Functional characterization of plasmodium falciparum reticulocyte binding like protein homologue 1 (PfRH1)

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NANYANG
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**FUNCTIONAL CHARACTERIZATION OF
PLASMODIUM FALCIPARUM RETICULOCYTE
BINDING LIKE PROTEIN HOMOLOGUE 1
(PfRH1)**

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SCHOOL OF BIOLOGICAL SCIENCES
2014

Functional Characterization of *Plasmodium
falciparum* Reticulocyte Binding Like Protein
Homologue 1 (PfRH1)

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List of Abbreviations

A

AAA+ proteins	ATPases associated with various cellular activities
ACT	Artemisinin-based Combination Therapy
ADP	Adenosine diphosphate
AMA-1	Apical membrane antigen 1
AMP	Adenosine monophosphate
ATP	Adenosin-5'- triphosphate

B

BSG	Basigin
BSA	Bovine serum albumin

C

CD	Circular Dichroism
CFTR	Cystic fibrosis transmembrane conductance regulator
CR1	Complement receptor 1

D

DBL	Duffy-binding-like
DBP	Duffy binding protein
DMSO	Dimethyl sulfoxide

E

E	Glutamic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EBD	Erythrocyte-binding domain
EBL	Erythrocyte binding like
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

F

FBS	Fetal bovine serum
FCS	Fluorescence Correlation Spectroscopy
FRET	Fluorescence Resonance Energy Transfer

G

GAPs	Glideosome associated proteins
GDP	Gross domestic product
GPI	Glycosylphosphatidylinositol

H

HAT Hypoxanthine-aminopterin-thymidine medium

I

I Isoleucine
IFA Immunofluorescence assay
IgG Immunoglobulin G
IMC Inner membrane complex
IP Immunoprecipitation
IPTG Isopropyl-1-thio- β -galactopyranoside
IRS Indoor spraying with residual insecticides
ITC Isothermal titration calorimetry
ITNs Insecticide-treated mosquito nets

L

L Leucine
LB Luria-Bertani
LB-Kan Luria-Bertani media with Kanamycin
LLINs Long lasting insecticides impregnated nets

M

mAbs Monoclonal antibodies
MSPs Merozoite surface proteins
MTIP Myosin A tail domain interacting protein

N

NBD Nucleotide binding domain

P

PBS Phosphate-buffered saline
PCR Polymerase chain reaction
Pf *Plasmodium falciparum*
PMSF phenylmethanesulfonylfluoride
PfNBR Nucleotide-binding region of PfRH1
PfRHs Reticulocyte binding like protein homologue of *Plasmodium falciparum*
PfRONS Rhoptry neck proteins of *Plasmodium falciparum*
Pk *Plasmodium knowlesi*
PV Parasitophorous vacuole
Pv *Plasmodium vivax*
PVM PV membrane
Py *Plasmodium yoelii*

R

RBR Reticulocyte binding region
RDTs Rapid diagnostic test

RH	Reticulocyte binding like protein homologue
rNBR	Recombinant nucleotide-binding region of PfRH1

S

SAXS	Small-Angle X-ray Scattering
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SERA	Serine repeat antigen
SUB1	Subtilisin-like protease 1
SUB2	Subtilisin-like protease 2

T

<i>T. gondii</i>	<i>Toxoplasma gondii</i>
TRAP	Thrombospondin-related anonymous protein

Abstract

Human malaria parasites, *Plasmodium falciparum*, invade human erythrocytes through multiple ligand-receptor interactions. One of the protein families involved in invasion, reticulocyte binding like protein homologues (PfRHs) has been suggested to be involved in host cell selection. Erythrocyte intracellular ATP is essential for merozoite invasion as merozoites fail to invade ATP depleted-erythrocyte, suggesting that ATP is a potential signal in host cell selection. In this work, a novel ATP/ADP binding region (PfNBR) which binds ATP at a higher affinity was identified in PfRH1, a member of PfRHs. IP-coupled photoaffinity labeling assay and Mass spectroscopy data suggest that PfNBR binds nucleotides without the conserved ATP-binding P-loop or A-loop. The determined ATP binding constant of the recombinant PfNBR is in the micromolar (μM) range. Merozoite surface exposure of PfNBR occurs when merozoites are in close contact to erythrocytes, suggesting that PfRH1 could act as an ATP sensor to detect near-membrane environmental ATP released from a deforming erythrocyte during invasion. Epitope mapping of invasion and Ca^{2+} signaling inhibitory antibodies against Erythrocyte binding domain (EBD) reveals a number of short linear epitopes within the predicted coiled-coils region of the EBD. PfNBR is detected with coiled-coils structures based on the bioinformatic prediction and Circular Dichroism (CD) measurement. Co-presence of PfNBR and EBD in the same processed fragment of PfRH1 implies a close working relationship between these two domains. PfRH1 plays multiple roles along the invasion process including 1) host receptor binding, 2) ATP binding, and 3) signaling transduction but the interplay between these functional roles during invasion remain unclear. A proposed model of the possible molecular mechanism of PfRH1-dependent merozoite invasion may provide us more insights about the invasion mechanisms of malaria parasites and, thus, how these processes might lead to development of new strategies for malaria intervention.

Chapter 1 Introduction

1.1 An Overview of Human Malaria

1.1.1 Malaria: A Major Global Killer

Malaria has been noted for more than 4, 000 years (CDC, 2012) in the history of human infectious disease. It is caused by *Plasmodium spp.*, an obligate intracellular parasite that is transmitted by *Anopheles* mosquitoes. Malaria has a broad distribution in both the subtropics and tropics. Today, there are 109 countries in four regions (Africa, Asia Pacific, the Americas, Middle East and Eurasia) affected by malaria. About 3.3 billion people, almost half of the world's population, are at risk of malaria. In 2009, there were about 250 million cases of malaria and almost one million deaths worldwide, in which most of the cases and deaths occurred in sub-Saharan Africa. In Africa, this disease accounts for 20% of all childhood deaths with a child dying every 45 seconds from malaria. Besides that, the infection during pregnancy causes around 200,000 deaths of infants annually and a maternal death rate of 10-50%. The high mortality rate caused by malaria has created a heavy social and economic burden on the affected countries particularly in Africa. The disease accounts for up to 40% of public health expenditures and the decrease of gross domestic product (GDP) of as much as 1.3% in countries with high levels of transmission (WHO, 2011).

About 60 of the 400 species of *Anopheles* mosquitoes in the world are malaria vectors under natural conditions (Tuteja, 2007). More than 100 species of the protozoan parasite genus *Plasmodium* can infect a wide range of animals from human and other mammals to birds and reptiles (Sherman, 1998a). There are six known species of *Plasmodium* that can infect human which are *Plasmodium falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, *P. knowlesi* and *P. cynomolgi* (Kean, 1978; Sherman, 1998b; Singh et al., 2004; Ta et al., 2014). *P. falciparum* known as the deadliest human malaria parasite causes severe anemia and obstructions of small blood vessels in patients. The obstructions in brain or placenta result in cerebral malaria and placental malaria, the two complications that contribute to high mortality of children and

pregnant women in Africa. *P. vivax* contributes a lower parasite biomass in infection as compared to *P. falciparum* infections (Ross and Thomson, 1910). However, it is responsible for the major morbidity and mortality in *vivax*-endemic areas (Anstey et al., 2012) and large series of severe or fatal cases of infection has been reported from different areas such as Indonesia, Papua New Guinea and other countries (Rogerson and Carter, 2008; Anstey et al., 2012). *P. ovale* infections rarely lead to severe illness or life-threatening complications. Similar to *P. vivax*, it has a dormant liver stage known as hypnozoites that may remain in the liver for weeks to years, causing relapses of the disease. The non-fatal *P. malariae* is able to develop long lasting blood stages infections, which can persist asymptomatically in the host for decades (Tuteja, 2007). *P. knowlesi* naturally infects long-tailed and pig-tailed macaques (Knowles and Das Gupta, 1932) while *P. cynomolgi* naturally infects various macaques (Di Fiore et al., 2009). Naturally acquired human infection for both species was thought to be rare. Today, *P. knowlesi* is widely recognized as the fifth species that causes malaria in humans as cases of infection have been described throughout Southeast Asia (Singh and Daneshvar, 2013). *P. knowlesi* infection causes a full spectrum of diseases which are similar to those of *P. falciparum* and *P. vivax* infections (Daneshvar et al., 2009; Barber et al., 2013). The infection may develop to severe disease that is potentially fatal. Several cases of laboratory-acquired infections by *P. cynomolgi* have been reported in 1960s (Eyles et al., 1960; Schmidt et al., 1961; Baird, 2009). However, it is only until recently that first case of naturally acquired *P. cynomolgi* infection in humans has been identified from Peninsular Malaysia (Ta et al., 2014) but the possibility of having undiagnosed or misdiagnosed cases cannot be ruled out because *P. cynomolgi* is morphologically indistinguishable from *P. vivax* (Ta et al., 2014).

1.1.2 Symptoms, diagnosis and treatment of Malaria

In general, the onset of malaria might be gradual or fulminate, depending on the parasite species and immune status of the patient. Symptoms of malaria in humans usually develop 10-15 days after the infective mosquito bite for *P. vivax* and *P. falciparum* malaria, but longer incubation period may occur. The early signs are often described as resembling a flu-like illness, except for one distinct difference that is the

absence of a 'sore throat' in malaria. Fever, headache, nausea, fatigue, muscle and joint aches are the common symptoms. A thick blood smear is usually required to verify a diagnosis of malaria. A typical malaria fever is a well-described entity, which consists of two distinct stages. A "cold stage" of sharply rising body temperature often leads to frank rigors, and a "hot stage" with flushes, tachycardia that is sustained for a few hours. The "hot stage" is followed by a defervescence stage when sharply falling body temperature, profuse sweating and symptomatic orthostatic hypotension occurs. Cycles of chills, fevers, and sweating typically repeat every 48 hours in infections by *P. falciparum*, *P. vivax* or *P. ovale*, but 72 hours and 24 hours in the case of *P. malariae* and *P. knowlesi* respectively. Transition from an uncomplicated malaria to a life-threatening complicated condition would be expected if no treatment is given (Compans et al., 2005) in *P. falciparum* infection. Patients would be experiencing associated complications like severe anemia, hemoglobinuria with renal failure, acute pulmonary oedema, cerebral malaria and metabolic acidosis.

Diagnosis of malaria comprises of clinical diagnosis or clinical suspicion and parasitological diagnosis. Due to the non-specificity of malaria symptoms, clinical suspicion of malaria should be confirmed by parasitological diagnosis, based on the detection of parasites in the blood. There are two methods in routine use for parasitological diagnosis, light microscopy and rapid diagnostic tests (RDTs). RDTs detect parasite-specific antigens or enzymes in the sample and some have a certain ability to differentiate species (2010).

Today, artemisinin and its derivatives represent the preferred drugs to treat malaria. Artemisinin is commonly known as *Qinghaosu* in Chinese, a traditional Chinese medicine used to treat many illnesses for thousands of years (Group, 1979). Artemisinin rapidly reduces parasitaemia in patients, and also the number of mature parasites that potentially sequester in capillaries and venules. The effect of killing all the sexual and asexual parasite stages (including young ring-stage parasites) by artemisinin in *P. falciparum* infections is remarkable (Udomsangpetch et al., 1996), which is not shown by other popular antimalarial drugs such as quinine and chloroquine (White, 1997). Artemisinin is used in combination with other antimalarial drugs in treatment known as ACT (Artemisinin-based Combination Therapy) to

prevent the development of drug resistance in parasites. It has been shown an outstanding efficacy in treating Chloroquine-resistant and Sulfadoxine-pyrimethamine-resistant malaria, thus it is now the first line treatment against *P. falciparum* malaria as recommended by WHO (WHO, 2010).

1.1.3 Prevention and Control

Vector control is the primary health intervention to reduce malaria transmission at the community level. In fact, this strategy together with application of antimalarial drugs was proven very effective especially in eliminating malaria in low-transmission areas. In high-transmission areas, it can reduce child mortality rates (WHO, 2014). Larva source management (LSM) is known as a very effective approach in vector control, as shown by the success in Italy against malaria in the early 20th century (Majori, 2012). LSM targets the immature stage of mosquitoes (the larvae and pupae) through four types of methods: habitat modification, habitat manipulation, larviciding and biological control (WHO, 2013a). Larviciding represent the most widely use approach in LSM which involves addition of insecticide to water bodies to kill the larvae and pupae. However, it is recommended only in settings where the mosquito breeding sites are “few, fixed and findable” to be cost-effective (WHO, 2013a).

To protect individuals from mosquitoes carrying *Plasmodium spp.*, two forms of interventions are widely used currently, insecticide-treated mosquito nets (ITNs) and indoor spraying with residual insecticides (IRS) (WHO, 2013b). In regions of Africa, long lasting insecticides impregnated nets (LLINs) are the preferred choice of insecticide treated nets for the public to accomplish universal vector control. Ideally, everyone in high transmission areas should sleep under a LLIN every night but the distribution of such nets remains a logistical challenge. Nevertheless, ITNs has been proven effective to kill large scale of adult mosquitoes in certain areas of Africa (WHO, 2006). IRS is the most powerful way so far to rapidly reduce malaria transmission by applying residual insecticides to the inner surface of dwellings including animal shelters. This method is to target the vectors that resting on the wall

after a blood meal. It is effective for average 3-6 months, depending on the type of insecticide used (WHO, 2011). Similar to ITNs, the effectiveness of IRS is often compromised by limitations such as cross-resistance among insecticides and technical issue during the implementation. IRS requires stringent planning, management and supervision. It must be delivered by well-equipped vector control services, which currently do not exist in many countries (WHO, 2008). In Southeast Asia, ITNs and IRS are not effective in reducing human-vector contact throughout the region due to high biodiversity of *Anopheles* species in the domestic environment. Different *Anopheles* species in Southeast Asia has been observed with different biting rhythm and resting behavior (Trung et al., 2005). For example, in central Vietnam, the major vector *Anopheles dirus* A in that area bites before the bedtime of the residents (Trung et al., 2005). ITNs and IRS are not effective in this case as most of the residents are still engage in various indoor and outdoor activities before bedtime. Recently, use of bacterial toxins (Cry and Cyt toxins from *Bacillus spp.*) has been implemented in LSM as a complementary intervention to ITNs and IRS (WHO, 2013a). These toxins specifically target the midgut epithelial cells of larvae, leading to termination of feeding and insect death without any toxic effect to human and the environment (Federici et al., 2003; Bravo et al., 2007).

Extensive research has been done on the vector to develop new strategies for malaria control. Transgenic mosquitoes have been produced by expressing anti-parasitic genes in their midgut epithelium, leading to their inefficiency in transmitting the disease (Ito et al., 2002). Recently, introduction of stable infection of maternally transmitted symbiotic bacterium, *Wolbachia* into a malaria vector has rendered *Anopheles stephensi* resistant to *P. falciparum* (Bian et al., 2013).

1.2 Biology of Human Malaria

1.2.1 The Complex Life Cycle of *P. falciparum*

The life cycle of *P. falciparum* is complex, involving both vertebrate and invertebrate hosts in which asexual and sexual replication occur respectively (Figure 1.1). Parasites from the salivary gland of the *Anopheles* mosquito are injected into human host skin in an infective form known as sporozoites. Model studies using *P. yoelii* have indicated that an average of 15-123 sporozoites were injected by a single infected mosquito (Medica and Sinnis, 2005) and majority of sporozoites actually remain at the injection site for hours before slowly releasing into the blood circulation (Yamauchi et al., 2007). However, this phenomenon has not been confirmed in the case of *P. falciparum*. It is thought that *P. falciparum* sporozoites, once in the human bloodstream, migrate rapidly from the site of injection to the liver. Sporozoites are able to pass through the Kupffer cells and cross the sinusoidal cell layer to enter hepatocytes. In hepatocytes, it takes 6-14 days for sporozoites to differentiate and divide mitotically into approximately 40, 000 liver merozoites for *P. falciparum* (Despommier et al., 2000). Each of them is competent to invade an erythrocyte once it is released into the blood circulation. The release of liver merozoites marks the beginning of the asexual blood stage, an essential stage for parasite propagation in the host. Each invading merozoite develops through ring, trophozoite and schizont stages within the erythrocyte, producing 16 to 32 daughter merozoites. Forty-eight hours after invasion, all the daughter merozoites are released from the current host cells in a process known as egress. Egress in *P. falciparum* involves a sudden increase of intracellular pressure, followed by the destruction of the parasitophorous vacuole (PV) membrane and erythrocyte membrane (Glushakova et al., 2005).

Members of the serine repeat antigen (SERA) family that localized to the PV are important in mediating the egress process (Pang et al., 1999; Miller et al., 2002; Hodder et al., 2003). Merozoites egress after osmotic swelling is a very fast process (as short as 400 milliseconds) that involves an ordered sequence of events that happens on the infected erythrocyte membrane (curling–buckling–eversion–vesiculation) (Abkarian et al., 2011). Merozoites are released during the eversion and are free to invade new erythrocytes. Some of the intraerythrocytic stages are committed to develop into sexual forms, the male or female gametocytes that are taken up by the mosquito during a blood meal (Sinden, 1998). Gametocytes will differentiate into gametes which then fuse in the mosquito's gut to form zygotes. The zygote further develops into a motile ookinete that penetrates and traverses the midgut

epithelial cells, where oocyst is formed. The oocyst undergoes sporogony to produce sporozoites that will be released eventually. The invasive sporozoite migrates to the salivary gland where they are ready to be injected into a new human host during a mosquito bite (Beier and Vanderberg, 1998).

1.2.2 A Closer Look at the Erythrocyte Invasion Process of *P. falciparum*

1.2.2.1 *The Merozoite*

The genus of *Plasmodium* belongs to the phylum Apicomplexa, in which all members share a specialized apical complex structure in their invasive forms. Merozoites, the invasive form of the *Plasmodium* asexual blood stage are the smallest of the parasite stages. For *P. falciparum*, they are in an elliptical shape with an average size of 1.5µm in length and 1.0 µm in diameter. The major ultrastructural features of the merozoite are shown in Figure 1.2.

It is surrounded by three membranous layers, which are a plasma membrane as the outermost layer and two underlying membranes (also known as inner membrane complex, IMC). The outer membrane is covered with a surface coat of about 20 nm in thickness, which is formed by bundles of fibril (Bannister et al., 1986). This coat is composed of several integral and peripheral membrane proteins known as merozoite surface proteins (MSPs) and some minor coat proteins (Pf12, Pf38 and Pf92). These surface proteins are responsible for the early, weak contact between merozoite and erythrocyte (Cowman and Crabb, 2006; Kadekoppala and Holder, 2010). During the erythrocyte invasion, this fuzzy surface coat is mostly shed as the merozoite moves inwards (Aikawa et al., 1978). The propulsive force that drives merozoite along the

Figure 1.1. The life cycle of *P. falciparum*.

Parasites (sporozoites) are injected into the human host by the *Anopheles* mosquito. They migrate to the liver where they invade hepatocytes, within which they develop into liver merozoites. The merozoites, once released are competent to invade erythrocyte to enter their asexual blood stage in the life cycle. They develop through ring, trophozoite and schizont stages inside the erythrocyte. The infected erythrocyte ruptures upon maturation of daughter merozoites and those merozoites are free to invade new erythrocytes. The sexual form of the parasite (female and male gametocytes) that developed in the asexual blood stage are taken up by the mosquito during feeding. They develop into macrogamete and microgamete. The microgamete fertilizes the macrogamete, forming a motile ookinete, which traverse the midgut wall of the vector to transform into oocyst on the midgut wall. Sporozoites are released from the oocyst and migrate to the salivary glands of the vector for the next injection.

Figure 1.2. Cellular organization of a *Plasmodium falciparum* merozoite.

invasion process is powered by an acto-myosin complex, known as glideosome which lies in between the IMC and plasma membrane. A row of subpellicular microtubules originating from the polar ring at the apical end is attached right beneath the IMC. In addition to serving as a cytoskeleton for the merozoite, these microtubules are likely connected to the motor complex which lies in between the IMC and merozoite plasma membrane via an uncharacterized, family of proteins (Baum et al., 2008).

The apical end of merozoite is a cone-shaped projection defined by three polar rings, where a cluster of specialized secretory structures is located. These invasive structures are thought to secrete proteins in order to select, interact and invade host cell in a tightly regulated temporal manner. Rhoptries were the first invasive organelle of *Plasmodium* to be described by electron microscopy 50 years ago (Garnham et al., 1960), as they appeared as electron dense compartments. In *P. falciparum*, two rhoptries are the largest secretory apical organelles arranged in pairs. This club-shaped organelle is divided into two subcompartments: rhoptry neck and rhoptry bulb. The rhoptry neck represents the apical duct of the organelle in which parasite proteins involving host cell adhesion and invasion are located (Counihan et al., 2013; Hanssen

et al., 2013). Proteins secreted from the rhoptry bulb have been found to contribute to downstream events of invasion including parasitophorous vacuole (PV) formation, a parasite-derived vacuole that encloses the invading merozoite within the infected erythrocyte which has been discussed in detail by Counihan *et al.* (Counihan et al., 2013). Micronemes were first described as neighboring “dense bodies” that were closely associated with rhoptries (Hepler et al., 1966). A single merozoite typically contains up to 40 micronemes that release crucial players in the invasion, such as surface-destined proteases and erythrocyte-binding proteins (Iyer et al., 2007; Singh and Chitnis, 2012).

Unlike rhoptries and micronemes, dense granules are the secretory organelle that are scattered throughout the cytoplasm. Their timing of secretion at the later stage of invasion (Bannister et al., 1975) has indicated their role in post-infection of host cell, most probably responsible for the PV formation in the infected cell (Baum et al., 2008). However, some of these “dense granules” have been identified to be distinct in structure, contents and secretory behavior, at least in merozoites. They were renamed as exonemes, as an indication of the suggested role of being important in the exit of merozoites from schizonts (Yeoh et al., 2007). Other cell organelles that are commonly found in animal cells were observed in merozoites as well. The nucleus is positioned at the posterior end (Aikawa, 1977) while a mitochondrion and an apicoplast are localized beside the nucleus towards the edge. Ribosomes are abundant in the cytoplasm but little endoplasmic reticulum is observed (Torii and Aikawa, 1998).

1.2.2.2 *The four distinct phases of invasion*

Most of the early studies regarding merozoite ultrastructures and sequence of the invasion steps had been done on *P. knowlesi*, a primate malaria parasite, due to its bigger size (Dvorak et al., 1975; Aikawa, 1977; Aikawa et al., 1978). With the advance of imaging technology, real-time imaging studies on merozoite egress and the multistep invasion process of *P. falciparum* (Glushakova et al., 2005; Gilson and Crabb, 2009) and *P. yoelii* (Yahata et al., 2012) have been performed recently. The

notable findings of these studies are the remarkable similarities in kinetics of invasion between *P. falciparum* with the distantly related *P. knowlesi* while *P. yoelii* remains different from these two species in invasion kinetics and merozoite morphology.

Erythrocyte invasion by free merozoites is a fast and efficient process that greatly reduces the risk to the merozoites from being exposed to the host immune system. Time-lapse imaging of invasion by two separate groups has shown that recognition of a new target erythrocyte is completed within one minute, and the completion of *P. falciparum* entry falls into the range of 27.6s (Gilson and Crabb, 2009) to 30s (Yahata et al., 2012). Despite the very short time frame for merozoite to invade, the invasion process is very complex consisting a sequence of ordered events. For decades, the underlying mechanism and linkage between each step of invasion are poorly understood but recent work focusing on signaling transduction pathways (Gaur and Chitnis, 2011; Lim et al., 2012) has shed some lights to our understanding of the regulations of the invasion events.

There are mainly four distinct phases (Figure 1.3) in the process of invasion: i) Reversible, primary attachment to the target host, ii) Erythrocyte deformation and parasite reorientation, iii) Irreversible tight junction formation and iv) Active parasite entry and PV formation. Multiple specific ligand-receptor recognitions and interactions are shown crucial for invading merozoite to propel itself into the erythrocyte while the precise mechanism is not clearly known. A dissection of the coordinated events in *P. falciparum* invasion using high-resolution microscopy and electron tomography (Riglar et al., 2011; Hanssen et al., 2013) has provided new insights about different stages of invasion where merozoite attachment is probably the master switch triggering all the downstream events in the following order: secretion of rhoptry neck followed by rhoptry bulb, tight junction establishment and then shedding of the surface proteins, finally the motor activation to penetrate the erythrocyte.

1.2.2.2.1 Reversible, primary attachment to the target host

The initial contact of the free merozoite to an erythrocyte is low affinity and reversible, as the parasite must select a suitable new host cell before proceeding into cell invasion. The primary attachment may occur at any point on the merozoite surface (Dvorak et al., 1975) (Figure 1.3A) and merozoites have been described to be able to “fling and wave” the targeted erythrocyte around until the irreversible interaction (tight junction) is formed between the merozoite and erythrocyte (Gilson and Crabb, 2009).

A number of merozoite surface proteins (MSPs) have been identified and some are thought to be the important players mediating the primary contact during invasion. MSPs are generally either membrane bound through a glycosylphosphatidylinositol (GPI) anchor or loosely associated with GPI-anchored proteins as peripheral proteins. MSP-1, -2, -4, -5, -10, Pf12, Pf38 and Pf92 are the membrane bound MSPs in *P. falciparum*, while MSP-3, -6, -7, MSPDBL-1 and -2 are known as peripheral proteins (Wickramarachchi et al., 2009; Kadekoppala and Holder, 2010; Cowman et al., 2012). MSP-1 forms a complex with MSP-6 and -7 on the merozoite surface and the complex undergoes a primary processing by subtilisin-like protease (SUB1) just prior to merozoite release. The secondary processing by SUB2 occurs when the merozoite invades an erythrocyte and the cleaved fragments are shed during the invasion process (Holder, 2009). Evidence from Wickramarachchi and co-workers has proven that two MSPs, MSPDBL-1 and -2 bind to erythrocyte (Wickramarachchi et al., 2009). The interaction is mediated via the Duffy-binding-like (DBL) domain (Hodder et al., 2012). Antibodies against several MSPs have been shown to inhibit parasite invasion (Pirson and Perkins, 1985; Lazarou et al., 2009; Woehlbier et al., 2010). Among all, MSP-1 is the most well-studied MSP and it has long been considered a major vaccine candidate due to several reasons. First, it is the most abundant protein that is evenly distributed all over the merozoite surface and it binds to Band 3, a major erythrocyte surface antigen (Goel et al., 2003), thus suggesting it is the candidate to mediate the initial adherence (Cowman and Crabb, 2006). Second, MSP-1 is essential for parasite survival since it cannot be knocked out at the blood stage (O'Donnell et al., 2000). More importantly, antibodies against the C terminal-EGF domain of MSP-1 block erythrocyte invasion by *P. falciparum* *in vitro* (Blackman et al., 1990). Besides, the recombinant EGF domain has been shown to provide protection against infection of blood stage rodent parasites after being introduced into mice (Daly and Long, 1993;

Ling et al., 1994). However, the outcome of early phase clinical trials of MSP-1 vaccines have not been encouraging (Ogutu et al., 2009) and the current challenges and considerations are well discussed in a review by Anthony Holder (Holder, 2009).

1.2.2.2.2 Erythrocyte deformation and parasite reorientation

After the primary attachment, the erythrocyte immediately experiences rapid deformation to different degrees (Figure 1.3B) (Dvorak et al., 1975; Gilson and Crabb, 2009; Harvey et al., 2012; Yahata et al., 2012). Interestingly, the deformation of the erythrocyte is short-lived as it lasts only a few seconds. The cell resumes its normal shape with merozoite attached on it before the invasion commences (Figure 1.3B and C). Lew and Tiffert have suggested that a localized influx of calcium ions triggered by merozoite contact could activate the cytoskeleton changes of the erythrocyte, eventually lead to deformation of erythrocyte (Lew and Tiffert, 2007). The merozoite then re-orientates to position its penetrative apical end to the host cell. This step is crucial to drive a closer, committed interaction between the apical organelle of the merozoite and erythrocyte, by which the downstream invasion events are probably triggered (Riglar et al., 2011). While the mechanism of merozoite reorientation is not clearly known, enrichment of parasite ligands that interacts with erythrocyte receptor in high affinity has been suggested to be the driving force of apical reorientation (Singh and Chitnis, 2012). The potential candidates of such parasite ligands could be Reticulocyte binding like protein homologue (RH) and Erythrocyte binding like (EBL) protein which represent two key protein families of parasite adhesins at the apical end. RHs are believed to be crucial in erythrocyte recognition, selection and invasion. They have been proposed to trigger tight junction formation following merozoite reorientation by recruiting high-affinity parasite ligands from the EBL family (Galinski et al., 1992; Barnwell and Galinski, 1998; Duraisingh et al., 2003) whereas EBLs have been demonstrated to be critical in erythrocyte recognition and more importantly, tight junction formation (Miller et al., 1979; Barnwell and Galinski, 1998; Riglar et al., 2011). The role of AMA-1 (Apical

Figure 1.3. The four distinct phases of erythrocyte invasion of *P. falciparum*.

Important merozoite proteins involved at each stage of invasion are listed in blue box. **(A)** Primary attachment of merozoite can occur at any point on the merozoite surface. The attachment is reversible so that the merozoite can select a suitable host cell. **(B)** Erythrocyte experiences deformation, which probably triggered by primary attachment of merozoite. At the same time, merozoite needs to reorientate itself to position its apical end to the erythrocyte. **(C)** Erythrocyte resumes its normal shape. Irreversible tight junction formation mediated by proteins localized at the apical end of merozoite. These proteins are secreted from the micronemes and rhoptries of the merozoite. Electron-dense thickening at the erythrocyte membrane can be observed at the point of contact between merozoite and host cell. Parasite proteins highlighted in red are involved in erythrocyte binding and invasion but their presence at tight junction is yet to be shown. **(D)** Active entry of the merozoite starting from the site of contact. Erythrocyte membrane first invaginates and AMA1-RON complex (the moving junction, black arrows) moves around the invading merozoite from the anterior end to the posterior end. Merozoite surface proteins like MSPs are cleaved by PfSUB2 and shed during this process (blue fragments).

membrane antigen 1) in parasite reorientation remains controversial as different studies showed incompatible findings (Mitchell et al., 2004; Treeck et al., 2009; Richard et al., 2010). However, the most recent study on *P. falciparum* and *T. gondii* has proven that AMA-1-deficient merozoite and tachyzoite have defect in erythrocyte adhesion and apical positioning instead of host penetration, further supporting the importance of AMA-1 in preinvasive orientation step (Bargieri et al., 2013).

1.2.2.2.3 Irreversible tight junction formation

Merozoites are committed to invasion once the apical adhesins are engaged in irreversible interactions with host receptors that then stabilize into a tight junction (Riglar et al., 2011). The tight junction was first described 33 years ago, as a prominent structure observed during erythrocyte invasion in *P. knowlesi* (Aikawa et al., 1978). It is characterized by an increased electrone-dense thickening at the erythrocyte membrane at the intimate point of contact between the merozoite apical end and erythrocyte which is reminiscent of a tight junction in mammalian cells (Aikawa et al., 1978; Miller et al., 1979) (Figure 1.3C). It marks the entry point of penetrating merozoites as well as the starting site of shedding of the surface coat, and finally where the PV is generated from invaginated erythrocyte membrane as the merozoite moves inward (Bannister et al., 1975). Secretion of micronemes and rhoptries are crucial for tight junction formation because the junction's core molecular constituents are parasite-derived (Singh et al., 2010; Riglar et al., 2011). PfAMA-1 and rhoptry neck proteins (PfRONs) from micronemes and rhoptries respectively interact and form a ring-like structure around the circumference of merozoite at the tight junction (Alexander et al., 2006; Cao et al., 2009; Richard et al., 2010). The AMA1-RON complex creates a tight molecular seal between the merozoite and erythrocyte membrane with parasite proteins embedded into the host membrane (Baum and Cowman, 2011). This molecular anchor has been suggested to be assembled by translocation of PfRON4 together with other RONs to the cytosolic side of the host during rhoptry secretion, followed by interaction between PfAMA-1 on the parasite surface and PfRON2 which spans through the host membrane (Besteiro et al., 2009; Straub et al., 2009; Tyler et al., 2011).

The secretion of microneme and rhoptry proteins has been demonstrated to be induced by Ca^{2+} signaling (Singh et al., 2010; Gao et al., 2013). It has been demonstrated that the low level of K^+ in blood plasma probably triggers the rise in the merozoite cytosolic Ca^{2+} level, leading to the translocation of EBA-175 and PfAMA-1 to the merozoite surface. The engagement of EBA-175 to its receptor further induces secretion of rhoptry proteins such as CLAG3.1 and PfRH2b (Singh et al., 2010). A separate report shows that antibodies against the erythrocyte binding domain of PfRH1 inhibit Ca^{2+} signaling in merozoite (Gao et al., 2013). The absence of Ca^{2+} signals prevents translocation of EBA-175 to the surface and merozoite invasion. These two studies are in line with the work done by Riglar and colleagues (Riglar et al., 2011). The correct rhoptry release is dependent on the committed attachment that is likely initiated by PfRHs/EBLs receptor binding, in the presence of PfAMA1-RON complex at the tight junction (Collins et al., 2009; Richard et al., 2010).

1.2.2.2.4 Active parasite entry and PV formation

As merozoite enters the erythrocyte, the flexible tight junction moves like a ring-like zone around the penetrating merozoite from the anterior end and finally fuses at the posterior end of merozoite together with the “shedding” of invasion proteins (Figure 1.3D) (Blackman et al., 1990; Blackman and Holder, 1992; Holder et al., 1992; Howell et al., 2001; Howell et al., 2003). However, the moving junction which consists of AMA1-RON complex has been suggested to have a rather passive role in host penetration, for example as a passive molecular sieve that facilitates the sieving of certain erythrocyte receptors or lipid moieties to be brought into the PV (Baum and Cowman, 2011; Tyler et al., 2011). The hypothesis is supported by the recent study in which AMA1-deficient merozoites form a normal constriction and RON ring at the tight junction and their active entry into the host is as normal as wild type merozoites (Bargieri et al., 2013).

The movement of the parasite is powered by a conserved actin-myosin motor complex that lies between the parasite plasma membrane and the IMC (Sibley et al., 1998; Keeley and Soldati, 2004; Baum et al., 2006). A model for the gliding motor

machinery or glideosome of merozoite has been proposed in which interaction of Myosin A, myosin A tail domain interacting protein (MTIP) and glideosome associated proteins (GAPs, GAP45 and GAP 50) helps to transfer the driving force of myosin into merozoite movement (Baum et al., 2008; Besteiro et al., 2011). Research on *T. gondii* has shown that recombinant TgAMA1 interacts with aldolase in a pull-down assay (Sheiner et al., 2010). Mutation of the FW motif in its cytoplasmic tail domain disrupts aldolase binding and invasion (Sheiner et al., 2010). In *P. falciparum*, there is no direct evidence of an interaction between PfAMA-1 and aldolase so far. However, the FW motif from the C-terminal part of PfAMA-1 has been demonstrated to be essential for invasion (Treeck et al., 2009). The current finding suggests that merozoite surface adhesins function as a bridging component that connects glideosome to the moving junction complex during merozoite invasion (Besteiro et al., 2011). Such interaction allows coupling of host recognition to the actin-myosin motor during host penetration whereby the merozoite motility is dependent on the tread milling along the adhesins that engaged to the host receptor. The process of internalization of merozoites take ~17 seconds to be completed (Harvey et al., 2012).

After the completion of invasion, the internalized parasite resides in a specialized vacuole known as parasitophorous vacuole (PV) within the erythrocyte (Bannister et al., 1975; McLaren et al., 1979; Aikawa et al., 1981). The formation and development of PV remains controversial to date but it is clear that nascent PV membrane (PVM) emerges from the joining point of the duct of secreting rhoptries and erythrocyte membrane (Zuccala and Baum, 2011). The contribution from parasite and host towards the lipid composition of PVM is yet to be confirmed as conflicting data have been shown by different research groups (Dluzewski et al., 1992; Haldar and Uyetake, 1992; Ward et al., 1993). Nevertheless, it is clear that the PVM contains non-erythrocyte membrane-derived lipids and certain subsets of erythrocyte membrane proteins are found to be associated with PVM (Dluzewski et al., 1995; Zuccala and Baum, 2011). The parasite contributes significantly to the establishment of the PVM and vacuole architecture primarily through rhoptries bulb secretion as reported by various sources (Bannister et al., 1986; Stewart et al., 1986; Dluzewski et al., 1989; Riglar et al., 2011; Zuccala and Baum, 2011).

1.2.3 The Two Protein Families, RHs and EBLs: Important Molecular Players in Invasion

Erythrocyte invasion by *Plasmodium* merozoites has been shown to involve specific interaction between parasite ligands and host cell surface receptors. Members from two parasite protein families, RHs and EBLs have been identified as key adhesins involved at different stages of invasion including host cell selection, invasion pathway determination and tight junction formation. Different combination of parasite ligands and host cells receptors used define different invasion pathways used by merozoites. Removal of a certain group of receptor leads to parasite-host interaction through an alternative ligand-receptor combination. RHs and EBLs are either micronemal or rhoptry proteins which function cooperatively during invasion. It has been reported that loss of function of one particular adhesin can be compensated by the action of other member from the same family or the other family (Stubbs et al., 2005; Lopaticki et al., 2011; Riglar et al., 2011). Hence, RHs and EBLs possibly contribute to parasite phenotypic variation to adapt to the polymorphic nature of the erythrocyte in human population as well as to evade the host immune system (Cowman and Crabb, 2006; Iyer et al., 2007).

1.2.3.1 *Reticulocyte binding like protein homologues (RHs)*

1.2.3.1.1 *P. yoelii*

The first member of the RH family was identified in *P. yoelii* as Py235, named after its molecular weight of 235 kDa. To date, 14 copies of Py235 have been identified in the *P. yoelii* genome (Carlton et al., 2002; Iyer et al., 2006). High level of Py235 transcription has been associated with increased severity of the disease (Iyer et al., 2007). The protein was first identified using monoclonal antibodies from mice that are resistant to YM infection (Freeman et al., 1980). YM is a virulent strain of *P. yoelii* that is able to invade all range of erythrocytes including normocytes and reticulocytes. Association of Py235 to parasite virulence has been proven when vaccination of mice using immunopurified Py235 and passive transfer of antibodies against Py235

protected the mice from YM strain infection by restricting the parasite invasion to only reticulocytes (~1% of the blood population) (Freeman et al., 1980; Holder and Freeman, 1981). Interestingly, Py235 in the virulent (YM) and avirulent (YA) strain is different in the transcription profile and expression repertoires (Preiser and Jarra, 1998; Khan et al., 2001). Merozoites released from single schizont are found to transcribe distinct Py235 members, indicating that Py235 could be a determinant of merozoite invasion properties and immunogenicity (Preiser et al., 1999). This form of clonal phenotypic variation allows merozoites to evade the host immune system and ensures parasite survival and propagation. Py235 binds to a specific erythrocyte receptor via an erythrocyte-binding domain (EBD) (Figure 1.4) (Ogun and Holder, 1996; Gruber et al., 2011). Recently, the dominantly expressed member, PY01365 has been shown to display nucleotides (ATP and ADP) binding properties and binding of ATP induces protein conformational changes that possibly leads to exposure of the EBD (Ramalingam et al., 2008). This finding suggests that Py235 or other RH proteins act as sensor to detect or select suitable host cells.

1.2.3.1.2 *P. vivax*

The functional roles of RH members in *P. vivax* are thought to be involved in host cell selection. This concept is closely associated to early observation that *P. vivax* only invades reticulocytes that are Duffy antigen positive (Culleton et al., 2008). Duffy-negative phenotype is highly prevalent to African people who are described as being resistant to *P. vivax* infection. The recognition of Duffy antigen is mediated by the Duffy binding protein (DBP) which is a member of the EBL protein family. Recently, it has been reported that Duffy-negative individuals are now susceptible to *P. vivax* infection (Cavasini et al., 2007; Menard et al., 2010; Mendes et al., 2011; Wurtz et al., 2011), suggesting that *P. vivax* has evolved to invade erythrocyte via alternative receptors. Parasite preference to reticulocytes has been shown to be mediated by the expression of two homologues of the Py235 family, PvRBP1 and PvRBP2 which bind to reticulocytes only (Galinski et al., 1992). In *P. vivax* (strain Belem), PvRBP1 and PvRBP2 are colocalized at the apical end of merozoites and may function in a complex form. Since DBP is crucial in tight junction formation and invasion, PvRBPs

have been suggested to act prior the formation of tight junction which is most likely during the host cell selection (Galinski et al., 1992; Barnwell and Galinski, 1998).

Almost two decades after the discovery of PvRBP1 and PvRBP2, 10 additional PvRH members (5 full length genes, 3 partial genes and 2 pseudogenes) have been identified with the completion of *P. vivax* Salvador 1 (Sal 1) genome sequencing (Carlton et al., 2008). They are further categorized into two groups, PvRBP1 subgroup and PvRBP2 subgroup (Li and Han, 2012) (Table 1.1). Functional domain identification and functional studies on PvRBP1 and PvRBP2 have been challenging and problematic due to their large size (over 300 kDa), the absence of an optimal recombinant protein expression and a reliable *ex vivo* invasion assay before 2010 (Tsuboi et al., 2010; Russell et al., 2011; Li and Han, 2012). Four putative reticulocyte binding region (RBR) have been identified in PvRBP1 by screening a library of synthetic peptides (Urquiza et al., 2002) while little is known of other members. PvRBP1 and PvRBP2 are 99% and 83% identical to PvRBP1a and PvRBP2c respectively at the protein level. All PvRBPs shows low similarity (not more than 35%) to their homologues in *P. falciparum* (Carlton et al., 2008). PvRBP2 subgroup members (*Pvrbp2*, *Pvrbp2a*, *Pvrbp2b*, *Pvrbp2d*) have been determined to have a high polymorphism rate with *Pvrbp2* being the most diverse gene. This suggests that *P. vivax* may utilize different PvRBPs in alternate invasion pathway, which has also been observed in *P. falciparum* invasion.

1.2.3.1.3 *P. falciparum*

In *P. falciparum*, RH members are more extensively studied as compared to PvRBPs or Py235. They are large Type I transmembrane proteins (Figure 1.4) with a molecular weight above 200 kDa except for PfRH5 (Table 1.2). Six members have been identified to date which are PfRH1 (Rayner et al., 2001), PfRH2a and 2b (Rayner et al., 2000; Triglia et al., 2001a), PfRH3 (Taylor et al., 2001), PfRH4 (Kaneko et al., 2002; Stubbs et al., 2005; Baum et al., 2009) and PfRH5 (Rodriguez et al., 2008; Baum et al., 2009). PfRH3 is a pseudogene found in a number of laboratory

Table 1.1 General information of *Plasmodium vivax* reticulocyte binding-like proteins (PvRBPs)

Name	Strain	Chromosome No.	No. exons	CDS length	MW (kDa)	IP	Length	No. TM	SP
PvRBP1	Belem	7	Unknown	8502	303.3	5.57	2833	0	Yes
PvRBP1a	Sal I	7	3	8454	324.4	4.74	2817	0	Yes
PvRBP1b	Sal I	7	1	7818	303.3	5.75	2605	0	Null
PvRBP1-P ^c	Sal I	Not assigned	1	2361	90.9	4.99	786	1	Null
PvRBP2	Belem	5	Unknown	8604	331.4	5.44	2867	1	Yes
PvRBP2a	Sal I	14	2	7464	286.7	7.26	2487	0	Yes
PvRBP2b	Sal I	8	2	7994	308.3	5.63	2652	0	Null
PvRBP2c	Sal I	5	1	8340	321.3	5.42	2779	1	Null
PvRBP2-P1 ^c	Sal I	5	2	1989	77.4	8.21	662	0	Null
PvRBP2-P2 ^c	Sal I	14	1	1926	74.1	9.35	641	0	Null
PvRBP2hB ^c	Sal I	12	1	3958	124.0	9.57	1061	0	Null
PvRBP2d ^d	Sal I	14	1	8495	308.4	10.34	2900	0	Null
PvRBP3 ^d	Sal I	14	1	8702	318.2	12.35	2831	1	Null

Abbreviations: CDS length, coding sequence length; MW, molecular weight; Length, length of amino acid; IP, isoelectric point; SP, signal peptide; TM, transmembrane domain. PvRBP1 subgroup in yellow; PvRBP2 subgroup in blue

^c Partial fragments.

^d Pseudogene.

(Table adapted and modified from *Li and Han, 2012*)

Figure 1.4. The RH protein family in *P. falciparum*, *P. yoelii* and *P. vivax*.

Sequence alignment of the PfrH members showed a homology region within the first 500 amino acids (blue shade). Green bars represent homology region based on the coding sequence alignment of PfrH2a, Py235 and PvRBP2. Nucleotide (ATP/ADP) binding region has been confirmed in PY01365, the dominant member of Py235. Red bars indicate region of each PfrH members to which invasion inhibitory antibodies have been raised against. The figure is published as part of the review paper “The role of the reticulocyte-binding-like protein homologues of *Plasmodium* in erythrocyte sensing and invasion.” by Gunalan *et al.*, 2013

parasite isolates but it does not appear to be expressed by the parasite (Triglia et al., 2001a; Cowman and Crabb, 2006). Most members of RHs are stored in the rhoptries before being secreted to the apical end of merozoite for host cell invasion but their functional roles in invasion are not clearly understood. Unlike EBLs, PfrHs share a low level of similarity in protein sequence. Nevertheless, a homology region with conserved cysteine residues which is located within the first 500 amino acids from the N-terminal end has been identified across all PfrH proteins (Figure 1.4) (Baum et al., 2009). In this family, each member is involved in distinct ligand-receptor interactions during erythrocyte binding (Rayner et al., 2001; Baum et al., 2009; Gunalan et al., 2011; Triglia et al., 2011). The specificity of the interaction was determined by treating erythrocytes with different enzymes such as neuraminidase, trypsin or chymotrypsin. Enzyme treatment removes specific groups of receptors on the erythrocyte surface that are sensitive to specific enzyme. For example, neuraminidase treatment on erythrocytes removes sialic-acid moieties from the glycoproteins on the cell surface, leading to the disruption of erythrocyte binding of specific PfrH members which involve glycoproteins (Rayner et al., 2001; Duraisingh et al., 2003; Triglia et al., 2005; Gaur et al., 2006; Gaur et al., 2007; Rodriguez et al., 2008; Baum et al., 2009). This method is very useful to narrow down the list of potential receptors for a particular parasite ligand (Camus and Hadley, 1985; Gaur et al., 2007; Baum et al., 2009). Different strains of *P. falciparum* parasites utilize different PfrH members in erythrocyte binding for invasion. The binding is generally divided into two types, sialic-acid dependent and sialic-acid independent (Table 1.2). Sialic-acid moieties found on the erythrocyte surface have been demonstrated to be essential for ligand-receptor interaction in a sialic-acid dependent invasion pathway (Camus and Hadley, 1985). Besides, differential expression of PfrH members (PfrH1 and PfrH4) has been linked to the switching of invasion pathway from sialic acid-dependent to sialic acid-independent (Stubbs et al., 2005; Gao et al., 2008). The wide variety in host cell recognition and redundancy in invasion pathway clearly explain the ability of the highly virulent *P. falciparum* to invade all ranges of erythrocytes, as well as their ability to escape from the host immune system.

Studies to date have shown that PfrH1, PfrH2a, PfrH2b and PfrH4 bind to erythrocytes except PfrH3 via an erythrocyte-binding domain near the N-terminus

Table 1.2. Characteristics of *Plasmodium falciparum* reticulocyte binding-like protein homologue (PfRH) members.

PfRH Members	Residing organelle ^c	Chromosome	Features ^a	Receptor	Inhibitory Antibodies ^b
PfRH1 (360 kDa)	Rhoptry neck ¹	4	Sialic-acid dependent erythrocyte binding; Nm-S, T-R, C-R. Being processed before and/or during invasion. Present at tight junction.	Unknown, receptor Y	Y
PfRH2a (370 kDa)	Rhoptry neck ²	13	Erythrocyte binding; Being processed before and/or during invasion; Full length protein and each of the processed forms display distinct sensitivity towards different enzymes*. Present at the moving junction	Unknown	Y
PfRH2b (382 kDa)	Rhoptry neck ²	13	Erythrocyte binding (85kDa processed form); Nm-R, T-R, C-S Being processed before and/or during invasion; cAMP-independent phosphorylation on the cytoplasmic tail prior host cell egress.	Unknown	Y
PfRH3	NA	12	Pseudogene	NA	NA
PfRH4 (220 kDa)	Rhoptry neck ³	4	Sialic-acid independent erythrocyte binding; Nm-R, T-S, C-S Being processed before and/or during invasion.	Complement 1 (CR1)	Y
PfRH5 (63 kDa)	Rhoptry ⁴	4	Sialic-acid independent erythrocyte binding; Nm-R, T-R, C-R Being processed before and/or during invasion. Essential gene. Present at tight junction. Interacts with PfRH5-interacting protein	Basigin	Y

^a Nm, neuraminidase; T, trypsin; C, chymotrypsin; -S, sensitive; -R, resistant; ^b Y, Antibodies raised against the protein inhibit invasion; ^c location before secretion to the apical end of merozoite

¹ Triglia *et al.*, 2001 and Gunalan *et al.*, 2011; ² Triglia *et al.*, 2011; ³ Stubbs *et al.*, 2005; ⁴ Baum *et al.*, 2009

* Details stated in Gunalan *et al.*, 2011

NA, not applicable

(Figure 1.4) (Gaur et al., 2007; Gao et al., 2008; Rodriguez et al., 2008; Triglia et al., 2011). The recombinant form of these binding regions have been demonstrated to bind erythrocytes in the same manner as their native protein that is released into the parasite culture supernatant after processing. PfRH5 is different from the other members in two aspects: i) it is much smaller in size and without a transmembrane domain (Figure 1.4) and ii) it is essential for parasite survival as *PfRH5* gene is refractory to deletion in various parasite strains (Baum et al., 2009). Erythrocyte receptors for PfRHs remain unknown so far except for PfRH4 and PfRH5 (Table 1.2). Complement receptor 1 (CR1) and Basigin have been identified as the receptor for PfRH4 and PfRH5 respectively (Tham et al., 2010; Crosnier et al., 2011). Erythroid CR1 is a single-chain transmembrane glycoprotein of which the main function is to transport C3b/C4b-opsonized immune complexes (Birmingham and Hebert, 2001). PfRH4 binds to the N-terminal site of CR1 but the interaction does not interfere with the binding of CR1 to complement fragment C3b or C4b. Instead, PfRH4, CR1 and C4b form a ternary complex of which the actual biological role is yet to be confirmed (Tham et al., 2011). The amount of CR1 on the erythrocyte surface directly determines the binding level of PfRH1 to host cell (Tham et al., 2010). PfRH4-CR1 interaction is important in a sialic acid-independent invasion pathway that is used by *P. falciparum* including some field isolates (Spadafora et al., 2010; Tham et al., 2010; Awandare et al., 2011). PfRH5 binds specifically to Basigin (BSG) that belongs to the Ok blood group antigens (Spring et al., 1997; Crosnier et al., 2011). The binding site is located at the receptor protein backbone since removal of the glycans from BSG does not affect binding. Interestingly, antibodies against BSG are able to inhibit merozoite invasion by various *P. falciparum* strains, confirming that PfRH5 is essential for parasite invasion (Crosnier et al., 2011). Polymorphism have been observed in PfRH5 and amino acids substitutions in the sequence leads to changes in receptor binding property and specificity. Such polymorphisms in PfRH5 allow the human malaria parasite, *P. falciparum* binds to both human and *Aotus* monkey erythrocyte (Hayton et al., 2008).

Processed-size fragments of PfRHs have been observed in the parasite culture supernatant (O'Donnell et al., 2006) or in some cases, at the schizont stage (Triglia et al., 2009). Hence it is believed that PfRHs are cleaved before and/or during merozoite

invasion and such proteolytic processing is critical to their function (Triglia et al., 2009). PfRH1, PfRH2a, PfRH2b and PfRH4 have been shown to go through step-wise processing throughout merozoite maturation and invasion (Triglia et al., 2009; Triglia et al., 2011), indicating that the processing of RHs follows a conserved pathway. Different erythrocyte binding properties by individual processed forms of PfRH2a has suggested that PfRHs are likely to engage to different receptors at different invasion stages (Gunalan et al., 2011). *P. falciparum* rhomboid 4 (PfROM4), a rhomboid protease, is believed to mediate the cleavage of PfRH1, PfRH2a, PfRH2b and PfRH4 (Baker et al., 2006; Triglia et al., 2009). PfROM4 cleaves the protein at the site of the transmembrane domain to release the ectodomain into the culture supernatant during invasion (Triglia et al., 2009; Triglia et al., 2011). PfRH5 is cleaved to smaller fragments during invasion as well but the protease responsible for the processing is yet to be identified (Hayton et al., 2008; Baum et al., 2009). To date, detailed study of protein processing has only been done on PfRH1. Full length PfRH1 (360 kDa) is processed to smaller fragments of 240 kDa and 120 kDa at the schizont stage. It has been observed that these two processed fragments of PfRH1 colocalized with EBA-175 at the tight junction but they do not form a complex after cleavage (Triglia et al., 2009). Some of the 240 kDa fragments are further processed to a 140 kDa fragment during invasion and both fragments are released to the culture supernatant (Gao et al., 2008; Triglia et al., 2009).

1.2.3.2 Erythrocyte Binding-Like Proteins (EBLs)

EBL proteins are members of the Duffy binding-like family. They have been suggested to be important in merozoite invasion and they contain a conserved cysteine-rich domain named Duffy Binding Like (DBL) domain that binds directly to host cell receptors (Chitnis et al., 1996). This domain was first described in *P. vivax* Duffy binding Protein (PvDBP) (Fang et al., 1991) and it have been found across other *Plasmodium* species such as *P. knowlesi*, *P. falciparum*, *P. reichenowi*, *P. yoelii* etc (Iyer et al., 2007). Each EBL member has four exons in their gene structure except for PkDBP α and Pf-MAEBL, which contain an additional exon and intron. Further characterization of EBLs has shown that all members shared an extracellular

region (region II) containing conserved cysteines residues (Adams et al., 1992). In PvDBP and PkDBP, the extracellular domain can be divided into six regions (I to VI) and region II has been reported to bind specifically to the Duffy antigen on the erythrocyte surface (Chitnis and Miller, 1994; Chitnis et al., 1996; Ranjan and Chitnis, 1999).

In *P. falciparum*, EBLs do not bind to the Duffy antigen but they bind to a number of other erythrocyte surface receptors mainly glycoproteins. All of them especially EBA-175 play a direct role in merozoite attachment during invasion (Riglar et al., 2011; Tham et al., 2012). The overlapping binding function of EBLs and RHs has given flexibility to *P. falciparum* in invasion by providing multiple invasion pathways. To date, six members of EBL have been identified in *P. falciparum*, which are EBA-175 (Camus and Hadley, 1985), EBA-140 (Mayer et al., 2001), EBA-165 (Triglia et al., 2001b), EBA-181(Adams et al., 2001; Gilberger et al., 2003), EBL-1 (Peterson and Wellems, 2000) and MAEBL (Kappe et al., 1998) . The protein architecture of EBL members is divided to six regions in their extracellular domain (Howell et al., 2006) (Figure 1.5). Region II which consisted of duplicated DBL domains represent the erythrocyte-binding domain. Each DBL domain consisted of F1 and F2 region (Sim et al., 1994). MAEBL is the only member that contains no DBL domain (Kappe et al., 1998), indicating that MAEBL does not utilize the DBL domain for binding. EBA-165 is known as a transcribed pseudogene and is not expressed in any *P. falciparum* parasite (Triglia et al., 2001b).

Figure adapted from Howell et al., 2006

Figure 1.5. Domain arrangement of the EBLs.

EBA-175 is the best-studied parasite adhesins among the members of these two protein families. It binds to Glycophorin A on the erythrocyte and the binding is critically dependent on the sialic-acid residues and part of the glycoprotein backbone (Camus and Hadley, 1985; Sim et al., 1994). Dimerization of region II forms an interdomain channel that constitutes the binding cleft (Tolia et al., 2005). Each monomer contains F1 and F2 region but the binding site lies within the F2 region only (Sim et al., 1994). More recent crystallography studies on the region VI of EBA-175 have suggested that the dimerization of region VI may induce or facilitate region II dimerization at the parasite-host cell interface (Withers-Martinez et al., 2008). Receptor binding specificity is also seen in other members where Glycophorin B and C have been identified as the receptors for EBL-1 and EBA-140 respectively (Lobo et al., 2003; Maier et al., 2003; Mayer et al., 2009). The receptor of EBA1-181 remains unknown but sialic acids have been shown to be essential in the ligand-receptor interaction (Gilberger et al., 2003; Mayer et al., 2004).

1.3 Aims and objectives

Erythrocyte invasion by malaria parasites is extremely complex and involves numerous parasite proteins at the distinct phases. Extensive work has been done to investigate the coordinated events of invasion, from primary attachment to the active entry of the merozoite. However, the molecular basis of host cell selection at the early stages of invasion is poorly understood. Intracellular ATP of erythrocytes has been shown to be essential for parasite invasion. Erythrocyte encounters waves of deformation during invasion whereby mechanical-induced deformation causes intracellular ATP release from erythrocyte under normal physiological condition. RHs have been shown to play important roles in merozoite invasion. Among all PfRHs, little is known about PfRH1. Recently, an ATP/ADP binding domain (NBD94) has first been identified in Py235, the homologue of PfRHs in *P. yoelii* and a similar nucleotide binding domain has been predicted in PfRH1.

The principle aim of this work is to investigate the relationship between PfRH1 and ATP, followed by a dissection on the working mechanism of PfRH1 during merozoite invasion. To address these questions, the following research objectives have been identified:

1. To determine interaction between PfRH1 and ATP
2. To delineate a nucleotide binding region in PfRH1
3. To characterize the interaction between ATP and PfRH1 using various biochemical and biophysical analyses
4. To identify the critical ATP binding residues in PfRH1
5. To generate monoclonal antibodies against various region of PfRH1 as specific protein labeling tools for various purposes including microscopy imaging
6. To investigate the biological role of ATP on merozoite invasion

Chapter 2 Methods and materials

2.1 Cloning of recombinant PfNBR (rNBR) into *Escherichia coli*

DNA sequence of PfNBR was amplified from *P. falciparum* 3D7 genomic DNA (gene ID PF3D7_0402300) by Polymerase Chain Reaction (PCR) using a forward primer 5' GTGAG TccatggGAAAACAAGTACAA GAAAATGTTGAGAA 3' and a reverse primer 5' AATTACgagctcTTACTTAAAAACATCGCTCA GTGTATTT 3'. The PCR product was digested with restriction enzymes, NcoI-HFTM and SacI-HFTM (New England BioLabs) and ligated into a modified expression vector pET-9d (Novagen) in which an additional SacI restriction digestion site has been introduced to produce a N-terminal 6X His-tag (from Prof. Gerhard Gruber's lab, NTU). Ligation was done using the LigaFastTM Rapid DNA Ligation System (Promega) and the construct was chemically transformed into BL21(DE3) competent cells for recombinant protein expression.

2.2 Site-directed mutagenesis in PfNBR

Double point mutation in PfNBR was done using GeneArt® *Site-Directed Mutagenesis* System (Life Technologies) (Appendix 1) to convert double Tyrosine residues (at the position of 233 and 234 in rNBR) to double Alanine residues. Forward primer 5' TCTTTAGATAAATTAAAAAAgctgcGAAGAAGCGCTC TTTCAAAG 3' and reverse primer 5' TTGAAAGAGCGCTT CTTCggcagcTTTT TTTAATTTATCTAA 3' were used to amplify the target plasmid in the mutagenesis reaction. Mutated plasmid was transformed into BL21(DE3)pLysS (Promega) for recombinant protein expression.

2.3 Induction Assay

Transformed *E. coli* cells of both rNBR and NBR-YYAA were inoculated into fresh Luria-Bertani (LB) media containing 30 µg/ml of Kanamycin (Sigma-Aldrich) (LB-

Kan). Overnight bacteria culture was diluted with fresh LB-Kan media to OD_{600nm} 0.2 before incubating at 37°C, 220 r.p.m. For rNBR, once the OD_{600nm} has reached the range of 0.6-0.8, culture was divided into two aliquots with equal volume, one added with Isopropyl-1-thio-β-galactopyranoside (IPTG) (induced culture) and the other one with no IPTG added (non-induced culture). Both aliquots were incubated under the same condition. Concentration of IPTG was adjusted according to the incubation hours and temperature as the following: (i) 1 mM of IPTG for 37°C, 3 hours of incubation, (ii) 0.3 mM of IPTG for 25°C, 5 hours of incubation, (iii) 0.2 mM of IPTG for 16°C, 16-20 hours of incubation. After completion of incubation, each of the induced culture and non-induced culture was standardized to OD_{600nm} 1.0 before they were centrifuged at 16,200 g for 1 minute. Supernatant was discarded and cell pellet was washed once with 1ml of 1X phosphate-buffered saline (PBS), pH7.4. Then 50 µl of 2X protein sample loading dye (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8) was added to the pellet for SDS-PAGE. For NBR-YYAA, induction was done at one condition using 0.2 mM of IPTG and bacteria culture was incubated at 16°C for 16-20 hours in a shaking incubator.

2.4 Solubility Assay

Induced culture was dispensed into nine tubes, at 5 ml per tube. Cultures were pelleted down at 622 g, 10 minutes at 4°C. Each cell pellet was fully resuspended with 2 ml of lysis buffer containing 1X cOmplete™, EDTA-free, Protease Inhibitor Cocktail Tablets (Roche Applied Science) dissolved in water or Pefabloc SC (Biomol). One millilitre of the cell suspension was sonicated on ice using a Bandelin Sonoplus Ultrasonic Sonicator HD2200, with the probe KE72. The amplitude was set at 10-12% for 3 cycles of sonication, with 1 minute per cycle and 2 minutes pause on ice. Suspension was centrifuged at 9,168 g for 5 minutes at 4°C after sonication to separate the soluble fraction (supernatant) from the insoluble fraction (pellet). The pellet was fully resuspended with equal volume of designated lysis buffer and 5X

protein sample loading dye was added to both supernatant and pellet suspension for SDS-PAGE analysis.

2.5 Purification of rNBR and NBR-YYAA

Induced bacteria culture was grown at 16°C for 16-20 hours at 220 r.p.m. and pellet down at 4,500 g, 10 minutes at 4°C. Cell pellet (from 1 L culture) was resuspended with 50 ml of lysis buffer (50 mM TrisHCl, 500 mM NaCl pH 9.0 with 2 mM Pefabloc SC (BioMol) and 2 mM of phenylmethanesulfonylfluoride (PMSF) (Sigma). Cell lysate was dispensed equally into two tubes and sonicated immediately on ice for 3 cycles, with 1 minute for each cycle using the Bandelin Sonoplus Ultrasonic Sonicator HD2200 (probe KE76). Each tube was allowed to rest on ice for 2 minutes before the next cycle of sonication. After that, cell suspension was centrifuged at 13,000 g, 20 minutes at 4°C and supernatant was filtered with Millex® syringe filter, 0.45 µm (Merck Millipore). 1.5 ml of TALON® Metal Affinity Resins (Clontech) was equilibrated with the lysis buffer before incubating with the filtered supernatant at 4°C for 1 hour. His-tagged rNBR and NBR-YYAA were eluted at 4°C by an Imidazole gradient. Elution buffer with increasing Imidazole concentration was applied to the resins in a stepwise manner from the lowest concentration to the highest concentration (0 mM, 10 mM, 25 mM, 50 mM, 75 mM, 100 mM, 125 mM, 200 mM, 250 mM, 400 mM). Before protein elution, EDTA was added into the collection tubes to achieve a final concentration of 10 mM in a 4 ml or 10 ml of eluate.

Eluted fractions that contained the protein of interest were pooled and concentrated using Millipore Amicon® Ultra-4 centrifugal filter concentrators. A second step purification was done using a gel filtration column, Superdex75 10/300 GL (GE Healthcare), which was connected to the AKTA-FPLC system (GE Healthcare) with running buffer 50 mM TrisHCl, 500 mM NaCl, pH 9.0, with 1 mM Pefabloc SC and 10 mM EDTA. Two millilitre of concentrated protein sample was injected into the column and high purity fractions were collected.

2.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Coomassie Blue Staining

12% SDS-PAGE running gel was used for rNBR and NBR-YYAA and 6% SDS-PAGE running gel for PfRH1 in all electrophoresis-related assays. SDS-PAGE gels were immersed in Coomassie Blue Stain (0.1% Coomassie Blue powder, 50% of methanol, 20% acetic acids) for 10-20 minutes on a shaker. Coomassie Blue stain was drained and replaced by destain solution (30% methanol, 20% acetic acids). Incubation with the destain solution was at least 20 minutes until the background has fully destained.

2.7 Western blot

rNBR and NBR-YYAA on SDS-PAGE gel were transferred onto a Trans-Blot® nitrocellulose membrane (BioRad Laboratories Inc) using a Bio-Rad Trans-Blot SD Semi-Dry Blotting Apparatus (BioRad Laboratories Inc). The transfer was done at 18 V for 40 minutes. The membrane was blocked with 5% skimmed milk for 1 hour at room temperature and it was incubated overnight at 4°C with 6xHis Monoclonal Antibody (Albumin free) (1:5000) (Clonotech). Membrane was washed with 1XPBST (phosphate-buffered saline with 0.05% Tween-20) for 4 times before one-hour incubation with Amersham ECL anti-mouse IgG, Horseradish peroxidase (HRP)-linked whole antibody (1:5000) (GE Healthcare) at room temperature. Four washes were performed after the incubation and membrane was incubated with SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology) for protein detection.

To detect the presence of PfRH1, 10 µl of culture supernatant was loaded into 6% SDS-PAGE running gel and protein was transferred to nitrocellulose membrane using Mini Trans-Blot cell in Tank Transfer System (BioRad Laboratories Inc). Transfer was done using 1X Tris-Glycine buffer with 20% methanol, at 50 V for 2 hours at room temperature. Blocking and washing steps were the same as described above. Primary antibody (Table 2.2) against PfNBR and Pf-RVIII (Gao et al., 2008), PfRH1-

Table 2.1. Different antibodies against invasion proteins of *P. falciparum* used in this work

Antibody	Polyclonal / Monoclonal	Species	Working Dilution / concentration	Source
α -PfNBR (N205)	Monoclonal	Rat	1/2000-4000 (WB) 1/100 (IFA) from stock of 5 mg/ml	This work
α -PfNBR (N208)	Monoclonal	Rat	1/2000 (WB) 1/100 (IFA) from stock of ~5 mg/ml	This work
α -PfNBR (N209)	Monoclonal	Rat	1/2000 (WB) 1/100 (IFA) from stock of 5 mg/ml	This work
α -PfNBR (N206)	Monoclonal	Rat	1/100 (WB) 1/50 (IFA) from stock of 5 mg/ml	This work
α -PfNBR (N207)	Monoclonal	Rat	1/100 (WB) 1/50 (IFA) from stock of 5 mg/ml	This work
α -PfRH1-EBD (C41)	Monoclonal	Mouse	1/5000 (WB) 1/1000 (IFA) from stock of 5 mg/ml 2.5 μ g/ml (ELISA)	Gao Xiaohong (NTU)
α -PfRH1-EBD (C49)	Monoclonal	Mouse	2.5 μ g/ml (ELISA)	Gao Xiaohong (NTU)
α -PfRH1-EBD (C2)	Monoclonal	Mouse	2.5 μ g/ml (ELISA)	Gao Xiaohong (NTU)
α -PfRH1-EBD (C50)	Monoclonal	Mouse	2.5 μ g/ml (ELISA)	Gao Xiaohong (NTU)
α -PfRVIII (R8201)	Monoclonal	Rat	1/500-1000 (WB) 1/100 (IFA) from stock of 4 mg/ml	This work
α -PfRVIII	Polyclonal	Mouse	1:500 (WB)	Gao Xiaohong (NTU)
α -PfEBA-175	Polyclonal	Rabbit	1/5000 (WB)	MR4
α -MSP-1	Polyclonal (serum)	Rabbit	1/500 (IFA)	Gift from Mike Blackman

WB, western blot ; IFA, immunofluorescence assay ; ELISA, enzyme-linked immunosorbent assay

EBD (Gao et al., 2013) or PfEBA175 (Sim et al., 2011) were used together with compatible secondary antibodies HRP goat anti-rat IgG Minimal X-reactivity antibody (1:5000) (Biolegend), Amersham ECL sheep anti-mouse IgG (1:5000) or ECL[™] HRP donkey anti-rabbit antibody (1:5000) (GE Healthcare) respectively.

Amersham™ ECL Select™ Western Blotting Detection System (GE healthcare) and Amersham Hyperfilm ECL (GE Healthcare) were used for protein detection. In the validation of selected hybridoma clones, culture supernatant from hybridoma subclones (PfNBR and RVIII) culture was incubated directly with the membrane as primary antibody overnight at 4°C.

2.8 Bioinformatic online tools

Protein secondary structure was predicted using PredictProtein (Rost et al., 2004). Coiled-coils prediction was done using COILS/PCOILS (Lupas et al., 1991). Results of two matrices, MTIDK and MTK (weighted or no weight on residues at position *a* and *d*) were compared in win-28. Amino acids residue composition was analysed by ProtParam (Gasteiger et al., 2005).

The baseline-corrected Circular Dichroism (CD) spectra were further analyzed using an online program, Dichroweb (Mao et al., 1982; Whitmore and Wallace, 2008; Abdul-Gader et al., 2011) in which a algorithms named K2D (Andrade et al., 1993) was applied.

2.9 Fluorescence Correlation Spectroscopy (FCS)

FCS was performed in reference to the procedures described in details in *Ramalingam et al., 2008*. It was done on an LSM 510 Meta/ConfoCor 3 (Zeiss, Jena, Germany) using the fluorescently labelled ATP analogue EDA-ATP ATTO-647N (ATTO-TEC, Siegen, Germany). For the FCS experiment 50 mM TrisHCl, 500 mM NaCl, pH 9.0 with 1 mM Pefabloc SC, 10 mM EDTA and 1 mM DTT was used. The temperature was adjusted to 25°C in an incubation chamber (Zeiss). The 633 nm laser line of a HeNe633 laser were attenuated to 5 mW and focused into the aqueous solution by a water immersion objective (40 x / 1,2 W Korr UL-VIS-IR, Zeiss). Fluorescence signal was measured in 15 µl droplets of the diluted fluorescent derivatives of ATP, which

were placed on Nunc 8 well chambered cover glass. Before usage, the cover glasses were treated with 3% of gelatin, in order to prevent unspecific binding and removed by washing steps with H₂O. The following filter sets were used: MBS: HFT 514/633, EF1: BP 655-710 IR, EF: None, DBS: None. Out-of-focus fluorescence was rejected by a 90 µm pinhole in the detection pathway. Solution of Cyanine 5 in water was used as references for the calibration of the confocal microscope. Variable concentrations of protein solutions were mixed after addition of MgCl₂ and fluorescently labelled nucleotide. The drops were incubated on the glass slip surface for 3 minutes, and monitored during this time by FCS. The fluorescence autocorrelation functions were determined by measurements of at least 10 repetitions at 30 second each. The calculations of the bound fractions and the dissociation constants were done by the ConfoCor 3-software 4.2, Excel2003 and OriginPro 8 SR4. A 'Standard AC normalized triplet' model was used to determine the diffusion time of the fluorescent nucleotides. The determined value was fixed for the fitting in the titration experiments, whereby the 'Standard AC2 diffusion coefficients normalized triplet' model has been used, which takes two components in consideration. The second parameter was determined and fixed during the calculations to determine the bound fractions and autocorrelation functions which were analyzed in Windows Excel and the figures were generated in OriginPro 8. The resulting sigmoidal non-linear curve fit was used to calculate the binding constant.

2.10 Circular Dichroism (CD) spectroscopy and ATPase assay

To obtain CD spectra of rNBR, 2 mg/ml of purified protein in 50 mM TrisHCl, 500 mM NaCl, pH 9.0 with 1 mM Pefabloc SC and 10 mM EDTA was prepared and incubated with or without 2 mM nucleotides (ATP or ADP) (Sigma) and 2 mM MgCl₂, on a rocker for 10 minutes at 4°C. Individual sample was applied into a 0.1 mm quartz cell (Hellma) and measured in far-UV light region (180-260 nm) using Chirascan spectropolarimeter (Applied Photophysics). Each sample was measured three times and all measurements were set with 1 nm bandwidth and step-point of 1 nm. The final CD spectrum was obtained by subtracting average buffer spectrum from the average protein spectrum. All CD spectrum (rNBR, rNBR in binding to ATP or

ADP) was normalized to the number of residue in the protein for comparison. The helical content was calculated using the formula:

$$fH = ([\theta]_{208\text{nm}} - 4,000) / (-33,000 - 4,000) \quad (\text{Greenfield and Fasman, 1969})$$

whereby fH = fractional helicity and $[\theta]$ = mean molar residue ellipticity

ATPase assay was done using a colorimetric ATPase assay kit (Innova Biosciences) and all procedures were done as described in the manufacturer manual. ATPase activity of purified rNBR was tested in buffer 50 mM TrisHCl, 500 mM NaCl, pH 9.0 while ATPase activity of Apyrase (Sigma) was tested in distilled water in the presence of 2.5 mM CaCl_2 . Free phosphates released from ATP hydrolysis were detected by absorbance at 590 nm using Benchmark PlusTM Microplate Spectrophotometer (BioRad Laboratories Inc). For ATPase assay of immunoprecipitated native PfRH1, agarose resins with PfRH1 attached were washed with 1XTBS (25 mM Tris, 150 mM NaCl at pH 7.2). 100 μl 1XTBS was used to resuspend the resins and ATPases activity was tested in the same buffer with the presence of 2.5 mM MgCl_2 . Free phosphates released from ATP hydrolysis were detected by absorbance at 620 nm using Benchmark PlusTM Microplate Spectrophotometer (BioRad Laboratories Inc).

2.11 Parasite Culture and Synchronization

Plasmodium falciparum asexual stages (T9/94 strain or T9/94 Δ RH1 strain) (Triglia et al., 2005) were cultured *in vitro* with human erythrocytes from healthy blood donor using complete culture medium. Complete culture medium contains RPMI 1640 medium (Life Technologies) (with L-glutamine and HEPES, without sodium bicarbonate) supplemented with 27.4 mM sodium bicarbonate, 0.25% AlbuMAX® II (Life Technologies) and 0.005% hypoxanthine (Sigma) dissolved in 1 M NaOH. Cultures were incubated at 37°C and supplied with mixed gas (5% CO_2 , 3% O_2 and 92% N_2). To synchronize the parasites, culture at the ring stage was added with equal

volume of 5% L-Sorbitol (Sigma-Aldrich) and then incubated at 37°C for 7 minutes. After the incubation, the mixture was centrifuged at 622 g (acceleration, 9; deceleration, 2, Eppendorf centrifuge 5810 R) for 3 minutes. Supernatant was discarded and the cell pellet was washed once with incomplete RPMI medium which contains RPMI 1640 medium (with L-glutamine and HEPES, without sodium bicarbonate) (Life Technologies) with 27.4 mM sodium bicarbonate.

2.12 Purification of Schizonts and Culture Supernatant

Synchronized ring stage-parasite culture was incubated at 37°C for over 20 hours, whereby schizonts would be developed. Parasite culture was diluted with incomplete RPMI medium in 1:5 dilution before subjected to Percoll® (Sigma-Aldrich) density gradient centrifugation. Percoll® stock solution was diluted to 68% working solution with 10X PBS and incomplete RPMI medium before use. 10 ml of 68% Percoll was added into a 30 ml Sterillin tube (Sterilin) and 5 ml of diluted culture was gently added on top. Centrifugation was set at 622 g (acceleration, 9; deceleration, 0, Eppendorf centrifuge 5810 R) for 11 minutes. Four layers were formed after the centrifugation and the first two layers from top containing only schizonts and medium were transferred into a separate tube using a Pasteur pipette. The schizont containing-suspension was centrifuged again at 622 g for 3 minutes and supernatant was discarded. The pellet was washed once with incomplete RPMI medium before complete culture medium was added. Five millilitre of culture medium was added to schizonts purified from a 3 ml parasite culture of 3% haematocrite with 5-7% parasitemia.

To prepare culture supernatant, purified schizonts in complete culture medium were incubated at 37°C for 20 hours in the absence of uninfected erythrocytes. The invasion-related proteins were cleaved and released into the culture medium after the rupture of the schizonts. The culture medium was centrifuged at high speed of 48,000 g at 4°C for 30 minutes to remove cell debris. The supernatant was collected and stored at -80°C until used.

2.13 Isolation of free merozoites from *P. falciparum* *in vitro* culture

Purified schizonts were added with complete culture medium and incubated at 37°C for 2 hours (Gao et al., 2013). Schizonts were allowed to rupture in the absence of uninfected erythrocytes. After that, schizonts culture was centrifuged at 622 g for 3 minutes at room temperature to separate free merozoites from unruptured schizonts and uninfected erythrocytes. Supernatant containing free merozoites was centrifuged again at 3214 g for 3 minutes. The pellet (free merozoites) was resuspended immediately in warm complete culture medium to be used in the subsequent assays.

2.14 Invasion Inhibition Assay and Flow Cytometry

In a 96-well microtitre plate, purified late schizonts were mixed with fresh human erythrocytes and purified IgGs in 1XPBS at different concentration to obtain a final volume of 50 µl/well. After 24 hours post invasion, the plate was centrifuged at 622 g for 3 minutes and supernatant was discarded. 3 µl of the blood sample from each well was added into tubes containing 300 µl of 1X SYBR® Green I Nucleic acid gel stain (Invitrogen) in 1XPBS with 0.5% BSA. The samples were incubated at room temperature in dark for 20 minutes. 1.5 ml of cold 1XPBS was added into each tube right before sample acquisition using LSR II flow cytometer (Becton Dickinson). Blue laser (488 nm) was used to detect 1X SYBR® Green I positive erythrocytes (infected erythrocyte) and parasitemia was calculated. For all samples, 100,000 events were recorded for each measurement and FlowJo was used for all flow cytometer data analyses. Gating strategy used in FACS measurement has been shown in Appendix 2. The following formulas were used in this assay:

Formula 1:

$$\text{Parasitemia (\%)} = \frac{\text{Total no. of infected erythrocyte}}{\text{Total no. of erythrocyte}} \times 100$$

Formula 2:

$$\text{Changes in Parasitemia (\%)} = \frac{\text{Parasitemia of A} - \text{Parasitemia of B}}{\text{Parasitemia of B}} \times 100$$

where **A** = schizont + uninfected erythrocyte + purified IgG

B = schizont + uninfected erythrocyte + medium alone

A positive value indicates an enhancing effect of IgG while a negative value indicates an inhibitory effect.

2.15 Real-time cytosolic Ca²⁺ level measurement of merozoites

Fresh isolated merozoites were first stained with 6 μM Fluo-4 AM (Invitrogen) in complete culture medium at 37 °C for 20 minutes (Gao et al., 2013). Fluo-4 AM preloaded merozoites were washed once and then immediately resuspended in warm complete culture medium. Merozoites were added with fresh erythrocyte or complete culture medium containing ATP (Adenosine 5'-triphosphate disodium salt hydrates (Sigma) dissolved in distilled water) at various concentrations (0 μM , 5 μM , 10 μM , 40 μM , 80 μM and 100 μM). All conditions were set in a Greiner 96 Black Flat Bottom Polystyrol plates (Greiner Bio-one). Changes in cytosolic Ca²⁺ levels of merozoites under different conditions were examined at 37 °C by using a fluorescence plate reader, Infinite 200® PRO (Tecan) and the supporting software TECAN-i-control 1.8.20.0. Ca²⁺ fluorescence intensity was measured for 600 seconds with excitation at 488 nm and emission at 530 nm as described in previous publication (Gao et al., 2013). The maximum increase of Ca²⁺ green fluorescence (F) during a period of 600 seconds after the start of the stimulation was normalized to the fluorescence intensity before stimulation (F₀). Changes in cytosolic Ca²⁺ levels at different time point (t) after stimulation ($\Delta F(t)$) were expressed as changes in fluorescence intensity over the starting fluorescence levels: $\Delta F(t) = (F - F_0) / F_0$ where F = fluorescence intensity (t), F₀ = starting fluorescence signal. The total changes of

Ca²⁺ levels ($\sum \Delta F(t)$) in merozoites along the 600 seconds were summed up and plotted against time (s).

2.16 Photoaffinity Labeling and Mass Spectroscopy Analysis

Culture supernatant of T9/94 was incubated with 5 mM MgCl₂ (Sigma) and 5 mM unlabelled nucleotides (ATP or ADP) (Sigma) in competition to 5 mM 8-N₃-3'-biotinyl-ATP (Schafer et al., 2001). The mixtures were incubated on ice on a rocker for 10 minutes before transferring to a 96-well plate. The plate was exposed to UV light (Konrad Benda N-6L, $\lambda=366\text{nm}$) in dark for 15 minutes on ice with gentle shaking. 6X sample loading dye (0.5 M TrisHCl, 0.4% SDS, 30% glycerol, 0.6 M DTT, 0.012% bromophenol blue) was added to all mixtures for Western Blot analysis. Monoclonal antibody C41 (1:5000) was used to determine the presence of PfRH1 in each mixture while Streptavidin-Peroxidase (1:500, Sigma) was used to detect 8-N₃-3'-biotinyl-ATP. Culture supernatant of T9/94 was also applied on 6% SDS-PAGE gel and proteins were stained with Coomassie Blue Staining. To do MALDI-TOF analysis, the 8-N₃-3'-biotinyl-ATP-labeled and unlabeled gel bands were cut out from the gel. In-gel tryptic digestion and sample preparation were performed as described by Ramalingam *et al.* (Ramalingam et al., 2008).

2.17 Immunoprecipitation (IP)

For IP of PfRH1 in photoaffinity labeling assay, culture supernatant of T9/94 or T9/94 Δ RH1 was incubated with 5 μg of monoclonal antibody C2 and 50 μl of $\mu\text{MACS}^{\text{TM}}$ ProteinA/G Microbeads (Miltenyi Biotech) overnight at 4°C, in rotating motion. The mixture was then incubated with 5 mM MgCl₂ and 5 mM 8-N₃-3'-biotinyl-ATP for sample crosslinking. Samples were loaded on μ Column in the magnetic field of the $\mu\text{MACS}^{\text{TM}}$ Separator and washed with 4 x 200 μl of 20 mM TrisHCl at pH 7.5. Immunoprecipitated PfRH1 was eluted by using 50 μl of hot 1X

SDS-PAGE gel loading dye (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue and 0.125 M TrisHCl, pH 6.8). IP elutions (T994 or T9/94ΔRH1) were loaded on 6% denaturing SDS-PAGE gel and probed with Streptavidin-Peroxidase (1:500, Sigma) or monoclonal antibody against PfRH1 (C2).

For IP of PfRH1 in ATPase assay, Pierce Crosslink IP Kit (Pierce) was used. 70 µg of monoclonal antibody C50 was immobilized and crosslinked to 140 µl of Protein A/G beaded agarose resins slurry. Parasite culture supernatant of both T994 and T9/94ΔRH1 were standardized to total protein concentration of 16.2 mg as determined by PierceTM BCA Protein Assay Kit (Pierce) before incubating with immobilized C50. 100 µl of elution buffer was used to elute PfRH1 for Western Blot.

2.18 Immunofluorescence Assays

Synchronized late schizonts were smeared and fixed with fixation solution (90% acetone, 10% methanol) at 4°C for 10 minutes followed by blocking with 3% BSA in 1XPBS at room temperature for 10 minutes. For free merozoite staining, synchronized schizont culture was incubated at 37°C for 1-3 hours to allow egress. Culture was smeared and fixed once free merozoites in abundance were seen in Giemsa-stained smear. Fixed smears were incubated with monoclonal antibodies (N205 or N208 or N209) together with mouse anti-PfRH1-EBD monoclonal antibody (C41) or rabbit antiserum against full length MSP-1 (Holder and Freeman, 1984)(Table 2.2) in 3% BSA in 1XPBS at 37°C for 1 hour or overnight at 4°C. Three times of washing were performed using 1XPBS. All smears were then incubated for 1 hour with the mixture of DyLightTM 488 Goat anti-rat IgG (minimal X-reactivity) Antibody (1:500) (Biolegends) and Alexa Fluor® 594 Goat Anti-Mouse IgG (H+L) (1:800) (Molecular Probes®, Life Technologies) or Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody (1:500) (Molecular Probes®, Life Technologies) to achieve double staining of EBD and NBR or NBR and MSP-1. Slides were washed twice and then incubated with Hoechst 33342 (1 µg/ml in 1XPBS) (Sigma) for 10 minutes at 37°C. Slides were washed and mounted using VECTASHIELD mounting

medium without DAPI (Vector Labs) before sealing. Fluorescence images were captured using Nikon microscope ECLIPSE 50i (Nikon) with running software DP Controller version 2.2.1.227 (Olympus). All images were post-processed using ImageJ (Rasband, 1997-2012; Schneider et al., 2012).

2.19 Surface staining of parasite ligands on merozoites

Purified synchronized late schizonts were added with fresh erythrocytes and incubated for 1-2 hours at 37°C. Culture was smeared once free merozoites or merozoites attached to erythrocyte were in abundance as seen in Giemsa-stained smear. To collect free merozoites devoid of contact to erythrocyte, free merozoites were isolated using the method described in section 2.13 and smeared. All smears were fixed with 4% paraformaldehyde (PFA) (Sigma) in 1XPBS at room temperature for 10 minutes. Fixed smear was washed with 1XPBS and allowed to dry. All subsequent procedures were the same as described in section 2.18. Secondary antibodies used against monoclonal antibodies (N205 or N208 or N209 or C41) and MSP-1 antiserum were DyLight™ 488 Goat anti-rat IgG (minimal X-reactivity) Antibody (1:500) (Biolegends), Alexa Fluor® 488 Goat Anti-Mouse IgG (H+L) (1:800) (Molecular Probes®, Life Technologies) and Alexa Fluor® 594 Goat Anti-Rabbit IgG (H+L) Antibody (1:500) (Molecular Probes®, Life Technologies). For cell permeabilization, 0.1% Triton X-100 in 1XPBSA (1XPBS + 1% BSA (w/v) + 0.5% Tween-20 (v/v) was applied on smear after 4% PFA fixation for 10 minutes at room temperature. 3% BSA (w/v) in 1XPBS was replaced with 3% BSA in 1XPBSA in cell permeabilized-IFA.

For surface staining of PfRH1 under the effect of exogenous ATP, isolated free merozoites were resuspended in 100 µl complete culture medium supplemented with different concentration of ATP (Adenosine 5'-triphosphate disodium salt hydrates (Sigma) dissolved in distilled water). Incubation was done at room temperature for 7 minutes before centrifuging at 3214 g for 3 minutes. Total exposure time to ATP was limited to 10 minutes. Merozoite pellet was washed with fresh complete culture medium once and smeared for IFA.

All fluorescence images were captured using Olympus 1X71 inverted microscope (Olympus) with running software DP Controller version 2.2.1.227 (Olympus) or Nikon inverted microscope ECLIPSE Ti (Nikon) with running software NIS-Elements viewer software (Nikon). All images were post-processed using ImageJ (Rasband, 1997-2012; Schneider et al., 2012).

2.20 Recombinant RVIII purification

E. coli cells transformed with RVIII expressing plasmid (Gao et al., 2008) was inoculated into Luria-Bertani (LB) media containing 50µg/ml of Kanamycin (Sigma-Aldrich). Bacteria culture was induced with 0.2 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated in the shaking incubator for 20 hours at 16°C. Purification was done using lysis buffer 50 mM TrisHCl, 500 mM NaCl pH 9.0 with 2 mM Pefabloc SC (BioMol) and 2 mM of phenylmethanesulfonyl fluoride (PMSF). The protein was first purified using Ni-NTA agarose resins (Qiagen) and eluted by applying elution buffers containing an increasing concentration of Imidazole. Eluted fractions were concentrated followed by injection into a gel filtration column, Superdex75 10/300 GL (GE Healthcare) to be further purified.

2.21 Rat Immunization

Purified rNBR and recombinant RVIII were dialyzed against 1XPBS, pH 7.4. After dialysis, they were concentrated using Vivaspın 20 Centrifugal Concentrator (Sartorius Stedim Biotech). To immunize rats, 100 µg of protein was used in primary injection with complete Freud's adjuvant (Sigma) and 50 µg of protein with incomplete Freud's adjuvant (Sigma) in each boosting subsequently. Total of three times of boosting were performed every two weeks.

2.22 Hybridoma Production and Culture Subcloning

Draining lymph nodes were removed from the rat and fat tissues were cleared from the lymph nodes. B-cells were removed by squashing the lymph nodes in a petri dish with complete Iscove's Modified Dulbecco's Media (IMDM with L-glutamine and phenol red) supplemented with 5% Fetal Bovine Serum (FBS) (Gibco). Medium containing B-cells was centrifuged at 350 g for 8 minutes. After that, both B-cells and Sp2/0 cells were washed with incomplete IMDM before mixing (1:4 in volume). Three washes were performed on the mixed cells before fusion. Fusion of B-cells and Sp2/0 cells was done by adding 1.5ml of Polyethylene glycol (PEG) (Sigma) drop by drop to the mixed cells while swirling gently in a 37°C waterbath. 400 ml of IMDM containing 1X Hypoxanthine-aminopterin-thymidine medium (HAT medium) and 7% Fetal Bovine Serum (FBS) (Gibco) were added to the fused cells and 200 µl/well was seeded in 96-well tissue culture plates (NUNC). They were incubated at 37°C, supplied with 5% CO₂. Viable hybridoma clones were identified 10-12 days after fusion and they were subjected to ELISA screening. Positive clones were further subcloned with IMDM containing 1X HAT and FBS (Gibco) to obtain single clone that was subsequently validated by ELISA.

2.23 Screening of Hybridoma Clones by ELISA

Purified rNBR (0.2 µg/well) and RVIII (0.15 µg/well) were coated in 96-well plates (NUNC) overnight at 4°C. 1% BSA (in 1XPBS pH 7.4) was used as blocking solution and blocking was done at 37°C for 1 hour. Five microlitre of supernatant from each hybridoma clone was diluted in 95 µl of 1% BSA. The mixture was incubated with the coated antigen for 1 hour at 37°C. HRP goat anti-rat IgG (minimal X-reactivity) antibody (Biolegend) was diluted in 1% BSA (1:5000) for incubation. All plates were washed twice with 1XPBST (1XPBS with 0.05% Tween-20) in between incubations of this assay. Fifty microlitre per well of TMB High Sensitivity Substrate Solution (Biolegend) was added and incubated for 1.5-2 minutes. Signal reading was

performed using Benchmark Plus™ Microplate Spectrophotometer (BioRad Laboratories Inc) at 370 nm.

2.24 IgG Purification

Hybridoma culture supernatant was harvested by mixing cell culture with Hyflo® Super Cel® (Sigma) and filtered through a 0.45 µm filtration membrane. IgG was purified from the filtered culture supernatant using HiTrap™ Protein G HP column (GE Healthcare). 20 mM sodium phosphate buffer pH 7.0 and 0.1 M glycine-HCl pH 2.7 were used in IgG-protein G binding and IgG elution respectively. Purified IgG was later buffer-exchanged from 0.1 M glycine-HCl to 1XPBS for subsequent assays.

2.25 Statistical test

To compare the effect of various mAbs on parasite invasion between T9/94 and T9/94ΔRH1, unpaired t-test was performed with two-tailed confidence level at 95%. To compare the % of PfNBR- or EBD- positive merozoite population across different (early) stages of invasion, One-way ANOVA was performed with post hoc test “Tukey test” (95% confidence intervals) to compare all pairs of groups. To compare PfNBR- and EBD-positive merozoite population under the effect of exogenous ATP, unpaired t-test was performed with two-tailed confidence level at 95%. To compare PfNBR- and EBD-positive merozoite population at different early phases of invasion, unpaired t-test was performed with two-tailed confidence level at 95%.

2.26 Expression of seven different regions of PfRH1-EBD in *Escherichia coli*

Six overlapping regions in PfRH1-EBD were amplified by PCR (Table 2.3) and cloned into expression vector pET24a(+) (Novagen) before chemically transforming into BL21 (DE3) (Stratagene) cells. All of them were tagged with 6X His-tag at the C-terminal and expressed at 16°C (>16 hours) with 0.2 mM IPTG after optimizing the induction condition. The optimization of the induction assay was the same as described in section 2.3. Total lysate of each recombinant protein was prepared by centrifuging 1 ml of the bacteria culture. Pellet was washed with 1XPBS, pH 7.4 before adding with 2X protein sample loading dye. For epitope mapping assay, 12% SDS-PAGE gel was used for all related analysis. Monoclonal antibody against 6X His-tag (Clontech) was used to detect His-tagged recombinant protein.

2.27 Peptides generation and epitope mapping of PfRH1-EBD

All biotinylated overlapping synthetic peptides were synthesized by Peptide Synthesis Core facilities, SBS, NTU. The lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO). Peptide stocks were diluted in 1XPBS+0.1% BSA to make up 2.5 µg/ml of sample. The Streptavidin-coated 96-Well plate, pre-blocked with Superblock blocking buffer (Thermo Scientific) was washed 3 times with 200 µl of wash buffer (25 mM Tris, 150 mM NaCl, pH 7.2, 0.1% BSA, 0.05% Tween-20). One hundred microlitre of the peptide solution was added into each well of the Streptavidin-coated plate and incubated for 2 hours at room temperature with gentle shaking. The plate was washed four times before the addition of 100 µl of primary antibody (C2, C41, C49 and C50 in 25 mM Tris, 150 mM NaCl, pH 7.2, 0.1% BSA) at the concentration of 2.5 µg/ml into each well (Table 2.2) and incubated for 1 hour at room temperature. The wells were washed four times followed by the addition of 100 µl of secondary antibody (Biolegend) (HRP goat anti-mouse IgG diluted 1:3000). After six times of washing, 50 µl of TMB high sensitive substrate solution (Biolegend) was added into each well. The plates were incubated for 3-5 minutes before analyzing by Benchmark PlusTM Microplate Spectrophotometer (BioRad Laboratories Inc) at 370 nm. To derive the cut off value which determined the background value, the lowest 25 percentile of all readings of each screening was identified (as Y) and the standard

deviation (SD) of the values of these 25 percentile was calculated (as X). The cut off value was calculated by using a formula : (average of Y + 3 X Z).

Table 2.2. Primers used in PCR amplification of different regions in PfRH1

Recombinant Proteins	Forward Primers	Reverse Primers	Restriction Digestion Enzymes
BA2		5' - AACctcgagATCTTCTTTCATTTTTATGATTTC - 3'	
BA4	5' -	5' - AACctcgagTTGCTTTTGTATTAAGTCATCGAG - 3'	
N1	GACcatatgTTAC	5'- AACctcgagAGAAATAAGTTGAATCGTCTCATTATTTT- 3'	NdeI (NEB) and XhoI (NEB)
N2	AAATAGTACAA	5'- AACctcgagGGATTTAAGAAGATTTTGGATGTTTT - 3'	
N3	CAAAAACCTTTT	5'- AACctcgagTTGTTTTAATATATATTTAGAAATTGTGTCTATGAA - 3'	
N4	AGAAATC -3'	5'- AACctcgagGTTTTGTTCATTTTTTATTTCTTCAAGAT - 3'	

Note: Restriction digestion sites are denoted in lower cases.

Chapter 3 Epitope mapping of invasion inhibitory monoclonal antibodies against PfRH1

This chapter has been published as part of the work “Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*. Nat Commun. 2013, by Xiaohong Gao, Karthigayan Gunalan, Sally Shu Lin Yap & Peter R. Preiser” (Appendix M)

3.1 Introduction

All members of PfRHs bind to erythrocyte through specific ligand-receptor interaction. Each of the members has its own characterized binding property that is sensitive to particular enzyme treatment. Binding of PfRH1 to the erythrocyte is neuraminidase-sensitive, chymotrypsin- and trypsin-resistant, indicating that PfRH1 receptor is a glycoprotein with sialic acid moieties. A 334 amino acids long region near the N-terminus of PfRH1 has been identified as the erythrocyte-binding domain (EBD) that mediates host cell binding during invasion (Gao et al., 2008). Four monoclonal antibodies (mAbs) (Table 2.2) have been generated against this region and their effect on merozoite invasion has been investigated. Two mAbs (C41 and C49) inhibit parasite invasion whereas the other two exhibit no effect on invasion. Interestingly, the inhibitory mAbs do not block PfRH1 binding to erythrocyte. Live video imaging of parasite invasion shows that in the presence of inhibitory mAbs, merozoite attaches to erythrocyte but rapidly detaches itself and fails to proceed to penetration. Further investigations have proved that inhibitory mAbs, C41 and C49, block invasion by inhibiting elevation of merozoite intracellular Ca^{2+} , leading to failure of EBA-175 surface expression.

In order to determine the basis of the differential effect displayed by mAbs against the same antigen as well as the importance of PfRH1 in invasion, epitope mapping for all four mAbs was carried out by using a collection of overlapping peptides.

3.2 Results and discussions

To narrow down the recognition site of distinct mAbs in EBD, six overlapping regions (BA2, BA4, N1, N2, N3 and N4) derived from EBD (Figure 3.1A) (Appendix A) were cloned into expression vector pET24a(+) to express the recombinant protein with a C-terminal 6X His tag. The total lysate of *E. coli* expressing the recombinant protein was loaded on a denaturing SDS-PAGE gel and probed using different monoclonal antibodies in Western blot. None of the PfRH1 mAbs recognizes N1. C2, C41 and C49 showed a similar recognition pattern to full length EBD and N4 in Western Blot. Interestingly, C50 displayed strong recognition to full length EBD, N2, N3 and N4 (Figure 3.1A), suggesting that a different epitope was targeted by C50 which is upstream of the epitope of the other three mAbs.

Based on the Western blot results, ten overlapping peptides were generated (Figure 3.2A) to further distinguish the individual epitopes of each mAb using ELISA. None of the peptides from P1 to P6 was recognized by C50 which was consistent with the Western Blot results. C2 and C41 bound to P3 but not to P2 or P4, indicating ⁷⁶²NDYLE⁷⁶⁶ as the possible linear epitope of C2 and C41 (Figure 3.2B). As most of the linear epitopes involve five amino acids residues (Geysen et al., 1988), any five amino acids residues in P3 including one or two residues from the overlapping sequence between P3 and P2 or P4 was potential epitope as well (Table 3.1). Different levels of recognition to the same peptide were shown by the mAbs. C49 recognized P3 at a much weaker level as compared to C2 and C41. The mapping of the linear epitope of C50 was unsuccessful due to the insolubility of the peptides. Nevertheless, C50 recognized a different epitope from those of the inhibitory mAbs, which explains its limited effect on parasite invasion.

Generally, an epitope is 5-8 amino acids long and most of the linear epitopes involve five amino acids residues (Geysen et al., 1988). It is surprising that the non-inhibitory mAb (C2) and the inhibitory mAb (C41) bound to the same peptide (P3). However, as an epitope could be as long as 5–8 amino-acid long, it is possible that multiple distinct epitopes (at least five epitopes) lie within the same peptide as described above. It has

A

B

Figure 3.1. Epitope mapping in PfrH1 using recombinant proteins and biotinylated peptides. (A) Six regions of the erythrocyte binding domain (EBD, ~40 kDa) of PfrH1 were selected and expressed as recombinant proteins. Numbers indicate the position of amino acid residue in the full length PfrH1. BA2, 12 kDa; BA4, 21 kDa; N1, 24 kDa; N2, 28 kDa; N3, 30 kDa; N4, 34 kDa. Two regions in EBD that were recognized by each antibody have been narrowed down (highlighted in red and green with corresponding monoclonal antibodies. N, N-terminal; C, C-terminal. (B) Recognition of monoclonal antibodies towards different recombinant proteins. NBD6, a Py235 fragment from *P. yoelii* was not recognized by the PfrH1 monoclonal antibodies. Anti His-tag monoclonal antibody (α His) was used as loading control.

A

Region 693 - 727 aa (C50)

50a GQEDKHESNP**FNHIQ**
 50b **FNHIQ**TYIQQ**KDTQN**
 50c **KDTQN**KNIQ**NLLKS**
 50d **NLLKS**LYNGNINTFI

Region 747- 774 aa (C2, C41 and C49)

P1 SKYILKQ**KDIEL**
 P2 **KDIEL**TQHVV**TDEKI**
 P3 **TDEK**INDYLE**EIKNE**
 P4 **EIKNE**QNKID**KTIDD**
 P5 **KTIDD**IKIQ**ETL**
 P6 **ETL**KQITHIV

B

Figure 3.2. Identification of a 5-amino acids linear epitope in PfrH1.

(A) Biotinylated peptides with overlapping sequences were designed according to the corresponding regions in Figure 3.1A. (B) C2, C41 and C49 exhibited strong recognition to peptide P3. The Y-axis indicates absorbance at 370 nm. The X-axis shows the list of peptides used. The cut off value is indicated in red. Any value that is below the cut off value is considered background.

Table 3.1. Potential epitopes for C41, C49 and C2

Amino acids position in PfRH1	Amino acids sequences
759 – 763	EKIND
760 – 764	KINDY
761 – 765	INDYL
762 – 766	NDYLE
763 – 767	DYLEE
764 – 768	YLEEI
765 – 769	LEEIK

Amino acids in the non-overlapping region in P3 are highlighted in blue.

been reported that mAbs against epitopes in very close proximity exhibit different effects on cellular or biological functions in other systems (Martin et al., 1983; Chopineau et al., 1993; Imakiire et al., 2004). Different critical binding amino acid residues may be responsible for the different effect exhibited by inhibitory and non-inhibitory mAbs. It is possible to identify an immunogenic epitope to a resolution of single amino acids by replacement net analysis (Appendix 4) (Geysen et al., 1984; Sumar, 2001) in future work. Each of the five amino acids in ⁷⁶²NDYLE⁷⁶⁶ could be substituted by the 19 common amino acids in the peptide. Comparison between modified peptide and parent peptide in binding to mAb would confirm the critical residues at specific position recognized by each of the three mAbs. To further narrow down the epitope for C50, a redesign of a new overlapping peptides library with shorter peptide sequence (less than 10 amino acids) may be helpful to identify the epitope of C50. A phage display library expressing random peptides of the C50-targeting region (Figure 3.2A) could be used as an alternative method to search for the epitope of C50 which does not involved peptide synthesis as described in this work (Fack et al., 1997; Hay and Westwood, 2001).

Calcium is an important second messenger in cell signaling of an eukaryotic cell to support various cellular functions (Berridge et al., 2003). In Apicomplexans such as *Plasmodium*, intracellular Ca²⁺ stored in the endoplasmic reticulum (ER) and acidocalcisomes is essential for survival and development of the parasite (Wasserman et al., 1982; Lovett and Sibley, 2003). Recently increase in Ca²⁺ level has been shown

to be important in host cell invasion of *P. falciparum* and *Toxoplasma gondii* (*T. gondii*) by triggering release of micronemal invasion protein (Carruthers and Sibley, 1999; Singh et al., 2010). The epitope of the invasion inhibitory mAbs against PfRH1 has been identified in this work by screening through a collection of peptides. However, the actual molecular basis of blocking of a short sequence leading to an inhibition of intracellular Ca^{2+} signaling remains unknown.

PfRH1, a Type I transmembrane protein, has a cytoplasmic tail at its C-terminus. The biological role of the cytoplasmic tail is yet to be determined except that it was carried into the ring stage-infected erythrocyte after the completion of invasion (Triglia et al., 2009). Phosphorylation of the cytoplasmic tail by different class of kinases on two invasion proteins in *P. falciparum*, AMA-1 and PfRH2b, exerts different effects on parasite invasion, suggesting that a relay of signal transduction by invasion proteins may take place along the process but the sequential order of signaling events is not fully understood (Leykauf et al., 2010; Engelberg et al., 2013). Binding of C41 and C49 to the 5 amino acid-epitope probably induce a conformational change in PfRH1 that results in incorrect positioning or conformation of the cytoplasmic tail for downstream signaling transductions which is important to complete invasion process, whereas binding of C2 possibly induces little change in protein conformation through binding to a different critical residue. A second possibility of the working mechanism of the inhibitory mAbs is by affecting protein multimerization. Homo-dimerization or oligomerization of EBD is commonly observed in *Plasmodium* adhesins such as EBA-175 and PvDBP (Tolia et al., 2005; Batchelor et al., 2011a). The Ca^{2+} signaling-inhibitory mAb targeted-site, ⁷⁵⁹EKINDYLEEIK⁷⁶⁹ falls in the sequence of EBD where a coiled-coil region has been predicted (Gao et al., 2008). This coiled-coil region has been suggested to be responsible for the multimerization of EBD, which possibly influences the binding avidity and specificity (Gao et al., 2008). EBD multimerization could be important as a trigger to the downstream Ca^{2+} signaling since protein dimerization is common in signaling pathway (Klemm et al., 1998). To address those questions, native PfRH1 or recombinant protein of EBD are required for the experiment. Unfortunately, all attempts to obtain soluble recombinant EBD in high concentration or to purify native PfRH1 (>200 kDa) were not successful in this work. Recombinant EBD was mainly insoluble under native condition and prone to precipitation during protein concentration. Purification of native PfRH1 from parasite

culture supernatant using immunoprecipitation or ion-exchange chromatography led to extremely low yield of native protein. In both cases, the starting concentration of both types of materials (proteins) was too low to be visibly assessed by Coomassie Blue staining for purity, as well as to allow any protein characterization assay to be done.

Chapter 4 Identification of a novel ATP/ADP- binding domain in PfRH1

4.1 Introduction – ATP and Merozoite Invasion

4.1.1 Adenosin-5'- triphosphate (ATP), a molecule with diverse functions

Adenosin-5'- triphosphate (ATP) is a multifunctional nucleotide that is widely used in cells. It consists of an adenosine (an adenine ring and a ribose sugar) and three phosphate groups (Figure 4.1). The phosphate groups are attached to the ribose sugar at the 5' carbon atom. As implied by its name, Adenosine diphosphate (ADP)

Figure adapted and modified from http://library.thinkquest.org/C004535/cellular_currency_atp.html

Figure 4.1. Chemical structure of ATP.

ATP consisted of an adenine ring, a ribose sugar and three phosphate groups. Phosphate groups are connected via high-energy phosphoanhydride bonds.

contains an adenosine as ATP but only two phosphate groups. ATP, ADP and Adenosine monophosphate (AMP) are inter-convertible through the addition or removal of the phosphate group. Removal of the γ phosphate group from ATP produces ADP, while removal of both γ and β phosphate groups yields AMP. ATP is required as a building block for DNA and RNA synthesis in all cells. It is also the most abundant active carrier in cells, which supplies energy for many biochemical reactions, molecular pumps, molecular motors and intracellular transportation system. The basis of ATP as a convenient and versatile store of energy is the high-level

energy that lies within the phosphoanhydride bonds (Figure 4.1). ATP is synthesized in an energetically unfavorable phosphorylation reaction in which a phosphate group is added to ADP. Hydrolysis of an ATP to ADP and inorganic phosphate is energetically favorable thus it is coupled to many unfavorable reactions through which the other molecules are synthesized. Most of the reactions involve the transfer of the terminal phosphate group to another molecule.

4.1.2 Merozoite invasion and erythrocyte ATP

The discovery of intracellular ATP level of erythrocyte being an important determinant of the Malaria infection severity was first made in 1960s, when studies on *P. falciparum* and *P. cynomolgi* (rhesus monkey malaria parasite) showed a positive correlation between the level of erythrocyte ATP and malaria infections: the higher level of erythrocyte ATP, the more rapidly parasitemia develops (Brewer and Powell, 1965; Eaton and Brewer, 1969). Lower ATP content has been suggested to cause diminished erythrocyte membrane integrity which potentially affects parasite maturation within the host cell (Eaton and Brewer, 1969). The requirement of ATP for *Plasmodium* infection has been shown later to be more relevant in merozoite invasion rather than in parasite development by several studies using resealed erythrocyte ghosts in which the ATP content is depleted (Dluzewski et al., 1981, 1983b, a). Resealed erythrocyte ghosts are a type of modified erythrocyte produced by dialyzing normal human erythrocyte against ionic strength buffer to remove the cytoplasmic contents including ATP and the cell content can be replaced by solutes of one's choice (Schwoch and Passow, 1973). Susceptibility of resealed erythrocyte ghost to parasite invasion is greatly reduced or abolished in the absence of intracellular Mg^{2+} -ATP whereas addition of extraneous Mg^{2+} -ATP but not ADP or non-hydrolyzable AMP-PNP fully restores the host cell susceptibility to invasion (Dluzewski et al., 1981, 1983b). Intracellular ATP has been suggested to regulate the phosphorylation of membrane-associated cytoskeleton proteins such as spectrin. Reduced phosphorylation level on spectrins could directly affect erythrocyte cell shape and rearrangement of the membrane cytoskeleton, leading to reduced parasite invasion (Olson and Kilejian, 1982; Jinbu et al., 1984a; Rangachari et al., 1986). This assumption is further supported by the observation of which invading merozoites

attach to resealed erythrocyte ghost but rapidly detach, and fail to invade the cell (Miller, 1977). It is probably because the active entry of merozoite is not well complemented by host cell membrane invagination which requires major rearrangement of membrane cytoskeleton.

4.1.3 Purinergic signaling in merozoite invasion

ATP is involved in an extracellular signaling system known as purinergic signaling which utilizes purine-based nucleotides (ATP, ADP, UTP and UDP) as the signaling molecule (Corriden and Insel, 2010). Almost all human cell types including erythrocyte rely on purinergic signaling for their physiological function. Intracellular ATP will be released from the cell due to all kinds of stimuli varying from mechanical to biochemical or chemical stimuli (Praetorius and Leipziger, 2009). The released ATP is recognized by a specific class of receptors, purinergic receptors which have been proposed as the most abundant receptors in mammalian tissues (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998; Abbracchio et al., 2009). They are plasma membrane receptors which mediate diverse cellular responses in mammalian cells after stimulated by extracellular nucleotides (Burnstock, 1978).

Under normal physiological condition, as much as ATP is required in maintaining the cell shape and deformability of erythrocyte, it will be released from erythrocyte in response to stimuli such as hypoxia and mechanical deformation (Bergfeld and Forrester, 1992). Under reduced oxygen tension, ATP acts as signaling molecule in a paracrine manner to regulate vascular tone by activating the purinergic receptors on the vascular endothelium, leading to increased oxygen delivery (Sprague et al., 2007). Erythrocytes encounter hydrodynamic shear stress *in vivo* on passing through small capillaries and releases ATP upon deformation (Wan et al., 2011). It has been suggested that deformed erythrocyte releases ATP via three main pathways 1) membrane transporters such as cystic fibrosis transmembrane conductance regulator (CFTR) (Sprague et al., 1996; Sprague et al., 1998a; Sprague et al., 1998b) 2)

voltage-dependent anion channels (VDAC), (Sridharan et al., 2012) and 3) Pannexin or Connexin channels (Bao et al., 2004; Locovei et al., 2006; Wan et al., 2011). Recently, Wan and colleagues have provided experimental evidences for an ATP-releasing model based on CFTR-actin pathway involving the spectrin network within erythrocyte (Figure 4.2A) (Gov and Safran, 2005; Wan et al., 2011) in which defects in the spectrin network in erythrocytes provides a signal for ATP release.

Figure 4.2. Deformation-induced ATP release and erythrocyte deformation during invasion. (A) A model of the CFTR-actin based ATP release model, membrane skeleton defect due to deformation induces defect in spectrin network followed by the binding of exposed actin filament to CFTR, leading to ATP release. Figure adapted from *Wan et al., 2011*. (B) Erythrocyte encounters different levels of deformation (red arrow) in the early phase (0-10 sec) of invasion in contact with merozoite. Merozoite begins to invade starting from 12 sec. Figure adapted and modified from *Gilson and Crabb, 2009*.

Host-pathogen interactions in diseases including *P. falciparum* infection have been reported to trigger ATP release from infected mammalian cells (Tanneur et al., 2006; Piccini et al., 2008; Corriden and Insel, 2010) whereas purinergic signaling remains an important system in regulation of immune cells activity during infection (Junger,

2011). Interestingly, it is not widely known that the mechanical cause of erythrocyte deformation may not be limited to shear stress alone. In *Plasmodium* infection, erythrocyte encounters waves of deformation during contact with the parasite at the early phase of merozoite invasion (Figure 4.2B) (Miller, 1977; Gilson and Crabb, 2009; Yahata et al., 2012). Recently purinergic signaling has been demonstrated to play a role in parasite invasion. Extracellular ATP elicits an increase of cytosolic Ca^{2+} level in parasites that are at the trophozoite and schizont stages (Levano-Garcia et al., 2010). Furthermore, the addition of purinergic receptor blockers or extracellular ATP scavenger to *in vitro* cultures of *P. falciparum* significantly reduced parasite invasion (Levano-Garcia et al., 2010). In the same report, Levano-Garcia and colleagues have provided new evidences of the presence of purinergic receptors in late-stage parasites which upon activation trigger intracellular Ca^{2+} signaling. However, it is not clear that the reduction in parasite invasion was due to interference to merozoite or erythrocyte as both of them express purinergic receptors on their surfaces. Therefore it is too early to conclude that ATP signaling modulates parasite invasion through regulation of intracellular Ca^{2+} signaling of parasite as they claimed. Two rodent *Plasmodium* species, *P. yoelii* and *P. berghei* have shown elevation in intracellular Ca^{2+} level in response to extracellular ATP as well but the specific class of purinergic receptors on parasites remain unknown (Cruz et al., 2012). Cruz and colleagues made the first hint indicating purinergic signaling is important in invasion by acting on merozoites. Extracellular ATP is required for the proteolytic processing of MSP-1, a key parasite adhesin in invasion and blocking of purinergic receptors of three *Plasmodium* species (*P. falciparum*, *P. yoelii* and *P. berghei*) inhibits parasite proteases activity which is important in regulating parasite protein functions (Fleck et al., 2003; Cruz et al., 2012).

4.1.4 ATP and Reticulocyte binding like protein homologues (RHs)

As one of the important invasion protein families, RHs are differed from MSPs and EBLs in the localization within merozoites. These three protein families also show difference in their domain arrangement in linear sequence. However, little is known

about their actual function during invasion except for being a host cell-binding ligand. Recently, all members of Py235 as well as PfRHs have been predicted to contain a nucleotide binding region which is not found in MSPs and EBLs (Ramalingam et al., 2008). The function of RH members during invasion is likely driven by a more complex working mechanism which involves Ca^{2+} signaling (Gao et al., 2013). The discovery of an ATP/ADP binding domain in PY01365, the dominantly expressed member of the Py235 family (Ramalingam et al., 2008) has suggested a distinct role of RHs from the other parasite ligands. ATP binding in RHs supports the previous suggested role for RHs in host cell selection (Barnwell and Galinski, 1998) seeing that erythrocyte ATP is one of the important determinants of a susceptible host cell. Erythrocyte binding of Py235 has been shown to be triggered by ATP binding to the nucleotide binding domain (Ramalingam et al., 2008). Hence, RHs at the apical end of merozoite have been proposed to act as an ATP sensor when in close contact to deforming erythrocyte during invasion. Here in this work, the study of the role of ATP-binding RHs has expanded to the most virulent *Plasmodium* species, *P. falciparum* where the ATP binding property of PfRH1 and its biological relevance in invasion were investigated.

4.2 Results

4.2.1 Delineating a small Nucleotide Binding Region (PfNBR) in PfRH1 (done in conjunction with Prof. Gerhard Gruber and Dr. Annie Gao)

Previous work on *P. yoelii* has shown the importance of a novel nucleotide binding domain (NBD94) in Py235, in which ATP/GTP binding-Walker A and B motifs have been identified. A nucleotide binding domain has been predicted in PfRH1, a protein homologue of Py235 in *P. falciparum* (Ramalingam et al., 2008). To further investigate the nucleotide binding properties of PfRH1, a photoaffinity labeling assay was performed using 8-N₃-3'-biotinyl-ATP (a photolabeled ATP analogue with a biotin residue) (Schafer et al., 2001) (Appendix 5). In this assay, 5 mM photoreactive 8-N₃-3'-biotinyl-ATP was incubated *in vitro* with parasite culture supernatant containing invasion proteins that were released from merozoites during invasion (Figure 4.3A). Upon UV radiation, 8-N₃-3'-biotinyl-ATP was cross-linked to interacting protein via the photoreactive azido group. 8-N₃-3'-biotinyl-ATP bound proteins were detected by Streptavidin-Peroxidase in Western Blot whereby biotin residue was bound by Streptavidin that was coupled to Horseradish peroxidase. In Figure 4.3B, a ~220 kDa band was clearly detected by Streptavidin-Peroxidase in Western Blot, which has the similar size to PfRH1. The corresponding location of PfRH1 on denaturing SDS-PAGE was shown by Western blot using C41, a monoclonal antibody against PfRH1 (Gao et al., 2013) in a separate blot. No protein was detected when 5 mM unlabeled ATP was pre-incubated with parasite culture supernatant before addition 8-N₃-3'-biotinyl-ATP, suggesting that unlabeled ATP shared an identical binding site with 8-N₃-3'-biotinyl-ATP. Pre-incubation of 5 mM unlabeled ADP significantly reduced the intensity of the ~220 kDa band, suggesting that the presence of an interaction between PfRH1 and 8-N₃-3'-biotinyl-ATP and PfRH1 showed binding preference to ATP over ADP.

A

B

C

Figure 4.3. (on page 71) Binding of 8-N₃-3'-biotinyl-ATP to PfRH1 in photoaffinity labeling assay.

(A) Brief outline of the working procedures of photoaffinity labeling assay. The interaction between PfRH1 and 8-N₃-3'-biotinyl-ATP was analyzed in three different methods separately. Route 1 (red) shows the working steps of a standard photoaffinity labeling assay, which corresponds to the results in **(B)**. Route 2 (black) denotes procedures for Immunoprecipitation (IP) of PfRH1 after cross-linking with labeled-ATP. Mass Spectroscopy analysis (route 3, blue) that confirmed the ATP binding site in PfRH1 is highlighted in the shade of beige. No unlabeled ATP or ADP was pre-added for Mass Spectroscopy. **(B)** In the right panel, 8-N₃-3'-biotinyl-ATP was detected on proteins at ~220 kDa in the absence of unlabeled ATP and ADP. Addition of 5 mM ATP or ADP reduced the binding of 5 mM 8-N₃-3'-biotinyl-ATP at different degrees. 8-N₃-3'-biotinyl-ATP was completely undetectable when unlabeled ATP was added. Left panel shows the same sample loading probed by monoclonal antibody (C41) against PfRH1. **(C)** Western Blot analysis of PfRH1-IP elution. Equal amount of IP elution was loaded into each panel and probed separately by antibody against 8-N₃-3'-biotinyl-ATP and monoclonal antibody against PfRH1. 8-N₃-3'-biotinyl-ATP was detected from culture supernatant of T9/94 parasites which express PfRH1.

To further confirm the identity of the 8-N₃-3'-biotinyl-ATP bound protein as PfRH1, immunoprecipitation (IP) of PfRH1 and Mass spectroscopy analysis (MALDI-TOF) were performed after parasite culture supernatant was cross-linked with 8-N₃-3'-biotinyl-ATP (Figure 4.3A, route 2 and 3). Prior to ATP-crosslinking, T9/94 parasite culture supernatant was incubated with monoclonal antibody (C2) against the erythrocyte binding domain (EBD) of PfRH1 and ProteinA/G conjugated microbeads. After IP, PfRH1 was then eluted out using SDS-PAGE gel loading buffer and analyzed by Western Blot. As shown in Figure 4.3C, 8-N₃-3'-biotinyl-ATP bound PfRH1 was detected by either Streptavidin-Peroxidase or PfRH1 monoclonal antibodies on the same blot in the IP elution, strongly indicating the specific interaction between 8-N₃-3'-biotinyl-ATP and PfRH1. There was no protein and 8-N₃-3'-biotinyl-ATP detected in the IP elution of T9/94ΔRH1 parasite culture supernatant further confirming the specific interaction between the ATP and PfRH1.

The interaction of PfRH1 and ATP was further confirmed by Mass Spectroscopy of T9/94 parasite culture supernatant in the absence or presence of 8-N₃-3'-biotinyl-ATP followed by MALDI-TOF analysis. Table 4.1 shows the PfRH1 peptides that were detected by Mass Spectroscopy after in-gel tryptic digestions. In the presence of 8-N₃-3'-biotinyl-ATP, all peptides containing the sequence of ¹⁹²⁶KYYEEALFQK¹⁹³⁵ were observed with an additional mass of 1079 dalton, indicating the binding of 8-N₃-3'-biotinyl-ATP as a triethylammonium salt derivative as described in previous report (Schafer et al., 2001).

Table 4.1. Peptides of 8-N₃-3'-biotinyl-ATP-bound PfRH1 detected by MALDI-TOF

Measured mass (Da)	Peptide sequence	Starting residue ^b	Ending residue ^c
1620.4415	QELKQVQENVEKVK	10	20
920.8468	QVQENVEK	11	18
1590.3344	INDKTFEHIKTSK	77	90
5104.0741	NFMLDKSKKIDEEKKLDILKVNISNINNSLDKLKK YY EEALFQK ^a	197	241
6714.9811	NFMLDKSKKIDEEKKLDILKVNISNINNSLDKLKK YY EEALFQKVKEK ^a	197	245
2579.1630	KYY EEALFQK ^a	232	241
3680.4633	ALFQKVKEKAEIQKENIEKIKQEINTLSDVFK	237	268
3565.9949	KTLYHKSNVLHNQINFNEDAFINLLINIEK	NA	NA
805.7802	NEMEQLK	NA	NA
2875.7101	SNVLHNQINFNEDAFINLLINIEK	NA	NA
1380.7020	NINNILVLTNYK	NA	NA

^a Peptide labeled with 8-N₃-3'-biotinyl-ATP; ^b and ^c Position of the peptide in the rNBR, numbering includes 6X His-tag at the N-terminus; NA, not applicable where the peptide is not within the predicted nucleotide binding domain shown by *Ramalingam et al. (2008)*. The Tyrosine residues (Y) which was suggested to be the critical ATP binding residues were highlighted in red. They were mutated to Alanine in the mutant protein NBR-YA.

No additional mass was detected in the corresponding PfRH1 peptides from the sample in the absence of any 8-N₃-3'-biotinyl-ATP. Interestingly, the ATP binding-¹⁹²⁶KYYEEALFQK¹⁹³⁵ was found within the nucleotide binding domain (1461-2121 aa) predicted by the previous work (Ramalingam et al., 2008) (Figure 4.4A). The listed peptides from MALDI-TOF analysis were mapped to the full length PfRH1 sequences. A region of 259 amino acids (1704-1962 aa) was narrowed down and delineated as a putative nucleotide-binding region (PfNBR) PfRH1 (Figure 4.4B) based on several considerations: 1) The region overlapped with the sequence of the previously predicted nucleotide binding domain in PfRH1. 2) The region included the ATP binding-¹⁹²⁶KYYEEALFQK¹⁹³⁵ sequence. This region served as the basis for producing recombinant protein of putative nucleotide binding region for further biochemical characterization. To increase the likelihood of getting soluble and biologically active recombinant protein, the N- and C- boundaries of this region was carefully determined to include the domain of interest without disrupting the predicted secondary structure element of the protein (Figure 4.4A).

A

1461
 QKDKAAKYME NIDTYKNNIE IISKQINPEN YVETLNKSNM YSYVEKANDL FYKQINNIII NSNQLKNEAF
 TIDELQNIQK NRKNLLTKKQ QIIQYTNEIE NIFNEIKNIN NILVLTNYKS ILQDISQININ HVSITYTEQLH
 NLYIKLEEEK EQMKTLYHKS NVLHNQINFN EDAFINNLLI NIEKIKNDIT HIEKKTNIYM IDVNKSKNNA
 QLYFHNTLRG NEKIEYLKNL KNSTNQQITL QELKQVQENV EKVKDIYNQT IKYEEEIKKN YHIITDYENK
 INDILHNSFI KQINMESSNN KKQTKQIID INDKTFEEDI KTSKTKINML KEQSQMKHID KTLLEQALK
 LFVDINSTNN NLDNMLSEIN SIQNNIHTYI QEANKSFDKF KIICDQNVND LLNKLSLGDL NYMNLKLNQ
 NEIRNMNLEK NFMLDKSKKI DEEEKKLDIL KVNISNINNS LDKLKYYEE ALFOKVKEKA EIQKENIEKI
KQEINTLSDV FKPKFFFIQL NTDSSQHEKD INNNVETYKN NIDEIYNVFI QSYNLIQKYS SEIFSSTLNY
 IQTKEIKEKS IKEQNQLNQN EKEASVLLKN IKINETIKLF KQIKNERQND VHNKEDYNL LQQYLYNMKN
 EMEQLKKYKN DVHMDKNYVE NNGEKEKLL K
 2121

B

Figure 4.4. Location of the nucleotide-binding region (PfNBR) in PfrH1.

(A) The amino acid sequences of the predicted nucleotide binding domain in PfrH1 by Ramalingam et al., 2008. The position of the starting and ending residue in full length PfrH1 sequences is specified. 8-N₃-3'-biotinyl-ATP bound peptides detected by MALDI-TOF are highlighted in yellow and the shortest ATP binding peptide is underlined. PfNBR (red lined box) is further characterized in this work. Blue bars denotes predicted α -helical structures with a reliability index (0-9) >5. Dotted lines denotes region with no secondary structure predicted. (B) Pictorial representation of PfrH1 and Py235 and the location of the various functional and structural domains.

4.2.2 Expression of soluble recombinant Nucleotide Binding Region (rNBR) of PfRH1 using *E.coli*

Expressing malaria parasite (AT-rich genome) proteins in *E. coli* has always been challenging in various aspects of Malaria research. Even so, *E. coli* is still the preferred host for expression of recombinant malaria proteins due to various reasons such as cost, speed and ease of manipulation. PfNBR was expressed in *E. coli* using a modified pET 9d protein expression vector to place a 6X Histidine tag at the N-terminal of the recombinant protein, which has been shown to improve the protein yield by making the recombinant protein more resistant to intracellular proteolysis (Varshavsky, 1997; Waugh, 2005). Amplification of PfNBR from *P. falciparum* 3D7 genomic DNA was successful as shown by the 777 bp PCR product (Figure 4.5A). Cloning of the PfNBR into the expression vector was verified using restriction digestion enzymes, *NcoI* and *SacI*. Digested plasmid gave rise to two bands on an agarose gel which represented the pET9d expression vector backbone and the PfNBR insert (Figure 4.5B) and the insert was further verified by DNA sequencing. Another one of the greatest challenges of heterologous expression of malarial protein in *E. coli* system is the poor solubility of target protein (Flick et al., 2004; Birkholtz et al., 2008; Tsuboi et al., 2008). In most cases, the proteins expressed are improperly folded and accumulate as insoluble inclusion bodies in *E. coli*. Hence, the expression condition was further optimized in an induction assay to achieve optimum yield of soluble recombinant protein. Several factors such as IPTG concentration, temperature and duration of induction influence the solubility of the target protein (Tolia and Joshua-Tor, 2006). Lower temperatures and IPTG concentrations have been shown to increase protein solubility (Tolia and Joshua-Tor, 2006) because such conditions favor slower expression rates that might allow better folding of target protein. Three different expression conditions varying in temperatures and IPTG concentrations were tested (Figure 4.6A). Recombinant PfNBR protein (rNBR) was shown to be expressed under all conditions in the presence of IPTG, an inducer for protein expression. However, lower expression temperature (25°C and 16°C) with lower IPTG concentration (0.3 mM and 0.2 mM) significantly reduced the production of truncated rNBR, which was confirmed by Western blot analysis (Figure of 4.6B). To overcome the problem of low yield of rNBR due to a slower expression rate, longer incubation

time (up to 20 hours) was applied along with lower temperature and IPTG concentration. Basal expression of rNBR was detected even in the absence of the IPTG under all conditions (Figure 4.6B). It is a widely recognized problem of the *lac* promoter that was used for expression of rNBR. The *lac* promoter is commonly known to be leaky as transcription occurs even in the absence of inducer and the presence of repressor (EMBL, 2009-2014; Penumetcha et al., 2010). Leaky transcription of target protein may exert toxic effect to the host cell that leads to low or null expression of target protein. However, as shown in Figure 4.6B, rNBR expression was not compromised by leaky transcription before induction even at the optimum growth temperature of 37°C and the basal expression was greatly reduced by a slower expression rate. Taken altogether, it was clear that expression condition at 16°C with 0.2 mM IPTG minimized the basal expression of rNBR and formation of truncated rNBR without affecting the protein yield. Thus these parameters were chosen to express rNBR throughout this project.

Figure 4.5. Cloning of PfNBR into a protein expression vector.

(A) PCR product of PfNBR (777 bp) amplified from 3D7 genomic DNA. (B) Presence of the PfNBR insert in the expression vector, pET9d (4341 bp) was confirmed by plasmid digestion using restriction enzymes.

Besides optimizing the expression conditions, different lysis buffer conditions were tested to improve the solubility of rNBR. rNBR was detected only in the insoluble fraction in the buffers with pH 6.5 (Figure 4.7A, lane 4 and 5). An increased solubility of protein was observed in 50 mM Tris buffer with higher pH, starting from pH 8.0 (Figure 4.7A, lane 1 to 3) and rNBR was most soluble in the Tris buffer at pH 9.0. By taking this buffer (50 mM TrisHCl, 200 mM NaCl at pH 9.0) as reference, the effect of salt in solubilizing the protein was further investigated in Tris buffer at a pH range of 8.5 to 9.0 since protein solubility (vary from one protein to another) is strongly dependent on the ionic strength of the solvent. Increased ionic strength (not more than 1 M) was found to increase protein solubility in most cases (Whitford, 2005). The concentration of salt was therefore increased from 200 mM up to 500 mM (Figure 4.7B). Three hundred millimolar (300mM) of salt concentration in 50 mM Tris buffer, pH 9.0 was shown to be the best buffer composition for rNBR solubilization. However, buffer at a salt concentration of 500 mM was found to significantly reduce contaminants as well as maintaining the protein solubility in the purification process (Figure 4.8), which will be described in details in the coming section 4.2.3.1. Hence, 50 mM TrisHCl, 500 mM NaCl at pH 9.0 was chosen as the designated buffer for rNBR.

Figure 4.6. Expression of rNBR (32 kDa) under different conditions.

(A) Coomassie Blue Staining of the total lysate of the *E. coli* expressing rNBR. Different concentrations of IPTG was added to induce protein expression under different incubation temperatures. Black arrow denotes rNBR. (B) Same loading of the total lysate as in (A) was blotted and probed by anti-6XHis tag antibody. Truncated rNBR was expressed by *E. coli* at 37°C but the amount was greatly reduced at lower incubation temperature. All samples were standardized to OD_{600nm} 1.0 before added with sample loading dye. Equal volume of total lysate was loaded in all lanes.

Legends:

1. 50 mM Tris-HCl, 200mM NaCl pH 8.0
2. 50 mM Tris-HCl, 200mM NaCl pH 8.5
3. 50 mM Tris-HCl, 200mM NaCl pH 9.0
4. 50 mM MES-NaOH, 250mM NaCl pH 6.5
5. 50 mM MOPS-KOH, 250mM NaCl pH 6.5
6. 50 mM Tris-HCl, 300mM NaCl pH 8.5
7. 50 mM Tris-HCl, 300mM NaCl pH 9.0
8. 50 mM Tris-HCl, 500mM NaCl pH 8.5
9. 50 mM Tris-HCl, 500mM NaCl pH 9.0

Figure 4.7. Effect of different buffers in solubilizing rNBR.

Coomassie Blue Staining (upper panels) of the soluble and insoluble fractions of rNBR after sonication. Same loading of each fraction was confirmed by anti-6XHis tag monoclonal antibody in Western Blot (lower panels). Black arrowhead indicates rNBR at the correct size. **(A)** rNBR was completely insoluble in buffers at pH 6.5. Increased solubility was observed at higher pH. rNBR was most soluble in 300 mM NaCl, pH 9.0. **(B)** In 50 mM Tris buffer at pH 8.5 and pH 9.0, solubility of rNBR increased when salt concentration was increased from 200 mM to 300 mM. No further increase in protein solubility when 500 mM salt was used. S, supernatant; P, pellet.

4.2.3 Optimization of purification of rNBR

The 6X Histidine (His)-tagged rNBR was purified using a two-step purification procedure (Figure 4.11B). Immobilized-metal affinity chromatography (IMAC) was used as a primary step to separate the 6X His-tagged rNBR from *E. coli* proteins. Product from the first purification was then further purified using size exclusion chromatography (gel filtration), whereby non-specific contaminant proteins were separated from rNBR pool based on their differences from rNBR in molecular size. The whole process was optimized at different levels to ensure the end product was in high purity and quality for various biochemical assays.

4.2.3.1 *Increased salt concentration in buffer improves rNBR purity*

Based on the results of solubility assay as described in section 4.2.2, rNBR was initially purified in 50 mM Tris buffer with 300 mM NaCl. However, the two-step purification process was unable to remove contaminant proteins with high molecular weight ranging 72 kDa to over 130 kDa (Figure 4.8A). Size exclusion chromatography was not able to remove them despite the huge difference in molecular size between contaminant proteins and rNBR. This could be caused by the binding of nonspecific protein to the chromatography matrix in both purification steps through nonspecific ionic interaction. To overcome this problem, ionic strength of the selected buffer was further optimized by increasing the salt concentration to 500 mM. The adjustment successfully reduced most of the nonspecifically bound contaminant proteins with molecular weight higher than 72 kDa (Figure 4.8B). However, the increase in salt concentration did not prevent degradation of rNBR as degraded rNBR was co-purified with rNBR in the presence of 500 mM NaCl. Concentration of proteases inhibitors was increased in the process to decrease the degradation of rNBR.

Figure 4.8. Effect of salt concentration of purification buffer on rNBR purity.

Purified rNBR was stained with Coomassie Blue staining after a two-step purification process using 50 mM Tris buffers with different salt concentrations. **(A)** At 300 mM NaCl, rNBR (black arrowhead) was co-purified with other contaminant proteins or degraded rNBR (red arrowheads). **(B)** At 500 mM NaCl, contaminant proteins with higher molecular weight were significantly reduced. Degradation products of rNBR (≤ 28 kDa) were co-purified with rNBR in both cases.

Figure 4.9. Different metal affinity chromatography resins shows different binding specificity to rNBR.

Elution profile of rNBR (red arrowhead) from the primary purification step. Two different immobilized-metal affinity resins were used: **(A)** Ni-NTA (Nickel-charged) resins and **(B)** TALON (Cobalt-charged) metal resins. Less contaminant proteins were co-eluted with rNBR from 25 mM – 100 mM Imidazole when TALON metal resins **(B)** was used as compared to Ni-NTA resins **(A)**.

4.2.3.2 *Co²⁺-charged TALON resin shows higher rNBR-binding specificity than Ni-NTA resin*

The purification process was also optimized at the primary step by comparing two types of IMAC matrices, Ni-NTA resin and TALON Metal Affinity resin. The interaction between rNBR and the matrix is based on the high affinity binding of 6X His tag to the bivalent ions (Ni²⁺ for Ni-NTA or Co²⁺ for TALON) on the resins. Imidazole was used to elute bound rNBR as it displaced the His tag from metal ion co-ordination. In Ni-NTA purification, rNBR was eluted by Imidazole at the concentration range of 50 mM to 200 mM with the peak elution at 75 mM and 100 mM (Figure 4.9A). However, many other proteins were co-purified with rNBR in these elutions as well. On the other hand, cobalt charged-TALON resin showed a lower binding affinity but higher binding specificity to rNBR (Figure 4.9B). rNBR was eluted from TALON resin at Imidazole range of 25 mM to 125 mM. The peak elution occurred at 50 mM and 75 mM with improved purity. Most of the contaminant proteins that co-eluted with rNBR in Ni-NTA purification were not detected in TALON resin purification, as shown by the elutions at 75 mM and 100 mM Imidazole. TALON resins showed a higher binding specificity to rNBR because its Co²⁺-containing core offered a strict requirement for spatial positioning of Histidine residues, whereby only neighbouring Histidines with specific positioning were allowed for binding (Quinn et al., 2010). Other Histidine-rich proteins with Histidine residue located outside the 6X His tag were unable to bind to the Co²⁺-containing core.

4.2.3.3 *Lower injection concentration (< 5 mg/ml) of rNBR for gel filtration improves rNBR purity*

To increase the purity of rNBR, purified rNBR from IMAC was further purified using another method known as gel filtration. Gel filtration separates molecules according to their sizes as they pass through the porous matrix packed in a column. Molecules will diffuse in and out of the pores of matrix as they move through the column. Thus, bigger molecules leave the column first followed by smaller molecules because smaller molecules tend to move further into the pores, leading to delayed passage

through the column. Proteins leaving the column are detected by an automated system based on their UV absorbance at 280 nm, which will be translated into distinct peaks in a chromatogram (Figure 4.10). Each peak represents one population of proteins at a particular molecular size, allowing collection of eluted protein in fractions according to UV peaks. All fractions were then assessed by Coomassie Blue staining on denaturing SDS-PAGE gels to verify the size and purity of each fraction after purification.

To purify rNBR using gel filtration, IMAC elution fractions containing abundant rNBR were selected, combined and concentrated before loading onto the gel filtration column. Gel filtration chromatogram of rNBR consisted of two main peaks (Figure 4.10). The first peak (peak 1) appeared at retention volume of 9.52 ml followed by the second peak (peak 2) at retention volume of 11.54 ml. The resolution of the two peaks determined the purity of purified rNBR and is dependent on the rNBR concentration of the injection sample and the duration of purification (section 4.2.3.4). At injection concentration of 4-5 mg/ml, majority of rNBR were eluted as peak 2 as shown by Coomassie Blue staining of the elution fractions (Figure 4.10A). Only rNBR were detected in low abundance from peak 1 suggested that rNBR formed oligomers. On the other hand, injection sample with protein concentration above 5 mg/ml gave a different purification profile in gel filtration (Figure 4.10B), as shown by the poorly resolved shoulder peak 2 in fusion to peak 1. Peak 2 was less prominent and merged into peak 1. Increased amount of rNBR was detected in peak 1 in SDS-PAGE analysis, which was consistent with the increased UV absorbance of peak 1 in gel filtration. The result further strengthened the suggestion that rNBR forms oligomers at higher protein concentration. The high molecular weight contaminant proteins were confirmed to be aggregates of rNBR when rNBR sample was over-concentrated to above 10 mg/ml (4.10C) in a separate purification. This suggested that rNBR formed irreversible aggregates when the protein was over-concentrated to above 5 mg/ml. In these cases, gel filtration at the subsequent step was not able to separate rNBR monomer from its aggregates, leading to low yield of purified rNBR monomer.

Figure 4.10. Protein concentration of the injection sample affects purity of eluted rNBR from gel filtration.

Gel filtration chromatograms of rNBR purification with different injection sample concentrations. **(A)** At 4-5 mg/ml, most of the rNBR (black arrowhead) was eluted from peak 2. **(B)** At > 5 mg/ml, rNBR (black arrowhead) was co-eluted with increased amount of other contaminant proteins (red arrowheads) with higher molecular weight. The two populations of protein were not separable in elution as the two peaks were overlapped. Green and yellow panels shows the corresponding elution fractions as shown in the Coomassie Blue-stained gels (Peak 1 and Peak 2) on the right. Dotted blue lines denote extrapolated lines of each distinct peak. **(C)** Co-eluted proteins with higher molecular weight were confirmed as aggregates of rNBR (black arrowhead) by anti-His tag monoclonal antibody in Western Blot (left panel), in parallel with Coomassie Blue (CB) staining (right panel) when rNBR sample was over-concentrated to up to 10 mg/ml. Equal amount of sample was loaded on denaturing SDS-PAGE gel in both panels.

4.2.3.4 *Shorter duration of purification process improves rNBR purity*

The purity of purified rNBR was also greatly dependent on the peak resolution of the gel filtration chromatogram, whereby the purity increased when peak 1 and peak 2 were well separated. A well-separated peak 2 from peak 1 lead to increased homogeneity in rNBR monomer population. As described in the previous section, an asymmetric peak caused by fusion of two peaks (Figure 4.10B) reflected inhomogenous and partially aggregated or oligomerized rNBR in the sample and selective collection of these different populations from an asymmetric peak was not possible. The resolution of gel filtration chromatogram in rNBR purification was also influenced by the duration of the whole process of purification (Figure 4.11A), starting from cell lysate preparation to protein storage after two steps of purification. The shorter the duration of the process, the better the separation of rNBR monomer from aggregates, as shown by the increased resolution of the two peaks in the chromatogram (Figure 4.11A). More aggregation and degradation occurred when purification was completed after more than 5 hours. Aggregates and degraded rNBR were co-eluted with rNBR monomer in gel filtration, as clearly shown by the Coomassie Blue-stained fraction from peak 2 (rNBR monomer). Aggregation and degradation of rNBR were associated with the stability of the protein, which decreased over time. To shorten the purification duration, several changes were made to the purification procedures as shown in Figure 4.11B. Among all steps, protein concentration before gel filtration was the critical step that often determined the total hours required for rNBR purification. This step was influenced by the starting concentration of rNBR. High starting concentration promotes irreversible precipitation or aggregation of the protein on the filtration membrane during the process, which in turn increased the concentration duration to achieve to the required sample volume for gel filtration injection.

Taken altogether, it was clear that rNBR purification required a tightly controlled working procedure in order to obtain the protein in high purity. The best procedure to purify rNBR was by applying immobilized metal affinity chromatography using cobalt-charged resins before gel filtration (Figure 4.12) and the whole process must be completed within 5-5.5 hours. Concentration of the protein sample loaded onto gel

filtration was maintained at a concentration lower than 5 mg/ml. All these parameters were carefully maintained to achieve consistency in every purification.

Figure 4.11. Duration of the purification process influences purity of eluted rNBR from gel filtration.

Purification efficacy of gel filtration increased in a shorter duration of the two-step purification. (A) Peak 1 and peak 2 were better separated when purification was completed within 5.5 hours, leading to increased purity of eluted rNBR. (B) Brief outline of working procedures of rNBR purification. Left panel represents the procedures that took more than 7 hours to complete. Right panel shows revised procedures in order to shorten the purification duration. Changes are highlighted in red.

Figure 4.12. An optimized two-step purification process for rNBR purification.

(A) Elution profile of rNBR (black arrowhead) of immobilized metal affinity chromatography. Asterisks denotes elution fractions that were combined and pooled to be concentrated for the next step. (B) Chromatogram of rNBR purification by gel filtration. rNBR monomers in high purity were eluted from peak 2 which is highlighted in yellow. (C) Coomassie Blue staining of all elution fractions from (B). (D) Quality of the purified rNBR that was used in all protein functional and characterization assays was confirmed by Coomassie Blue staining (CB) and Western Blot. Protein was thawed from storage at -80°C and concentrated to the required working concentration for Circular Dichroism (2 mg/ml, lane 1) and Fluorescence Correlation Spectroscopy (10 mg/ml, lane 2). Same sample as lane 2 was probed with anti-His tag monoclonal antibody (α -His) in Western Blot. rNBR purity was maintained at above 95% in both cases as determined by gel analysis using ImageJ (Appendix E).

4.2.4 rNBR binds to ATP with a lower K_d value as determined by Fluorescence Correlation Spectroscopy (FCS) (done in conjunction with Dr. Cornelia Hunke)

Native PfRH1 showed a preferential binding to ATP over ADP in a photoaffinity labeling assay (Section 4.2.1) whereby preincubation of unlabeled ADP partially abolished the binding of biotinylated-ATP. The difference in ATP/ADP-binding of PfRH1 was further examined using the recombinant form of PfNBR (rNBR) in FCS. FCS is a correlation analysis of the fluctuation of the detected fluorescence intensity (Appendix 3). This technique is coupled with a confocal microscope allowing fluorescently labeled biomolecules diffusing in and out of a focal volume to be detected. FCS makes use of the information extracted from the fluctuating fluorescence signals of the molecule to obtain quantitative information like diffusion coefficients, average concentration, kinetics reaction rate, protein-protein interactions, etc (Krichevsky and Bonnet, 2002).

The characteristic mean diffusion time, τ_D of the fluorescence-labeled (ATTO 647N labeled) nucleotide was first determined and the changes of its diffusion time after the addition of the rNBR were observed. Here in this work, binding of two nucleotides, ATP ATTO-647N and ADP ATTO-647N were investigated. The τ_D of ATP ATTO-647N and ADP ATTO-647N were $83.4 \pm 4.4 \mu s$ and $84.3 \pm 3.3 \mu s$ respectively. The addition of purified rNBR to the nucleotide resulted in a significant change in the mean diffusion time of ATP ATTO-647N whereby the τ_D value increased with increasing concentrations of rNBR, as illustrated by the autocorrelation curves of the ATP ATTO-647N in binding with rNBR (Figure 4.13A). An identical trend was shown in the case of ADP ATTO-647N (Figure 4.13B) in which the mean diffusion time of the nucleotide increases in the presence of rNBR. An increased diffusion time of the labeled nucleotide was due to an elevation in molecular weight of the labeled nucleotide caused by the binding of nucleotide to rNBR.

The percentage of bound nucleotides was analyzed by FCS using a two-component binding scheme, $[A] + [B] \leftrightarrow [AB]$. The binding constant K_d of the nucleotide to the protein was calculated from the best-fitted curve (Figure 4.13C). A higher K_d value,

$103.6 \pm 3.7 \mu\text{M}$ was calculated in ADP ATTO-647N binding. rNBR showed a higher affinity and stronger binding to ATP ATTO-647N shown by lower K_d value of $90.1 \pm 3.9 \mu\text{M}$. This showed that a lower concentration of rNBR was required to achieve 50% of nucleotide binding. ATP binding to rNBR did not require Mg^{2+} as similar binding trend (as shown in figure 4.13C) was observed in FCS in the absence of Mg^{2+} .

Figure 4.13. Fluorescence Correlation Spectroscopy of Fluorescence-labeled Nucleotides and rNBR.

(A) The normalized autocorrelation functions of ATP ATTO-647N in the absence and presence of increasing concentration of rNBR. (B) The normalized autocorrelation functions of ADP ATTO-647N in the absence and presence of increasing concentration of rNBR. Black arrows denote the shift of diffusion time to a longer diffusion time along with the addition of increasing concentration of rNBR. (C) Concentration-dependent binding of nucleotides to rNBR. Empty squares represent ATP ATTO-647N and empty triangles represent ADP ATTO-647N.

4.2.5 rNBR is rich in alpha-helical secondary structure and forms coiled-coils

The secondary structure of rNBR was determined by far UV-Circular Dichroism (CD) spectroscopy. Far UV measurement (below 240 nm) allows estimation of protein secondary structure due principally to absorption by the peptide bonds (Kelly et al., 2005). Based on the far UV spectrum, rNBR was shown to be predominantly alpha-helical with signature double minima points of the molar ellipticity at 208 nm and 222 nm in (Figure 4.14A). The result was consistent with the secondary structure prediction of PfNBR (Figure 4.14B) by a bioinformatics online tool, PredictProtein (Rost et al., 2004). The CD data was further analyzed using an online algorithm, *k2d* (Andrade et al., 1993) to estimate the percentage of protein secondary structure of rNBR. The *k2d* result of 81% of alpha helical content was consistent with the calculated percentage (79%) using the formula derived by Greenfield and Fasman (Greenfield and Fasman, 1969). The ratio $(\theta)_{222\text{ nm}}/(\theta)_{208\text{ nm}}$ is often used as an indicator for the presence of stable alpha-helical coiled coils whereby a value ≥ 1.0 indicates coiled-coils formation and a value of ≤ 0.86 indicates isolated helices (Lau et al., 1984; Zhou et al., 1992). The ratio $(\theta)_{222\text{ nm}}/(\theta)_{208\text{ nm}}$ of rNBR was 1.02, suggesting the protein forms a coiled-coils structure. By using Bioinformatic tools COILS/PCOILS, (Lupas et al., 1991), rNBR was analysed and again predicted with coiled-coils with high probability (probability score 0.950 to 1.0) between residues 103 to 145 and between residues 171 to 257 (Figure 4.15). Leu and Ile were predominantly detected at the *a* and *d* positions in the two 'heptad' repeat motif predicted in rNBR (Appendix B). Coiled-coils structure is commonly associated with oligomerization of protein (either homo- or hetero-oligomer) due to its unique arrangement of amino acid residues. The presence of coiled-coils structure in rNBR further supported the evidence of the formation of rNBR oligomer based on the size exclusion chromatography result earlier.

rNBR contained uneven distribution of amino acids with excessive Lys (15.3%), Asn (13.8%) and Ile (11.9%), very similar to the overall amino acids distribution in full length PfRH1 (Asn, 14.2%; Lys, 13.9%; Ile, 13.4%) based on ProtParam computation (Gasteiger et al., 2005). However, rNBR differs from the Erythrocyte binding domain

(EBD) of PfRH1 whereby the EBD contained large excess of Ile (16.2%), Lys (14.7%), Gln (12.3%) and Leu (10.2%) residues (Gao et al., 2008), suggesting a dissimilar structure and functions of these two domains of PfRH1.

A

B

Figure 4.14. Far UV Circular Dichroism Spectra of rNBR.

(A) rNBR is rich in an alpha-helical secondary structure based on the signature double minima points at 208 nm and 222 nm. (B) Secondary structure prediction by PredictProtein. SUB_sec is a subset of the prediction for secondary structure prediction with a reliability $n \geq 5$, which corresponds to an expected accuracy $> 82\%$. AA, amino acids; H, helix; L, loop; ".": means that no prediction is made for this residue, as the reliability index is < 5 . The percentage of α -helical structure was predicted to be $\sim 98\%$ in PredictProtein.

Figure 4.15. Coiled-coils prediction in rNBR.

Two regions in rNBR were predicted to form coiled-coils structure by COILS/PCOILS. Prediction using a 28 residues window with weighted matrix was shown. Y-axis indicates obtained probabilities in prediction.

4.2.6 No significant secondary structural changes in rNBR upon nucleotide binding

It is of great interest to assess the changes in secondary structure of PfNBR upon nucleotide binding. The 94kDa nucleotide binding domain (NBD94) of Py235 experienced significant changes in secondary structure with a decreased alpha helical content upon binding of nucleotide. The structural changes triggered by ATP binding has been proposed to expose the erythrocyte binding domain (EBD) of the protein which facilitates the host cell binding during parasite invasion (Ramalingam et al., 2008; Gruber et al., 2010; Basak et al., 2011). However, unlike NBD94, rNBR did not undergo changes in secondary structure composition upon binding with ATP or ADP based on the CD spectrum (Figure 4.16). Analyses of CD measurement by Dichroweb (Whitmore and Wallace, 2008) showed a similar result, further supporting the absence of changes in PfNBR secondary structure after nucleotide binding (Appendix C & D).

Figure 4.16. Secondary structure of rNBR in binding to nucleotides.

Secondary structure of rNBR remained unchanged in binding to different types of Adenosine nucleotides.

4.2.7 PfNBR shows no ATP hydrolysis activity

ATP/ADP binding is a fundamental feature of many proteins performing their biological functions by gaining energy from ATP hydrolysis such as membrane transporters and motor proteins. Although rNBR binds to ATP with a higher affinity than ADP, it did not hydrolyze ATP in a colorimetric based ATPase assay at any working protein concentration ranging from 0.25 μM to 10 μM (Figure 4.17A). In this assay, absorbance at 590 nm is in linear relationship with the amount of free phosphate (P_i) produced from ATP hydrolysis whereby the higher amount of P_i released from ATP hydrolysis, the higher value of absorbance at 590 nm (Appendix 6). rNBR were supplied with 2.5 mM MgCl_2 and 10 mM ATP for effective ATP hydrolysis. Free phosphates (P_i) produced from the hydrolysis reaction, ($\text{ATP} \rightarrow \text{ADP} + \text{P}_i$) can be detected with an Orange dye forming a P_i -dye complex which is green in color and the intensity of the color was determined by measuring the absorbance at 590 – 660 nm. rNBR, as a monomer did not hydrolyze ATP even at concentration up

A **B** **Figure 4.17. ATP hydrolysis activity undetected in rNBR.**

(A) rNBR did not hydrolyze ATP even at a high concentration of 10 μM and in longer assay time of 25 minutes. Apyrase, a positive control, hydrolyzed ATP at a concentration as low as 0.05 μM within 5 minutes. The absorbance signal for the concentrations higher than 0.20 μM was beyond the detection limit of the reader due to signal saturation. **(B)** Native PfRH1 or PfRH1 protein complex from IP did not hydrolyse ATP in a 5-minute ATPase assay in the presence of Mg^{2+} . ATP hydrolysis activity for both IP product of T9/94 and T9/94 ΔRH1 supernatant were similar. Apyrase hydrolyze ATP in the presence of agarose resins effectively at a concentration as low as 0.01 μM .

to 10 μM . Increase in total assay time from 5 minutes to 25 minutes showed similar results. rNBR used in the ATPase assay was purified in 50 mM Tris buffer with 10 mM EDTA, a Mg^{2+} chelator to reduce protein proteolysis as described in section 2.5 (Chapter 2). As Mg^{2+} ion is essential for many catalytic of ATPases as it forms a correct substrate (Mg-ATP) complex for the enzymes (Pasternak et al., 2010), rNBR purified in 50 mM Tris buffer without EDTA was also tested under the same condition to ensure that the the negative results was not due to the chelation of Mg^{2+} by EDTA. rNBR showed no ATP hydrolysis in the absence of EDTA as well.

Catalytic activity of ATP by some ATPases involves multiple structural subdomains within the enzyme (Hanson and Whiteheart, 2005) whereby nucleotide binding and nucleotide hydrolysis are carried out by different subdomains. In addition, many ATPases function as homo- or hetero-oligomers whereby interconnections between several nucleotide binding domains is necessary for ATP hydrolysis (Wendler et al., 2012). To further assess the ATPase activity of PfRH1, immunoprecipitation (IP) of native PfRH1 and/or PfRH1-protein complexes from *in vitro* T9/94 parasite culture supernatant was performed. Monoclonal antibody against the Erythrocyte binding domain at the N-terminal part of PfRH1 (C50) was crosslinked to Protein A/G conjugated-agarose resins before incubated with parasite culture supernatant of parasite strain T9/94 and T9/94ΔRH1. To preserve the native structure of the native PfRH1 or PfRH1 protein complex, IP product was not eluted from the resins using low pH elution buffer or denaturing protein sample loading dye following the standard IP procedures. Native PfRH1 attached to C50 conjugated-agarose resin was tested directly in an ATPase assay in the presence of Mg^{2+} . As observed in figure 4.17B, native PfRH1 or PfRH1 protein complex from IP-T9/94 showed a similar result as IP product of T9/94ΔRH1 which contains no PfRH1, evidenced that native PfRH1 or PfRH1 protein complex does not hydrolyse ATP. Apyrase hydrolysed ATP in the presence of equal volume of resins, showing that agarose resins did not affect any ATPase activity in this assay. To confirm the IP efficiency, a separate set of IP was conducted with both T9/94 and T9/94ΔRH1 parasite culture supernatant following the same procedure and IP product was eluted with low pH buffer for Western Blot analysis (Figure 4.27A. Section 4.2.13). PfNBR was detected in the IP elution by monoclonal antibody (N205) raised against this region. No PfRH1 was detected in T9/94ΔRH1 culture supernatant and IP elution, verifying the specificity of the IP in this assay.

4.2.8 Generation of a mutant rNBR, NBR-YYAA with point mutation on Tyrosine residues (done in conjunction with Lim Hwee Min)

In a photoaffinity labeling assay, 8-N₃-3'-biotinyl-ATP was shown to bind to a short peptide of PfRH1, ¹⁹²⁶KYYEEALFQK¹⁹³⁵ (Table 4.1) in which the double Tyrosine

residues might be the critical ATP binding residues. The postulation was made based on two recent findings. First, based on the NMR structure analysis of the critical ATP binding peptide of Py235 (Basak et al., 2011), it was proposed that Tyrosine 493 within the NBD is the important ATP binding residue. Secondly, conserved aromatic residue (commonly Tyrosine) has been known to directly interact with ATP in many ATP-binding cassette (ABC) transporters (Ambudkar et al., 2006). In this work, the double Tyrosine residues of PfRH1 (at the position of 1927 and 1928) were mutated to two alanine residues to investigate their importance in ATP binding. Taking the rNBR-expressing plasmid (4341 bp) as the template, site-directed mutagenesis was done in a PCR reaction from which linearized daughter plasmids were produced (Figure 4.18). Restriction digestions and DNA sequencing were performed to select plasmids with the correct point mutations.

The expression and purification condition for the mutant protein, NBR-YYAA remained the same as for rNBR since they were different from each other by only two amino acid residues. Based on a series of assessments including induction assay and solubility assay, these same conditions were effective in producing NBR-YYAA in high-purity as well (Figure 4.19). NBR-YYAA was expressed at 16°C for 20-22 hours, with 0.2 mM IPTG to yield comparable quantity to wild type rNBR and only trace amount of truncated NBR-YYAA was expressed under such condition. 50 mM TrisHCl, 500 mM NaCl pH 9.0 was used throughout the entire purification process of NBR-YYAA. Likewise, the elution profile of NBR-YYAA in cobalt charged-resin affinity chromatography and gel filtration were very similar to rNBR (Figure 4.20).

Figure 4.18. Site-directed mutagenesis of NBR.

(A) The linearized daughter plasmid amplified from the parental plasmids (4341 bp) after a site-directed mutagenesis. (B) The mutant plasmid was confirmed to contain the insert of interest (777 bp) after restriction digestion. The double point mutations in the insert was then verified by DNA sequencing,

Figure 4.19. Induction assay and solubility assay of NBR-YYAA.

(A). Coomassie Blue Staining and Western Blot analysis of the total lysate of NBR-YYAA under the expression temperature of 16°C with 0.2 mM IPTG. (B). Solubility of NBR-YYAA in 50 mM TrisHCl, 500 mM NaCl, pH 9.0.

Figure 4.20. Two-step purification process to produce NBR-YYAA in high purity.

(A) Coomassie Blue Staining of the elution profile in Co^{2+} -resins affinity chromatography purification. Arsteriks indicate two fractions that were pooled and concentrated to be further purified by size exclusion chromatography. (B) The chromatogram of NBR-YYAA purification by size exclusion chromatography using a Superdex75 10/300 GL column. High-purity NBR-YYAA was eluted with retention volume at 11.5 ml. The precise area was highlighted in yellow. (C) Coomassie Blue staining of the purified NBR-YYAA.

4.2.9 Point mutation on double Tyrosine in PfNBR does not affect protein binding affinity to ATP (done in conjunction with Saw Wanyu)

The nucleotide binding ability of the mutant protein, NBR-YYAA was investigated using FCS in parallel with the wild type rNBR under the same experimental condition as described earlier in Section 4.24. Similar to rNBR (Figure 4.13A), addition of the purified NBR-YYAA increased the mean diffusion time of the ATP ATTO-647N, indicating an interaction between the two molecules (Figure 4.21A). The mutation of two putative ATP-binding Tyrosine residues did not compromise the ATP binding to the protein as represented in the percentage of bound nucleotides to the two proteins (Figure 4.21B). NBR-YYAA showed a very similar ATP binding affinity with wild type rNBR, as demonstrated by two nearly overlapping curves. The K_d value of the

wild type rNBR and its mutant was determined to be 72 μM and 68 μM respectively. In this FCS, the K_d value obtained for the wild type rNBR was different from the one obtained in the previous FCS in section 4.2.4. It was likely due to the variation in different batches of protein samples or ATP ATTO-647N used in these two separately conducted experiments. The result indicates that these two Tyrosine residues are unlikely to form the crucial molecular interaction to ATP during binding. Other residues in PfNBR may be involved in ATP binding including Lysine (K), Isoleucine (I), Leucine (L) and Glutamic acid (E). Lysine was shown to form the critical interaction with the phosphate moieties of ATP in other nucleotide binding proteins such as actin and myosin (Holmes et al., 1993; Cope et al., 1996) while Isoleucine, Leucine and Glutamic acid have been found to replace aromatic residues in nucleotide binding in many other ATP-binding proteins (Gaudet and Wiley, 2001; Mao et al., 2004).

Figure 4.21. Fluorescence Correlation Spectroscopy of Fluorescence-labeled Nucleotides and NBR-YYAA.

(A) The normalized autocorrelation functions of ATP ATTO-647N in the absence and presence of increasing concentration of NBR-YYAA containing double point mutation (YY $\rightarrow$ AA) in the sequence. Black arrows denote the shift of diffusion time to a longer diffusion time along with the addition of increasing concentration of rNBR. Black arrows denote the shift of diffusion time to a longer diffusion time along with the addition of increasing concentration of rNBR. (B) Comparison of rNBR and NBR-YYAA in ATP binding. NBR-YYAA binds to ATP with a similar affinity. Black line and black triangles represent NBR-YYAA. Red line and empty circles represent rNBR.

4.2.10 Generation and purification of monoclonal antibodies (mAbs) against different regions of PfRH1

PfRHs are subjected to a series of processing steps besides interacting with other partner proteins throughout the invasion process (Triglia et al., 2009; Chen et al., 2011; Gunalan et al., 2011). However, the importance and connections between these events remain an unresolved gap in our current understanding of the invasion process. The identification of a novel PfNBR in this work suggests that PfRH1 may have a complex function with at least two functional domains. To understand the biological relevance of the nucleotide binding region, as well as its working relationship with the N-terminus Erythrocyte binding domain (EBD) throughout the different stages of the invasion process, mAbs against PfNBR and an additional region, RVIII (Gao et al., 2008) (Figure 4.23A) were generated in this work. RVIII at the C-terminal end of the ectodomain of PfRH1 was chosen because polyclonal antibodies against this region were previously reported to show no effect on merozoite invasion (Gao et al., 2008), thus making it a good site for protein labeling without affecting the function of PfRH1. Hence, together with the available monoclonal antibodies against PfRH1-EBD, different regions throughout PfRH1 from N- to C-terminus could be specifically labeled for imaging, immunoprecipitation of different fragments and other purposes.

Figure 4.22. Purified RVIII for rat injection.

Lane 1, Coomassie Blue staining of the protein. Lane 2, RVIII probed with anti 6XHis tag monoclonal antibody. Lane 3, RVIII probed with anti-RVIII polyclonal antibody. Black arrowhead denotes full length RVIII, (40kDa).

Figure 4.23. Generation of monoclonal antibodies (mAbs) against PfnBR and RVIII.

(A) Schematic representation of the monoclonal antibodies (black) generated in this work against different regions of PfRH1. Monoclonal antibodies generated in previous work that were used in this work are highlighted in red. (B) Work flow of the complete process of mAb generation. Black arrows represent NBR mAbs. Red arrows represent RVIII mAbs. Asterisk denotes the part of work done in conjunction with Dr. Andrea Ravasio. (C) Coomassie Blue staining of the six purified IgGs against PfnBR and RVIII on the denatured SDS-PAGE gel. The heavy chains and light chains are shown by the 55 kDa bands and the 25 kDa bands respectively.

In this work, antibodies against PfNBR and RVIII were raised in rats. Unlike rNBR as described in section 4.2.3, purified recombinant RVIII prepared for immunization was not homogenous. Proteins with a lower molecular weight than the full length recombinant RVIII (~40 kDa) were unable to be removed by various purification methods including gel filtration and ion exchange chromatography (Figure 4.22). Nonetheless, it was used for rat immunization since the contaminants were proven as truncated RVIII fragments by Western Blot. They were recognized by both anti-His tag mAbs and polyclonal antibody against RVIII (Gao et al., 2008) with the same pattern. Screening of hybridoma clones after the completion of rat immunization was done following a series of ordered steps (Figure 4.23B) to obtain mAbs against PfNBR and RVIII. A total of five NBR monoclonal antibodies in the form of Immunoglobulin G (IgG) were successfully purified (Appendix F-J) as shown by the two bands on the denatured SDS-PAGE gel, representing the heavy chain (50 kDa) and the light chain (25 kDa) (Figure 4.23C). For RVIII mAbs, IgG from clone R8201 was obtained and purified in this work (Appendix K). Culture supernatant containing IgG of the rest of the 4 clones (R8202 to R8205) were harvested and stored. They have yet to be purified and validated in this work.

4.2.11 Validation of monoclonal antibodies (mAbs) against PfNBR and RVIII by indirect immunofluorescence assay (IFA)

The specificity of the mAbs against PfRH1 was assessed in an immunofluorescence assay whereby parasite strain T9/94, a laboratory strain which expresses and utilizes PfRH1 in host cell invasion (Triglia et al., 2005) was used. Late-stage schizonts and free merozoites after egress were smeared and fixed for immunostaining. PfRH1 of each individual merozoite in schizont stage was labeled by two different mAbs, C41 (Gao et al., 2013) targeting the EBD and mAbs targeting PfNBR or RVIII (Figure 4.23A). All mAbs generated in this work specifically targeted PfRH1 except for N206 and N207, as clearly demonstrated by the positive IFA signal on T9/94 parasites and the negative IFA signals on T9/94 Δ RH1 parasites that do not express any PfRH1 (Figure 4.24 and 4.25). N206 and N207 did not show any signal in all IFA attempts in this work. IFA on free merozoites showed consistent results whereby

Figure 4.24. Immunofluorescence staining of T9/94 and T9/94ΔRH1 schizonts using generated mAbs against PfNBR and PfRH1-RVIII.

Late-schizonts were smeared and probed with different mAbs. Parasite nuclei were stained with Hoechst 33342 (blue); PfRH1-EBD was probed with mAb C41 (red); PfNBR and RVIII were probed with five NBR mAbs and mAb R8201 respectively (green). Yellow colour in the merge panel was resulted from the overlapping signals from both green and red signal. White bar denotes a scale bar of 5 μ m.

Figure 4.25. Immunofluorescence staining of T9/94 and T9/94ΔRH1 free merozoites using generated mAbs against PfNBR and PfRH1-RVIII.

Free merozoites were smeared and probed with different mAbs. Parasite nuclei were stained with Hoechst 33342 (blue); MSP-1 was probed with rabbit antiserum against full length MSP-1 (red); PfNBR and RVIII were probed by monoclonal antibodies generated in this work (green), which showed a small cap-like pattern near the apical end of merozoites. White bar denotes a scale bar of 5 μ m.

positive signals were only detected in T9/94 parasites. The signals were observed to be elongated dots in irregular shape, resembling a cap-like pattern over the conoid apical end of merozoites (Figure 4.25). This cap-like staining pattern is in line with the immunostaining of PfRH1 in previous reports (Rayner et al., 2001; Triglia et al., 2009). Signals from mAbs against PfNR and RVIII varies from a complete cap-like structure to a two-dot staining near the apical end, which was likely due to the different orientations of merozoites fixed on the focal plane. Rabbit antiserum against Merozoite surface protein-1 (MSP-1) (Holder and Freeman, 1984) stained the circumference of individual free merozoites for both T9/94 and T9/94 Δ RH1 parasites since it is the major merozoite surface antigen expressed by both parasite strains.

4.2.12 Validation of monoclonal antibodies (mAbs) against PfNBR and RVIII by Western blot

It has been previously demonstrated that full length PfRH1 is cleaved into two fragments with sizes of ~240 kDa and ~120 kDa. The N-terminal-240 kDa fragment is further processed into a ~140 kDa fragment while the C-terminal-120 kDa fragment is further processed into a ~110 kDa fragment (Triglia et al., 2009; Gao et al., 2013). All three processed forms of PfRH1 are released into the culture supernatant during invasion (Figure 4.26A). The specificity of the generated mAbs against PfRH1 was further validated in a Western Blot by using the parasite culture supernatant. In figure 4.26B, N205, N208 and N209 recognized PfRH1 from the T9/94 parasite culture supernatant whereby two dominant fragments from the N-terminus were detected, similar to the recognition of PfRH1 by C41, a monoclonal antibody against PfRH1-EBD (Gao et al., 2013). In this Western blot validation, the relative migration distance of these two dominant fragments (from the blots of N205, N208, N209 and C41) in reference to the migration distance of protein standards was plotted into a graph. The molecular weight of these fragments was then determined to be approximately 220 kDa and 170 kDa respectively. The observed difference between the estimated fragments size in this work and the estimated size in previous reports was likely due to the variation in the percentage of SDS-PAGE gel used in Western blot. Monoclonal antibodies against PfNBR recognized two additional low-abundance fragments of

PfRH1 at the sizes of ~210 kDa and ~150 kDa which were not detected by C41. These fragments could be the degraded products from the ~220 kDa and ~170 kDa fragments or additional processed fragments. No PfRH1 product of any expected size was seen in the culture supernatant of the RH1 knock out strains (T9/94ΔRH1) (Triglia et al., 2005). Purified IgG of N206 and N207 did not recognize PfRH1 which was different from the validation results in the screening process earlier using hybridoma culture supernatant. This could be due to the compromised quality of the IgGs caused by the low pH elution buffer (at pH 2.7) in the purification process.

Monoclonal antibody R8201 against RVIII specifically recognized three fragments in the parasite culture supernatant (Figure 4.26B). The ~110 kDa fragment represented one of the cleaved products of the C-terminal part of PfRH1 which has been previously reported by Triglia and co-workers (Triglia et al., 2009). The low abundant ~135 kDa fragment could be the degraded product of the dominant ~170 kDa fragment. As a potential C-terminal fragment encompassing the PfNBR and RVIII would yield a molecular weight of approximately 130kDa, detection of fragments with size of >110 kDa by R8201 suggests the possibility of co-presence of PfNBR and RVIII region in one processed fragment.

4.2.13 Co-presence of PfNBR with EBD in processed fragments of PfRH1

In a proposed model, the processed fragments of PfRH1 (Figure 4.26A) are anchored to the merozoite surface through a binding partner protein containing a transmembrane domain (Triglia et al., 2009). All processed fragments are eventually released to the surrounding during merozoites invasion. As shown in previous result section, mAbs against PfNBR detected two dominant processed fragments (~220 kDa and ~170 kDa) in parasite culture supernatant (Figure 4.26B). Similar fragments were also recognized by mAbs against EBD in schizont extract, merozoite extract and culture supernatant (Gao et al., 2013). To confirm that PfNBR and EBD were co-present in these two fragments, monoclonal antibody C50 against EBD was used to immunoprecipitate the released fragments from culture supernatant. Two compatible

Figure 4.26. Western Blot validation of mAbs against PfNBR and RVIII.

(A) Summary of the processing events of PfrH1 during invasion as reported by Triglia *et al.*, 2009 and Gao *et al.*, 2013. Red crosses denote the proposed cleavage sites. The size of the fragments (white bars) was determined to be 220 kDa and 170 kDa respectively in this work. (B) N205, N208 and N209 specifically recognized two dominant fragments of PfrH1 in the parasite culture supernatant, a ~220 kDa fragment and a ~170 kDa fragment, similar to the PfrH1 recognition by C41 against the EBD. Two additional fragments in low abundance (~210 kDa and ~150 kDa, red arrowheads) were detected only by N205, N208 and N209, which were possibly the degraded products of the dominant fragments. NBR206 and NBR207 showed no signal in any Western Blot validation. R8201 specifically recognized two dominant fragments (~110 kDa and ~170 kDa, black arrowheads) and ~135 kDa fragment in low abundance (red arrowhead) in the culture supernatant. EBA-175 was used as a loading control as it was expressed in both T9/94 and T9/94ΔRH1 parasites. This validation was done in conjunction with Loh Han Ping.

bands were detected by monoclonal antibody N205 in the IP elution, with the size of ~220 kDa and ~170 kDa (Figure 4.27A). The processing of PfRH1 into these two fragments occurred at the schizont stage before their release from the merozoite to the ambient media during invasion (Triglia et al., 2009; Gao et al., 2013). Hence, detection of both domains in these processed fragments suggests that EBD and PfNBR are likely to function closely during merozoite invasion. More importantly, the IP result here also confirms that one of the pre-determined cleavage sites of PfRH1 should be located upstream of EBD, instead of downstream of EBD as proposed by Triglia and co-workers earlier (Figure 4.27B).

Figure 4.27. Co-presence of EBD and PfNBR in one processed fragment of PfRH1.

(A) Immunoprecipitation of the processed fragment of PfRH1 from the parasite culture supernatant. mAb C50 recognized two fragments in the size of ~220 kDa and ~170 kDa before IP (left panel). Fragments in the same size were detected in the IP elution (E) by mAb N205 (red arrowheads, right panel). Sup, supernatant; FT, IP flow through. (B) Simplified pictorial representation of the released processed fragments of PfRH1 during invasion. Detection of a ~110 kDa fragment by R8201 supported the locations of the two cleavage sites (red crosses) proposed by Triglia and colleagues (Triglia et al., 2009). Red arrow denotes a possible location of the third cleavage site based on the finding in this work.

4.2.14 Monoclonal antibodies against PfNBR do not inhibit parasite invasion *in vitro*

Two mAbs against the EBD of PfRH1, C41 and C49 have been reported to exhibit invasion inhibitory effect by blocking the calcium signaling cascade which is essential to trigger the subsequent invasion steps (Gao et al., 2013). To further investigate the biological importance of PfNBR, the effect of N205/8/9 on parasite invasion was investigated using T9/94 parasites and its PfRH1 knock out strain (T9/94 Δ RH1) in an *in vitro* invasion system. T9/94 parasites express and utilize PfRH1 for invasion in a sialic-acid dependent pathway (Triglia et al., 2005). Purified schizonts were incubated with uninfected erythrocytes in a microtitre plate in which merozoites released after schizont egress were allowed to invade host cells in the presence of increasing IgG concentration or medium alone. After approximately 24-30 hours post invasion, all samples were incubated with SYBR® Green which stained parasite nucleic acids. SYBR® Green positive erythrocytes represented newly infected cells at ring to early trophozoite stage, which was a direct indicator of the occurrence of merozoite invasion. Quantitative data from FACS measurement showed that all three antibodies (N205, N208 and N209) exhibited no inhibition on T9/94 merozoite invasion even at a concentration up to 500 μ g/ml (Figure 4.28). No differences in changes in parasitemia were observed between T9/94 and T9/94 Δ RH1 except for the case of N208. Addition of N208 IgG increased T9/94 merozoite invasion, showing ~20 % increase of parasitemia relative to medium alone. However, such enhancement in parasitemia was not statistically different from the parasitemia changes (%) shown in the case of T9/94 Δ RH1 parasites. Monoclonal antibody C41 inhibited parasite invasion in a dose-dependent manner whereby addition of 500 μ g/ml of purified IgG inhibited invasion up to 40%. This result was consistent with the previous report testing the inhibitory effect of C41 on parasite invasion (Gao et al., 2013).

Multiple merozoite invasions into a single erythrocyte is a common phenomenon in malaria infection including *P. falciparum* infection (Simpson et al., 1999). Despite the inevitable competition for nutrients or other resources in the host cell, parasites seem to have a preferred target to invade through an unknown selection mechanism. Preference of a parasite for differential cell subpopulation can be measured by the

Figure 4.28. Effect of PfNBR mAbs on merozoite invasion.

The parasite invasion efficiency of T9/94 and T9/94ΔRH1 parasites are compared. The Y-axis represent changes in parasitemia (%) caused by different mAbs as compared to culture medium alone. mAbs against NBR did not inhibit merozoite invasion. Addition of N208 enhanced parasitemia by ~20% for T9/94 as compared to T9/94ΔRH1 parasites but the difference was not statistically significant. The bars denote mean (SD) and biological replicates were shown, $n = 3$ for all mAbs. * $p = 0.0061$, + $p = 0.0179$, # $p = 0.0238$ and ^ $p = 0.0013$ denote the inhibition of C41 on T9/94 merozoite invasion was statistically significant as compared to T9/94ΔRH1 based on unpaired t-tests.

multiplicity of infection (Bei and Duraisingh, 2014). Preferential invasion to particular erythrocyte has commonly been associated with the age class of erythrocytes (Mons, 1990; Antia et al., 2008). Reticulocytes and matured erythrocytes are different from each other in various aspects, such as surface antigen and cytoplasmic content including ATP level (Mohandas and Groner, 1989;

Ataullakhanov and Vitvitsky, 2002; Mohandas and An, 2012). To date, the molecular basis of the differences that drives preferential invasion remains unclear. Although the generated PfNBR mAbs had no effect on invasion in terms of parasitemia (%), it is worthwhile to investigate its impact on host cell selection. This was done using the same system used to study invasion in which all samples from the assay were smeared and examined under light microscope about 24 hours post invasion. Single invasion and multiple invasions (≥ 2 invaded parasites in one host cell) in the presence of mAbs or medium alone were counted and quantified (Figure 4.29). Deviation of the frequency distribution of single or multiple invasions in the presence of mAbs from the expected frequency distribution (invasion in the absence of mAbs) would imply the occurrence of preferential invasion. The percentage of both infected cell populations showed insignificant differences among each other in the presence of antibodies. Cells with multiple invasions for all cases were maintained in the range of 20-30% out of the total infected cells. The invasion preference in the control sample, T9/94 Δ RH1 remained similar, indicating the dispensable effect of PfNBR mAbs in parasite host cell selection as well as invasion efficiency.

4.2.15 Exposure of PfNBR on merozoite only in contact to erythrocyte

To further understand the biological relevance of PfNBR during invasion, merozoite surface staining of PfRH1 was performed by using mAbs against different functional domains of the protein. 4% paraformaldehyde (PFA) was used in parasite fixation, unlike the previously described IFA (Figure 4.25) in which the organic solvent fixative used removes lipid (membrane permeabilization) while precipitating antigen on the cellular architecture during fixation. PFA at low percentage (4%), on the other hand, immobilized antigen by forming cross-links between proteins while rendering cells in a static condition that was as close to its *in vivo* state as possible (Renshaw, 2007). In Figure 4.30, EBD of PfRH1 was detected on the surface of the free merozoite by mAb C41. However, PfNBR and RVIII were not exposed on the free merozoite surface, as shown by negative fluorescence signals from the validated mAbs against these two regions. A separate IFA was conducted by applying detergent

A **B**

Figure 4.29. Effect of PfNBR mAbs on host cell preference.

(A) Mixed infected cell populations were observed in the *in vitro* invasion assay in the presence of PfNBR mAbs. Parasite culture was smeared and stained with Giemsa stains. Left panel, single merozoite invasion into single erythrocyte. Right panel, multiple invasion (double invasion) in a single erythrocyte. Black arrows denote parasites at ring stage. Red bar denotes a scale bar of 10 μM. (B) Proportion (%) of single invasion and multiple invasions into a single host cell in the presence of mAbs. The bars denote mean (SD) and biological replicates were shown, n = 3. Frequency distribution of the single or multiple invasions remained unchanged in the presence of mAbs against PfNBR.

Figure 4.30. Immunofluorescence surface staining PfRH1 of T9/94 free merozoites.

Free merozoites were smeared and fixed with 4% PFA. Parasite nuclei were stained with Hoechst 33342 (blue); MSP-1 was probed with rabbit antiserum against full length MSP-1 (red); PfRH1-NBR and PfRH1-RVIII were probed by monoclonal antibodies generated in this work (green). Only EBD was exposed on the surface of free merozoite after egress, as shown by a small cap-like staining pattern near the apical end of merozoites. White bar denotes a scale bar of 5 μ m.

(0.1% Triton-X 100) after PFA fixation to permeable cell membrane. All three domains of PfRH1 were detected by designated mAbs after permeabilization.

Screening of merozoites at different invasion phases after schizont egress was performed next. To do this, purified schizonts were mixed with erythrocytes and allowed to egress at 37°C. The culture was smeared and fixed with 4% PFA only for surface staining of merozoite proteins. Merozoites at different invasion phases were determined and categorized into four groups based on their MSP-1 surface coat staining and their contact to host cell. Newly released merozoites (group 1) were identified as the population that showed a complete MSP-1 staining at the circumference of individual merozoite and near to or surrounded hemazoin crystals (Figure 4.31, panel A). Free merozoites devoid of host cell contact (group 2) were recognized by intact MSP-1 coat and nuclei staining. They are isolated from the purified schizont culture that was allowed to egress in the absence of fresh erythrocyte (Figure 4.31, panel B). Merozoites in primary reversible attachment to erythrocyte (group 3) were recognized with intact MSP-1 surface coat staining and random merozoite orientation in contact to erythrocyte based on the position of nucleus (Figure 4.29, panel C). Committed and invading merozoites (group 4) were identified by incomplete or partial MSP-1 surface coat staining and positioning of the apical end of merozoite towards the host cell (Figure 4.29, panel D). Merozoites with surface staining for either EBD or PfNBR were examined and counted. Positive staining signal was shown by irregular, connecting dots forming a cap-like pattern over the apical end, which was described in previous IFA result in Figure 4.25. EBD was exposed on the merozoite surface throughout the early stages of invasion (70-80% of all merozoites counted), from group 1 to group 3 (Figure 4.29E). However, PfNBR was not exposed on the newly released and free merozoites, only 5.08% and 7.29% of merozoites from the respective group were found with a positive fluorescence signal. Positive signal was only detected on those merozoites in close contact to host cell (group 2) whereby 43.54% of merozoites were detected with positive PfNBR staining (Figure 4.29E), suggesting that unknown factor from host cell triggered the exposure of PfNBR during invasion. Early and continual surface exposure of EBD on merozoites was likely associated with its function for host cell receptor binding during invasion, which is likely to occur before the function of PfNBR. Host cell receptor binding by EBD could serve as a trigger to expose PfNBR for ATP detection

Figure 4.31. (on page 114) Surface exposure of PfRH1-EBD and PfNBR on merozoites at different stages of invasion.

(Panel A-D) Surface staining of the EBD (green) and PfNBR (green) by mAb C41 and N205 respectively. Parasite nuclei were stained with Hoechst 33342 (blue); MSP-1 was probed with rabbit antiserum against full length MSP-1 (red). White bar denotes scale bar of 10 μ m. **(A)** Newly released merozoites surrounded the hemozoin crystal. **(B)** Free merozoites. **(C)** Merozoites in reversible contact to the red blood cell (RBC), merozoites in random orientations were spotted. Middle panel shows a deformed RBC before merozoite reorients its apical end towards the RBC. White boxes at the left bottom corner show the enlarged merozoite in contact to the RBC. **(D)** Committed and invading merozoites at the later stage of the invasion process. MSP-1 surface coat was shed partially during the active entry of merozoite into RBC. **(E)** Percentage of the EBD- and PfNBR-exposed merozoite population at different (early) stages of invasion. Bar denotes mean (SD); n = 4 for PfNBR and n = 3 for PfRH1-EBD. *** denotes $p < 0.0001$ based on One-way ANOVA comparing 3 groups of PfNBR-positive merozoites. Colored # denotes significant difference between % of EBD- and PfNBR-exposed merozoites at each stage based on three independent unpaired t-test.

via protein conformational changes. This could make sense as local concentration of ATP released from deforming erythrocyte was detected only near the cell membrane (Beigi et al., 1999; Joseph et al., 2003). The controlled and restricted exposure of PfNBR supported the “just-in-time” model of parasite ligand exposure proposed earlier (Adams et al., 1990; VanBuskirk et al., 2004). In this model, invasive merozoites are believed to release or expose invasion ligand “just-in-time” to reduce their exposure to immune system.

4.2.16 Host cell contact instead of extracellular ATP triggers surface exposure of PfNBR and Ca^{2+} signaling in free merozoites

Erythrocyte experiences waves of deformation during the reversible attachment of merozoite during invasion. It resumes to its original disc-like shape when a merozoite positions its apical end at the cell surface and is ready to invade (Gilson and Crabb, 2009; Yahata et al., 2012). Erythrocyte releases ATP upon deformation and PfNBR was shown to be exposed only when merozoites are in close contact to erythrocyte. To further dissect the working sequence of the two functional domains of PfRH1 (EBD and PfNBR) during invasion, the surface exposure of both domains was examined under the effect of exogenous ATP in the absence of erythrocytes. Surface exposure of EBD was not affected by increasing concentration of ATP, indicating that

merozoite were ready for host cell binding right after egress in order to complete the invasion process efficiently in a very short time frame. No increase of PfNBR exposure on the merozoite surface was seen due to ATP, demonstrating again that the contact to the host cell was a pre-requisite for its exposure.

Figure 4.32. Differential surface exposure of PfRH1-EBD and PfNBR on free merozoites under the effect of exogenous ATP alone.

Percentage of the EBD- and PfNBR-exposed merozoite population under different concentrations of ATP in the absence of the host cell. Exogenous ATP up to 10 μM did not increase the number of PfNBR-exposed merozoite. Presence of the EBD on merozoite surface did not affected by the ATP as well. Bar denotes mean(SD); n = 3 for all groups. * $p = 0.0123$; ** $p = 0.031$ and 0.020 for 5μM and 10 μM ATP respectively.

Calcium is an important second messenger in cell signaling of eukaryotic cell including *Plasmodium* to support various cellular functions (Berridge et al., 2003) (Wasserman et al., 1982; Lovett and Sibley, 2003). Increase in cytosolic Ca^{2+} level has been shown to be important in host cell invasion of *P. falciparum* and *T. gondii* to trigger the release of micronemal invasion protein such as EBA-175 (Carruthers and Sibley, 1999; Singh et al., 2010; Gao et al., 2013). Recently it has been suggested that activation of purinergic receptors on the parasite surface is important to trigger Ca^{2+} signaling for invasion (Levano-Garcia et al., 2010). On the other hand, by using a high-throughput fluorescence scanning approach, antibodies against PfRH1 have been demonstrated to inhibit the rise in cytosolic Ca^{2+} level in merozoites, leading to

failure of invasion (Gao et al., 2013). A strong correlation between Ca^{2+} signaling and merozoite invasion has been shown by this approach.

Here, the effect of extracellular ATP on changes of merozoite Ca^{2+} level was investigated using the same approach. Free merozoites (strain T9/94) were isolated freshly from *in vitro* culture and preloaded with Ca^{2+} binding fluorescent indicator, Fluo-4 AM. Changes in Ca^{2+} level in merozoites were monitored in real-time as indicated by the detected fluorescence intensity. Cumulative increase of Ca^{2+} signal was observed in merozoites that were resuspended in ATP free-complete culture medium within the first 10 minutes in contact to erythrocytes, which was consistent with the previous report (Figure 4.31A) (Gao et al., 2013). In previous report, it has been shown that human erythrocyte releases intracellular ATP upon deformation and the released ATP concentration has been determined to be up to 5 μM (Sprague et al., 1998a). For merozoites supplemented with exogenous ATP in various concentrations (0 to 10 μM), there was no increase in cytosolic Ca^{2+} level in a 10 minute-measurement. The decrease of the fluorescence signal throughout the measurement in all conditions with ATP were consistent with the sample media containing Fluo-4 AM indicator alone. This could be due to photobleaching of the unbound Fluo-4 AM.

After the first measurement, merozoites supplemented with or without exogenous ATP were added with fresh erythrocytes and subjected to an immediate second measurement. In the presence of erythrocytes, ATP supplemented-merozoites showed an increase in Ca^{2+} level (Figure 4.31B), as what was seen in merozoites that were resuspended in complete culture medium in the first 10 minute-measurement (Figure 4.31A). The increase in Ca^{2+} level from in the second measurement was not dependent on ATP concentration, demonstrating the redundancy of exogenous ATP for invasion in the presence of the host cell. The data showed that extracellular ATP alone was unable to trigger Ca^{2+} signaling in merozoites, indicating that activation of purinergic receptors on merozoite surface is unlikely to be important for invasion as previously suggested by Levano-Garcia and co-workers (Levano-Garcia et al., 2010).

EBD was exposed on the free merozoite surface before parasite made contact to host cell. Antibody against EBD was previously shown to inhibit Ca^{2+} signaling in

merozoites (Gao et al., 2013). However, addition of exogenous ATP alone triggers neither intracellular Ca^{2+} level nor the surface exposure of PfNBR. Addition of the host cell triggered both events. Taken together, it strongly suggests that EBD function before PfNBR during invasion.

Figure 4.33. Effect of exogenous ATP on merozoites cytosolic Ca^{2+} level.

(A) Cytosolic Ca^{2+} level of the freshly isolated merozoites in the presence of erythrocytes or exogenous ATP alone in various concentrations. Exogenous ATP (up to 10 μM) did not trigger Ca^{2+} signals. Merozoites Ca^{2+} level remained unchanged under the effect of ATP alone. (B) Cytosolic Ca^{2+} level measurement of merozoites from (A) after the addition of fresh erythrocytes. Merozoites in all conditions (0-10 μM ATP) showed an increase in Ca^{2+} level in 600 s. All merozoites were preloaded with Fluo-4 AM and resuspended in ATP free-complete culture medium before the addition of erythrocytes or exogenous ATP. Experimental data were presented as the mean (SD); $n = 3$.

4.3 Discussions

RHs have been proposed to be involved in parasite host cell selection since the discovery and characterization of Py235 and PvRBPs (Galinski et al., 1992; Barnwell and Galinski, 1998). Extensive work on RHs in *P. falciparum* has revealed specific ligand-receptor interaction between RH members and the erythrocyte surface receptors that determines the parasite invasion pathway (Gaur et al., 2004; Tham et al., 2012). The requirement of specific erythrocyte receptors allows parasites to select suitable host cells but at the same time not limiting the repertoire of potential host cells as different strains of parasites express different combination of ligands (Iyer et al., 2007). By utilizing different RH members, parasites are able to invade a wider range of erythrocytes through a phenomenon known as invasion pathway switching (Stubbs et al., 2005). Pathway switching is observed in parasite strains that are able to switch between sialic acid-dependent and –independent invasion pathways by the differential expression of RH members. W2mef parasites utilize PfRH1 to invade erythrocyte through sialic acid-dependent pathway. Under the selection of neuraminidase-treated erythrocytes where the sialic acid-containing receptors are removed, W2mef parasites are able to switch to sialic acid-independent invasion pathway through upregulation of gene expression of *PfRH4* (Stubbs et al., 2005). In addition, genetic disruption of *PfRH1* in T9/94 parasites also leads to an increased invasion into neuraminidase-treated erythrocytes as compared to the wild type parasites (Triglia et al., 2005).

Recently, intracellular ATP of the host cell has been suggested as a potential selection criterion by parasites based on several findings. Erythrocyte ATP is essential for parasite invasion because merozoites fail to invade ATP depleted-erythrocyte (known as resealed erythrocyte ghost) but addition of external ATP restores merozoite invasion (Olson and Kilejian, 1982; Dluzewski et al., 1983a). Secondly, time-lapse imaging of merozoite invasion of different *Plasmodium* species clearly showed that erythrocyte experiences waves of deformation during the initial phases of invasion in contact with the reorientating merozoite (Gilson and Crabb, 2009; Yahata et al., 2012). Erythrocyte releases intracellular ATP upon mechanical deformation as part of

the purinergic signaling system under physiological condition (Wan et al., 2011). The recent discovery of a 94 kDa ATP/ADP binding domain (NBD94) in Py235 suggests that the Py235 as well as RHs act as an ATP sensor which could detect the gradient of the released ATP when merozoite is in close contact with erythrocyte (Ramalingam et al., 2008). In *P. yoelii*, ATP binding of NBD94 activates Py235 binding to the erythrocyte (Gruber et al., 2011). In the work here, we show that PfRH1 binds ATP and a corresponding PfNBR has been identified. Various assays including biochemical and biophysical tests have been performed to understand the biological role of this domain during invasion.

4.3.1 PfNBR: Structure and function

4.3.1.1 *Comparison of PfNBR to PyNBD94 and other ATP-binding proteins*

4.3.1.1.1 *The nucleotide binding site*

Until this work, there was no clear evidence of ATP binding to any PfRHs, the protein homologue of Py235 in *P. falciparum* except a prediction of a similar nucleotide binding domain (NBD) (Ramalingam et al., 2008). Here for the first time, a new functional domain which interacts with adenosine nucleotides was identified in PfRH1. Different analyses provided strong evidences of ATP binding to PfRH1 via a NBD, known as PfNBR without the need of a phosphate-binding loop. The phosphate-binding loop, commonly known as P-loop or Walker A motif is commonly found in adenosine nucleotide-binding proteins and best known for its function in ATP or GTP binding (Walker et al., 1982; Saraste et al., 1990; Kumar et al., 2010). Walker B motif is crucial in nucleotide binding and ATP hydrolysis by coordinating the β and γ phosphates of ATP and the magnesium ion (Walker et al., 1982).

Walker A motif is conserved in many ATP/GTP binding proteins like α - and β -subunits of ATP synthases, *ras* proteins, myosin head piece and various kinases including adenylate kinases. However, it is not an essentiality as there are proteins

known to bind ATP without using the P-loop such as glycolytic kinases, E1/E2-type ATPases, actin, tubulin and aminoacyl-tRNA synthetases (Saraste et al., 1990). Submission of the primary sequences of full length PfRH1 or PfNBR to various bioinformatic tools has shown no detection of any Walker A motif or AAA+ related domain which is present in NBD94 of Py235 (data not shown). However, it should be noted that the actual ATP binding site in NBD94 does not correspond to the Walker A and Walker B motif identified in Py235 (Ramalingam et al., 2008) (Appendix K).

Since a Glycine-rich Walker A is not required by PfRH1 in ATP binding, a different type of nucleotide binding pocket would be expected to facilitate nucleotide binding. Conserved aromatic amino acids residues (especially Tyrosine) outside of the Walker A and Walker B motif have been found to be involved in ATP binding in NBD of ATP binding cassette (ABC) proteins based on X-ray crystallography data (Hung et al., 1998; Mao et al., 2004). The Tyrosine residue represent a conserved subdomain, known as the A loop which is the integral part of many ABC protein in ATP adenine ring recognition (Ambudkar et al., 2006). The contact between the conserved Tyrosine and ATP is through a π - π interaction in which the aromatic residue stacks against one side of the adenine ring. It is reasonable to assume that the nucleotide binding site of PfNBR could be confined to the Tyrosine residues in the designated ATP binding peptide “KYYEEALFQK” (Table 4.1) as Tyrosine has been proposed to be the ATP-binding residue in Py235 (Basak et al., 2011). However, a double point mutation on Tyrosine residues does not abolish ATP binding in PfNBR based on the FCS results. The result shows that Tyrosine residues are not essential in ATP binding as in the cases of most of the ABC proteins. Other residues in PfNBR may be involved in ATP binding as different nucleotide binding protein interacts with nucleotide base in a different manner. In other nucleotide binding proteins such as actin and myosin, lysine forms the critical interaction with the phosphate moieties of ATP (Holmes et al., 1993; Cope et al., 1996). Isoleucine (I), Leucine (L) and Glutamic acid (E) have also been found to replace aromatic residues for nucleotide binding in other ATP-binding proteins (Gaudet and Wiley, 2001; Mao et al., 2004).

Besides the absence of a P-loop, recombinant PfNBR (rNBR) is different from most of the ATP binding protein as Mg^{2+} ion is found unnecessary in nucleotide binding. Metal ions such as Mg^{2+} are essential cofactor in catalyzing ATP hydrolysis and

synthesis by many enzymes (Williams, 2000). rNBR in buffer containing 5-10 mM EDTA (a Mg^{2+} chelator that is commonly used as a protease inhibitor in protein purification) was still able to bind to ATP and ADP with a binding constant, K_d in the μM range. This is consistent with the absence of Walker B motif in PfNBR as Walker B motif is essential for Mg^{2+} coordination which is required for ATP hydrolysis in AAA+ (ATPases associated with various cellular activities) proteins (Hanson and Whiteheart, 2005). This observation is supported by the ATPase assay result whereby PfNBR is not involved in ATP hydrolysis in either monomeric (rNBR) or possible homo-oligomer form (protein complex in culture supernatant). The finding is similar to NBD94 as it does not hydrolyze ATP as well (Ramalingam, 2010).

4.3.1.1.2 Secondary structure and structural architecture

NBD94 has a direct impact on the protein function whereby host cell binding of full length Py235 is compromised by the addition of the ADP, a binding site competitor (Ramalingam et al., 2008). Further dissection of NBD94 identified three important subdomains: 1) N-terminal NBD94₁₋₁₉₄ as the Erythrocyte binding domain (EBD), 2) a minimal NBD (NBD₄₈₃₋₅₉₂ retaining ATP binding capacity and 3) a C-terminal hinge-like segment (Gruber et al., 2010; Gruber et al., 2011). Data obtained from low resolution structural analyses (Small-Angle X-ray Scattering, SAXS) on EBD and NBD of Py235 provided some insights and suggests a model on the structural interplay between the two functional domains in order to fulfill both the sensing and binding function of Py235 during invasion (Figure 4.34) (Gruber et al., 2012). ATP binding to the NBD induces conformational changes which induce movement of the hinge-like structure leading to the rearrangement of NBD94, including exposure and dimerization of the neighboring EBD for host cell binding (Gruber et al., 2012).

Figure adapted and modified from Gruber et al., 2012

Figure 4.34. A proposed model of Py235 binding of *P. yoelii* to host cell.
The detailed structural arrangement of NBD and EBD are shown.

The three important subdomains are in close proximity to each other within the full length NBD94 (Figure 4.34) and such arrangement would permits a direct signal transduction from NBD to EBD via structural alterations (Gruber et al., 2012) which have been observed within the full length recombinant protein NBD94. Currently, we have little knowledge on the structural interaction between EBD and PfNBR within PfRH1 because neither a high resolution crystallographic analysis nor low resolution SAXS examination of PfRH1 has been done to date due to the large size of the native protein (>200 kDa) and its low abundance in *in vitro* culture. Circular Dichroism (CD) spectroscopy of rNBR shows that the secondary structure of PfNBR undergoes insignificant changes upon nucleotide binding, unlike NBD94 which shows a decrease in alpha helical content after binding (Ramalingam et al., 2008). Nevertheless, we could reasonably assume that any structural rearrangement during the interplay between EBD and PfNBR likely involve sequences between the two domains, as supported by the evidence where surface exposure of PfNBR occurs only in contact with erythrocyte. Thus it is not surprising to observe unnoticeable structural alterations within rNBR alone. More importantly, ligand binding to PfNBR may not

necessary trigger similar structural changes as observed in Py235, but induces other events such as protein processing of PfRH1 during invasion.

4.3.1.2 *Coiled-coils structure and oligomerization of rNBR*

PfNBR forms a stable coiled-coils structure based on the COILS/PCOILS prediction and CD measurement. Coiled coils are bundles of α -helices that wind around each other to form a “supercoil” structure, most commonly consisted of two, three or four helices (Lupas and Gruber, 2005). They are important for protein complex assembly or oligomerization in many cases (Lupas and Gruber, 2005). Coiled-coils protein complexes have been reported to be dynamic structures that are important for pathogen-host interaction in viral infection, for example, the gp 41 of HIV virus (Garg and Blumenthal, 2008) and the F protein of parainfluenza virus 5 (SV5)(Yin et al., 2006).

Based on the size exclusion chromatography, rNBR forms an oligomer since it is the only protein eluted in the two consecutive peaks as seen in the denaturing SDS-PAGE gel. Hence, the oligomerization of rNBR is likely promoted by the coiled-coils structure within this region, similar to EBD which has been reported to form an oligomer as well (Gao et al., 2008). Co-presence of PfNBR and EBD in the processed fragments of PfRH1 implies the possibility of homo-oligomerization of PfRH1 on the merozoite surface during invasion. The oligomerization (dimerization) associated with the function of invasion proteins have been demonstrated in the cases of *Plasmodium spp.* (Withers-Martinez et al., 2008; Batchelor et al., 2011b) and *T. gondii* (Cerede et al., 2002; Jewett and Sibley, 2004). The monomeric host cell binding domain (RII) of Duffy binding protein of *P. vivax* (PvDBP) has been shown to dimerize upon receptor binding (Batchelor et al., 2011b). This receptor induced-ligand dimerization has been proposed to transduce a signal to initiate host cell invasion. Region VI of PvDBP also shares sequence similarity with Region VI of EBA-175 that forms a homodimer in its crystal structure (Withers-Martinez et al., 2008; Batchelor et al., 2011b).

4.3.2 PfRH1 in invasion: binding, sensing and signaling

RHs have been suggested to play an important role in the preinvasive phase of invasion which involves host cell selection and attachment (Barnwell and Galinski, 1998). Little is known about PfRH1 as compared to other RH members of *P. falciparum* as research has focused more on PfRH4 and PfRH5 (Tham et al., 2012). PfRH1 has been reported to be present at the apical end of merozoite and to mediate erythrocyte binding in the invasion process. Sialic acids moieties from the host receptor are required for successful binding which eventually leads to sialic-acid dependent merozoite invasion (Triglia et al., 2001a; Triglia et al., 2005). A short region (334 amino acids) near the N-terminus has been identified as the erythrocyte-binding domain (Gao et al., 2008) whereas the host receptor remains currently unknown. Antibodies against this domain inhibit parasite invasion by preventing intracellular Ca^{2+} signaling that triggers secretion of EBA-175 for tight junction formation (Gao et al., 2013), indicating that the host cell binding property of PfRH1 could be associated with signaling transduction during invasion. Understanding the function of PfRH1 in invasion is made complicated by multiple proteolytic processing steps at different time point during invasion. Two processed fragments of the extracellular domain of PfRH1 (220 kDa and 170 kDa fragments) are thought to be associated with unknown binding partner protein that are anchored to the parasite surface (Triglia et al., 2009). In this work, the identification of a novel ATP-binding domain indicates an additional functional role of PfRH1 in parasite invasion. The interplay between the two functional domains and possible working mechanism of PfRH1 during invasion are discussed in the following sections.

4.3.2.1 *PfNBR as an ATP-binding domain in PfRH1*

Merozoite invasion is a fast and efficient process which takes less than a minute to be completed (Gilson and Crabb, 2009). A possible reason for such a short invasion window is that merozoites need to minimize their exposure to the host immune

defense system for survival. Being able to detect or sense a suitable host cell beforehand would be a great advantage for parasite survival and propagation. Although the list of the determinants of a suitable host cell are yet to be completely identified, host cell receptors and deformability have been shown to be two important factors (Dvorak et al., 1975; Tham et al., 2012). Host receptors are for host-parasite interaction whereas erythrocyte deformability driven by major membrane/cytoskeleton rearrangement is essential for parasite entry. ATP is important in maintaining erythrocyte deformability and shape change, possibly through phosphorylation of erythrocyte cytoskeleton protein (Jinbu et al., 1984b). Erythrocyte releases intracellular ATP due to various stimuli including mechanical deformation (Wan et al., 2011) whereby reversible contact of merozoites elicit deformation of the erythrocyte during invasion (Gilson and Crabb, 2009; Yahata et al., 2012). The erythrocyte resumes its original shape as merozoite position its apical end towards the host cell and invade.

With the identification of PfNBR in this work, a novel nucleotide binding property is now observed in one of the RHs in *P. falciparum*. This further supports the earlier suggestion of the presence of a conserved ATP sensing function in RH members across species (Ramalingam et al., 2008). The biochemical analyses of PfNBR indicate that PfRH1 is a non-ATPases, unconventional ATP-binding protein that is likely to form an oligomer. PfNBR of PfRH1 could be an ATP-sensing domain during invasion although this is not confirmed to date. In most cells including erythrocytes, the local concentration of extracellular ATP near the cell membrane has been determined to be in low micromolar (μM) range and such a low concentration is sufficient to trigger functional changes (Beigi et al., 1999; Joseph et al., 2003; Sprague et al., 2007; Corriden and Insel, 2010). The low near-membrane environmental ATP of a deforming erythrocyte creates an ATP gradient for paracrine and autocrine signaling purposes. The surface exposed-PfNBR of merozoite that is in close contact to erythrocyte could serve as an ATP-sensing domain to determine a suitable host cell. The controlled and restricted exposure of PfNBR is in line with the “just-in-time release” model proposed earlier (Adams et al., 1990; VanBuskirk et al., 2004). Contact to host cell provides signal to trigger exposure of PfNBR, making the detection of the local ATP concentration possible. On the other hand, early exposure of the erythrocyte binding domain (EBD) on the newly released merozoites coincides

with its function to bind to host cell receptor. Its prolonged exposure also allowed recognition by the host immune system, as shown by the development of highly potent inhibitory monoclonal antibodies against this domain (Gao et al., 2013).

4.3.2.2 *A possible working mechanism of PfRH1*

With the identification of PfNBR in this work, at least two functional domains have now been identified in PfRH1 but the actual order and working relationship of receptor binding, ATP binding and signaling of PfRH1 in invasion remains to be determined. Nevertheless, further dissections and speculations on the working sequence relating the three known functions of PfRH1 are possible with the available evidences. Recently, the available technique to isolate viable merozoites has allowed the assessment of changes in merozoite cytosolic Ca^{2+} level during invasion (Boyle et al., 2010; Singh et al., 2010; Gao et al., 2013). In the presence of erythrocytes, increase of Ca^{2+} in merozoites triggers the downstream events of the invasion cascade. mAbs against EBD inhibit the increase of Ca^{2+} level and leads to the failure of invasion (Gao et al., 2013). However, the mAbs that inhibits Ca^{2+} signaling and merozoite invasion exhibit no effect on PfRH1 protein binding to host cell based on the erythrocyte binding assay, strongly suggests that receptor binding occurs before the signaling event. Epitope mapping of the inhibitory mAbs (in Chapter 4) shows that they target a short linear amino acids sequence which lies within a predicted coiled-coil region. Blocking of Ca^{2+} signaling could be caused by the disruption of homo-multimerization of PfRH1 which is required to transmit the extracellular triggers to the intracellular environment, as commonly seen in signal transduction pathway (Klemm et al., 1998). Such possibility is supported by receptor binding–induced protein dimerization that has been observed in host cell binding domain of EBL members in *Plasmodium spp.* (Withers-Martinez et al., 2008; Batchelor et al., 2011b).

In previous work, depletion of ATP in culture supernatant reduced Py235 binding to erythrocyte and addition of exogenous ATP restores the binding, suggesting that the ATP binding acts before host receptor binding in Py235 (Ramalingam et al., 2008).

However, for PfRH1, addition of exogenous ATP alone does not affect or trigger 1) Ca^{2+} signaling in free merozoites in the absence of erythrocytes and 2) surface exposure of both EBD and PfNBR. These observations indicate that additional factor is required to elicit the signaling process during invasion, possibly the binding of PfRH1 to host receptor. Surface exposure of PfNBR occurs only after merozoite attachment to the erythrocyte implies that PfNBR functions after EBD during invasion. This proposal is supported by the observation that binding of PfRH1 in the culture supernatant to erythrocyte was not affected by the absence or presence of ATP (Gao XiaoHong, personal communication). In addition, merozoites have been observed to attach to resealed erythrocyte ghosts lacking intracellular ATP but then rapidly detach from them (Miller, 1977).

EBD binding to host cell receptor is likely to induce protein conformational changes, possibly through homodimerization that in turn trigger the exposure of PfNBR for ATP detection. Co-presence of EBD and PfNBR in the same processed fragment of PfRH1 allows a direct signal transmission via alteration in protein structure. Up to this stage, it is unclear what are the subsequent events that is triggered by the ATP binding to PfNBR. Nonetheless, a few speculations could be made for the possible function of ATP binding to PfNBR during invasion: 1) to activate (PfRH1) protein complexes or oligomer disassembly as generally seen in the cases of AAA+ ATPases (Hanson and Whiteheart, 2005) or 2) to trigger merozoite Ca^{2+} signaling or 3) to trigger subsequent processing of PfRH1 that eventually leads to the release of the fragments.

Figure 4.35. A model of biological role of PfRH1 in host cell selection and host-parasite interaction.

(1) Primary reversible contact of merozoite to erythrocyte elicits erythrocyte deformation. MSP-1 and AMA-1 on the merozoite surface may facilitate the primary contact. (2) ATP released from the deformed erythrocyte forms an ATP gradient near the cell membrane. (3) & (4) PfRH1 interacts with the host receptor as the merozoite reorientates its apical end towards the host cell. Binding of PfRH1 to receptor induces oligomerization and triggers Ca^{2+} signaling in merozoite. Contact to the host cell triggers exposure of PfNBR. Receptor binding holds the merozoite in place in close proximity to erythrocyte, increasing the chances of detection of the near-membrane environmental ATP in low concentration. ATP binding to PfNBR induces subsequent steps for invasion (5) Secretion of EBA-175 to the merozoite surface to bind to Glycophorin A to commit invasion.

4.3.3 Future perspectives

PfRHs have been proposed to be involved in host cell selection but the selection mechanism is poorly understood. The identification of a novel ATP-binding domain of PfRH1 in this work further supports this hypothesis since intracellular ATP of erythrocytes have been shown to be essential for invasion. Based on the current data, it strongly suggests that PfRH1 carries multiple functions during invasion such as host

cell binding, ATP binding and cellular Ca^{2+} signaling transduction. However, many important aspects are to be investigated to verify the hypothesis above and to understand the working mechanism of PfRH1 during merozoite invasion: How does the erythrocyte ATP influence merozoite (preferential) invasion? How does it relate to PfRH1 during invasion? Does ATP binding associated with Ca^{2+} signaling? How does ATP binding to PfNBR affect the invasion protein machinery involving PfRH1? More work needs to be done to answer these questions. To answer the first three questions, merozoite invasion to reticulocyte with higher ATP content or modified host cells (resealed erythrocyte ghosts with and without ATP content or erythrocyte with defected ATP-releasing channels) could be examined in the presence or absence of mAbs against PfNBR. Merozoite Ca^{2+} signaling could be studied using these modified host cells in the high-throughput fluorescence scanning approach available (Gao et al., 2013) to further understand the relationship PfRH1-ATP binding and Ca^{2+} signaling in merozoites. More PfNBR mutants could be generated by mutating each of the potential ATP binding residues within the minimal ATP binding peptide (Table 4.1). With the identification of critical ATP binding residues, generation of a mutant parasite strain that is defective in ATP-binding would be possible and it will be useful to study the function of ATP binding to PfRH1 during invasion.

With the available monoclonal antibodies against at least 3 regions of PfRH1 (including two identified functional domains, EBD and PfNBR), it would be possible to tag and trace different fragments of PfRH1 at different stages of invasion in the future using super resolution imaging technique. By labeling each of the domains of PfRH1, their interactions at different stages of invasion could be examined by Fluorescence Resonance Energy Transfer (FRET). The monoclonal antibodies will also allow further studies on the processing of PfRH1.

The proposed model of the erythrocyte ATP sensing by merozoite also raises interesting questions on the host-pathogen interaction prior to irreversible junction formation during invasion. Does the cell deformation phase serve as a communication phase between merozoite and the host cell? Does merozoite contact or reorientation trigger erythrocyte deformation and ATP release? How does merozoite trigger erythrocyte deformation? With the available well-established techniques to isolate

purified merozoites (Gao et al., 2013) and to study erythrocyte deformability and ATP release (Wan et al., 2011), it would be possible to explore this area of interest.

Chapter 5 Conclusion

PfRHs have been shown to mediate host cell binding by interacting to specific surface receptors of erythrocyte. They have also been suggested to be involved in host cell selection. In this work, a novel ATP/ADP binding region (PfNBR) which binds ATP at a higher affinity has been identified in PfRH1, a member of PfRHs. IP-coupled photoaffinity labeling assay and Mass spectroscopy data suggest that PfNBR binds nucleotides without the conserved ATP-binding P-loop or A-loop, which is different from most of the ATP/GTP-binding proteins. PfNBR shows no similarity to most of the ATPases and it does not hydrolyze ATP. However, the determined ATP binding constant of PfNBR in the micromolar (μM) range suggests that PfRH1 could act as an ATP sensor to detect near-membrane environmental ATP released from a deforming erythrocyte during invasion. PfNBR is exposed on merozoite surface only when merozoites are in close contact to erythrocytes. Co-presence of PfNBR and Erythrocyte binding domain (EBD) in the same processed fragment of PfRH1 implies a close working relationship between these two domains. A model proposed in this work shows that a possible receptor binding-induced oligomerization of PfRH1 may act as a trigger for the exposure of PfNBR and the Ca^{2+} signaling in merozoite. In conclusion, PfRH1 plays multiple roles along the invasion process including 1) host receptor binding, 2) ATP binding, and 3) signaling transduction.

Chapter 6 References

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Appendix 1. GeneArt® Site-Directed Mutagenesis System

Description of the System, continued

Workflow

Figure adapted from the manual of GeneArt® Site-Directed Mutagenesis System (Life Technologies).

An illustration of work flow of using GeneArt® *Site-Directed Mutagenesis* System (Life Technologies) to create a double point mutation in PfNBR, producing a mutant protein, NBR-YYAA.

Appendix 2. FACS gating strategy for parasite culture parasitemia measurement using FlowJo

Gating strategy for infected red blood cells (RBC) measurement follows the order of **A** → **B** → **C/D**. **C** represents parasite infected RBC sample. **D** represents the negative control which is the SYBR Green stained-uninfected RBC alone.

Appendix 3. Working principle of Fluorescence Correlation Spectroscopy (FCS)

(Figures adapted from http://www.dkfz.de/Macromol/teaching/files/fcs_practical.pdf
<http://www.dkfz.de/Macromol/teaching/files/SingleMolSpec.pdf>
http://www.cellmigration.org/resource/imaging/imaging_approaches_correlation_microscopy.shtml#fcs)

FCS is a technique that allows measurement of the dynamical properties of biomolecules (usually fluorescently-labeled) such as diffusion coefficient of a biomolecule. (A). It is coupled with a confocal microscope so that fluorescently labeled biomolecules diffusing in aqueous buffer solution are detected within a volume less than 1 fL (known as focal volume). When the labeled molecules diffuse within the focal

Appendix 3. Working principle of Fluorescence Correlation Spectroscopy (FCS)

(Continued)

volume, the fluorescence intensity detected is high, while when it is out of the focal volume, the signal detected will be low, thus a series of signal fluctuations will be shown. **(B)**. Biomolecules with different diffusion coefficient display distinct patterns of signal fluctuation. Molecules with different mass will have different pattern of signal fluctuation. When the labeled molecules bound to another molecules, the increased mass directly affects its diffusion coefficient and a broader peak is seen. **(C)**. By using mathematical calculation, autocorrelation function curves can be obtained from the detected fluctuating signal. The autocorrelation function reveals the time required for a molecule to diffuse through an open observation volume. A slow fluctuation indicates an increasing diffusion time.

Appendix 4. Identification of epitopes within a protein region using Multiple Pin Peptide Scanning (Pepscan)

Identification of epitopes within a protein region.

Figure adapted from Sumar, 2001

Appendix 5. Structure of the photoactivatable 8-N₃-3'-biotinyl-ATP

Figure adapted and modified from Schafer et al., 2001

A biotin group was added to 8-N₃-ATP with a photoreactive compound (azido group). This compound forms covalent bonds with interacting protein in close proximity when activated by light.

Appendix 6. A linear relationship between free phosphate (P_i) and absorbance at 590 nm in a colorimetric based ATPase assay

Appendix A. Amino acids sequence of six overlapping region in
Erythrocyte-binding Domain (500-833 aa) of PfRH1

500	L QIVQQKLLEIKQKKNDITHKVQLINHIYKNIHDEILNKKNNEITKIIINNIKD
	HKKDLQDLLLLFIQQIKQYNILTDHKITQCNNYYKEIHKMKE D INHIHIYIQPIL
	NNLHTLKQVQNNKIKYEEHIKQILQKIYDKKESLKKIILLKDEAQLDITLLDD
	LIQK Q TKKQTQTQTQTQKQTLIQNNETIQLI S GGQEDKHESNPFNHIQTYIQQ
	KDTQNKNIQNLLK S LYNGNINTFIDTISKYILK Q KDIELTQHVYTDEKINDY
	LEEIKNEQ N KIDKTIDDIKIQETLKQITHIVNNIKTIKKDLLKEFIQHLLIKYMN
	ERYQNMQQGYNNLT N
	833

L = starting residue for all six constructs

D = ending residue of BA2

Q = ending residue of BA4

S = ending residue of N1

S = ending residue of N2

Q = ending residue of N3

N = ending residue of N4

Appendix B. Coiled-coils prediction of PfNBR by COILS/PCOILS

```
PCOILS v1.0.1
Input Matrix : MTIDK
Using weights
Input Sequence:
>Sat_Oct_11_06:08:13_+0200_2014
QELKQVQENVEKVKDIYNQTIKYEEIKNYHIITDYENKINDILHNSFIKQINMESSNNKKQTKQIIDIIINDKTFEHHIKTSKTKINMLKEQSQMKHID
KTLLNEQALKLFVDINSTNNLDNMLSEINSIQNNIHTYIQEANKSPDKFKIICDQNVNDLLNKLSDLGDLNMYMHLKKNLQNEIRNMNLEKNFMLDKSKKI
DEEEKKLDILKVNISNNSLDKLKKYEEALFQKVKEAEIQKENIEKIKQEINTLSVDFK
```

1 Q	f 0.777	f 0.257	b 0.028
2 E	g 0.777	g 0.257	c 0.028
3 L	a 0.777	a 0.257	d 0.028
4 K	b 0.777	b 0.257	e 0.028
5 Q	c 0.777	c 0.257	f 0.028
6 V	d 0.777	d 0.257	g 0.028
7 Q	e 0.777	e 0.257	a 0.028
8 E	f 0.777	f 0.257	b 0.028
9 N	g 0.777	g 0.257	c 0.028
10 V	a 0.777	a 0.257	d 0.028
11 E	b 0.777	b 0.257	e 0.028
12 K	c 0.777	c 0.257	f 0.028
13 V	d 0.777	d 0.257	g 0.028
14 K	e 0.777	e 0.257	a 0.028
15 D	f 0.716	f 0.257	b 0.028
16 I	g 0.179	g 0.257	c 0.028
17 Y	d 0.042	a 0.257	d 0.028
18 N	e 0.042	b 0.257	e 0.028
19 Q	f 0.042	c 0.257	f 0.028
20 T	g 0.042	d 0.257	g 0.028
21 I	a 0.042	e 0.257	a 0.028
22 K	b 0.031	f 0.220	b 0.028
23 Y	c 0.011	d 0.157	c 0.028
24 E	d 0.011	e 0.157	d 0.028
25 E	e 0.011	f 0.157	e 0.028
26 E	f 0.011	g 0.157	f 0.028
27 I	g 0.011	a 0.157	g 0.028
28 K	a 0.011	b 0.157	a 0.028
29 K	b 0.011	c 0.157	b 0.027
30 N	c 0.011	d 0.157	c 0.021
31 Y	e 0.017	e 0.157	d 0.004
32 H	f 0.033	f 0.157	f 0.003
33 I	g 0.033	g 0.157	g 0.003
34 I	a 0.138	a 0.157	a 0.003
35 T	b 0.138	b 0.157	b 0.003
36 D	c 0.138	c 0.157	c 0.003
37 Y	d 0.138	d 0.157	d 0.003
38 E	e 0.138	e 0.157	e 0.003
39 N	f 0.138	f 0.157	f 0.003
40 K	g 0.138	g 0.157	g 0.003
41 I	a 0.138	a 0.157	a 0.003
42 N	b 0.138	b 0.157	b 0.003
43 D	c 0.138	c 0.147	c 0.003
44 I	d 0.138	d 0.062	d 0.003
45 L	e 0.138	e 0.013	e 0.003
46 H	f 0.138	f 0.005	f 0.003
47 N	g 0.138	b 0.007	g 0.003
48 S	a 0.005	c 0.007	a 0.003
49 F	d 0.002	d 0.007	d 0.002
50 I	e 0.003	e 0.007	e 0.002
51 K	f 0.005	f 0.007	f 0.004
52 Q	g 0.005	g 0.007	g 0.004
53 I	a 0.005	a 0.007	a 0.004
54 N	b 0.005	b 0.007	b 0.004
55 M	c 0.005	c 0.007	c 0.004
56 E	c 0.007	d 0.007	d 0.004
57 S	d 0.007	e 0.007	e 0.004
58 S	e 0.007	f 0.007	f 0.004
59 N	f 0.010	g 0.007	g 0.004
60 N	g 0.010	a 0.007	a 0.004
61 K	a 0.010	b 0.007	b 0.004
62 K	b 0.010	c 0.007	c 0.004
63 Q	c 0.010	d 0.007	d 0.004

Appendix B. Coiled-coils prediction of PfNBR by COILS/PCOILS (Continued)

64	T	d 0.010	e 0.007	e 0.004
65	K	e 0.010	f 0.007	f 0.004
66	Q	f 0.010	g 0.007	g 0.004
67	I	g 0.010	a 0.007	a 0.004
68	I	a 0.010	b 0.007	b 0.004
69	D	b 0.010	c 0.007	c 0.004
70	I	c 0.010	d 0.007	d 0.004
71	I	d 0.010	e 0.007	e 0.004
72	N	e 0.010	f 0.007	f 0.004
73	D	f 0.008	g 0.005	g 0.004
74	K	g 0.008	b 0.015	a 0.004
75	T	c 0.006	c 0.016	b 0.004
76	F	d 0.006	d 0.016	c 0.004
77	E	e 0.090	e 0.016	e 0.006
78	E	f 0.090	f 0.016	f 0.006
79	H	g 0.090	g 0.016	g 0.006
80	I	a 0.192	a 0.016	a 0.011
81	K	b 0.192	b 0.016	b 0.011
82	T	c 0.192	c 0.016	c 0.011
83	S	d 0.192	d 0.016	d 0.011
84	K	e 0.192	e 0.016	e 0.096
85	T	f 0.192	f 0.016	f 0.096
86	K	g 0.192	g 0.016	g 0.096
87	I	a 0.192	a 0.016	a 0.096
88	N	b 0.192	g 0.018	b 0.096
89	M	c 0.192	a 0.018	c 0.096
90	L	d 0.192	b 0.018	d 0.096
91	K	e 0.192	c 0.018	e 0.096
92	E	f 0.192	d 0.018	f 0.096
93	Q	g 0.192	e 0.018	g 0.096
94	S	f 0.011	f 0.018	a 0.096
95	Q	g 0.013	g 0.018	b 0.096
96	M	a 0.013	a 0.018	c 0.096
97	K	b 0.013	b 0.018	d 0.096
98	H	e 0.022	c 0.018	e 0.221
99	I	f 0.022	d 0.018	f 0.306
100	D	g 0.022	g 0.034	g 0.427
101	K	a 0.022	a 0.045	a 0.647
102	T	b 0.032	b 0.210	b 0.899
103	L	c 0.072	c 0.453	c 0.964
104	L	d 0.143	d 0.804	d 0.986
105	N	e 0.143	e 0.804	e 0.986
106	E	f 0.143	f 0.804	f 0.986
107	Q	g 0.143	g 0.804	g 0.986
108	A	a 0.143	a 0.804	a 0.986
109	L	b 0.143	b 0.804	b 0.986
110	K	c 0.143	c 0.804	c 0.986
111	L	d 0.143	d 0.804	d 0.986
112	F	e 0.143	e 0.804	e 0.986
113	V	f 0.143	f 0.804	f 0.986
114	D	g 0.143	g 0.804	g 0.986
115	I	a 0.196	a 0.814	a 0.986
116	N	b 0.196	b 0.814	b 0.986
117	S	c 0.196	c 0.814	c 0.986
118	T	d 0.196	d 0.814	d 0.986
119	N	e 0.331	e 0.895	e 0.986
120	N	f 0.430	f 0.895	f 0.986
121	N	g 0.430	g 0.895	g 0.986
122	L	a 0.430	a 0.895	a 0.986
123	D	b 0.430	b 0.895	b 0.986
124	N	c 0.430	c 0.895	c 0.986
125	M	d 0.430	d 0.895	d 0.986
126	L	e 0.430	e 0.895	e 0.986
127	S	f 0.430	f 0.895	f 0.986
128	E	g 0.430	g 0.895	g 0.986
129	I	a 0.430	a 0.895	a 0.986
130	N	b 0.430	b 0.895	b 0.986
131	S	c 0.430	c 0.895	c 0.986
132	I	d 0.430	d 0.895	d 0.980
133	Q	e 0.430	e 0.895	e 0.980
134	N	f 0.430	f 0.895	f 0.980
135	N	g 0.430	g 0.895	g 0.980
136	I	a 0.311	a 0.895	a 0.980

Appendix B. Coiled-coils prediction of PfNBR by COILS/PCOILS (Continued)

137	H	b 0.138	b 0.895	b 0.980
138	T	c 0.110	c 0.895	c 0.980
139	Y	d 0.110	d 0.895	d 0.980
140	I	e 0.110	e 0.781	e 0.980
141	Q	f 0.110	f 0.781	f 0.980
142	E	g 0.110	g 0.781	g 0.980
143	A	a 0.057	a 0.351	a 0.951
144	N	b 0.057	b 0.309	b 0.951
145	K	c 0.057	c 0.280	c 0.951
146	S	d 0.014	d 0.034	d 0.861
147	F	a 0.004	e 0.021	e 0.367
148	D	b 0.004	f 0.021	f 0.285
149	K	c 0.004	g 0.013	g 0.282
150	F	d 0.004	a 0.001	a 0.011
151	K	e 0.004	b 0.001	b 0.011
152	I	b 0.014	b 0.001	c 0.003
153	I	c 0.029	c 0.001	d 0.001
154	C	d 0.031	d 0.001	d 0.003
155	D	e 0.031	e 0.001	e 0.007
156	Q	f 0.031	f 0.001	b 0.172
157	N	g 0.031	g 0.001	c 0.172
158	V	a 0.031	a 0.001	d 0.172
159	N	b 0.031	e 0.004	e 0.288
160	D	c 0.031	f 0.005	f 0.288
161	L	d 0.031	g 0.007	g 0.288
162	L	e 0.031	a 0.027	a 0.447
163	N	f 0.031	b 0.027	b 0.447
164	K	g 0.031	c 0.027	c 0.447
165	L	a 0.031	d 0.027	d 0.447
166	S	b 0.031	e 0.027	e 0.447
167	L	c 0.031	f 0.027	f 0.447
168	G	d 0.003	g 0.027	g 0.447
169	D	e 0.006	a 0.065	a 0.447
170	L	b 0.898	b 0.978	b 0.724
171	N	c 0.985	c 0.997	c 0.952
172	Y	d 0.985	d 0.997	d 0.952
173	M	e 0.985	e 0.997	e 0.952
174	N	f 0.995	f 0.997	f 0.952
175	H	g 0.995	g 0.997	g 0.952
176	L	a 0.998	a 0.997	a 0.983
177	K	b 0.998	b 0.997	b 0.983
178	N	c 0.998	c 0.997	c 0.983
179	L	d 0.998	d 0.997	d 0.983
180	Q	e 0.998	e 0.997	e 0.983
181	N	f 0.998	f 0.997	f 0.983
182	E	g 0.998	g 0.997	g 0.983
183	I	a 0.998	a 0.997	a 0.983
184	R	b 0.998	b 0.997	b 0.983
185	N	c 0.998	c 0.997	c 0.983
186	M	d 0.998	d 0.997	d 0.983
187	N	e 0.998	e 0.997	e 0.983
188	L	f 0.998	f 0.997	f 0.983
189	E	g 0.998	g 0.997	g 0.983
190	K	a 0.968	a 0.997	a 0.983
191	N	b 0.956	b 0.997	b 0.983
192	F	c 0.764	c 0.992	c 0.983
193	M	d 0.231	d 0.992	d 0.983
194	L	e 0.071	e 0.992	e 0.983
195	D	c 0.280	f 0.992	c 0.995
196	K	d 0.280	g 0.992	d 0.996
197	S	e 0.893	e 0.947	e 1.000
198	K	f 0.966	f 0.976	f 1.000
199	K	g 0.966	g 0.984	g 1.000
200	I	a 0.966	a 0.984	a 1.000
201	D	b 0.966	b 0.984	b 1.000
202	E	c 0.966	c 0.984	c 1.000
203	E	d 0.966	d 0.984	d 1.000
204	E	e 0.966	e 0.999	e 1.000
205	K	f 0.966	f 0.999	f 1.000
206	K	g 0.966	g 0.999	g 1.000
207	L	a 0.966	a 0.999	a 1.000
208	D	b 0.966	b 0.999	b 1.000
209	I	c 0.966	c 0.999	c 1.000

Appendix B. Coiled-coils prediction of PfNBR by COILS/PCOILS (Continued)

210	L	d 0.966	d 0.999	d 1.000
211	K	e 0.966	e 0.999	e 1.000
212	V	f 0.827	f 0.999	f 1.000
213	N	g 0.858	g 0.999	g 1.000
214	I	a 0.858	a 0.999	a 1.000
215	S	b 0.858	b 0.999	b 1.000
216	N	c 0.858	c 0.999	c 1.000
217	I	d 0.858	d 0.999	d 1.000
218	N	e 0.858	e 0.999	e 1.000
219	N	f 0.858	f 0.999	f 1.000
220	S	g 0.858	g 0.999	g 1.000
221	L	a 0.858	a 0.999	a 1.000
222	D	b 0.858	b 0.999	b 1.000
223	K	c 0.858	c 0.999	c 1.000
224	L	d 0.858	d 0.999	d 1.000
225	K	e 0.858	e 0.998	e 1.000
226	K	f 0.858	f 0.998	f 1.000
227	Y	g 0.764	g 0.967	g 1.000
228	Y	a 0.764	a 0.967	a 1.000
229	E	b 0.764	b 0.967	b 1.000
230	E	c 0.764	c 0.967	c 1.000
231	A	d 0.764	d 0.883	d 1.000
232	L	e 0.496	e 0.669	e 0.999
233	F	f 0.161	f 0.404	f 0.989
234	Q	g 0.161	b 0.897	g 0.989
235	K	f 0.201	c 0.897	c 0.981
236	V	g 0.201	d 0.897	d 0.981
237	K	a 0.201	e 0.989	e 0.981
238	E	b 0.201	f 0.989	f 0.981
239	K	c 0.201	g 0.989	g 0.981
240	A	d 0.201	a 0.989	a 0.981
241	E	b 0.505	b 0.989	b 0.981
242	I	c 0.505	c 0.989	c 0.981
243	Q	d 0.505	d 0.989	d 0.981
244	K	e 0.987	e 0.989	e 0.981
245	E	f 0.987	f 0.989	f 0.981
246	N	g 0.987	g 0.989	g 0.981
247	I	a 0.987	a 0.989	a 0.981
248	E	b 0.987	b 0.989	b 0.981
249	K	c 0.987	c 0.989	c 0.981
250	I	d 0.987	d 0.989	d 0.981
251	K	e 0.987	e 0.989	e 0.981
252	Q	f 0.987	f 0.989	f 0.981
253	E	g 0.987	g 0.989	g 0.981
254	I	a 0.987	a 0.989	a 0.981
255	N	b 0.987	b 0.989	b 0.981
256	T	c 0.987	c 0.989	c 0.981
257	L	d 0.987	d 0.989	d 0.981
258	S	e 0.953	e 0.969	e 0.907
259	D	f 0.915	f 0.947	f 0.907
260	V	g 0.625	g 0.773	g 0.864
261	F	a 0.048	a 0.290	a 0.864
262	K	b 0.035	b 0.223	b 0.859

Appendix C. Reference protein lists used in Dichroweb analyses of PfNBR

Ref set 4
A-Bangarotoxin
Alcohol dehydrogenase
Adenylate kinase
Azurin
β -lactoglobulin
Bence Jones Protein
Bovine Pancreatic Trypsin Inhibitor Carbonic Anhydrase
A-Chymotrypsin
Colicin A
Concanavalin A
Carboxypepsidase A
Cytochrome C
EcoR1 Endonuclease
Elastase
Flavodoxin
G-Crystallin
GFP
GPD
Glutathione reductase
Hemoglobin
Hemerythrin
Rat Intestinal Fatty Acid Binding protein Insulin
Lactate dehydrogenase
Lysozyme
Myoglobin
Papain
Parvalbumin
Phosphoglycerate kinase
Pepsinogen
Prealbumin
Rhodanase
Ribonuclease A
Subtilin BPN
Subtilin novo
Superoxide dismutase
T4 lysozyme
Thermolysin
Tumor Necrosis Factor
Triose Phosphate Isomerase

Appendix D. Dichroweb analyses of PfNBR CD spectra using *k2d*

K2D				
	Ref set 4			
	Alpha helix	Beta sheet	Random Coil	NRMSD ^a
rNBR	0.81	0	0.19	0.082
rNBR+ATP	0.81	0	0.19	0.085
rNBR+ADP	0.81	0	0.19	0.086

^a normalized root-mean-square deviation (goodness-of-fit parameter)

Appendix E. Analysis of protein purity of purified rNBR by ImageJ

Concentrated purified rNBR at 2 mg/ml (left panel) and 10 mg/ml (right panel) was loaded on denaturing SDS-PAGE gel and stained by Coomassie Blue. Gel photos were analyzed by using ImageJ to quantify protein purity. Intensity of all bands in the sample were calculated and compared. Purified rNBR maintained at a purity of above 95% at both working concentrations for Circular Dichroism (CD) (2 mg/ml) and fluorescence correlation spectroscopy (FCS) (10 mg/ml).

Appendix F. Elution chromatogram of N205 in IgG purification

Fractions A1 to A4 contained IgG with high purity

Appendix G. Elution chromatogram of N206 in IgG purification

Fractions A1 to A5 contained IgG with high purity.

Appendix H. Elution chromatogram of N207 in IgG purification

Fractions A1 to A5 contained IgG with high purity.

Appendix I. Elution chromatogram of N208 in IgG purification

Fractions A1 to A4 contained IgG with high purity

Appendix J. Elution chromatogram of N209 in IgG purification

Fractions A1 to A4 contained IgG with high purity.

Appendix K. Elution chromatogram of R8201 in IgG purification

Fractions A1 to A4 contained IgG with high purity.

Appendix L. Location of Walker A and Walker B in NBD94 of Py235

1 SDKNEYVKKA IDLKKVVENN NAYIDELAKI SPYQVTEYVK NKDKIYSTIK₅₀
 51 SELSKIYQGD LDALYNELSS IVKENAVDNT EDKAKLEDLK SKIDKEYDKI₁₀₀
 101 QNMETETVKL NLSTIENKKN ELLSIIVEMK KHIHSELNNE LNKIVEDFKS₁₅₀
 151 KEKQLSSNIN DYSNYKDELN KYKSKISEIK SQYNDQSNID NIKDEDAKQN₂₀₀
 201 YEKSKEYIKT ISVKEDEIFK IINEMKFMKD DILNKVNVFV NLENNHKEKI₂₅₀
 251 NSGHESFAEL VNKIKNEISD DQLNDYEKNV NDSKSLINEI TKSIEEEYQN₃₀₀
 301 INTLKKVNGY LKICKNTTES IEKFRNKQNK LNEILNKNID VIKNSNLIEK₃₅₀
 351 SYTNQFDNTL TDKKTELEKI FTELSLSSYE AKNNELIKYF NDLKENLGTP₄₀₀
 401 KGNTLYQQFD ENEKATNDIE QKSVNANKNV SNIEMVIHTS IYNIIDEIEK₄₅₀
 451 LIGKNIELLN KEILKEAEIS ITNFNEIK **EK LKHYNFDDFV** KEENIKYAD₅₀₀
 501 INKIKNDIKT LDQKV₅₅₀DN₅₅₀NIK ALTEIKNKSE NYINEIKTQI SDLEDVTD₆₀₀KT₆₀₀

(Figure adapted from Ramalingam, 2008)

Position of Walker A motif (red line) and Walker B motif (blue line). Black shading denotes the ATP binding site.

Appendix M. Publication

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Triggers of key calcium signals during erythrocyte invasion by *Plasmodium falciparum*

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Invasion of erythrocytes by *Plasmodium falciparum* merozoites is a complex multi-step process mediated by specific interactions between host receptors and parasite ligands. Reticulocyte-binding protein homologues (RHs) and erythrocyte-binding-like (EBL) proteins are discharged from specialized organelles and used in early steps of invasion. Here we show that monoclonal antibodies against PfRH1 (an RH) block merozoite invasion by specifically inhibiting calcium signalling in the parasite, whereas invasion-inhibiting monoclonal antibodies targeting EBA175 (an EBL protein) have no effect on signalling. We further show that inhibition of this calcium signalling prevents EBA175 discharge and thereby formation of the junction between parasite and host cell. Our results indicate that PfRH1 has an initial sensing as well as signal transduction role that leads to the subsequent release of EBA175. They also provide new insights on how RH–host cell interactions lead to essential downstream signalling events in the parasite, suggesting new targets for malaria intervention.

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P*lasmodium falciparum* (*P. falciparum*) is the most virulent malaria parasite and accounts for the vast majority of deaths (WHO, <http://www.rbm.who.int>, 2010). Erythrocyte invasion is a complex multi-step process that is mediated by specific interactions between host receptors and parasite ligands. Junction formation between the merozoite and erythrocyte is a crucial step that allows the parasite to irreversibly bind to the host followed by the activation of its motor machinery system that drives the parasite completely into the erythrocyte¹. Previous studies suggest the involvement of apical secretory organelles termed rhoptries and micronemes². In *Plasmodium* species, a number of key parasite ligands that mediate critical interactions with host receptors during invasion are localized in these apical organelles³. Two protein families are used in early steps of invasion in *P. falciparum*: reticulocyte-binding protein homologues (RHs) and erythrocyte-binding-like proteins (EBLs)³. RHs and EBLs have been shown to have a crucial role in host cell recognition and sensing as well as virulence⁴. Members of both protein families can be found in all *Plasmodium* species, further highlighting their importance in malaria parasite biology. In EBLs, a duffy-binding-like (DBL) domain mediates specific binding to different host cell receptors⁵ including glycoporphins A, B and C as well as the Duffy blood group antigen³. In RHs, the precise erythrocyte-binding regions are still being delineated^{4,6–13}; complement receptor 1 (CR1) and basigin have been identified to be the receptors for PfRH4 (ref. 14) and PfRH5 (ref. 15), respectively. Although RHs have been proposed to have an early role in host cell sensing^{16,17}, the EBLs are thought to have a direct role in junction formation¹⁸. This suggests that RH sensing and subsequent interaction with a suitable host erythrocyte sends a signal to the merozoite that triggers the subsequent steps of invasion⁴.

Here we generate monoclonal antibodies (mAbs) against the erythrocyte-binding region RII-3 of PfRH1 (a member of the RH family in *P. falciparum*⁷). We show that PfRH1 mAbs block merozoite invasion by specifically inhibiting calcium signalling in the parasite rather than by directly interfering with the erythrocyte binding of the parasite. We further show that the inhibition of this calcium signalling prevents EBA175 discharge and thereby prevents junction formation (a key step that enables the parasite to penetrate the erythrocyte using an actin-myosin motor²). Our results demonstrate that PfRH1 and EBA175 have

distinct functions, with PfRH1 having an initial sensing and signal transduction role that leads to subsequent EBA175 release from the microneme and to junction formation. These new insights might help to identify new targets for malaria intervention.

Results

Specific PfRH1 mAbs inhibit merozoite invasion. In this work, we use PfRH1 to study key intracellular signalling events that lead to successful erythrocyte invasion. We have previously shown that antibodies targeting an erythrocyte-binding region (RII-3) of PfRH1 (Fig. 1a) can inhibit merozoite invasion⁷. Here we generated four monoclonal antibodies (mAbs: C49, C41, C2 and C50) against this region and validated their specificity using western blot and immunofluorescence assays. All antibodies recognize the expected 240- and 140-kDa-processed form of PfRH1 in T994 parasite culture supernatant, merozoite and schizont extracts, whereas no equivalent bands were detected in the PfRH1 knockout line T994ΔRH1 (Supplementary Fig. S1a–c). Immunofluorescence assays showed that all mAbs give punctuate patterns that are partially localized with EBA175 (a known microneme protein) at the apical end of merozoites in T994, consistent with the expected location of PfRH1 (Supplementary Fig. S1d). No staining was observed in T994ΔRH1 (Supplementary Fig. S1e), further confirming the specificity of the mAbs.

To establish whether the different mAbs were able to inhibit merozoite invasion, inhibition assays with increasing concentration of antibodies were carried out using T994 and T994ΔRH1 (Fig. 1b,c). We found that mAbs C2 and C50 have no significant effect on invasion, whereas both mAbs C49 and C41 inhibit invasion in a dose-dependent manner in T994. In contrast, none of the antibodies has any impact on T994ΔRH1, in line with their specificity to PfRH1. Similar results (Supplementary Fig. S2a,b) were obtained when using the parasite lines W2mef and W2mef/NM, which are known to differ in their requirement for PfRH1 in invasion^{7,19}, suggesting that both C49 and C41 are invasion-inhibiting mAbs. To ensure that the invasion inhibitory effect is not because of the large size of the mAb (which could lead to a secondary blocking effect), we purified the Fab fragment of mAb C41 (Supplementary Fig. S2c, insert) and performed invasion

Figure 1 | Anti-PfRH1 mAbs significantly block parasite invasion. (a) Full-length PfRH1 including signal sequence (SS, red), erythrocyte-binding region RII-3 (blue, encompassing amino acids 500–833) and transmembrane domain (TM, yellow). (b,c) Invasion inhibition assays using anti-PfRH1 mAbs (IgG) in T994 (b) and T994ΔRH1 (c). Bar charts show mean $\pm$ s.e.m.; $n = 3$. * $P \leq 0.0003$ by one-way ANOVA, indicating that C49 successfully blocked the invasion in T994 compared with the invasion in the presence of C2 and C50. † $P \leq 0.0003$ by one-way ANOVA, indicating that C41 significantly inhibited the invasion compared with C2 and C50.

inhibition assays^{20,21}. The purified C41 Fab fragment significantly blocks invasion in a dose-dependent manner in T994 parasites (Supplementary Fig. S2c), confirming that mAb C41 directly targets PfPRH1.

Inhibitory mAbs block invasion before junction formation.

Merozoite invasion inhibition by antibodies is often caused by antibodies directly interfering with the binding of the parasite to its receptor. We therefore assessed the ability of the different mAbs to inhibit PfPRH1 binding to erythrocytes. Both non-inhibitory mAbs (C2 and C50) and inhibitory mAbs (C49 and C41), at concentrations (0.3 mg ml^{-1}) shown to effectively inhibit merozoite invasion (Fig. 1b), have no impact on erythrocyte binding by PfPRH1 (Fig. 2a,b), suggesting that invasion-blocking mAbs act independently of erythrocyte binding. To further determine the epitope specificity, four N-terminal overlapping fragments (N1–N4) derived from RII-3 were expressed as

recombinant proteins and screened using the mAbs (Supplementary Fig. S3). The size of each fragment was confirmed using anti-His-tag mAbs (Supplementary Fig. S3f). None of the PfPRH1 mAbs recognizes N1, whereas C2, C41 and C49 recognize N4 only. C50 is able to detect N2, N3 and N4 (Supplementary Fig. S3b–e), suggesting that C50 binds to a different epitope from that of C2, C41 or C49. On the basis of the western blot results, we further synthesized six overlapping peptides (Supplementary Fig. S3g). The inhibitory mAbs C41 and C49, as well as the non-inhibitory mAb C2, recognize the same 15-mer peptide, whereas another non-inhibitory mAb C50 recognizes a different peptide (Supplementary Fig. S3h). It is somewhat surprising that the non-inhibitory and inhibitory mAbs recognize the same peptide; however, as an epitope is 5–8 amino-acid long, it is possible that multiple distinct epitopes lie within the same peptide. In fact, previous studies have shown that mAbs against epitopes that are very close to each other exhibit different effects on cellular or biological functions^{20–22}.

Figure 2 | Anti-PfPRH1 mAbs inhibit invasion before junction formation. (a,b) Erythrocyte binding assays of T994 parasite culture supernatants using the C49 (a) and C2 (b) mAbs. Neither C49 nor C2 ($0.035\text{--}0.3 \text{ mg ml}^{-1}$) was able to inhibit the erythrocyte binding of PfPRH1. T994 parasite culture supernatants (S/N) were used as a quality control. Molecular sizes are indicated on the left (in kDa). (c,d) Snapshots taken from time-lapse live movie microscopy of invasion of W2mef by merozoites in the absence (c) or presence (d) of C49 mAb. Time-elapsed post schizont rupture is indicated in each snapshot in seconds (sec, white). A white dotted circle indicates a rupturing schizont. Green arrows point to merozoites. A red dotted circle around an erythrocyte is added to help follow the infection. Scale bars = $5 \mu\text{m}$. (c) In the absence of mAb, the merozoite released from a mature schizont (white circle) attaches, apically reorients and penetrates into an uninfected erythrocyte. Following successful invasion, deformation of infected erythrocytes occurs (echinocytosis) and by 2 min the erythrocytes recover back to its normal shape. (d) In the presence of C49, the merozoite released from a mature schizont attaches and apically reorients, but it is unable to penetrate the erythrocyte even after 3 min. Also see Supplementary Movies 1,2. (e) Anti-PfPRH1 inhibitory mAbs significantly reduce merozoite junction formation. T994 or T994ΔRH1 schizonts were pretreated with Cyto D and allowed to rupture in the absence or presence of anti-PfPRH1 mAbs (0.2 mg ml^{-1}). Junction-arrested merozoites were counted microscopically. Bar chart indicates the counting of junction-arrested merozoites with mAbs C2, C41, C49 and C50 in T994 and T994ΔRH1 compared with the arrested parasites in the absence of mAbs. Inhibitory mAbs C49 and C41 significantly blocked merozoite junction formation in T994. The error bar indicates the s.e.m.; $n = 3$. *** $P \leq 0.00014$ by one-way ANOVA indicates the significant differences between the effects of inhibitory mAbs (C49 and C41) and those of non-inhibitory mAbs (C2 and C50).

Live video microscopy is a powerful tool to dissect merozoite invasion, and it has been effectively used to demonstrate the role of another merozoite invasion protein, PfAMA1, during merozoite re-orientation step in invasion²³. Using time-lapse live video microscopy, we followed the process of merozoite invasion both in the presence and absence of invasion inhibitory antibodies (Fig. 2c,d, Supplementary Fig. S4a,b and Supplementary Movies 1–7). We found that, in the absence of antibodies, newly released merozoites rapidly attach, reorient and invade erythrocytes (Fig. 2c and Supplementary Movie 1). Immediately after invasion, the erythrocyte shrinks and echinocytosis occurs, with the infected erythrocyte recovering its shape within 2 min. Interestingly, in the presence of the invasion inhibitory antibody C49 (Fig. 2d and Supplementary Movie 2,6) or C41 (Supplementary Fig. S4a and Supplementary Movie 3,7), merozoites are able to attach and reorient themselves but are unable to penetrate the erythrocyte even after 3 min, despite extensive distortions of the erythrocyte being observed. In contrast, invasion similar to that seen in the absence of any antibody is observed in the presence of the non-inhibitory antibodies C2 (Supplementary Fig. S4b and Supplementary Movie 4) or C50 (Supplementary Movie 5). The inability of the merozoite to penetrate the erythrocyte despite reorientation and clear binding suggests that the inhibitory antibodies interfere with a subsequent step of the invasion process. Junction formation is known to be linked to the irreversible commitment of the merozoite to invasion, and recent studies have shown that PfRH1, PfRH2a/2b and PfRH5 localize at the tight junction, suggesting a specific role for the PfRH proteins during this part of the invasion process^{6,24–26}. Therefore, to assess whether the inhibitory mAbs prevented junction formation, merozoites were arrested at the tight junction by treatment with cytochalasin D (Cyto D)²⁷. Experiments were carried out in the absence and presence of PfRH1 mAbs, and junction-arrested merozoites were counted. Similar to the invasion inhibition assay, inhibitory mAbs C49 and C41 significantly reduce junction-arrested merozoites as compared with C2 and C50 in both T994 and W2mef (Fig. 2e, Supplementary Table S1 and Supplementary Fig. S4c). In contrast, no effect is seen in T994ΔRH1 and W2mef/NM, in line with PfRH1 not having a role in these parasites. Taken together, these data clearly demonstrate that inhibitory mAbs inhibit the invasion before junction formation and suggest that PfRH1 could have a key signalling role ahead of junction formation.

PfRH1 inhibitory mAbs block Ca^{2+} signalling during invasion. Previous work has suggested that secretion of EBA175 from the micronemes onto the surface of the merozoite is a prerequisite for junction formation and is triggered by calcium (Ca^{2+}) release in the merozoite²⁸. Here we carried out real-time measurement of Ca^{2+} signalling in the presence/absence of Cyto D during merozoite invasion using freshly isolated free merozoites from T994 parasite. We found that Ca^{2+} signal increases both in the presence and absence of Cyto D (Fig. 3a, Supplementary Data 1–6), demonstrating that the signal originates before or at junction formation. This allowed us to further elucidate whether the invasion inhibitory mAbs targeting PfRH1 directly had an impact on Ca^{2+} signalling. We observed that the cumulative intracellular Ca^{2+} signal during merozoite invasion is significantly reduced in the presence of the inhibitory mAbs C41, as compared with the non-inhibitory mAbs C2 or no antibody control (Fig. 3b, Supplementary Data 1–6). No antibody effect on Ca^{2+} signalling is seen in T994ΔRH1 (Fig. 3c, Supplementary Data 1–6). These results suggest that PfRH1 inhibitory mAbs inhibit invasion by blocking Ca^{2+} signalling.

Control experiments using the Ca^{2+} ionophore A23187 and the Ca^{2+} chelator BAPTA-AM showed that treatment of merozoites with A23187 leads to a significant increase in the Ca^{2+} signal over time, whereas pretreatment with BAPTA-AM significantly reduces Ca^{2+} signals (Supplementary Fig. S5, Supplementary Data 1–6 and Supplementary Table S2). Although the Ca^{2+} signal observed during invasion assays is only derived from successfully invading merozoites, all viable merozoites are expected to give Ca^{2+} signals after A23187 treatment, explaining the higher signal obtained (compare Fig. 3b with Supplementary Fig. S5). Importantly, functional C41 Fab fragments also block Ca^{2+} signalling in T994 parasite during invasion (Fig. 3d, Supplementary Data 1–6) as effectively as mAbs C41. This further confirms that the specific binding of inhibitory mAbs to PfRH1 is important for blocking Ca^{2+} signalling.

We performed similar experiments using flow cytometry to measure Ca^{2+} levels during invasion of fresh erythrocytes by free merozoites in the presence of Cyto D, as well as in the presence/absence of inhibitory mAbs. Invading merozoites will be arrested by Cyto D after junction formation, whereas merozoites in the presence of inhibitory antibodies will either detach or remain attached to the RBC without forming a junction. On the basis of these criteria, there are two distinct populations: free merozoites and RBC-bound merozoites. Staining for DAPI (+) events identifies all merozoites, whereas gating for size enables us to distinguish between free and RBC-bound merozoites (Fig. 3e,f). An inhibitory antibody C41 can reduce the number of invading merozoites to 56.6% (Fig. 3g-blue) and decrease the overall Ca^{2+} level to 32.42% (Fig. 3g-red) during invasion, when compared with the absence of inhibitory mAbs. This result is consistent with our previous conclusion, supporting the accessibility and reproducibility of real-time measurement of Ca^{2+} signalling by using a fluorescence plate reader. Taken together, these results strongly support the conclusion that PfRH1 has a direct or indirect role in the regulation of Ca^{2+} signalling during invasion. Hence, we propose that binding of the mAbs to PfRH1 directly inhibits a signal to trigger Ca^{2+} signal, or it has an indirect effect by preventing the recruitment of other proteins important for the initiation of the signal.

Merozoite viability. To ensure that the observed Ca^{2+} signal was indeed due to merozoite invasion, each merozoite preparation in parallel to Ca^{2+} signalling studies was assessed for their ability to invade fresh erythrocytes. T994 and T994ΔRH1 merozoites from each preparation successfully invade erythrocytes, and T994 invasion is successfully inhibited by C41 but not C2. In contrast, none of the antibodies has any impact on invasion in the case of T994ΔRH1 (Supplementary Table S3). Importantly, there is a clear correlation ($R^2 = 0.836$) between invasion and Ca^{2+} signal, with low invasion (due to antibody blocking) leading to low Ca^{2+} signal (Fig. 4a). In addition, we followed the kinetics of merozoite invasion using heparin treatment²⁹ and observed that almost 75% of isolated merozoites invade erythrocytes in a linear manner ($R^2 = 0.991$) over the first 10 min (Fig. 4b), which is in line with time-dependent Ca^{2+} data. Only a small proportion (25%) of the total invading merozoites have not invaded within 10 min, confirming that the observed increase in Ca^{2+} signal was contributed by the majority proportion of invading merozoites over the first 10 min. Taken together, these data clearly indicate that viable invasion-competent merozoites contribute to the signals.

An EBA175 inhibitory mAb has no effect on Ca^{2+} signalling. In line with published data, our results indicate that PfRH1 has a direct role in Ca^{2+} release, which then triggers microneme and

Figure 3 | Detection of cytosolic Ca^{2+} levels during merozoite invasion. (a–d) Cytosolic Ca^{2+} levels were detected using a fluorescence plate reader. (a) Preloaded T994 merozoites with Fluo-4AM were incubated with erythrocytes in the absence (RBC) or presence of Cyto D (RBC + Cyto D). (b,c) Preloaded T994 (b) or T994 Δ RH1 (c) merozoites were incubated with erythrocytes in the absence (RBC) and presence of either C41 (RBC + C41 (0.2 mg ml $^{-1}$)) or C2 (RBC + C2 (0.2 mg ml $^{-1}$)). (d) Preloaded T994 merozoites were incubated with erythrocytes in the absence (RBC) or presence of mAb C41 Fab fragment (C41 Fab (0.2 mg ml $^{-1}$)). The cumulative changes of Ca^{2+} levels ($\Sigma\Delta F(t)$) in merozoites over 600 s were summed up and plotted against time (s). Experimental data were presented as the mean $\pm$ s.e.m.; $n = 3$. * $P \leq 0.0001$ by one-way ANOVA, indicating that C41 successfully blocked the Ca^{2+} signalling in T994 when compared with the absence of C41. # $P \leq 0.0001$ by one-way ANOVA, indicating that C41 successfully blocked the Ca^{2+} signalling in T994 when compared with the presence of C2. + $P \leq 0.0001$ by one-way ANOVA, indicating that C41 Fab fragment significantly inhibited the Ca^{2+} signalling in T994, when compared with the absence of C41 Fab fragment. (e–g) Cytosolic Ca^{2+} levels detected by flow cytometry. Representative FACS contour plots (e,f) were gated by size (FSC-A) versus mean fluorescence (MF) of Fluo-4 AM of merozoite (DAPI (+)) incubated with erythrocytes in the absence (e, Positive) or presence of C41 (f, C41). Vertical gating is to separate the free merozoites with low FSC from the RBC-attached merozoites with high FSC, whereas horizontal gating is to differentiate intercellular Ca^{2+} levels. Q1 (green) represents free merozoites with Fluo-4 AM signals. Q2 (green) represents invading merozoites with increasing Fluo-4 AM signals. The number (blue) in Q2 represents % population of attached merozoites. Q3 (peach) represents free merozoites with no signal. Q4 (pink) represents attached merozoites with no signal. (g) Statistical analysis of the effect of C41 on Ca^{2+} signalling during T994 merozoite invasion by FACS. C41 was able to reduce the number of invading merozoites to 56.6% (blue) and to decrease the overall Ca^{2+} level to 32.42% (red) during invasion, as compared with that in the absence of C41. Experimental data are presented as the mean $\pm$ s.e.m.; $n = 3$.

EBA175 secretion. If this is indeed correct, it would be expected that blocking EBA175 function and thereby junction formation would have no impact on Ca^{2+} signalling. To investigate this further, we used the invasion-blocking mAb R215, which targets EBA175 region II (ref. 30). As previously described, mAb R215 is able to dose-dependently block invasion, with maximum inhibition around $47.7\% \pm 1.11$ at 0.25 mg ml^{-1} (Fig. 5a)³⁰. However, this antibody has no effect on intracellular Ca^{2+} signalling during invasion (Fig. 5b, Supplementary Data 1–6).

This result confirms that invasion-blocking antibodies targeting EBA175 act after Ca^{2+} signalling, whereas those targeting PfPRH1 act before Ca^{2+} signalling, and clearly highlights the different roles that are performed by PfPRH1 and EBA175.

PfPRH1 inhibitory mAbs block EBA175 expression. To investigate whether the inhibition of Ca^{2+} signalling by PfPRH1 inhibitory mAbs has a downstream effect on the surface

Figure 4 | Correlation between invasion and Ca^{2+} signal. (a) T994 merozoites isolated from each Ca^{2+} measurement experiment (Fig. 3b) were also used in parallel to test invasion (Supplementary Table S3). The percentage of invasion (Parasitemia (%), horizontal axis) is plotted against the Ca^{2+} signals at $t = 600$ s ($\Sigma\Delta F(t = 600s)$, vertical axis) (Fig. 3b). Blue diamonds represent merozoites incubated with erythrocytes in the absence of mAbs (RBC). Green triangles represent merozoites incubated with erythrocytes in the presence of inhibitory mAb C41 (RBC + C41 (0.2 mg ml^{-1})). Purple circles represent merozoites incubated with erythrocytes in the presence of non-inhibitory mAb C2 (RBC + C2 (0.2 mg ml^{-1})). The R -square (R^2) with value of 0.836 indicates strong correlation between invasion and Ca^{2+} signalling. (b) Merozoite invasion kinetics assay under heparin treatment. Freshly isolated T994 merozoites were allowed to invade erythrocytes followed by treatment with heparin ($200 \mu\text{g ml}^{-1}$) at different time points (2–10 min with 2 min interval) to inhibit invasion. After the final time point, cultures were incubated for 40 h, and resulting parasitemias were analysed by flow cytometry. The proportion of merozoites that have invaded with increasing time is shown as % of maximum invasion obtained in uninhibited culture. The rate of merozoite invasion over time is linear ($R^2 = 0.991$). Experimental data are presented as the mean $\pm$ s.e.m.; $n = 3$.

Figure 5 | The R215 mAb against EBA175 region II inhibits invasion but not Ca^{2+} signalling. (a) Invasion inhibition assay using anti-EBA175 mAb R215 in T994 parasites. Bar charts show invasion inhibition of different concentrations (0.25 – 0.008 mg ml^{-1}) of the EBA175-specific mAb R215 in T994. Bar charts show mean $\pm$ s.e.m.; $n = 3$. (b) Cytosolic Ca^{2+} levels were detected by using a fluorescence plate reader. Preloaded T994 merozoites with Fluo-4 AM were incubated with erythrocytes in the absence (RBC) or presence of R215 (RBC + R215 (0.2 mg ml^{-1})). Ca^{2+} ionophore A23187 (RBC + A23187) was used as positive control. Changes in cytosolic Ca^{2+} levels during merozoite invasion were assessed over time using a fluorescence plate reader. $\Sigma\Delta F(t)$ that reflects the total changes of cytosolic Ca^{2+} levels in merozoites were plotted against time (s). Experimental data were presented as the mean $\pm$ s.e.m.; $n = 3$.

expression of EBA175, we assessed the translocation of EBA175 onto the merozoite surface. Merozoites arrested by Cyto D during erythrocyte invasion in the presence/absence of invasion inhibitory mAbs were surface stained with either antibodies against EBA175 or the merozoite surface marker MSP1. Surface expression of EBA175 was observed under fluorescence

microscopy or measured under the fluorescence plate reader. Merozoite protein EBA175 of impermeabilized merozoites will not be stained unless they are translocated to the surface of merozoites. Immunofluorescence assays clearly showed that the invasion inhibitory antibody C41 blocks surface expression of EBA175 in T994 (Fig. 6a lower panel), whereas it has no effect on T994 Δ RH1 parasites (Fig. 6b lower panel). In the absence of C41, EBA175 is expressed on the surface of both T994 and T994 Δ RH1 (upper panels of Fig. 6a,b). In contrast, the inhibitory antibody has no effect on the detection of MSP1 on the surface of merozoites (Fig. 6c,d). Quantification of the overall impact of EBA175 surface expression in the parasite population was assessed using a fluorescent plate reader. In T994, overall EBA175 surface expression is reduced by $>35\%$ by the inhibitory antibody C41, whereas no impact is seen in T994 Δ RH1 or on the surface expression of MSP1 (Fig. 6e and Supplementary Table S4). Streptolysin O (SLO) is often used to selectively permeabilize the plasma membrane of *P. falciparum*-infected erythrocytes by forming stable proteinaceous pores without altering the orientation of membrane proteins³¹. Permeabilization of T994 merozoites with SLO before staining with EBA175 antibody showed no difference in overall EBA175 expression (internal and surface) between free and junction-arrested merozoites in C41-treated cultures as compared with the control. This result was confirmed by performing immunofluorescence assays and by measurement using the fluorescent plate reader (Supplementary Fig. S6 and Supplementary Table S5). To further determine whether SLO could effectively permeabilize merozoite membrane to distinguish protein expression internally or on the surface, nucleoporin 100 (Nup 100; a nuclear periphery marker) and histone marker H3K9ac were used³². In merozoite permeabilized with SLO, Nup 100 localizes in the nuclear periphery outside the DAPI-stained area, whereas H3K9ac tends to co-localize with DAPI-stained area (Supplementary Fig. S7a,b). No staining was found in impermeabilized merozoites (Supplementary Fig. S7c,d). Taken together, these data confirm that C41 blocks EBA175 surface expression on merozoites during invasion.

We provide additional support for the surface expression of EBA175 on junction-arrested T994 merozoites using flow cytometry. Isolated merozoites were allowed to invade fresh erythrocytes in the presence of Cyto D as well as in the presence/absence of inhibitory mAbs. In addition, labelling for EBA175 surface expression with anti-EBA175 antibodies allowed us to identify the population of free and arrested merozoites that express EBA175 on their surface. Analysis of $\sim 5,000$ DAPI (+) merozoites showed that, in the absence of inhibitory antibodies, free merozoites make up 47.1% of population, of which 0.2% show EBA175 staining. In contrast, 40.7% of the 52.9% RBC-bound merozoites show EBA175 staining. In the presence of invasion inhibitory antibodies, free merozoites make up 52% of population, of which 0.5% are EBA175 positive, whereas only 17% of the 47.8% RBC-bound merozoites are EBA175 positive (Supplementary Fig. S8). The data show that there is an $\sim 45\%$ inhibition of EBA175 surface expression in RBC-bound merozoites in the presence of the invasion inhibitory antibody (Fig. 6f), in line with the data obtained using the fluorescence plate reader (Fig. 6e). In addition, these results show that free merozoites hardly express EBA175 on the surface; furthermore, it also shows that in the absence of invasion inhibitory antibodies more RBC-attached merozoites are expressing EBA175, in line with them having formed a junction. Taken together, our data provide the first evidence that PfRH1 has a crucial role in triggering intracellular Ca^{2+} signal, which then leads to the subsequent release of EBA175 to form tight junction.

Figure 6 | Effect of anti-PfPRH1 inhibitory mAbs on merozoite surface expression of EBA175 during invasion. (a–d) Surface expression of EBA175 and MSP1 on T994 and T994ΔRH1 was detected by immunofluorescence assays. Isolated T994 (a,c) or T994ΔRH1 (b,d) merozoites were directly incubated with erythrocytes containing 4 μM Cyto D in the absence (Control) and presence (C41) of C41 (0.2 mg ml⁻¹) before analysis of expression of EBA175 and MSP1 at the junction. Both differential interference contrast (DIC) and fluorescence images were captured. Nuclear DNA was counterstained with DAPI (blue). EBA175 is shown in green, whereas MSP1 is shown in red. Fluorescence images, merged fluorescence images (DAPI and green or DAPI with red) and merged fluorescence images with DIC images are shown. Scale bars = 10 μm. (e) EBA175 surface expression on merozoites was detected by using a fluorescence plate reader. Surface expression of EBA175 and MSP1 on merozoite in the presence of C41 was compared with their controls. Experimental data are presented as the mean ± s.e.m.; $n = 3$. *** $P \leq 0.000025$ by one-way ANOVA, indicating that C41 significantly decreases the surface expression of EBA175 in T994. (f) T994 merozoite EBA175 surface expression was also detected by flow cytometry during invasion (detail shown in Supplementary Fig. S8). Statistical analysis of the effect of C41 on merozoite EBA175 surface expression is shown. Merozoite attached to RBCs surface expression of EBA175 in the presence of C41 was compared with its positive control. Experimental data are presented as the mean ± s.e.m.; $n = 4$. (g) Schematic diagram of Ca^{2+} signalling in junction formation during erythrocyte invasion. After the merozoite initially attaches to the erythrocyte (RBC) and reorients itself, PfPRH1 released from rhoptry (Rh) is responsible for sensing the apical end interaction (I). The binding of PfPRH1 to its receptor triggers an increase of intracellular Ca^{2+} (II). Rise in cytosolic Ca^{2+} levels results in release of microneme (Mn) proteins such as EBA175 to bind to glycophorin A (GPA), which leads to tight junction formation (III).

Discussion

Ca^{2+} is a second messenger in eukaryotic cell signalling and is typically released from intracellular stores in response to an

external stimulus (chemical, electrical or mechanical) that triggers a signalling cascade, which leads to a rise in cytoplasmic-free calcium³³. Apical secretion of micronemal and rhoptry proteins

has been observed upon contact of the apicomplexan parasite *Toxoplasma gondii*^{34,35} as well as *P. falciparum*^{36,37} with their host cells, thereby mediating invasion. In *T. gondii* increased intracellular Ca^{2+} has been shown to trigger both microneme protein secretion as well as parasite egress from host cell^{35,38}, and recently it has been shown that a rise in cytosolic Ca^{2+} because of the Ca^{2+} ionophore A23187 triggers secretion of microneme proteins such as EBA175 to the merozoite surface²⁸. The precise signalling mechanisms that lead to the release of invasion proteins in a coordinated manner during invasion are not clear. Recent work by Singh *et al.* has shown that increase in the intracellular Ca^{2+} is the key step leading to the release of EBA175 from the microneme. EBA175 binding to its glycoprotein receptor subsequently leads to the release of rhoptry proteins CLAG3.1 and PfRH2b. This is in line with our findings that PfRH1-induced Ca^{2+} signalling triggers EBA175 release. Previous work has shown that RH proteins appear to be located at the rhoptry neck, and our data would indicate that this enables at least some of the RHs to mediate the initial interactions with the erythrocyte thereby triggering Ca^{2+} release and microneme discharge. The bulk of the rhoptry content, including the majority of RH, is discharged only after microneme release. It will now be interesting to establish any additional roles for RHs beyond the initial sensing. In the study by Singh *et al.*, Ca^{2+} release is triggered by changing the concentration of K^{+} in the buffer from a high to a low to reflect difference in the concentration of K^{+} inside erythrocyte as compared with the blood plasma. In contrast, in our study, merozoites are kept in normal growth media that require PfRH1 engagement with erythrocytes to trigger Ca^{2+} release. In future, it is desirable to further address the effect of K^{+} level changes compared with PfRH1-mediated changes for eliciting Ca^{2+} level increases. This could determine whether the K^{+} -mediated signalling is upstream or independent of the PfRH1-mediated signalling during invasion. The relative importance of the two mechanisms *in vivo* also needs to be resolved. On the basis of our data, we hypothesize that the complex invasion process begins with a relatively small amount of PfRH1 exposed at the rhoptry duct interacting with its receptor on the erythrocyte surface, thereby either directly or indirectly activating a signalling cascade that leads to the release of intracellular Ca^{2+} stores, followed by triggering microneme and rhoptry discharge and junction formation (Fig. 6g). This is consistent with a recent finding showing that Ca^{2+} -mediated microneme discharge is important for invasion in both *T. gondii* and *P. falciparum*³⁹. Our results also demonstrate a function for RHs that is independent of rhoptry discharge and occurs after microneme secretion, possibly indicating that RHs are moved to an active site ahead of the release of the rest of the rhoptry content.

In future, it will now be important to further determine the mechanism by which the RH-mediated signal is passed to the merozoite to trigger Ca^{2+} release. Overall, our results provide new insights on the finely regulated signalling cascade that is required to ensure successful invasion of the merozoite and to open up a path for the analysis of signalling pathways involved in regulated secretion of apical organelles during invasion. Together this will provide us with a better understanding of the signalling mechanisms important in merozoite invasion and how these processes might be a new target for malaria intervention.

Methods

***P. falciparum* strains and culture.** *P. falciparum* parasite lines T994, W2mef (MR4) and T994ARH1 (ref. 40) were cultured in fresh erythrocytes from healthy human donors (after obtaining informed consent) and RPMI supplemented with 5% albumax (Invitrogen)⁴¹. W2mef parasites were allowed to grow in erythrocytes treated with 10 mU ml⁻¹ neuraminidase (Nm; Roche). After 2–3 weeks, the

W2mef parasite adapts and grows in neuraminidase-treated erythrocytes (sialic acid independent)^{7,26}. This switched parasite was named W2mef/NM parasite.

PfRH1 monoclonal antibodies generation and purification. PfRH1 hybridoma cells were developed by BioGenes (Germany) using purified soluble recombinant protein PfRH1-RII-3 (ref. 7). Positively selected hybridoma cells were cultured in 2% FBS-DMEM (Invitrogen). Purification of PfRH1 mAbs IgG from cell culture medium was carried out by using standard purification procedures (HiTrap Protein G HP, GE healthcare). Monoclonal antibody C41 Fab fragment was generated and purified by using standard procedures (Pierce Fab Micro Preparation Kit, Thermo Scientific). The authenticity of the Fab fragment was confirmed by 12% SDS-PAGE (Bio-Rad) followed by coomassie blue staining (Sigma), which yielded one band of ~50 kDa under nonreducing conditions.

Western blot of parasite extracts and culture supernatants. Parasite culture was synchronized at the ring stage with 5% D-sorbitol (Sigma) and cultured to mature schizont stages^{42,43}. Tightly synchronized late schizonts were purified using 68% Percoll gradient centrifugation⁴⁴. Merozoites were purified from schizont extract by filtration under nitrogen cavitation^{45–48}. Schizonts and merozoite extracts were washed twice with PBS, lysed directly into sample buffer and frozen and thawed three times, followed by separation on 12% SDS-PAGE. Mouse mAb gl2 (1:1,000, MR4)⁴⁹ was used as a loading control for merozoite extracts, whereas rabbit α -actin polyclonal antibodies (1:1,000, Sigma) were used for schizont extracts. To make parasite culture supernatant, purified schizonts were allowed to grow in the absence of RBC at 37 °C with shaking. Cells were collected by centrifugation after 16 h, and supernatants were either stored in aliquots at –80 °C or directly separated on 6% SDS-PAGE. Micronemal marker EBA175-specific antibody⁵⁰ (1:5,000, MR4) was a loading control for parasite culture supernatant. Following electrophoresis, gels were transferred onto nitrocellulose membranes (0.2 μ m) (Bio-Rad). Specific proteins were detected by using PfRH1 mAbs (1:5,000), followed by HRP-linked secondary antibodies (1:10,000) and enhanced chemiluminescence (GE healthcare).

Invasion inhibition assay. Synchronized late-stage schizonts were purified, and 160 μ l of parasite suspension was added in duplicate in a 96-well flat bottom microtitre plate containing PfRH1 mAbs (0.008–0.5 mg ml⁻¹), purified C41 Fab fragment (0.008–0.5 mg ml⁻¹) or EBA175 mAb R215 (0.008–0.25 mg ml⁻¹), respectively. A total of 1,000 erythrocytes were scored for presence of rings on Giemsa-stained smears after 24 h for reinvasion. Invasion (Inv) was assessed as (% parasitemia). (% Parasitemia = (total number of ring-infected RBCs/total number of RBCs) $\times$ 100. Invasion in the presence of mAbs was compared with positive controls of invasion of the same parasite lines in normal complete RPMI. Invasion inhibition efficiencies were determined as follows: (% inhibition efficiency = (1 – Inv(mAbs)_{obs}/Inv(positive)) $\times$ 100. Data shown are from three biological replicates. Each biological replicate has two technical replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Erythrocyte binding assays. Hundred microliters of T994 parasite culture supernatant was incubated with 100 μ l of packed normal erythrocytes in the presence of PfRH1 mAbs (at final concentration ranging from 0.3 down to 0.035 mg ml⁻¹) at 37 °C for 1 h^{7,26}.

Junction merozoite blocking assay. Merozoites were arrested in the erythrocyte junction by treatment with cytochalasin D (Cyto D) (Sigma)^{26,27}. Purified late-stage schizont parasites were added to the completed RPMI containing fresh erythrocytes in 96-well plates. Cyto D was added to the final concentration of 0.1 μ M. PfRH1 mAbs were also present at 0.2 mg ml⁻¹ concentration if required. After 8 h incubation, Giemsa (Sigma)-stained smears were prepared for counting merozoites arrested at junction formation by using light microscopy. A total of 1,000 erythrocytes were scored. The number of junction-arrested merozoites in the presence of mAbs was compared with that in a positive control of the same parasite. Junction merozoite inhibition efficiencies were determined as follows: % inhibition of junction merozoite = (1 – total number of junction merozoites (mAbs)/total number of junction merozoites (positive control)) $\times$ 100. Data shown are from three biological replicates. Each biological replicate has two technical replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Expression of four overlapping regions from RII-3. Four N-terminal overlapping fragments from RII-3 were amplified by PCR, expressed as His-tag recombinant proteins by BL21 (DE3) (Stratagene)⁷, named N1 (500–692 amino acid (aa)), N2 (500–726 aa), N3 (500–746 aa) and N4 (500–773 aa), followed by 12% SDS-PAGE and probed with mAbs C49, C41, C2 and C50, respectively (1:5,000). A mAb against 6X His-tag (zHis, 1:5,000, Clontech) was used as loading control. For the PCR, the forward primer was 5'-GACCATATGTTACAAATAG TACAACAAAACTTTTGAATC-3' and the reverse primers were 5'-AACCT CGAGAGAAAAATGTTGAATGCTCTCATTATTTT-3' (N1), 5'-AACCTCGA GGGATTTAAGAAGATTGTTGATGTTT-3' (N2), 5'-AACCTCGAGTTGT

TTTAATATATATTTAGAAATTGTGTCTATGAA-3' (N3), and 5'-AACCTC GAGGTTTGTTCATTTTATTCTTCAAGAT-3' (N4).

Peptide generation and epitope mapping of PfPRH1. Peptide design was based on the mAbs recognition of four His-tag recombinant overlapping fragments derived from RII-3 by western blot (Supplementary Fig. S3a–e). All biotinylated overlapping synthetic peptides were synthesized by Peptide Synthesis Core facilities, SBS, NTU. The lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO, Sigma). Peptide stocks were diluted in PBS + 0.1% BSA to make up $2.5 \mu\text{g ml}^{-1}$. The streptavidin-coated 96-well plate, pre-blocked with Superblock blocking buffer (Thermo Scientific), was washed three times with 200 μl wash buffer (25 mM Tris, 150 mM NaCl, pH 7.2, 0.1% BSA, 0.05% Tween 20). Hundred microliters of the peptide solution was added into each well of the streptavidin-coated plate and incubated for 2 h at room temperature (RT) with gentle shaking. After four washes, 100 μl of mAb (C49, C41, C2 or C50 in 25 mM Tris, 150 mM NaCl, pH 7.2, 0.1% BSA) at a concentration of $2.5 \mu\text{g ml}^{-1}$ was added into each well and incubated for 1 h at RT. The peptide was washed four times followed by the addition of 100 μl of HRP goat anti-mouse IgG (13,000; Biologend). After six washes, 50 μl of 3,3',5,5'-Tetramethylbenzidine (TMB) high sensitive substrate solution (Biologend) was added into each well. The plates were incubated for 3–5 min before analysing by using a Benchmark PlusMicroplate Spectrophotometer (BioRad Laboratories Inc) at 370 nm.

Live video microscopy. For live video microscopy²³, we used an Axiovert 200M cell observer with a 37 °C chamber with a continuous supply of 5% CO₂. Five to ten percentage of the late-schizont stage parasites were diluted at 1:100 in complete RPMI. Live video was performed using 35 mm glass bottom dishes. The video was captured in the presence or absence of PfPRH1 mAbs (C2, C41, C49 and C50) at a final concentration of 0.2 mg ml^{-1} . AxioCam HRm camera and Axiovision software, or Cool SNAP camera and MetaMorph software, were used for image acquisition. The video files were processed using Image J.

Isolation of *P. falciparum* merozoites. Purified *P. falciparum* schizonts were resuspended in warm complete RPMI and allowed to rupture and release merozoites over a period of 2 h at 37 °C. Cultures containing unruptured schizonts and released merozoites were centrifuged at 2,200 r.p.m. (942 g) on an Eppendorf 5810R centrifuge for 3 min to separate released merozoites from unruptured schizonts and uninfected erythrocytes. Supernatants containing free merozoites were then centrifuged at 5,000 r.p.m. (4,500 g, Sigma 3K15) for 3 min to collect merozoites. The merozoites were immediately resuspended in warm complete RPMI medium for further studies.

Viability assay of free merozoites. Erythrocyte invasion assays were performed in parallel to assess the viability of isolated free merozoites tested for Ca²⁺ measurement. After 24–30 h post-invasion, newly invaded ring-infected erythrocytes were scored by Giemsa (Sigma, USA) staining. Invasion rates were determined as (%) parasitemia. Data were shown from three biological replicates.

Kinetics of merozoite invasion by heparin treatment. To define the kinetics of merozoite invasion, the invasion inhibitor heparin was used to block merozoite invasion²⁶. In brief, freshly isolated T994 merozoites were allowed to invade erythrocytes in the 96-well plate followed by addition of heparin ($200 \mu\text{g ml}^{-1}$) at different time points (2–10 min with 2 min interval) to inhibit further invasion. After the final time point, cultures were incubated at 37 °C for 40 h. The plate was centrifuged at 2,200 r.p.m. (942 g) for 3 min to separate free merozoites from uninfected and infected erythrocytes. The culture was washed with complete RPMI twice and stained with 40 nM 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, USA). The resulting parasitemias were analysed by flow cytometry. The cells were first gated by size in SSC/FSC, followed by further gating in FSC-A/FSC-H to discriminate doublets from single cells. Uninfected erythrocytes were gated as baseline by DAPI fluorescence in FSC-A/DAPI-A. The proportion of merozoites that have invaded with increasing time is shown as % of maximum invasion obtained in uninhibited culture. Data shown were from three biological replicates. Experimental data were presented as the mean $\pm$ s.e.m.; $n = 3$.

Real-time Ca²⁺ measurement in merozoites during invasion. Fresh isolated merozoites were preloaded with 6 μM Fluo-4 AM (Invitrogen) in complete RPMI at 37 °C for 20 min. Preloaded merozoites were washed twice with warm complete RPMI and then immediately resuspended in fresh erythrocytes or in the presence of 4 μM Cyto D, C41, C2, C41 Fab fragment or EBA175 mAbs R215 at 0.2 mg ml^{-1} , or 10 μM Ca²⁺ ionophore A23187 (Calbiochem, USA), or pretreated with 50 μM Ca²⁺ chelator BAPTA-AM (Calbiochem, USA) followed by A23187 (ref. 28), and cultured in Greiner 96 Black Flat Bottom Polystyrol plates. Changes in cytosolic Ca²⁺ levels during merozoite invasion were timely assessed using a fluorescence plate reader (Infinite M200; Tecan). Fluorescence intensity was measured for 600 s with excitation at 488 nm and emission at 530 nm by TECAN-*i*-control 1.8.20.0 software. The maximum increase of Ca²⁺ green fluorescence (F)

during a period of 600 s after the start of the stimulation was normalized to the fluorescence intensity before stimulation (F_0). Changes in cytosolic Ca²⁺ levels ($\Delta F(t)$) were expressed as changes in fluorescence intensity as a percentage of the starting fluorescence levels: $\Delta F(t) = (F - F_0)/F_0$ where F = fluorescence intensity (t), F_0 = starting fluorescence signal. The total changes of Ca²⁺ levels ($\sum \Delta F(t)$) in merozoites along the 600 s were summed up and plotted against time (s). Data shown were from three biological replicates. Experimental data were presented as the mean $\pm$ s.e.m. TECAN scanning was performed at 37 °C.

Flow cytometry Ca²⁺ measurement in merozoites during invasion. Isolated T994 merozoites were preloaded with Fluo-4 AM as well as labelled with DAPI as described above, followed by incubation with fresh warm erythrocytes containing 4 μM Cyto D in the absence and presence of mAb C41 (0.2 mg ml^{-1}). The culture was immediately processed using a BD LSR II flow cytometer (BD Biosciences). Merozoites that are invading will be arrested by Cyto D after junction formation, whereas merozoites in the presence of inhibitory antibodies will either detach or remain attached to the RBC without forming a junction. Each culture was excited with light at 488 and 355 nm, and fluorescence signal was detected with a 530/30 and 440/40 nm band pass filters. In each experiment, data were acquired for $\sim 20,000$ DAPI (+) merozoites with the same settings for each condition. A histogram representing two distinct cell populations among these 20,000 cells indicates free merozoites and RBC-bound merozoites. Preloaded merozoites alone were measured as the baseline for detection of Fluo-4 AM signals. The cells were gated by FSC-A versus Fluo-4 AM mean fluorescence (MF). We observed the % parent population of attached merozoites with increasing Ca²⁺ signal from the culture in the absence (positive) or presence of C41 (C41) and investigated the effect of C41 on signalling during invasion. Data were analysed by using BD FACS Diva software. (%) Inhibition of attached merozoites = $(1 - \% \text{ parent population (C41)}) / \% \text{ parent population (positive)} \times 100$. (%) Decrease of Fluo-4 AM mean fluorescence (MF) = $(1 - \text{MF (C41)}) / \text{MF (positive)} \times 100$. Data shown are from three biological replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Measurement of intracellular Ca²⁺ levels on free merozoites. Merozoites preloaded with Fluo-4 AM as described above were immediately resuspended in complete RPMI only or treated with 10 μM A23187 for 15 min at 37 °C with or without pretreatment with 50 μM BAPTA-AM for 15 min at 37 °C (ref. 28). Changes in cytosolic Ca²⁺ levels were assessed using flow cytometry. Preloaded merozoites in complete RPMI were measured as baselines. Merozoites were excited at 488 nm, and fluorescence signal is detected with a 530/30 nm band pass filter for periods of 2 min on BD LSR II (BD Biosciences). Merozoites were gated on the basis of their forward scatter (FSC) and side scatter (SSC). Data were evaluated by using dot plot of Fluo-4 AM mean fluorescence (MF) versus time using BD FACS Diva software to visualize the cytosolic Ca²⁺ levels in merozoites. The effect of BAPTA-AM (MF_{BAPTA-AM}) on free merozoites Ca²⁺ signalling was compared with that of A23187 (MF_{A23187}). (%) Inhibition of BAPTA-AM = $(1 - (\text{MF}_{\text{BAPTA-AM}} / \text{MF}_{\text{A23187}})) \times 100$. Data shown are from three biological replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Immunofluorescence assays and microscopy. Synchronized late-stage schizonts were smeared and air-dried followed by fixation with acetone (Merck) for 5 min at RT. Smears were incubated with PfPRH1 mAbs (1:800) and co-incubated with rabbit α -EBA175 (1:800) for 1 h in 3% BSA/PBS buffer, followed by three times for 5 min washes in 1 $\times$ PBS. They were then incubated with a mixture of Alex Flour 594 goat anti-mouse IgG (H + L) and Alex Flour 488 goat anti-rabbit IgG (H + L) secondary antibodies (1:1,000, respectively, Molecular Probe) for 1 h. Slides were washed in 1 $\times$ PBS for three times for 5 min. Slides were air-dried, mounting medium containing DAPI was applied (Vector laboratories), and the coverslips were sealed. The fluorescence images were captured using a LSM510 Confocal Microscope (Carl Zeiss).

Immunofluorescence assay for merozoites. Isolated T994 merozoites were incubated with or without streptolysin O (SLO, Sigma)³¹. The culture was washed three times with incomplete RPMI and then fixed with 1% p-formaldehyde (Sigma) for 45 min at RT followed by centrifugation at 5,000 r.p.m. (4,500 g, Sigma 3K15) for 3 min. After two washes, the culture was incubated with either rabbit α -Nucleoporin 100 (α -Nup 100, 1:500), a nuclear periphery marker, or rabbit α -acetyl-Histone H3 (Lys9) antibody (α -H3K9ac, 1:500, Millipore), a histone marker³² for 45 min at 37 °C. The culture was washed three times in incomplete RPMI followed by incubation with Alex Flour 594 goat anti-rabbit IgG (H + L) (1:1,000) or Alex Flour 488 goat anti-rabbit IgG (H + L) (1:1,000) together with DAPI for 45 min at RT. The culture was washed three times and then applied to the slides. The images were captured by Nikon TE2000-U Inverted Fluorescence Microscopy.

Detection of EBA175 on merozoite surface by fluorescence-based methods.

Isolated merozoites from T994 and T994ARH1 were incubated with fresh erythrocytes containing 4 μM Cyto D in the absence and presence of mAb C41

(0.2 mg ml⁻¹) at 37 °C for 20 min followed by incubation with or without Streptolysin O (SLO, Sigma)⁵¹. The culture was washed three times with 1% BSA/PRS and then fixed with 1% p-formaldehyde (Sigma) for 45 min at RT followed by centrifugation at 5,000 r.p.m. (4,500 g, Sigma 3K15) for 3 min. After two washes, the culture was incubated with rabbit α -EBA175 (1:500) for 45 min at 37 °C. The culture was washed three times with incomplete RPMI followed by incubation with Alex Fluor 488 goat anti-rabbit IgG (H + L) (1:1,000) together with DAPI for 45 min at RT. The culture was washed three times with incomplete RPMI. The culture was finally resuspended in 100 μ l complete RPMI and transferred into Greiner 96 Black Flat Bottom Polystyrol plates for analysis by using a fluorescence plate reader using the program TECAN-i-control 1.8.20.0. The duplicated culture (except SLO treated culture) was also tested using rabbit α -MSP1 (1:1,000, MR4) followed by incubation with Alex Fluor 594 goat anti-rabbit IgG (H + L) (1:1,000) together with DAPI. The samples were applied to the slides. The images were captured by Nikon TE2000-U Inverted Fluorescence Microscopy. To further confirm and quantify the effect of inhibitory mAbs C41 on the surface expression of EBA175, fluorescence intensity was also measured by using a fluorescence plate reader. Fluorescence signal of EBA175 (F_{EBA175}) was excited at 488 nm and emitted at 519 nm, MSP1 surface staining (F_{MSP1}) was excited at 590 nm and emitted at 617 nm and nuclear signal ($F_{EBA175-DAPI}$ or $F_{MSP1-DAPI}$) was excited at 375 nm and emitted at 461 nm. Fluorescence signal was normalized to nuclear DAPI signal as follows: $EBA175 \text{ ratio} = F_{EBA175}/F_{EBA175-DAPI}$ and $MSP1 \text{ ratio} = F_{MSP1}/F_{MSP1-DAPI}$. Protein expression on merozoite surface in the presence of mAbs C41 was compared with its control of the same culture incubated in the absence of C41. Surface expression inhibition efficiencies were determined as follows: (%) inhibition of EBA175 surface expression = $(1 - EBA175 \text{ ratio (C41)}/EBA175 \text{ ratio (control)}) \times 100$ and (%) inhibition of MSP1 surface expression = $(1 - MSP1 \text{ ratio (C41)}/MSP1 \text{ ratio (control)}) \times 100$. Protein expression on merozoite permeabilized with SLO in the presence of mAbs C41 were compared with its control of the same culture in the absence of C41. EBA175 expression inhibition efficiencies were determined as follows: (%) inhibition of EBA175 expression = $(1 - EBA175 \text{ ratio (C41 - SLO)}/EBA175 \text{ ratio (control - SLO)}) \times 100$. Data shown are from three biological replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Detection of EBA175 on merozoite surface by flow cytometry. The culture prepared for detection of EBA175 by fluorescence microscopy, mentioned above, was resuspended in complete RPMI and used for analysis by BD LSR II (BD Biosciences). In each experiment, data were acquired for 5,000 DAPI (+) merozoites with the same settings for each condition. A histogram representing two distinct cell populations among these 5,000 cells indicates free merozoites (Free Mero) and RBC-bound merozoites (Attached Mero + RBCs). We observed these two populations individually and investigated the effect of C41 on EBA175 surface expression in T994. Each culture was excited with light at 488 and 355 nm, and fluorescence signal was detected with 530/30 and 440/40 nm band pass filters. The cells were gated on the basis of their FSC and SSC. Data were analysed using BD FASCDiva software. Histograms showing logarithmic green fluorescence intensities (FITC-A) versus blue fluorescence intensities (DAPI-A) were plotted. For each experiment, fixed untreated cultures were stained only with FITC-conjugated anti-rabbit IgG to establish background staining and to set up a gate before calculating the percentage of FITC(+) merozoites (that is, those expressing EBA175 on their surface) in the parent population. EBA175 surface expression on junction-arrested merozoites in the presence of mAb C41 was compared with a positive control in the absence of C41. EBA175 surface expression inhibition efficiencies were determined as follows: (%) inhibition of EBA175 surface expression = $(1 - \% \text{ parent population (C41)}/\% \text{ parent population (positive)}) \times 100$. Data shown are from four biological replicates. Experimental data are presented as the mean $\pm$ s.e.m.

Statistical analysis. Statistical comparison was done using one-way ANOVA as appropriate. The significance level was set at $P < 0.05$. The P -value is provided for some individual experiments.

Raw data of absolute measurement Ca^{2+} signalling. Absolute measurement raw data of all Ca^{2+} signalling experiments have been provided in Supplementary Data 1–6.

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Author contributions

X.G. and K.G. contributed extensively to the experiments, analysed the data and wrote the manuscript. X.G. and P.R.P. conceived the experiments. S.S.L.Y. performed the epitope mapping experiment and analysed the data. P.R.P. supervised the project, analysed the data and wrote the manuscript.

Additional information

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