Analysis of 6-cys proteins and calcium fluxes during erythrocyte invasion by *Plasmodium falciparum* parasites

Tana Taechalertpaisarn

Submitted in total fulfilment of the requirements of the degree of
Doctor of Philosophy

June 2015

Burnet Institute & The Walter and Eliza Hall Institute of Medical Research
Department of Medical Biology, The University of Melbourne

Produced on Archival Quality Paper
ABSTRACT

*Plasmodium* parasites amplify their population within the human host by invading, growing and replicating within the body’s erythrocytes. When the population becomes high enough, the damage caused produces symptomatic malaria disease. To develop new drugs and vaccines against malaria it is therefore important to know as much possible about how parasites grow within the human host and particularly about how the extracellular merozoite stage invades erythrocytes, since this short-lived stage is highly vulnerable. This thesis provides new information from the most deadly human malaria pathogen *P. falciparum*, on the biochemical characteristics of a little known family of merozoite surface proteins which were thought to facilitate erythrocyte invasion as well revealing with unprecedented resolution, new details about how merozoites enter erythrocytes.

P12, P38, P41, and P92 comprise a group of blood-stage merozoite surface proteins that belong to the 6-cys family and all except P41 are predicted to have membrane anchors. To functionally characterize the proteins, specific antibodies were made and were then employed to block merozoite invasion by interfering with the binding of 6-cys to erythrocytes. The effect of the antibodies was very weak and therefore not indicative of a major role for 6-cys in invasion. The antibodies were then used as localization probes and indicated that P12 and P41 were at the merozoite periphery with some concentration towards the apex. In addition, the non-anchored P41 was held on the merozoite surface through heterodimerization with the membrane anchored P12. Despite the P12/P41 heterodimer being in prime position to bind erythrocytes during invasion no evidence for binding could be established.

Characterisation of P92 was next conducted and revealed that like the P12/P41 heterodimer, it was tightly associated with the parasite membrane and later cleaved off possibly during invasion. On the other hand, P38 did not shed from the merozoite surface, and it was carried into the erythrocyte. P92 was strictly localised to the apical end of the merozoite while P38 displayed both apical and surface localisation. Similar to the P12/P41 heterodimer, P92 does not appear to bind erythrocytes. In a final attempt to derive a function for the blood stage 6-cys, their genes were individually knocked out but none of the mutants produced any defective growth or invasion phenotypes suggestive of function.
To further study invasion, the morphology and kinetics of this process in *P. falciparum* merozoites was examined with high-speed live-cell microscopy. With greater temporal resolution, novel cellular actions of the merozoites were observed. For example, during the 7.5 s pre-invasion phase the merozoite deforms the erythrocyte plasma membrane multiple times whilst re-orientating. After a brief rest, the merozoite invaded over a ~17 s period forming a vacuole mainly from wrapping the erythrocyte’s membrane around itself. About 18.5 s after entry, the merozoite began spinning in a clockwise direction to possibly to help disconnect itself from the erythrocyte membrane.

After spinning had commenced the host erythrocyte began to develop a spiculated appearance called echinocytosis. Suspecting that calcium influx into the erythrocyte during invasion might be responsible for the echinocytosis, the appearance of these fluxes was monitored during invasion by live cell imaging. These observations confirmed for the first time, that a calcium flux originated as an intense spot emanating from the area of contact between the merozoite and erythrocyte suggestive of pore formation between the cells. Further experiments with modified levels of calcium indicated the ion is required for efficient invasion and may play role in causing echinocytosis. Other work using the calcium flux as a visual marker indicated that pore formation coincided with the deployment of tight adhesive proteins from the merozoite that commit it to invasion. The live cell imaging work presented therefore sheds considerable light on many details of merozoite invasion that could inform future drug and vaccine development.
DECLARATION

This is to certify that the thesis comprises only my original work except where indicated in the preface, due acknowledgement has been made in the text to other material used, and the thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies and appendices.

Tana Taechalertpaisarn

The work presented in this thesis was performed at the Burnet Institute under the supervision of Dr. Paul R. Gilson, Professor Brendan S. Crabb, Dr. Anthony N. Hodder, and Professor Alan F. Cowman.
I duly acknowledge the contribution of individuals who assisted in the work presented in this thesis.

Paul Gilson – (Burnet Institute) for overall guidance in preparing this thesis and performing the immunoprecipitation experiment (Fig. 3.4A)

Anthony Hodder – (The Walter and Eliza Hall Institute of Medical Research) for production of *E. coli* recombinant P12 and P41 for anti-P12 and anti-P41 antibody production (Fig. 3.1B).

Jenny Thompson – (The Walter and Eliza Hall Institute of Medical Research) for providing Δp12, Δp41, Δp38, and Δp92 mutant parasites for analyses and Southern blot analyses (Figs. 3.10B and 4.6B)

Danny Wilson – (The Walter and Eliza Hall Institute of Medical Research) for performing the invasion inhibition assays (Figs. 3.12B and 4.7B)

Paul Sanders – (Burnet Institute) for assistance in performing size exclusion chromatography showing the interaction of recP12 and recP41 (Fig. 3.3)

Marcel Hijnen – (past Burnet Institute) for assistance in performing size exclusion chromatography to purify recombinant 6-cys domain of P92 (Fig. 4.1C)

Cecile Crosnier, Gavin Wright, and Julian Rayner – (Wellcome Trust Sanger Institute, Cambridge, UK) for providing recombinant P12 and P41 expressed in mammalian cell system for analysis and supporting results in appendices.
PUBLICATIONS

Scientific publications resulting from studies undertaken in this thesis are listed below:


* Joint first authors
ACKNOWLEDGEMENTS

Firstly I would like to thank my supervisors, Dr. Paul Gilson, Professor Brendan Crabb, Dr. Anthony Hodder, and Professor Alan Cowman, for accepting me under your supervision. It has been a great experience as an international student to receive an opportunity to conduct scientific research abroad. This would not be possible if I had not been awarded the Melbourne International Fee Remission Scholarship and the Melbourne International Research Scholarship from The University of Melbourne, for which I am deeply grateful.

To Paul Gilson, especially, I am greatly thankful for your help and guidance during my PhD course. I have learnt so many invaluable things from you that I am certain will be very helpful in my future career.

To fellow lab members with whom I also become good friends; Hayley Bullen, Sarah Charnaud, Paul Sanders, Catherine Nie, Katherine Harvey, Brendan Elsworth, Greta Weiss, Mauro Azevedo, Kerstin Leykauf, thank you very much for your friendship and sharing all the good and sometimes not-so-good moments during my 4-year stay in Melbourne. They are unforgettable memories. I would also like to extend my gratitude to Ulrike Ruch (Uli), Kazuhide Yahata, and Nienke de Jong who share my passion in microscopy.

I would also like to thank Candida da Fonseca Pereira, Stephen Cody, and Monash Micro Imaging for providing technical support regarding microscopy. Without your help I would not have been able to accomplish the work in this thesis.

I also appreciate all of the help and friendship from everyone at the Burnet Institute and the Walter & Eliza Hall Institute of Research whom I do not mention their name here.

My PhD study would not happen if I did not receive encouragement from Dr. Chairat Uthaipibull and Dr. Sumalee Kamchonwongpaisan from the National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand and I am deeply grateful for their endorsement.

Lastly I would like to thank my family, my girlfriend, and friends for always believing in me and supporting me during my tough times. I would not be able to accomplish this without everyone.
TABLE OF CONTENTS

ABSTRACT i
DECLARATION iii
PREFACE iv
PUBLICATIONS v
ACKNOWLEDGEMENTS vi
TABLE OF CONTENTS vii
LIST OF FIGURES x
LIST OF TABLES xii

CHAPTER 1 Literature Review 1
Malaria 1
Malaria control, cure, and prevention 2
The Plasmodium life cycle 4
Merozoite invasion of erythrocytes 8
Merozoite surface proteins 9
Erythrocyte binding antigens and reticulocyte binding protein homologs 12
Apical membrane antigen and rhoptry neck proteins 18
The 6-cys protein family 22
Signaling during erythrocyte invasion 26
Aims of research 30
Functional analysis of 6-cys domain proteins in blood-stage malaria parasite 31
Live-cell microscopic analysis of $\text{Ca}^{2+}$ signaling and its role in erythrocyte invasion 31

CHAPTER 2 Materials and Methods 41
Parasite media and solutions 41
Bacterial media 43
Molecular biology reagents 44
Recombinant protein expression reagents 46
Zeiss AxioObserver Z1 filter sets 47
Experimental procedures 48
Parasite culture techniques 48
Molecular biology techniques 51
CHAPTER 3  Biochemical and Functional Analysis of Blood-Stage 6-Cys Proteins: P12 and P41

Introduction 63

Results 64

Expression of recombinant P12 and P41 in E. coli and HEK293E mammalian cell systems 64

P12 and P41 are expressed in late asexual parasite stages and shed into the culture supernatant 65

P12 resides on the merozoite surface 66

P12 and P41 form a heterodimer on the merozoite surface 67

Recombinant P12 and P41 associate as a heterodimer 67

Native P12 and P41 form a heterodimer 67

P12 and P41 from culture supernatants do not bind to erythrocytes 69

P12 and P41 from culture supernatants do not bind to heparin 70

P12 and P41 are not essential in the blood-stage parasites 71

Parasites lacking of P12 and P41 do not have altered growth phenotypes 72

P12 and P41 antisera do not significantly obstruct parasite growth and invasion efficiency 73

Discussion 74

CHAPTER 4  Biochemical and Functional Analysis of Blood-Stage 6-Cys Proteins: P38 And P92

Introduction 105

Results 106

Expression of recombinant 6-cys domain of P92 in E. coli for polyclonal antibody production 106

Biochemical characterisation of P38 and P92 in blood-stage P. falciparum 107

Localisation of P38 and P92 in late-stage P. falciparum and the evidence of P38 being carried into the erythrocyte 108

P92 may contribute to the interaction of merozoites and host erythrocytes during invasion but not through heparin-like molecules 109

P38 and P92 are not essential in blood-stage P. falciparum 110
The absence of P38 and P92 in the blood-stage P. falciparum do not significantly alter the growth rates of the parasites in vitro.

Blocking P38 and P92 with antigen-specific antibodies do not substantially inhibit the invasion of merozoites into the host erythrocytes.

Discussion

CHAPTER 5  Live-Cell Imaging of P. falciparum Merozoite Invading Human Erythrocyte and A Role of Calcium During Invasion

Introduction

Results

High-speed acquisition live-cell microscopy provides better resolution of morphology and kinetics of the erythrocyte invasion by P. falciparum merozoites.

Visualisation of the P. falciparum merozoite invading the erythrocyte labelled with a fluorescent plasma membrane dye.

During invasion a calcium signal was observed near the merozoite entry point followed by an influx into the erythrocyte.

Microscopic observation suggests extracellular calcium might contribute to the efficiency of erythrocyte invasion by P. falciparum merozoites.

The influx of calcium ions into the infected erythrocyte may trigger the onset of echinocytosis.

Inhibiting erythrocyte invasion by interrupting the tight junction formation does not prevent the calcium fluxes and prolongs the echinocytosis phase.

Interfering with the parasite actin-myosin motor prolongs echinocytosis of the infected erythrocytes without disturbing calcium fluxes.

Discussion

Supplementary Videos

CHAPTER 6  Concluding Remarks

REFERENCES

APPENDICES
LIST OF FIGURES

Figure 1.1. The *Plasmodium* life cycle. 34
Figure 1.2. The 6-cys protein family in *Plasmodium falciparum*. 36
Figure 1.3. A morphology and kinetics model of erythrocyte invasion by *Plasmodium falciparum* merozoites. 38
Figure 1.4. Ligand-receptor interactions taking part in erythrocyte invasion by *Plasmodium falciparum* merozoite. 40
Figure 3.1. Recombinant expression of P12 and P41 in *Escherichia coli*. 82
Figure 3.2. P12 and P41 are expressed in late asexual stage parasites and are shed into the culture supernatant. 84
Figure 3.3. Recombinant P12 and P41 can form a heterodimer. 86
Figure 3.4. Native P12 and P41 associate as a heterodimer. 88
Figure 3.5. Protein-crosslinking analysis confirms the interaction of native P12 and P41. 90
Figure 3.6. Identification of other binding partners of the P12/P41 complex. 92
Figure 3.7. Typical erythrocyte-binding assays show no binding activity for P12 and P41. 94
Figure 3.8. Depletion assays also demonstrate a lack of erythrocyte binding of P12 and P41. 96
Figure 3.9. P12 and P41 do not bind to heparin. 98
Figure 3.10. P12 and P41 genes can be deleted in *P. falciparum* asexual stage. 100
Figure 3.11. P12 and P41 are not expressed in the Δp12 and Δp41 parasites. 102
Figure 3.12. Genetic deletion and invasion inhibition assays suggest a non-essential role of P12 and P41 in *P. falciparum* asexual stage. 104
Figure 4.1. Expression of the E. coli recombinant 6-cys domain of P92 for polyclonal antibody production. 121
Figure 4.2. Biochemical characteristics of the *P. falciparum* P38 and P92. 123
Figure 4.3. Localisation of P38 and P92 in *P. falciparum*. 125
Figure 4.4. Depletion assay suggests P92 may bind to erythrocytes. 127
Figure 4.5. P92 does not bind to heparin-like molecules. 129
Figure 4.6. P38 and P92 genes can be deleted from the asexual stage *P. falciparum*. 131
Figure 4.7. Growth comparisons and invasion inhibition assays suggest a non-essential role of P38 and P92 in *P. falciparum* asexual stage.

Figure 5.1. High-speed acquisition of live-cell microscopy demonstrates continuous erythrocyte deformation in pre-invasion phase of erythrocyte invasion by *P. falciparum* merozoite.

Figure 5.2. High-speed acquisition of live-cell microscopy reveals a short resting phase following erythrocyte deformation and improves on temporal resolution of the invasion period.

Figure 5.3. Merozoite rotation was observed after complete internalisation.

Figure 5.4. Comparison of the kinetics in each step of erythrocyte invasion by *P. falciparum* merozoite between high-speed acquisition and the previous low-speed study.

Figure 5.5. Live-cell imaging of the invasion into the erythrocytes fluorescently labeled with a lipid dye supports the intake of the erythrocyte plasma membrane by the invading merozoite.

Figure 5.6. Calcium fluxes into erythrocytes were observed during merozoite invasion.

Figure 5.7. Depletion of extracellular calcium partly reduces merozoite invasion.

Figure 5.8. Extracellular calcium does not play a crucial role in the invasion kinetics.

Figure 5.9. Influx of calcium ions into infected erythrocytes may play a role in triggering the onset of echinocytosis.

Figure 5.10. Inhibiting invasion with the R1 peptide did not block the invasion calcium flux but extended the echinocytosis period of infected erythrocytes.

Figure 5.11. Inhibiting erythrocyte invasion with cytochalasin D does not prevent a calcium flux but extends the echinocytosis period.

Figure 6.1. A modified model of erythrocyte invasion by the *P. falciparum* merozoite.
LIST OF TABLES

Table 2.1. Primers used to generate knockout plasmids and Southern blotting. 60
Table 2.2. Primers used to validate homologous recombination in the knockout parasites by PCR. 61
Table 2.3. Primers used to amplify 6-cys domain of P92 62
CHAPTER 1

Literature Review

Malaria

Malaria remains one of the deadliest diseases to affect human beings. It is also considered to be one of the most ancient infectious diseases since the characteristic symptoms of malaria were found documented in Chinese medical writings dated back to 2700 BCE. Despite a very long history of the disease, an estimated 3.3 billion people remained at risk of getting malaria in 2010 and it was reported to cause over 200 hundred million episodes worldwide mostly in Sub-Saharan African and South-East Asian regions (World Health Organization. Global Malaria Programme, 2011). Even though the mortality rates have dropped by 25% over the last decade (between 2000 and 2010), there were still approximately 0.8 - 1.2 million deaths annually (Murray et al., 2012; World Health Organization. Global Malaria Programme, 2011). The majority of those deaths (86%) were of children under the age of 5 (World Health Organization. Global Malaria Programme, 2011), however another study suggested that there were more deaths in individuals ≥ 5 than the number reported by WHO (Murray et al., 2012).

Malaria is caused by an intracellular protozoan parasite named Plasmodium, which infects reticulocytes and/or mature erythrocytes depending on the species. Plasmodium parasites belong to the phylum Apicomplexa which includes Cryptosporidium parasites that causes diarrhea in humans, as well as Toxoplasma gondii, an opportunistic parasite infecting humans. Plasmodium parasites are transmitted through infected female Anopheles mosquitoes feeding on a variety of hosts such as birds, reptiles, rodents, primates, etc. There are 5 major species currently known to affect humans; Plasmodium falciparum, P. vivax, P. malariae, P. ovale, and P. knowlesi. The first two are the most common species responsible for symptomatic disease and P. falciparum accounts for the majority of deaths. P. vivax on the other hand has been reported to cause non-severe form of malaria, however, recently there have been a growing number of fatalities from P. vivax infection (reviewed in (Price et al., 2009)). P. ovale and P. malariae infections are generally considered not life-threatening. P. knowlesi, which was thought to mainly infect non-human primate, has
recently been reported to be responsible for hundreds of cases, which were misdiagnosed in the past as *P. malariae* infection, largely in Southeast Asian countries (Kantele and Jokiranta, 2011).

Clinical features of malaria disease arise from the propagation of the parasites within erythrocytes. The symptoms usually involve having fever, chills, headaches, diaphoresis, and anemia (Trampuz et al., 2003). The more serious complications from *P. falciparum* infection include cerebral malaria, severe anemia, acute renal failure, hypovolaemia, metabolic acidosis, respiratory distress, hypoglycaemia, coma, and ultimately death (Mackintosh et al., 2004; Miller and Good, 1994; Trampuz et al., 2003). Another unique clinical feature of *P. falciparum* infection is malaria in pregnancy which often results in serious outcomes of both mothers and babies such as placental infection, low birth weight, or even fetal loss (Mackintosh et al., 2004; Miller and Good, 1994). The pathogenesis of most severe clinical symptoms mentioned above is generally due to the ability of *P. falciparum* infected erythrocytes to bind to uninfected and infected erythrocytes, called rosettes, and the ability to adhere to the endothelial cells causing the blockage of small blood vessels. Since the infected cells are also able to sequester in blood vessels and escape from splenic clearance, this results in higher parasite density within the infected individuals leading to other complications.

**Malaria control, cure, and prevention**

Attempts to eradicate malaria have been pursued for a long time but still have not managed to accomplish this goal. In 2008, the Roll Back Malaria Partnership (RBM) launched the Global Malaria Action Plan aiming to reduce deaths from malaria to near zero by 2015 (World Health Organization. Global Malaria Programme, 2011). To achieve this ambitious target, a number of guidelines and policies to control and eradicate malaria have been implemented globally.

The recommended strategies needed to accomplish the malaria eradication include malaria vector control, malaria diagnostic testing and treatment, and chemoprevention. The WHO recommended method to control malaria vector and transmission of the disease is to provide people at risk with insecticide-treated mosquito nets (ITNs) as well as indoor residual spraying (IRS). The situation has improved since 2000 with more than 50 % households in sub-Saharan Africa in 2011 being reported to have access to at least one ITN compared to 3 % in the past. A
similar trend was also observed regarding the number of people who were protected by IRS having risen from less than 5 % in 2005 to 11 % in 2010 (World Health Organization. Global Malaria Programme, 2011). Another crucial approach to fulfill the malaria elimination goal is to robustly detect infected people and treat them appropriately to avoid unnecessary usage of antimalarial drugs. The current recommended procedure is to test a suspected malaria patient by either microscopy or rapid diagnostic test (RDT) before beginning the treatment. These guidelines have been adopted quite effectively and there is an increasing number of suspicious malaria cases being tested before antimalarial drugs are administered to the patients (World Health Organization. Global Malaria Programme, 2011). These achievements have in part been supported by the growing amounts of RDTs being distributed worldwide as well as the better quality of the tests. Confirmed \textit{P. falciparum} infected patients are recommended to receive artemisinin-based combination thearapies (ACTs) as first-line treatments while those infected with \textit{P. vivax} are administered with chloroquine, or ACTs if chloroquine-resistant parasites are present in the area. Primaquine is also used in combination with either of these drugs to prevent relapse of \textit{P. vivax} infection. Recently, however, there have been reports concerning the occurrence of artemisinin-resistant parasites in Southeast Asian countries especially around Thailand-Cambodia and Thailand-Burma border areas (Denis et al., 2006; Dondorp et al., 2009; Phyo et al., 2012; Vijaykadga et al., 2006).

Another policy WHO has put in place is to use the intermittent preventive treatment for pregnant women (IPTp) and infants (IPTi) who are prone to serious outcomes from malaria infection. So far there have only been less than 50 % of \textit{P. falciparum} endemic countries which have adopted the IPTp policy and no country has yet implemented the IPTi program as a national policy (World Health Organization. Global Malaria Programme, 2011). Data from surveys during 2009-2011 indicated the percentage of pregnant women in 12 African countries who received the recommended 2 doses of IPTp treatment ranged from 5 % - 69 % (World Health Organization. Global Malaria Programme, 2011).

Although many tools have been employed to fight this deadly disease, there is still a long way to go to achieve the goal of elimination. New ways to combat \textit{Plasmodium} parasites are definitely needed and one of them is to develop a protective vaccine. Despite a long history of malaria, there has never been an effective vaccine being licensed for commercial use. The most advanced malaria vaccine to date is the
RTS,S/AS01 vaccine which is currently in phase 3 clinical trials. The first results in children 5 to 17 months of age showed an efficacy of the vaccine around 50% protection against clinical episodes while an efficacy against severe malaria in this group of children was around 45% (Agnandji et al., 2011). The follow-up report however suggested rather disappointing outcome of the vaccination in younger children at the age of 6 to 12 weeks. In the younger children the efficacy of the RTS,S/AS01 vaccine against malaria episodes was reduced to around 30% and the protective efficacy against severe malaria dropped to 26% - 36% (RTS,S Clinical Trials Partnership et al., 2012). Alternative vaccines are also being explored with the goal set by WHO to have a vaccine with at least 80% efficacy against clinical malaria by 2025 (World Health Organization. Global Malaria Programme, 2011).

The *Plasmodium* life cycle

The complexity of the *Plasmodium* parasite’s life cycle which involves asexual and sexual life stages in two different hosts, contributes towards the difficulty of malaria elimination. It is therefore critical to thoroughly understand this complex life cycle since this may provide novel ways to eliminate the pathogen by disrupting its cycle.

The life cycle begins when an infected female *Anopheles* mosquito bites a human host during blood meal and sporozoites inside the salivary gland are released into the dermis at the injection site. Most sporozoites move through the host skin cells using their gliding motility until they find the blood vessel. They then enter the host bloodstream and travel passively via the blood circulation system to the liver. Once the sporozoites reach there, they penetrate through the sinusoidal endothelial cells, primarily via Kupffer cells, and traverse many other hepatocytes before establishing infection inside the cells within a parasitophorous vacuole that they form as they invade. The complex motility of the sporozoite is mainly governed by a protein called thrombospondin-related anonymous protein (TRAP) as well as other TRAP-like molecules that migrate over the parasite surface. The extracellular part of the TRAPs acts as an anchoring point on host cells while the intracellular part connects to the parasite's actin-myosin motor driving the parasite forward (Keeley and Soldati, 2004). It has been proposed that the mechanism by which the sporozoite recognises liver endothelial cells is by interacting with heparin sulfate proteoglycans (HSPGs) uniquely expressed on hepatocytes. To do that, the parasite utilises another abundant
surface protein named circumsporozoite protein (CSP), to attach to the liver sinusoids (Frevert et al., 1993). The binding to highly sulfated HSPGs in the liver thus switches the sporozoites from 'migratory mode' to 'invasion mode' through a calcium signaling pathway (Coppi et al., 2007). One of the downstream events is the proteolytic cleavage of CSP by a yet to be identified cysteine protease, to expose CSP’s cell-adhesion domain. This process appears to be regulated by a calcium-dependent protein kinase 6 pathway (Coppi et al., 2011; 2007).

The parasite develops inside the hepatocyte as a stage called exoerythrocytic schizogony, for a minimum of roughly 6 - 7 days depending on the number of sporozoites that reach the liver (Hermsen et al., 2004; Murphy et al., 1989). Each exoerythrocytic schizont is able to produce up to 40,000 daughter cells termed merozoites waiting to be released into the host bloodstream (Miller et al., 2013). These hepatic merozoites, when matured, bud from the infected cell and are delivered back to the liver sinusoidal lumen in a hepatocyte-derived vesicle called a merosome (Sturm et al., 2006). Since the membrane of merosome originates from the host cell, it also acts as a shield for the merozoites from being recognised and engulfed by resident macrophages. Work performed with P. yoelii, a rodent malaria parasite, has suggested that these merosomes are carried along the cardiovascular vessels to the lung capillaries where the merozoites are subsequently released to begin the blood-stage infection (Baer et al., 2007).

The blood-stage infection, which causes the disease in the host, is also called the asexual stage of the parasite life cycle since at this point the parasite propagates by several rounds of nuclear multiplications to produce haploid merozoites for following infections without forming gametes. The asexual cycle begins when the merozoites invade erythrocytes after being released from the merosomes. Similar to the sporozoite infection in hepatocyte, the invaded merozoite makes its own home inside the parasitophorous vacuole separating itself from the erythrocyte cytoplasm. The morphology of the parasite then changes into an amoeboid-like shape for a few hours before it rounds up and appears as ring-shaped parasite when it is stained and observed under a light microscope, hence the name of the ring stage. Afterwards, the parasite begins to increase in size and takes up more space within the erythrocyte progressing into the next stage called a trophozoite, around 18 hours post invasion (hpi) (Arnot et al., 2011). As the parasite grows it digests hemoglobin, which not only provides the nutrients but also makes room for the growing parasite. The problem for
the parasite at this stage is that the heme molecules released during hemoglobin
digestion are toxic to the parasite. The parasite has solved this problem by
polymerising the heme molecules into hemozoin crystals to detoxify them (Pisciotta
and Sullivan, 2008). The hemozoin crystals can be easily seen as dark material inside
the digestive vacuole first detectable from the trophozoite stage. Another important
biological aspect at this life stage is that the parasite creates its own protein export
system for delivery of its proteins into the erythrocyte which help modify it. Amongst
the most important modifications is to deploy adhesive PfEMP1 proteins out onto the
erythrocyte surface so the infected cell can bind to host endothelial cells and stop
circulating. This then prevents the parasitised erythrocyte from passing through the
spleen and being cleared thereby allowing the parasite to mature. The parasite begins
multiple rounds of nuclear division from around 28 hpi onwards, entering the next
stage termed schizogony, followed by the formation of merozoites (Arnot et al.,
2011). The number of merozoites produced from a schizont may vary from 8 to 32
merozoites, which is a result of its unique asynchronous mitotic division (Arnot et al.,
2011). Once the merozoites have matured, they egress from the infected cell through
breakdown of the enveloping PVM and erythrocyte plasma membrane assisted by a
number of proteases (reviewed in (Wirth and Pradel, 2012)). The merozoites then
invade new erythrocytes and continue their asexual proliferation. Overall the asexual
stage in P. falciparum takes roughly 44 - 48 hours to complete a cycle.

The Plasmodium parasite also has to make sure that it is transmitted to a new
host in case its current host dies. This is achieved by transmission through female
anopheline mosquitoes when they feed. Transmission serves an additional function
which is to facilitate sexual recombination between different parasites so that fitter
alleles can spread through the population. In preparation for transmission, a portion of
blood-stage parasites escape the asexual cycle and begin to form gametocytes. There
are many factors that have been proposed to initiate the gametocytogenesis; for
instance, human serum and lymphocytes, erythrocyte lysate, mammalian hormones,
and high level of reticulocytes (reviewed in (Baker, 2010)). Two recent studies have
revealed that the parasites are able to communicate with one another through
exosome-like vesicles secreted from the infected cells (Mantel et al., 2013; Regev-
Rudzki et al., 2013). These microvesicles could be involved in triggering
gametocytogenesis since it has been shown that the number of parasites undergoing
this process is directly proportional to the amount of microvesicles present in the
culture. This is to some extent in agreement with the earlier study which suggested that the commitment to sexual development of *P. falciparum* is programmed before merozoites egress from a schizont (Bruce et al., 1990). The signal, yet to be identified, that is received from exosome-like vesicles could prime the parasites for sexual development in the next cycle. The gametocytogenesis of *P. falciparum* takes place roughly two weeks after the blood stage has established in the human host and it takes about 10 days to accomplish through 5 developmental stages (reviewed in (Baker, 2010; Pradel, 2007; Sinden, 1983)). Only the stage V gametocytes circulate in the bloodstream waiting to be taken up by the mosquito during a blood meal. Sensing the changes of environment, e.g. the drop of temperature and the presence of xanthurenic acid in the mosquito midgut, the gametocytes further develop into either microgametes or macrogametes. The male gametocyte produces 8 flagellar bearing microgametes by replicating its genome 3 times, in a process called exflagellation. These cells then adhere to other erythrocytes forming an exflagellation center. Once they are released from the rosette, they can move freely to find and fertilise with a macrogamete forming a zygote. The diploid zygote further develops into the next ookinete stage which is motile and able to penetrate the chitinaceous peritrophic matrix-coated midgut epithelium through to intercellular space between the basal lamina and the midgut epithelium where it advances to the next oocyst stage (reviewed in (Vlachou et al., 2006)). This process, including receptor-ligand interactions between the parasite and the host cell as well as the translocation of the ookinete, resembles the hepatocyte invasion of sporozoites, although the underlying molecular machineries might be different. Several thousand haploid sporozoites are developed from multiple nuclear divisions followed by cytokinesis within the oocyst. When the sporozoites are mature, they are released into the mosquito's haemolymph and travel to the salivary glands. Whether this migration is mediated by haemocoel circulation alone (Hillyer et al., 2007) or involves chemotaxis (Akaki and Dvorak, 2005) remains to be confirmed. Invasion of sporozoites into the salivary glands is thought to be one of the critical steps in *Plasmodium* life cycle. Only 19 % of released sporozoites are able to penetrate the salivary glands and this has to happen within 8 hours (Hillyer et al., 2007). Similar to the hepatocyte invasion, the sporozoite utilises its surface proteins to first recognise the basal lamina of the salivary glands. Searches for receptor-ligand interactions between the host and the parasite have come up with multiple candidates responsible for the process (Ghosh and Jacobs-Lorena, 2009;
Mueller et al., 2010). One pair of the receptor-ligand interactions that is quite well studied is the binding between saglin on the salivary gland and TRAP of the sporozoite (Ghosh and Jacobs-Lorena, 2009). Another sporozoite surface protein, CSP, is also suggested to bind to heparan sulfates on the salivary gland surface and thus this interaction may also play a role in gland recognition (Sinnis et al., 2007). The sporozoites then begin penetration into the salivary gland where they eventually accumulate in the secretory cavity waiting to be injected into the mammalian host during the next feeding event (Mueller et al., 2010; Rodriguez and Hernández-Hernández, 2004).

**Merozoite invasion of erythrocytes**

Since the discovery of the causative pathogen of malaria, we have only relied on using chemotherapeutic treatments to fight against the disease without any long-term preventive method such as vaccination. This is simply due to the fact that *P. falciparum* parasites spend most of their roughly 48-hour asexual cell cycle, in human erythrocytes where they are able to hide from the immune system. The parasites are only directly and briefly exposed to host defenses when merozoites leave the erythrocyte to invade new cells. Thus, the extracellular merozoite and its invasion functions are potential targets for the development of effective vaccines. It is therefore important to clearly understand the underlying mechanism of how a parasite invades an erythrocyte to identify possible candidates for vaccine development.

The first recordings of live merozoites invading erythrocytes using light microscopy were published in 1975 by Dvorak and colleagues (Dvorak et al., 1975). They successfully captured the invasion of *P. knowlesi* merozoites into rhesus monkey erythrocytes, and were able to describe important characteristics of the process. In comparison to *P. knowlesi*, the merozoites of *P. falciparum* are smaller and more short-lived and are therefore more technically challenging to perform live-cell microscopy on. For this reason it had taken another 34 years for Gilson and Crabb to accomplish a similar study with *P. falciparum* (Gilson and Crabb, 2009). Not long after that Yahata et al. successfully recorded the invasion events of rodent malaria parasite *P. yoelii* merozoites (Yahata et al., 2012). Despite some morphological differences of the merozoites between species, these studies have shown that kinetics and manifestation of the erythrocyte invasion process are remarkably similar.
Microscopy has revealed the following details about the invasion process. After the merozoites are released from the host erythrocyte, they disperse by Brownian motion until they recognise and attach to new erythrocytes. Under the microscope where the cells are spread out as a monolayer this can take several seconds to minutes however within the host’s circulatory system, where the merozoites are carried along by bulk flow, contact would occur more rapidly. After initial contact the invasion process can be divided into 3 distinct phases (Gilson and Crabb, 2009). Firstly, during an ~11 second ‘pre-invasion’ phase, merozoite contact often causes pronounced deformation of the erythrocyte membrane followed by a quiescent recovery phase. This primary contact is also considered reversible because merozoite detachment from the erythrocyte can occur. One of the purposes of the pre-invasion phase might be to facilitate merozoite reorientation so that its penetrative apical end becomes juxtaposed to the erythrocyte plasma membrane. Once the merozoite successfully attaches its apical end to the membrane, it stops moving and remains in a non-motile ‘resting’ phase for a few seconds. The second phase begins when the merozoite starts penetrating into the erythrocyte and this step lasts for about 17 seconds. By differential interference contrast microscopy a dark band, likely to be a tight junction, can clearly be seen at the interface between the invading merozoite and erythrocyte membrane, and this band moves rearward as the merozoite internalises into the erythrocyte. The last phase occurs ~36 seconds after merozoite successfully invades the erythrocyte when the cell rounds up and develops protuberances on its surface. This phenomenon is called echinocytosis and is probably due to dehydration caused by a breakdown of ionic homeostasis. As ionic homeostasis returns after several minutes the cell begins to recover its normal biconcave shape. With the morphology and kinetics of invasion now comparatively well characterised this has become a framework to understand the roles of many merozoite proteins responsible for each phase of the process.

**Merozoite surface proteins**

The plasma membranes of Plasmodium parasites are typical of most other eukaryotes in that they contain special areas commonly referred to as lipid rafts or detergent-resistant membranes (DRM) (Wang et al., 2003). These membrane microdomains are rich in cholesterol and sphingolipids that make them rigid and are resistant to non-ionic detergent in the cold. The proteins that are able to insert
themselves into the DRMs usually undergo post-translational modification with either myristate/palmitate acylation or a glycosylphosphatidyl inositol (GPI) anchor, and these proteins usually participate in cell signaling and cell adhesion (reviewed in (Brown and London, 2000)). Both resident merozoite surface proteins and those that translocate from apical organelles, especially rhoptries, typically locate to lipid rafts. Most GPI-anchored merozoite surface proteins have been detected in isolated DRMs as expected along with other multimembrane-spanning proteins (Wang et al., 2003); (Sanders et al., 2005).

It was in 1969 when Ladda and the team first mentioned the granular coating of the outer membrane of avian malaria merozoites (Ladda et al., 1969). The follow-up study led by Aikawa et al. described the surface coat of extracellular merozoites as having a uniform thickness of around 20 nm (Aikawa et al., 1978). During invasion this fibrillar coat disappeared on the intraerythrocytic part of merozoite while the remaining extracellular part still had the coat similar to free merozoites (Aikawa et al., 1978). This observation has since triggered decades of studies that have led to the discovery that the merozoite surface coat is of parasite origin and comprised of proteins generally called merozoite surface proteins (MSPs).

MSPs are present on the parasite membrane not only as integral membrane proteins, mostly affixed via GPI anchors, but also as soluble proteins peripherally attached to the membrane proteins. The most abundant merozoite surface protein is MSP1 which is synthesised as a ~195 kDa GPI-anchored protein in the schizont stage (Holder, 1982). MSP6 and MSP7 are co-expressed and become attached to MSP1 forming a protein complex on the merozoite surface (Pachebat et al., 2001; Trucco et al., 2001). Prior to merozoite egress, MSP1 is primarily cleaved by a subtilisin-like protease, SUB1, into 4 major polypeptide fragments, MSP183, MSP130, MSP138, and MSP142, while N-terminal ends of MSP6 and MSP7 are also truncated by this protease (Koussis et al., 2009). These MSP1 fragments remain non-covalently attached to each other (Kauth et al., 2006). A secondary processing step takes place during erythrocyte penetration by another protease SUB2 (Harris et al., 2005). This action removes the MSP1/6/7 complex off the plasma membrane leaving only a GPI-anchored stub of MSP119 which is carried into the erythrocyte with the merozoite (Blackman et al., 1990). The MSP1/6/7 complex function is suggested to be responsible for low-affinity reversible interactions with the erythrocyte either via sialic acid on the surface (Perkins and Rocco, 1988) or a protein receptor (Goel et al.,
A recent report has also demonstrated that it is MSP1$_{33}$, the secondary-processed fragment of MSP1$_{42}$, which interacts with heparin-like molecules on the erythrocyte surface mediating the attachment of the merozoite to the erythrocyte (Boyle et al., 2010a).

There are also many other merozoite surface proteins identified and characterised for their role in erythrocyte invasion. MSP2 is the second most abundant GPI-anchored protein present on the surface and has been extensively studied (Adda et al., 2012; 2009; Gilson et al., 2006; MacRaild et al., 2012). Despite that, its function remains unknown apart from that the central variable region of the protein was able to induce humoral immune responses (Adda et al., 2012; Ekala et al., 2002; Scopel et al., 2007; Weisman et al., 2001). This potential capability of this antigen has led scientists to include MSP2 in trial vaccine formulations, Combination A and Combination B (Chauhan et al., 2010).

MSP3 is a six-member protein family, including MSP6 mentioned previously, whose genes are tandemly located on chromosome 10 (Singh et al., 2009). MSP3 was first discovered by genome-wide screening for antigens which could trigger natural protective immunity via antibody-dependent cellular inhibition (ADCI) activity, a parasite killing mechanism mediated by monocytes (Oeufray et al., 1994). Subsequent work also demonstrated the same antigenicity for MSP6 (Singh et al., 2005). However, the role of MSP3 and MSP3-like proteins remain unclear since the parasites that have some of these genes knocked out individually are still viable in vitro (Mills et al., 2002; Pearce et al., 2005). The most apparent effect was from the disruption of MSP3 which led to mislocalisation of MSP9 and also showed reduction in invasion efficiency (Mills et al., 2002).

MSP4 and MSP5 are the other two GPI-anchored surface proteins whose function has not been revealed to date (Marshall et al., 1998; Wang et al., 1999). While MSP4 appeared to be refractory to genetic deletion, MSP5 could be knocked out without a defective phenotype being observed (Sanders et al., 2006).

MSP7 on the other hand is a peripheral membrane protein that forms part of the MSP1 complex mentioned above. It belongs to a six-member protein family where other members have been knocked out but showed no effect on parasite growth and invasion efficiency (Kadekoppala and Holder, 2010). MSP7 itself could also be disrupted and the mutant parasites demonstrated a decrease in invasion competency (Kadekoppala et al., 2008).
MSP8 is classified as a merozoite surface protein because of its structural similarity to MSP1 (Black et al., 2001). However it was found to be expressed and localised to the plasma membrane of early ring-stage parasites instead (Drew et al., 2005). The MSP8 gene has been disrupted, and no growth or invasion-related defects were observed (Black et al., 2005; Drew et al., 2005).

MSP9 was first described as an acidic basic repeat antigen (ABRA) and is peripherally associated on the merozoite membrane (Stahl et al., 1986; Weber et al., 1988). Functional studies of this protein indicated that it could interact with band 3 on the erythrocyte surface and therefore could be serving as an invasion ligand possibly working together with MSP1 (Goel et al., 2003; Kariuki et al., 2005; Kushwaha et al., 2002; Li et al., 2004). MSP10, despite being classed as a merozoite surface protein, was shown to have localisation distinct from the others namely, apically concentrated with a minor portion around the merozoite surface (Black et al., 2003).

It is clear that a lot more remains to be accomplished to elucidate the roles of the MSPs. The challenge to understand their functions by reverse genetic approaches is that either they are essential and cannot be knocked out or they can be knocked out but display no phenotype. The latter can be explained by the fact that many invasion proteins have degenerate functions probably as an immune evasion strategy and when one is knocked out others appear to compensate. Nonetheless, a lot of studies have reported that many of these proteins are immunogenic and that they could become potential vaccine candidates.

Erythrocyte binding antigens and reticulocyte binding protein homologs

Once the merozoite comes into contact with the erythrocyte presumably via the interactions of merozoite surface proteins and heparin-like molecules on the erythrocyte surface, the parasite subsequently establishes a strong attachment with the erythrocyte readying itself for invasion. Studies of this mechanism have divided it into 2 so-called invasion pathways; sialic acid-dependent and sialic acid-independent pathways, depending on which ligand the particular parasite strain relies on the most. Two groups of proteins have been assigned for their roles in facilitating these invasion mechanisms, namely erythrocyte-binding like (EBL) protein family and reticulocyte-binding homologs (Rhs).

The EBL family of proteins, also called erythrocyte-binding antigens (EBAs), comprises of 4 functional members; EBA175 (Camus and Hadley, 1985), EBA140
There is also *eba*165 gene present in the parasite genome but it does not appear to be translated into a protein (Triglia et al., 2001a). The EBAs were localised to apical organelles of the merozoite called micronemes, small vesicles containing numerous proteins involved in invasion (Gilberger et al., 2003; Mayer et al., 2001; 2009; Sim et al., 1992; Thompson et al., 2001a). The EBA proteins are type I transmembrane proteins and the major characteristic of this protein family is their Duffy-binding like (DBL) domain that is used to bind to the erythrocyte receptor. The conventional Duffy binding proteins discovered in *P. knowlesi* and *P. vivax*, which bind to Duffy blood group antigen on the erythrocyte surface, contain only one DBL domain, while the EBAs of *P. falciparum* have two domains, designated as F1 and F2, at their amino end (Adams et al., 1992).

EBA175 is the first erythrocyte binding protein in *P. falciparum* identified and has been very well characterised. Its binding activity to the erythrocyte was found to be blocked when the erythrocytes were enzymatically treated with neuraminidase and trypsin, which removed sialic acid molecules and some of the proteins on the erythrocyte surface respectively (Camus and Hadley, 1985). The identity of the receptor for EBA175 was later discovered to be glycophorin A and both the protein backbone and the sialic acids that decorate it were required for the interaction (Sim et al., 1994). Selective region binding study as well as crystal structure of EBA175 revealed that F2 domain was the major contributor to the interaction of the protein with glycophorin A, and the binding pocket required the dimerisation of EBA175 (Sim et al., 1994; Tolia et al., 2005). Erythrocyte binding activities of other EBAs and the identification of their receptors have also been studied using similar approaches. The interaction of EBA140 and its receptor was also dependent on sialic acids and sensitivity to trypsin treatment (Mayer et al., 2001; Thompson et al., 2001a). EBA140’s binding activity was reduced when tested with Gerbich mutation erythrocytes, which expressed shortened form of glycophorin C and glycophorin D on the surface (Mayer et al., 2001; Walker and Reid, 2010) and it was later confirmed that glycophorin C was the actual receptor for EBA140 (Maier et al., 2003). However, unlike EBA175, EBA140 seemed to require both F1 and F2 domains for binding and it did not form a dimer (Lin et al., 2012; Malpede et al., 2013). Glycophorin B was recently discovered to be a receptor for EBL1 and the invasion through this pathway.
was also dependent on sialic acids (Mayer et al., 2009). The final EBA ligand EBA181, also utilised sialoglycoprotein receptors on the erythrocyte surface to invade (Gilberger et al., 2003). The identity of the EBA181 receptor however remains unknown even though some reports suggest that EBA181 binds to 4.1R protein, an intracellular erythrocyte membrane protein associated with the cytoskeleton, which seems unlikely to play a role in parasite invasion (Lanzillotti and Coetzer, 2006; Lauterbach et al., 2003).

The Rh proteins in *P. falciparum* are named after their homologs firstly identified in *P. yoelii* and *P. vivax*, Py235 and PvRBP respectively (Freeman et al., 1980; Galinski et al., 1992). These proteins in the two species are heavily involved in host cell selection and invasion, hence the homologs in *P. falciparum* are believed to play a similar role (Galinski et al., 1992; Ogun and Holder, 1996). The Rh protein family in *P. falciparum* comprises of several members including Rh1, Rh2a, Rh2b, Rh4, and Rh5. PfRh3 was also annotated in the genome but appears to be a pseudogene (Taylor et al., 2001). Similar to the EBA protein family, the Rh1-4 are type I transmembrane proteins and they, except for the PfRh4, share a conserved 500-amino-acid region signifying them as part of the same protein family (Iyer et al., 2007). Again like the EBAs, the Rhs appear to be localised to the apical area of the merozoite, except not to the micronemes but the rhoptry neck, which is consistent with their role as invasins (Cowman and Crabb, 2006).

PfRh1, originally known as normocyte binding protein 1 (PfNBP1), is expressed as ~390 kDa protein and its protein sequences are very conserved among multiple laboratory strains, except for 3D7 and 7G8 which have mutations causing early termination of the transcribed gene product (Rayner et al., 2001). Its ability to bind to the erythrocytes has been examined and it appears to also be involved in a sialic-acid dependent invasion pathway, although its receptor has not been verified and is therefore termed Receptor Y (Rayner et al., 2001).

PfRh2a and PfRh2b are almost identical in their N-terminal region but vary greatly in their C terminal regions (Triglia et al., 2001b). PfRh2a and -b were found to be expressed at different levels in various parasite lines (Duraisingh et al., 2003b). A binding study of PfRh2b has shown that the protein is responsible for the sialic-acid independent invasion pathway (Duraisingh et al., 2003b), however the function and essentiality of PfRh2a have found to vary among laboratory strains (DeSimone et al., 2009; Duraisingh et al., 2003b; Dvorin et al., 2010a). The receptor for PfRh2b has not
yet been identified except for its characteristic of being sensitive to chymotrypsin treatment but resistant to trypsin and it therefore has been referred to as Receptor Z (Duraisingh et al., 2003b). The differences between PfRh2a and PfRh2b function may reside in their dissimilar cytoplasmic domains implying that the downstream consequences after binding to their receptor might be different (Dvorin et al., 2010a).

Another member of the PfRh protein family, PfRh4, was genetically identified and characterised by Kaneko et al. and the gene product was detected as ~220 kDa protein (Kaneko et al., 2002). PfRh4 is expressed in the parasite lines which mainly rely on the sialic acid-independent invasion pathway including those that have just switched from the other pathway (Gaur et al., 2007; Stubbs et al., 2005). Both native PfRh4 and recombinant protein are able to bind to the untreated and neuraminidase-treated erythrocytes, confirming the role of PfRh4 in a sialic acid-independent invasion mechanism (Gaur et al., 2007). Furthermore, the erythrocyte receptor of PfRh4 is sensitive to both trypsin and chymotrypsin treatments (Gaur et al., 2007; Tham et al., 2009). This information led to the discovery of the PfRh4 receptor to be complement receptor 1 (CR1), a member of the regulators of complement activation that protects host-tissue from self complement-mediated immunity (Tham et al., 2010).

Selective utilisation of these ligands for particular invasion pathways is strain-specific preference (Dolan et al., 1990). For example, the W2mef laboratory line was demonstrated to rely on a sialic acid-dependent pathway by using EBA175 as a dominant ligand for invasion (Reed et al., 2000). As a consequence, less than 10 percent of W2mef cultivated in neuraminidase-treated erythrocytes could grow when compared to the group tested with untreated erythrocytes (Duraisingh et al., 2003a). However, W2mef parasites continuously cultured in sialic acid-null erythrocytes could restore their invasion efficiency back to a similar level as parasites cultivated in untreated cells illustrating that they were able to switch to sialic acid-independent invasion pathway (Stubbs et al., 2005). Upregulation of PfRh4 was shown to be responsible for this alternative pathway as its expression increased by ~60 - 80 fold and when the pfhrh4 gene in W2mef was deleted the mutant parasites could no longer grow in neuraminidase-treated erythrocytes (Stubbs et al., 2005). The ability to alternatively utilise different ligands for invasion was supported by successful genetic deletions of these proteins, which also caused a switch in invasion pathway of the mutant parasites. For instance, the eba175-deleted W2mef was able to invade
neuraminidase-treated erythrocytes through the compensatory upregulation of PfRh4 (Stubbs et al., 2005). The genes of both EBL and Rh protein family, except for EBL-1, have already been disrupted successfully and various phenotypes of the mutant parasites were observed (Cowman and Crabb, 2006). Some resulted in switching invasion pathway, as described earlier, or changing the receptor binding profile, while some remained unchanged suggesting that the deleted gene played a minor role in a particular parasite strain as in the case of *pfhr1* disruption in 3D7 (Duraisingh et al., 2003a; 2003b; Triglia et al., 2005). Furthermore, there was evidence suggesting that these ligands were hierarchically utilised, whereby the secondary machinery was already present to compensate for the loss of the primary one (Baum et al., 2005; Duraisingh et al., 2003a; Iyer et al., 2007). The benefit of this organisation would be to permit the parasites to continue to efficiently invade erythrocytes even when the host protective immunity arises to particular invasion ligands. Overall it appeared that EBA175, PfRh2b, and PfRh4 were the dominant ligands responsible for the two invasion pathways since the combination of antibodies to these three proteins gave the strongest invasion inhibition (Lopaticki et al., 2011).

PfRh5 is the most unique member of the Rh protein family and its essential role in erythrocyte invasion has recently been demonstrated. PfRh5 is the smallest member of the family being only 63 kDa in size compared to the other much larger Rh proteins that have molecular weights of ~200 - 400 kDa. Furthermore, PfRh5 shares sequence similarity with the other members only in N-terminal region and lacks the C-terminal part including the transmembrane domain and cytoplasmic tail (Hayton et al., 2008). Like other merozoite invasion ligands, PfRh5 is subjected to proteolytic cleavage and sheds a 45 kDa fragment into the culture supernatant (Baum et al., 2009). Its localisation was confirmed by both immunofluorescence and immuno-electron microscopy to be in the rhoptry bodies, slightly different from its related proteins, and the protein was released out on to the merozoite surface during invasion (Baum et al., 2009; Rodriguez et al., 2008). PfRh5 was observed to follow the tight junction suggestive of its function as parasite ligand (Baum et al., 2009). Indeed it was demonstrated to possess an ability to bind to host erythrocytes with the unique receptor characteristics of being resistant to all enzymatic treatments normally used to determine the binding profiles (Baum et al., 2009). More importantly the antibodies raised to the full-length PfRh5 of one strain could cross-inhibit erythrocyte invasion of many other laboratory strains as well as culture-adapted field isolates.
illustrating its unique characteristic of being universal antigen, unlike the other ligands mentioned above which exhibit strain-specific variation (Bustamante et al., 2013; Douglas et al., 2011). PfRh5-specific antibodies also acted synergistically with other merozoite surface protein antibodies to inhibit merozoite invasion in in vitro assays thereby raising the possibility of including these antigens into a future combination vaccine (Williams et al., 2012). The fact that pfrh5 could not be disrupted in multiple laboratory lines strongly supports its essential role in parasite invasion (Baum et al., 2009).

Since PfRh5 does not have either a transmembrane domain or GPI anchor modification to embed itself on to the merozoite surface it seems likely to bind to another surface anchored protein. Chen et al. was able to discover its binding partner and named that protein P. falciparum Rh5 interacting protein (PfRipr) (Chen et al., 2011). This protein was localised to micronemes meaning that the two proteins associated as a complex only when they were secreted to the surface (Chen et al., 2011). Interestingly, PfRipr does not contain a membrane anchor suggesting that it must also bind to a membrane-anchored protein that has just recently been identified to be Cysteine-rich protective antigen (CyRPA), a GPI-anchored protein localised to micronemes (Reddy et al., 2015). Antibodies targeting to the PfRipr and CyRPA were also able inhibit parasite invasion across multiple strains likely by interfering PfRh5 function since PfRipr and CyRPA themselves did not bind to erythrocytes (Baum et al., 2009; Chen et al., 2011; Reddy et al., 2015).

As for the erythrocyte receptor of PfRh5, it was revealed to be the Ok blood group antigen called basigin (Crosnier et al., 2011). Basigin (BSG) is a glycoprotein belonging to the immunoglobulin superfamily commonly found on leucocyte membrane and other human hematopoietic cells (Spring et al., 1997). In accordance with PfRh5 studies, blocking the PfRh5-basigin interaction either by adding soluble BSG or anti-BSG antibodies strongly inhibited invasion in multiple laboratory strains and fresh isolates (Crosnier et al., 2011). In agreement with this was that merozoite invasion into erythrocytes expressing reduced levels of BSG was substantially reduced (Crosnier et al., 2011).

Taken together, PfRh5 appears to be a master invasion ligand utilised by all strains of P. falciparum along with other varieties of EBAs and Rhs selectively employed by the individual parasites. With this range of high-affinity binding proteins, the merozoites ensure that they strongly bind to the new erythrocytes by
their apex and ready themselves for internalisation. Similar to those merozoite surface proteins mentioned earlier, these EBAs and Rhs are also potential candidates to be developed into malaria vaccine, especially PfRh5 for its essentiality and universal usage by most if not all parasite variants in the field.

*Apical membrane antigen and rhoptry neck proteins*

Following successful reorientation and apical attachment to the erythrocyte, the merozoite is set for penetration. To achieve this important step, the invasive merozoite, instead of relying more on erythrocyte receptors, prepares its own machinery for setting up a ring of close apposition with the erythrocyte surface through which the parasite moves during internalisation. This ring called a tight junction, was first demonstrated in detail many years ago in a series of electron micrographs by Aikawa et al. (Aikawa et al., 1978). The images showed circumferential attachment between the parasite and the erythrocyte started to form at the entry point and it was anchored there until the internalisation finished. This junction appeared to move to the posterior end of the merozoite as it was invading the erythrocyte, hence this ring is also called a moving junction. The knowledge of molecular basis of this moving junction was first established in *T. gondii* and this has informed further studies in *Plasmodium* indicating this part of the invasion process is well conserved in the phylum.

The first molecule that is believed to be responsible for junction formation is a micronemal protein named Apical Membrane Antigen 1 (AMA1). This protein is present in most Apicomplexan parasites suggestive of its conserved role in erythrocyte invasion. Being a type I transmembrane protein, AMA1 was first characterised and given its name according to its location at the apex of the merozoite before spreading out onto the merozoite surface (Peterson et al., 1989). Its ectodomain is divided into 3 parts, namely domains I, II, and III, while its short cytoplasmic tail is very conserved within the phylum (Hodder et al., 1996; Leykauf et al., 2010; Pizarro et al., 2005). AMA1 is expressed late in asexual stage as an 83 kDa protein and is subjected to proteolytic cleavage to 66 kDa protein just prior to schizont rupture (Healer et al., 2002; Howell et al., 2001). This is also when AMA1 begins to translocate onto the merozoite surface. The protein is then cleaved off the membrane, presumably during invasion similar to other merozoite surface proteins, into 48 kDa form (Howell et al., 2003). The protease responsible for this processing is PfSUB2,
the same protease that sheds MSP1 (Harris et al., 2005). PfAMA1 is refractory to genetic deletion and antibodies to PfAMA1 can block erythrocyte invasion by merozoites indicative of an essential role for the protein (Hehl et al., 2000; Mitchell et al., 2004; Triglia et al., 2000). Recently, however, success has been reported in deleting *ama1* in *T. gondii* tachyzoite as well as *P. berghei* sporozoites and merozoites (Bargieri et al., 2013). The tachyzoite and merozoite mutants demonstrated a similar defect in host cell attachment that led to a decrease in invasion rate. In contrast, the tight junction ring was observed to form normally and the AMA1-null zoites could invade into their respective host cells as usual. Not long afterwards Yap et al. were able to delete 80% of the AMA1 genes in *P. falciparum* merozoites thereby knocking down the expression of AMA1 and reported that the mutants failed to successfully invade the host erythrocytes and develop into ring-stage parasites (Yap et al., 2014). Detailed observations by live-cell microscopy further revealed that a proportion of AMA1-deficient merozoites were still able to penetrate the erythrocytes but could not properly reseal the erythrocyte membrane as determined by the prolonged echinocytosis period. The outcome of these findings leads to some controversy as to whether or not AMA1 is actually required for tight junction formation. A new model regarding AMA1 function as a tight junction component from these recent findings was proposed (Harvey et al., 2014). Otherwise AMA1 might have some other yet to be confirmed role during invasion (Bargieri et al., 2013; Mitchell et al., 2004).

Upon close examination of AMA1 structure Bai et al. has discovered that there is a hydrophobic groove which is surrounded by highly polymorphic residues (Bai et al., 2005). This suggests that the trough may have an important function, likely to act as a binding pocket, since it is protected from the host immune system. The binding sites of invasion blocking monoclonal antibodies to PfAMA1, 1F9 and 4G2, were mapped to this same area of the protein supporting the important role of this region (Coley et al., 2001; 2006; Collins et al., 2007; Kocken et al., 1998).

There was some inconsistency whether AMA1 could bind directly to the erythrocyte since the whole recombinant ectodomain of the protein did not show erythrocyte binding activity (Ghai et al., 2002) but selective regions of AMA1 were able to do so (Fraser et al., 2001; Urquiza et al., 2000). However, it was subsequently revealed in *T. gondii* that AMA1 interacts with the parasite's own proteins, the RON complex, at the moving junction (Alexander et al., 2005; Lebrun et al., 2005) and this
interaction was later confirmed in *Plasmodium* parasites (Collins et al., 2009; Richard et al., 2010). The RON complex comprises of RON2, RON4 and RON5, with an additional RON8 found only in *T. gondii* (Besteiro et al., 2009). The parasite utilises this RON complex as an invasion receptor by first secreting them into the erythrocyte where RON4, RON5, and RON8 were found to localise to the erythrocyte side of the tight junction (Besteiro et al., 2009; Riglar et al., 2011). Members of the complex appear to cross the erythrocyte membrane and perhaps interact with the cytoskeleton (Besteiro et al., 2009). Topology studies have revealed that TgRON2 which is predicted to be a transmembrane protein, likely has its N-terminal on the cytosolic side of the host cell and an exposed extracellular loop between transmembrane domains 2 and 3 (Lamarque et al., 2011). Although this has not been formally proved for PfRON2 there is enough similarity between the sequences to suggest PfRON2 likely has the same orientation.

AMA1 appears to binds directly to RON2 at the loop between the transmembrane domains, and the fact that a peptide derived from this region of RON2 can inhibit invasion strengthens the essentiality of this interaction (Lamarque et al., 2011; Srinivasan et al., 2011). During erythrocyte penetration it was observed that the junction expands into concentric rings, with AMA1 at the inner ring and the RON complex in the outer, through which the merozoite passes to gain access to the erythrocytic cytosol (Riglar et al., 2011). Since AMA1 could be deleted in some parasites without any defect seen in tight junction formation, this might imply that the RON complex alone can establish a gateway for erythrocyte invasion or binds to another proteins as reported for *T. gondii* (Bargieri et al., 2013; Lamarque et al., 2014). The strong association between RON2 and AMA1 seen at the junction might have other role waiting to be discovered.

It was also shown during merozoite entry that a ring of erythrocyte F-actin rapidly formed beneath the tight junction in invading *Toxoplasma* tachyzoites and sporozoites of the rodent parasite *P. berghei* (Gonzalez et al., 2009). Inhibition of F-actin ring formation with actin destabilising drugs significantly reduced invasion efficiency indicating the ring appears to assist invasion by acting as a solid anchoring point for the tight junction. Actin polymerisation appears to be stimulated by recruitment of F-actin nucleating factors such as ARP2/3 and cortactin. Which factors act to recruit the F-actin nucleators is unknown but the possibility of parasite proteins being involved has been raised (Gonzalez et al., 2009). These may well be members
of the RON complex that are exposed on the cytoplasmic side of the erythrocyte. Although demonstrated for Toxoplasma and sporozoites, this mechanism is yet to be shown for merozoite invasion of human erythrocytes.

In addition to the crucial role the AMA1 ectodomain plays in invasion, its cytoplasmic tail has also been demonstrated to have a vital function during invasion. The tail sequences of AMA1s are so well conserved across Plasmodium, that tails from P. vivax and P. berghei can complement the function of the P. falciparum one (Treeck et al., 2009). It has been demonstrated that the serine residue at position 610 (S610) in the tail is phosphorylated by protein kinase A (PKA) and this phosphorylation can be enhanced by calcium ion (Ca\(^{2+}\)) (3-fold) as well as cyclic adenosine monophosphate (cAMP) (17-fold) suggesting that AMA1 might be involved in invasion-related cell signaling (Leykauf et al., 2010). The phosphorylation event appears important since transgenic parasites carrying a S610 to alanine mutation invaded erythrocytes at greatly reduced efficiency (Leykauf et al., 2010). Mutation of other conserved residues in the AMA1 cytoplasmic tail also prevented erythrocyte invasion (Treeck et al., 2009). The degree of tail phosphorylation appears to increase during schizogony and has been proposed to trigger interaction with the actomyosin invasion motor thereby helping move the merozoite through the tight junction during invasion (Leykauf et al., 2010). While binding of the AMA1 tail to aldolase has been demonstrated in vitro, phosphorylation was not necessary for this interaction so the role of phosphorylation remains unclear (Srinivasan et al., 2011).

The significance of aldolase is that it has been shown to act as a bridge between the actin component of the actomyosin invasion motor and the tails of TRAP proteins involved in the motility of zoite parasite stages (Jewett and Sibley, 2003). This type of complex formation was originally thought to be similar for AMA1, however it has recently been demonstrated that tachyzoites expressing a mutated form of TgAMA1 was unable to bind to aldolase but still display normal movement in both gliding and invasion (Shen and Sibley, 2014). If this was also the case in Plasmodium merozoites, the hypothesised role of the AMA1 tail in directly linking the tight junction complex with the parasite actomyosin motor to create a forward propelling force facilitating erythrocyte invasion might be incorrect. The function of the AMA1 tail particularly in its active phosphorylated form, therefore still awaits discovery.

PKA is stimulated by fluxes of cAMP but what triggers the adenylate cyclase that makes cAMP which then in turn initiates the phosphorylation of AMA1 tail and
the following downstream effects remains unknown. Given the mild increase in AMA1 tail phosphorylation caused by Ca\(^{2+}\) ions it is likely that intracellular Ca\(^{2+}\) signaling is playing a role as well. A significant boost in AMA1 phosphorylation could arise after merozoite egress since this event has been shown to stimulate an increase in intracellular levels of Ca\(^{2+}\) (see below)(Singh et al., 2010).

The 6-cys protein family

The 6-cys domain proteins are a family of cell surface proteins expressed in all of the extracellular stages of *Plasmodium*. Sequence homology searching in the available databases revealed that this protein family share orthologous groups within the *Plasmodium* genus. 6-cys-like proteins are found in many other Apicomplexan species such as the SAG proteins of *Toxoplasma*. So far 14 genes for 6-cys domain containing proteins have been annotated in the *P. falciparum* genome database (Aurrecoechea et al., 2009), and 8 of them are predicted to encode GPI-anchors (Annoura et al., 2014; Arredondo et al., 2012; Gilson et al., 2006; Kappe et al., 2001; Sanders et al., 2005; Templeton and Kaslow, 1999; Thompson et al., 2001b). Their genes usually locate in pairs next to each other on the chromosomes. While those members that have GPI-anchors are attached to the outer leaflet of cell membrane exposed to the extracellular environment, other members that do not have anchors similarly localise to the surface probably by peripherally interacting with other membrane proteins, likely to be the GPI-anchored 6-cys protein partners (Kumar, 1987; Williamson, 2003).

The 6-cys proteins are so called because they possess a unique motif comprising of 6 cysteine residues, which form 3 disulfide bonds in a conserved pattern between C1 & C2, C3 & C6 and C4 & C5 (Arredondo et al., 2012; Carter et al., 1995; Gerloff et al., 2005). Structural studies of this *Plasmodium* unique cysteine-rich motif have found that it resembles the folding of SAGs, an immunodominant surface protein family in *T. gondii* (Arredondo et al., 2012; Gerloff et al., 2005). Each of the members of the 6-cys protein family mostly possesses two 6-cys modules but some can have up to 14 domains (Arredondo et al., 2012).

Many functional studies have revealed an important role of the proteins in this family relating to cell-cell recognition throughout parasite life cycle. Two members of the 6-cys proteins, P230 and P48/45, which are expressed on the surface of both male and female gametocytes have been shown to be essential for the fertilisation process
(Eksi et al., 2006; van Dijk et al., 2001). They were firstly identified as antigens for transmission-blocking monoclonal antibodies, which has raised the possibility of using both proteins as vaccines (Quakyi et al., 1987; Vermeulen et al., 1985). P230, a non GPI-anchored protein, is retained on the surface of gametes via an interaction with the GPI-anchored P48/45 (Kumar, 1987). Genetic deletion of the p48/45 in both P. falciparum and P. berghei resulted in a drastic decrease of zygote formation and eventually the reduction of oocyst production (van Dijk et al., 2001). Interestingly, this defect appeared to be restricted to only male gametes, which failed to penetrate and fertilise the fertile females. Detailed observation has revealed that P230 is no longer retained on the surface of the Δp48/45 male gametes suggestive of their cooperative role (Eksi et al., 2006). In P. falciparum lacking P230 the male gametes could not bind to uninfected erythrocytes and form exflagellation centres leading to poor fertilisation with the female gametes (Eksi et al., 2006). These findings support the notion that non GPI-anchored P230 has to interact with the GPI-anchored P48/45 partner to remain on the male gamete surface for proper function. P230 and P48/45 are also found to be associated with the filamentous tubules formed by the gametes which are believed to play a role in cell-cell communication between the parasites in the mosquito gut (Rupp et al., 2011).

Another member of the 6-cys protein family found in the sexual stage is P47, a paralog of P48/45 that is expressed only in female gametocytes and does not appear to be essential, at least, in P. falciparum (van Schaijk et al., 2006). In rodent malaria parasite P. berghei, however, disruption of pb47 gene revealed its function as a fertility factor for female gametes (van Dijk et al., 2010). The difference in P47 roles between the two species may be partly due to their distinct insect hosts. Interestingly P47 also plays a crucial role in gamete survival in A. gambiae, a major mosquito specie found in Africa, by inhibiting the activation of complement-like system in the mosquito (Molina-Cruz et al., 2013). Although this function appears to be specific to some strains of parasites, it could contribute to the huge problem of malaria transmission in Africa. Since the three members of the 6-cys domain proteins demonstrate such important functions in sexual life stage of the parasite, they have become major candidates in transmission-blocking vaccine development (Sutherland, 2009; Williamson, 2003). Another 6-cys domain protein recently identified in gametocyte and ookinete stages, PSOP12, has also been reported to be a promising transmission-blocking vaccine candidate (Sala et al., 2015).
Analogous to P230 and P48/45 but instead expressed in sporozoites are the GPI-anchored P36p, also known as P52, and the non-anchored P36. Although yet to be formally demonstrated, it is presumed P36 localises to the membrane by interacting with P36p. Both p36 and p36p can be disrupted, individually or simultaneously, in rodent and human Plasmodium species without any phenotype observed in blood-stage, sexual-stage, and salivary gland sporozoites (Ishino et al., 2005; Labaied et al., 2007; van Dijk et al., 2005; van Schaijk et al., 2008; VanBuskirk et al., 2009). However, infectivity of these knockouts in hepatocytes was impaired because although they could invade the hepatocytes normally they failed to develop into exo-erythrocytic forms. The flaw most often seen was that these mutant sporozoites could not form a parasitophorous vacuole membrane (PVM) properly to protect them from being recognised by the hepatocytes (Labaied et al., 2007; van Dijk et al., 2005). The lack of PVM was suggested to trigger apoptosis of the infected hepatocytes leading to activation of host immunity and protection from subsequent sporozoite infection (van Dijk et al., 2005).

This result prompted various groups to prove the usefulness of these ∆p36 and ∆p36p knock out parasites as a genetically attenuated live vaccine (Labaied et al., 2007; Vaughan et al., 2010). A study in BALB/c mice showed promising results as the double deletion of p36 and p36p genes in P. yoelii sporozoites could induce complete protection from wild-type sporozoite challenges via intravenous injection or mosquito bites (Labaied et al., 2007). However this protective effect of ∆p36/∆p36p sporozoites appeared to be dependent on specific parasite strain and host species since another study showed that there was a blood-stage infection breakthrough from ∆p36/∆p36p P. berghei sporozoites in C57BL/6 mice (Annoura et al., 2012). This could be due to the unusual ability of the ∆p36/∆p36p P. berghei sporozoites to develop and mature inside hepatocytes without forming the PVM (Ploemen et al., 2012). A phase 1/2a clinical trial with P. falciparum mutants also produced a similar result whereby breakthrough blood-stage infection in volunteers was detected after immunisation with the ∆p36/∆p36p sporozoites (Spring et al., 2013).

Recently, two new liver-stage 6-cys proteins, liver-specific protein 2 (LISP2; also formerly known as sequestrin) and B9, have recently been identified and reported to play crucial roles in sporozoite development in hepatocytes (Annoura et al., 2014). Whilst sporozoites lacking LISP2 struggled at late liver-stage development (Annoura et al., 2014; Orito et al., 2013), ∆b9 sporozoites arrested soon after hepatocyte
invasion (Annoura et al., 2014). The role of B9 was therefore proposed to be crucial for the formation of PVM similar to P36 and P36p mentioned earlier (Annoura et al., 2014). Consequently, parasites lacking these b9 and lisp2 genes, like the Δp36 and Δp36p mutants, could also show promise as genetically attenuated live vaccines. Indeed van Schaijk et al. (2014) have recently reported the usefulness of deleting b9 in combination with slarp in P. berghei, to produce attenuated Δb9/Δslarp sporozoites that provided complete protection from subsequent challenge with wild-type P. berghei in BALB/c and C57BL/6 mice (Ben C L van Schaijk et al., 2014). Specifically, the mice were completely protected from breakthrough and blood-stage infection with wild-type parasites after vaccination with the mutant Δb9/Δslarp sporozoites (Ben C L van Schaijk et al., 2014).

Despite a relatively advanced state of knowledge regarding the importance of 6-cys proteins in mosquito and liver stages, little is known about members of this protein family expressed in the blood-stage. The first blood-stage 6-cys domain protein discovered was P12 due to its recognition by human malaria immune sera when expressed in a library of recombinant malaria antigens on the surface of mammalian COS-7 cells (Elliott et al., 1990). The other members of blood-stage 6-cys proteins, namely P38, P41, and P92, were identified as bona fide blood stage proteins in a proteomic study of detergent resistant membranes of developing merozoites (Sanders et al., 2005). The merozoite surface location of these 6-cys proteins was experimentally confirmed either by immunofluorescence microscopy with protein-specific anti-sera or by tagging these proteins with green fluorescent protein (Sanders et al., 2005). In addition, P12, P38 and P92 were proved to possess GPI-anchors as predicted (Gilson et al., 2006). The surface localisation of the blood stage 6-cys proteins raises the possibility that they may serve as ligands for cell-cell interaction during erythrocyte invasion, analogous to the roles of the other members of 6-cys proteins in gamete fusion and the invasion of hepatocytes. The proteins were also recognised by malaria immune sera supporting their value as potential vaccine candidates (Sanders et al., 2005). Studying these blood-stage 6-cys domain proteins in P. falciparum could be beneficial in terms of gaining more knowledge of merozoite biology, particularly during erythrocyte invasion, as well as indicating if they could be developed further as vaccine candidates. For these reasons functional analysis of P12, P38, P41, and P92 will be the main aim of this thesis.
Signaling during erythrocyte invasion

Another interesting research area of merozoite invasion of erythrocytes is how the parasite regulates such a complex process to achieve a successful invasion in a restricted timeframe. This control should function as a universal mechanism for all of the *Plasmodium* parasites since the kinetics of invasion in different species appears to be very similar as mentioned earlier (Dvorak et al., 1975; Gilson and Crabb, 2009). In eukaryotes calcium ions (Ca$^{2+}$) act as secondary messengers that help regulate normal cellular activities. In Apicomplexan parasites, Ca$^{2+}$ fluxes are involved in regulating cell differentiation, cell motility, egress, secretion, and invasion. In a normal resting eukaryotic cell the concentration of cytosolic Ca$^{2+}$ is usually within the low nanomolar range while extracellular Ca$^{2+}$ level is generally higher namely, in the micromolar to millimolar range (reviewed in (Moreno et al., 2011)). This typical characteristic has been proved to be true in *Plasmodium* parasites despite that fact that Ca$^{2+}$ level in the erythrocyte cytosol is very low. Parasites manage to manipulate external Ca$^{2+}$ levels by taking up the ion from an extracellular source and concentrating it at micromolar levels within the PV space (Gazarini et al., 2003). This allows the parasites to maintain the Ca$^{2+}$ gradient between outside and inside of the cells necessary for signaling functionality. Indeed it was shown that lowering the Ca$^{2+}$ level in the PV space impaired parasite growth and interfered with the internal Ca$^{2+}$ stores (Gazarini et al., 2003). There are 3 major Ca$^{2+}$ stores in the parasites which are the endoplasmic reticulum (ER), mitochondrion, and acidocalcisomes, the electron-dense acidic organelles found in both prokaryotes and eukaryotes (Docampo et al., 2005; Nagamune et al., 2008). The release of Ca$^{2+}$ from these stores can be stimulated by many molecules, e.g. nucleotides, melatonin, xanthurenic acid, and potassium ions (K$^+$), which trigger different downstream cellular activities depending on the stimuli (Garcia, 2012). Moreover, the activation of cellular functions through Ca$^{2+}$ release was found to interplay with another important signaling pathway having cyclic adenosine monophosphate (cAMP) as a second messenger (Beraldo et al., 2005).

One aspect of the *Plasmodium* biology that has gained a lot of understanding recently is how the mature merozoites egress from their erythrocytes. The process involves multiple steps, most of which are controlled by Ca$^{2+}$ fluxes. It was clearly demonstrated through microscopic observation using cell-permeable calcium fluorescence sensor Fluo-4 AM that there was an increasing accumulation of Ca$^{2+}$ in the maturing schizonts (Agarwal et al., 2013; Garg et al., 2013; Glushakova et al.,
This rise was shown to be sensitive to a phospholipase C (PLC) inhibitor, U73122. PLC produces inositol 1,4,5-triphosphate (IP3) to trigger the release of Ca\(^{2+}\) from ER and treatment with U73122 inhibited merozoite egress providing a link between Ca\(^{2+}\) release from the ER and schizont rupture (Agarwal et al., 2013). Supportive of this finding is that BAPTA AM, a chelating agent of intracellular Ca\(^{2+}\), prevented merozoite egress also (Agarwal et al., 2013).

How calcium might stimulate egress was shown when increases in intracellular Ca\(^{2+}\) levels initiated the release of *P. falciparum* subtilisin-like protease 1 (PfSUB1) from apical organelles called exonemes to the PV (Agarwal et al., 2013; Yeoh et al., 2007). PfSUB1 is required for maturation of serine-repeat antigen (SERA) protease family, particularly SERA5 and SERA 6 which have been shown to be essential for parasite egress (Blackman, 2008; McCoubrie et al., 2007). The mechanism of action of these SERA proteins is still not clearly understood however. Another protein that has recently found to participate in the merozoite egress is the *P. falciparum* perforin-like protein 1 (PfPLP1), which localises to micronemes and is discharged upon intracellular Ca\(^{2+}\) activation (Garg et al., 2013). PfPLP1 is thought to form pores in both PVM and the erythrocyte membrane to either destabilise the membrane or create gateways for the release of other parasite molecules required for egress. PfPLP1’s membrane binding and membrane lytic activities were demonstrated to be calcium-dependent (Garg et al., 2013).

Calcium ions are also responsible for the function of the other two molecules very much involved in merozoite egress which are *P. falciparum* calcium-dependent protein kinase 5 (PfCDPK5) and erythrocyte calpain-1 (Chandramohanadas et al., 2009; Dvorin et al., 2010b). CDPKs are generally inactive at resting levels of intracellular Ca\(^{2+}\) due to the occupation of the kinase’s active site with an auto inhibitory domain. Upregulation of intracellular Ca\(^{2+}\) causes the auto inhibitory domain to release the active site thereby switching the CDPK on. PfCDPK5 is believed to be involved in schizont egress since knocking its expression levels down prevents the egress of mature viable merozoites (Dvorin et al., 2010b). The kinase is believed to act separately and downstream of the protease cascade initiated by PfSUB1 described earlier (Dvorin et al., 2010b). Another protein involved in egress is calpain-1. Interestingly, calpain-1 is not parasite derived but is an erythrocyte protein residing in the cytosol. Similar to CDPKs, calpain-1 only functions when it binds to calcium ions. This coincides with the release of Ca\(^{2+}\) accumulated in the PV, possibly
by the action of PfPLP1 and/or mechano-sensitive cation channel, into the erythrocyte cytosol (Gazarini et al., 2003; Glushakova et al., 2013; Millholland et al., 2013). The activated calpain-1 then becomes membrane-associated and disassembles the erythrocyte cytoskeletal proteins especially ankyrin-1 and α/β - spectrins leading to merozoite egress (Chandramohanadas et al., 2009; Millholland et al., 2011).

Adducin-1, another erythrocyte cytoskeletal protein connecting actin and spectrin together, was also removed from the erythrocyte cytoskeletal network beginning from late trophozoite stage by the protein kinase C (PKC) phosphorylation pathway (Millholland et al., 2011; 2013). Taken together the disintegration of multiple erythrocyte structural proteins triggered mainly by calcium signaling cascades weakens erythrocyte membrane integrity and eventually allows the parasites to exit from the erythrocyte. Assisting merozoite egress is the apparent build-up of internal osmotic pressure followed by curling and buckling of the ruptured erythrocyte membrane (Abkarian et al., 2011; Glushakova et al., 2005).

Following on from egress there is much evidence suggesting that calcium signaling also plays a role in the invasion of new erythrocytes. It was initially established for the host cell invasion of *T. gondii* that changes in cytosolic Ca$^{2+}$ levels governed gliding motility and discharge of microneme proteins, both of which were essential for successful invasion (Carruthers and Sibley, 1999; Lovett and Sibley, 2003). The increased intracellular Ca$^{2+}$ was then restored to normal level while the tachyzoite was entering the host cell (Lovett and Sibley, 2003). This rise of cytosolic Ca$^{2+}$ and its consequences were later demonstrated to be similar in erythrocyte invasion by *Plasmodium* merozoites (Singh et al., 2010). The release of Ca$^{2+}$ from internal stores in both parasites appeared to be triggered by the same cue which was the drop of extracellular K$^+$ concentration which happens following egress (Moudy et al., 2001; Singh et al., 2010). In addition, it was also discovered in merozoites that PfRh1 was also involved in triggering the increase of cytosolic Ca$^{2+}$ because when PfRh1 was inhibited with specific antibodies the release of Ca$^{2+}$ was reduced and downstream events were obstructed (Gao et al., 2013). Whether K$^+$ levels and PfRh1 ligand binding initiate the Ca$^{2+}$ signaling in concert or independently remain to be tested.

One consequence of increased cytosolic Ca$^{2+}$ levels is the release of microneme proteins, e.g. EBA175 and AMA1, on to the merozoite surface (Singh et al., 2010). This downstream effect was suggested to be mediated through the
activation of calcium-dependent protein kinase 1 (CDPK1) (Singh et al., 2012). Another study also suggested that a calcium-dependent phosphatase, calcineurin, is a downstream effector involved in controlling the microneme protein release upon calcium activation (Singh et al., 2013). This was achieved by regulating actin polymerisation dynamics at the apical end of the parasites. Calcineurin was activated when the merozoites were exposed to a low K\(^+\) environment concurrent with the increase of intracellular Ca\(^{2+}\) in free merozoites (Singh et al., 2010). Following merozoite contact with its target erythrocyte, microneme adhesins engage with their respective receptors on the erythrocyte surface. This event appears to trigger the restoration of cytosolic Ca\(^{2+}\) back to resting levels which in turn stimulates the release of rhoptry proteins such as CLAG3.1 and PfRh2b allowing invasion to proceed (Singh et al., 2010). Despite some progress much remains to be unraveled about the mechanisms that convey the cue changes, both the drop of extracellular K\(^+\) and the engagement of microneme proteins with their counterparts, to control the intracellular Ca\(^{2+}\) levels. The activation of PfCDPK1 also leads to phosphorylation of a few members of actin-myosin motor complex, namely the myosin A tail domain-interacting protein (MTIP) and glideosome-associated protein 45 (GAP45) (Green et al., 2008). Whether the importance of this phosphorylation is to regulate the formation of the invasion motor complex or actually control the movement of the motor remains unsolved (Holder et al., 2012).

The participation of Ca\(^{2+}\) in erythrocyte invasion has also been hypothesised to extend to the erythrocyte. Lew and Tiffert have proposed that the first wave of erythrocyte deformation triggered after merozoite contact in the pre-invasion period could be caused by a localised influx of Ca\(^{2+}\) into the cell which triggers modification of the erythrocyte cytoskeleton (Lew and Tiffert, 2007). The mechanism proposed postulates that weak initial contact of the merozoite with the erythrocyte induces a temporary influx of Ca\(^{2+}\) causing phosphorylation-dependent rearrangement of erythrocyte cytoskeletons and membrane wrapping around the merozoite. This mechanism repeats when a new contact point is formed until strong irreversible attachment at the apical end is established, marking the end of merozoite reorientation and the start of penetration. The Ca\(^{2+}\) that entered the erythrocyte at contact points is then pumped back out by plasma membrane calcium pumps to restrict the activation to within the area of contact. This hypothesis stemmed from a number of studies led by Wasserman and colleagues which demonstrated the change in phosphorylation
patterns of the cytoskeletal proteins in infected erythrocytes from the influx of Ca$^{2+}$ (Wasserman and Chaparro, 1996; Wasserman et al., 1982; 1990).

Calcium ions are also believed to have a role in the second wave of erythrocyte deformation, which is observed about 30 seconds after the internalisation. The drastic change in morphology of the infected erythrocyte at this stage resembles the appearance of dehydrated erythrocytes and is termed echinocytosis. It is usually found in many types of hemoglobinopathies especially sickle erythrocytes, as well as cold-stored erythrocytes in citrate-dextrose preservation (Tiffert, 2005). In both instances there is an increase in intracellular Ca$^{2+}$ level which subsequently activates the Ca$^{2+}$-sensitive K$^+$ channels called Gardos channels. This results in the loss of K$^+$ followed by chloride ions and water from the cells (Freeman et al., 1987; Tiffert et al., 1988). These dehydrated erythrocytes have been shown to be moderately protective from *P. falciparum* infection hence a number of abnormal traits causing echinocytic erythrocytes are selectively retained in human population living in malaria endemic regions (Tiffert, 2005; Williams, 2006).

It is evident that Ca$^{2+}$ is a major player in many steps of merozoite egress and invasion of erythrocytes. However, most of these reports were based on indirect observations whereby calcium ion levels were artificially modified and the subsequent effects examined. It will therefore be interesting to observe Ca$^{2+}$ fluxes directly during the invasion process in order to confirm the hypotheses mentioned above and provide new insights.

**Aims of research**

With the emergence of resistant malaria parasites to the ACTs, our last line of chemotherapeutic defense, and the unavailability of an effective vaccine, the need for new tools to combat against this deadly disease is extremely urgent. This thesis aims to gain more insights into the invasion of erythrocytes by *P. falciparum* merozoites that we then might be able to translate into novel disease interventions. Since we have the tools to microscopically observe the invasion in real-time as well as expertise in molecular analyses, we have attempted to examine morphology and kinetics of invasion in more details and combine these physical characteristics with the molecular actions studied. Two main aims for my thesis are as follows.
Functional analysis of 6-cys domain proteins in blood-stage malaria parasites

The work from our group showed that the four members of 6-cys domain proteins, P12, P38, P41, and P92, were relatively abundant on the raft-like plasma membrane of the merozoites (Sanders et al., 2005). Their expression and localisation were already demonstrated but the function(s) of these proteins had not been addressed yet. As shown for other stages, 6-cys domain proteins play an important role in cell-cell recognition and are required for efficient infection and fertilisation (Eksi et al., 2006; van Dijk et al., 2010; van Schaijk et al., 2008). I therefore hypothesised that 6-cys proteins expressed in blood-stage parasites could also function similarly and be involved in host-parasite recognition during the pre-invasion phase on invasion. To resolve this recombinant 6-cys proteins were expressed and used to generate antibodies for molecular and functional analyses. I examined binding efficacy of the proteins to erythrocytes and heparin in order to discover their functions. I also explored the interaction between the 6-cys proteins themselves; particularly looking for the binding partner of non-GPI-anchored P41, using both recombinant and parasite-derived proteins. Genetic deletion and functional inhibition by specific antibodies were performed to verify their essentiality for parasite survival.

Live-cell microscopic analysis of Ca\(^{2+}\) signaling and its role in erythrocyte invasion

With the 6-cys proteins possibly being involved in invasion-related signaling events I decided to directly observe signaling events namely calcium fluxes, in live merozoites. Our group had developed the necessary expertise to observe erythrocyte invasion in real time by live *P. falciparum* merozoites under transmission illumination (brightfield, phase contrast, DIC). The morphology and kinetics of the whole process has been previously described in detail (Gilson and Crabb, 2009). Here I improved the technique to give us higher temporal resolution of the invasion process. I also combined fluorescence with brightfield methods to observe real time Ca\(^{2+}\) fluxes in merozoite egress and erythrocyte invasion. The change of calcium ions during the course of invasion was visualised by using the membrane-permeable fluorescent calcium sensor Fluo-4 AM. I then manipulated intra- and extraerythrocytic calcium levels to study the role of the ion by observing its fluxes in real-time. Inhibitors known to interfere with the invasion, i.e. R1 peptide and cytochalasin D, which obstruct the merozoite at different stages were added along with Fluo-4 AM in an attempt to overlay the Ca\(^{2+}\) signaling with the invasion machineries. This part of the
thesis provides substantially improves our understanding of the complex process of erythrocyte invasion by the merozoite.
**Figure 1.1. The *Plasmodium* life cycle.**

An infected female *Anopheles* mosquito injects *Plasmodium* salivary gland sporozoites into its animal host during a blood meal. The malaria sporozoites are passively carried in the blood circulation until they reach the liver. Once in the liver the parasites invade liver cells and develop into the pre-erythrocytic stage producing thousands of merozoites. The merozoites are released into the bloodstream and start the asexual cycle by invading host erythrocytes. The parasites grow from a ring-shaped stage to trophozoite and then into the replicative schizont stage. The mature schizonts release more merozoites that invade new host erythrocytes continuing the cycle. Each cycle, some blood stage parasites differentiate into male and female gametocytes waiting to be taken up by mosquitoes in their next blood meal. The sexual stage begins in the mosquito gut where the male and female gametocytes differentiate into gametes that fertilise and form zygotes. These subsequently develop into motile ookinetes which penetrate the mosquito midgut and form oocysts between the midgut epithelium and basal lamina. Multiple rounds of nuclear divisions within the oocysts produce thousands of sporozoites that are then released into the mosquito haemolymph. The sporozoites proceed to the salivary gland and remain in there until being introduced into a new animal host to continue the life cycle. (Modified from (Ménard, 2005)).
Figure 1.2. The 6-cys protein family in *Plasmodium falciparum*.

The diagram illustrates the members of the 6-cys domain proteins identified in the *P. falciparum* genome. The colored circles represent the timing of protein expression; blood stage (red), gametocyte (green), and sporozoite (blue). The characteristics of the protein structure are depicted in various color-coding; ER signal sequence (white box), 6-cys domain (red box), cysteine residue (red line), spacer region (yellow), and GPI-anchoring signal sequence (black). The gene identifiers are from the PlasmoDB database (Aurrecoechea et al., 2009).
Figure 1.3. A morphology and kinetics model of erythrocyte invasion by *Plasmodium falciparum* merozoites.

A free merozoite initiates a primary contact, which is a weak and reversible interaction, with a new erythrocyte. The parasite causes erythrocyte membrane deformation while it reorientates to establish a strong irreversible interaction with the erythrocyte at its apex. This 'pre-invasion' period takes about 11 s. The next phase is the internalisation of the merozoite into the host erythrocyte and this process finishes within approximately 17 s. The third period occurs about 36 s after the invasion is completed when the infected erythrocyte morphology appears dehydrated, often called echinocytosis. The infected cell remains echinocytic for the next 20 s or so before it starts to recover back to normal biconcave shape which takes between 5 - 11 min. The diagram is modified from Gilson and Crabb (Gilson and Crabb, 2009).
Figure 1.4. Ligand-receptor interactions taking part in erythrocyte invasion by *Plasmodium falciparum* merozoite.

The illustration (not to scale) depicts parasite ligands (right) shown to function in erythrocyte invasion with their corresponding erythrocyte receptors if known (left). The parasite proteins' names are indicated together with their gene identifications (brackets) given in the PlasmoDB database. White boxes represent the N-terminal signal sequences for exported proteins. GPI anchors and transmembrane domains are rendered in black and grey boxes respectively. Protein regions believed to be involved in the interactions with the binding partners are illustrated in red. The parasite proteins are coloured to indicate whether they originate from the merozoite plasma membrane (yellow), micronemes (green), or rhoptries (blue). Glycosylated erythrocyte receptors are indicated with the decorated green dots. Merozoite surface protein (MSP); Erythrocyte binding antigen (EBA); Erythrocyte binding ligand 1 (EBL1); Apical membrane antigen 1 (AMA1); Merozoite thrombospondin-related adhesive protein (MTRAP); Reticulocyte binding protein homolog (Rh); Rh5-interacting protein (Ripr); Cysteine-rich protective antigen (CyRPA); Rhoptry neck protein (RON); Glycophorin (GYP); Semaphorin-7A (SEMA7A); Complement receptor 1 (CR1).
CHAPTER 2

Materials and Methods

Parasite media and solutions

*RPMI-HEPES medium*

52 g RPMI 1640 powder (Gibco), 0.25 g hypoxanthine (200 µM) (Sigma Aldrich), 29.7 g HEPES (25 mM) (Life Technologies), and 3.125 mL gentamicin (20 µg/mL) (Gibco) were dissolved in Milli-Q water up to 5 L. pH was adjusted to 6.72, then filter-sterilised with 0.22 µm filter (Millipore) in laminar flow hood and stored at 4°C.

*5 % Albumax II stock solution*

Albumax II powder (Gibco) was dissolved in RPMI-HEPES medium to 5 % and was filter-sterilised with 0.22 µm filter (Millipore) and stored at 4°C.

*3.6 % Na$_2$CO$_3$ stock solution*

18 g of Na$_2$CO$_3$ (AnalR) was dissolved in 500 mL reverse osmosis purified water, filter-sterilised with 0.22 µm filter (Millipore) in laminar flow hood, and stored at 4°C.

*Complete RPMI-HEPES medium*

Each litre of RPMI-HEPES medium was supplemented with 58 mL of 3.6 % Na$_2$CO$_3$ w/v and 100 mL of 5 % Albumax II w/v stock solutions.

*Complete Dulbecco's Modified Eagle Medium (DMEM)*

DMEM liquid (Gibco) was supplemented with 25 mM HEPES, 200 µM hypoxanthine, 20 µg/mL gentamicin, and Na$_2$CO$_3$ and Albumax II stock solutions as indicated above. It was 0.22 µm filter-sterilised (Millipore) and stored at 4°C.

*Complete Ca$^{2+}$-free Dulbecco's Modified Eagle Medium*

Ca$^{2+}$-free DMEM liquid (Gibco) was supplemented and prepared as per the complete DMEM media.
Cytomix
120 mM KCl (Univar), 0.15 mM CaCl₂ (AnalR), 2 mM EGTA (Sigma Aldrich), 5 mM MgCl₂ (Sigma Aldrich), 10 mM K₂HPO₄/KH₂PO₄ (LabServ) pH 7.6 and 25 mM HEPES (Gibco) were prepared in reverse osmosis purified water. pH was adjusted to 7.6 and filter-sterilised with 0.22 µm filter (Millipore) and stored at 4°C.

Malaria freezing solution
37.8 g sorbitol (Sigma Aldrich) and 8.1 g NaCl (Sigma Aldrich) were dissolved in reverse osmosis purified water up to 900 mL. 350 mL glycerol (LabServ) was added and the solution was filter-sterilised using 0.22 µm filter (Millipore) and stored at 4°C.

Malaria thawing solution
3.5 g NaCl (Sigma Aldrich) was dissolved in 100 mL reverse osmosis purified water, sterilised by autoclaving, and stored at 4°C.

0.15 % Saponin
0.75 g saponin (Kodak) was dissolved in 500 mL RPMI-HEPES, filter-sterilised using 0.22 µm filter (Millipore) and stored at 4°C.

5 % Sorbitol
25 g sorbitol (Sigma Aldrich) was dissolved in 500 mL reverse osmosis purified water, filter-sterilised with 0.22 µm filter (Millipore) and stored at 4°C.

Heparin
Heparin sodium salt powder (Sigma, H-3149) was dissolved in RPMI-HEPES to 100 mg/mL as a stock solution, filter-sterilised with 0.22 µm filter (Millipore) and stored at 4°C.

WR99210
WR99210 (Jacobus Pharmaceutical Company) powder was dissolved in DMSO (Sigma Aldrich) to make a 20 mM stock solution. This was diluted to 20 µM in RPMI-HEPES, filter-sterilised with 0.22 µm filter (Millipore) and stored at 4°C. It
was used at 2-4 nM final concentration in parasite culture, i.e. 1-2 µL of the 20 µM working solution was added per 10 mL culture dish.

*5-fluorocytosine*

5-fluorocytosine (Ancotil) was diluted 1:100 in sterilised PBS to make a 100 µg/mL working solution in laminar flow hood. 5 µL of the working solution were added to the 10 mL culture dish.

*Parasite lysis buffer*

10 mM Tris-HCl pH 8.0, 0.4 M NaCl, 1 mM EDTA, and 1 % SDS were prepared in reverse osmosis purified water prior to use.

*Bacterial media*

*Luria broth*

15.5 g Millers modified Luria broth powder (Sigma Aldrich) was dissolved in 1 L of reverse osmosis purified water and was sterilised by autoclaving.

*2× YT broth*

16 g BactoTryptone (Difco), 10 g Yeast Extract (Fermtech), and 5 g NaCl (Sigma Aldrich) were dissolved in 1 L reverse osmosis purified water and was sterilised by autoclaving.

*Super broth*

32 g BactoTryptone (Difco), 20 g Yeast Extract (Fermtech), and 5 g NaCl (Sigma Aldrich) were dissolved in 1 L reverse osmosis purified water. 1 mL of 5 M NaOH (Sigma Aldrich) was later added and the broth was sterilised by autoclaving.

*Ampicillin*

Ampicillin sodium salt (Sigma Aldrich) was dissolved in Milli-Q water at 50 mg/mL. The solution was sterilised with 0.22 µm filter (Millipore) in laminar flow hood and stored at -20°C. It was used at 100 µg/mL final concentration.
**LB Agar**

5.3 g microbiology grade agar (Fermtech) was added to 400 mL Luria broth and the agar was dissolved by autoclaving.

**Molecular biology reagents**

*Antibodies*

- Rabbit anti-P12: see (Taechalertpaisarn et al., 2012)
- Rabbit anti-P41: see (Taechalertpaisarn et al., 2012)
- Rabbit anti-P38: a gift from Dr Anthony Hodder
- Rabbit anti-P92: made by the Monoclonal Production Facility, Walter & Eliza Hall Institute from antigen prepared by myself.
- Mouse anti-MSP1: 4H9/19 (Cooper et al., 1992)
- Anti-MSP1 mouse monoclonal: 17b6 made by the Monoclonal Production Facility, Walter & Eliza Hall Institute.
- Anti-AMA1 mouse monoclonal: 1F9 (Coley et al., 2006)
- Rabbit Anti-EBA175: see (Reed et al., 2000)
- Rabbit Anti-GAPDH: a gift from Professor Leann Tilley
- Pooled malaria non-immune Melbourne sera
- Pooled malaria-immune PNG IgG: see (O'Donnell et al., 2001)

**1 % Casein**

Casein sodium salt (Sigma) was added to PBS at 1 % and the solution was stirred on heat block until the casein was completely dissolved. It was stored at 4°C. 0.05 % sodium azide was optionally added to the solution for long-term storage.

**Bovine serum albumin (BSA)**

BSA was dissolved in PBS, aliquoted, and stored at -20°C.

**10× phosphate-buffered saline (PBS)**

370 g NaCl (Sigma Aldrich) was dissolved in 2 L reverse osmosis purified water. 142.5 g Na₂HPO₄·2H₂O (AnalR) and 27.58 g NaH₂PO₄·H₂O (AnalR) was added to 2 L heated reverse osmosis purified water and stirred until dissolved. The two solutions were combined and made up to 5 L with reverse osmosis purified water. 1×
PBS working solution was prepared by 1:10 dilution in reverse osmosis purified water.

**6× SDS-PAGE sample buffer**
0.3 M Tris-HCl pH 6.8 (AnalaR), 60 % glycerol (Labserv), 12 mM ethylenediaminetetraacetic acid (EDTA) (Research Organics), 12 % sodium dodecyl sulfate (SDS) (Amresco), and 0.05 % bromophenol blue (BioRad) were mixed together in reverse osmosis purified water and heated to dissolve to make non-reducing sample buffer (NRSB). It was stored at room temperature. To prepare reducing sample buffer (RSB), 100 mM dithiothreitol (DTT) (Astral) was added.

**10× Tris-glycine transfer buffer**
40.4 g Tris (Sigma Aldrich) and 133 g glycine (BioRad) were dissolved in 1 L reverse osmosis purified water.

**50× TAE buffer**
242 g Tris (Sigma Aldrich), 100 mL of 0.5 M EDTA pH 8.0 (Research Organics), and 57.1 mL glacial acetic acid (Labserv) were combined to make 1 L solution with reverse osmosis purified water.

**0.7 % TAE-agarose**
3.5 g agarose (Bioline) was added to 500 mL 1×TAE buffer and heated in microwave until agarose was completely dissolved.

**TE buffer**
10 mM Tris-HCl pH 8.0 and 1 mM EDTA were prepared in reverse osmosis purified water, autoclaved, and stored at room temperature.

**Protein gel destain buffer**
100 mL acetic acid (LabServ), 400 mL ethanol (Thermo Fisher Scientific), and 500 mL reverse osmosis purified water were mixed together and stored at room temperature.
1 % Triton X-100
Triton X-100 (Sigma Aldrich) was made up in PBS to 1 % v/v and stored at room temperature.

RIPA buffer
1 % Triton X-100 (Sigma Aldrich), 1 % sodium deoxycholate (Sigma Aldrich), 0.1 % SDS (Amresco), 150 mM NaCl (Sigma Aldrich), and 50 mM Tris-HCl pH 7.6 (Sigma Aldrich) were dissolved in PBS and stored at 4°C.

2D-sample buffer
7 M Urea (Sigma Aldrich), 2 M thiourea (Sigma Aldrich), 2 % ASB-14 (Calbiochem), and 0.05 % Bromphenol blue (BioRad) were prepared in reverse osmosis purified water, aliquoted, and stored at -20°C.

Quenching solution (for chemical crosslinking)
120 mM NaCl and 25 mM Tris-HCl pH 7.5 were prepared in reverse osmosis purified water and stored at room temperature.

Semi-dry protein transfer buffer
2× Tris-glycine buffer (1:5 dilution from 10× buffer), 10 % methanol (Thermo Fisher Scientific), and 0.05 % SDS (Amresco) were made up to 1 L with reverse osmosis purified water. For high molecular weight proteins methanol was decreased to 5 % and SDS was increased to 0.5 % for better transfer.

Coomassie Brilliant Blue R250 staining solution
For 1 L staining solution 3 g Coomassie Brilliant Blue R250 powder was dissolved in 450 mL methanol. 100 mL of glacial acetic acid was added to 450 mL reverse osmosis purified water and the solution was then combined with the dye mixture. The dye was filtered prior to use.

Recombinant protein expression reagents
Resuspension buffer (RB)
15 mM sodium phosphate was mixed with 450 mM NaCl and the pH was adjusted to 7.4 in reverse osmosis purified water.
**Solubilisation buffer (SB)**

7 M Urea was dissolved in RB.

**Washing buffer**

20 mM imidazole was dissolved in SB.

**Elution buffer**

500 mM imidazole was dissolved in SB.

**Refolding buffer**

20 mM sodium phosphate, 1 mM reduced glutathione (GSH), and 0.05 mM oxidized glutathione (GSSG) were prepared in reverse osmosis purified water and the pH was adjusted to 8.0.

**Zeiss AxioObserver Z1 filter sets**

<table>
<thead>
<tr>
<th>Filter Set</th>
<th>Excitation BP</th>
<th>Emission BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>365 nm</td>
<td>445/50 nm</td>
</tr>
<tr>
<td>HE eGFP</td>
<td>470/40 nm</td>
<td>525/50 nm</td>
</tr>
<tr>
<td>HQ TexasRed</td>
<td>560/40 nm</td>
<td>630/75 nm</td>
</tr>
</tbody>
</table>
Experimental procedures

Parasite culture techniques

P. falciparum continuous culture
Parasites were cultivated according to a modified version of Trager and Jensen (Trager and Jensen, 1976). In short, parasites were grown in complete media with 4 % human O+ erythrocytes from Red Cross blood bank donations. Cultures were put in a box gassed with 1 % O$_2$ and 5 % CO$_2$ gas mixture in N$_2$, and incubated at 37°C. Parasite cultures were sub divided regularly when the parasitemia reached 5 % as monitored by Giemsa staining.

Parasite synchronisation with sorbitol treatment
Infected erythrocytes containing ring stage parasites (0 - 24 h) were pelleted by centrifugation at 1000 \( \times g \) and the culture supernatant was removed. The pelleted cells were resuspended with 5 \( \times \) pellet volume of sterile 5 % sorbitol in reverse osmosis purified water and the mixture was incubated for 15 min at 37°C. The cells were pelleted by centrifugation and were washed once in incomplete RPMI medium (no Albumax II) then returned to normal culture conditions.

Parasite synchronisation with heparin
Heparin was added to 5 % parasitemia cultures at 100 \( \mu \)g/mL final concentration (1:1000 dilution of stock solution) to prevent invasion until the majority of the parasites were at late schizont stage. The cells were then washed once with incomplete medium to remove heparin and were put back into normal culture conditions with heparin-free complete medium. Parasites were allowed to invade for a certain window, typically 4 h, and heparin was then added back to cultures to prevent further invasions.

Parasite purification using magnet separation
A column containing ferromagnetic fibers (CS or D column (Miltenyi Biotec)) was firstly placed in a large magnet, either VarioMacs or SuperMACS II (Miltenyi Biotec). The column was washed with 2 column volumes of incomplete culture medium to remove air bubbles prior to a slow flow of parasite culture through the
column. Parasites at trophozoite and schizont stages, approximately 24 - 48 h post invasion, containing hemozoin crystals were retained in the column while the ring stage parasites and uninfected erythrocytes flowed through. The column was washed inside the magnet with 2 column volumes of culture medium to completely remove ring stage parasites and uninfected erythrocytes. The column was then removed from the magnet and the trapped parasites were eluted with 2 column volumes of culture medium.

*Parasite transfection*

Parasites were cultivated in fresh erythrocytes and synchronised 2 days before transfection to obtain ~5 % parasitemia of ring stage parasites on the day of transfection. 200 µL of packed cells were required per transfection. 100 µg plasmid DNA was resuspended in 30 µl sterile TE buffer and was added to 370 µl sterile cytomix. The 400 µl mixture was then added to the 200 µL cell pellet and it was thoroughly mixed by pipetting. The plasmid/infected erythrocyte mixture was transferred to a 0.2 cm gap sterile cuvette (BioRad) and electroporation was performed at 310 V, 950 µF, and ∞ ohms. The electroporated material was immediately resuspended in 10 mL complete media with 2 % fresh erythrocytes and the transfected culture was cultivated per normal procedure. Positive drug selection began 48 h later by adding 1 – 2 µL WR99210 working solution to the 10 mL transfected culture. The transfected parasites should be visible in Giemsa-stained blood smears 3 - 4 weeks after transfection.

*Positive/negative drug selection of transfected parasites with homologous recombination*

The transfected parasites were cultivated without WR99210 selection for 3 weeks and the drug was reapplied to the parasite culture until the transfected parasites were detectable by Giemsa-stained blood smears again. Once the selected parasites were firmly established, 5 µL 5-FC working solution, the negative selection drug, was added to the 10 mL parasite culture to eliminate the transfected parasites harbouring episomal plasmids. This process was repeated for 1 – 2 more cycles until the transfected parasites having the transgenic DNA integrated into their genome by double recombination to remove the negative selection cytosine deaminase marker were confirmed by PCR or Southern blotting.
Parasite cloning by limiting dilution

Synchronised parasites were cultivated to around 1 % parasitemia at trophozoite stage. An accurate parasitemia was counted from thin Giemsa-stained blood smears by light microscopy. 1 μL of the cell pellet was firstly added to 10 mL complete medium. Further dilution was done by adding 60 μL of the first mixture, after thoroughly mixed, to 10 mL complete medium. ‘100 % parasitemia’ μL of the second dilution was added to 10 mL of 2 % hematocrit in complete medium. 100 μL of this mixture was then added to each well of a 96-well U-bottom plate (Nunc). The culture medium was changed every 3 days without adding drug selection during the first 10 days. Parasite positive wells could be observed due to a slight yellowing of the medium after being left for a few days without changing the medium, and it was later confirmed by observing Giemsa-stained blood smears under light microscope. These parasites were expanded for further storage and analyses. If the dilution of the parasites was accurate a maximum of roughly 1/3 of the 96-well plate should become positive.

Saponin lysis

Parasites were grown to the required stage at roughly 5 % parasitemia, and harvested by centrifugation at 1,000 × g. Culture medium was removed and the cell pellet was resuspended in 1.5× pellet volume 0.15 % saponin supplemented with complete protease inhibitor cocktail tablet (Roche). The mixture was left on ice for 10 min to allow the lysis of erythrocyte membrane and parasitophorous vacuole membrane. Parasites were pelleted by centrifugation at 2,500 × g at 4°C and were washed twice in ice-cold PBS supplemented with complete protease inhibitor cocktail tablet (Roche). Parasite samples were kept at -80°C until required.

Parasite growth rates

Synchronous parasite cultures were adjusted to 1 % parasitemia and were subsequently diluted 100,000 fold in duplicate. They were continuously cultured for about 6 cycles, until one of the parasite lines grew back up to approximately 1 % parasitemia. The parasitemias of all lines were derived by counting total of 2,000 cells by light microscopy of Giemsa-stained blood smears. The amplification rates of the parasites per cycle were then calculated and subjected to One-way ANOVA analysis using Prism software (GraphPad).
Parasite invasion inhibition assay

The invasion inhibition assays were performed as previously described in (Boyle et al., 2010b). Briefly, purified merozoites were made by passing late schizonts through a 1.2 µm filter that allowed the merozoites to pass through and retained the unbroken schizonts. The merozoites were mixed with uninfected erythrocytes at appropriate parasitemia and hematocrit and were rapidly were added to a 96-well U-bottom plate (Nunc) containing purified rabbit IgGs made to *E. coli* recombinant proteins at 2 mg/mL final concentration. Non-immune rabbit IgG was used as a non-inhibitory control. All samples were run in duplicate. The 96-well plates were placed in humidified gassed chamber at 37°C for an hour to allow invasion to occur. The cells were then washed twice with culture medium to remove the antibodies. 40 h later the cells were prepared for flow cytometry to count the parasitemia by resuspension in 100 µL of PBS with 10 µg/mL ethidium bromide (EtBr, Bio-Rad). They were stained for one hour prior to centrifugation, removal of the supernatant and resuspension in 200 µL of PBS. Parasitemia was measured on a Becton Dickinson FACSCalibur flow cytometer using a 488 nm laser for excitation of EtBr stained (FL-2) parasites. Samples were analysed using FlowJo software (Tree Star Inc.) by first gating for intact erythrocytes with side scatter and forward scatter parameters, and subsequently determining the positive cells in FL2 channel. Invasion inhibition by the antibodies was calculated as percentages of pre-immune rabbit IgG.

Invasion inhibition assays using rabbit polyclonal anti-P12 and anti-P41 IgGs generated against the HEK293E expressed proteins were performed as described in (Theron et al., 2010).

Molecular biology techniques

Amplification of DNA

DNA was amplified by polymerase chain reaction (PCR) from appropriate templates for each experiment. Each reaction contained 200 ng template, 100 µM dNTPs, 100 nM each forward and reverse primers, 2.5 mM MgSO₄, and 1 unit of *Taq* DNA polymerase or Platinum® *Taq* DNA polymerase High Fidelity (Invitrogen) where appropriate. PCR products were fractionated by gel electrophoresis in 0.7 % TAE-agarose prior to cleaning up using QIAquick PCR purification kit (QIAGEN) per manufacturer’s protocol.
Plasmid construction

5′ and 3′ sequences of the 6-cys genes were amplified from 3D7 parasite genomic DNA and cloned into pCC1 plasmid (Maier et al., 2008). The 5′ flanks were cloned into the plasmid via SacII/Spel restriction sites while the 3′ flanks were cloned into the plasmid via EcoRI/AvrII sites. The ligation was performed using Rapid ligation kit (ThermoScientific) per manufacturer’s protocol. An equal volume of chloroform was subsequently added to the ligation reaction to remove components of the rapid ligation buffer that reduce transformation efficiency (polyethylene glycol). After centrifugation at 13,000 × g for 5 min the aqueous phase was used for bacterial transformation. Bacterial colonies grown on the selective LB-agar plate were screened for the constructed plasmid by PCR.

Bacterial transformation

45 µL electrocompetent E. coli cells were mixed with 2 µL of the ligation reaction or 10 ng of plasmid DNA prepared in TE buffer. The mixture was transferred to a 0.2 cm gap cuvette (BioRad) and the cells were transformed with the following settings: 2.5 kV, 200 Ω, and 25 µF. Electroporated cells were immediately resuspended in 1 mL Super broth and incubated at 37°C for an hour. 10 % of the transformed cells were subsequently spread onto an LB agar plate supplemented with 100 µg/mL Ampicillin, and the rest of the cells were spread onto another plate. The plates were incubated for approximately 16 h at 37°C and were examined for growth of the transformed cells. E. coli strain XL1 Gold (Stratagene) was used for general plasmid cloning and strain BL21 DE3 (New England Biolobs) was used to express the 6-cys domain of P92 from pPROEX HTb based vector.

Plasmid DNA extraction

Small-scale preparation of plasmid DNA was performed using QIAGEN Plasmid Mini Kit (QIAGEN) from a 3 mL bacterial culture grown overnight in Super broth. For large-scale preparation of plasmid DNA for parasite transfection a bacterial culture was grown in 500 mL Super broth overnight and the plasmid DNA was extracted using QIAGEN Plasmid Maxi Kit (QIAGEN).
**DNA sequencing**

To prepare the required DNA fragment for sequencing plasmids isolated from bacterial colonies containing the correct DNA fragment were put in a reaction involving 300 ng of plasmid DNA, 3.2 pmol primer, 2 µL BigDye reaction mix, 3 µL BigDye reaction buffer, and Milli-Q water to 20 µL final volume. The reaction was put through thermal cycling as follows: 96°C for 1 min followed by 25 cycles of ‘96°C for 10 s, 50°C for 5 s, 60°C for 4 min’, and it was held at 15°C. Sample was precipitated by adding 80 µL of 80 % isopropanol into the reaction and the pellet was collected by centrifugation at 13,000 × g for 15 min. The pellet was washed once with 80 % isopropanol and air-dried. The sample was sent to the Australian Genome Research Facility (AGRF) in Melbourne for sequencing. The results were analysed using Sequencher software (Gene Codes Corporation USA).

**Parasite genomic DNA extraction**

Saponin-lysed parasite pellet from 10 mL culture at ~5 % parasitemia of schizonts was resuspended well in 1 mL parasite lysis buffer. 100 µg RNAse was added and the mixture was incubated at 37°C for 30 min. 100 µg proteinase K was further added and it was incubated at 37°C for another 45 min. The mixture was then subjected to 3 rounds of phenol/chloroform extraction. The aqueous phase containing genomic DNA was collected and 3 M sodium acetate (pH 5.2) was added at 1/10 volume of the solution followed by 2.5 volumes of ice-cold absolute ethanol. The genomic DNA was left to precipitate at -20°C overnight. The DNA was pelleted by centrifugation at 13,000 × g at 4°C for 10 min and the pellet was washed twice with ice-cold 70 % ethanol. The DNA pellet was left to dry at room temperature for 5 min and was carefully dissolved in 50 – 100 µL sterile TE buffer.

**Southern blotting**

Genomic DNA of knockout parasites were extracted and digested with specified restriction enzymes, and transferred to a Hybond-N nylon membrane (GE Healthcare Life Sciences) after electrophoresis. The membrane was probed with dioxygenin (DIG)-labelled 5' flank and 3' flank, amplified using the primers listed in Table 2.1, followed by chemiluminescent detection per manufacturer’s instructions (Roche).
Parasite protein extraction

*P. falciparum* parasites were harvested at the required stage and were subjected to saponin lysis. The parasite pellet was then solubilised in either 1 % Triton X-100 in PBS, RIPA buffer, or 2-D sample buffer with complete protease inhibitor cocktail (Roche) as indicated. The soluble and insoluble materials were separated by centrifugation (13,000 × g) and the protein samples were prepared in sample buffer prior to SDS-PAGE or kept at -20°C or -80°C until use.

**SDS-PAGE and western blotting**

Parasite proteins were prepared in either non-reducing or reducing sample buffer as indicated prior to loading onto pre-cast 4 – 12 % acrylamide gradient NuPAGE® Bis-Tris gels (Invitrogen) together with Precision Plus Protein Standard (BioRad) molecular weight markers. The proteins were electrophoresed in 1× NuPAGE® MOPS SDS running buffer (Invitrogen) per manufacturer’s instructions. To visualise separated proteins gels were stained with GelCode™ Blue Safe Protein Stain (Thermo Scientific) per manufacturer’s instructions. For western blotting separated proteins were blotted onto nitrocellulose membrane (BioRad) using the Trans-Blot® SD Semi-Dry Transfer Cell (BioRad) or the iBlot system (Invitrogen) according to manufacturer’s instructions. The membrane was subsequently soaked in 1 % casein in PBS blocking solution for an hour followed by probing with specific primary antibodies. Goat anti-mouse IRDye™ 700 and goat anti-rabbit IRDye™ 800 secondary antibodies (Rockland Immunochemicals) were used to visualise the protein bands with a LI-COR Odyssey FC instrument.

Production of antibodies to recombinant P12 and P41 in Escherichia coli

From *P. falciparum* genomic DNA *p12* sequence corresponding to H26 through to S321 was amplified so that the N-terminal secretion signal sequence and C-terminal GPI-anchor signal sequence were excluded. The *p12* DNA fragment was ligated into the SacII and NcoI sites of pASK45(+) (ABI) in frame with a N-terminal Strep II tag and C-terminal 6×His tag. A fragment of *p41* excluding the N-terminal secretion signal sequence (K21 to S378), was similarly amplified and ligated into the pASK45(+) as per *p12*. After inducing the expression of the 6-cys proteins in *E. coli* with 0.2 mg/L anhydrotetracycline (as per manufacturer’s instructions) the bacteria were harvested and their inclusion bodies containing insoluble recombinant proteins....
were isolated as per (Hodder, 2003). The inclusion bodies were solubilised in 8 M urea and the 6×His tagged proteins were purified over nickel-nitrilotriacetic acid (Ni\({\text{2+}}\)-NTA) agarose under denaturing conditions (QIAGEN). The proteins were then greatly diluted and refolded in the presence of glutathione redox pair and were further purified by anion exchange chromatography. P12 and P41 polyclonal rabbit sera and P12 mouse monoclonal antibodies were produced at the Walter & Eliza Hall Institute Monoclonal Antibody Facility.

Expression of the recombinant 6-cys domain of P92 in E. coli for polyclonal antibody production

The predicted 6-cys domain of P92 (G574-P758) was amplified from parasite gDNA using primers listed in Table 2.3 and cloned into the pPROEX HTb expression plasmid (Invitrogen) via BamHI/SpeI restriction sites. The recombinant 6-cys domain of P92 was expressed in the BL21 E. coli strain with the induction of 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Bacterial cells were harvested by centrifugation at 6,000 \(\times\) g for 15 min at 4°C, and were washed twice with cold sterile reverse osmosis purified water. The cell pellet was thoroughly resuspended in resuspension buffer (RB; 15 mM sodium phosphate, 450 mM NaCl pH 7.4) (10 mL per gram starting cell weight) and lysozyme (Sigma) was added to 1 mg/mL final concentration. Cells were lysed by sonication and homogenizer, and soluble and insoluble fractions were separated by centrifugation at 12,000 \(\times\) g for 45 min at 4°C. The 6×His-tag recombinant protein in the inclusion bodies was resuspended in solubilisation buffer containing 8 M urea (6.6 mL per gram starting cell weight) and incubated at room temperature for 20 min. After centrifugation at 12,000 \(\times\) g for 45 min at 10°C, the supernatant was mixed with Ni\({\text{2+}}\)-NTA agarose pre-equilibrated in solubilisation buffer for an hour at room temperature. The resin was put into a column and was washed with 10 column volumes of washing buffer. The bound protein was eluted with 5 column volumes of elution buffer and was subjected to a rapid refolding process in a refolding buffer at room temperature overnight. The refolded protein was purified again on Ni\({\text{2+}}\)-NTA agarose and was subject to size-exclusion chromatography to further purify the P92 protein. The purified protein was sent to Walter & Eliza Hall Institute Monoclonal Antibody Facility for rabbit polyclonal antibody production.
Biochemical techniques

Rabbit IgG purification

1 volume of Protein G beads was washed twice in 5 volumes of filter-sterilised PBS and was collected by centrifugation at $500 \times g$ for 1 min. 1 volume of serum was diluted in 4 volumes of filter-sterilised PBS and mixed with the beads at room temperature for an hour. The beads were washed twice with filter-sterilised PBS and IgG was eluted with 5 volumes of 0.1 M glycine pH 2.8 in 1 volume fractions. Each fraction was eluted into 0.1 volume of 1 M Tris-HCl pH 8.0 to neutralise the glycine buffer. Eluted fractions were pooled and the IgG was concentrated using a 100 kDa molecular weight cut-off spin concentrators (Pierce). The purified IgG was stored at -20°C.

Recombinant P12-P41 interaction analysis using size-exclusion chromatography

The ability of HEK293E cell line expressed recombinant P12 and 41 (Taechalertpaisarn et al., 2012) to form a heterodimer was examined by column shift assay. Equimolar amounts of recP12-Cd4d3/4-6H (150 µg at 2.0 mg/mL) and recP41-Cd4d3/4-6H (180 µg at 3.5 mg/mL) were co-incubated for 1 h at 37°C in phosphate buffer containing 250 mM imidazole. Following incubation, samples were centrifuged for 10 min at 18,000 $\times g$ at 4°C prior to filtration through 0.2 µm Acrodisc 13 mm Syringe Filters (Pall Corporation). This material underwent gel filtration chromatography using a Superdex 200 10/300 GL column (GE Healthcare) in PBS on an ÄKTA Purifier (GE Healthcare). recP12-Cd4d3/4-6H (300 µg) and recP41-Cd4d3/4-6H (360 µg) were also subjected to gel filtration (Superdex 200 10/300 GL) independently to determine their individual retention volumes as a point of comparison with the heterodimer.

Immunoprecipitation for P12-P41 interactions

To capture potential interactions of P12 and P41 with other parasite proteins, a column was prepared containing IgGs to these proteins. Rabbit pre-immune, anti-P12, and anti-P41 IgGs were firstly crosslinked to Protein G agarose as per manufacturer’s instructions (Pierce). Parasite lysates were incubated with IgG-immobilised Protein G agarose overnight at 4°C. The unbound lysates were collected for further analysis and the IgG-agarose complexes were then washed with 1 % Triton X-100 in PBS to
remove unbound proteins. Bound proteins were eluted off the IgG-agarose using 0.1 M glycine pH 2.8 solution or as indicated. The pH of the samples were adjusted to 7.0 using 1 M Tris-HCl pH 9.0 and were prepared in non-reducing sample buffer for western blot analysis. For protein identification by LC-MS/MS sequencing, samples were prepared in reducing sample buffer for SDS-PAGE. Protein gels were stained with GelCode™ Blue Safe Protein Stain (Thermo Scientific) per manufacturer’s instructions.

**Chemical crosslinking**

Schizont-stage parasites were purified by magnetic separation and were divided into 5 equal aliquots and resuspended in 5 mL PBS with cOmplete protease inhibitor cocktail (Roche). Dithiobis(succinimidylpropionate), DSP (Thermo), prepared in dimethyl sulfoxide (Sigma) was added to the final concentration of 0 µM, 62.5 µM, 125 µM, 250 µM, and 500 µM, and parasites were crosslinked for 30 min at room temperature. Parasite material was subsequently pelleted and crosslinking reactions were quenched for an additional 30 min at room temperature by the addition of 5 mL quenching solution (120 mM NaCl, 25 mM Tris HCl pH 7.5). The crosslinked samples were washed and subsequently lysed by 0.09 % saponin. They were then solubilised in 1 % Triton X-100 in PBS and prepared for western blot analysis in non-reducing sample buffer.

**Erythrocyte-binding assay**

To produce culture supernatant, the magnet-purified schizonts were incubated overnight until all had released merozoites that subsequently shed their surface coats. The culture supernatant was clarified by pelleting the merozoites followed by concentration of the supernatant proteins through 10 kDa molecular weight cut-off spin concentrators (Millipore). 250 µL of culture supernatant was then mixed with 50 µL of packed fresh erythrocytes for 60 min at room temperature. The erythrocytes were separated from supernatant by spinning through 400 µL of dibutyl phthalate (Aldrich) at 12,000 × g for 30 s. Proteins bound to the erythrocytes were eluted by incubating with 10 µL of 1.6 M NaCl in PBS at room temperature for 10 min. Eluted proteins were obtained after 4 min of centrifugation at 12,000 × g and mixed with equal volume of 2× non-reducing sample buffer. The eluates were analysed by western blotting.
Depletion assay
Depletion assays were performed to determine the erythrocyte-binding activity of the 6-cys proteins. Culture supernatant containing shed merozoite surface proteins, was serially incubated 5 times with batches of uninfected erythrocytes at 20 % hematocrit to deplete any erythrocyte binding proteins. Each incubation was performed at room temperature for an hour and a small aliquot of supernatant from each round of binding was retained for by western blot analysis.

Heparin-binding assay
Heparin binding assays were performed as described in (Boyle et al., 2010a) using culture supernatant as a protein source for 6-cys proteins. Bound proteins were eluted using non-reducing sample buffer prior to western blot analysis. Ni²⁺-NTA resin was used as a control for heparin-agarose beads (Sigma).

Microscopy techniques

Immunofluorescence Microscopy
Parasites were fixed with 4 % EM grade paraformaldehyde and 0.0075 % EM grade glutaraldehyde (ProSciTech) as described previously in (Tonkin et al., 2004), and the cells were smeared onto slides and dried. Alternatively, live parasites were smeared onto slides, dried and fixed with ice-cold methanol. The cells fixed with both methods were then labelled with mouse and rabbit antibodies to specific proteins as indicated followed by secondary antibodies Alexa Fluor® 488 goat anti-rabbit IgG and Alexa Fluor® 568 goat anti-mouse IgG (1:2000 dilution, Molecular Probes®). After antibody labeling the cells were mounted in VECTASHIELD® with DAPI (VECTOR laboratories). Cell images were captured using Zeiss AxioObserver Z1 fluorescence microscope with the respective filter sets indicated above and analysed with ImageJ software.

Live-Cell Microscopy
Highly synchronous parasite cultures at 4 % hematocrit were diluted to 0.16 % in a specified complete medium and 2 mL of this was allowed to settle onto a 35 mm Fluorodish (World Precision Instruments) to produce a cell monolayer. Custom dishes holding smaller volumes of 25 or 50 µL were used for experiments containing
invasion inhibitory compounds. All live-cell experiments were performed at 37°C on a Zeiss AxioObserver Z1 fluorescence microscope equipped with humidified gas chamber (94 % N₂, 1 % O₂, and 5 % CO₂). Late stage schizonts were observed until they looked ready to rupture (described in (Crick et al., 2013)) and time-lapse videos were recorded with a AxioCam MRm camera for general acquisition or EMCCD QuantEM camera for high-speed acquisition. ImageJ and Prism (Graphpad) were used to perform image and statistical analyses. A value of P ≤ 0.05 was used as the determinant of statistical significance for all tests.

Cell labelling for live-cell microscopy

The erythrocyte membrane was labelled with 5 µM BODIPY® FL C₁₂-sphingomyelin (Molecular Probes®). Fluo-4 AM (Molecular Probes®) was used at 5 µM for calcium imaging. R1 peptide (Mimotopes) and cytochalasin D (Sigma) were used to inhibit invasion at 100 µg/mL and 1 µg/mL final concentration respectively. Calcium within erythrocytes was chelated using 60 µM BAPTA-AM (Sigma). Any labelling prior to imaging was done by incubation at 37°C for 1 h followed by washing in complete media twice.

Live-cell fluorescence imaging

When erythrocytes were separately labelled with a fluorescent indicator, magnet-purified late schizont stage parasites were added later to a 35 mm Fluorodish (World Precision Instruments). The parasites were imaged alternately between brightfield and fluorescence channels with the HE eGFP filter set (Zeiss).
Table 2.1. Primers used to generate knockout plasmids and Southern blotting.

<table>
<thead>
<tr>
<th>Gene regions</th>
<th>Primers</th>
<th>Sequences (5′ - 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ p12 (3D7)</td>
<td>Forward</td>
<td>ATCCCGCGGCCACCCACATGTGGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATACTAGTCATACAACATGGATG</td>
</tr>
<tr>
<td>3′ p12 (3D7)</td>
<td>Forward</td>
<td>ATCGAATTCTTACAACGAAGCCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATCCTAGGTTGAGTGTCTTCA</td>
</tr>
<tr>
<td>5′ p41 (3D7)</td>
<td>Forward</td>
<td>AATCCGCCGTCATATACATAATAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATTACTAGTCATCGTCATCTT</td>
</tr>
<tr>
<td>3′ p41 (3D7)</td>
<td>Forward</td>
<td>ATTGAATTCCCAAATGATAATACTATG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAAACTAGGCTTCATCCAGGTT</td>
</tr>
<tr>
<td>5′ p41 (CS2)</td>
<td>Forward</td>
<td>ATCCCGCGGTGTGATGCCAATTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATACTAGTCACAAGATATCCTT</td>
</tr>
<tr>
<td>3′ p41 (CS2)</td>
<td>Forward</td>
<td>ATCGAATTCTGTAATCTGACAGTCAT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATCCTAGGAAATCCAATATT</td>
</tr>
<tr>
<td>5′ p38 (W2mef)</td>
<td>Forward</td>
<td>TATCCCGGGAATTTAAAAGGAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TATACTAGTGTACCACATAAGGAC</td>
</tr>
<tr>
<td>3′ p38 (W2mef)</td>
<td>Forward</td>
<td>TTAGAATTTCGTAATATGAGTCCT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAACTAGGAAATAAAGTATTGA</td>
</tr>
<tr>
<td>5′ p92 (W2mef)</td>
<td>Forward</td>
<td>ATCCCGCGGTACACATGCTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATACTAGTCCAGAAGTCG</td>
</tr>
<tr>
<td>3′ p92 (W2mef)</td>
<td>Forward</td>
<td>ATCGAATTCCAGGAAGGATATTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GATCCTAGGTTACAGTATC</td>
</tr>
</tbody>
</table>

60
Table 2.2. Primers used to validate homologous recombination in the knockout parasites by PCR.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Primers</th>
<th>Sequences (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’ p12</td>
<td>Forward</td>
<td>TAGTGGAAGGACAATATAAAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTTAAGACAGATCTTCGGACTAG</td>
</tr>
<tr>
<td>3’ p12</td>
<td>Forward</td>
<td>GGGATAGCGATTTTTTTTACTGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TATCTGCTATTTAAAACTATGAAATTTAACC</td>
</tr>
<tr>
<td>5’ p41</td>
<td>Forward</td>
<td>TTATGTGTGATGCCAAATTTACAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTTAAGACAGATCTTCGGACTAG</td>
</tr>
<tr>
<td>3’ p41</td>
<td>Forward</td>
<td>GGGATAGCGATTTTTTTTACTGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTTTTGAAATCTTAATAGATGTCACATAC</td>
</tr>
</tbody>
</table>
Table 2.3. Primers used to amplify 6-cys domain of P92

<table>
<thead>
<tr>
<th>Sites</th>
<th>Primers</th>
<th>Sequences (5′ - 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5′ p92</td>
<td>Forward</td>
<td>TAGTGGAAAGGACAATATAAAGGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTTAAGACAGATCTTCGGACTAG</td>
</tr>
<tr>
<td>3′ p92</td>
<td>Forward</td>
<td>GGGATAGCGATTTTTTTTACTGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TATCTGCTATTTAAAAACTATGAAATTAACC</td>
</tr>
</tbody>
</table>
CHAPTER 3

Biochemical and Functional Analysis of Blood-Stage 6-Cys Proteins: P12 and P41

Introduction

The 6-cys protein family in *P. falciparum* comprises of 14 members some of which have been shown to play important roles during the parasite's life cycle especially in the sexual and sporozoite stages. P230, P48/45, and P47 are three members of the family that facilitate the fertilization between the male and female gametes in the mosquito gut (van Dijk et al., 2010). The P52 and P36 proteins on the other hand, are expressed in sporozoites and are involved in hepatocyte invasion (VanBuskirk et al., 2009). The final member whose role has been partly addressed is the newly discovered 6-cys protein B9, that appears to function late during the development of the exoerythrocytic (liver) stage (Annoura et al., 2014). Despite reasonable progress in understanding the functions of the 6-cys proteins in other stages, the precise role(s) of blood-stage 6-cys proteins remains elusive.

There are 4 members of the 6-cys protein family that are expressed in the asexual blood stage, namely P12, P38, P41, and P92 (Sanders et al., 2005). P12 was the first to be discovered by using human immune sera to immuno-screen a *P. falciparum* genomic library transiently expressed in COS-7 mammalian cells (Elliott et al., 1990). P12 was later identified, along with P41 for the first time, in a proteomic study of merozoite surface proteins present in detergent-resistant membranes, which are typically occupied by proteins involved in cell-cell interaction and cell signaling (Sanders et al., 2005). The predicted GPI anchoring characteristics of P12 was later confirmed in another proteomic study focusing only on the GPI-anchored proteins in developing merozoites (Gilson et al., 2006). The fact that P41, which does not possess GPI anchoring sequences, was detected on the merozoite surface suggested that it could be attached there via an interaction with other protein(s).

In this chapter I have characterised the biochemical properties of P12 and P41 and attempted to discover the possible functions that these proteins may play in the blood-stage *P. falciparum*. These studies first involved expressing recombinant P12 and P41 to produce polyclonal antibodies for detecting the proteins in parasites.
Molecular interaction studies were also performed with these recombinant proteins along with native proteins, and revealed that P41 binds to P12 on the merozoite surface. Both P12 and P41 were found to be shed from the merozoite surface after egress which pointed to their possible role as invasion ligands. Unexpectedly however, they did not demonstrate any erythrocyte-binding capacity and so attempts were made to individually knock the genes of P12 and P41 out to potentially reveal a phenotype indicative of function. The gene disruptions were successful and as part of the phenotypic analysis of the mutants their growth rates were measured. The growth rates of the Δp12 and Δp41 parasites were however comparable to the parental control parasite lines. Invasion inhibition assays were also performed to determine if the anti-P12 and anti-P41 antibodies could inhibit merozoite invasion into the erythrocytes thereby suggesting an invasion role for the proteins. Weak inhibition was observed at high antibody concentrations.

The fact that P12 and P41 are present throughout the *Plasmodium* genus and are exclusively expressed in the blood-stage parasites suggests they have a conserved and important function in this part of the parasite’s life cycle since their genes have never been lost. Many approaches were attempted here to reveal their function but have not provided a definitive role for the proteins. However the biochemical studies indicated that since the two proteins work as a pair, future studies should focus in this direction.

**Results**

*Expression of recombinant P12 and P41 in E. coli and HEK293E mammalian cell systems*

The genes of P12 and P41 were amplified from *P. falciparum* genomic DNA of the 3D7 strain and cloned into protein expression vectors. N-terminal secretion signal sequences and C-terminal GPI-anchoring sequences were excluded for the p12 (H26 to S321, Fig. 3.1A). Similarly the N-terminal signal sequences of p41 were left out from the amplification (K21 to S378, Fig. 3.1A). For *E. coli* expression the p12 and p41 were fused with N-terminal Strep II and C-terminal 6×His tags for purification purpose. The insoluble recombinant P12 and P41 present in the isolated *E. coli* inclusion bodies were solubilized in 8 M urea solution. The proteins were subsequently purified with Ni²⁺-NTA agarose beads under denaturing conditions. The extracted proteins were then subjected to refolding process using a glutathione redox
pair followed by anion exchange chromatography to enrich for the full length proteins and remove fragments and contaminating material. Equal amounts of the purified recombinant P12 and P41 were separated in the SDS-PAGE under reducing and non-reducing conditions prior to western blot analysis. The pooled malaria-immune human sera were used to probe the proteins, and both the P12 and P41 protein bands were detected at the expected sizes of 37 and 45 kDa respectively (Fig. 3.1B). The fact that human immune sera could recognise the recombinant proteins, particularly the non-reduced forms suggested the proteins were mimicking native epitopes and so polyclonal antibodies were raised in rabbit and mice to these materials.

For the expression of recombinant P12 and P41 in HEK293E mammalian cells, which was conducted by our collaborators (details in Taechalertpaisarn et al., 2012), the DNA sequences of the predicted extracellular regions of both proteins were chemically synthesised using a codon bias that was optimised for expression in mammalian cells. They were cloned and C-terminally fused with domain 3 and 4 of the rat Cd4 (Cd4d3/4) followed by 6×His tag (6H). The secreted recombinant P12 and P41 were collected and purified using Ni²⁺-NTA agarose resin. They were highly pure and migrated at the expected sizes in SDS-PAGE (Appendix I). Rabbit polyclonal antibodies were also produced from these mammalian expressed P12 and P41.

P12 and P41 are expressed in late asexual parasite stages and shed into the culture supernatant

The immunoglobulin G (IgG) component of the rabbit polyclonal sera raised against the recombinant E. coli expressed P12 and P41 proteins was purified on protein G sepharose prior to further utilization. To thoroughly explore the expression of P12 and P41 in the P. falciparum asexual stage, parasites were synchronized and then collected at every 8-10 hour intervals post invasion covering a 48-hour life cycle. Parasite proteins from each time-point were prepared in 1 % Triton X-100 in phosphate-buffered saline (PBS) solution, and equal amounts of the proteins were separated by SDS-PAGE under non-reducing and reducing conditions. They were then blotted onto nitrocellulose membrane and probed with rabbit anti-P12 and anti-P41 IgGs. The constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also probed to ensure equal loading of total proteins from each sample. P12 and P41 were first detected around 30-40 hours post invasion (hpi) and their expression peaked in the mature schizont stage, 40-48 hpi (Fig. 3.2A). Interestingly
both P12 and P41 were also detected in the culture supernatant suggesting that they were shed from the merozoite surface. Two forms of P12 were noticeably detected in both reducing and non-reducing conditions, but only the smaller size was observed in the culture supernatant implying that proteolytic cleavage may be responsible for this event. Since P12 is a GPI-anchored protein, it is possible that the cleavage takes place in close proximity to the anchor resembling the processing of MSP1 (Blackman et al., 1990). Several merozoite surface proteins are cleaved by a protease called subtilisin 2 (Green et al., 2006; Harris et al., 2005) and thus P12 might be one of its substrates. P41 and GAPDH were also seen as doublets in non-reducing condition, but in reducing condition they were detected as a single band. There were small amounts of P12 and P41 detected in the younger stage parasites of 12-20 and 20-30 hpi, however the transcriptome data of p12 and p41 indicated that they were not transcribed at these periods (Le Roch et al., 2004). The faint bands were therefore interpreted as a result of low level contamination from older parasites during sample preparation.

P12 resides on the merozoite surface

In a previous study from my laboratory, it was demonstrated that P12 and P41 when expressed episomally as N-terminal GFP-fusion proteins, were both localised to the merozoite surface (Sanders et al., 2005). This surface localisation was also confirmed by immunofluorescence microscopy but only for P41 with an antibody raised to the linker region between the 6-cys domains (Sanders et al., 2005). The fact that GFP-tagging has been known to sometimes alter the trafficking of fusion proteins (Lenassi Zupan et al., 2004), prompted the location of native P12 to be re-examined with the P12 IgG. Schizonts and free merozoites were fixed and labelled with rabbit anti-P12 IgG (50 µg/mL) and mouse anti-MSP1\textsubscript{19} (10 µg/mL). These IgGs were then detected with secondary goat antibodies conjugated with Alexa Fluor\textsuperscript{®} 488 and Alexa Fluor\textsuperscript{®} 568 respectively. When observed under the fluorescence microscope P12 was observed around the segmented schizonts and on the merozoite surface as expected (Fig. 3.2B). Interestingly P12 also displays concentrated localisation towards the apical end of the merozoite, represented by the dark area seen in the phase contrast microscopy, similar to the published localisation of P41 (Fig. 3.2B, white arrow) (Sanders et al., 2005). This is different from the validated surface resident protein, MSP1, which is uniformly distributed on the merozoite surface (Fig. 3.2B).
P12 and P41 form a heterodimer on the merozoite surface

Recombinant P12 and P41 associate as a heterodimer

It has been established that non-GPI and GPI-anchored 6-cys proteins usually function as a pair as seen in sexual and sporozoite stages (Eksi et al., 2006; VanBuskirk et al., 2009). For example, it was demonstrated that the non-GPI protein, P230, interacts with its GPI-anchored partner, P48/45, on the gamete surface so that they can perform their roles (Kumar, 1987). For this reason potential dimerization between the GPI-anchored P12 and non-anchored P41, was examined.

To begin with the mammalian-expressed recombinant P12 and P41 proteins fused with Cd4d3/4 and 6H (recP12-Cd4d3/4-6H and recP41-Cd4d3/4-6H), as described earlier, were subjected to a column shift assay which fractionates proteins based on size. Equimolar amounts of both proteins were co-incubated in PBS at 37°C for an hour prior to gel filtration chromatography analysis comparing them to the individual proteins. The co-incubated proteins eluted as a single major peak with the retention volume of 13.2 mL. The chromatogram also displayed a small shoulder possibly representing an excessive monomeric protein that had not formed a complex (Fig. 3.3). For individual proteins, recP12-Cd4d3/4-6H was resolved with a retention volume of 14.69 mL while recP41-Cd4d3/4-6H was eluted with the retention volume of 14.34 mL (Fig. 3.3). To establish how large the proteins and their complexes were their chromatograms were plotted against the molecular weight standards (linear log MW curve). The elution volumes of recP12-Cd4d3/4-6H and recP41-Cd4d3/4-6H corresponded to a size of ~90 kDa and a ~105 kDa respectively, indicative of being monomeric. When the proteins were co-incubated however, the retention volume was equivalent to a ~180 kDa protein indicative of a heterodimer formation. The physical properties and specificity of the interaction between recP12-Cd4d3/4-6H and recP41-Cd4d3/4-6H were also examined by means of surface plasmon resonance which was carried out by our collaborators (details in Appendix II). The results also emphasise the heterodimerisation characteristics between the two proteins since no homophilic interactions were observed for both recP12-Cd4d3/4-6H and recP41-Cd4d3/4-6H.

Native P12 and P41 form a heterodimer

Having established heterodimer formation between recombinant P12 and P41 I then went on to explore if this held true for the native proteins in parasites. This was
first examined by immunoprecipitating P12 from the 3D7 schizont lysates in 1 % Triton X-100 in PBS using rabbit anti-P12 IgG crosslinked to the Protein G agarose resin. The bound proteins were serially eluted with increasing stringency buffers, namely 0.1 M glycine pH 2.8, 0.1 M glycine 1 M NaCl, and SDS sample buffer. The eluted proteins were subjected to SDS-PAGE followed by colloidal Coomassie staining. Strongly stained bands were excised from the gel and the proteins were identified by LC-MS/MS sequencing. P12 was identified from a ~32 kDa band, while another larger protein band, ~37 kDa, that was co-eluted with P12 in a glycine pH 2.8 buffer was discovered to be P41 (Fig. 3.4A). This indicated that native P12 and P41 might bind to each other. Other proteins were also co-eluted with P12 under the more stringent conditions, i.e. ER-resident HSP70 (Pf3D7_0917900), 60s ribosomal protein (Pf3D7_0307200), bovine serum albumin, and stomatin (Fig. 3.4A). These proteins are very abundant and were considered to be non-specific contaminants.

To validate the mass spectrometry identifications, western blot analysis was performed on the immunoprecipitated samples. Specifically, rabbit anti-P41 IgG was used to probe for P41 in the eluates immunoprecipitated by rabbit anti-P12 IgG. This confirmed the interaction between native P12 and P41 in parasites was genuine (Fig. 3.4B). Reciprocal experiments using anti-P41 IgG to immunoprecipitate P41 also co-precipitated P12 confirming the same heterodimer formation between the two proteins. Western blot analysis for other merozoite surface proteins, i.e. MSP1 and EBA175, did not detect these in the eluate samples indicating the interaction between these two 6 cys proteins was specific.

Whether P12 and P41 only formed a single heterodimer or a higher order complex was further explored by performing chemical-crosslinking experiments. Schizonts of CS2 parasites were magnet-purified and the extracted proteins were crosslinked by adding various concentrations of dithiobis(succinimidylpropionate), DSP, followed by solubilisation in 1 % Triton X-100 in PBS. Crosslinked proteins were then separated in non-reducing SDS-PAGE and transferred to the membrane for immunoblotting. Probing for P12 and P41 with specific antibodies revealed the bands corresponding to the expected monomeric sizes for P12 and P41 (monomer; Fig. 3.5). Additional bands unique to the crosslinked samples were observed at ~70 kDa (red arrows; Fig. 3.5), coincided with the estimated size of a single P12-P41 heterodimer. No larger complex that was obviously specific to both P12 and P41 was identified. We also found that in the absence of P41 (Δp41 CS2, described in another section)
P12 does not form a homodimer which is consistent with the results obtained from the gel filtration chromatography (Fig. 3.3) and surface plasmon resonance analysis (Appendix II).

To identify what the binding targets of the P12/P41 dimer might be and what this might infer about the possible function of this complex, other proteins derived from the P41 immunoprecipitation described above were examined. As already mentioned these included the HSP70 chaperone and the 60s ribosomal proteins which since they do not localise to outside of merozoite were likely non-specific contaminants. No other proteins identified by the mass spectrometry appeared likely to be interacting partners of the P12-P41 dimer and so it was considered that the interaction of the dimer with its partners might be weak or transient and therefore may not have survived the immunoprecipitation process. Therefore to stabilise these weak or transient interactions with the P12-P41 complex the immunoprecipitations were performed on parasite lysates that has been DSP-crosslinked first. Using specific antibodies to P12 and P41, the eluates were examined by SDS-PAGE in reducing conditions to reduce the crosslinks followed by colloidal Coomassie staining (Fig. 3.6). Protein samples from the Δp41 parasite line were also included to help identify unique bands co-purified with the P12-P41 heterodimer. Protein bands that appeared only in the crosslinked samples were excised from the gel and identified by LC-MS/MS protein sequencing (red boxes; Fig. 3.6). The merozoite surface proteins MSP1, MSP9, and SERA5 were found to co-precipitate with the complex, however we consider it likely that these proteins are non-specific binders likely caused by random crosslinking of the densely packed merozoite surface proteins (Gilson et al., 2006). Thus we conclude that native P12 and P41 forms a single heterodimeric species on the merozoite surface which does not appear to bind strongly to other parasite proteins.

**P12 and P41 from culture supernatants do not bind to erythrocytes**

It remained possible that the P12 and P41 heterodimer possessed a binding pocket that interacted with an erythrocyte protein and thus served as a ligand for an erythrocyte receptor similar to other known merozoite proteins, e.g. EBAs and Rhs (Tham et al., 2012). As P12 and P41 were also discovered in parasite culture supernatants like other invasion ligands, it was decided to explore their erythrocyte-binding capability by using erythrocytes as bait to pull out the parasite ligands.
Known ligands such as EBA175 would serve as a positive control. Synchronized schizonts were allowed to rupture and the culture supernatants were collected for the experiments following erythrocyte invasion by these merozoites. Fresh erythrocytes were mixed with the culture supernatant for an hour at room temperature to allow the invasion ligands to bind to the cells. The erythrocytes were then separated from the culture supernatants by centrifugation through an oil, dibutyl phthalate, and the cells were washed for a few times to completely remove unbound components. The erythrocyte-bound proteins were then eluted with high salt solution. The culture supernatant input, unbound proteins, and bound proteins were subjected to SDS-PAGE analysis followed by western blotting. EBA175, a well-known erythrocyte binding protein, was detected in the bound fraction as expected. P12 and P41, on the other hand, were only found in the unbound fraction indicating that they probably did not bind to the erythrocytes.

Since the conventional erythrocyte-binding assay involves centrifugation of the erythrocytes through oil and subsequent multiple washing steps which could possibly remove weakly interacting proteins, it was decided to use a gentler approach to explore the erythrocyte-binding action of P12 and P41. In this method uninfected erythrocytes were serially added to the invasion supernatant and then removed to gradually deplete the invasion ligands from the culture supernatant. Samples of supernatant collected from each round of incubation were compared as well as the initial input and the mock incubated control (Fig. 3.8). Immunoblots of these samples clearly show the decline of EBA175, as a positive control, from the culture supernatant to less than 10 % after 5 rounds of incubation with the erythrocytes (Fig. 3.8). The depletion of MSP142, a weaker-interacting ligand which was demonstrated to bind to heparin-like molecules on the erythrocyte surface (Boyle et al., 2010a), was also observed to decline to ~30 % of its initial level (Fig. 3.8). P12 and P41, however, were not substantially reduced by multiple exposures to the fresh erythrocytes (Fig. 3.8). It was therefore concluded that the heterodimeric P12/P41 shed from the merozoite surface into the culture supernatant does not have a direct function in erythrocyte binding during invasion.

**P12 and P41 from culture supernatants do not bind to heparin**

Since P12 and P41 do not appear to strongly bind to the erythrocytes as demonstrated by typical erythrocyte-binding assays and depletion assays, I speculated
that these two proteins might contribute to the invasion process differently from the known invasion ligands, i.e. EBAs and some Rhs, which have their own specific protein receptors on the erythrocyte surface (Tham et al., 2012). MSP1 is an example of invasion ligand that is recognised to facilitate the primary attachment to the erythrocytes by interacting with heparin-like molecules covering the erythrocyte surface (Boyle et al., 2010a). As demonstrated by the depletion assays, the amount of MSP1$_{42}$ was moderately declined after multiple exposures to uninfected erythrocytes validating the relatively weak interactions the proteins had with the cells. P12 and P41, on the other hand, only showed minimal decrease from the experiments suggesting that they may display even weaker interactions to the cells compared with MSP1$_{42}$. I decided to explore the role of P12 and P41 in binding to the heparin-like molecules directly as has previously been performed for MSP1 (Boyle et al., 2010a). Culture supernatants were mixed with the heparin-agarose beads, followed by washing before the bound proteins were eluted. Ni$^{2+}$-NTA resin, which also has agarose as its matrix, was used as a control. Western blots clearly showed that MSP1$_{42}$ is the only tested protein able to bind to heparin (Fig. 3.9). All of the other proteins examined were found binding to the Ni$^{2+}$-NTA resin, which was interpreted as being due to non-specific interactions. This result corroborates that P12/P41 heterodimer is less likely to have a direct role as an invasion ligand binding to erythrocytes.

**P12 and P41 are not essential in the blood-stage parasites**

Genetic deletion studies of the 6-cys members in sexual and liver stages have revealed pronounced phenotypes in mutants lacking the expression of the corresponding 6-cys protein specific for that particular stage (van Dijk et al., 2010; VanBuskirk et al., 2009). These findings suggest that the 6-cys family plays a crucial role in each step of parasite development. To examine whether P12 and P41 could play such important role in blood-stage parasite, genetic deletion of these 2 proteins was carried out in 3 different laboratory parasite lines, 3D7, CS2, and W2mef. The transfection plasmids were designed to homologously recombine into the *p12* and *p41* loci, and replace the genes with the positive selectable marker, human dihydrofolate reductase (hDHFR), as depicted in the Fig. 3.10A. The negative drug selection marker, cytosine deaminase (CD), was also used to remove parasites from the population that were maintaining the plasmid as an episome or which had integrated...
the CD gene into the chromosome via a single recombination event. After the parasites were transfected with *p12* and *p41* gene replacement plasmids, they were subjected to positive/negative drug selection cycles with WR99210 and 5-fluorocytosine (5-FC), respectively. Genomic DNA of the wildtype and transfected parasites were extracted for Southern blot analysis to prove the replacement of *p12* and *p41* (Fig. 3.10B, only for 3D7).

The results showed that, for the Δ*p12* 3D7 parasites, after two rounds of drug selections *p12* was disrupted in the majority of the parasites if not all. However, in the case of Δ*p41* 3D7 parasites, the blots indicated that the wildtype and Δ*p41* parasites were both present in the culture after three rounds of positive/negative drug selection cycles requiring the generation of clonal lines by limiting dilution. Several clonal lines of both Δ*p12* and Δ*p41* parasites were selected for PCR screening to validate deletion of the *p12* and *p14* genes. The primers were designed to amplify across the 5′ and 3′ crossover sites as illustrated in Fig. 3.10A to ensure that the genetic replacement had occurred. The Δ*p12* 3D7 clone 1 and Δ*p41* 3D7 clone 1, which correctly showed the expected recombination, were chosen for further validation (Fig. 3.10C).

Western blotting of the total proteins extracted from schizont stages of both clonal lines verified that there was no expression of P12 and P41 in the Δ*p12* clone 1 and Δ*p41* clone 1 parasites, respectively (Fig. 3.11A). Immunofluorescence microscopy was also carried out to confirm the absence of P12 and P41. Schizont stage of Δ*p12* clone 1 and Δ*p41* clone 1 parasites were fixed and probed with primary and secondary antibodies as described earlier. As shown in Fig. 3.11B, the results confirm the loss of P12 and P41 expression in the knockouts. Therefore, similar to the 6-cys proteins expressed in insect and liver stages, the genes for P12 and P41, can be deleted in the blood stages. A major difference however is that P12 and P41 are expressed in the blood stages but do not appear to be essential.

*Parasites lacking of P12 and P41 do not have altered growth phenotypes*

It is clear that P12 and P41 are dispensable in blood-stage parasites as Δ*p12* and Δ*p41* mutant parasites could be generated. Although not essential it could be that the knockout parasites might display subtle phenotypic changes that might indicate what the function(s) of the P12-P41 complex were. For example the P12 and P41 knockout parasites might display slower growth and/or a reduction in invasion
efficiency. The Δp12 and Δp41 parasites were therefore compared with the 3D7 wild-type parasites and another mutant, the Δp36 3D7 parasite, which was transfected with the same backbone construct and had the same hDHFR selection cassette integrated into its genome. P36 is also a 6-cys protein that is expressed and functions specifically in sporozoites (VanBuskirk et al., 2009), therefore the parasites without p36 were unlikely to display any phenotype in the blood-stage. 3D7, Δp12 clone 1, Δp41 clone 1, and Δp36 clone 4.9 parasites were tightly synchronised, adjusted to 1 % parasitemia, and diluted 100,000 fold in duplicate. They were cultured until the fastest growing line reached ~1 % parasitemia and then the parasitemias of all of the lines were counted. The amplification rates per asexual cycle were then calculated from 2 independent experiments (Fig. 3.12A, Fig. 3.12B). 3D7, Δp12 clone 1, Δp41 clone 1, and Δp36 clone 4.9 parasites had the growth rates of 6.77 ± 0.29, 6.67 ± 0.36, 6.56 ± 0.10, and 7.02 ± 0.21 fold per cycle, respectively. The growth rates of the Δp12 clone 1 and the Δp41 clone 1 parasites were then compared in a pair-wise manner with the parental 3D7 and the Δp36 clone 4.9 transfected parasite control using Mann-Whitney analysis (Fig. 3.12A, Fig. 3.12B). The tests indicated that the growth rates of the Δp12 clone 1 and the Δp41 clone 1 parasites were not significantly different from the control parasites. This finding suggested that P12 and P41 may not play a crucial part in parasite growth and erythrocyte invasion in the blood-stage, since without them the parasites were still able to grow at a similar rate as the wildtype.

P12 and P41 antisera do not significantly obstruct parasite growth and invasion efficiency

It is well established that antisera to various merozoite proteins can halt parasite proliferation by inhibiting the erythrocyte invasion mechanism, and this approach is being pursued in search of an effective vaccine. It is not always necessary for the antisera to directly interrupt the interaction of the merozoite surface protein with its erythrocyte receptors to have an inhibitory role. For example, antibodies that inhibit the interaction of AMA1 with RON2, two proteins of parasite origin, potently inhibits invasion (Coley et al., 2006; Healer et al., 2004; Richard et al., 2010; Riglar et al., 2011). Despite the fact that it was not established that P12 and P41 were invasion ligands or strongly interacted with other merozoite proteins, we decided to examine an inhibitory effect of anti-P12 and anti-P41 antisera upon erythrocyte invasion. The IgG-purified rabbit antisera raised to E. coli expressed recombinant P12 and P41 were
tested in invasion inhibition assays in comparison with pre-immune control IgG. The results showed that anti-P12 and anti-P41 IgGs, used at relatively high concentration of 2 mg/mL, could slightly inhibit the erythrocyte invasion by 15 - 20 % (Fig. 3.12B). Since the amounts of antibodies used were quite high, it was considered the moderate inhibition observed could partly be due to steric obstruction of the accumulated IgG on the merozoite surface. Our collaborators also witnessed similar lack of potent invasion inhibition using antisera raised against mammalian expressed recombinant P12 and P41 with 3D7 and Dd2 parasite lines in their invasion inhibition assays (Appendix III).

It remained possible that the binding of P12 to P41 might be important for invasion efficiency and so dimerization of these proteins was examined in the presence of protein specific IgGs. It was found the pretreatment of the monomers inhibited dimerization, however, once formed the dimer was resistant to antibody mediated disruption (Appendix IV). This result is similar to that observed between native AMA1 and RON2 which do not normally associate until just before merozoite invasion when the tight junction forms (Collins et al., 2009). That this occurs for P12 and P41 however seems unlikely because using the crosslinkers it was found that the two proteins had already formed a dimer in the schizont stage before merozoite egress (Fig. 3.5).

**Discussion**

The 6-cys protein family has attracted considerable attention because of their important roles in the *Plasmodium* life cycle. Important roles have been shown for certain 6-cys proteins exclusively expressed in the insect and liver stages by various genetic and functional studies. It appears that, in general, the 6-cys proteins are involved in recognition and/or interactions between cell types whether they are the male and female gametes or the sporozoites and host hepatocytes (van Dijk et al., 2010; VanBuskirk et al., 2009). Since there have not been many studies that have attempted to characterise the functions of the blood-stage 6-cys proteins, it was decided to explore their possible roles beginning with the P12 and P41. I hypothesised that P12 and P41 would play a similar role as other family members by acting as the invasion ligands facilitating erythrocyte invasion for the merozoites. I was able to demonstrate that P12 and P41 were both located as a heterodimer on the merozoite surface particularly around the apical region. Although these characteristics seem to
suggest that the P12/P41 heterodimer could likely be an invasion ligand, other
evidence presented here does not support this notion.

To investigate if the P12/P41 dimer served as an invasion ligand methods were
employed that had been successfully used to characterise other invasion ligands
involved in erythrocyte-binding. Firstly erythrocytes were exposed to culture
supernatants containing invasion ligands. The cells were then centrifuged through the
dibutyl phthalate oil followed by multiple washing steps before elution and analysis of
the erythrocyte binders. This approach however failed to yield any P12 and P41
suggesting they were not high affinity erythrocyte binders. It remained possible
however that erythrocyte-bound proteins could dissociate during the washing process,
especially those with low-affinity binding. As it was believed the blood-stage 6-cys
proteins might play a role in initial recognition and interaction of the erythrocyte, it
was possible that the proteins were low affinity binders. Consequently, a milder
deployment assay was used to test the erythrocyte-binding capacity of the two proteins
but again I did not observe significant binding of P12 and P41 to erythrocytes. This
was somewhat surprising since particular members of a family of *Toxoplasma gondii*
proteins related to the 6-cys, have shown to bind their host cells (Gerloff et al., 2005;
He et al., 2002). Called the SRS (SAG1-related sequence) superfamily, these surface
antigens of *T. gondii* are known to interact with sulfated proteoglycans of the
nucleated host cells. The crystal structure of SAG1, the archetypal SRS, has revealed
that it forms homodimer which then creates a binding groove between the N-terminal
modules of the two monomers (He et al., 2002). The properties of this binding pocket,
e.g. shape and charge, appear to be heterogenous among the SRS proteins suggestive
of their diverse carbohydrate ligands (Crawford et al., 2009; 2010; He et al., 2002).
Demonstration of P12 and P41 heterodimerisation has led to the proposition that the
complex might have similar binding characteristics to the SAGs, but so far the ligands
of the P12 and P41 dimer have not been identified.

That P12 and P41 form a heterodimer has been independently validated by
Tonkin et al. who solved the crystal structure of full-length *P. falciparum* P12 which
indicated probable heterodimerization with P41 (Tonkin et al., 2013). For their
interaction studies they chose to express the recombinant P12 and P41 in insect cells
without large tags unlike our recombinant proteins in which bacterial and mammalian
expression systems were used. Although we expressed the proteins with a relatively
large tag, Cd4d3/4-6H (~25 kDa), we have shown that it did not interfere with the
interaction of recombinant P12 and P41 in our size-exclusion chromatography (Fig. 3.3) and the SPR studies (Appendix II). Tonkin et al. (2013) further attempted to verify the dimerisation of the two proteins by chemical-crosslinking the recombinant P12 and P41 prior to proteolytic digestion and peptide identification. Their result suggested that P41 possibly interacted with P12 in an antiparallel orientation unlike the parallel homodimerisation of the SAGs (He et al., 2002; Tonkin et al., 2013). Because the crystal structure of recombinant P41 produced by the insect cells could not be solved, our mammalian-expressed recombinant protein might provide alternative source for crystallisation studies. It is important to solve the structure of the P12/P41 heterodimer since it may reveal the nature of potential binding pockets which in turn might help to identify its binding partner(s).

After failure to establish that the P12/P41 heterodimer bound to erythrocytes and may therefore have had an invasion role an alternative approach was attempted. Antibodies against P12 and P41 were used to attempt to inhibit erythrocyte invasion to support a role in the invasion process. IgG-purified rabbit polyclonal antibodies raised against *E. coli* expressed P12 and P41 were used at a relatively high concentration of 2 mg/mL, yet only achieved a slight invasion inhibitory effect, of around 10 - 20 % for both proteins. In comparison, polyclonal antibodies to a known essential invasion ligand, namely AMA1, could inhibit invasion up to 50 % at the maximum of 0.5 mg/mL (Coley et al., 2006; Healer et al., 2004). Anti-Rh5 rabbit IgG could achieve a similar rate in invasion inhibition at the same concentration and this increased to 60 % at higher IgG concentrations (Douglas et al., 2011). Our colleagues also produced polyclonal antibodies to the mammalian-expressed recombinant proteins and tested them against various laboratory strains. The poor invasion inhibitory effects were similarly detected even though the combination of anti-P12 and anti-P41 rabbit IgGs were used (Appendix III). Since the recombinant P12 and P41 from mammalian cells could robustly form a heterodimer, this suggested that the proteins were correctly folded and the antibodies would be able to recognise the native epitopes. Moreover, these antibodies were successful in preventing the recombinant P12 and P41 from forming heterodimer if they were mixed with their respective proteins prior to co-incubation with the other dimer partner. The antibodies however, could not disrupt the heterodimer once it was already established (Appendix IV). This observation implied the antibodies were unable to disrupt the P12/P41 heterodimer in *vivo* and hence this could be the reason why a noticeable phenotype
was not observed when using the antibodies to disrupt the proteins’ function in parasites. It was concluded that the modest invasion inhibition effects from using high levels of anti-P12 and anti-P41 IgGs at 2 mg/mL were due non-specific activity, possibly caused by coating the merozoite surface and interfering with the functions of other merozoite surface proteins' as opposed to neutralising an invasion-related function for P12 and P41.

Next an attempt was made to examine the essentiality of P12 and P41 for the blood-stage development of the *P. falciparum* parasites by genetic knockout studies. This approach has been proved useful to identify the functions of many proteins including invasion-related components. Although some the genes for some merozoite proteins are refractory to the genetic deletion because they are essential for invasion, most invasion proteins, despite their important contributions can be knocked out because they have redundant functions (Cowman and Crabb, 2006). We were able to individually remove *p12* and *p41* from 3D7, CS2, and W2mef laboratory lines and the subsequent growth measurement of the selected ∆p12 and ∆p41 3D7 clones did not display any obvious phenotype resulting from the loss. This was rather unexpected since the 6-cys proteins characterised in insect and liver stages exhibit prominent phenotypes when their genes were deleted. Removal of *p48/45*, *p230*, and *p47* from the parasite genome greatly reduces the fertilisation of male and female gametes in the mosquitoes (van Dijk et al., 2010). Strong effects were likewise seen in *p36* and *p52* deletion mutants whereby the sporozoites failed to develop into mature exo-erythrocytic schizont forms (Ishino et al., 2005; Labaied et al., 2007; van Dijk et al., 2005; van Schaijk et al., 2008; VanBuskirk et al., 2009).

It remains possible that ∆p12 and ∆p41 parasites were able to grow at a normal rate because of compensatory changes in the expression of other genes that substituted for lost P12/P41 function. The ability to upregulate gene expression to substitute for the loss of another gene is well known in *Plasmodium* parasites particularly for invasion ligands. This functional redundancy probably evolved as a way of overcoming host immunity to certain invasion ligands and as an adaptation to changes in the expression and genetic variability of erythrocyte receptors. An example of redundancy occurs in the W2mef parasite line that heavily relies on EBA175 for sialic-acid dependent invasion. When *eba175* was deleted from this parasite, PfRh4 expression was up-regulated which uses a sialic-acid independent invasion pathway (Stubbs et al., 2005). To identify the upregulation of PfRh4 in the ∆eba175 mutant,
microarray analysis of the parasite’s transcriptome was performed (Stubbs et al., 2005). In the future such an approach could be similarly used in the $\Delta p12$ and $\Delta p41$ mutants to discover if other genes have become upregulated to substitute for P12 and P41.

It is worth noting that in the $\Delta p12$ parasites we were able to detect P41 in the protein samples prepared from saponin-lysed schizonts that had been washed to remove the contents of the infected erythrocyte cytoplasm and the PV (Fig. 3.11). Since P41 does not have a GPI-anchor and has been shown to interact with the GPI-anchored P12 for surface localisation, this finding suggests that P41 could be retained on the merozoite surface through binding to other surface proteins. However, initial attempts to identify other possible interacting partners of P12 and P41 were not successful (Fig. 3.6). Since we only individually deleted $p12$ and $p41$, the remaining dimer partner could possibly compensate for the loss of other partner and this could be a reason why we did not see any phenotype in the knockouts we generated.

Another possibility for why we could not observe a phenotype in the $\Delta p12$ and $\Delta p41$ parasites might relate to how the mutants were produced. Generally the protocol requires months of culturing for 2 to 3 rounds of positive/negative drug selection to allow the homologous recombination to take place followed by limiting dilution to obtain single clones. The 30-40 blood-stage cycles that the transfected parasites have to go through possibly allowed them enough time to adapt for the loss of the 6-cys genes and restore their growth rates to normal. This can be considered a limitation of knockout studies in blood-stage expressed proteins compared to proteins of the mosquito and pre-erythrocytic stages where the phenotypes are examined in the first cycle that they have gone through. Here the mutants have not had enough time to compensate for their deficiency and the deleterious phenotypes can be strong. It is worth postulating that if the $\Delta p36$ and $\Delta p36p$ sporozoites of which a small number breakthrough and establish a blood-stage infection (Ploemen et al., 2012; Spring et al., 2013) had been passaged through multiple life cycles, they could adapt and regain normal growth similar to what could be happening with the blood-stage 6-cys mutants.

One new method that could be used to study loss of P12 and P41 expression is the conditional knockdown approach. Conditional strategies have been developed and increasingly been performed in blood-stage parasites to examine the roles of proteins that were refractory to genetic deletions in the past (Azevedo et al., 2013; Collins et
al., 2013; Yap et al., 2014). This procedure allows us to examine the phenotypes resulting from the loss of a particular protein in the first cycle when its gene is inducibly removed or its expression curtailed after the addition of a chemical inducer. A number of conditional methods exist but two stand out as being suitable of P12 and P41. The first is to flank the genes with loxP sites that are targets of an introduced DiCre recombinase which can delete the genes upon addition of rapamycin (Collins et al., 2013; Yap et al., 2014). The second approach that might be suitable for the 6-cys proteins is to insert the glmS ribozyme into the 3’ untranslated region of the genes which can be induced to cleave and destabilise the mRNA upon the addition of the inducer, glucosamine (Prommana et al., 2013). By subjecting the parasites to a sudden loss of P12 and P41 it might possible to induce a strong phenotype such as marked reduction in invasion efficiency for example, that would help establish the role of these proteins.

In summary, this chapter describes the biochemical and functional characterisation of two blood-stage 6-cys proteins, P12 and P41. We found that P12 and P41 robustly form a stable heterodimer and that the complex is shed from the merozoite surface during invasion since both proteins are present in the culture supernatant. However, despite trying two different assay methods we were not able to establish if the P12/P41 heterodimer could bind to host erythrocytes. Both p12 and p41 can be disrupted individually but no change in parasite growth rate was detected. Rabbit polyclonal antibodies to P12 and P41 show little parasite invasion inhibition activity also indicating the proteins are important for invasion. Despite failure to shed light on the function of P12 and P41, it is still possible that P12/P41 could be involved in binding with a low-affinity interaction to erythrocytes, given the roles of the related 6-cys proteins in other stages. Alternatively the 6-cys proteins may help the parasites recognise the blood types other than the O-type erythrocytes used in our experiments, or they may even facilitate the binding to different cell types, such as reticulocytes, which also support the growth of *P. falciparum* *in vitro* (Tamez et al., 2009). Interaction with glycan chains on the erythrocyte surface is also a possible role for P12/P41 heterodimer and testing this using our high quality mammalian expressed recombinant P12/P41 dimer on glycan arrays is a future goal (Geissner et al., 2014). On the other hand, the P12/P41 dimer may be playing a host immunomodulatory role, as has recently been demonstrated for the P47 (Molina-Cruz et al., 2013), which cannot be proved by our *in vitro* approaches. The fact that the P12/P41 heterodimer is
exclusively expressed in the blood-stage and is conserved across the *Plasmodium* genus implies that it plays some important but not essential role that we were not able to ascertain with the strategies employed here. New approaches are therefore required to find out what function(s) of P12 and P41 might be.
A

P12

P41

- ER signal sequence
- 6-cys domain
-Spacer
- GPI-anchor

B

<table>
<thead>
<tr>
<th>kDa</th>
<th>non-reduced</th>
<th>reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P12</td>
<td>P41</td>
</tr>
<tr>
<td>50</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>25</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

pooled malaria immune human serum
Figure 3.1. Recombinant expression of P12 and P41 in *Escherichia coli*.

(A) An illustration shows regions of P12 and P41 that were expressed in *E. coli* (black line). Features of the proteins are depicted as follows; secretion signal sequence (white), protein spacers (yellow), 6-cys domains (red), and the GPI-anchoring signal sequence (black). (B) Immunoblots of *E. coli* expressed recombinant P12 and P41 probed with pooled malaria human immune serum. The proteins were evaluated in non-reduced and reduced forms. Molecular mass protein markers are labelled on the left.
Figure 3.2. P12 and P41 are expressed in late asexual stage parasites and are shed into the culture supernatant.

(A) Parasite protein extracts collected at various times throughout the blood-stage life cycle were fractionated by SDS-PAGE and blots of these samples were probed with anti-P12 and anti-P41 rabbit IgGs (10 µg/mL) raised to *E. coli* recombinant proteins in both reducing and non-reducing conditions. The proteins exhibit expression in late stage parasites with maximum level in schizont stage. P12 and P41 are also detected in the culture supernatant indicating that they are shed from the parasite surface. Rabbit anti-GAPDH antibodies were also used to probe the blots to ensure that equal amounts of the total protein samples were loaded. (LR = late ring, ET = early trophozoite, T = trophozoite, ES = early schizont, Sc = schizont) (B) The surface localisation of P12 is determined by immunofluorescence microscopy. Fixed schizonts and merozoites were labelled with rabbit anti-P12 IgG (50 µg/mL), and anti-MSP1 monoclonal antibodies (10 µg/mL) as a control for a merozoite surface protein. Alexa Flour® 488 goat anti-rabbit IgG and Alexa Fluor® 568 goat anti-mouse IgG antibodies were used as secondary antibodies to visualise the two proteins respectively. In merozoites, P12 also demonstrated an apical concentration (white arrows) in contrast with MSP1 which mostly shows uniform surface localisation.
Figure 3.3. Recombinant P12 and P41 can form a heterodimer.
P12 and P41 were recombinantly expressed in the HEK293E cell system and were C-terminally tagged with domain 3 and 4 of rat Cd4 followed by 6×His (Appendix I). The recP12-Cd4d3/4-6H (blue line), recP41-Cd4d3/4-6H (red line) and the co-incubated proteins (green line) were resolved on a Superdex 200 10/300 GL column for size-exclusion chromatography. The chromatogram shows a single peak of the co-incubated material at lower retention volume, which has a size corresponding to a P12/P41 heterodimer, in contrast to the smaller individual proteins.
Figure 3.4. Native P12 and P41 associate as a heterodimer.

(A) To identify the binding partner(s) of P12 in the parasites, total proteins from a schizont extract in 1 % Triton X-100 in PBS were immunoprecipitated with rabbit anti-P12 IgG crosslinked to Protein G agarose beads. Bound proteins were sequentially eluted off the beads with increasing stringency buffers, namely 0.1 M glycine, then 0.1 M glycine with 1 M NaCl, and then 2 % SDS. Eluted proteins from each fraction as well as the unbound material were fractionated by SDS-PAGE and stained with colloidal Coomassie dye. Bands of proteins that were co-immunoprecipitated with P12 were excised from the polyacrylamide gel and analysed by LC-MS/MS sequencing. The parasite proteins identified were P41, HSP70, and 60s ribosomal proteins. Considering the nature of these three proteins, P41 was the most likely binding partner of P12. (B) The interaction between P12 and P41 was confirmed by western blotting. Protein extract from schizonts were immunoprecipitated using rabbit pre-immune (PI), anti-P12, and anti-P41 IgG coupled to Protein G agarose. Soluble (S) and insoluble (I) protein extracts from 1 % Triton X-100 in PBS were included as input controls. The blot was also probed with anti-EBA175 and anti-MSP119 antibodies to validate the specificity of the P12/P41 interaction.
Figure 3.5. Protein-crosslinking analysis confirms the interaction of native P12 and P41.

Synchronised CS2 parasites at the schizont stage were magnet-purified and subjected to protein crosslinking with various concentrations of dithiobis(succinimidylpropionate), DSP. The crosslinked proteins were separated by SDS-PAGE in non-reducing condition and transferred to the membrane for western blotting. An approximate 70 kDa protein band (red arrow), which was comparable to the size of a predicted P12/P41 heterodimer, appeared in the crosslinked samples when the blot was probed for P12 (upper panel). There was no heterodimeric proteins detected in the non-crosslinked sample (-DSP) whereas the monomeric form of P12 were seen in all of the samples. Probing the blot for P41 showed two bands of ~70 kDa protein species (lower panel). To resolve which band corresponded to the P12/P41 heterodimer, crosslinked protein samples from Δp41 CS2 parasites were probed. The species denoted with an arrow in the CS2 blot two disappeared from the crosslinked Δp41 CS2 when probed with both antibodies denoting that this band was the P12/P41 heterodimer.
Figure 3.6. Identification of other binding partners of the P12/P41 complex.

Magnet-purified schizonts of CS2 and Δp41 CS2 parasites were subjected to protein crosslinking by DSP. Crosslinked and non-crosslinked protein extracts from these parasites were immunoprecipitated using anti-P12 and anti-P41 IgG coupled with Protein G agarose. Eluates were reduced by the addition of dithiothreitol (DTT) prior to SDS-PAGE and colloidal Coomassie staining. The bands specific for the crosslinked samples were excised from the polyacrylamide gel (red boxes) for LC-MS/MS sequencing. The proteins identified were MSP1, MSP9, and SERA5, as indicated.
Figure 3.7. Typical erythrocyte-binding assays show no binding activity for P12 and P41.

(A) Parasite culture supernatant containing the shed merozoite proteins including P12 and P41 were used in the erythrocyte-binding assay. The culture supernatant was mixed with the uninfected erythrocytes to allow the proteins to bind to before centrifugation through oil and elution of the bound proteins by high salt solution. The input supernatant (S/N), unbound material, and the bound proteins were analysed by SDS-PAGE and western blotting. Both P12 and P41 were not found to bind to erythrocytes whereas the positive control, EBA175, did as expected.
A

<table>
<thead>
<tr>
<th>kDa</th>
<th>250-</th>
<th>150-</th>
<th>37-</th>
<th>37-</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>50-</td>
<td>25-</td>
<td>37-</td>
<td>37-</td>
</tr>
<tr>
<td>b</td>
<td>50-</td>
<td>25-</td>
<td>37-</td>
<td>37-</td>
</tr>
</tbody>
</table>

Rounds of exposure to RBCs

EBA175
P12
P41
MSP1_{42}

a = CS2 invasion supernatant
b = mock control

B

CS2 invasion supernatant depletion

<table>
<thead>
<tr>
<th>relative band intensity (percentage)</th>
<th>rounds of exposure to RBCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBA175</td>
<td>0  1  2  3  4  5</td>
</tr>
<tr>
<td>P12</td>
<td>120 100 80 60 40 20</td>
</tr>
<tr>
<td>P41</td>
<td>100 80 60 40 20 0</td>
</tr>
<tr>
<td>MSP1_{42}</td>
<td>120 100 80 60 40 20</td>
</tr>
</tbody>
</table>
Figure 3.8. Depletion assays also demonstrate a lack of erythrocyte binding of P12 and P41.

(A) Another gentler depletion assay was performed to assess the erythrocyte binding deficiency of P12 and P41. Culture supernatant was mixed with the uninfected erythrocytes for several rounds and an aliquot from each exposure was examined by SDS-PAGE and immunoblot analysis. EBA175 and MSP1_{42} were probed for as positive erythrocyte-binding controls. The starting sample (a) and the mock control (i.e. no erythrocyte; b) were included for comparison. (B) The band intensities were quantified using ImageJ software and the relative amounts calculated as percentages of the starting material. The intensity of each band was plotted and the lines were drawn using nonlinear regression analysis in the Graphpad Prism software. The graph clearly displayed the decrease of EBA175 (black), which has a specific erythrocyte receptor, glycosphorin A, down to ~10 % of the original level. MSP1_{42} (green) was also depleted by interacting with heparin-like molecules on the erythrocyte surface to ~30 % of its starting material. P12 (blue) and P41 (red) showed slight decreases after 5 rounds of exposure to the erythrocytes suggesting that they may not have specific receptors.
Figure 3.9. P12 and P41 do not bind to heparin.
Culture supernatant (S/N) containing merozoite surface proteins including P12 and P41 were combined with heparin-agarose beads and the bound proteins were examined by SDS-PAGE and western blot. The proteins eluted from mixing the sample with nickel-nitrilotriacetic acid (Ni$^{2+}$-NTA) resin were used as a control. MSP1_{42} was found to bind to the heparin-agarose beads as expected, but both P12 and P41 did not. EBA175 as a negative control also showed no binding to the heparin-agarose beads. All of the proteins probed for were found in the Ni$^{2+}$-NTA bound samples which presumably represented non-specific interactions.
**A**

Knockout plasmid

\[ \text{cytosine deaminase} \]

\[ \text{pOC1} \]

\[ 5' p12/p41 \]

\[ \text{hDHFR} \]

\[ 3' p12/p41 \]

WT p12/p41 locus

\[ \text{p12/p41 gene} \]

\[ \text{WT PCR} \]

\[ 5' \text{flank} \]

\[ 3' \text{flank} \]

Δp12/Δp41 locus

\[ \text{Δp12/Δp41} \]

\[ \text{hDHFR} \]

\[ \text{Δp12/Δp41} \]

\[ 5' \text{flank} \]

\[ 3' \text{flank} \]

\[ \text{Δp12/Δp41} \]

\[ \text{Δp12/Δp41} \]


**B**

**Δp12 3D7**

\[ \text{kb} \]

\[ \text{WT} \]

\[ 0 \text{ cyc} \]

\[ 2 \text{ cyc + 5-FC} \]

\[ 5' \text{flank} \]

\[ 3' \text{flank} \]

XbaI/AvaI digestion

\[ \text{WT} = 3 \text{ kb} \]

\[ \text{plasmid} = 3.8 \text{ kb} \]

\[ \text{KO} = 2 \text{ kb} \]

**Δp41 3D7**

\[ \text{kb} \]

\[ \text{WT} \]

\[ 0 \text{ cyc} \]

\[ 2 \text{ cyc + 5-FC} \]

\[ 5' \text{flank} \]

\[ 3' \text{flank} \]

HindIII/SphI digestion

\[ \text{WT} = 1.8 \text{ kb} \]

\[ \text{plasmid} = 4.3 \text{ kb} \]

\[ \text{KO} = 3.3 \text{ kb} \]

\[ \text{WT} = 0.6 \text{ kb} \]

\[ \text{plasmid} = 1.7 \text{ kb} \]

\[ \text{KO} = 1.1 \text{ kb} \]


**C**

\[ \text{kb} \]

\[ 3D7 \]

\[ Δp12 C1 \]

\[ Δp41 C1 \]

\[ 5' \text{site p12} \]

\[ 3' \text{site p12} \]

\[ 5' \text{site p41} \]

\[ 3' \text{site p41} \]

**Expected sizes:**

\[ 5' \text{site p12} = 909 \text{ bp} \]

\[ 3' \text{site p12} = 1,064 \text{ bp} \]

\[ 5' \text{site p41} = 904 \text{ bp} \]

\[ 3' \text{site p41} = 1,001 \text{ bp} \]
Figure 3.10. P12 and P41 genes can be deleted in *P. falciparum* asexual stage.

(A) The diagram depicts the plasmid outline and strategy used for disrupting the *p12* and *p41*. Regions from P12 and P41 genes designed to be integrated into via double homologous recombination were cloned and put in a pCC1 integration plasmid having a positive drug selection encoding a human dihydrofolate reductase (hDFHR) gene. A negative drug selection cytosine deaminase gene was included to eliminate parasites from the transfected population which remained having the plasmid backbone. (B) To validate the successful integration events genomic DNA of wildtype 3D7, Δ*p12* 3D7, and Δ*p41* 3D7 were extracted for Southern blotting analysis. The samples were digested with the designated restriction enzymes and probed with the labelled 5′ flank and 3′ flank DNA fragments. Genomic DNA from Δ*p12* and Δ*p41* parasites after 2 rounds (2cyc + 5-FC) and 3 rounds (3cyc + 5-FC) of drug cycling displayed the expected sizes of DNA fragments corresponding to parasites that had undergone double crossover recombination. Δ*p41* parasites still contained a subpopulation of wild-type parasites, therefore the Δ*p41* lines were cloned by limiting dilution. (C) The extracted gDNA from 3D7, Δ*p12* clone 1, and Δ*p41* clone 1 were also confirmed for homologous recombination by PCR with double crossover specific primers. All primer pairs gave expected results for both 5′ and 3′ integration sites of Δ*p12* and Δ*p41* parasites indicative of successful disruption of the genes.
Figure 3.11. P12 and P41 are not expressed in the Δp12 and Δp41 parasites.

(A) Total proteins extracted in 1 % Triton X-100 in PBS from schizont stage of the Δp12 and Δp41 clones were separated in SDS-PAGE and probed for P12 and P41 in western blots. The result indicates that Δp12 and Δp41 lines no longer express P12 and P41 respectively, as expected. (B) Δp12 and Δp41 clone lines were also examined for the loss of P12 and P41 expression by immunofluorescence microscopy. The fixed parasites were probed with rabbit anti-P12 and anti-P41 IgGs in combination with the merozoite surface marker MSP1 mAb followed by corresponding secondary antibodies as described in the text. Together with the positive control parental 3D7 line the results confirm the absence of protein expression in the mutants aside from minor cross-reactivity.
A

Parasites

growth rates (fold per cycle)

307  Δ36 Cl.4.9  Δp12 Cl.1

P = 0.6667

P = 0.6667

B

Parasites

growth rates (fold per cycle)

307  Δ36 Cl.4.9  Δp4 Cl.1

P = 0.6667

P = 0.3333

C

Rabbit IgG made to E. coli recombinant 6-cys proteins (2 mg/mL)

growth (% pre-immune rabbit IgG 2 mg/mL)

anti-P12  anti-P41

All stages  Late-stage
Figure 3.12. Genetic deletion and invasion inhibition assays suggest a non-essential role of P12 and P41 in *P. falciparum* asexual stage.

Growth rates of parental 3D7, Δp12 clone 1, Δp41 clone 1, and Δp36 clone 4.9, as a transfected parasite control, were measured. The parasites were synchronised and adjusted to 1 % parasitemia followed by 100,000 fold dilution in duplicate. They were monitored for 6 cycles until one of the parasite lines reached 1 % parasitemia. The parasitemia for all of the lines was then counted and their amplification rate per cycle calculated (n = 2). Parental 3D7, Δp12 clone 1, Δp41 clone 1, and Δp36 clone 4.9 had similar growth rates of 6.77 ± 0.29, 6.67 ± 0.36, 6.56 ± 0.10, and 7.02 ± 0.21 fold per cycle, respectively, as shown in the graph (mean with SD). The growth rates of the Δp12 clone 1 (A) and Δp41 clone 1 (B) were compared in a pair-wise manner with the parental 3D7 and the Δp36 clone 4.9 transfected parasite control and they were found to be not statistically different (P-values: 3D7 vs Δp12 clone 1 = 0.6667, Δp36 clone 4.9 vs Δp12 clone 1 = 0.6667, 3D7 vs Δp41 clone 1 = 0.6667, Δp36 clone 4.9 vs Δp41 clone 1 = 0.3333; Mann-Whitney Test). (C) Invasion inhibition assays with rabbit IgGs raised against *E. coli* recombinant P12 and P41 demonstrated only modest inhibition. Purified merozoites were incubated with 2 mg/mL of polyclonal rabbit anti-P12 and anti-P41 IgGs, and the parasitemia was measured 40 h later by flow cytometry. The percentage of invasion inhibition was calculated for total parasites and schizont-stage parasites by using rabbit pre-immune IgG as a control. The results showed that rabbit anti-P12 and anti-P41 IgGs could inhibit parasite invasion by 15 - 20 %.
CHAPTER 4

Biochemical and Functional Analysis of Blood-Stage 6-Cys Proteins: P38 And P92

Introduction

The asexual stage *P. falciparum* parasites express 4 members of the 6-cys protein family (Sanders et al., 2005). Two of them, P12 and P41, have been substantially studied and reported in the previous chapter. Since a definitive role for P12 and P41 in blood-stage parasites could not be established, two other members of the 6-cys family, P38 and P92, were studied in the hope that they may provide functional insight into the family.

P38 was discovered from a comparative genomic study using the 6-cys domain to search for other members of the family, and was found to be conserved throughout the genus (Thompson et al., 2001b). Like most other 6-cys proteins, P38 contains the two 6-cys domains (P12, P41, P47, P48/45, P36 and P36p). The expression of P38 in *P. falciparum* was confirmed in the proteomic study of detergent-resistant membrane proteins along with other blood-stage 6-cys proteins (Sanders et al., 2005). P38 was predicted to be a GPI-anchored protein due to the presence of a conserved GPI-anchor signal sequence at its C-terminal end (Sanders et al., 2005). This was later confirmed by biochemical means (Gilson et al., 2006). To localise P38 in developing merozoites it was expressed as a GFP-tagged chimeric protein and was found to concentrate at the merozoite’s apical end. Bioinformatic analysis of P38’s transcriptional profile indicates that it is also present in gametocytes and sporozoites (Gilson et al., 2006). An attempt to delete *p38* has been successful in the rodent malaria parasite, *P. berghei*, but did not reveal any altered phenotype compared to the wild type parental parasites during asexual growth, sexual stage development, and oocyst maturation (van Dijk et al., 2010).

P92, originally annotated as a putative GPI-anchored protein, was firstly identified to be a part of merozoite detergent-resistant membrane proteins, and was confirmed to be membrane bound via a GPI anchor (Gilson et al., 2006; Sanders et al., 2005). Unlike other blood-stage 6-cys proteins, P92 has other cysteine-rich domains in addition to a single unusual 6-cys domain that comprises only 4 cysteine...
residues corresponding to the C3 - C6 in the typical domain. Similar to P12, an N-terminal fusion of GFP to P92 demonstrated the protein was targeted to the merozoite surface (Sanders et al., 2005). Deletion of p92 in the CS2 strain of *P. falciparum* appeared lethal to the parasites suggesting that P92 might play an important role in the asexual stage (Sanders et al., 2006). Nonetheless, there has been no further study with respect to P92 so far.

In this chapter I describe biochemical and functional studies of the other two blood-stage 6-cys proteins, P38 and P92, in *P. falciparum*. Firstly the rabbit polyclonal anti-P92 antibody was generated from the recombinant protein of the predicted 6-cys module of P92 for the further experiments. The expression and the shedding of P38 and P92 from the merozoite surface were then studied as this information might hint at the proteins’ possible functions. The localisation of both proteins in the parasites was re-examined by immunofluorescence microscopy. There localisation of the surface and apical region of the merozoite supported their role as invasion ligands and thus functional studies were performed to prove this hypothesis. Erythrocyte-binding and heparin-binding assays were used to study since these could be possible roles for the proteins. To further validate whether P38 and P92 might facilitate the entry of the parasite into the host erythrocyte, invasion inhibition assays using rabbit anti-P38 and anti-P92 polyclonal antibodies was done. The essentiality of P38 and P92 for the blood-stage parasites was also examined by a genetic approach. Attempts to delete p38 and p92 were made in multiple laboratory parasite strains and the growth rates of the mutants were measured to determine if their invasion rates had declined after losing the proteins.

**Results**

*Expression of recombinant 6-cys domain of P92 in E. coli for polyclonal antibody production*

To begin characterising P92, protein-specific antibodies needed to be raised. Since P92 was predicted to have a unique 6-cys domain, this region of the protein (G574 - P758) was selected for recombinant expression in *E.coli* (Fig. 4.1A). The forward DNA primer containing a BamHI restriction site and the reverse DNA primer containing a SpeI restriction site were synthesised and used to amplify the 6-cys domain region of P92 from genomic DNA of the 3D7 parasite line by PCR. The DNA fragment was digested and ligated into the pPROEX HTb expression vector that had
been digested with the same enzymes. After transformation of the ligated products into XL1 Gold (Stratagene) plasmid DNA was grown from several clones and the presence of a P92 insert was validated by restriction digest analysis and PCR. The pPROEX-P92 plasmid was then transformed into the BL21 DE3 *E. coli* strain and the recombinant protein was expressed with a 6×His-tag at its C-terminus. After inducing expression with isopropyl-β-D-thiogalactopyranoside (IPTG) the bacterial cells were harvested and lysed to check the expression of the protein. Both soluble and insoluble fractions from the lysis were sampled for SDS-PAGE and western blotting analyses in reducing and non-reducing conditions. Pooled malaria-immune Papua New Guinean IgG was used to probe for the expressed 6-cys domain of P92 and the protein was found in the insoluble material as ~25 kDa protein as expected (Fig. 4.1B). Sera from malaria non-immune individuals in Melbourne did not react with the P92 fragment confirming that the detection was specific for a recombinant protein from the malaria parasite. The expressed protein in the inclusion bodies was then subjected to denaturation and purification processes using urea and Ni²⁺-NTA agarose resins, respectively. The purified protein was subsequently put through a refolding process with a glutathione redox pair followed by another purification step with size-exclusion chromatography. The refolded protein was pooled and used for antibody production.

*Biochemical characterisation of P38 and P92 in blood-stage P. falciparum*

The basic biochemical properties of P38 and P92 were next determined. As P38 and P92 were thought to be components of the merozoite’s surface protein coat, their expression and cleavage from the plasma membrane were studied. Total proteins at schizont stage were prepared from the saponin-lysed parasites and solubilised with 1 % Triton X-100 in PBS solution. Proteins in the soluble and insoluble fractions were separated by SDS-PAGE under non-reducing conditions followed by western blotting. P38 was detected as a soluble protein from this preparation as expected (Fig. 4.2A; left panel). For the characterisation of P92, total proteins from the Δp92 parasites (described below) were also included in the analysis to ascertain the specificity of the newly generated antibodies to the native P92 protein band. The expression of P92 was firstly evaluated by using 1 % Triton X-100 in PBS solution to solubilise the schizont proteins as was done with other 6-cys proteins. Interestingly
P92 was mainly detected in the insoluble fraction. This result suggested that P92 might have strong association with membranes and/or cytoskeleton and might require more stringent solubilisation methods. Proteins from saponin-lysed parasites were then prepared in increasingly stringent buffers, namely Triton X-100, RIPA and 2D respectively, and both soluble and insoluble fractions of wild-type and Δp92 parasites from the three different solubilisation methods were fractionated by SDS-PAGE. Western blots of these were probed for P92 and the protein appeared soluble only in the highest stringency 2D buffer demonstrating its strong association with membrane/cytoskeleton (Fig. 4.2B; left panel). Culture supernatant collected post-invasion was also subjected to SDS-PAGE and western blotting in non-reducing condition to examine whether P38 and P92 were cleaved from the merozoite surface similar to the P12/P41 heterodimer described in the previous chapter. Surprisingly P38 was not found in the culture supernatant indicating that it remained on the merozoite surface and could possibly be carried into the newly invaded erythrocytes (Fig. 4.2A; right panel). P92 was shed from the merozoite surface as anticipated (Fig. 4.2B; right panel).

*Localisation of P38 and P92 in late-stage P. falciparum and the evidence of P38 being carried into the erythrocyte*

A previous study had shown that P38-GFP and P92-GFP fusion proteins expressed from episomes localised to the apical end and surface of merozoites respectively (Sanders et al., 2005). To ensure that the GFP-fusion proteins localisation remained the same as the native proteins, antibodies specific to P38 and P92 were used in the immunofluorescence microscopy studies. Dried blood films on glass slides containing schizont, merozoites and young ring stage parasites were fixed using ice-cold methanol. The cells were primarily probed with anti-P38 or anti-P92 rabbit IgGs together with a mouse anti-AMA1 monoclonal antibody (1F9) or a mouse anti-MSP1<sub>19</sub> antibody as controls. Secondary fluorophore-conjugated goat antibodies specific to either mouse or rabbit primary antibodies were used to visualise the location of the stained proteins under fluorescence microscope. The images obtained for P38 were not of great quality, nonetheless the results showed that some P38 located to the merozoite surface with most having an apical concentration similar to the localisation pattern of AMA1 (Fig. 4.3A). This is a little different than previous observation using a GFP-P38 fusion protein which demonstrated the fusion
concentrated almost entirely at merozoite apex with none on the surface (Sanders et al., 2005). Since P38 was not found in the culture supernatant, this raised the possibility of the protein being carried into the erythrocyte after invasion. Therefore newly formed ring-stage parasites were examined for the presence of P38 and it was indeed detected supporting the notion that it remained on the merozoite surface during invasion (Fig. 4.3A).

Regarding the localisation of P92, the protein was found to concentrate at merozoite apical organelles differing from the micronemes since the P92 did not co-localise with AMA1 (Fig. 4.3B). After the egress of free merozoites P92 remained inside its apical organelles and did not redistribute to the merozoite surface like AMA1 does (Fig. 4.3B). Given the extreme apical location of P92 it could reside in the rhoptry neck but time did not permit validation of this with antibodies to the rhoptry neck marker RON4. The location of P92 was contrary to a previous finding which showed that the GFP-P92 chimeric protein localised around the merozoite surface and indicates the N-terminal GFP partner may have prevented P92 from being correctly trafficked (Sanders et al., 2005).

**P92 may contribute to the interaction of merozoites and host erythrocytes during invasion but not through heparin-like molecules**

Since P38 remained on the merozoite surface and was carried into the host erythrocytes, it was impossible to assay for its erythrocyte-binding activity in its native form like other known invasion ligands which are shed into the culture supernatant. As a result only P92 was tested to determine if it could facilitate the binding of merozoites to the erythrocytes. Culture supernatant collected from the synchronous parasite culture following invasion was mixed repeatedly for 6 times with fresh erythrocytes. Identical aliquot volumes from each round of incubation were taken and prepared for SDS-PAGE and western blotting in non-reducing condition. The initial input and the mock control without erythrocytes were included in the analysis as the references for original level of the proteins in the culture supernatant. Upon serial incubation with fresh erythrocytes, it was evident that the amount of P92 was continually decreased when exposed to the erythrocytes (Fig. 4.4). This depletion of P92 was comparable to the reduction of MSP142, but was less pronounced than the decrease of EBA175. MSP142 is a known invasion ligand which was demonstrated to interact with heparin-like molecules on the erythrocyte surface (Boyle et al., 2010a),
on the other hand EBA175 specifically binds to an erythrocyte membrane protein, glycophorin A (Sim et al., 1994). The similar level of depletion of P92 to MSP142 implied that P92 might aid the initial binding of the merozoite to the host erythrocyte through low-affinity interaction with heparin-like molecules rather than having a specific receptor protein on the erythrocyte surface like EBA175. To further validate if P92 could be binding to heparin-like molecules, a heparin-binding assay was performed to prove this hypothesis. The culture supernatant was incubated with heparin-agarose beads and the bound proteins were eluted with non-reducing SDS sample buffer. Western blotting confirmed the interaction of MSP142 with heparin molecules as a positive control, however there was no P92 observed binding to the heparin beads (Fig. 4.5). Ni²⁺-NTA agarose beads were intended to be used as a negative control, but every protein probed for appeared to stick to the beads presumably via non-specific interactions. Despite having found that P92 was depleted upon multiple exposures to the fresh erythrocytes suggestive of its role as an invasion ligand, the identity of its interacting partner remains unknown.

**P38 and P92 are not essential in blood-stage *P. falciparum***

Since the localisation and erythrocyte binding studies did not provide definitive information regarding the functions of P38 and P92 in blood-stage parasites, a genetic knockout approach was adopted. It was hoped that by disrupting the p38 and p92 genes phenotypic changes in parasite growth or merozoite invasion might be informative as to the proteins’ functions. Primers were designed to amplify the 5′ and 3′ regions of the p38 and p92 from the parasite's genomic DNA. These 5′ and 3′ gene fragments of both genes were subsequently cloned into the pCC1 construct flanking the gene expression cassette of positive drug selection marker, human dihydrofolate reductase (hDHFR) (Fig. 4.6A). This construct also comprises the negative drug selection cassette expressing cytosine deaminase, which helps in the process of selecting the double recombination knockout parasites in favour of parasites containing plasmid only or integrations that had chromosomally inserted via single cross-over recombination.

The plasmids were transfected into two laboratory strains of *P. falciparum*, W2mef and CS2. Once drug-resistant transfecants appeared they were subjected to 2 - 3 cycles of positive/negative drug selection to obtain parasites that had lost their plasmids and undergone successful double homologous recombination removing the
$p38$ or $p92$ from their genomes. Genomic DNA of the transfected parasites at each cycle of drug selection was extracted and analysed by Southern blotting to confirm that gene replacement had occurred. Shown in the Fig 4.6B are the Southern blot results of the $\Delta p38$ and $\Delta p92$ in W2mef parasite lines. The genomic DNA of wild-type W2mef, $\Delta p38$ W2mef before the drug cycling, and after 2 and 3 cycles of drug selection were digested with PstI and SpeI, and were subsequently probed with 5' flank DNA fragment. The band at around 3.2 kb was evidently observed only after drug cycling indicative of successful homologous recombination as predicted. To confirm successful $p38$ gene replacement the same genomic DNA samples were digested with SpeI and HpaII and were probed with 3' flank DNA fragment. The Southern blot result showing the DNA band around 3.4 kb ascertained that the endogenous $p38$ was successfully replaced by the hDHFR gene expression cassette. Since there was still a 2.8 kb band corresponding to the transfection plasmid, this meant that not all of the parasites had undergone homologous recombination. The $\Delta p38$ W2mef culture was therefore subjected to cloning by limiting dilution to obtain a pure lineage of the knockout. The Southern blot analysis of the $\Delta p92$ W2mef was performed in a similar way. The genomic DNA of the wild type and transfected W2mef, before and after drug selections, were digested with XmnI or ClaI restriction enzymes for the detection by 5' flank and 3' flank DNA fragments respectively. The blot clearly showed the DNA bands around 5.2 kb for the 5' probe and 5.1 kb for the 3' probe after 2 cycles of drug selection indicative of successful deletion of the $p92$. As shown previously, the band matching the predicted sizes of the transfection vector, around 9 kb and 8.8 kb in 5' and 3' probe detections respectively, remained visible, hence the $\Delta p92$ W2mef parasites were subjected to limiting dilution to clone out the individual knockout line as well.

To confirm that the Southern blot results were correct and P38 and P92 proteins were no longer being expressed western blots of these parasites were probed with rabbit anti-P38 and anti-P92 IgGs. In $\Delta p38$ parasites a band detected in the wild-type parental line migrating at ~40 kDa was absent confirming loss of protein expression (Fig. 4.6C). Western blot of $\Delta p92$ parasite protein extract confirming loss of protein expression were performed previously (Fig. 4.2B). With the knockout of both $\Delta p38$ and $\Delta p92$ ascertained by western blots it is evident that neither of these proteins is essential for in vitro blood-stage growth.
The absence of P38 and P92 in the blood-stage P. falciparum do not significantly alter the growth rates of the parasites in vitro

Measurement of the growth rates in vitro was carried out to examine whether losing P38 or P92 would affect the fitness of the blood-stage P. falciparum. The wild type W2mef, Δp38 W2mef clone 1, Δp92 W2mef clone 1, and Δrh4 W2mef, as a control for the transfected line known to have a normal growth phenotype (Stubbs et al., 2005), were tightly synchronised and their parasitemia was adjusted to 1%. All parasite cultures were then diluted 100,000 fold in duplicate and their parasitemias were followed until one of them recovered back to 1%, which took around 6 asexual growth cycles. The parasitemia of all the lines were counted and the average amplification rates for 6 cell cycles were calculated from 2 independent experiments. The wild-type W2mef had the highest amplification rate of 7.63 ± 0.07 fold per cycle, while the Δrh4, Δp38, and Δp92 W2mef had 6.43 ± 0.08, 6.66 ± 0.24, and 6.91 ± 0.05 fold per cycle, respectively (Fig. 4.7A, Fig. 4.7B). Mann-Whitney analysis was applied to measure whether these differences were significant in a pair-wise manner between the controls and the Δp38 and Δp92 parasites. As shown in the Fig. 4.7A and 4.7B the P-values indicated that the growth rates of the Δp38 and Δp92 parasites were not significantly slower than the controls. These findings infer that neither P38 nor P92 are necessary for the normal blood-stage growth of P. falciparum, at least in vitro.

Blocking P38 and P92 with antigen-specific antibodies do not substantially inhibit the invasion of merozoites into the host erythrocytes

By taking the genetic approach and removing p38 and p92 from the parasite genomes and assessing their necessity for the asexual growth, it can be concluded that the mutant parasites appear to grow normally without these genes. As the process of producing transfected parasites requires long-term culturing, this could give the parasites time to adapt to their gene losses and regain normal growth rates. Examining the role of P38 and P92 directly and immediately in wild-type parasites could reveal if these proteins are important for growth and invasion. Invasion inhibition assays with antibodies raised to these proteins were therefore carried out to test this hypothesis. The purified merozoites were allowed to invade in the presence of either the pre-immune, anti-P38, or anti-P92 rabbit IgG at the final concentration of 2 mg/mL, and the number of parasites were measured 40 h later by flow cytometry. Successful
invasions of the parasites blocked by anti-P38 and anti-P92 IgGs were calculated and presented as the growth percentages of pre-immune rabbit IgG control (Fig. 4.7B). Flow cytometry revealed that late stage and total parasites numbers had declined by 15 – 20% following treatment with anti-P38 and anti-P92 rabbit IgG relative to the pre-immune control. Given the relatively high concentration of the antibodies used in the assays, the inhibitory effect was small. The weak effect of the antibodies upon invasion was probably indirect and mostly like due to them coating the merozoites and interfering with important invasion ligands.

**Discussion**

This chapter reports the biochemical characterisation and the attempts to demonstrate the function of another two blood-stage 6-cys proteins, P38 and P92, in *P. falciparum*. Both P38 and P92, like their P12 and P41 relatives, are found conserved throughout the *Plasmodium* genus with the exception that P92 is restricted to the primate *Plasmodium* species. This characteristic raises the possibility of them having a crucial role in the parasite life cycle.

The properties of native P38 and P92 in blood-stage parasites were firstly examined. Since P92 had not been characterised before, anti-P92 antibodies required for the study were unavailable. To produce these antibodies a recombinant fragment of P92 was expressed in *E. coli*. Due to the cysteine-rich nature of P92, expression of full-length protein would have been very difficult and therefore only P92’s predicted 6-cys domain was chosen for *E. coli* expression. P92 is the most unique 6-cys protein among the other blood-stage members as it is predicted to have only one 6-cys domain that contains only 4 cysteine residues, corresponding to C3 - C6 of a typical 6-cys domain. The antibodies raised to this region, after being IgG-purified, could detect a band consistent with the size expected for P92. Unfortunately the rabbit antibodies also detected other bands suggesting the antibody reagent was not extremely specific. To ensure the band suspected of being P92 was the genuine protein samples from the Δp92 parasite line were also probed and an ~80 kDa band was absent confirming the antibody reagent did recognize a protein of the expected size corresponding to P92 (discuss below).

From blood-stage parasite extracts P92 was remarkably found insoluble in PBS buffer containing 1% Triton X-100. Other GPI-anchored 6-cys proteins, P12 and P38, were all soluble using this solubilisation method (Fig. 3.2A and Fig. 4.2A).
therefore this finding suggests diversity in the nature of how P92 associates with cytoskeletons and membranes compared to other 6-cys proteins. To further explore how strongly P92 was membrane associated, saponin-lysed parasites were solubilised in RIPA buffer and 2D sample buffer which both have higher stringency than the 1 % Triton X-100 buffer used previously. In addition to Triton X-100, RIPA buffer includes ionic detergents, i.e. deoxycholate and SDS, to help solubilise the proteins. 2D sample buffer on the other hand, contains urea which also denatures sample proteins, thereby enhancing its solubilisation effectiveness. The fact that the solubility of P92 was low and required the strongest 2D buffer for solubilisation suggests a strong association of P92 with the membrane and cytoskeleton. This is rather surprising since P92 is just a GPI-anchored protein (Gilson et al., 2006), unlike integral or membrane-spanning proteins that require strong detergents to dissolve (Bullen et al., 2009). It is also possible that P92 is a part of a larger protein complex that is tightly associated with the plasma membrane and/or cytoskeleton of parasites.

Localisation of P38 and P92 were previously investigated by Sanders et al. using GFP-fusion chimeric proteins expressed episomally in the parasites (Sanders et al., 2005). They showed that GFP-P38 was located in apical organelles, while GFP-P92 was on the periphery of the merozoites. Because the chimeric proteins were not expressed under the control of native P38 and P92 promoters (Meissner et al., 2005; Sanders et al., 2005), it was possible that the GFP-P38 and GFP-P92 were transported to a different location than their native forms. Moreover, high expression of green fluorescent proteins could also lead to an incorrect trafficking of the proteins (Lenassi Zupan et al., 2004). In this study the location of the native proteins were re-examined using antigen-specific antibodies. In contrast to published results, P38 was observed localised on both the surface of the merozoites and to the apical end, while P92 was strictly localised to the apical organelles in schizonts and free merozoites (Fig. 4.3). The resolution of the images obtained for P38 were not of high quality, therefore further studies using various other methods of cell fixation are required to confirm the localisation of P38 in the merozoite. The majority of GPI-anchored proteins are targeted to the plasma membrane (e.g. MSP1, MSP2, P12), however P92 appears to be an exception as it was strongly detected only at the apical ends of the merozoite. Another Plasmodium GPI-anchored protein demonstrated to completely localise to the rhoptry neck is Pf34 (Proellocks et al., 2007). Pf34 was carried into the newly invaded erythrocyte possibly facilitating formation of the parasitophorous vacuole in
the young ring-stage parasite (Proellocks et al., 2007). However P92 was detected in
the culture supernatant implying that P92 was secreted at some point, presumably to
function during the pre-invasion period, and was subsequently cleaved by a protease
like other shed surface proteins (Fig. 4.2B).

Surprisingly P38 was undetectable in the culture supernatant suggesting that it
was not shed from the merozoite plasma membrane. Most surface proteins that are
cleared from the merozoite plasma membrane appear to act as invasion ligands and
the parasites need to eliminate a large proportion of these proteins in order to
complete the invasion. Other surface proteins however have been found to be carried
into the newly invaded erythrocytes where they are displayed on the surface of ring-
stage parasites (Boyle et al., 2014). It has been speculated that proteins carried into
the erythrocytes might have a role during internalisation or after invasion assisting in
intraerythrocytic development of the ring-stage parasites. For example MSP2 was
found to be rapidly degraded soon after the invasion, while MSP4 remained
detectable for a longer period of time post-invasion (Boyle et al., 2014). Both proteins
were taken in without processing, unlike MSP1 which has only a small part, MSP119,
carried into the erythrocytes after proteolytic processing (Blackman et al., 1990).
MSP119 has been suggested to play a role in facilitating the formation of the early
stages of the digestive vacuole (Dluzewski et al., 2008). Although a role for MSP4
has not been revealed, it is also thought to take part in an early developmental stage
of the parasites since it is completely carried in. Because P38 was not detectable in the
culture supernatant and the localisation study revealed that P38 was present in the
young ring-stage parasites (Fig. 4.2A and Fig. 4.3A), it could be assumed that P38
was carried into the newly invaded erythrocytes fully intact. As a result, it is
reasonable to speculate that P38 may play a role in an early ring-stage development
similar to MSP4. The sporozoite 6-cys proteins, namely P52 and B9, have also been
demonstrated to play their post-invasion role in facilitating the maintenance of
parasitophorous vacuole membrane (Labaied et al., 2007; van Dijk et al., 2005;
Annoura et al., 2014), hence it is possible that P38 might play a similar function. It
will also be interesting to examine how long P38 remains detectable in the ring-stage
parasites as it may be informative for determining the role of P38.

The fact that P92 was detected in the culture supernatant suggested a role of
P92 in some pre-invasion event, presumably involved in binding to erythrocytes.
Consequently the shed P92 was tested for erythrocyte-binding activity. The gentler
depletion assay was adopted rather than performing the typical erythrocyte-binding assay because it does not include centrifugation of the erythrocytes through a dibutyl phthalate oil layer. P92 demonstrated moderate level of erythrocyte-binding activity of similar to MSP142. The erythrocyte-binding ability of MSP142 was clearly illustrated by Boyle et al. as well as the molecules that MSP142 binds to, namely heparin and heparin-like molecules (Boyle et al., 2010a). The binding efficacy appears to be mainly dependent on the level and pattern of sulfation and less so on the type of sugar (Boyle et al., 2010a). Heparin-like sugars were reported to be present on the human erythrocyte surface (Vogt et al., 2004), thereby supporting the notion that MSP142 facilitates the binding of merozoites to the host erythrocytes. Since P92 demonstrated a comparable erythrocyte-binding level as MSP142, P92 was tested for the heparin-binding activity. P92, however, did not seem to bind to heparin and it is possible that P92 might bind to a different type of sugar and/or different level and pattern of sulfation or even to an erythrocyte surface protein. As suggested for the P12 and P41 heterodimer, the identification of sugar/protein molecules P92 might be binding to is worth pursuing as this might be further developed into a therapeutic agent against malaria. Glycan array experiments are a possible way to reveal whether P92 is able to bind to any sugar molecules at all (Geissner et al., 2014). Otherwise P92 may act in a completely different role yet to be explored.

Having mentioned the probable roles of P38 and P92, attempts to block the proteins from functioning were performed by means of invasion inhibition assays. Since P38 and P92 appear on the merozoite surface/apical organelles and in just the apical organelles respectively, it was assumed that the two proteins might be involved in erythrocyte invasion. The purified rabbit anti-P38 and anti-P92 polyclonal IgGs were incorporated into the invasion assays to bind to and presumably inhibit the functions of their target proteins. The 15 – 20 % reduction in invasion efficiency following antibody treatment compared with the control is interpreted to be from non-specific activity of the high concentrations of antibodies used that probably coat the merozoite surface and interfere with other invasion ligands. In comparison to our antibodies, rabbit anti-Rh5 IgG can inhibit invasion by up to 50 % when it was used at 0.5 mg/mL (Douglas et al., 2011), compared to the 2 mg/mL concentration used here. A similar invasion inhibition efficacy to anti-Rh5 was achieved with anti-AMA1 polyclonal antibodies (Coley et al., 2006; Healer et al., 2004). This could be reflective of the less important role of that P38 and P92 play during invasion compared to Rh5
and AMA1 (Crosnier et al., 2011; Douglas et al., 2011; Yap et al., 2014). The fact that p38 and p92 can be knocked out in blood-stage parasites without displaying any major phenotype also supports this scenario (discuss below). Furthermore, as P38 appeared to be carried into the erythrocyte suggests it may have a post-invasion role and the observed invasion inhibitory effect of anti-P38 IgG may alternatively support the non-specific action of the antibodies during the invasion event. There is evidence that antibodies to some merozoite surface proteins can be carried into the host erythrocytes during invasion (Boyle et al., 2014) raising the possibility that the anti-P38 IgG may follow the same path and interfere with the parasite development at post invasion stage.

Since the functional assays described above did not provide much information regarding the role of P38 and P92, the genetic deletion experiments were performed to ascertain the essentiality of the two proteins. The p38 gene was removed from W2mef and p92 was deleted from W2mef and CS2. The growth rates of the knockouts in W2mef were compared with the wild-type and another knockout line, Δrh4 W2mef, known to have a normal rate of growth in the asexual stage (Stubbs et al., 2005). The knockouts seemed to have a slight decrease in growth compared with the wild-type, however the rates were still quite high among the knockouts. This observation could be explained by the fact that the transfection process, especially to obtain a knockout parasite via homologous recombination, requires a considerably long duration of roughly 2 - 3 months, equivalent to 30 - 40 cell cycles, at the very least to complete the whole procedure. This may allow potentially impaired parasites sufficient time to adapt and compensate for their gene loss. By the time these knockout parasites were tested for their amplification rates, their growth could have been restored back to almost normal levels.

One reason the Δp38 and Δp92 parasites might have behaved normally is because the roles were compensated for by the upregulation of other proteins. This is a well-established mechanism in Plasmodium parasites whereby a functionally related substitute protein is upregulated after the loss of another protein. A widely known example is Rh4, an invasion ligand responsible for the sialic acid independent invasion pathway, which is highly expressed after the W2mef parasite loses its main invasion ligand EBA175 (Stubbs et al., 2005). Microarray analysis was used in the aforementioned study to identify the upregulation of the compensatory genes/proteins,
and this approach could also be used to determine the changes that have occurred in the Δp38 and Δp92 mutants.

To overcome the drawback of this knockout strategy, a rapid deletion of a particular gene could be performed to observe the effect of losing a gene in one asexual cycle. A number of new technologies have been developed over the last few years to achieve such goal. One method introduces loxP sites to either side of the gene of interest which are the targets of two part DiCre recombinase. Upon dimerisation and activation of the DiCre recombinase with rapamycin, the targeted gene is then excised (Collins et al., 2013; Yap et al., 2014). This strategy was shown to be rapid and highly efficient at deleting a gene in one asexual cycle. Another approach that could be employed is to append the 3’ untranslated region of a gene of interest with the glmS ribozyme. When the inducer, glucosamine, is added, the 3’ untranslated region is cleaved and the mRNA is destabilised (Prommana et al., 2013). This results in the knockdown of protein levels and the phenotype can be readily observed. This approach has been recently used to knock down expression of PTEX150 and resulted in substantial reduction in protein export into host erythrocytes (Elsworth et al., 2014). If the DiCre and glmS techniques had available a few years ago they might have been successfully employed by me to dissect the role of P38 and P92.

In summary this chapter demonstrates the initial characterisation of another two members of the 6-cys protein family, P38 and P92, in the blood-stage P. falciparum. The rabbit polyclonal antibodies were raised against P92 by expressing its unique 6-cys domain. The expression and localisation of both proteins were examined and discovered to be distinctive. P92 was detected in the apical organelles whereas P38 was seen there and also on the merozoite surface, suggestive of potentially different roles for the two proteins. Discovering of P38 in the newly formed ring-stage parasites and the absence of it in the culture supernatant indicate that P38 is carried into the host erythrocytes with the merozoites presumably to function in a post-invasion event. P92, on the other hand, is shed into the culture supernatant during invasion. Although this characteristic suggests that P92 could be an invasion ligand, binding experiments performed in this study provide little additional information regarding its role. It is possible that P92, as has been suggested for the P12/P41 heterodimer, could facilitate low-affinity binding of the merozoite to the host erythrocyte. Inhibiting P38 and P92 during invasion using antigen-specific antibodies does not however support these proteins having significant roles. Genetic knockout
approaches proved P38 and P92 were not essential in the asexual stage since both genes can be deleted. The mutants demonstrated comparable growth rates to the wild-type and another knockout control implying that other proteins could possibly compensate for their loss. The changes in mRNA transcription and potential compensatory proteins remain to be determined in these knockouts. The fact that these proteins are found in many Plasmodium species hints they perform a common function in the parasites. Since P92 is only present in the species infecting primates, its function is likely constrained to a limited number of host organisms. These unique features therefore make efforts to discover their functions even more important. Additional experimentations are therefore needed to clarify the functions of P38 and P92 and if these proteins can be targeted in novel therapeutics for malaria disease.
Figure 4.1. Expression of recombinant 6-cys domain of P92 for polyclonal antibody production.

(A) An illustration that depicts the characteristics of the P92 protein; ER signal sequence (white box), cysteine residues (red lines, not drawn to scale), protein spacer regions (yellow boxes), 6-cys domain (red box), and GPI-anchor (black box). The predicted 6-cys domain of the protein (G574-P758; black underline) were amplified and cloned into the pPROEX HTb expression plasmid. (B) The recombinant 6-cys domain of P92 was expressed in the BL21 *Escherichia coli* strain, and after the cell lysis both soluble (S) and insoluble fractions (I) were tested for the protein production. The extracted lysates were prepared for SDS-PAGE followed by western blotting in reducing and non-reducing conditions. The samples were probed with either pooled non-immune Melbourne (Melb) serum control or the pooled malaria-immune Papua New Guinean (PNG) IgG. The 6-cys domain of P92 was detected in the insoluble fraction indicating that the protein was expressed as inclusion bodies and required refolding. (C) The 6×His-tag recombinant protein in the inclusion bodies was solubilised and purified using Ni²⁺-NTA agarose followed by a rapid refolding process with a glutathione redox pair. The refolded protein was subsequently examined by SDS-PAGE and western blotting. The Coomassie-stained polyacrylamide gel of the non-reduced and reduced forms of the refolded recombinant protein is shown (left). The refolded 6-cys domain of P92 was recognised only by the pooled malaria-immune PNG IgG suggesting that the recombinant protein was appropriate for antibody production (right).
### A

<table>
<thead>
<tr>
<th>kDa</th>
<th>S</th>
<th>I</th>
<th>S/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td></td>
<td></td>
<td>P38</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>kDa</th>
<th>1% Triton X-100</th>
<th>RIPA</th>
<th>2D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>I</td>
<td>S/N</td>
</tr>
<tr>
<td>100</td>
<td>WT Δρ92 WT Δρ92</td>
<td>WT Δρ92</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>WT Δρ92 WT Δρ92</td>
<td>WT Δρ92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT Δρ92 WT Δρ92</td>
<td>WT Δρ92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WT Δρ92 WT Δρ92</td>
<td>WT Δρ92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S/N</td>
<td></td>
<td>P92</td>
</tr>
</tbody>
</table>
Figure 4.2. Biochemical characteristics of *P. falciparum* P38 and P92.

(A) Expression and shedding of P38 in late-stage *P. falciparum* were examined. Total proteins from schizont stage parasites were extracted in 1 % Triton X-100 in PBS. Soluble (S) and insoluble (I) materials as well as culture supernatant (S/N) containing cleaved merozoite proteins were subjected to SDS-PAGE in non-reducing conditions, and were then transferred to membrane for western blotting. Rabbit polyclonal anti-P38 IgG could detect P38 in the soluble fraction at the expected size. Surprisingly P38 was not present in the culture supernatant unlike other blood-stage 6-cys proteins. (B) Expression, solubility, and shedding of P92 in late-stage *P. falciparum* was investigated. Total proteins from magnet purified schizonts were extracted with 3 different methods; 1 % Triton X-100 in PBS, RIPA buffer, and 2D sample buffer, which are increasingly stringent, respectively. To ensure the correct identification of P92, total proteins extracted from the Δp92 parasites were used as controls. Rabbit polyclonal IgG raised to the 6-cys domain of P92 could detect the protein in insoluble samples extracted using 1 % Triton X-100 and RIPA buffers. Only the highest stringency 2D sample buffer was able to solubilise the P92 suggestive of tight association of P92 with the parasite plasma membrane and/or cytoskeleton. P92 was detected in the culture supernatant indicating that it was cleaved from the surface during invasion.
Figure 4.3. Localisation of P38 and P92 in *P. falciparum*.

(A) Immunofluorescence microscopy reveals surface and apical localisation of P38 in merozoites and that it remains present in the newly formed ring-stage parasites. Mixed populations of schizont, merozoite, and ring stages were fixed and incubated with mouse anti-AMA1 monoclonal antibody (1F9) and rabbit anti-P38 IgG as indicated followed by corresponding secondary antibodies. Another sample of schizonts and merozoites were probed with mouse anti-MSP119 antibody and rabbit anti-P38 IgG. P38 appears around the surface of the individual merozoites and also concentrates at the apical end. Unexpectedly P38 is detectable in the ring-stage parasite suggesting that it is not cleaved from the merozoite surface during erythrocyte invasion consistent with the protein analysis (Fig. 4.2A). (B) Location of P92 in schizonts and merozoites was examined by immunofluorescence microscopy. Rabbit anti-P92 IgG was used to probe fixed schizonts and merozoites, with the mouse anti-AMA1 monoclonal antibody (1F9) used as a control. The localisation of P92 was in one respect like P38 in that it localised to the merozoite apex but different in that no P92 seemed to be at the merozoite surface. P92 probably not localise to the micronemes since it did not precisely superimpose with AMA1 localisation (insets). No P92 was seen in ring-stage parasites (not shown).
**Figure 4.4. Depletion assay suggests P92 may bind to erythrocytes.**

Culture supernatant containing cleaved merozoite proteins was serially incubated with fresh erythrocytes for six times and the samples from each round were collected for western blot analysis. The starting material and the mock control (i.e. no erythrocytes) were included. EBA175 and MSP1$_{42}$ were probed for as the positive controls for erythrocyte-binding proteins. P92 was continuously depleted from the samples in every round of exposure to the erythrocytes indicating it may participate in low-affinity binding similar to that of MSP1$_{42}$ but unlike EBA175 that binds to a specific erythrocyte receptor with high-affinity.
<table>
<thead>
<tr>
<th>kDa</th>
<th>S/N</th>
<th>Control</th>
<th>Heparin bound</th>
<th>Ni-NTA bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td></td>
<td></td>
<td>MSP1</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
<td>P92</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
<td>EBA175</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.5. P92 does not bind to heparin-like molecules.
Culture supernatant (S/N) containing cleaved merozoite proteins were mixed with heparin-agarose beads, and were also incubated with Ni$^{2+}$-NTA agarose beads as a bead control. The bound proteins were eluted off the beads in non-reducing SDS sample buffer before being separated in SDS-PAGE. Western blotting was performed to analyse the heparin-bound proteins along with the controls. MSP1_{42} was clearly detected as a heparin-bound protein whereas EBA175 and P92 were not. Note that all proteins were found to bind to the Ni$^{2+}$-NTA agarose beads which was unexpected and could be due to non-specific interactions.
A

Knockout plasmid

[Diagram showing knockout plasmid and WT p38/p92 locus]

Δp38/Δp92 locus

B

Δp38 W2mef

PstI/SpeI digestion

WT = 4.6 kb
plasmid = 2.6 kb
KO = 3.2 kb

SpeI/HpalI digestion

WT = 1.3 kb
plasmid = 2.8 kb
KO = 3.4 kb

Δp92 W2mef

XmnI digestion

WT = 3.4kb
plasmid = 9kb
KO = 5.2kb

C

[Diagram showing protein bands for W2mef, Δp38 W2mef, and P38]
Figure 4.6. P38 and P92 genes can be deleted in the asexual blood stage of *P. falciparum*.

(A) A schematic depicts the strategy used to disrupt the *p38* and *p92* in blood-stage parasites. The 5' and 3' regions of the genes, were amplified and cloned into the pCC1 plasmids flanking the positive selectable marker, human dihydrofolate reductase (hDHFR), used to select for the transfected parasites. The negative selectable marker, cytosine deaminase, was included to help eliminate the transfected parasites harbouring the episomal plasmids and single crossover integrations. This resulted in a population of parasites with successful double crossover integrations that replaced the gene target and removed the cytosine deaminase. (B) The transfected parasites were put through 2 - 3 cycles of positive/negative drug selection, and the genomic DNA of the parasites from each cycle was collected for Southern blotting to confirm the correct integration. For the Δ*p38* W2mef (left panel), the DNA samples from wild-type (WT), 0, 2, and 3 selection cycles of the knockout were digested with PstI/SpeI for 5' flank probing, and SpeI/HpaII for 3' flank probing. Both southern blots confirm the presence of successful double homologous recombination at expected sizes (KOs; 3.2 kb and 3.4 kb for 5’ and 3’ flanks respectively). The verification of Δ*p92* W2mef was performed similarly (right panel). Genomic DNA of the wild-type W2mef (WT), 0 and 2 cycles of drug selection of the Δ*p92* W2mef were digested with XmnI and ClaI for the detection with 5’ and 3’ probes respectively. DNA fragments corresponding to the successful homologous recombination were observed (KOs: 5.2 kb and 5.1 kb for 5’ and 3’ flanks respectively). (C) Protein extracts in 1 % Triton X-100 in PBS from wild-type W2mef and Δ*p38* W2mef clone 1 were subjected to SDS-PAGE and western blotting. The result confirms the loss of P38 expression in the knockout parasites.
Figure 4.7. Growth comparisons and invasion inhibition assays suggest a non-invasion role of P38 and P92 in *P. falciparum* asexual blood stage.

Growth rates of $\Delta p38$ W2mef clone 1 and $\Delta p92$ W2mef clone 1 parasites were compared with the parental W2mef and $\Delta rh4$ W2mef parasites as a transfection control (Stubbs et al., 2005). All of the tested parasites were tightly synchronised and adjusted to 1% parasitemia. They were subsequently subjected to 100,000-fold dilution in duplicate and were monitored for roughly 6 cycles until one of the lines regrew to 1% parasitemia. The parasitemia of each line was then counted and the amplification rate per cycle was calculated ($n=2$). W2mef parental line, $\Delta rh4$ W2mef, $\Delta p38$ W2mef clone 1, and $\Delta p92$ W2mef clone 1 had the growth rates of $7.63 \pm 0.07$, $6.43 \pm 0.08$, $6.66 \pm 0.24$, and $6.91 \pm 0.05$ fold per cycle, respectively. The growth rates of the $\Delta p38$ clone 1 (A) and $\Delta p92$ clone 1 (B) were compared in a pair-wise manner with their parental W2mef and the $\Delta rh4$ W2mef transfected parasite control and they were found to be not statistically different (P values: W2mef vs $\Delta p38$ clone 1 $= 0.3333$, $\Delta rh4$ vs $\Delta p38$ clone 1 $= 0.6667$, W2mef vs $\Delta p92$ clone 1 $= 0.3333$, $\Delta rh4$ vs $\Delta p92$ clone 1 $= 0.3333$; Mann-Whitney Test). (C) Invasion inhibition assays were performed using anti-P38 and anti-P92 rabbit IgGs at a concentration of 2 mg/mL. The results were presented as percentages of the growth compared to the pre-immune rabbit IgG of either all parasite stages or only late-stage parasites measured 40 h later by flow cytometry. Both anti-P38 and anti-P92 demonstrated slightly inhibitory effect, around 15 – 20%, on erythrocyte invasion compared to the pre-immune control.
CHAPTER 5

Live-Cell Imaging of *P. falciparum* Merozoite Invading Human Erythrocyte and A Role of Calcium During Invasion

Introduction

The invasion of erythrocytes by the *Plasmodium* merozoites has been an interesting field of research for a very long time. A major breakthrough in our understanding of how erythrocyte invasion occurred in the asexual blood stages of the parasite occurred in 1975 when Dvorak et al. successfully recorded this event in real-time (Dvorak et al., 1975). They were able to capture and describe major characteristics of *P. knowlesi* merozoites invading rhesus monkey erythrocytes. Merozoites of the major human pathogen *P. falciparum*, proved more difficult to image due to them being more short-lived and smaller in size than the *P. knowlesi* merozoites. Gilson and Crabb (2009) were eventually able to develop methods for the live-cell imaging of *P. falciparum* invasion, using parasites maintained on the microscope under normal growth conditions of 37°C and supplied with humidified tissue culture gas (Gilson and Crabb, 2009). Subsequently Yahata et al. also reported successful live recordings of *P. yoelii* merozoites invading rodent erythrocytes (Yahata et al., 2012). Although some characteristics of the merozoites of the three species are quite diverse, the invasion process generally shares similar morphology and kinetics.

For *P. falciparum*, the invasion event begins when the newly released merozoite recognizes and attaches to a new erythrocyte. The first 'pre-invasion' phase, usually takes over ~11 s and involves the deformation of the host erythrocyte membrane while the merozoite re-orientates itself to juxtapose its apical end to the erythrocyte. The second phase begins with penetration of the merozoite into the erythrocyte which normally takes 17 s to complete. The third phase begins about 36 s later when the infected erythrocyte changes from its normal biconcave shape to a stellate shape. This process is called echinocytosis and it takes several minutes before the erythrocyte recovers its biconcave shape (Fig. 1.3).

To efficiently orchestrate such complex events in a very short time it is thought that calcium ion (Ca^{2+}) signaling plays a role in the merozoite. It was firstly
observed in a related parasite, *T. gondii*, that the host cell invasion by the tachyzoite required an increase of intracellular Ca\(^{2+}\) levels to control gliding motility and the release of microneme proteins, both of which are crucial for proper invasion (Carruthers and Sibley, 1999; Lovett and Sibley, 2003). Singh et al. later demonstrated a similar increase of cytosolic Ca\(^{2+}\) in *P. falciparum* merozoites upon egress supporting the importance of Ca\(^{2+}\) signaling for the invasion process (Singh et al., 2010). It was shown that the increase of cytosolic Ca\(^{2+}\) in the merozoites triggered the release of microneme proteins, such as EBA175 and AMA1, on to the merozoite surface (Singh et al., 2010). The restoration of Ca\(^{2+}\) back to basal levels and subsequent invasion steps were triggered when the merozoite contacted the erythrocyte and its EBA175 ligand bound to the glycophorin A receptor (Singh et al., 2010).

Lew and Tiffert (2007) also proposed calcium ions might function in the erythrocyte during the invasion. They hypothesised that the deformation of the erythrocyte membrane was a result of a temporary influx of Ca\(^{2+}\) from the media when the merozoite weakly interacted with the erythrocyte (Lew and Tiffert, 2007). The localised increase of Ca\(^{2+}\) levels was proposed to trigger the phosphorylation-dependent rearrangement of the erythrocyte's cytoskeleton causing the membrane to deform and wrap around the merozoite (Lew and Tiffert, 2007). Furthermore Ca\(^{2+}\) was also believed to be involved in triggering the onset of echinocytosis of the infected erythrocyte following invasion. The echinocytosis of erythrocytes is usually found in sickle erythrocytes and cold-stored erythrocytes in citrate-dextrose preservative (Tiffert, 2005). The increase in cytosolic Ca\(^{2+}\) levels is thought to activate the Ca\(^{2+}\)-sensitive K\(^{+}\) channels called Gardos channels, which allow K\(^{+}\) to exit the erythrocyte followed by Cl\(^{-}\) and water leading to dehydration and the characteristic stellate appearance.

This chapter describes the study of erythrocyte invasion with *P. falciparum* merozoites by live-cell microscopy using improved methodology and equipment. For example, invasion was recorded using a high-speed camera in order to achieve better resolution of the morphology and kinetics of invasion in *P. falciparum* merozoites. The results obtained here were compared with the published data from Gilson and Crabb (2009) (Gilson and Crabb, 2009). For the first time imaging has been combined with fluorescent indicators and invasion inhibitors to obtain more knowledge about this process. Fluorescent plasma membrane dyes were included to better observe the
changes of the erythrocyte membrane during contact and invasion by the merozoites. The role of Ca\(^{2+}\) in live cells was also studied using microscopy-based experiments after modifying extra- and intracellular Ca\(^{2+}\) levels to examine the importance of Ca\(^{2+}\) from each source. The changes in Ca\(^{2+}\) during invasion were also visualised by the use of fluorescent calcium indicator in an attempt to prove the hypothesis proposed by Lew and Tiffert (Lew and Tiffert, 2007). Two invasion inhibitors, R1 and cytochalasin D (cytD), were added to block invasion to determine at which stage these inhibitors acted by observing the changes in morphology of the cells and the kinetics in each step of invasion. Both inhibitors were also examined to see whether they had any effect on the calcium signaling during invasion. The results provide a better understanding of each erythrocyte invasion phase on both the molecular and cellular levels.

**Results**

*High-speed acquisition live-cell microscopy provides better resolution of morphology and kinetics of the erythrocyte invasion by P. falciparum merozoites*

In the previous study reported by Gilson and Crabb (2009), *P. falciparum* merozoites invading the erythrocytes was observed by time-lapse live-cell microscopy, acquiring an image every 2 s (Gilson and Crabb, 2009). Considering that invasion events can occur quite rapidly it was possible that some features could have been missed using a 2 s time-lapse frame rate. My study aimed to improve the temporal resolution of erythrocyte invasion by imaging at a faster frame rate in order to capture more detail about the movements occurring during this event. The Zeiss Axio Observer microscope was equipped with a high-speed EMCCD QuantEM camera that has very high sensitivity and very high quantum efficiency meaning that it can operate at low light intensity suitable for live-cell imaging. Furthermore this camera has no mechanical shutter, and it can acquire images and transfer the data to the computer simultaneously, all of which are essential for imaging at high frame rates. To prepare *P. falciparum* parasites for imaging, the 3D7 strain was synchronised using heparin and sorbitol treatments, and then grown to late schizont stage. The schizonts at 5 % parasitemia were diluted from 4 % to 0.16 % hematocrit in 2 mL of the RPMI medium. The culture was then placed in a FluoroDish cell culture vessel that had a glass bottom suitable for microscopy. The dish was placed on a microscope stage heated to 37°C and covered with a humidified gas chamber.
provided with 1 % O₂, 5 % CO₂ in N₂, that maintained suitable conditions for long-term observation. The transmission light source of the microscope was set to the lowest intensity possible to continuously capture the images for 3 min at 40 frames per second without photo-damaging the cells. The Zeiss time-lapse image files were opened with the open source ImageJ software, and the regions of interest were cropped and a time stamp inserted into the video files corresponding to the real-time speed (Video 1). The morphology and kinetics of erythrocyte invasion was re-examined. Fig. 5.1A shows the still images from Video 1 representing the pre-invasion phase beginning with the deformation of the erythrocyte plasma membrane. The waves of deformation appeared subtler than was previously perceived from the 2 s time-lapse videos. Moreover, the waves of erythrocyte deformation were more frequent, with the median of 4 waves, versus only 2 waves when observed in the earlier setting (Fig. 5.1B). Similar to the previous study though, the degree of deformation varied each time the merozoite attempted to push against the erythrocyte membrane. High-speed imaging allowed the re-orientation process to be more easily followed while the merozoite was deforming the erythrocyte membrane (Fig. 5.1A; blue arrows). After the last deformation, as the erythrocyte membrane recovered to the normal shape, the sides of the merozoite detached from the membrane leaving only the apical end attached to the erythrocyte. This marked the end of erythrocyte deformation and re-orientation of the merozoite, and the irreversible tight junction interaction was established. Afterwards, the merozoite appeared steady on the erythrocyte membrane for roughly 2 s, referred to the resting stage (Fig. 5.2A; Video 1) (Gilson and Crabb, 2009).

High-speed imaging revealed other phenomena during and after merozoite invasion that were not evident in the previous study. Firstly, full internalisation and sealing of the erythrocyte plasma membrane was indicated by the disappearance of the entry pore at the invasion point (Fig. 5.2B; Video 1). The second phenomenon occurred when the merozoite was observed to start spinning (Fig. 5.3; Video 1). The rotation began about 18.5 s after the end of penetration period. In some cases the posterior end of the merozoite appeared anchored to the inner plasma membrane of the erythrocyte while the apical end seemed to rotate freely in a broad clockwise circular motion. The speed of the rotation was initially slow with around 1 round/s and then increased to 2 rounds/s (Video 1). Observation of 2 other merozoites in
which the spinning motion was clearly seen, indicated they could rotate faster, up to 3.3 rounds/s.

Because high-speed imaging of erythrocyte invasion provided more detail for each movement the merozoite and erythrocyte made, the kinetics of each phase of invasion was re-examined and compared with published results. The entire invasion event was divided into 4 stages corresponding to the previous report, which were the pre-invasion, internalisation, time to echinocytosis, and time to reach the maximum echinocytosis (Gilson and Crabb, 2009). Essentially the kinetics of each phase of invasion measured from the high-speed imaging in this study was comparable to the previous study of Gilson and Crabb (2009) (Fig. 5.4). The length of the pre-invasion phase lasted an average of 9.15 ± 3.73 s in this study compared to the previous 10.67 ± 2.87 s. The pre-invasion phase could be further broken down into the erythrocyte deformation/merozoite re-orientation and the resting stage, whereby the latter phase took an average of 1.6 s as a part of 9.15 s pre-invasion period. The subsequent invasion phase took 16.82 ± 4.17 s measuring from the high-speed acquisition versus 16.92 ± 8.19 s from the previous report. It took another 31.92 ± 21.88 s for the infected erythrocytes to start the onset of echinocytosis. The previous study reported that this duration took 35.92 ± 17.14 s. The degree of echinocytosis of the infected erythrocyte became stronger and reached the maximum level 34.45 ± 20.67 s following the beginning of echinocytosis. This phase was reported to take 23.41 ± 10.65 s previously (Gilson and Crabb, 2009). Because the time to reach the maximum echinocytosis varied greatly, no substantial difference was observed between this study and the previous study.

*Visualisation of the P. falciparum merozoite invading the erythrocyte labelled with a fluorescent plasma membrane dye*

To better visualise the erythrocyte plasma membrane dynamics in the pre-invasion and internalisation phases, a lipid fluorescent dye, BODIPY FL C₁₂-sphingomyelin, was used for live-cell fluorescence microscopy. Fresh erythrocytes were incubated with 5 μM of the fluorescent dye prior to mixing with the magnet-purified late schizont stage parasites. The parasite culture was prepared for live-cell microscopy as per normal procedure. Because extended exposure of the parasites to excitation illumination was toxic to the cells and also bleached the fluorescence signal, it was not possible to record the invasion event in real-time at a high frame
rate. Invasion images were instead captured more slowly, once every second, alternating between the brightfield and fluorescence channels, for about 2 min. Representative snap shots from the recording (Video 2) are shown in the Fig. 5.5. To show both the merozoite and erythrocyte membranes together I attempted to superimpose the brightfield images onto the fluorescence images but this resulted in loss of detail. They were also slightly out of phase due to cell movements between the frames. I have therefore shown diagrams of the merozoite and its host in the corner of each panel to illustrate the relationships between the cells. The unlabelled merozoite was evidently seen denting the erythrocyte plasma membrane causing the membrane to wrap around it. The fluorescent dye aided the observation of membrane-wrapping action as the periphery of the plasma membrane was clearly visualised. The erythrocyte recovered from the deformation at 7 s after the primary contact. About a second after, the merozoite began to penetrate into the erythrocyte. The images show a fluorescence ring surrounding the invading merozoite as it entered the cell. Since the merozoite was not labelled with the fluorescent lipid dye from the beginning, these signals probably represent the nascent parasitophorous vacuole membrane (PVM). The fact the PVM was strongly labelled with the dye likely indicates membrane from the erythrocyte is making a substantial contribution to the PVM. It is worth noting that some free merozoites were seen fluorescing with the lipid dye which could be due to the contamination from leftover dye remaining after labelling the erythrocytes.

*During invasion a calcium signal was observed near the merozoite entry point followed by an influx into the erythrocyte*

Since the erythrocyte invasion involves the sequential deployment of ligands from merozoite microneme and rhoptry compartments that must then function cooperatively, it has been speculated that calcium signaling plays an important role in controlling these events. This has been shown by Singh et al., where a decrease in external potassium levels following merozoite egress triggers an increase in cytosolic Ca$^{2+}$. This in turn leads to the release of microneme proteins responsible for interacting with the host erythrocyte (Singh et al., 2010). The deformation of the erythrocyte membrane in the pre-invasion period has also proposed to be caused by an influx of Ca$^{2+}$ into the erythrocyte at the attachment points (Lew and Tiffert, 2007). This hypothesis was tested here, by observing Ca$^{2+}$ fluxes during invasion using live-
cell fluorescence microscopy of cells labeled with the membrane permeable Ca\(^{2+}\)-sensitive fluorescent dye, Fluo-4 AM. Late-schizont stage parasite culture was incubated with 5 μM Fluo-4 AM followed by washing steps to remove unincorporated dye. The invasion events were captured every 2 s for about 2 min alternately between the brightfield and fluorescence channels. The released merozoites were observed having a strong fluorescence signal in their cytoplasm corresponding to the up-regulation of cytosolic Ca\(^{2+}\) upon egress as reported previously (Singh et al., 2010). During the pre-invasion phase while the merozoite was deforming the erythrocyte membrane, no Ca\(^{2+}\) signals were observed indicating that influxes of the ion into the erythrocyte were likely not occurring and therefore not responsible as had previously been suggested (Fig. 5.6, Video 3) (Lew and Tiffert, 2007). However, seconds before the start of merozoite internalisation, a peak calcium signal was spotted emanating from the point of contact between the parasite and the erythrocyte (Fig. 5.6). Immediately after the apical calcium spike was observed the calcium signal was then seen increasing in the cytoplasm of the erythrocyte. The cytosolic Ca\(^{2+}\) level in the infected erythrocyte remained high throughout the recordings, from invasion to the start of echinocytosis (Fig. 5.6, Video 3). Although these findings did not demonstrate a role for Ca\(^{2+}\) during deformation in the pre-invasion phase as has been proposed, they suggested that Ca\(^{2+}\) might play a later role that starts just before invasion.

**Microscopic observation suggests extracellular calcium might contribute to the efficiency of erythrocyte invasion by P. falciparum merozoites**

Having visual evidence that calcium ions might be important for erythrocyte invasion by *P. falciparum* merozoites, the source of calcium was examined. To test if an extracellular calcium source was important for the invasion process, invasion events were recorded either in RPMI culture medium supplemented with 2 mM BAPTA to chelate its Ca\(^{2+}\) or in Ca\(^{2+}\)-free DMEM medium. Note the reason I had to use Ca\(^{2+}\)-free DMEM medium was that it could be purchased as a standard item unlike Ca\(^{2+}\)-free RPMI that would have required custom synthesis. To ensure the suitability of DMEM medium standard DMEM containing calcium supported parasite growth just as well as RPMI (data not shown). The modified media were used to wash the parasite culture two times prior to preparation for microscopy. The time-lapse images covering the pre-invasion and internalisation stages were recorded, and the
number of merozoites that successfully invaded erythrocytes in each type of media, including the normal RPMI medium as a control, was counted and compared. Of 34, 10, and 21 of schizont ruptures in the RPMI, BAPTA-supplemented RPMI, and Ca$^{2+}$-free DMEM media, respectively, the average number of successful invasions was 2.79, 1.90, and 2.05, respectively. Although invasion efficiency of the merozoites in the modified media appeared to be reduced relative to the control medium, the differences were not significant at 95 % confidence level (Fig. 5.7).

To further investigate the role of extracellular calcium in erythrocyte invasion in more detail, the kinetics of the various invasion steps occurring in the Ca$^{2+}$-free DMEM medium were compared to those in control RPMI medium. The kinetics of five distinct stages of invasion, namely erythrocyte deformation, resting, invasion, time to echinocytosis, and time to maximum echinocytosis, from each experimental condition were measured for comparison and statistical analysis. As shown in the Fig. 5.8, there were no differences in invasion kinetics observed across all five stages examined. This finding implied that, for those merozoites that successfully invade the erythrocytes in a low calcium environment, they do so with similar efficiency and kinetics as those with normal levels of the ion.

The influx of calcium ions into the infected erythrocyte may trigger the onset of echinocytosis

Following investigation of extracellular calcium’s role in the invasion process, I next examined the function of the rise in intra-erythrocytic Ca$^{2+}$ observed during merozoite internalisation. The erythrocytes were first prepared by incubating them with 60 µM BAPTA-AM, a membrane-permeable calcium chelator, prior to mixing them with purified schizonts. Live-cell microscopy of erythrocyte invasion was done in the Ca$^{2+}$-free DMEM medium to minimize the chance of having external Ca$^{2+}$ leaking into the erythrocytes and exceeding the chelating capacity of the pre-loaded BAPTA. The invasion data acquired from previous experiments using the untreated erythrocytes in Ca$^{2+}$-free DMEM medium was used as a control for this study. The number of successful invasions per schizont rupture into the BAPTA-treated erythrocytes appeared similar to the number of invasions/rupture in the untreated erythrocytes (data not shown). The kinetics of invasion in both conditions were next compared and revealed the average duration for each stage of invasion was not significantly different for most steps. The exception was in the BAPTA-treated
erythrocytes where the time taken from complete internalisation to the beginning of echinocytosis was longer, increasing from an average of 26.87 s in without treatment to 83.52 s with BAPTA treatment ($P = 0.0002$, student's t-test analysis; Fig. 5.9). This finding supports a role for elevated cytosolic Ca\textsuperscript{2+} levels in the infected erythrocytes to facilitate the rapid onset of echinocytosis after invasion. The underlying mechanism for this phenomenon was proposed to result from the Ca\textsuperscript{2+}-induced reorganisation of the erythrocyte cytoskeletal proteins (Wasserman et al., 1990). The benefits for the merozoites of inducing rapid echinocytosis in infected erythrocytes is not known.

Inhibiting erythrocyte invasion by interrupting the tight junction formation does not prevent the calcium fluxes and prolongs the echinocytosis phase

With the discovery of calcium signal near the merozoite apex before and during invasion, I next investigated if this event was linked to any particular molecular action during the various stages of invasion. The first calcium spike occurred at the area where the merozoite established the firm apical interaction with the erythrocyte a few seconds before the start of internalisation. Thus the timing of this calcium signal was likely to correspond to the period when the tight junction was forming. To test whether interrupting tight junction would affect the calcium signaling, its formation was blocked using the R1 peptide to inhibit the binding of AMA1 to RON2 (Srinivasan et al., 2011). To do this the parasite culture was labeled with Fluo-4 AM prior to the live-cell imaging in the presence of 100 $\mu$g/mL R1 peptide. Invasion imaging was performed by alternately acquiring the brightfield and GFP-filtered fluorescence channels every 2 s for 2 min which was long enough to cover the pre-invasion and internalisation periods. Shown in the Fig. 5.10A are the still images captured from a representative video (Video 4) and the calcium spike and subsequent flux in the erythrocyte cytoplasm were observed as per normal despite the fact that the merozoite was inhibited from invading. This finding demonstrates that although the AMA1 and RON2 interaction was disrupted thereby halting the progress of invasion, the peripherally attached merozoites were still able to trigger calcium fluxes by some means that is likely upstream of tight junction formation. Because the merozoites treated with the R1 peptide usually remained attached to the erythrocyte membrane for a long period, it is speculated that the calcium fluxes might also be activated for a longer duration than normal. Assuming that rises in intracellular Ca\textsuperscript{2+}
were triggering the reorganisation of cytoskeletal proteins in the infected erythrocytes leading to echinocytosis as suggested by Lew and Tiffert (2007), the live-cell imaging in R1 treated cultures was then extended to over 20 min to cover the post-invasion period. Fluorescence imaging was only performed for the first 2 min with only brightfield thereafter to prevent phototoxic damage to the cells. Surprisingly the imaging revealed that the echinocytic erythrocytes did not recover their normal biconcave shape after 20 min, compared to a maximum of 10 min for invaded untreated erythrocytes (Fig. 5.10B; Video 5) (Gilson and Crabb, 2009). These findings suggest that merozoites might release a factor upstream of tight junction formation that causes the apical calcium spike and the subsequent flux into the infected erythrocyte. The apical calcium fluxes normally cease after the merozoite completely invades and reseals itself into the erythrocyte. However when merozoite invasion was inhibited by R1 peptide the flux appeared to continue because the targeted erythrocyte had a greatly prolonged echinocytosis phase.

Interfering with the parasite actin-myosin motor prolongs echinocytosis of the infected erythrocytes without disturbing calcium fluxes

It is well established that inhibiting the actin-myosin motor of merozoites disrupts their ability to invade erythrocytes (Miller et al., 1979). Hence it was of interest to observe how the actin inhibitor, cytochalasin D (cytD) could potentially change calcium fluxes in invading merozoites. To focus the effect of cytD inhibition upon the merozoites, they were purified as late-schizonts from uninfected erythrocytes and treated with 1 µg/mL of cytD before being added to Fluo4-loaded erythrocytes for live-cell fluorescence microscopy. The invasion events were initially filmed every 2 s for 90 s to examine the cell morphology as well as the calcium fluxes in the pre-invasion period. It was evident from the imaging that the merozoite was not able to strongly deform the erythrocyte as it normally did indicating that deformation is powered by the actin-myosin motor (Fig 5.11A, Video 6). The cytD-treated merozoite was able to maintain contact with the erythrocyte surface for an extended period of time before it triggered a calcium spike at the apical tip. This was markedly different from the normal invasion when apical calcium spikes were observed about 10 s after the parasites made initial contact with the erythrocytes. In the presence of cytD, on the other hand, the calcium spikes were observed more than 20 s after the initial contact had been made (Fig. 5.11A). Unfortunately it was impossible to
examine the calcium fluxes in the erythrocytes since the resting calcium level was quite high from the beginning in this particular recording. As the apical calcium spikes were observed in cytD, it was assumed that the echinocytosis period would follow as usual. Live-cell microscopy imaging was therefore extended to around 20 min only in the brightfield channel to observe the echinocytosis stage. Similar to what occurred with the R1 peptide inhibition, the erythrocytes bound by cytD-treated merozoites underwent echinocytosis for a period longer than normal, often well beyond 15 min (Fig. 5.11B; Video 6). In addition, the commencement of the echinocytosis phase was delayed and began about 5 min after the initial attachment, compared to the 1 min average in untreated parasites. These findings suggested that the actin-myosin motor of the merozoite could be responsible for the pushing motion force that causes deformation of the erythrocyte membrane. Although the propelling force of the merozoite was hindered by cytD, other mechanisms relating to invasion appeared to function normally, albeit delayed as demonstrated by the occurrence of calcium spike and the subsequent echinocytosis. The fact that these downstream events happened later than normal emphasises the role that the parasite actin-myosin motor plays in quickly setting the stage for rapid invasion.

Discussion

Live-cell microscopy is increasingly being regarded as a powerful tool for use in cell biology studies. The first account of using live-cell microscopy to study invading malaria parasites was published in 1975 and focused on the monkey parasite, *P. knowlesi* (Dvorak et al., 1975). This study opened up our understanding of the basic steps of merozoite invasion, namely that there is a strong deformation of the host erythrocyte by the merozoite and that entry is rapid and causes echinocytosis of the erythrocyte. The spinning of newly invaded merozoites within the erythrocyte was also noted (Dvorak et al., 1975). A detailed study of live merozoite invasion in *P. falciparum* was not achieved until 2009 by Gilson and Crabb probably because the merozoites of these human parasites are more difficult to image. My study here, builds upon the earlier *P. falciparum* study by employing three new innovations. The first was that imaging was done at a much higher frame rate (up to 40 fps) than the earlier study which only managed 0.5 fps. A more sensitive camera, greater computational power and dedicated high speed imaging software enabled these improvements. The second innovation was the use of fluorescent probes, namely the
lipid and Ca\(^{2+}\)-sensitive fluorescent indicators, which enabled visualisation of the movement and formation of membrane structures and to uncover calcium fluxes during invasion, respectively. The final innovation was to use invasion inhibitors, namely the R1 peptide and cytD, to study the invasion process in real-time in live cells to unravel the molecular events that underpin the invasion process.

As mentioned above, the first video recordings of the erythrocyte invasion were accomplished with *P. knowlesi* merozoites (Dvorak et al., 1975). The merozoites of this *Plasmodium* species are easier to observe in live-cell microscopy than the *P. falciparum* merozoite since they are bigger, being 2.5 \(\mu\text{m}\) long and 1 \(\mu\text{m}\) wide compared with 1.3 \(\mu\text{m}\) long and 0.9 \(\mu\text{m}\) wide in the case of *P. falciparum* (Mitchell and Bannister, 1988). Furthermore *P. knowlesi* merozoites maintain viability nearly 30 min post rupture (Dennis et al., 1975), which is much longer than those of *P. falciparum* that have a half life of only 5 min at 37°C post rupture (Boyle et al., 2010b). These characteristics of *P. falciparum* merozoites have presented a technical challenge for microscopists who want to capture live erythrocyte invasion. These problems however have been overcome by the development of microscopes equipped with the humidified-gas units that can maintain a low oxygen environment and temperature-controlled chambers suitable for live-cell experiments. The ability to maintain parasites for extended periods enabled us to maintain schizonts in a healthy state for long enough to observe them rupturing, and releasing their merozoites and subsequent invasion. Due to their short half-life it was not practical to add purified merozoites to erythrocytes and observe invasion. A challenge with the schizont approach was to select highly mature schizonts that looked as they were going to rupture within several minutes. The selection criteria for rupture were empirically arrived at and were that the schizont had to be rounded up into a spherical shape with a smaller diameter than the broader flattened discs typical of less mature schizonts. The hemozoin in mature schizonts just prior to rupture was highly condensed and did not contain smaller oscillating particles found in younger cells. Other features that were sometimes observed were the movement of the merozoites inside the schizont prior to egress and short fine projections from the surface of the schizont. The observations that signified imminent rupture, have been noted by others (Crick et al., 2013; Glushakova et al., 2005). The ability to predict schizont rupture was very important for fluorescence imaging for it allowed observations to be made using less
damaging low level brightfield illumination until rupture occurred where upon the fluorescence imaging could commence.

The other important feature employed in the study was the use of new high-speed acquisition camera with specialised software for handling the data which enabled erythrocyte invasion by *P. falciparum* merozoites to be recorded at a high frame rate of up to 40 fps. This technology allowed for a higher resolution observations of invasion events in terms of pixel density compared with the original video tape recording of the *P. knowlesi* study (Dvorak et al., 1975). The higher frame rate achieved here compared to the first *P. falciparum* study (Gilson and Crabb, 2009) allowed certain events to be observed which were not obvious before.

The first of these phenomena was how the erythrocyte membrane deformation progresses through the pre-invasion phase. As expected, the deformation appeared smoother with the much higher frame rates being acquired. The deformation occurred many times more when observed at high temporal resolution during the pre-invasion period, starting from a mild deformation resembling a gliding movement on the erythrocyte surface. The next wave came with a strong push causing the erythrocyte membrane to wrap around the merozoite almost entirely. Following a few more waves of membrane deformation was the resting phase whereby the merozoite apically attached to the erythrocyte waiting to begin internalisation. This phase happened for only a brief period and high-speed acquisition provided the possibility to clearly define this moment during invasion.

Second phenomenon observed was the rotating movement of the merozoite. The merozoite has to initially re-orientate in order to prepare itself for invasion into the host erythrocyte. With a typical time-lapse imaging protocol, the merozoite appears to be changing its orientation from frame to frame, however the direction to which the parasite turns cannot be revealed. High-speed acquisition, on the other hand, was able to provide this information from when the merozoite first began wiggling on the erythrocyte surface through to strong deformations leading up the apical reorientation of the merozoite (Video 1). The most notable observation regarding the spinning motion of the merozoite happened after the parasite completely entered into the erythrocyte. This phenotype was also seen in the recordings of the *P. knowlesi* merozoite during invasion, however no further analysis was mentioned (Dvorak et al., 1975). It was seen here that the merozoite apex rotated in a circular motion, mostly in a clockwise direction with its posterior end anchored to the
erythrocyte inner-membrane. The speed of the rotation fluctuated but generally began slowly before increasing. It is worth noting that nearly all the merozoite invaded on the upper erythrocyte surface and so the merozoite apex was directed towards the centre of the erythrocyte. It would be interesting to observe merozoites invading the underside of the erythrocyte because their direction of rotation would be expected to be counter clockwise. This however rarely occurred because the erythrocytes were resting on a glass surface.

It is unknown what drives the merozoite spinning. It could be Brownian motion although the predominant clockwise rotations observed suggest an active process perhaps driven by the merozoite’s actin-myosin motor. CytD treatment could be used to test involvement of the motor in rotation but unfortunately it also blocks merozoite internalisation. It is interesting that Toxoplasma gondii when anchored to a surface via their posterior ends wave their tips in a clockwise direction and when on their sides glide in anticlockwise circles (Leung et al., 2014). It is possible the asymmetry of the apicomplexan cytoskeletons might control the arrangement of glideosome components and hence the direction of movement. The molecular purpose behind the rotation is unknown but I speculate that the merozoite might require a mechanical action to pinch the parasitophorous vacuole membrane off the erythrocyte membrane and the fact the merozoite appeared anchored at its basal end supports this (Aikawa et al., 1978). Another possibility is that the spinning could be a part of the release of dense granule contents required to quickly spread into the parasitophorous vacuole space to facilitate the progression into the ring stage (Riglar et al., 2011; Torii et al., 1989).

The ability to specifically probe and visualise molecules of interest in live-cell experiment could dramatically improve our understanding in the mechanisms of merozoite invasion. However, live-cell fluorescence microscopy is a challenging technique especially for the P. falciparum merozoites since they are prone to damage from the high-power of probe excitation light. The first attempt carried out in this study was to examine the fate of the erythrocyte membrane during the invasion period. The erythrocytes were labeled with the BODIPY FL C_{12}-sphingomyelin which was maximally excited at 505 nm. This fluorophore has a number of advantages over the NBD fluorophore used by many studies in the past, i.e. greater fluorescence output due to higher molar absorptivity and quantum yield, and improved photostability (Johnson et al., 1991). Thus it does not require the excitation light to be
at full power to visualise the dye, which in turn has less impact on the merozoite longevity. The results presented here suggest that the PVM originates, in part if not entirely, from the erythrocyte membrane since the PVM remains virtually as fluorescent as the erythrocyte during invasion which is in agreement with previous experiments with both *P. knowlesi* and *P. falciparum* merozoites (Haldar and Uy et al., 1992; Pouvelle et al., 1994; Ward et al., 1993). The PVM of *T. gondii* tachyzoite was also found to be host-derived (Suss-Toby et al., 1996), further supporting the general concept that the PVM of Apicomplexan parasites primarily comprises of the lipids from host cell membrane (Lingelbach and Joiner, 1998). The lipid contents stored in the rhoptries were also believed to get injected into the erythrocyte membrane during invasion suggestive of their contribution to the formation of nascent PVM, which dye molecules could then diffuse into from the erythrocyte (Bannister and Mitchell, 1989; Bannister et al., 1986; Dluzewski et al., 1995; Riglar et al., 2011). If some PVM lipids were of parasite origin and were mixing with erythrocyte lipids in the PVM, we would expect the PVM to be less bright due to dilution of the dye. Nonetheless it was estimated that the surface area of nascent PVM would be around 4 μm$^2$ for each merozoite corresponding to 2 - 3 % of total erythrocyte surface area (Dluzewski et al., 1995). Any significant changes in fluorescent intensity from the few percentages loss of the fluorescent dye would then be quite difficult to accurately measure. A detailed analysis of the lipid composition in the PVM compared to the erythrocyte membrane would clarify the origin of the PVM, however methods to isolate pure PVM are extremely challenging.

Another application for live-cell fluorescence microscopy is to examine the temporal and spatial occurrence of calcium ion fluxes involved in cell behavior. For *P. falciparum*, I therefore examined the involvement and essentiality of calcium ions in the invasion process. This work initially focused on the pre-invasion period following the hypothesis proposed by Lew and Tiffert regarding the underlying mechanism that was responsible for erythrocyte deformation (Lew and Tiffert, 2007). They proposed that calcium ions were locally entering the erythrocyte through the channels induced to open by weak contact with the merozoite. Localised increases of intracellular Ca$^{2+}$ would then trigger the reorganisation of the erythrocyte cytoskeletal proteins facilitating the membrane deformation (Lew and Tiffert, 2007). Therefore, the fluorescence calcium dye, Fluo-4, was used to report on changes in calcium levels which were expected to appear during the pre-invasion period. Surprisingly there
were no calcium fluxes observed in the erythrocyte during merozoite induced deformations, however Ca$^{2+}$ signals were instead seen in the two downstream stages. A calcium signal firstly appeared between the apical tip of the merozoite and the juxtaposed erythrocyte surface at the end of the deformation period. The calcium ions were then observed to spread into the erythrocyte a few seconds following the first signal (discussed below). These observations were not only made when both erythrocytes and parasites were labeled with the dye, but were also apparent when only the erythrocytes were loaded with the calcium indicator and mixed with the unlabeled purified schizonts. This led to speculation that Fluo-4 loaded into the erythrocytes gained access to the merozoite apical organelles, most likely the rhoptries, through a pore that possibly opened when the tight junction was formed. Supporting this was the observation that the apical calcium spike occurred just after erythrocyte deformation ended coinciding with the establishment of tight junction. Since there has not been any study reporting that the rhoptries also store calcium ions, it remains speculative as to whether the observed calcium spike corresponds to Ca$^{2+}$ stores in the rhoptries or from extracellular Ca$^{2+}$ in the medium that has somehow gained access to the tight junction region formed between the merozoite and erythrocyte.

To further explore the role of external calcium, live merozoite invasions were imaged in Ca$^{2+}$-free medium and with BAPTA Ca$^{2+}$ chelator. A number of previous studies have documented that extracellular Ca$^{2+}$ is required for erythrocyte invasion by the Plasmodium parasites (McCallum-Deighton and Holder, 1992; Wasserman and Chaparro, 1996; Wasserman et al., 1990). Live-cell microscopy however indicated that although there was a reduction in the mean number of invasions per schizont rupture with reduced free Ca$^{2+}$, this was not significant at the 95 % confidence interval. Close examination of the kinetics of each step of invasion further indicated that depletion of free Ca$^{2+}$ did not change the kinetics of any step. (Fig. 5.7, Fig. 5.8). My results here appear to be at odds with previous studies and more recent experiments conducted by my colleagues that indicated external calcium was required for efficient invasion (Weiss et al., 2015). There are two possible explanations for this discrepancy. The first is that studies in the past and in Weiss et al. (2015) were mainly performed by measuring invasion efficiency by means of invasion inhibition assays where hundreds of thousands of merozoites were tested for their ability to invade the erythrocytes in the absence of extracellular Ca$^{2+}$. On the other hand, live microscopy
observations are time-consuming which limits the amount of data that can be gathered. Had time permitted and more invasion movies to be analysed then the reduced invasion rates I reported without free Ca$^{2+}$ may have been significant. The other difficulty with attempting to image in Ca$^{2+}$-free and chelated media is that the ion can contaminate the media from a number of sources and influence the results. For example, in schizonts the vacuole space around the merozoite is rich in calcium (Glushakova et al., 2013) and so every time a schizont ruptures calcium is introduced into the media. In addition, Albumax, which is a bovine sourced serum supplement added to the media, contains undetermined amounts of calcium but its presence is needed for proper egress (Glushakova et al., 2005). In response to these problems my colleagues in Weiss et al. (2015) imaged in Ca$^{2+}$-free media with added BAPTA to strongly suppress increases calcium levels as well as using Albumax stock solutions that had been extensively dialysed against calcium-free PBS. In conclusion, it appears that external calcium is required for efficient invasion and although I probably reduced the levels enough to reduce invasion efficiency it was not enough to be significant. When rigorous methods are used to deplete free calcium then merozoite invasion is stalled at the point just prior to tight junction formation (Weiss et al., 2015). The merozoites can vigorously deform their target erythrocytes but cannot seem to release their rhoptries, form a tight junction and trigger echinocytosis. External calcium may therefore have a role in events that trigger rhoptry release.

Wasserman et al. (1990) proposed that Ca$^{2+}$ may function inside the infected erythrocytes. They observed that Ca$^{2+}$ increased in the recently infected erythrocytes and that invasion was inhibited when intracellular calcium was chelated. In addition, there was a change in phosphorylation patterns of cytoskeletal proteins that resembled the modifications induced by adding calcium ionophore to the erythrocytes (Wasserman and Chaparro, 1996; Wasserman et al., 1990). They proposed that there might be an influx of calcium into the infected erythrocytes during invasion and the only way to confirm this result was to observe the event directly (Wasserman et al., 1990). As discussed above, the live-cell fluorescence microscopy performed here has confirmed there was an influx of Ca$^{2+}$ during invasion and their report was correct. To further validate the role of Ca$^{2+}$ in the erythrocytes as proposed by them, the merozoites were observed invading BAPTA-loaded erythrocytes. The Ca$^{2+}$-free DMEM medium was chosen for this experiment since the inhibitory effect caused by intracellular calcium chelator was shown to also depend on the amount of calcium
available in the extracellular medium (Wasserman and Chaparro, 1996). In the presence of excess extracellular Ca$^{2+}$, it was demonstrated that the invasion efficiency was similar between the untreated and chelator-loaded erythrocytes since the chelator acted as a calcium sink and caused intracellular and extracellular Ca$^{2+}$ to reach a similar equilibrium as normal cells (Wasserman and Chaparro, 1996). As the invasion in this Ca$^{2+}$-free DMEM medium still occurred, it was possible that the low level of Ca$^{2+}$ left in this medium as discussed above might have slowed down the saturation of the chelator enough to observe effects upon invasion. A significant change was however only noted for the time taken from the end of merozoite entry to the beginning of echinocytosis. This observation seems to suggest that calcium ions are involved in activating downstream events that lead to the infected cell becoming an echinocyte. It was previously shown that Ca$^{2+}$ could induce echinocytosis in erythrocytes, however this phenomenon appeared to be irreversible despite multiple washes with calcium chelating agents and hours of incubation (Smith et al., 1981). Thus Ca$^{2+}$-induced echinocytosis, therefore, appears to be different from what occurs in malaria infection since the infected erythrocytes usually recovers from its echinocytotic state within 5-10 min (Gilson and Crabb, 2009). With the influx of Ca$^{2+}$ not likely responsible for echinocytosis it is seems contradictory that following invasion of chelated erythrocytes in Ca$^{2+}$-free medium echinocytosis was delayed which suggests it does play a role. Much more work therefore needs to be done with carefully controlled Ca$^{2+}$ levels to determine its true role in echinocytosis.

Another possible role for the injected Ca$^{2+}$ may be to cause the rearrangement of the erythrocyte cytoskeletal proteins during the internalisation period. It was observed from freeze-fracture electron micrographs of P. knowlesi merozoites invading erythrocytes that the cytoplasmic side of the infected cell where invagination of the erythrocyte membrane took place appeared bare lacking of the usual underlying membrane complex (Aikawa, 1981). It was assumed that cytoskeletal proteins at the entry point were disassembled in order for the merozoite to invade through the junction. This mechanism could be caused by a localised influx of Ca$^{2+}$ which triggers subsequent downstream events such as the phosphorylation/dephosphorylation of the target proteins, resulting in the dissociation of the cytoskeletal mesh. The changes in phosphorylation states of the skeletal proteins were indeed observed in the newly infected cells accompanied by the increase in intracellular calcium level (Wasserman
et al., 1990). The timing of the Ca\(^{2+}\) influx observed in the present study corresponds well with this hypothesis.

An important aspect in studying the invasion mechanism is to be able to correlate the roles of proteins that biochemical methods have implicated as functioning during invasion with physical roles during the multiple invasion steps observed by microscopy. The establishment of the tight junction, in particular, is an invasion step that is difficult to track. The discovery of calcium spike emanating from the apical area seconds before internalisation could potentially serve as a marker for the tight junction formation. The fact that Fluo-4 trapped inside the target erythrocyte produced a signal indicates the formation of an opening at the merozoite attachment site. This phenomenon became a useful tool for studying the morphology and kinetics of invasion in the presence of two inhibitory compounds, namely R1 peptide and cytochalasin D. In both cases, the apical calcium spike and influx of calcium into the erythrocyte were observed to still occur, but the invasion inhibitory characteristics were different.

The fact that the calcium spike and erythrocyte flux were not disrupted when the tight junction formation was inhibited with R1 peptide indicated that the establishment of hypothetical pore happens upstream of junction formation. It has been shown previously that the release of rhoptry contents was not disrupted when the AMA1-RON2 interaction was blocked (Riglar et al., 2011). The factor(s) responsible for initiating the apical calcium spike and the subsequent calcium influx in the erythrocyte, therefore, might possibly located in the rhoptries. The merozoites inhibited with R1 peptide also demonstrated a failure in recovering from an echinocytotic state after 20 min of observation, compared with the normal \(\sim\)10 min period. One possible reason for failure to recover from echinocytosis is that without proper tight junction formation in the presence of R1 peptide, the release of rhoptry contents from the non-invading merozoite creates an orifice in the erythrocyte that is not sealed off from the external environment. This could lead to the continuous flow of the factor(s), such as calcium ions, which constantly keep the erythrocyte in echinocytotic state.

Observation of cytD-treated merozoites indicated a lack of erythrocyte deformation, suggesting the actin-myosin motor might power the forceful movement of merozoites to deform the erythrocyte membrane during the pre-invasion period. Surprisingly the cytD-treated merozoites still exhibited apical calcium spikes, and
were able to cause the subsequent echinocytosis in the erythrocytes they were attached to, despite a 5-min delay. Similar to the R1-treated merozoites, the erythrocytes remained in an echinocytotic state for a longer period than usual. It was previously shown that the merozoites incubated with cytochalasin B were able to establish the tight junction normally (Miller et al., 1979). As invasion could not proceed due to the inhibitory effect of cytD, the junction though formed, might remain porous to ions and similar to R1-treated merozoites, not allow the erythrocyte to recover ionic homestasis and quickly return to its normal shape.

Thus far calcium ions have been the mentioned as the main contributor to echinocytosis since their entry into erythrocytes is known to trigger the dehydrated appearance of echinocytes (Tiffert, 2005). However, echinocytosis following invasion only takes 30 s, and happens much faster than calcium-mediated dehydration which can take several minutes. Since it was reported that the rhoptry contents were also released in the cytochalasin-treated merozoites (Bannister et al., 1986; Miller et al., 1979; Riglar et al., 2011), evidence points to factor(s) in the rhoptries that are likely to also contribute to echinocytosis. One such factor, are the lipids stored in the rhoptries (Bannister et al., 1986). Certain erythrocyte membrane shape changes have been proposed to be due to imbalances in the lipid composition between the outer or inner leaflets of the plasma membrane (Sheetz and Singer, 1974). The deposition of molecules onto the outer leaflet of the erythrocyte plasma membrane can induce the cell membrane to bend outwards and form protuberances like an echinocyte due to the expansion the outer layer in relative to the inner layer. As mentioned earlier, the rhoptry contents were released normally in the presence of R1 peptide, however they appeared to be deployed incorrectly. Riglar et al. have clearly demonstrated that the rhoptry contents, as marked by RAP1, were discharged onto the outside of the erythrocyte membrane when the merozoites were inhibited with R1 peptide (Riglar et al., 2011). Since the tight junction was not correctly formed, the release of rhoptry contents was not enclosed in the attachment area and continuously dispersed onto the erythrocyte surface. A large amount of rhoptry contents deposited onto the erythrocyte surface that would normally help form the PVM, could cause an imbalance of lipid components and make the membrane bend outwards. Due to excess deposition erythrocyte flippases could take a long time to restore the balance back to resting levels. This combined with a calcium influx could be why the erythrocytes
remained in the echinocytotic state for a longer period when merozoite invasion was blocked with R1 peptide.

This study has elegantly proved how powerful live-cell microscopy, in combination with fluorescence microscopy, are to help understand the erythrocyte invasion mechanisms used by *P. falciparum* merozoite. Although some of the data presented here may be preliminary due to time limitations, it has revealed novel features of the erythrocyte invasion mechanism such as the post invasion merozoite spinning and the pre-invasion pore. Further live cell imaging investigations using other inhibitors of invasion such as antibodies and peptides that bind to and block merozoite ligands, parasite mutants that lack particular ligands and enzymatic treatments that remove erythrocyte receptors, will help to complete our understanding of the invasion process that is essential for parasite survival.
Figure 5.1. Live-cell microscopy using high-speed image acquisition demonstrates continuous erythrocyte deformation in the pre-invasion phase of erythrocyte invasion by *P. falciparum* merozoites.

(A) Synchronous *P. falciparum* 3D7 late stage schizonts at 5 % parasitemia were prepared for live-cell microscopy in a 35 mm FluoroDish cell culture dish. The cells were kept in a humidified gas chamber (94 % N₂, 1 % O₂, and 5 % CO₂) of a Zeiss AxioObserver Z1 microscope. The high-speed camera, EMCCD QuantEM, was used to capture the erythrocyte invasion by the merozoites at 40 frames per second (fps). Snap shots from the Video 1 (42.501 s – 49.513 s) of the pre-invasion phase of a merozoite trying to invade the erythrocyte are presented (black arrow). It was seen that the merozoite deformed the erythrocyte in 4 waves (red numbers) while it was re-orientating (blue arrows). The action lasted around 7 s in this representative video. The comparative timing is shown on the top left and the timing corresponded to the Video 1 is shown at the bottom right. (B) The number of waves of erythrocyte membrane deformation observed was counted and compared between the previous setting of time-lapse acquisition and the high-speed acquisition in this study. The merozoites in the videos recorded with the settings in the earlier reports mostly displayed 2 waves of erythrocyte deformation (n = 15). On the other hand, the high-speed acquisition demonstrated that, in fact, the membrane deformation occurred more frequent having the median of 4 times (n = 15).
Figure 5.2. Live-cell microscopy using high-speed acquisition reveals a short resting phase follows erythrocyte deformation.

(A) Following the erythrocyte deformation, the merozoite (black arrow) appeared steady against the erythrocyte surface for the next ~2 s (in this example). This is marked as a resting stage before internalisation. Its purpose is unknown but it could be during this period that the tight junction is forming in preparation for invasion. Snap shots of the same merozoite in Fig. 5.1 were shown. (B) The merozoite (black arrows) penetrated into the erythrocyte through the ring-like tight junction, which could be observed as shadow around the periphery of the invading merozoite (red arrows). The disappearance of shadow posteriorly to the invading merozoite clearly indicated the moment when the internalisation finished, taken ~18 s for this particular merozoite (Video 1). The relative timing is shown on the top left and the timing corresponded to the Video 1 is shown at the bottom right.
Figure 5.3. Merozoite rotation was observed after complete internalisation. High-speed acquisition of the merozoite invading the host erythrocyte revealed that the merozoite began a spinning motion following internalisation. Using the posterior end of the merozoite anchored to the erythrocyte membrane as a reference point, the direction of the spinning appeared clockwise. The speed of spinning motion kept increasing once it started, roughly 18.5 s after complete internalisation. It started off around 1 rotation/s and increased to 2 rotations/s in this representative merozoite. Still images captured from the Video 1 showing the spinning action are shown with the timing from the video stamped at the bottom and the relative timing of the spinning event stamped at the top. Underneath each image is the illustration depicting the spinning motion of the merozoite.
Kinetics of *Plasmodium falciparum* merozoite invasion

![Graph showing time (s) for different stages of invasion](image)

### Table: Acquisition times

<table>
<thead>
<tr>
<th>Invasion stages</th>
<th>High-speed (n = 12)</th>
<th>Gillson and Crabb, 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-invasion</td>
<td>9.15 ± 3.73</td>
<td>10.67 ± 2.87</td>
</tr>
<tr>
<td>Invasion</td>
<td>16.82 ± 4.17</td>
<td>16.92 ± 8.19</td>
</tr>
<tr>
<td>Resting before spinning</td>
<td>18.49 ± 6.61</td>
<td>ND</td>
</tr>
<tr>
<td>Time to echinocytosis</td>
<td>31.92 ± 21.88</td>
<td>35.92 ± 17.14</td>
</tr>
<tr>
<td>Time to max echinocytosis</td>
<td>34.45 ± 20.67</td>
<td>23.41 ± 10.65</td>
</tr>
</tbody>
</table>
Figure 5.4. Comparison of the kinetics in each step of erythrocyte invasion by *P. falciparum* merozoite between high-speed acquisition and the previous low-speed study.

The time taken for each step of the invasion, namely pre-invasion, invasion, resting before spinning, time to echinocytosis, and time to maximum echinocytosis, was averaged from the 12 videos acquired by high-speed camera (table below). The data were compared with the kinetics presented by Gilson and Crabb (Gilson and Crabb, 2009), and plotted as a bar graph. The result illustrated that the timing of each stage of invasion acquired by different procedures was similar throughout the whole process. ND = not determined.
Figure 5.5. Live-cell imaging of the invasion into the erythrocytes fluorescently labeled with a lipid dye supports the incorporation of the erythrocyte plasma membrane into the nascent parasitophorous vacuole membrane. The human erythrocytes were labeled with the fluorescent lipid dye 5 µM BODIPY FL C_{12}-sphingomyelin, and were mixed with the purified *P. falciparum* late-schizont stages. The invasion events were captured every second in both brightfield and fluorescence channels. Each channel was taken alternately whereby the fluorescence channel was captured about 0.3 s after the brightfield channel. Snap shots from a representative invasion event (Video 2) were shown. A merozoite (black arrow; no fluorescent signal) firstly deformed the erythrocyte while it re-orientated. At 8 s after the initial attachment the merozoite started to penetrate the erythrocyte. The penetrating apical end of the merozoite appeared fluorescent as it was invading the host erythrocyte (white arrow), and the merozoite circumference was fully labeled with the dye once the invasion was complete. This observation may indicate intake or migration of the erythrocyte membrane into the nascent parasitophorous vacuole. Alternatively, the merozoite may be depositing lipid from its rhoptries into the nascent vacuole membrane and that dye molecules then diffuse into from the erythrocyte. The time stamps appeared in the Video 2 are presented at the bottom right. The relative timing of each action is shown at the top left. Simplified illustrations depicting what happened at each step are on the top right.
165
Figure 5.6. Calcium fluxes into erythrocytes were observed during merozoite invasion.

The parasite culture was incubated with 5 µM Fluo-4 AM, a membrane-permeable fluorescent calcium indicator. The invasion process was recorded roughly every 2 s for the brightfield and fluorescence channels. Both channels were alternately captured at each time point. Still images from representative Video 3 are presented (Top left: relative time stamps of the posed images; Bottom right: time stamp corresponding to the Video 3). The merozoite (black arrow) deformed the erythrocyte for nearly 6 s when a calcium signal appeared presumably at the point of contact between the two cells (white arrow). Subsequently an increasing level of calcium signal was observed while the merozite was invading throughout the erythrocyte cytoplasm shortly after the first apical calcium signal. The rise of cytoplasmic calcium in the infected erythrocyte persisted for the whole length of the recording as presented by the graph of fluorescence intensities over time below. Each event and invasion phases were labeled.
Figure 5.7. Depletion of extracellular calcium partly reduces merozoite invasion.
The concentration of free calcium ions in the culture medium were modified by either supplementing the RPMI medium with 2 mM BAPTA or using the Ca$^{2+}$-free DMEM medium. Erythrocyte invasion under these conditions was closely examined by live-cell imaging using brightfield illumination and compared to invasion in normal RPMI medium. The number of successful invasion per rupture was counted for each type of media. The average numbers of successful invasion per schizont rupture declined in media containing reduced free calcium, however they were not significantly different from the normal RPMI medium control (student's t-test analysis; $P = 0.1544$ for BAPTA-RPMI medium, $P = 0.1493$ for the Ca$^{2+}$-free DMEM medium).
Figure 5.8. Extracellular calcium does not play a crucial role in the invasion kinetics.

The kinetics of each stage of the invasion was compared between merozites in normal RPMI medium and in the Ca\(^{2+}\)-free DMEM medium whereby the level of extracellular calcium ions were kept to minimum. The student's t-test statistical analysis was performed to compare the differences in kinetics of each invasion phase between the two types of media and there were no significant differences. The numbers of observation for each condition and invasion stage are stated in the graph.
The graph illustrates the comparison between Normal RBCs and BAPTA-RBCs in various conditions, with the following time measurements in seconds:

- **RBC deformation**: 37 (Normal), 11 (BAPTA), varying n.
- **Resting**: 36 (Normal), 10 (BAPTA), varying n.
- **Invasion**: 36 (Normal), 10 (BAPTA), varying n.
- **Time to echinocytosis**: 36 (Normal), 7 (BAPTA), n = 7.
- **Time to max echinocytosis**: 36 (Normal), 6 (BAPTA), n = 6.

The P-value for the comparison is 0.0002, indicated by the asterisk (*) on the graph.
**Figure 5.9. Influx of calcium ions into infected erythrocytes may play a role in triggering the onset of echinocytosis.**

Fresh erythrocytes were incubated with the 60 µM BAPTA-AM, a membrane-permeable calcium chelator, prior to mixing with purified late- stage schizonts. Purified schizonts were also mixed with the normal erythrocytes as a control. Erythrocyte invasion was imaged in the Ca²⁺-free DMEM medium, and the kinetics of each invasion phase was measured. The differences in invasion kinetics between untreated and BAPTA-treated erythrocytes were validated using a student's t-test. Only the time required for the infected erythrocytes to start echinocytosis after the merozoite completely internalised was significantly delayed when compared with the invasion into untreated erythrocytes (P = 0.0002). The numbers of observation for each condition and each stage are shown on the graph.
Figure 5.10. Inhibiting invasion with the R1 peptide did not block the invasion calcium flux but extended the echinocytosis period of infected erythrocytes.

(A) Merozoite invasion of erythrocytes was observed in the presence of 5 μM Fluo-4 AM and 100 μg/mL R1 peptide, an invasion inhibitor that blocks AMA1 function. The live-cell images were recorded every 2 s for 2 min as alternating brightfield and fluorescence images. Still images from representative Video 4 were shown. The calcium spike at the presumed attachment site and the ensuing calcium influx into the erythrocyte were detected normally despite the fact that R1 peptide prevented the merozoite from entering the erythrocyte. The relative timings of the snap shots are on the top left, while the time stamps that appeared in Video 4 are at the bottom right.

(B) Still images of the brightfield channel from the Video 5 that followed the invasion event in the presence of Fluo-4 AM and the R1 peptide for up to 22 min. The fluorescence channel was captured for the first 2 min and only the brightfield channel was recorded for the rest of the invasion event. The images represented the beginning of the echinocytosis phase that lasted for longer than 20 min. The relative timings of the snap shots are on the top left, while the time stamps that appeared in the Video 5 are at the bottom right.
Figure 5.11. Inhibiting erythrocyte invasion with cytochalasin D does not prevent a calcium flux but extend the echinocytosis period.

(A) The purified late schizonts were treated with 1 µg/mL of cytochalasin D (cytD), an inhibitor of actin polymerisation known to block the invasion. The schizonts were then mixed with the erythrocytes labeled with 5 µM Fluo-4 AM. The live-cell images were captured every 2 s for 90 s by alternately recording the brightfield and fluorescence channels. Still images from representative Video 6 are shown. The cytD-treated treatment prevented the merozoite (black arrow) from deforming the erythrocyte as it normally would. A calcium spike presumably at the attachment site (white arrow) was detected as per normal, however the calcium influx into the erythrocyte did not ensue. It should be noted that the resting levels of intracellular calcium throughout this particular experiment seemed higher than normal. The relative timings of the snap shots are on the top left, while the time stamps that appeared in Video 6 are at the bottom right. (B) Selected still images Video 6 up to the 21 min time points are shown for the brightfield channel. The fluorescence channel was captured for the first 90 s and only the brightfield channel was recorded for the rest of the time. The images represented the beginning of the echinocytosis phase (red arrow) and show that it lasted for longer than normal at 14 min. The onset of the echinocytosis was also delayed. The relative timings of the snap shots are on the top left, while the time stamps appeared in the Video 6 are at the bottom right.
Supplementary Videos

Video 1. High-speed time-lapse acquisition of 3D7 merozoite invading the erythrocyte (40 fps).

Video 2. The 3D7 merozoite invading the BODIPY FL C₁₂-sphingomyelin labelled erythrocyte (2 fps, 2× real speed).

Video 3. The 3D7 merozoite invading the erythrocyte in the presence of Fluo-4 AM showing the punctate apical calcium and calcium influx in the infected erythrocyte (3 fps, 8× real speed)

Video 4. Fluo-4-stained 3D7 parasite culture showing merozoites attempting to invade in the presence of R1 peptide (3 fps, 8× real speed). The punctate apical calcium and influx in the attached erythrocyte were detectable.

Video 5. Fluo-4-stained 3D7 parasite culture showing merozoites attempting to invade in the presence of R1 peptide (variable speed). The echinocytotic erythrocyte had not recovered after ~20 min of recording.

Video 6. CytD-treated 3D7 merozoite attempting to invade the Fluo-4 labelled erythrocyte (variable speed). The punctate apical calcium was visible but the calcium influx was difficult to observe. The echinocytotic erythrocyte had not recovered after ~20 min of recording.
CHAPTER 6

Concluding Remarks

Combating malarial disease has been a perpetual mission for human kind. Modern malaria research began in the late 1800s with the discovery by Alphonse Laveran of the causative *Plasmodium* parasite. Ronald Ross soon after discovered that the parasite was transmitted by mosquito bite and despite more than a century of studying the causative microorganism, much still remains to be ascertained about parasite's biology. This thesis aimed to help fill in some small way gaps in our knowledge by focusing the invasive stage of the parasite in the human host where it causes disease. The research particularly concentrated on the mechanism of erythrocyte invasion by the *P. falciparum* merozoite and the biochemical and functional characterisation of 6-ey's proteins, a group of merozoite surface proteins that have been little studied in this species.

The mechanisms of erythrocyte invasion have always been an enigma in malaria research due to their molecular complexity and the fact they take place over a very short period of time of just a few minutes. Nonetheless a clearer picture of cellular actions during the invasion event has been achieved by means of various microscopy techniques. With improvements in temporal resolution and the use of fluorescent indicators, the microscopy techniques used in this study were able to provide more information about the invasion process which has been summarised below (Fig. 6.1). Following the explosive nature of merozoite egress from the schizont, erythrocyte invasion begins with the reversible interaction between the merozoite and host erythrocyte. It was observed that regardless of the merozoite’s orientation at first contact, the merozoite sometimes invaded the first erythrocyte contacted or detached and invaded the next erythrocyte. In measurements made by my colleagues, most wild-type *P. falciparum* strains invade the first or sometimes the second erythrocyte contacted whereas a mutant strain lacking the major ligand EBA175 tended to contact 2 - 3 erythrocytes before invading (Weiss et al., 2015). This possibly indicates the avidity of the merozoite for its erythrocyte host plays an important role in rapid erythrocyte selection.
Following first contact the next invasion step involves continuous waves of erythrocyte deformation which generally last for 7.5 s. By imaging this pre-invasion period at high-speed (40 fps) it was noticed here that the deformation occurred more frequently (avg. 4 waves) than it was previously reported (Gilson and Crabb, 2009), yet different degrees of deformation strength were similarly observed (Weiss et al., 2015). The deformation process is likely to involve the actin-myosin motor of the merozoite since the cytD-treated parasite cannot deform its erythrocyte (Weiss et al., 2015). Interestingly, the cytD-treated merozoites can still apparently release their rhoptries and trigger echinocytosis of the erythrocytes since they still undergo echinocytosis. The cytD-treated merozoites tend to contact more erythrocytes before ultimately selecting one to trigger echinocytosis upon. This suggests that deformation may help embed the merozoite into the erythrocyte surface leading to more rapid erythrocyte selection and downstream events (Weiss et al., 2015).

When the invasion events were imaged at high speed, the re-orientation of merozoite could be clearly followed whereas at a lower frame rate reorientation was not as obvious. Once the membrane deformation finished, a brief period of resting of 1.6 s commenced. This could be when the merozoite apically attached to the host erythrocyte because the resting period appears to be the time when the rhoptry neck RON complex contents are released and the tight junction with AMA1 is formed. Immediately following this internalisation of the merozoite into the erythrocyte commences. High-speed acquisition also helped clearly identify the penetration period, which lasts for about 17 s, from the beginning until the membrane is completely sealed behind the merozoite. About 18.5 s after this, the merozoite begins to spin in a clockwise motion using the posterior attachment to the erythrocyte inner membrane as an anchoring point. Although rotation has been previously reported for *P. knowlesi* (Dvorak et al., 1975), this is the first time such cellular action is reported for *P. falciparum* merozoites. The molecular mechanism behind this rotation and its purpose are not known although the fact it precedes differentiation into a ring-stage parasite, the rotation could play a role sealing the erythrocyte membrane and pinching off the PVM from it. This event was difficult to observe however, since the infected erythrocyte began to change its shape into an echinocyte. It took about 5-10 min for the infected erythrocyte to recover its biconcave shape by which time the merozoite had already transformed into a highly amoeboid ring stage parasite.
Another remarkable discovery happening during the invasion were the calcium fluxes which firstly appeared as a bright foci at the point of contact just before the beginning of internalisation then subsequently spread throughout the host erythrocyte. This finding provided visual evidence for the participation of calcium ions during the invasion process that has been previously proposed by several studies especially on the erythrocyte side. To determine the role of external calcium I imaged merozoite invasion in media with reduced levels of free calcium and found it to be important for efficient invasion but the reduction in the number of invasions per schizont rupture was not significant. My colleagues repeated these experiments taking extra measures to reduce external calcium as much as possible and were able to confirm it was crucial for erythrocyte invasion (Weiss et al., 2015). Under these conditions the merozoites could attach to and deform their erythrocytes but were not able to trigger echinocytosis or produce a calcium flux at the merozoite tip suggesting the rhoptries were not released. Although it is possible the source of the observed calcium in the fluxes is from the medium, given the timing and the initial punctate appearance, it is also possible that the ion could be coming from the rhoptries.

The calcium that enters the infected erythrocyte as examined in this study seems to participate in the echinocytosis phase of the infected erythrocyte probably by inducing the reorganization of the erythrocyte cytoskeleton. This notion requires further investigation with accurately regulated calcium levels to ascertain the function of the ion. The fact that interfering with the calcium levels in the infected erythrocyte disturbs the onset of echinocytosis suggests that calcium plays a role in triggering or maintaining echinocytosis. One possible explanation for why echinocytosis occurs is that the infected parasite tries to restrict other merozoites from invading into the same erythrocyte and competing for its resources since it was reported that the merozoites cannot infect crenated erythrocytes which have the same appearance of echinocytes (Tiffert, 2005).

The usefulness of the microscopy techniques established here are that they could lay the foundation for future studies to overlay the functions of merozoite ligands and erythrocyte receptors involved in invasion. The general idea would be to disrupt the function of molecules of interest with specific inhibitors and examine the changes in morphology and timing of events during invasion that could suggest roles of the molecules involved. Not only this would allow direct examination of the function of a particular molecule, but would also help reveal the order of actions
among the molecules associated with invasion. Two examples have been demonstrated here by using R1 peptide and cytD as inhibitors of the AMA1-RON2 interaction and merozoite actin-myosin motor, respectively. Both are well known for their invasion inhibitory activity but not much detail in terms of the appearance of impeded morphology has been described. Only the effect of R1 peptide has been reported by Treeck et al. using video microscopy, however the authors only pointed out that the internalisation was blocked by the peptide without disturbing the reorientation of the merozoite (Treeck et al., 2009). Herein the examination on the impact of R1 peptide was broadened to cover the emergence of calcium fluxes and the echinocytosis period. It is evident that the R1 peptide does not inhibit the activation of calcium fluxes, thus indicating that the tight junction formation is not required to trigger the calcium flux. Since the RON complex must be released from the rhoptries and cross the erythrocyte membrane to initiate tight junction formation it is possible that calcium flux occurs as a consequence of rhoptry release and erythrocyte permeabilisation.

CytD-treated merozoites too exhibit the calcium fluxes as normal but the flux is delayed. This could be a result of the preceding effect of cytD that prevents strong erythrocyte deformation thereby possibly slowing down the reorientation process. The fact that the flux occurs however suggests that motor driven deformation is not required for reorientation. Reorientation might instead be triggered by a gradient of EBA and Rh ligands originating from the merozoite apex as has been suggested previously (Farrow et al., 2011). Although the cytD-treated merozoites can eventually make apical attachment to the erythrocyte, they cannot further penetrate into the erythrocytes indicative of the requirement of propelling force from actin-myosin motor of the parasites for invasion. Another drastic change in morphology is the prolonged echinocytosis period observed in both R1 and cytD inhibition. It is likely that the echinocytosis is continuously activated by some factors, including calcium ions, through the hypothetical pore that is formed as determined by the emergence of apical calcium spike. Because the invasion and subsequent resealing cannot proceed due to the inhibitors, the invasion pore remains open and allows entry of extracellular ions such as calcium into the erythrocyte prolonging echinocytosis.

Using the microscopy methods developed by me, my colleagues have successfully revealed the order of action of several known invasion molecules (Weiss et al., 2015). First was an initial reversible attachment, possibly instigated by the
MSP1 complex that is responsible for weak erythrocyte deformation and progression of downstream ligands. When blocked by heparin which is thought to block a subunit of MSP1 (Boyle et al., 2010a), the merozoite can attach but cannot deform its target erythrocyte (Weiss et al., 2015). Blockage of EBA175 and Rh4 via neuraminidase treatment and addition of complement receptor 1 fragments respectively, similarly block strong deformation. This indicates that the erythrocyte binding antigens (EBAs) and some reticulocyte-binding like homologs (Rhs) appear to act next bridging their specific erythrocyte receptors with the merozoite’s actin-myosin motor to strongly deform the erythrocyte. Blockage of PfRh5 or its erythrocyte receptor basigin with specific IgGs, allow strong deformation but does not permit a calcium flux or echinocytosis. This suggests the interaction between PfRh5 and the basigin receptor creates an opening where small molecules such as calcium ions can flow into the erythrocyte and can trigger the echinocytosis. Subsequently the strong interaction between AMA1 and RON2 forms a tight junction through which the merozoite penetrates to get inside the erythrocyte.

The invasion ligands mentioned above are not the only ones present on the merozoite surface or in its apical organelles. It is possible that other proteins expressed in the merozoite also take part in the invasion process. This thesis also reports on the characterisation of a group of surface proteins that belongs to a protein family named 6-cys and their presence on the merozoite surface suggest an invasion role. The four members, P12, P38, P41, and P92, were identified by a past proteomic study (Sanders et al., 2005) but have not been studied in any great detail in P. falciparum yet. The protein expression and localisation analyses using specific antibodies confirm the presence of these molecules in late blood-stage parasites. P41 as the only member that does not possess a membrane anchoring modification forms a heterodimer with P12 to retain itself on the merozoite surface. This dimerisation between the non-anchored protein and the GPI-anchored protein expressed at the same stage of life cycle appears to be an interesting characteristic of the 6-cys family since a similar interaction has also been reported for P48/45 and P230, the GPI-anchored and non-anchored 6-cys members expressed in the insect stage, respectively (Kumar, 1987). It remains however to be demonstrated that this also holds true for the sporozoite stage GPI-anchored P52 binding the anchorless P36.

Since 6-cys proteins in other life stages have been demonstrated to function in cell-cell interactions, the blood-stage 6-cys proteins were initially thought to play a
similar function potentially between the merozoite and erythrocyte. However the various experiments performed to test this hypothesis proved otherwise. Although P12/P41 heterodimer and P92 are shed from the merozoite into the culture supernatant similar to other known invasion ligands, they do not appear to specifically bind erythrocyte receptors with high affinity like EBA175 for example. P38 on the other hand is not cleaved from the merozoite surface and it is carried into the erythrocyte with the merozoite. This is a characteristic that is not seen in many other important invasion ligands. Specific antibodies to the blood-stage 6-cys proteins are not able to substantially inhibit the merozoites from invading erythrocytes. Moreover the mutant parasites lacking each of the blood-stage 6-cys proteins demonstrate comparable growth to the wild-type. Similar lack of phenotypes was observed when some of the blood-stage 6-cys were deleted in P. berghei (except for P92 which is not found in rodent parasites) (van Dijk et al., 2010). Virtually all of the evidence presented here suggest that the 6-cys proteins expressed in the merozoite are not involved in the erythrocyte invasion. While the 6-cys proteins in the insect and liver stages display important functions and the fact that the blood-stage 6-cys proteins are conserved throughout the genus, strongly suggest that they should have a significant role in the blood-stage parasite as well but what could it be?

For the P12/P41 heterodimer, the small decrease of proteins levels after passage over several pools of erythrocytes suggests low-affinity binding of the heterodimer for erythrocytes similar to that observed for MSP142. The 6-cys homologous SAG proteins of Toxoplasma gondii, are thought to bind host glycan raising the possibility the 6-cys behave similarly (He et al., 2002). As the P12/P41 heterodimer does not bind to the glycan heparin, it is possible that it could bind to other types of glycans present on the erythrocyte surface facilitating the initial attachment of merozoites to the erythrocytes. Using recombinant P12 and P41, which readily form heterodimer, on glycan arrays would reveal whether they could bind to any glycan and which type of sugar it was. Furthermore the P12/P41 might assist the merozoites in selecting erythrocytes with other blood types other than the O-type used in this thesis and usual in vitro culture. There is also a possibility that this heterodimer could recognise reticulocytes which the P. falciparum also infects.

The shedding of P92 as well as the small depletion noticed when it was exposed to the erythrocytes multiple times suggests it may function similarly to the P12/P41 heterodimer as a weak erythrocyte binding protein. A breakthrough into the
function of P92 and potentially other 6-cys was recently achieved, whilst I was preparing my thesis. My colleagues in a search for host factors that may protect merozoites from host defenses, namely complement, discovered that P92 helps recruit Factor H to the merozoite surface (Kennedy et al., 2015; manuscript in preparation). Factor H is a soluble protein of human serum that acts as a part of the alternative pathway of the complement system and is generally recruited by the human cells to down regulate complement activation, thereby preventing the host from complement mediated membrane attack. Without their P92, the mutant merozoites are more easily prone to the complement mediated lysis after egress so this countermeasure is important for the parasite survival. Similar immunomodulatory roles of the 6-cys proteins have also been reported for P47 and P230 in blocking the progression of the sexual-stage malaria parasites in the mosquitoes (Healer et al., 1997; Molina-Cruz et al., 2013; Read et al., 1994; Williamson et al., 1995), hence protection from host defenses appears to be another major role for the 6-cys proteins in addition to cell-cell interaction.

The characteristic of P38 is the most unique among the four blood-stage 6-cys proteins, that it is carried into the erythrocyte during invasion. Recent new information has now reveled that several to many merozoite proteins might be taken into the newly infected erythrocyte during invasion. For example, MSP2 and MSP4 have been shown to be carried into the newly infected erythrocyte without processing (Boyle et al., 2014). MSP2 is rapidly degraded after invasion while MSP4 remains recognisable for a certain period after invasion. Nonetheless the role of MSP2 and MSP4 is still not known. Further investigation for the role of P38 should therefore focus on the development of ring-stage parasites. One possible function for P38 could be the maintenance of nascent parasitophorous vacuole membrane as has been reported for the sporozoite 6-cys proteins, i.e. P52 and B9 (Labaied et al., 2007; van Dijk et al., 2005; Annoura et al., 2014). In light of the P92 finding, it is also possible that P38 could help neutralise complement factors carried into the newly infected erythrocyte protecting the ring-stage parasite. The Δp38, Δp12, and Δp41 mutant parasites will be useful for discovering if these parasites are more susceptible to damage growing in the presence of active human complement thereby indicating that the primary function of these 6-cys proteins is for protection from host immune responses.
In summary this thesis demonstrates that live cell microscopy is very useful technique for studying erythrocyte invasion by the *P. falciparum* merozoite and is able to provide an in-depth understanding of these important steps in the malaria parasite’s life cycle. A more complete understanding of the invasion process could then be translated into the development of multivalent vaccines targeting crucial invasion ligands that act sequentially at each of the invasion steps providing a synergistic effect. Although my studies of blood-stage 6-cys proteins do not provide a definitive function for these proteins, the results suggest that they do not function as essential invasion ligands. Future experiments should instead focus on other possible functions such as having an immunomodulatory role and/or for low-affinity interactions with host erythrocytes. The probable role of the blood-stage 6-cys proteins in helping the merozoite to avoid the host immune system could additionally be blocked thereby boosting host defences to more effectively neutralise the merozoites. There is evidence showing that some blood-stage 6-cys proteins are under positive selection and induce strong host immune responses further supporting the idea of including these proteins into multivalent vaccines (Tetteh et al., 2009; Reeder et al., 2011; Amambua-Ngwa et al., 2012; Richards et al., 2013).
Ca²⁺ release into host

18.5 s

Internalisation

16.8 s

Invasion

1.6 s

Invasion Initiation

7.5 s

Reorientation & MN release

Primary Contact

Ring stage & Export

Spinning & DG release

Ca²⁺ enters host from merozoite

Host Actin Ring

Tight Junction

RON complex

Phospho AMA1

Micronemes

Rhoptries

Tight Junction Formation

Ron complex

Lipoprotein?

E-vacuoles?
Figure 6.1. A modified model of erythrocyte invasion by the *P. falciparum* merozoite.

Once a schizont ruptures, a number of merozoites are released and an environmental signal, presumably the low concentration of potassium ions, triggers the increasing level of intracellular calcium ions. The merozoite initially attaches to the erythrocyte membrane and starts to deform the erythrocyte. The actin-myosin motor of the parasite is believed to be responsible for this action since the cytD-treated merozoites could not deform the erythrocyte. It is during this 7.5 s pre-invasion phase that the merozoite re-orientates and releases its microneme (MN) proteins to prepare for the next stage of invasion. When the apical end of the merozoite establishes a tight interaction with the erythrocyte plasma membrane, it rests for a brief 1.6 s before the invasion begins. At this point a pore, or cytoplasmic continuity, forms between the cells because $\text{Ca}^{2+}$ released from the merozoite or media causes an intense spike as it is stained with fluorescent calcium indicator from the erythrocyte. The $\text{Ca}^{2+}$ spreads throughout the infected erythrocyte while the merozoite internalises through the tight junction. Blocking the AMA1 function with R1 peptide inhibits the invasion but does not stop the influxes of $\text{Ca}^{2+}$. When the merozoite completely internalises, which takes about 17 s, it remains attached to the inner membrane of the erythrocyte for the next 18.5 s or so before it begins to spin. The speed fluctuates over time but the direction appears clockwise. The spinning may facilitate the merozoite resolving the membrane junctions while the dense granule (DG) contents are released. Next the erythrocyte undergoes echinocytosis while the merozoite transforms into a ring stage parasite. After several minutes the erythrocyte recovers back to its normal biconcave shape.
REFERENCES


salivary gland infection by malaria sporozoites. J. Exp. Biol. 208, 3211–3218.


Boyle, M.J., Wilson, D.W., Richards, J.S., Riglar, D.T., Tetteh, K.K.A., Conway,


Apicomplexan parasites co-opt host calpains to facilitate their escape from infected cells. Science 324, 794–797.


Elliott, J.F., Albrecht, G.R., Gilladoga, A., Handunnetti, S.M., Neequaye, J.,


Gilson, P.R., and Crabb, B.S. (2009). Morphology and kinetics of the three distinct phases of red blood cell invasion by Plasmodium falciparum merozoites. Int J
Parasitol 39, 91–96.


Urquiza, M., Suarez, J.E., Cardenas, C., Lopez, R., Puentes, A., Chavez, F., Calvo,


Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes. Mol Biochem Parasit 75, 33–42.


APPENDICES
APPENDIX I

Expression of recombinant P12 and P41 fusion proteins in HEK293E mammalian cell system.

(A) The diagram depicts the characteristics of the recombinant P12 and P41 fusion proteins expressed in HEK293E cells. The predicted regions of the genes encoding the ectodomains of the proteins were synthesized and optimized for mammalian cell expression. P12, excluding the GPI signal sequences, and P41 were C-terminally fused with domain 3 and 4 of rat Cd4 (green) followed by 6×His tags (blue). (B) Coomassie-stained gel showed the pure soluble recombinant P12 and P41 migrated at expected sizes under reducing condition of the SDS-PAGE.
**A**

$$K_d = 0.31 \pm 0.03 \mu M$$

**C**

Graph showing blocking response (RU) over time (s) for P12-6H and P41-bio.

**B**

$$K_a = 1.336 \times 10^4 \pm 0.004 \text{ M}^{-1}\text{s}^{-1}$$

$$k_d = 0.02122 \pm 0.00007 \text{ s}^{-1}$$

**D**

Graph showing blocking response (RU) over time (s) for P41-6H, P12-bio, and P41-bio.
APPENDIX II

Biochemical analysis of the recombinant P12 and P41 interaction by surface plasmon resonance (SPR).

(A) The equilibrium dissociation constant ($K_D$) of the P12/P41 interaction was measured. The recP41-Cd4d3/4-6H was serially diluted, and each concentration of the proteins was injected through the flow cell containing either Cd4d3/4 alone, as a reference, or recP12-Cd4d3/4-bio captured on a streptavidin-coated sensor chip until the equilibrium had been reached (inset). The binding data between recP41-Cd4d3/4-6H and recP12-Cd4d3/4-bio were subtracted with the data from reference cell before being plotted into a binding curve. The $K_D$ was calculated using non-linear regression fitting of a simple Langmuir binding isotherm, which was equal to 0.31 ± 0.03 µM.

(B) Kinetic data of the interaction were also measured. Serial two-fold dilutions of 3.6 µM recP41-Cd4d3/4-6H were injected over the recP12-Cd4d3/4-bio and a reference flow cell at high flow rates (100 µL min$^{-1}$). The resulting sensograms displayed excellent fits for a simple 1:1 binding model (red lines) and were used to calculated for the association ($k_a$; $1.336 \times 10^5 \pm 0.004$ M$^{-1}$s$^{-1}$) and dissociation ($k_d$; $0.02122 \pm 0.00007$ s$^{-1}$) rate constants. (C) Purified 2.5 µM of recP12-Cd4d3/4-6H was injected over the recP41-Cd4d3/4-bio (blue line) and recP12-Cd4d3/4-bio (red dotted line) captured on streptavidin-coated sensor chips. The binding response was only seen between the recP12 and recP41. This result indicated that P12 does not interact homophilically. (D) The reciprocal assay whereby purified 2.5 µM of recP41-Cd4d3/4-6H was injected over the same two sensor chips confirmed the preferred heterophilic interaction between the two proteins.
APPENDIX III

Antibodies produced against mammalian cell expressed recombinant P12 and P41 do not inhibit 3D7 and Dd2 parasites in invasion inhibition assays.

Rabbit polyclonal anti-P12 and anti-P41 antibodies were tested individually and in combination against the 3D7 and Dd2 parasite lines as shown. Various concentrations of the antibodies, as indicated, were used. Late trophozoite stage parasites were incubated with the antibodies for 24 hours to allow the invasion to occur, and the parasitemia were measured by flow cytometry. Invasion efficiency was calculated relatively to the parasites cultured without the antibodies. The mean and SD of invasion efficiency from triplicates were plotted as shown. The findings indicate that the anti-P12 (green) and anti-P41 (blue) antibodies, even when they were used in combination (red), cannot inhibit the host cell invasion by the two laboratory strains tested.
APPENDIX IV

The interaction between recombinant P12 and P41 expressed from HEK293E cells can be interrupted by anti-P12 and anti-P41 antibodies but cannot once the complex formed.

The AVEXIS method was used to detect the interaction between the recP12 and recP41 from HEK293E mammalian cell expression system (Bushell et al., 2008). (A) The pentameric β-lactamase-tagged recP12 prey was incubated with the increasing concentration of the anti-P12 antibodies before adding to the monomeric biotinylated recP41 bait immobilized on a steptavidin-coated microtitre plate. The interaction was measured from the hydrolysis of β-lactamase substrate, nitrocefin, into the product that absorbs at 485 nm. The absorbance at 485 nm decreased relatively to the increasing concentration of the antibodies suggesting that the anti-P12 antibodies could interfere with the P12/P41 complex formation (B) The reciprocal assay was performed to measure the interaction of pentameric β-lactamase-tagged recP41 prey after being incubated with increasing concentration of the anti-P41 antibodies and the biotinylated recP12 bait captured on a streptavidin-coated microtitre plate. Again, upon increasing concentration of the anti-P41 antibodies, the reduced amount of the product formed from the hydrolysis of nitrocefin supporting the role of the antibodies to inhibit the formation of the P12/P41 heterodimer. (C) The recP12 (red) or recP41 (blue) prey was first allowed to interact with its respective bait prior to the addition of different concentration of the anti-P12 or anti-P41 antibodies to the formed complex. No decrease in the absorbance at 485 nm was observed indicating that once the complex formed, the antibodies could not disrupt it. All experiments were done in triplicate, and the mean and SD were plotted.