekoppala & Holder, 2010; Kadekoppala *et al.*, 2008). The SERA family of surface proteins carry a central protease domain with an active site cysteine or serine (Hodder *et al.*, 2009). The most abundant member of this family, SERA5, has been shown to be essential for parasite survival in the blood stage, but its recent crystal structure has cast doubt on its ability to function as a protease (Hodder *et al.*, 2009).

1.7 Merozoite proteins of the EBL and RH families play a critical role during erythrocyte invasion.

The erythrocyte-binding-like proteins (EBLs) and the reticulocyte-binding-like-homolog proteins (RHs) are two broad families of merozoite surface proteins secreted from the apical organelles. They are known to interact directly with erythrocyte receptors and are proposed to facilitate a step of the invasion pathway downstream from the initial contact with the erythrocyte, such as apical re-orientation or formation of the tight junction (Harvey *et al.,* 2012; Riglar *et al.,* 2011). In *P. falciparum* the EBL family includes EBA175 (MAL7P1.176), EBA140 (MAL13P1.60), EBA181 (PFA0125c) and EBL-1, all of which are type I transmembrane proteins with two cysteine-rich regions, Region II (RII) and Region VI (RVI), in the extracellular domain (Adams *et al*., 2001; Cowman & Crabb, 2006; Tham *et al*., 2012). The amino-terminal RII mediates erythrocyte binding and comprises two tandem Duffy-binding-like (DBL) domains, F1 and F2, which are homologous to the single DBL domain of the *P. vivax* Duffy-binding-protein (DBP) (Sim *et al*., 1994). RVI is proposed to play a role in the trafficking of the EBAs to the micronemes (Gilberger *et al*., 2003; Withers-Martinez *et al*., 2008). A gene, *eba165* has been identified that could potentially code for a fifth member of the EBA family, however, this gene carries a number of misssense mutations and a functional protein has not yet been identified in any *P. falciparum* strain (Triglia *et al.*, 2001 Tham *et al*., 2012).

The *P. falciparum* RH proteins are homologous to rhoptry proteins in *P. yoelii* (rodent parasite) and *P. vivax* (Rayner *et al*., 2000). This family consists of RH1 (PFD0110w), RH2a (PF13_0198), RH2b (MAL13P1.176), RH4 (PFD1150c) and RH5 (PFD1145c), of which only RH5 lacks a transmembrane region (Rayner *et al*., 2000; Rayner *et al*., 2001; Triglia *et al*., 2001; Taylor *et al*., 2001; Rodriguez *et al*., 2008). RH2a and RH2b are identical apart from a region

close to the carboxyl terminus indicating a possible gene duplication event followed by evolutionary drift (Rayner *et al*., 2000). Similar to *eba165*, the sixth *rh* paralog, *rh3* also has missense mutations and is a transcribed pseudogene (Taylor *et al*., 2001). All *ebl* and *rh* genes can be disrupted, with the exception of *rh5* suggesting that their encoded proteins perform functionally redundant roles (Duraisingh *et al*., 2003; Baum *et al*., 2009; Lopaticki *et al*., 2011). However, a minimal complement of these proteins appears to be necessary for merozoites to bind erythrocytes with sufficient affinity to activate the downstream processes that commit the parasite for invasion (Duraisingh *et al.*, 2003).

To date an erythrocyte receptor has been identified for three of the EBAs and two of the Rhs. EBA175, EBL-1 and EBA140 interact with the Glycophorins A, B and C respectively in a sialic acid-dependent manner (Camus and Hadely, 1985; Sim, 1995; Maier *et al*., 2002; Mayer *et al*., 2009), whereas RH4 and RH5 do not require sialic acid for the recognition of their respective receptors, CR1 and Basigin (BSG) (Crosnier *et al.*, 2011; Spadafora *et al.*, 2010). RH2a and RH2b have also been shown to bind to erythrocytes in a sialic acid independent manner, but their receptors have not yet been identified (Gaur & Chitnis, 2011). Interestingly only the RH5-BSG interaction has been shown to be essential for invasion, supporting the hypothesis that the other EBAs and RHs are functionally redundant (Crosnier *et al*., 2011; Tham *et al*., 2012). Having an array of ligands that can bind erythrocytes via different receptors probably enables the parasite to counter the highly polymorphic nature of the erythrocyte receptors, whilst reinforcing its ability to evade the human immune response using phenotypic variation (Harvey *et al*., 2012; Tham *et al*., 2012).

RH5 has also been shown to interact with another essential parasite protein, RIPR (RH5 interacting protein), which also lacks a transmembrane region (Chen *et al*., 2011). RIPR is a

micronemal protein that complexes with RH5 after secretion onto the merozoite surface, however, unlike RH5 it does not appear to interact directly with erythrocytes (Chen *et al*., 2011).

1.8 The micronemal protein AMA 1 is essential for tight junction formation.

AMA1 is a micronemal protein that is highly conserved across the *Apicomplexa* and in *P. falciparum* merozoites it is known to be essential for erythrocyte invasion (Triglia *et al*., 2000; Cowman & Crabb, 2006). AMA1 is not required for the initial attachment to erythrocytes or for apical re-orientation of the parasite (Mitchell *et al*., 2004; Treeck et al., 2009; Harvey *et al*., 2012), it is instead proposed to play a central role in tight junction formation via its interaction with the RON (rhoptry neck) complex, which occurs after the translocation of the latter to the erythrocyte surface (Riglar *et al.*, 2011). RON2, a membrane-spanning protein, has been identified as the immediate interacting partner of AMA1 within the RON complex and its binding site has been mapped to the hydrophobic groove on PAN (plasminogen, apple, nematode) domain I of AMA1 (Vulliez-Le Normand *et al.*, 2012; Bai *et al.*, 2005; Pizarro *et al.*, 2005).

1.9 Adhesins may be coupled to the molecular motor by MTRAP to allow movement of the tight junction.

In *P. falciparum* sporozoites, extracellular adhesins are coupled to the actin-myosin motor by the TRAP protein, which itself is linked to the parasite cytoskeleton via aldolase (Buscaglia *et al*., 2003). A similar membrane-spanning, thrombospondin type I repeats (TSR) domain-containing protein, MTRAP, is proposed to play the same role in merozoites (Baum *et al*., 2006). MTRAP is essential for the erythrocyte stage development of *P. falciparum* and its cytoplasmic tail has been shown to bind to aldolase *in vitro* (Baum *et al*., 2006; Morahan *et al*., 2009). Whereas, the

TSR-domain containing extracellular region of MTRAP has recently been demonstrated to bind erythrocytes via interaction with a putative receptor, Semaphorin 7A (Uchime *et al*., 2012; Bartholdoson *et al*., unpublished data). Other TSR domain containing proteins expressed at the merozoite stage include PTRAMP, SPATR and TLP, but the functional role of these proteins are not yet known (Baum *et al*., 2006; Heiss *et al*., 2008; Morahan *et al*., 2009).

1.10 *P. falciparum* merozoite surface proteins are important candidates for a blood-stage vaccine.

Clinical immunity to malaria develops slowly in response to a number of repeated exposures and is primarily associated with the presence of protective antibodies which act against blood-stage parasites (Cohen *et al.,* 1961; Geels *et al.,* 2011). The goal of an effective blood-stage vaccine is to mimic this natural immunity by inducing an immune response capable of inhibiting the erythrocytic development of the parasite (Greenwood & Targett, 2011). Merozoite surface proteins are the most likely candidates for such a blood stage vaccine as they are exposed to the immune system (albeit briefly) at each replication cycle (Section 1.3).

The blood-stage vaccine candidates that have so far been evaluated in epidemiological studies and clinical trials are limited in number and were almost all known before the completion of the *P. falciparum* genome sequence in 2002 (Conway *et al.,* 2000; Fowkes *et al.,* 2010; Osier *et al.,* 2008). To date, only seven merozoite surface proteins have been approved for clinical testing as vaccine candidates; MSP1, MSP2, MSP3, AMA1, EBA175, GLURP and SERA5 (Schwartz *et al.,* 2012). The most advanced of these, MSP1 and AMA1, did not afford significant protection from *P. falciparum* infection in recent phase II trials (Geels et al., 2011; Schwartz *et al.,* 2012). This has mainly been attributed to the highly polymorphic nature of these proteins resulting in antibodies raised against one haplotype failing to recognise others and has led to the usefulness

of MSP1 and AMA1 as vaccine candidates to be questioned (Geels et al., 2011; Hill, 2011; Schwartz *et al.,* 2012).

To increase the chance of developing an effective blood-stage vaccine, resources must be channelled towards identifying new candidates (Hill, 2011). Even though the sequencing of the *P. falciparum* genome has revealed a large number of merozoite surface proteins (Gardner *et al.,* 2002), determining which of these should be prioritised as vaccine candidates is a difficult task as a functional role in erythrocyte invasion is not known for the vast majority. Not only are these *P. falciparum* surface proteins hard to express recombinantly but identifying their extracellular interactions with erythrocyte receptors is difficult and beyond the scope of conventional biochemical methods for systematically identifying protein-protein interactions on a genome wide scale, such as yeast-2-hybrid screening and tandem affinity purification-mass spectrometry (TAP-MS) (Bei & Duraisingh, 2012).

1.11 Many prokaryotic and eukaryotic expression systems have been tested for the production of *P. falciparum* proteins in recombinant form.

Bioinformatic tools are often useful for identifying certain properties of selected proteins and for predicting their possible biological roles. However, such inferences are not possible in some cases (e.g. when the protein to be characterised has little sequence homology to any protein of known function) and in-depth structural and functional characterisation of any protein always requires experimentation with molecular biology techniques (Birkholtz *et al.*, 2008). Production of *P. falciparum* proteins in a recombinant form is a necessity as it is very difficult to isolate them in sufficient quantities from the parasite for *in vitro* experiments. A variety of heterologous and cell-free systems have been tested for expression of *P. falciparum* surface proteins with modest success (Birkholtz *et al.*, 2008). The difficulty in expressing *P. falciparum* proteins in

recombinant form is mainly attributed to the very high A+T content of the genes and the prevalence of repetitive amino acid sequences (Tsuboi *et al.,* 2008). Integral membrane proteins are in particular challenging to express in a biochemically-amenable manner due to their hydrophobic membrane-spanning domains and efforts have therefore been focused on the production of their soluble, truncated ectodomains.

The most widely used expression host for *P. falciparum* proteins is still *Escherichia coli* (Birkholtz *et al.,* 2008). Although cost effective, fast and easy to use, recombinant proteins expressed in these cells are often insoluble and sequestered in inclusion bodies. Such aggregated proteins can be solubilised and refolded to their native conformation (Pandey *et al*., 2002; Bai *et al*., 2005). However, protein refolding is a complicated process that requires several steps of optimisation and conditions which are suitable for one protein may not necessarily be appropriate for another, therefore its use in large scale, high throughput production of proteins is limited (Birkholtz *et al.,* 2008).

Two species of yeast, *Saccharomyces cerevisiae* and *Pichia pastoris* are also commonly used for production of *P. falciparum* proteins (Birkholtz *et al.,* 2008). Fragments of a number of merozoite surface proteins, including current vaccine candidates EBA175, AMA1, MSP1 and MSP3 have been successfully expressed in yeast in active, soluble form (Zang & Pan, 2005; Tolia *et al*., 2005; El Sahly *et al*., 2010). A major advantage of yeast over *E. coli* as an expression host is the secretion of recombinant proteins fused to yeast hormones into the growth media. Not only does this bypass the problem of over-expressed proteins aggregating in insoluble inclusion bodies, it also simplifies downstream protein purification. Recombinant proteins expressed in yeast are furthermore subjected to eukaryotic post-translational modifications, some such as disulphide-bond formation facilitate the correct folding of *P.*

falciparum surface proteins but others such as N- and O-glycosylation are potentially disadvantageous as plasmodial proteins are not glycosylated in the parasite (Gowda & Davidson, 1999). Additionally as yeast recognises some A+T containing codons as termination signals *P. falciparum* coding sequences need to be codon optimised for expression in these systems (Birkholtz *et al.*, 2008).

Other heterologous systems used for the production of *P. falciparum* proteins include expression from baculoviral vectors in insect cells (Birkholtz *et al.*, 2008). As the host cells recognise eukaryotic targeting signals and perform most post-translational modifications this system has been used successfully to produce immunologically active fragments of some *P. falciparum* proteins including EBA175 and AMA1 (Narum *et al*., 1993; Ockenhouse *et al*., 2001).

In vitro translation in cell-free systems has been used with some success for a number of *P. falciparum* surface proteins (Tsuboi *et al.*, 2008; Crompton *et al.*, 2010; Trieu *et al.*, 2011). Such systems can be easily manipulated for the production of correctly folded proteins and post-translational modifications can be facilitated by the use of eukaryotic cell extracts. They are also amenable to automation and hence high-throughput screening strategies. However, these systems have high-running costs and the availability of cell-free extracts is restricted as their preparation in laboratories is generally impractical (Farrokhi *et al*., 2009).

1.12 Studying extracellular interactions between *P. falciparum* merozoites and human erythrocytes using *in vitro* biochemical techniques is challenging.

Extracellular binding events between cell surface proteins are generally of very low affinity (equilibrium dissociation constants (K_D) in the μM to mM range) and the interactions between *P. falciparum* merozoite surface proteins and their erythrocyte receptors are no exception (Wright, 2009; Bei and Duraisingh, 2012). The highly transient nature of such interactions, with half lives

of less than 0.5 sec when measured in the monomeric state, limits the use of traditional biochemical assays based on affinity purification strategies, for their detection and characterisation (Bei and Duraisingh, 2012; Wright, 2009).

On the surface of cells, membrane proteins are locally concentrated in the context of the lipid bilayer and essentially displayed as multimeric arrays, which allows their interactions to occur with high avidity (Wright, 2009). Cell-based assays are therefore suitable for the identification of individually weak protein-protein interactions and have been used widely for investigating the binding of *P. falciparum* proteins to erythrocytic receptors.

Over the millennia, *P. falciparum* has exerted considerable selection pressure in the shaping of the human genome and a number of naturally occurring polymorphisms in erythrocyte proteins have been found to be associated with reduced risk of malaria (Evans and Wellems, 2002; Bei and Duraisingh, 2012). *In vitro* studies using polymorphic erythrocytes have facilitated the identification and/or verification of specific surface proteins as host receptors utilised by *P. falciparum* during invasion (Crosnier *et al*., 2011; Maier *et al.*, 2002; Mayer *et al*., 2009; Spadafora *et al*., 2010). The five known erythrocyte receptors of *P. falciparum* are all polymorphic blood group antigens (Bei and Duraisingh, 2012).

Interactions between erythrocyte receptors and their parasite ligands have also been biochemically characterised by pre-treatment of erythrocytes with specific enzymes, prior to performing the binding assays (Bei and Duraisingh, 2012). The requirement of some of the known interactions for sialic acid for example was identified by treatment of erythrocytes with the enzyme neuraminidase (Camus and Hadely, 1985; Thompson *et al*., 2001; Maier *et al*., 2002; Mayer *et al*., 2009). Treatment with the proteases trypsin and chymotrypsin has also been used

widely for classifying the different receptors used for invasion (Camus and Hadely, 1985; Thompson *et al*., 2001; Baum *et al*., 2009).

1.13 Work in this laboratory: expression of a library of *P. falciparum* merozoite surface proteins and avidity-based extracellular interaction screening (AVEXIS).

Mammalian expression systems are well-characterised, can aid the correct folding *P. falciparum* proteins in their secretory compartments and impart necessary post-translational modifications (Birkholtz *et al.,* 2008) . Despite these advantages, they have not been used for the preparative expression of *P. falciparum* surface proteins in the past, mainly due to the low yields of recombinant proteins obtained from traditional mammalian cell lines grown in adherent culture (Birkholtz *et al.,* 2008). However, many mammalian cell lines have now been adapted for growth at high densities in liquid culture and are routinely used in the research community for expressing various recombinant proteins in milligram to gram quantities (Tom *et al*., 2008). The suspension-adapted human embryonic kidney cell line stably expressing the Epstein-Barr virus nuclear antigen 1, HEK293E, is the most commonly used cell line for large-scale production of recombinant proteins (Tom *et al.*, 2008).

Our laboratory recently used HEK293E as the expression host for successfully producing the entire ectodomains of 50 *P. falciparum* merozoite surface proteins in soluble form (Table 1, Figure 4 A) (Cecile Wright-Crosnier, unpublished data). The majority of these proteins had previously not been expressed in an active form or produced as only small soluble fragments using heterologous and cell free systems. Successful expression of the *P. falciparum* proteins in HEK293E was achieved by codon optimisation of the native coding sequences for mammalian expression, replacement of the endogenous signal peptides with one from mouse to promote secretion and systematic removal of potential N-linked glycosylation sites. The proteins were

also expressed with the immunoglobulin-like domains 3 and 4 of rat Cd4 as a fusion partner. Cd4 was selected on the basis of its high levels of expression in mammalian systems, its well characterized structure and the availability of conformationally-sensitive anti-Cd4 monoclonal antibodies which can be used for quantitation and purification of the fusion proteins (Brown and Barclay, 1994).

The library of *P. falciparum* merozoite proteins was tested against a panel of 40 full-length ectodomains of single-pass erythrocyte receptors using AVEXIS (avidity-based extracellular interaction screening), an ELISA-based high-throughput screening platform developed in our laboratory (Cecile Crosnier, unpublished data). This screen identified the erythrocyte receptors for two *P. falciparum* proteins, RH5 and MTRAP, discussed previously (Crosnier *et al*., 2011; Bartholdoson *et al*., unpublished data). The detection of low affinity interactions is facilitated in the AVEXIS assay, as both 'bait' and 'prey' proteins are used in multimeric forms to enable potential binding events to occur with high avidity (Figure 4 B). The bait proteins, expressed with biotin tags, are multimerised by immobilisation on streptavidin-coated plates, whereas the prey proteins are produced as pentamers by fusion with the pentamerisation domain of the rat cartilaginous oligomeric matrix protein (COMP) (Bushell *et al.*, 2008).

1.14 Work described in this thesis

The three studies that were undertaken for this thesis are briefly described below. In all three, a number of different biochemical approaches were used to investigate the low-affinity interactions between *Plasmodium* surface proteins, produced recombinantly using the mammalian expression system optimised in our laboratory, and native and recombinant human erythrocyte receptors.

Sub-cellular location	Official nomenclature	Synonym/s	Accession Number	Region expressed	Len. (aa)
SURFACE (GPI-ANCHORED)	MSP1		PFI1475w	V20-S1701	1682
	MSP2		PFB0300c	I20-N246	227
	MSP4		PFB0310c	Y29-S253	225
	MSP5		PFB0305c	N22-S251	230
	MSP10		PFF0995c	H27-S503	477
	Pf12	P12	PFF0615c	H26-S323	298
	P12p		PFF0620c	Y21-T349	329
	Pf38	P38	PFE0395c	Q22-S328	307
	Pf92		PF13_0338	A26-S770	745
	Pf113		PF14_0201	Y23-K942	920
	PF3D7_1136200		PF11_0373	L19-G656	638
	PF3D7_1431400*		PF14_0293	N25-S968	944
SURFACE (PERIPHERAL)	MSP3	MSP3.1, SPAM	PF10_0345	K26-H354	328
	MSP6	MSP3.2	PF10_0346	Y17-N371	355
	H101	MSP3.3	PF10_0347	Q23-N424	402
	MSP11	H103, MSP3.7	PF10_0352	K27-Y405	379
	MSP3.4		PF10_0350	N26-K697	672
	MSP3.8		PF10_0355	Y23-N762	740
	MSP7	MSP7.1	PF13_0197	T28-M351	324
	MSRP1	MSP7.2	PF13_0196	Y22-T379	358
	MSRP2	MSP7.3	MAL13P1.174	K24-T280	257
	MSRP3	MSP7.4	PF13_0193	Q24-S298	275
	Pf41	P41	PFD0240c	K21-S378	358
	MSP9	p101, ABRA	PFL1385c	N24-S742	719
MICRONEME	AMA1	Pf83, RMA1	PF11_0344	Q25-T541	517
	EBA140	BAEBL	MAL13P1.60	I26-P1135	1110
	EBA175		MAL7P1.176	A21-P1424	1404
	EBA181	JESEBL	PFA0125c	I27-S1488	1462
	EBL1*		PF13_0115	K22-N2584	2563
	ASP		PFD0295c	A20-S708	689
	MTRAP		PF10_0281	I23-K432	410
	PTRAMP*	TRAMP, TSP-3	PFL0870w	N25-S306	282
	GAMA	PSOP9	PF08_0008	L22-P710	689
RHOPTRY	RH5		PFD1145c	F25-Q526	502
	RAP1*		PF14_0102	I23-D782	760
	RAP2*		PFE0080c	D22-L398	387
	RAP3*		PFE0075c	N23-K400	378
	CLAG3.2*	RhopH1(3.2)	PFC0110w	K21-H1416	1396
	RhopH2*		PFI1445w	L20-S1378	1359
	RhopH3		PFI0265c	K25-L897	873
	SPATR		PFB0570w	E22-C250	229
	AARP		PFD1105w	K18-P191	174
	Pf34	PV2	PFD0955w	N25-S306	282
	RON3*		PFL2505c	N22-N249	228
	RON6		PFB0680w	F16-T949	934
	RAMA		MAL7P1.208	Y17-K838	821
OTHER	TLP	TRAP2	PFF0800w	E24-P1306	1283
	PTEX150	Pf112	PF14_0344	A20-N993	974
	ETRAMP10.2	PfJ323	PF10_0323	R25-R52	28
	PF3D7_0606800		PFF0335c	V23-K299	277

Table 1. Members of the *P. falciparum* merozoite surface protein library. The 50 proteins are grouped and colour-coded according to their known/predicted sub-cellular localisation. Light blue-GPI-anchored surface proteins, dark blue- peripheral surface proteins, green-micronemal proteins, red-rhoptry proteins, orange-other proteins (no information about sub-cellular location). In the case of each protein, the full-length ectodomain was expressed, as indicated by the N-and C-terminal residues and their locations. Nine of the proteins from the library were expressed at very low levels and are indicated by *.

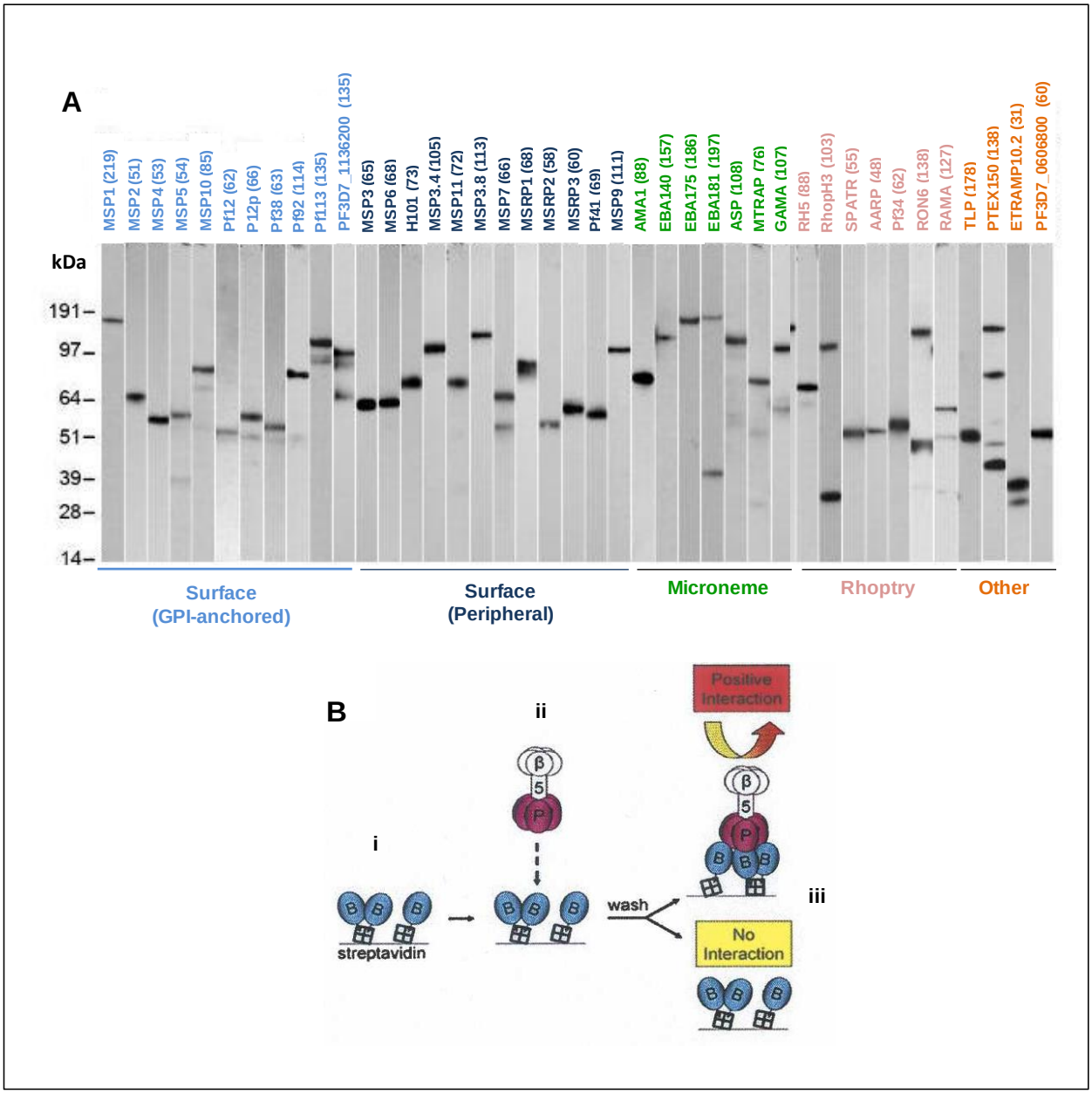
A
MSP1 (219)
MSP2 (51)
MSP4 (53)
MSP5 (54)
MSP10 (85)
Pf12 (62)
P12p (66)
Pf38 (63)
Pf92 (114)
Pf113 (135)
PF3D7_1136200 (135)
MSP3 (65)
MSP6 (68)
H101 (73)
MSP3.4 (105)
MSP11 (72)
MSP3.8 (113)
MSP7 (66)
MSRP1 (68)
MSRP2 (58)
MSRP3 (60)
Pf41 (69)
MSP9 (111)
AMA1 (88)
EBA140 (157)
EBA175 (186)
EBA181 (197)
ASP (108)
MTRAP (76)
GAMA (107)
RH5 (88)
RhopH3 (103)
SPATR (55)
AARP (48)
Pf34 (62)
RON6 (138)
RAMA (127)
TLP (178)
PTEX150 (138)
ETRAMP10.2 (31)
PF3D7_0606800 (60)
kDa
191
97
64
51
39
28
14
Surface (GPI-anchored)
Surface (Peripheral)
Microneme
Rhoptry
Other
B
i
ii
iii
streptavidin
wash
Positive Interaction
No Interaction

Figure 4. Previous work in this laboratory: a library of *P. falciparum* merozoite surface proteins was expressed and tested against a panel of erythrocyte receptor ectodomains by AVEXIS (avidity-based extracellular interaction screening). A) The full-length ectodomains of 50 *P. falciparum* surface proteins were expressed in soluble form using the mammalian HEK293E expression system. Only 41 of the proteins, expressed with a C-terminal Cd4 (25 kDa) tag, could be detected by western blotting (Cecile Crosnier, unpublished data). **B)** A simplified schematic representing the main steps of the AVEXIS assay, designed for detecting low affinity interactions between the soluble extracellular domains of membrane proteins expressed as mono-biotinylated 'baits' and pentameric β-lactamase-tagged 'preys'. i) Immobilisation of biotinylated 'bait' proteins on a streptavidin-coated plate. ii) Incubation of the immobilised 'baits' with the pentameric β-lactamase-tagged 'prey' proteins. iii) Detection of putative 'bait-prey' interactions by adding a colorimetric β-lactamase substrate after removal of non-bound 'preys' by stringent washing. (The schematic was adapted from Bushell *et al*., 2008).

Project A (Chapter 3): Functional validation of a mammalian-expressed EBA175 antigen and comparative analysis of its binding to MM and MN forms of human Glycophorin A.

P. falciparum EBA175 is the known ligand of the erythrocyte receptor Glycophorin A, which carries the antigenic determinants of the human MN blood group system. The goal of this study was to apply a range of biochemical and biophysical tools to investigate the EBA175-Glycophorin A interaction at a molecular level. The recombinant full-length ectodomain of EBA175 (*Pf*EBA175 FL), produced using the mammalian expression system optimised in our laboratory, was firstly confirmed to be functionally similar to native EBA175 isolated from parasite cultures by demonstrating its binding to human erythrocytes in a sialic acid- and Glycophorin A-dependent manner. The protein was then tested for direct binding to both native human Glycophorin A purified from erythrocytes and to the recombinantly-expressed full-length ectodomain of human Glycophorin A. Binding of *Pf*EBA175 FL was observed only to native Glycophorin A. The attempts to enhance the sialylation of recombinant Glycophorin A by co-expression with sialyl transferases and a sialic acid transporter were partially successful, but did not confer detectable binding of *Pf*EBA175 FL. Kinetic analysis of the interaction of *Pf*EBA175 FL with native Glycophorin A, by surface plasmon resonance (SPR), revealed the K_D to be ~ 0.24 µM. SPR analysis further revealed a slightly higher (x 1.3) affinity of *Pf*EBA175 FL for Glycophorin A from MN erythrocytes in comparison to that from MM erythrocytes. *Pf*EBA175 RII, a truncated derivative containing only the region known to be essential for interacting with erythrocytes, was observed to bind to native Glycophorin A with a ~10-fold lower affinity than *Pf*EBA175 FL, suggesting some role played by regions outside of RII to facilitate binding.

Project B (Chapter 4): Investigating the host-specificity of *Plasmodium* merozoite: primate erythrocyte interactions in the *Laverania* family.

The *Laverania* family of great ape parasites are stringently host-specific in their natural environment, with clades C1-C3 found only in chimpanzees and clades G1-G3 restricted to gorillas. *P. falciparum* falls within the G1 clade, and is hypothesized to have adapted to humans after a single cross-species transmission event from gorilla. The goal of this study was to investigate whether two known parasite ligand-host receptor interactions, EBA175-Glycophorin A and RH5-Basigin (BSG), could contribute towards the determination of host-specificity in *Laverania*. Both interactions studied are known to be important for the invasion of human erythrocytes by *P. falciparum*. EBA175 orthologues from three *Laverania* species, namely the human parasite *P. falciparum* and the chimpanzee parasites *P. reichenowi* and *P. billcollinsi* were all observed to bind to human erythrocytes in a sialic-acid dependent manner. SPR analysis of the binding of these EBA175 orthologues to native human Glycophorin A revealed only a ~2-fold lower affinity of the chimpanzee parasite proteins for the human erythrocyte receptor in comparison to the human parasite protein, suggesting that the EBA175-Glycophorin A interaction may not be a significant determinant of *Laverania* host-specificity. Investigating the binding of the *P. falciparum* and *P. reichenowi* RH5 proteins to human, chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*) Basigin on the other hand, revealed no binding of *P. reichenowi* RH5 to BSG. *P. falciparum* RH5 also did not recognise gorilla BSG and bound to chimpanzee BSG with a 15-fold lower affinity than to human BSG. Residues that confer host-specificity were then identified by generating site-directed mutants of the BSG orthologues and analysing their interactions with *P. falciparum* RH5.

Project C (Chapter 5): Development of high-throughput assays for characterising a library of *P. falciparum* merozoite surface proteins.

More than 250 *P. falciparum* merozoite proteins are now known but the vast majority of these have no identified function. To determine which of the novel *P. falciparum* antigens should be prioritised as potential vaccine candidates, their functional characterisation with the use of high-throughput strategies is a necessity. Traditional methods such as tandem affinity purification-mass spectrometry, used for identifying protein-protein interactions on a global scale, are not suitable for the detection of low affinity, extracellular interactions between merozoite surface proteins and their receptors on the erythrocyte surface. The ELISA-based, high-throughput screening platform, AVEXIS, developed in our laboratory is optimised for the identification of such interactions between recombinantly-expressed proteins, but cannot be used with multi-pass receptors, which are difficult to produce as correctly-folded soluble fragments. In this study, a library of 33 *P. falciparum* surface proteins was screened against erythrocytes and a panel of 41 erythrocytic multi-pass receptors, using a novel, flow-cytometry based, high-throughput approach. The *P. falciparum* proteins were multimerised by immobilisation on fluorescent beads for this purpose and each of the erythrocytic multi-pass receptors was expressed recombinantly on the surface of HEK293E cells. 13 putative interactions, between *P. falciparum* ligands and erythrocyte multi-pass receptors, were identified in the screen. Of these, the binding of the *P. falciparum* proteins, AARP and MSP11, to the erythrocyte proteins, Fatty acid transporter 4 and Plasma membrane $Ca2^{+}$ transporting ATPase 4, respectively, were the most significant. Nine of the *P. falciparum* proteins that showed binding to cells, were subsequently tested against a panel of 55 synthetic carbohydrate probes, using an ELISA-based approach, to identify whether their binding was glycan-dependent.

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