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Exported proteins of the malaria parasite *Plasmodium falciparum*

Characterization of blood-stage antigen 332

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The front cover is an immunofluorescence microscopy image of a *Plasmodium falciparum* parasitized red blood cell, illustrating the exported antigen Pf332 (red) beyond the confines of the intracellular malaria parasite (green and blue; EBA-175 and DNA, respectively). Image captured by Sandra Nilsson and designed by Sofie M. Nilsson.

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ABSTRACT

Plasmodium falciparum malaria is one of the most important infectious diseases in the world. Following invasion of the human red blood cell (RBC), the *P. falciparum* parasite dramatically remodels its host cell by introducing a parasite-derived trafficking machinery in RBC cytosol, interacting with the RBC cytoskeleton and expressing adhesins on the RBC surface. All host cell modifications are mediated by a subset of parasite-encoded proteins, which are exported beyond the confines of the parasite – a feature that is fundamental to the malaria pathogenesis. Central to this thesis is the Pf332 protein, the largest protein exported into the host cell cytosol. Although identified more than two decades ago, the function of Pf332 still remains elusive. Regardless, the location of Pf332 in close proximity to the RBC plasma membrane, its potential surface expression, characteristic protein structure and immunogenic nature make it an important antigen to study. We have revised the structure of the gene encoding Pf332, and identified a previously unknown first exon encoding an RBC-binding Duffy binding-like (DBL)-domain homologous to DBL-domains present in a family of invasion proteins. Studies on Pf332 have been hampered by the cross-reactive nature of antibodies generated against the molecule due to its high content of glutamic acid-rich repeats. In an attempt to evaluate the potential of the DBL-domain as a specific marker for Pf332, we set out to analyze the tertiary structure of the domain and the specificity of naturally acquired antibodies. Although the predicted structure of the DBL-domain was similar to that of the homologous domains present in invasion proteins, acquired antibodies were specific for Pf332. Thus, the DBL-domain can be used as a specific Pf332 marker and we expect this to facilitate further investigations of the antigen. Subunit vaccines based on recombinant proteins are often hampered by low antigenicity, thus adjuvants are of major importance. We set out to study the immunogenicity of a recombinant Pf332 DBL-domain in combination with adjuvants compatible for human use, in rodents and rabbits. The domain was found to be immunogenic and of the three adjuvants evaluated, Montanide ISA 720 appeared to be the most suitable adjuvant, as it induced a more long-lasting Th2-biased antibody response. Thus, the results support the use of Montanide ISA 720 for future immunization studies of other malaria vaccine candidates. To investigate the subcellular location and the solubility characteristic of Pf332, we employed a biochemical approach in combination with immunofluorescence microscopy. We found Pf332 to be a host cytoskeleton interacting protein that is synthesized as a peripheral membrane protein and associates with the cytosolic side of Maurer's clefts via protein-protein interactions throughout trophozoite maturation and schizogony. Importantly, our data show that Pf332 is not expressed on the surface of the host cell, but may have important functions in host cytoskeleton remodeling at the end of the intraerythrocytic developmental cycle. The gene encoding Pf332 is duplicated in the HB3 parasite, having only slight sequence variation between the two gene copies. This enabled us to develop a sensitive allelic discriminative assay, which can be used to study transcriptional activity of duplicated genes in the *P. falciparum* genome. We employed the assay to study the maternal malaria associated *var* gene *var2csa*, which is similarly found duplicated in the HB3 parasite. Both *var2csa* paralogs were simultaneously transcribed in a single cell, thus contradicting the mutually exclusive expression of *var* genes in *P. falciparum*. In conclusion, by using Pf332 as a model protein for studying malaria pathogenesis, we have not only obtained novel information regarding the protein itself, but gained important knowledge and developed versatile techniques, which can be used to study a wide array of other malaria antigens.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. Moll K., Chêne A., Ribacke U., Kaneko O., **Nilsson S.**, Winter G., Haeggström M., Pan W., Berzins K., Wahlgren M. & Chen Q. A novel DBL-domain of the *P. falciparum* 332 molecule possibly involved in erythrocyte adhesion.
PLoS ONE 2007, 2(5):e477.

II. **Nilsson S.**, Moll K., Angeletti D., Albrecht L., Kursula I., Jiang N., Sun X., Berzins K., Wahlgren M. & Chen Q. Characterization of the Duffy binding-like domain of *Plasmodium falciparum* blood-stage antigen 332.
Malaria Research and Treatment 2011, In Press.

III. Du C., **Nilsson S.**, Lu H., Yin J., Jiang N., Wahlgren M. & Chen Q. Immunogenicity of the *Plasmodium falciparum* Pf332 DBL-domain in combination with different adjuvants.
Vaccine 2010, 28(31).

IV. **Nilsson S.**, Moll K., Wahlgren M. & Chen Q. *Plasmodium falciparum* antigen 332 associates with the cytoplasmic side of Maurer's clefts via protein-protein interactions.
Submitted manuscript.

V. Brolin K.J., Ribacke U., **Nilsson S.**, Ankarklev J., Moll K., Wahlgren M. & Chen Q. Simultaneous transcription of duplicated *var2csa* gene copies in individual *Plasmodium falciparum* parasites.
Genome Biology 2009, 10(10).

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LIST OF ABBREVIATIONS

ARDS	Acute respiratory distress syndrome
ATS	Acidic terminal segment
BFA	Brefeldin-A
CIDR	Cysteine-rich interdomain region
CM	Cerebral malaria
CNP	Copy number polymorphism
CR1	Complement receptor 1
CSA	Chondroitin sulfate A
DBL	Duffy binding-like domain
DBP	Duffy binding protein
EBL	Erythrocyte binding-like
EqtII	Equinatoxin II
ER	Endoplasmatic reticulum
EXP1	Exported protein 1
GFP	Green fluorescent protein
IDC	Intraerythrocytic developmental cycle
IFA	Immunofluorescence microscopy assay
KAHRP	Knob-associated histidine-rich protein
MAHRP	Membrane-associated histidine-rich protein
MC	Maurer's cleft
MESA	Mature parasite-infected erythrocyte surface antigen
NPP	New permeation pathway
ORF	Open reading frame
p.i.	Post invasion
PAM	Pregnancy associated malaria
PEXEL	*Plasmodium* export element
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PM	Plasma membrane
PNEP	PEXEL-negative exported protein
pRBC	Parasitized red blood cell
PTEX	*Plasmodium* translocon of exported proteins
PV	Parasitophorous vacuole
PVM	Parasitophorous vacuole membrane
RBC	Red blood cell
RESA	Ring parasite-infected erythrocyte surface antigen
REX	Ring exported protein
RIFIN	Repetitive interspersed protein
RNA-FISH	RNA-fluorescence in situ hybridization
SBP1	Skeleton binding protein 1
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SP	Signal peptide
STEVOR	Subtelomeric variable open reading frame protein
TM	Transmembrane domain
TVN	Tubulovesicular network
TX-100	Triton X-100
VTS	Vacuolar transport signal

1 INTRODUCTION

1.1 THE GLOBAL BURDEN OF MALARIA

Malaria is one of the most important infectious diseases worldwide. In 2010, WHO estimated that 3.3 billion people (half the world's population) are at risk of contracting malaria in 106 countries (WHO, 2010). Every year this leads to 225 million clinical cases of malaria and close to 800 000 deaths. The disease burden is greatest in sub-Saharan Africa and the vast majority of the fatal cases occur in children under the age of five (Figure 1).

It is obvious that the burden of malaria extends well beyond morbidity and mortality, as the disease poses a major hindrance for economic development (Sachs and Malaney, 2002). In Africa today, malaria is recognized as both a disease of poverty and a cause of poverty (RBM, 2011). For low-income countries this has meant that the gap in wealth between countries with and those without malaria become wider each year. A number of direct and indirect costs can be attributed to the disease – for both families and households, and national economies. Examples are doctor's fees, antimalarial drugs, spending by government on maintaining health facilities and vector control, negative impact on trade, and productivity losses associated with malaria-attributed illness or death. Malaria also hampers children's schooling and cognitive development through absence from school and permanent neurological damages (Sachs and Malaney, 2002).

Although global control efforts have resulted in a reduction in the estimated number of deaths from nearly 1 million in 2000 to 781 000 in 2009, there has been evidence of an increase in malaria cases in some African countries (WHO, 2010). This highlights the fragility of malaria control and the need to maintain control programs even where the number of malaria cases is reducing.

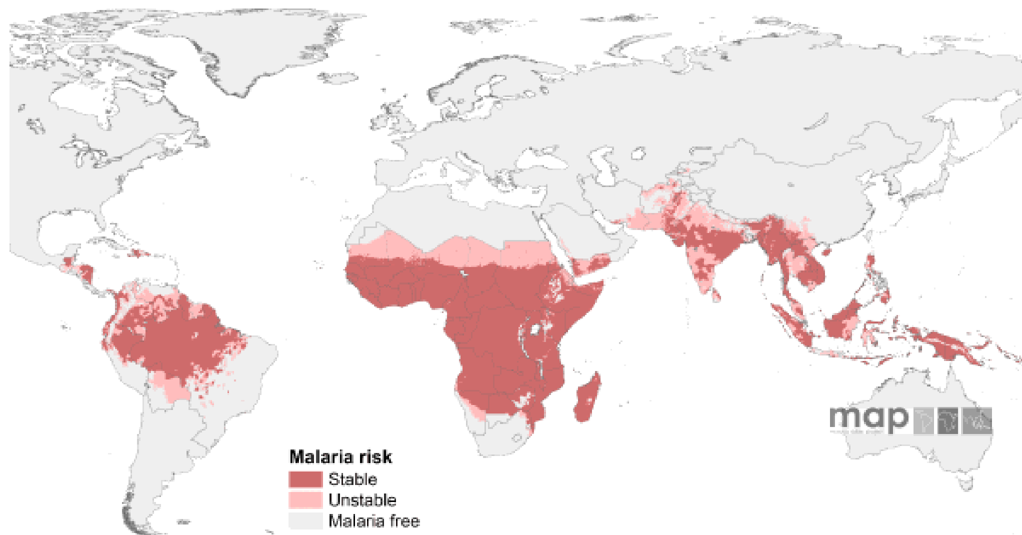

Figure 1. Malaria distribution. Although eliminated in wealthier countries, malaria is still persistently remaining in low-income countries. (Adopted from Malaria Atlas Project (MAP; www.map.ox.ac.uk) and published with permission from MAP under a Creative Commons Attribution 3.0 License (http://creativecommons.org/licenses/by-sa/3.0/)).

1.2 THE ORIGIN OF MALARIA

Malaria is one of the most ancient diseases of man. The first descriptions of the illness appear in the classical Chinese medical writing Nei Ching (2700 B.C.) and the Egyptian medical text Ebers Papyrus (1550 B.C.) (Desowitz, 1991, Sherman, 2007). Also the Greek physician Hippocrates recognized malaria, detailing the symptoms that hallmark the disease in his writings. He noted an association between the disease and people living close to marshes, and therefore considered the causative agent of malaria to be the Miasma (harmful or poisonous atmosphere; from Greek *Miasma* meaning "pollution").

From its origin in tropical Africa, malaria spread all across the globe to become one of the world's most important diseases (Sherman, 2007). It was probably introduced in Europe via the Nile Valley and the close contact between Europeans and the people of Asia Minor (Sherman, 2007). Over the centuries malaria spread across Europe and the disease was so prevalent in the marshlands of Roman Campagna that the condition was called the "Roman fever". The Romans believed that vapors and bad smells emanating from stagnant swamp water caused the disease and the Miasma theory is evident even today, as malaria literally means "bad air" (mal´aria) in Italian.

Although the Miasma theory is not accurate, it had some good consequences. It prompted efforts to improve housing and drain swamplands, and as a side effect, it reduced the reservoirs of stagnant water in which mosquitoes could breed. "Bad air" was considered the cause of malaria until 1880. That year, military physician Alphonse Laveran discovered crescent formed bodies (the sexual form of the malaria parasite) while examining blood samples from Algerian soldiers in his microscope. Laveran realized that he had found the cause of malaria: a small, living organism. It was, however, left for British physician Ronald Ross to solve the problem of malaria transmission. Ross had spent a great deal of time trying to find a definite link between malaria and the mosquito, and in India 1897, he discovered the oocyst of a malaria parasite in the gut wall of a female *Anopheles* mosquito.

In the 1900s, larvicides along with drainage were introduced to limit mosquito-breading sites in water. This was very successful in reducing malaria transmission in some parts of the world. In 1939, work by the Swiss chemist Paul Hermann Müller lead to the synthesis of the pesticide dichlorodiphenyltrichloroethane (DDT), and it was introduced as part of a malaria eradication campaign. As such it was very successful and DDT led to malaria elimination on many island areas. However, the use of DDT had to be interrupted due to the emergence of DDT-resistant mosquitoes and the negative environmental side effect of the pesticide. The work of Ronald Ross, Alphonse Laveran and Paul Hermann Müller was recognized in 1902, 1907 and 1948, respectively, when they were awarded the Nobel Prize in Physiology and Medicine for their important discoveries.

1.3 APICOMPLEXAN PROTOZOA

Malaria is caused by the infection of a protozoan parasite belonging to the phylum apicomplexa. This phylum comprises a wide spectrum of eukaryotic organisms causing major human and veterinary diseases, of which some of the most important are listed below.

- *Plasmodium* species (spp.) are the causative agents of malaria. There are over 100 *Plasmodium* spp. having vertebrates such as mammals, reptiles and birds as hosts; however, out of these only five are infective to man. *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* have for long been known to cause human malaria. The fifth species, *P. knowlesi*, causes malaria in macaques but has recently proven to also infect and cause disease in man and is therefore considered to be an important zoonotic human pathogen.
- *Toxoplasma gondii* causes toxoplasmosis in rodent, felids and humans. Congenital toxoplasmosis in humans can result in severe eye and brain damage in the fetus.
- *Theileria* spp. cause east coast fever or tropical theileriosis in cattle and pose a major constraint on the development of cattle industry and production.
- *Babesia* spp. cause babesiosis or red-water fever in cattle, horses, dogs and occasionally humans.
- *Eimeria* spp. infect birds and are a major cause of morbidity in poultry.
- *Cryptosporidum* spp. mainly infect the intestines of mammals, usually resulting in a self-limiting diarrhea. The effects of cryptosporidiosis can be fatal in immunocompromised individuals.

Common to the apicomplexan, is their complex life cycles often involving several species as hosts, with some, including *Toxoplasma*, *Eimeria*, and *Cryptosporidium*, passing directly between vertebrate hosts. In contrast, the life cycle of others, including *Plasmodium*, *Babesia* and *Theileria* involve an arthropod vector that transmits the parasite to a vertebrate host during blood feeding. Regardless of their host or mode of transmission, all apicomplexan parasites share features such as the presence of a specialized apical complex (for which the group is named), consisting of intricate structures that enable the parasite to penetrate the tissues of their hosts.

1.3.1 Life cycle of *Plasmodium* species

The life cycle of the malaria parasite is highly complex, involving a number of different asexual and sexual developmental stages in both the insect vector and the vertebrate host (Figure 2).

In the human host- The malaria parasite is transmitted to the human host when an infected female *Anopheles* mosquito takes a blood meal as a prelude to the reproductive process. At the same time, 15-120 sporozoite forms of the parasite are injected along with her saliva. Most sporozoites are injected into the dermal tissue and not directly into the circulation (Medica and Sinnis, 2005, Sidjanski and Vanderberg, 1997). Real time imaging using the *P. berghei* rodent model has revealed that sporozoites actively glide through the dermis until they encounter a blood vessel and move into the circulatory system, which will take them to the liver (Amino et al., 2006). Once in the circulatory system, the sporozoites reach the liver within minutes. After traversing the Kupffer cell lining of the liver sinusoids and the space of Disse, sporozoites migrate through several hepatocytes before invading a final hepatocyte in which a parasitophorous vacuole is formed (Frevert et al., 2005, Pradel and Frevert, 2001, Baer et al., 2007b, Mota et al., 2001, Mota et al., 2002). Over 5-15 days (depending on *Plasmodium* species), each sporozoite differentiates and divides into thousands of merozoite forms of the parasite. These are then released from the hepatocyte in merozoite-filled vesicles referred to as merosomes, which bud off from the parasitized cell into the lumen of the liver sinusoids (Sturm et al., 2006, Baer et al., 2007a). In *P.*

vivax and *P. ovale* infection, some sporozoites convert to dormant forms called hypnozoites, which can cause relapses after weeks, months or even years. These resting stages do not appear in *P. falciparum*, *P. malariae* or *P. knowlesi*.

Once released into the blood-stream, the merozoite quickly invades circulating red blood cells (RBC) and thereby initiates the blood-stage or the intraerythrocytic developmental cycle (IDC). Within the new host cell, the parasite undergoes a 24-72 h (depending on *Plasmodium* species) maturation process from a ring-stage trophozoite to a pigmented trophozoite before finally undergoing mitotic nuclear divisions into daughter merozoites at the schizont stage. At this point, the parasitized RBC (pRBC) ruptures and releases 8-16 daughter merozoites into the circulation to resume another round of asexual reproduction. This leads to an exponential growth in parasitemia that will continue until the parasite is controlled either by the host's immune response or by antimalarial medication.

A small subset of parasites develops into male or female sexual forms, termed gametocytes. This dramatic developmental switch may be predetermined genetically or reflect a response to some specific stimuli of host or parasitic origin (reviewed in (Day et al., 1998a, Talman et al., 2004)). Previous work in *P. falciparum* has demonstrated that all merozoites emerging from a single schizont either continue the asexual cycling or develop into gametocytes (Bruce et al., 1990). Furthermore, gametocytes originating from a single schizont become either all male or all female (Smith et al., 2000b, Silvestrini et al., 2000). This indicates that trophozoites from the preceding asexual generation are already committed to produce a progeny of parasites with the same developmental fate. The sexual forms of *P. falciparum* can remain in the circulation for a period of 10-15 days. The details of how gametocytes survive in the human body for such a prolonged period of time is not completely understood, but it is believed in part to be the result of immune evasion through sequestration (Rogers *et al.*, 2000, Day *et al.*, 1998b, Smalley *et al.*, 1981). When a feeding female *Anopheles* mosquito takes a blood meal from an infected individual, both male and female gametocytes may be ingested; hence the sexual forms of the parasite are responsible for parasite transmission.

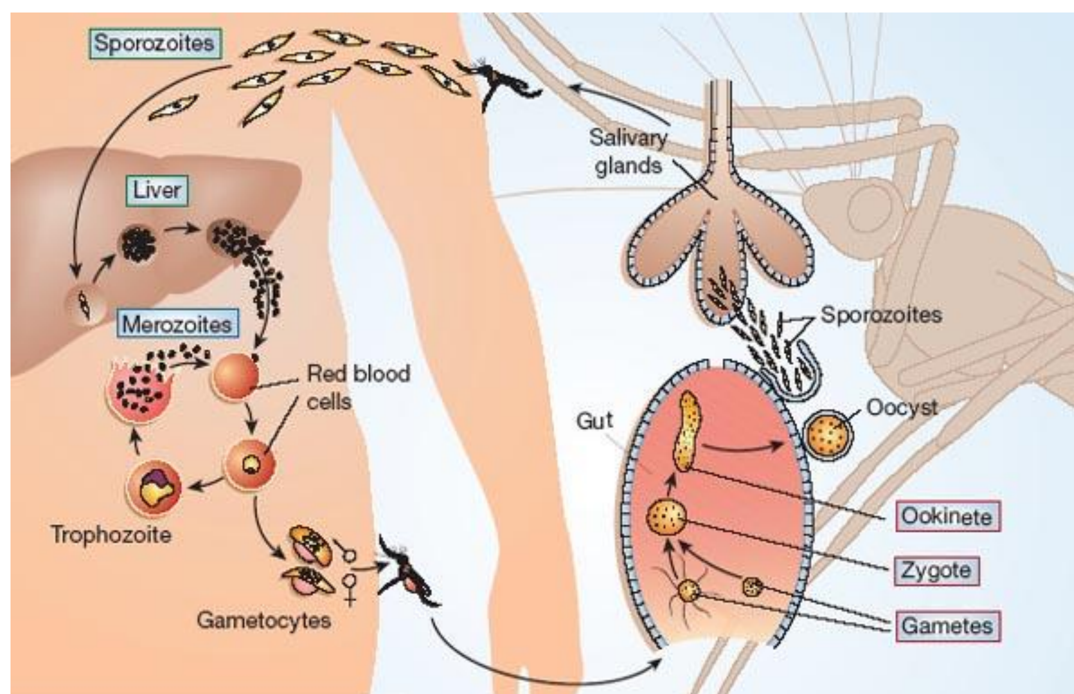

Figure 2. Life cycle of *P. falciparum*. (Adopted from Ménard, 2005 and published with permission from Nature Publishing Group).

In the mosquito vector- Ingested gametocytes rapidly mature into gametes within the mosquito gut. The male microgametocyte divide into eight flagellated microgametes in a process called exflagellation. The microgamete then break out of the RBC, become motile and fertilize the female macrogamete, resulting in a zygote, which develops into a motile and invasive ookinete that penetrates the mosquito gut wall. Upon reaching the outside wall of the mosquito stomach, the ookinete encysts in bodies known as oocysts. Yet again, the parasite undergoes an asexual expansion resulting in thousands of sporozoites. Following rupture of the oocyst, sporozoites migrate to the salivary glands where they become infective (Touray et al., 1992, Vanderberg, 1975). The mosquito stage is now completed and the new sporozoites are ready to be injected into a new human host at the next encounter.

1.4 DISEASE CHARACTERISTICS

Out of the five species of *Plasmodium* infective to man, *P. falciparum* is responsible for most of the malaria associated morbidity and mortality. In contrast, *P. ovale* and *P. malariae* generally give rise to a benign malaria. *P. vivax* has traditionally been considered a benign infection; however, although less often fatal, it is now evident that *P. vivax* constitute an important burden on public health (Anstey et al., 2009, Poespoprodjo et al., 2009, Barcus et al., 2007, Tan et al., 2008). *P. knowlesi* has often been misdiagnosed by microscopy as *P. malariae* (Cox-Singh et al., 2008, Singh et al., 2004). Most *P. knowlesi* cases respond well to treatment and resolve without complications; however, severe and fatal cases have been reported (Daneshvar et al., 2009, Cox-Singh et al., 2008).

1.4.1 General clinical manifestations

The liver-stage of the parasite's life cycle is clinically silent and all pathological manifestations of malaria are associated with the asexual blood-stage or IDC.

Plasmodium infection can exhibit non-specific symptoms a few days before the first febrile attack. These symptoms are usually described as flu-like, and include headache, slight fever, weakness, diarrhea, nausea, muscular discomfort and malaise and they tend to correlate with increasing numbers of parasites. These symptoms are followed by febrile attacks known as the malarial paroxysm, which is most notable for its periodicity; occurring every 24, 48 or 72 h (depending on the species of parasite; Table 1). The regularity of the fever is due to the synchronous development of the malaria parasite, where the onset of fever corresponds to the rupture of pRBC at the end of the IDC. The fever is believed to the cause of released proinflammatory cytokines, such as tumor necrosis factor (TNF) (Kern et al., 1989, Molyneux et al., 1991, Scuderi et al., 1986), which are liberated as a response to RBC destruction and parasite-derived pyrogens. The pattern of regular periodic fever, however, often does not occur until the illness has continued for a week or more.

The malaria paroxysm has a sudden onset and usually begins with chills in which the patient experiences vigorous shivering and a feeling of cold, despite having an elevated temperature. This lasts for an hour and is often referred to as the "cold stage". Immediately following the cold stage, the patient typically feels an intense heat in combination with headache, muscle pain and dizziness, which typically lasts for 2-6 h. This is referred to as the "hot stage". Vomiting and convulsions are common. Next a period of profound sweating will ensue and the fever will start to decline. This stage is commonly referred to as the "sweating stage", and is typically followed by exhaustion and weakness. Upon awakening, the patient generally feels well until the onset of the next paroxysm. Another typical feature of malaria infection is splenomegaly, where the

spleen enlarges in response to the infection. Also haemolytic anemia is often noted, and it is assumed to be the result of hemolysis in combination with failure of the erythropoesis to compensate for the RBC losses (Jakeman et al., 1999, Lamikanra et al., 2007).

Parasite (disease)	IDC, hours	Typical fever pattern
P. falciparum (Malignant tertian malaria/Semi-tertian malaria)	48 h	Peaks every 2nd day /irregular
P. vivax (Tertian malaria)	48 h	Peaks every 2nd day
P. ovale (Benign tertian malaria)	48 h	Peaks every 2nd day
P. malariae (Benign quartan malaria)	72 h	Peaks every 3rd day
P. knowlesi (Quotidian malaria)	24 h	Peaks every day/ irregular

Table 1. Disease characteristics exerted by different *Plasmodium* spp. The name of the fever (quotidian; daily, tertian; three and quartan; four) refers to the number of days from the beginning of the first fever attack to the end of the second. (IDC; intraerythrocytic developmental cycle).

1.4.2 Severe malaria

Complications and severe manifestations due to *P. falciparum* are numerous and diverse. A patient may progress from relatively minor symptoms to having severe disease within a few hours. This usually manifests itself with one of the following: severe anemia, unrousable come (cerebral malaria), pulmonary edema or acute respiratory distress syndrome (ARDS), multiple convulsions, renal failure, circulatory collapse, abnormal bleeding, hypoglycemia, acidosis and hyperlactamia. Severe and complicated malaria has a mortality rate of 15-30%, even with intensive care management. If left untreated, severe malaria is almost always fatal. *P. falciparum* also gives rise to pregnancy associated malaria (PAM), which is associated with both maternal and infant complications.

1.4.2.1 Severe anemia

Severe anemia, defined as having a hemoglobin level lower than 5 g/dl or a hematocrit beneath 15%, is the most common complication of severe malaria. It is seen most frequently in areas of high malaria transmission and most commonly among young children and pregnant women (Lamikanra et al., 2007). Severe anemia has been suggested a consequence of destruction of both parasitized and unparasitized RBC, in combination with erythropoetic suppression and bone marrow dyserythropoiesis (Clark and Chaudhri, 1988, Jakeman et al., 1999, Lamikanra et al., 2007).

1.4.2.2 Acute respiratory distress syndrome

Pulmonary edema or ARDS is a severe complication of malaria with a high mortality rate. It may develop at any time during the course of infection, either after some days of treatment or when the patient's general condition is improving and parasitemia has fallen. Pregnant women with severe malaria are particularly prone to develop ARDS (Taylor and White, 2002). Previously called "adult respiratory distress syndrome", the condition is now called "acute respiratory distress syndrome" as it can occur also in children, although this is considered to be rare (Mohan et al., 2008, Waller et al., 1995). Typical manifestations include abrupt onset of dyspnea, cough and tightness in the chest, which may progress rapidly over a few hours to cause life-threatening hypoxia. The pathogenesis of ARDS is not fully understood, but ultrastructural studies from individuals with fatal malaria have revealed sequestered pRBC in the capillaries, a marked interstitial edema of the alveolar septa,

mononuclear cells in the capillary lumen and endothelial swelling that caused narrowing of the capillary lumen (Duarte et al., 1985, MacPherson et al., 1985).

1.4.2.3 Cerebral malaria

Cerebral malaria (CM) is one of the most serious complications of *P. falciparum* malaria and has a mortality rate of 15-20% (Mishra and Newton, 2009). The prognosis of CM is particularly grave when presented in combination with other complications such as severe metabolic acidosis, ARDS, renal failure or hypoglycemia. The WHO definition of CM is unrousable coma in a patient where *P. falciparum* parasites have been demonstrated, after other causes of encephalopathy have been excluded (WHO, 2000). In children with CM, coma usually has a sudden onset after one to three days of fever, and often follows seizures. In adults, seizures are only occasionally observed and coma tends to have a more gradual onset (Mishra and Newton, 2009). Earlier studies have suggested that surviving patients fully recover from CM (Muntendam et al., 1996), but over the years it has become evident that many children sustain significant brain injury. Although some deficits (e.g., ataxia and cortical blindness) may improve with time, many children demonstrate long-term neurological or cognitive deficits, including memory disturbances, motor deficits, epilepsy, speech and language difficulties, and disorders of concentration and attention (Ngoungou and Preux, 2008, John et al., 2008, van Hensbroek et al., 1997). Indeed, CM has been associated with long-term cognitive impairments in as many as one out of four surviving children (John et al., 2008). In adults, neurological sequelae are less common (Mishra and Newton, 2009).

Parasite sequestration in the cerebral microvasculature is thought to be central to the CM pathogenesis and the resulting pathophysiological changes in tissue around the sequestered parasites (MacPherson et al., 1985, Pongponratn et al., 1991). The obstruction of blood flow caused by sequestered parasites could lead to hypoxia, reduction of metabolic exchange as well as release of proinflammatory mediators. Cytokines and chemokines play a complex role in CM pathogenesis and can be either protective or detrimental to the infected individual. Elevated levels of proinflammatory cytokines TNFα and IFNγ have been extensively studied, and have for a long time been implicated in the pathogenesis of CM both in humans and murine models (for review see (Hunt and Grau, 2003)). Anti-inflammatory cytokines, such as IL-10, have instead been proposed to have a protective role against CM (Hunt and Grau, 2003). Also nitric oxide has been implicated in CM pathogenesis (Clark et al., 1992, Anstey et al., 1996).

1.4.2.4 Pregnancy associated malaria (PAM)

Despite pre-existing protective immunity (see Section 1.4.3), women once again become susceptible to severe disease during pregnancy. Epidemiological data have shown that the susceptibility to malaria declines with increasing parity (McGregor, 1984, Brabin, 1983), hence, primigravidae are particularly at risk. PAM can severely affect the fetus and newborn and increases both maternal and infant mortality. PAM often leads to premature deliveries, low birth weight babies, miscarriages and stillbirths (Guyatt and Snow, 2004, Menendez et al., 2000, Fischer, 2003, Duffy and Fried, 2005). Women residing in areas of unstable or low transmission suffer an increased risk of severe syndromes like CM and respiratory distress, whereas women residing in areas of stable or high transmission commonly suffer from severe anemia (Duffy and Fried, 2005).

The increased susceptibility to malaria during pregnancy is believed to be dependent on the introduction of a new organ; the placenta, which presents a new niche for the parasite to sequester in. PAM is characterized by an accumulation of pRBC in

the intervillous space of the placenta (Walter et al., 1982, Moshi et al., 1995) where they bind to chondroitin sulfate A (CSA) present on placental syncytiotrophoblast cells (Fried and Duffy, 1996). Besides the damage caused by parasite sequestration through obstruction of blood flow, histological observations have demonstrated that malaria infected placentas are infiltrated by maternal monocytes and macrophages (Ismail et al., 2000, Rogerson et al., 2003, Walter et al., 1982). There is also evidence for increased levels of proinflammatory cytokines such as TNFα, IFNγ, and IL-2 (Rogerson et al., 2007).

1.4.3 Determinants of severe disease

The severity of disease depends on various factors, such as age, genetic constitution, state of immunity and general health and nutritional status of the infected individual. In general, severe anemia is more common among young children, whereas CM more often occurs in older children and adults (Snow et al., 1997). Furthermore, there are considerable differences in the manifestation of disease between areas with different rates of malaria transmission. In areas of high transmission, severe malaria is usually confined to children under the age of five, whereas in areas of lower transmission severe malaria may occur at all ages (Snow et al., 1997, Snow et al., 1994, Mbogo et al., 1993). The most plausible explanation for the observed pattern is the natural acquisition of clinical immunity, which is acquired faster in areas of intense malaria transmission as a consequence of more frequent exposure to the parasite. Accordingly, in areas of high malaria transmission, older children and adults rarely experience life-threatening complications. Clinical immunity can take years or even decades of exposure to develop and most likely never develops into a sterile immunity (Doolan et al., 2009).

1.4.4 Malaria vaccines

Few public health interventions have had such an impact on global health as vaccinations. It has been used to tackle diseases such as smallpox, polio, rabies, diphtheria, tetanus, yellow fever, measles, mumps, rubella and hepatitis B. While antimalarial drugs, insecticide-treated bednets and indoor residual spraying are currently being used to reduce the burden of malaria, the parasite is highly complex and adaptable. A safe, efficient and affordable vaccine would therefore provide a much-needed way of alleviating the toll of malaria in the world.

Vaccines can be classified into three general categories: modified live, killed/inactivated, or subunit vaccines. Inoculations with irradiated sporozoites can lead to protection against subsequent challenge (Nussenzweig et al., 1967, Hoffman et al., 2002); however, the cost, manufacturing problems and logistic difficulties in delivering such a vaccine to individuals in endemic areas are substantial. Subunit vaccines contain only a portion of the pathogen and can be based on peptides, recombinant proteins or nucleic acids. In general, subunit based vaccines are easier to produce, more cost efficient and considerably safer than live or inactivated vaccines. Moreover, they can be genetically or synthetically engineered to only include desired epitopes while at the same time excluding epitopes that are inducing non-protective antibodies. Despite these advantages, there are some major drawbacks associated with the development of subunit vaccines. In general, these are poor immunogens when used alone and require multiple doses and co-administrations of an adjuvant that can stimulate the immune system (Wilson-Welder et al., 2009). The choice of an adjuvant depends on the desired type of immune response in terms of humoral or cell-mediated immunity, as adjuvants can bias the response to either Th1 (cell mediated immunity) or Th2 (humoral immunity) type.

Today, several malaria subunit vaccine candidates are in clinical trials (Arevalo-Herrera *et al.*, 2010, Anders *et al.*, 2010, MVI, 2007). It is unlikely that any single antigen will meet all the criteria for a perfect vaccine. Thus, an effective malaria vaccine is likely to contain a combination of antigens from the same stage of the parasite's life cycle or from different stages. Furthermore, no single adjuvant will be effective for all vaccine applications, hence there is extensive research conducted also on the suitability of different adjuvants to be included in a malaria vaccine.

1.5 PARASITE INVASION

Blood-stage infection is initiated when the extracellular merozoite-stage parasite invades the RBC. The whole process of merozoite invasion can be divided into five main steps: (I) merozoite egress from the parasitized host cell, (II) initial attachment (reversible binding of the merozoite to a new host cell), (III) reorientation of the merozoite, (IV) formation of a junction (the irreversible commitment of the parasite to invade) and (V) parasite entry.

Merozoite egress - To invade the host cell, the parasite must first initiate egress from its host cell. Video microscopy have illustrated that merozoite egress is a rapid and therefore tightly regulated process (Glushakova et al., 2005). Particularly two models of egress are currently in discussion, the inside-out model, in which the parasitophorous vacuole membrane (PVM) ruptures before the RBC plasma membrane (RBC PM) (Wickham et al., 2003), and the outside-in model, in which the RBC PM is degraded first (Salmon et al., 2001, Soni et al., 2005). Proteases implicated in egress include the cytoskeleton-degrading cysteine protease falcipain-2 (Dua et al., 2001, Hanspal et al., 2002) and aspartic protease plasmepsin II (Le Bonniec et al., 1999), as well as a family of PVM degrading SERA proteins (Miller et al., 2002b, Hodder et al., 2003), which are regulated by serine protease PfSUB1 and cysteine protease DPAP3 (Yeoh et al., 2008, Arastu-Kapur et al., 2008). Also kinases are expected to play a role in merozoite egress and a plant like calcium-dependent protein kinase, PfCDPK5, has been shown to be essential in the process (Dvorin et al., 2010). Using high-speed video-microscopy and epifluorescence, a recent report has revealed a rather surprising new mechanism of RBC rupture (Abkarian et al., 2011). At the initial opening the RBC first curls back and then buckles, turning itself inside-out after which it spontaneously vesiculates.

Initial attachment - The reversible initial contact between the merozoite and the RBC may occur on any side of the extracellular parasite. The surface of the merozoite is covered by a coat mainly consisting of glycosylphosphatidylinositol (GPI) anchored membrane proteins and their associated partners (Sanders et al., 2005). Currently there are nine known GPI-anchored proteins on the merozoite surface, of which merozoite surface protein-1 (MSP1) is the most abundant antigen. MSP1 is thought to mediate the initial contact to the host RBC and is today a major vaccine candidate (Cowman and Crabb, 2006).

Reorientation and tight junction formation - After binding to the RBC, the merozoite reorients itself such that the apical end is in contact with the RBC membrane. Apical membrane protein-1 (AMA1), a protein that is highly conserved throughout the phylum, is thought to be essential in establishing this interaction (Triglia et al., 2000, Mitchell et al., 2004). Once reorientation has occurred, a tight junction is formed and the rhoptry and micronemal proteins are discharged, indicating the irreversible commitment of the merozoite to invasion. Two protein families, the

Erythrocyte binding-like (EBL)-family (Adams et al., 2001, Adams et al., 1992, Miller et al., 2002a) and *P. falciparum* reticulocyte binding protein homologs (PfRh) (Rayner et al., 2001, Duraisingh et al., 2003b, Stubbs et al., 2005, Triglia et al., 2005) are prime candidates in tight junction formation. Members of the EBL-family localize to the micronemes, but are believed to be exported to the merozoite surface during invasion (Adams et al., 1990, Sim et al., 1992). The EBLs will be discussed in more detail below. The PfRhs were identified as homologs of rhoptry proteins in *P. vivax* (Galinski et al., 1992) and *P. yoelii* (Preiser et al., 2002) and have been implicated in determining the specificity of host cell invasion. The PfRh family consists of six members in *P. falciparum*; PfRH1, PfRH2a, PfRH2b, PfRH3, PfRH4 and PfRH5 (Iyer et al., 2007, Gaur et al., 2004), where PfRh4 has been shown to bind complement receptor 1 on the RBC (Tham et al., 2010). While the EBLs and PfRh proteins are important in merozoite invasion, they are clearly not essential, as the corresponding genes can be disrupted without an apparent effect on parasite growth (Duraisingh et al., 2003a, Duraisingh et al., 2003b, Gilberger et al., 2003, Maier et al., 2003, Stubbs et al., 2005, Triglia et al., 2005, Lopaticki et al., 2011). However, they each mediate invasion through different receptors and thereby give rise to highly redundant invasion pathways, which is believed to guarantee parasite invasion.

Parasite-entry - The subsequent movement into the RBC involves an active actin-myosin motor. This motor complex has been studied most extensively in *T. gondii*, and the proteins involved appear to be highly conserved across the apicomplexa, including *Plasmodium* spp. (Baum et al., 2006). The link between the merozoite and the motor complex is not known; however, the trombospondin related apical protein (TRAP) appears to provide the crucial link in sporozoites (Sultan et al., 1997). Following invasion, *P. falciparum* resides within a membrane enclosed parasitophorous vacuole (PV). The PV membrane (PVM) is formed from invaginations of the RBC PM during invasion. Upon completion of parasite entry the PVM fuses and separates, hence forming a biochemical and physical barrier between the host cell cytosol and the parasite. The content of the dense granules are believed to be discharged only after the parasite has completed its entry, and to be implicated in the modification of the host cell (Torii et al., 1989, Culvenor et al., 1991). *Babesia* and *Theilera* quickly degrade their PVM resulting in a free moving parasite within the host cell cytosol. In contrast, *Toxoplasma* and *Plasmodium* remain enclosed within the PVM throughout parasite maturation.

1.5.1 The Duffy binding-like (DBL)-domain of the EBLs

Species of *Plasmodium* differ in their requirements for RBC surface molecules in host cell invasion. The Duffy blood group antigen (DARC) is obligatory for *P. vivax* and RBCs lacking the antigen are refractory to parasitic infection. This Duffy null phenotype, long known to be common among certain sub-Saharan African populations, provides an explanation for the evident absence of *P. vivax* among these populations (Langhi and Bordin, 2006). Interestingly, some cases of *P. vivax* infection have been reported in Duffy-negative individuals in Kenya (Ryan et al., 2006), Brazil (Cavasini et al., 2007) and Madagascar (Menard et al., 2010). The protein giving *P. vivax* such specific requirements for invasion was identified in the late 1980s (Wertheimer and Barnwell, 1989), and named Duffy binding protein (DBP). In a subsequent study, the region responsible for Duffy/DARC-binding was identified in PvDBP and its homolog in *P. knowlesi*, and the region was termed the Duffy binding-like (DBL)-domain (Chitnis and Miller, 1994). *P. falciparum* exhibits no such dependence on Duffy blood group antigen and parasites can utilize different receptors for invasion.

DBL-domains can be found in two distinct protein families in *Plasmodium* that together form a DBL-superfamily: (I) the EBL-family of invasion proteins (including PvDBP and PkDBP), and (II) the PfEMP1 (*var*)-family of antigenic variable sequestration proteins (discussed in more detail in Section 1.6.2). Common to all members is the presence of one or more DBL-domains, homologous to the Duffy/DARC-binding domain of PvDBP and PkDBP. In *P. falciparum* the EBL family has expanded by duplication and diversification and a repertoire EBL paralogs are present, including EBA-175, EBA-181 (JESEBL), EBA 140 (BAEBL), EBA-165 (PEBL) and EBL-1 (Adams et al., 2001). MAEBL is an additional EBL paralog; however, a distinct cysteine-rich region with similarity to AMA1 replaces the DBL-domains in this protein. The expansion of EBLs in *P. falciparum* provides ligand diversity and potential usages of different host receptors for the parasite. For example, the receptors of EBA-175, EBL-1, and EBA-140 is glycophorin A (Orlandi et al., 1992), glycophorin B (Mayer et al., 2009), and glycophorin C (Maier et al., 2003), respectively. Of the six EBLs present in *P. falciparum*, EBA-175 appears to be of most importance.

1.5.1.1 Gene structure of the EBLs

The *ebl* genes have a similar exon-intron structure with conserved splicing boundaries, indicating a common evolutionary origin (Adams et al., 1992). The four consensus exons encode: (I) an extracellular domain with a signal peptide (SP), a conserved 5′ cysteine rich-region and a conserved 3′ cysteine-rich region (referred to as c-cys); (II) a transmembrane domain (TM); (III) and (IV) a putative cytoplasmic domain. A separate exon encoding the SP is always present in the *maebl* gene and is commonly seen also in the *dbp* gene of *P. vivax* and *P. knowlesi* (Adams et al., 1992). The 5′ cysteine rich-region functions as the RBC-binding domain (Chitnis and Miller, 1994, Sim et al., 1994), and in *P. vivax* and *P. knowlesi* it consists of a single DBL-domain (PvDBL and PkDBL, respectively), whereas the *P. falciparum* EBLs and the homolog in *P. reichenowi* encode tandem copies of the DBL-domain (referred to as F1 and F2) (Adams et al., 1992, Michon et al., 2002), (Figure 3).

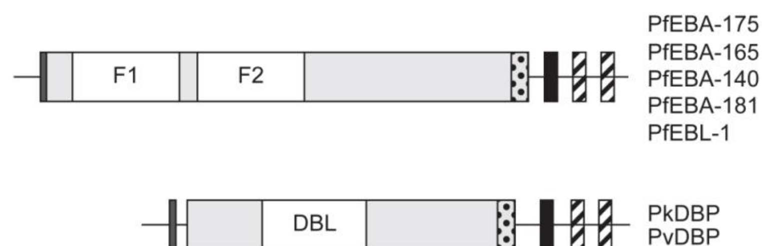

Figure 3. Simplified gene structure of the EBLs. Exon I: encodes the extracellular domain (light gray) including the SP (dark gray), DBL-domains (white, referred to as F1 and F2 in *P. falciparum*) and c-cys domain (dotty), Exon II: encodes the TM (black), Exon III and IV: the C-terminal domain (striped). In DBP, a separate exon encodes the signal peptide. Note that *maebl* have been excluded from the illustration. (The presence of the three last exons has not been defined in EBL-1 (Adams *et al.*, 2001)).

1.5.1.2 Protein structure of the DBL-domains

The DBL-domains of the EBLs possess twelve cysteine residues that are conserved in location for PvDBL, PkDBL and the tandem *P. falciparum* domains F1 and F2 (Adams et al., 1992). Out of these, ten align with cysteines in the *var*-DBLs (Smith et al., 2000a). The F1-domain of EBA-175 has one extra cysteine residue and the F2-domain two extra cysteine residues, of which none are seen in PvDBL and

PkDBL. One of the extra cysteines found in F2 is completely conserved in all *var*-DBLs and in all F2-domains of the EBL-DBLs (Michon et al., 2002, Smith et al., 2000a), whereas the second extra cysteine is located in a region not shared with the *var*-DBLs. Interestingly, all F1-domains of the EBL-DBLs appear more closely related to the single DBL-domain of PvDBP and PkDBP than to the F2-domain present in the same molecule (Adams et al., 1992, Michon et al., 2002). This has lead to speculations about the F2-domain being the progenitor of the *var* genes (Michon et al., 2002). Hence, the DBL-domains of PfEMP1 may have derived by duplication of the F2-domain in an ancestral *ebl* gene followed by sequential diversification (Michon *et al.*, 2002, Smith *et al.*, 2000a). The presence of several invariant cysteines implies a conserved tertiary structure of the DBL-domains. In 2005, the tandem F1/F2 of *P. falciparum* EBA-175 was crystallized (Tolia et al., 2005). The overall structure resembled an elongated molecule, comprising mainly α-helices as well as two β-hairpins. The F1/F2 crystallized as a dimer, in where the domains interacted with each other in an antiparallel orientation resembling a handshake. Glycan binding was shown to be scattered at the dimer interface, thus dimerization appear crucial for receptor recognition. The following year, the structure of the monomeric DBL-domain of PkαDBP was solved (Singh et al., 2006). Interestingly, the DBL-domain displayed a very similar overall structure consisting mainly of α-helices connected by loops. The disulfide bonding pattern of PkαDBL was found to be identical to that of the F1/F2 domains of EBA-175, although F2 has an additional disulfide bridge. Mapping the DARC binding sites, previously determined by domain deletion (Singh et al., 2003) and site-directed mutagenesis (VanBuskirk et al., 2004, Hans et al., 2005), illustrated that binding occurs on the opposite face of the molecule compared to EBA-175 F1/F2. In 2008, the tertiary structure of the DBL3x domain of VAR2CSA (a PfEMP1 variant implicated in PAM) was solved (Higgins, 2008, Singh et al., 2008). Again the structure was highly similar to that of EBA-175 F1/F2 and PkαDBL. The receptor binding region was observed on the face of the molecule corresponding to the glycan binding region of EBA-175 F1/F2, but separated from the DARC binding region of PkαDBL. Hence despite having poor sequence identity, the crystallized DBL-domains display a similar overall structure, illustrating the importance of the invariant cysteine residues in maintaining the DBL fold.

1.6 *PLASMODIUM FALCIPARUM* VIRULENCE

P. falciparum is by far the deadliest of the five species causing human malaria and two special aspects contribute to this virulence. Firstly, *P. falciparum* achieves much higher levels of parasitaemia than the other species due to high asexual multiplication rates and the ability of the parasite to invade RBC of all ages. Secondly, *P. falciparum* possesses the unique capacity to sequester in the deep vasculature and the ability to evade the host immune system by expressing variant parasite-derived adhesins on the RBC surface (Miller et al., 1994). The ability of the parasite to sequester and evade host immunity will here be reviewed.

1.6.1 Sequestration

Crossing the spleen is the most stringent challenge on RBC deformability in the human body as this is a site of RBC quality control. Senescent, rigid and parasitized cells are routinely retained in the spleen before being permanently removed from the blood circulation (Quinn and Wyler, 1979). In order to circumvent this event, the parasite sequesters in the deep tissue. As a consequence, only RBCs parasitized with young forms of the parasite are found in the peripheral circulation, whereas RBCs containing the more mature and rigid form of the parasite accumulate in the deep

vasculature. The sequestered mass of pRBC leads to microvascular obstruction, metabolic disturbances, and release of damaging inflammatory mediators, which can combine to cause severe disease and death of the human host. Two different events are thought to bring about the accumulation of pRBC in the vascular microciruclation; (I) the binding of pRBC to endothelial cells (cytoadhesion) and (II) the binding of pRBC to unparasitized RBC (rosetting).

1.6.1.1 Cytoadhesion

P. falciparum pRBC are capable of adhering to vascular endothelium seen in various organs such as brain, intestine, liver, lung, skin and the syncytiotrophoblast of the placenta. Via the cytoadhesion mechanism, pRBCs are not only removed from the peripheral circulation and thus prevented from splenic clearance, but also gain access to a relatively hypoxic environment preferred by the parasite for proliferation and RBC invasion. A number of endothelial receptors have been identified as targets for pRBC including CD36, intercellular adhesion molecule-1 (ICAM-1), chondroitin-sulfate A (CSA), thrombospondin (TSP), VCAM and E-selectin, PECAM-1/CD31, heparan sulfate and P-selectin. Although several studies have attempted to correlate the binding phenotype of pRBC with clinical outcome (Newbold *et al.*, 1997, Ho *et al.*, 1991b, Rogerson *et al.*, 1999), the importance of adhesion to specific endothelial receptors in severe malaria has so far not been proven. CSA may be the exception, as this receptor is intimately linked with PAM.

CD36 is a glycoprotein that is expressed on the endothelium of various organs (not the brain), platelets, monocytes and dendritic cells, and it was one of the first endothelial pRBC receptors described (Barnwell et al., 1989, Ockenhouse et al., 1989). The molecule seems to be widely used by the parasite since the vast majority of clinical *P. falciparum* isolates are capable of adhering to CD36 and the binding appears stable under *in vitro* flow conditions (Cooke *et al.*, 1994). However, CD36 binding is frequent in parasite isolates both from patients with mild and severe malaria (Turner et al., 1994), and there is subsequently no strong evidence for a specific role for CD36 in severe disease (Heddini *et al.*, 2001, Newbold *et al.*, 1997, Rogerson *et al.*, 1999).

ICAM-1 is a member of the immunoglobulin superfamily and is present on the surface of endothelial cells and monocytes. It is a candidate for pRBC binding to brain endothelial cells and fatal malaria has been associated with a widespread induction of ICAM-1 and E-selectin on brain endothelial cells (Turner et al., 1994). Expression of ICAM-1 is upregulated by proinflammatory cytokines TNFα, IL-1 and IFNγ (Berendt et al., 1994, Dustin et al., 1986). Although capable of supporting pRBC binding under static *in vitro* conditions, ICAM-1 cannot support pRBC binding under *in vitro* flow conditions, thus ICAM-1 binding appears to be in need of synergism with additional receptors (McCormick et al., 1997).

CSA is a sulfated glucosaminoglycan present on the syncytiotrophoblast in the intervillous space of the placenta where it normally functions as an immobilizer for cytokines, hormones and other molecules (Rogerson et al., 2007). CSA is the dominant receptor involved in PAM, which is illustrated by the observation that placental parasite isolates commonly bind CSA but not CD36, whereas non-placental isolates rarely bind CSA (Beeson *et al.*, 1999, Fried and Duffy, 1996, Maubert *et al.*, 2000). Furthermore, sera from multi-gravidae women are able to block CSA-binding (Maubert et al., 1999, Fried et al., 1998). It is by now well established that the adhesion of pRBC to CSA depends on a particular PfEMP1 variant called VAR2CSA (Salanti et al., 2004, Salanti et al., 2003, Viebig et al., 2005) and that the decreasing risk of malaria with subsequent pregnancies can be attributed to a parity-dependent acquisition of antibodies towards placental VAR2CSA expressing parasites (Ricke et al., 2000, Staalsoe et al.,

2004). Also hyaluronic acid has been demonstrated to serve as a receptor for pRBC in the placenta (Beeson et al., 2000, Rasti et al., 2006) and non-immune immunoglobulins (Igs) have been suggested to bridge pRBC to the syncytiotrophoblast cell-lining (Flick et al., 2001, Rasti et al., 2006).

1.6.1.2 Rosetting

The discovery that *P. falciparum* pRBC can bind to unparasitized RBCs to form rosette-like clumps, was first made in the late 1980s (Udomsangpetch *et al.*, 1989b, Handunnetti *et al.*, 1989). The phenomenon was first observed *in vitro* but later confirmed in *ex vivo* blood samples examined directly after sampling (Carlson et al., 1990, Wahlgren et al., 1992, Wahlgren et al., 1990, Ho et al., 1991a, Hasler et al., 1990). Defined as one mature trophozoite-stage pRBC binding two or more unparasitized RBCs, rosettes first become apparent approximately 16-18 h post invasion (p.i.), but persist throughout trophozoite maturation and schizogony until they finally disappear upon rupture (Treutiger et al., 1998). The occurrence of rosettes in small blood vessels in the brain and other vital organs is thought to contribute to malaria pathogenesis by causing obstruction to blood flow leading to hypoxia and tissue damage. In contrast to cytoadhesion, rosetting has repeatedly been associated with severe disease (Carlson et al., 1990, Heddini et al., 2001, Rowe et al., 1995, Rowe et al., 2002, Ringwald et al., 1993, Treutiger et al., 1992). A number of different RBC surface receptors as well as serum factors have been identified to be involved in rosetting.

Rosetting levels and the size of the rosette have been shown to vary with different RBC blood groups, and a preference to either A, B or AB over O RBC have been reported for both laboratory parasite strains and field isolates (Udomsangpetch et al., 1993, Barragan et al., 2000b, Rowe et al., 1995). In particular blood group A has been associated with severe malaria (Pathirana et al., 2005, Fry et al., 2008), whereas blood group O seems to give some protection to severe disease and is overrepresented in uncomplicated cases of malaria (Rowe et al., 2007, Loscertales et al., 2007).

Complement receptor 1 (CR1) is an immune regulatory molecule that is expressed by all peripheral blood cells except platelets, natural killer cells and most T lymphocytes. The importance of CR1 in rosetting was first demonstrated through the use of blood from CR1 deficient donors, in which a number of rosetting laboratory strains failed to form rosettes (Rowe et al., 1997). This finding was later confirmed by the use of soluble CR1 and monoclonal anti-CR1 antibodies, which were both shown to disrupt rosettes in laboratory strains and clinical isolates (Rowe et al., 2000). CR1 deficiency is highly frequent in populations in endemic region of Papua New Guinea and has been linked with protection against severe disease (Cockburn et al., 2004). Levels of CR1 on RBC have also been reported to influence the outcome of disease (Stoute et al., 2003, Waitumbi et al., 2004, Waitumbi et al., 2000).

Heparan sulfate (HS) and other sulfated glycans have been shown to inhibit rosette formation and to disrupt rosettes (Carlson et al., 1992, Barragan et al., 2000a). Modified heparin, devoid of its anti-coagulant activity, is capable of efficiently disrupting rosettes (Vogt et al., 2006).

Serum proteins are essential for the formation of rosettes both in laboratory strains and field isolates (Treutiger et al., 1999, Rogerson et al., 2000, Somner et al., 2000). Fibrinogen, von Willebrand's factor and non-immune Igs have all been reported to support rosette formation (Scholander et al., 1996, Clough et al., 1998, Treutiger et al., 1999, Flick et al., 2001, Heddini et al., 2001). Binding of non-immune Igs has also been reported as a common phenotype among field isolates from patients with severe

malaria (Scholander et al., 1998, Heddini et al., 2001). Although the role of IgM seems generally accepted, the role of IgG is more controversial.

1.6.2 Surface antigens of *P. falciparum*

1.6.2.1 PfEMP1

P. falciparum erythrocyte membrane protein 1 (PfEMP1) is the main surface-adhesin responsible for rosetting and sequestration of pRBC in the deep vasculature, and its surface expression coincides with the withdrawal of pRBC from the peripheral circulation. Early on it became evident that PfEMP1 is a target of protective antibodies and that acquired immunity develops in response to extended infections with pRBC expressing different PfEMP1 variants (Marsh et al., 1986, Bull et al., 1998).

PfEMP1 are large multi-domain proteins (ranging between 200 and 350 kDa), encoded by the hypervariable *var* gene family that undergoes antigenic variation and thereby allows for the generation of various adhesive phenotypes (Su et al., 1995, Baruch et al., 1995, David et al., 1983, Smith et al., 1995). The *P. falciparum* genome contains approximately 60 *var* genes mainly located in the highly polymorphic subtelomeric region but also in the central parts of the 14 chromosomes (Rubio *et al.*, 1996, Hernandez-Rivas *et al.*, 1997, Gardner *et al.*, 2002, Su *et al.*, 1995). *var* genes are between 6-14 kb and have a two-exon structure that is separated by a conserved intron. The first exon encodes a hypervariable extracellular binding region, which comprise the N-terminal segment (NTS), multiple adhesive domains of DBL-type or cysteine-rich interdomain region (CIDR)-type sometimes interspersed with C2 interdomains. The second exon encodes a more conserved acidic terminal segment (ATS), which also harbors a C-terminal TM. Although all *var* genes maintain this basic architecture, there is significant sequence variation when comparing PfEMP1 proteins among paralogs and across parasite isolates, indicating that there is an enormous repertoire of PfEMP1 variants. Gene conversions and recombination events within the family is probably held accountable for maintaining this high level of sequence diversity (Flick and Chen, 2004, Freitas-Junior et al., 2000)

The chromosomal location and transcriptional orientation of *var* genes have been shown to correspond to similarities in the 5′ upstream open reading frame of the genes. Based on this conservation, the 5′ promoter regions can be defined into four major upstream (Ups) sequence groups, UpsA, UpsB, UpsC, and UpsE (Lavstsen et al., 2003). The former UpsD is now grouped with UpsA (Kraemer et al., 2007). Interestingly, rosetting parasites more frequently express *var* genes belonging to group A, whilst both group A and B are more often transcribed in patients suffering from severe malaria (Jensen et al., 2004, Kaestli et al., 2006, Normark et al., 2007, Bull et al., 2005). Based on the Ups region and the domain architecture, yet another (although similar) grouping is used, with three major (A, B, and C) and two intermediate (B/A and B/C) groups. Two unique genes have been characterized in all sequenced isolates, which do not fit into the classification above. The highly conserved *var2csa* gene is flanked by a 5′ UpsE and have been linked to CSA binding and PAM. The conserved *varcommon* (also known as *var1csa*) is flanked by a 5′ UpsA2 and is transcribed in almost all clinical isolates but has an unusual transcription pattern (Kyes et al., 2003, Lavstsen et al., 2003, Winter et al., 2003).

Finer mapping of the extracellular domains of PfEMP1 has enabled the attribution of certain adhesive phenotypes to different domains. For example, DBL1 has been shown to bind CR1, blood group A and HS on both endothelial cells and RBCs (Vogt et al., 2003, Barragan et al., 2000a, Barragan et al., 2000b, Rowe et al., 1997), whereas CIDR1 has been shown to bind CD36 and IgM (Baruch et al., 1996,

Chattopadhyay et al., 2004). VAR2CSA contains six DBL-domains of which at least three (DBL2x, DBL3x, and DBL6ε) exhibit some affinity for CSA *in vitro*, whereas the other three domains show limited or no binding (for review see (Dahlbäck *et al.*, 2010)).

1.6.2.2 Other surface antigens

In addition to PfEMP1, other proteins of parasite origin have been suggested to be exposed on the pRBC surface. Surface iodination experiments have revealed a trypsin sensitive variant family with two TMs named RIFINs, encoded by the *repetitive interspersed family (rif)* genes (Kyes et al., 1999, Fernandez et al., 1999). The *rif* family holds approximately 160 copies in the genome and they can be subdivided into two sub-classes; group A and group B, primarily depending on a 25 nucleotide deletion in group A (Gardner et al., 2002, Joannin et al., 2008). Whereas A-type RIFINs appear to be exported into the host cell via Maurer's clefts (MCs), B-type RIFINs are mostly retained inside the parasite (Petter et al., 2007). The function of RIFINs remains elusive, but the high gene copy numbers and clonal variations imply that they are involved in immune evasion.

The RIFINs are structurally related to the *subtelomeric variable open reading frame* (STEVOR) family. There are approximately 30-40 *stevor* genes per haploid genome, and they are located to a large extent adjacent to the *rif* genes in the subtelomeric regions of the chromosomes (Gardner et al., 2002). *Stevor* and *rifin* show a very similar two-exon gene structure, where the short exon I encodes a signal peptide and the larger exon II codes for a polypeptide possessing two predicted TMs flanking a hypervariable region. Recently, STEVORs were demonstrated to be clonally variant on the surface of schizont-stage pRBC, indicating that they play a role in creating antigenic diversity of schizont-stage parasites (Niang et al., 2009). STEVORs have also been detected on the apical end of the merozoite (Khattab et al., 2008, Khattab and Meri, 2011).

The *surface associated interspaced gene* (SURFIN) family of proteins was identified by mass spectrometric analysis of peptides cleaved off the surface of live pRBC with trypsin (Winter et al., 2005). They are encoded by a family of ten *surf* genes, including three predicted pseudogenes, located within or close to the subtelomeres of five of the 14 chromosomes. SURFINs have been associated with merozoites, MCs, and the pRBC surface (Mphande et al., 2008, Winter et al., 2005). SURFINs share a tryptophan-rich region (WRD) with other proteins such as PfEMP1, Pf332, and PkSICA*var*, suggesting a potential ancestral relationship (Winter et al., 2005).

1.6.3 Antigenic variation

The expression of parasitic antigens on the pRBC surface renders the parasite vulnerable to the host immune system. However, the host's efforts to eliminate the malaria pathogen are constantly counteracted by the parasites capability of switching their surface expressed PfEMP1 molecules. Hence, *P. falciparum* infections are often characterized by waves of parasitemia, with each wave representing the rise and fall of distinct populations of parasites expressing a particular set of surface antigens (Miller et al., 1994). This fascinating strategy of immune evasion is a key survival mechanism employed by a wide range of infectious organisms including *Trypanosoma brucei* (Turner, 1999) and *Giardia lamblia* (Nash, 1997, Prucca et al., 2008), as well as by many others.

1.6.3.1 Molecular basis of var gene regulation

While multiple transcripts of the *var* genes can be seen in the early stages of parasite development, only one dominant transcript is believed to be present in more mature stages. Furthermore, only a singular PfEMP1 type is expressed on the pRBC surface at a time (Chen et al., 1998, Scherf et al., 1998). This phenomenon is referred to as mutually exclusive expression, and studies have revealed that it is transcriptionally regulated and independent on protein production (Dzikowski et al., 2006, Voss et al., 2006). Several lines of evidence suggest that regulation of *var* gene transcription is a multi-layered system involving: (I) DNA control elements and the regulatory proteins that bind them, (II) histone modifications and epigenetic memory, and (III) subnuclear positioning.

The first level of regulation involves the two promoters found in virtually all *var* genes; the 5′ Ups region and the intron (Deitsch et al., 2001). The upstream promoter is responsible for the mRNA transcription, whereas the intron promotor produces non-coding sterile RNA (Calderwood et al., 2003, Su et al., 1995). While a single *var* gene is transcribed from the Ups promotor and the rest of *var* genes are transcriptionally silent, most of the intron promoters seem to be active simultaneously (Calderwood et al., 2003). These introns are believed to function as transcriptional silencers via promoter pairing, thereby controlling antigenic variation (Frank et al., 2006, Dzikowski et al., 2007, Voss et al., 2006). While there is a paucity in transcription factors in the *P. falciparum* genome, a group of conserved proteins containing putative AP2 DNA-binding domains, now known as the apicomplexan AP2 (ApiAP2) protein family, was recently identified (Balaji et al., 2005). The ApiAP2 family may be important for regulation of *var* gene transcription since a member of the family has been shown to bind the regulatory upstream regions of UpsB *var* genes (De Silva et al., 2008).

The second level of regulation involves chromatin modifications, and among these have acetylated histone H3 and H4, and methylated H3K27 and H3K4 been found at active genes, whereas tri-methylated H3K9 has been observed at silent loci (Freitas-Junior et al., 2005, Lopez-Rubio et al., 2007, Duraisingh et al., 2005, Chookajorn et al., 2007). A *P. falciparum* homolog of the histone deacetylase SIR2 (PfSIR2) has been shown to associate with silent *var* 5′ promoter types UpsE and UpsB, but not UpsC (Freitas-Junior et al., 2005), consistent with a telomere-silencing association for this protein. Genetic disruption of the *PfSir2* gene resulted in activation of only certain subtelomeric *var* genes (UpsA and UpsE) (Duraisingh et al., 2005), which suggests that the UpsB-type *var* genes are subject to a further layer of silencing.

The third level of regulation involves perinuclear repositioning of *var* genes upon activation. Silent genes tend to localize to the periphery of the nucleus, which contain primarily heterochromatin, whereas active transcription generally takes place in euchromatic internal regions in which chromatin is loose and open for transcription (Ralph et al., 2005). However, the periphery of the nucleus contains distinct regions that are clear of heterochromatin, possibly representing active expression sites. Using RNA-FISH, Ralph and coworkers demonstrated that when *var2csa* is silent, it co-localizes with telomeric clusters, whereas upon activation it moves to another location of the nuclear periphery apart from these clusters (Ralph et al., 2005). Conflicting findings have however been observed when using transgenic parasite lines with drug inducible *var* gene promoters (Voss et al., 2006). There is also recent evidence for the existence of a *var* specific subnuclear expression site, which can accommodate more than one active gene at a time, indicating that mutually exclusive expression of *var* genes is regulated at a different level than simply nuclear architecture (Dzikowski and Deitsch, 2008).

1.7 MALARIA PATHOGENESIS BY HOST CELL REMODELING

P. falciparum is rather unusual in its choice of a host cell. In contrast to most pathogens, which invade and multiply in nucleated host cells, *P. falciparum* invades mature human RBC. These nearly metabolically inert cells are devoid of a nucleus, internal organelles, a functional trafficking machinery, and surface expressed major histocompatibility complex (MHC) molecules. At first, this may seem like a perfect hideaway from the host's immune system; however, it comes with a trade-off. Firstly, as the RBC does not readily take up nutrients, the parasite is forced to gain its own access to nutrients from the extracellular milieu. Secondly, as the parasite matures, the pRBC becomes vulnerable to splenic clearance mechanisms. To overcome these problems, the parasite dramatically remodels its host cell by: (I) introducing new permeation pathways in the RBC PM for nutrient uptake, (II) establishing a parasite-derived trafficking machinery in the host cell cytosol, (III) interacting with the host cell cytoskeleton, and (IV) expressing parasite-derived adhesins on the RBC surface (summarized in Figure 4). All these host remodeling properties are mediated by a subset of parasite-derived proteins, which are exported beyond the confines of the parasite – a feature that is central to the malaria pathogenesis.

1.7.1 New permeation pathways

Approximately 12-18 h p.i., the pRBC undergoes a profound increase in its permeability to low-molecular-weight solutes. This has been attributed to the induction of channels in the RBC PM, referred to as new permeation pathways (NPPs), which allow the uptake of nutrients and excretion of metabolic waste products (Ginsburg, 1994, Kirk, 2001). Using the patch-clamp technique, Desai and co-workers demonstrated that the membrane conductance of pRBC is 150 times greater than that measured in unparasitized RBC and that this increase in conductance results from activation of small anion channels (Desai et al., 2000). The origin of the NPPs is quite controversial and they have been suggested to be both parasite and host cell derived (reviewed in (Kirk, 2001)). NPPs are of interest as potential drug targets, since they are believed to play an important role in providing the parasite with essential nutrients, such as the vitamin pantothenic acid (Saliba et al., 1998) and the amino acid isoleucine (Martin and Kirk, 2007).

1.7.2 The tubulovesicular network

Transmission electron microscopy of pRBC has revealed a variety of membranous structures in the host cell cytosol. The most prominent structure is the tubulovesicular network (TVN) comprised of an interconnected network of tubular and vesicular membranes that extend from the PVM into the cytoplasm towards the RBC PM of trophozoite-stage pRBC (Elmendorf and Haldar, 1994, Behari and Haldar, 1994). The TVN harbors the Golgi marker sphingomyelin synthase and has therefore been suggested to possess secretory properties (Elmendorf and Haldar, 1994, Lauer et al., 1995). Other reports argue for the involvement of the TVN in nutrient import at junctions between the TVN and the RBC PM (Lauer et al., 1997).

1.7.3 Maurer's clefts

In 1898, using light microscopy, German physician Wilhelm Schüffner was the first investigator to describe a stippled pattern within the host cell cytoplasm of stained *P. vivax* pRBC. In honor of his work, the stippling were later termed Schüffner's dots. German physician Georg Maurer confirmed and extended the work by Schüffner, and in 1902, using a refined staining protocol, he described stipplings and dots in the RBC

cytosol of *P. falciparum* pRBC. William Trager, known for establishing the continuous *in vitro* culture conditions for *P. falciparum* parasites, was the first to associate the stipplings in *P. falciparum* with long and slender clefts, predominately located in close proximity to the RBC PM (Trager et al., 1966). These were subsequently named Maurer's clefts (MC). Since then, cleft-like membranous structures have been identified in most *Plasmodium* spp. (Aikawa, 1988b).

MCs first appear in the cytosol of late ring-stage pRBC (Langreth et al., 1978, Bannister et al., 2004) although, a recent study has demonstrated that they are present already at 2-6 h p.i. (Grüring et al., 2011). MCs appear as convoluted disk-like compartments with an electron-dense coat and an electron-lucent lumen (Hanssen *et al.*, 2008b, Aikawa, 1988b). Long, slender membrane extensions connect neighboring clefts and stalk-like "tethers" attach them to the RBC PM and PVM, although no continuum exists to allow molecules to freely flow between the three compartments (Hanssen et al., 2010, Spycher et al., 2006, Tilley et al., 2008, Hanssen et al., 2008b). The latter, however, has been heavily debated over the years. Also the molecular mechanism involved in MC biogenesis is a matter of discussion, but it is now generally accepted that MCs originate through budding from the PVM or the TVN (Spycher et al., 2006, Tilley et al., 2008). Using four-dimensional imaging, MCs were recently shown to be mobile in ring-stage pRBC, whereas they were fixed following transition into trophozoite-stage (Grüring et al., 2011). Moreover, MCs were shown to collapse a few hours before merozoite egress, implying that host cell modifications are disassembled prior to rupture, possibly with the aim of facilitating merozoite egress (Grüring et al., 2011). There is growing evidence that MCs are anchored to the host cell cytoskeleton in mature-stage pRBC. Firstly, MCs are often found in close proximity to the inner leaflet of the host RBC PM in mature-stage pRBC (Waterkeyn et al., 2000, Hinterberg et al., 1994b, Wickham et al., 2001). Secondly, MCs stay attached to pRBC ghosts after lysis by osmotic shock or merozoite egress (Martinez et al., 1998, Blisnick et al., 2000). Skeleton binding protein 1 (SBP1) has been suggested to anchor MCs to the RBC cytoskeleton in a phosphorylation-dependent manner and to prevent premature rupture of the host cell by interacting with the host protein LANCL1 (Blisnick et al., 2000, Blisnick et al., 2005, Blisnick et al., 2006). SBP1 has further been associated with the stalk-like structures that tether MCs to the RBC PM (Hanssen et al., 2008b).

There are several integral membrane proteins resident in MCs. These include previously mentioned SBP1 (Blisnick et al., 2000, Cooke et al., 2006), the membrane-associated histidine-rich protein-1 (MAHRP1) (Spycher et al., 2003), the ring exported protein 2 (REX2) (Spielmann et al., 2006), the MC two TM proteins (MC-2TM) (Sam-Yellowe et al., 2004), STEVORs (Kaviratne *et al.*, 2002, Przyborski *et al.*, 2005) and some members of the RIFINs (Khattab and Klinkert, 2006, Kyes et al., 1999). REX1 and MAHRP2 are two MC residents that appear to be peripherally associated with the cytoplasmic surface of the clefts (Dixon et al., 2008b, Hawthorne et al., 2004, Pachlatko et al., 2010, Spielmann et al., 2006). Interestingly, a truncation of REX1 resulted in distortion of MC morphology and stacking of the clefts, indicating that the protein plays a structural role in MCs (Hanssen et al., 2008a).

It has been postulated that MC are parasite-induced secretory organelles that concentrate and traffic parasite proteins beyond the confines of the parasite (Bhattacharjee et al., 2008, Lanzer et al., 2006). A number of exported proteins, including PfEMP1 (Knuepfer *et al.*, 2005), KAHRP (Rug et al., 2006), PfEMP3 (Waterkeyn et al., 2000) and Pf332 (Hinterberg et al., 1994b) transiently associate with the clefts en route to the RBC PM, consistent with this hypothesis. Furthermore, targeted gene disruption of residential proteins of the clefts, including SBP1 (Cooke et al., 2006, Maier et al., 2007) and MAHRP1 (Spycher et al., 2008) has been shown to

abrogate export of PfEMP1 to the RBC surface. Taken together, this points to a crucial role for MCs in protein trafficking across the host cell cytoplasm to the RBC PM. MCs may also play a role in merozoite egress, cell signaling and phospholipid biosynthesis (Lanzer et al., 2006).

1.7.4 Knobs

Electron-dense protrusions that typically measure 30-40 nm in height and 90-100 nm in width appear on the surface of the pRBC during trophozoite and schizont development (Aikawa, 1988b). These structures, termed knobs, have been shown to act as attachment points of sequestered parasites in the blood vessels (Aikawa, 1988b) and electron microscopy from autopsies of CM patients have demonstrated pRBCs that are attached to the endothelium via knobs (Aikawa, 1988a). Also RBCs parasitized with the monkey malaria parasite *P. brasilianum* display knob-protrusions on the pRBC surface; however, the function of the knobs in this *Plasmodium* species remains unclear since all stages of *P. brasilianum* pRBC are found in the peripheral circulation (Aikawa, 1988b).

On the cytoplasmic side of the RBC PM, knobs are composed of the *P. falciparum* knob-associated histidine-rich protein (KAHRP), which is essential for knob formation (Culvenor *et al.*, 1987, Crabb *et al.*, 1997, Pologe and Ravetch, 1986, Leech *et al.*, 1984) and serves as a platform to anchor PfEMP1 to the RBC cytoskeleton (Waller *et al.*, 1999). Parasites with a disrupted *kahrp* gene still display PfEMP1 on the surface; however, the cytoadhesion to CD36 under flow conditions is dramatically impaired (Crabb et al., 1997), implying that KAHRP is essential for anchoring of PfEMP1, but does not play a role in trafficking and assembly of the cytoadhesion complex.

1.7.5 Cytoskeleton remodeling

The primary function of the mature RBC is to carry oxygen from the lungs to the cells of the body, followed by the return of carbon dioxide from the tissues to the lungs. To be able to transport high amounts of oxygen and at the same time withstand the enormous pressure of being repeatedly squeezed through the small capillaries of the body, the RBC contains a high concentration of hemoglobin and has a strong but very deformable submembrane cytoskeleton.

1.7.5.1 The RBC cytoskeleton

The RBC cytoskeleton is organized as a polygonal network formed by spectrin tetramers that are cross-linked at junctional points by short actin filaments. Each junctional point (or junctional complex) is composed of actin, adducin, tropomyosin, and tropomodulin, where the latter two strengthen the network by preventing the actin filament from depolymerizing. The final outcome is a highly deformable actin-spectrin meshwork that underlies the inner leaflet of the RBC PM. The stability of the spectrin network is not only influenced by the component proteins but can also be modulated by the levels of protein phosphorylation (Ling et al., 1988, Manno et al., 1995). The spectrin-actin network is coupled to the lipid bilayer primarily by association of the central region of spectrin with peripheral membrane protein ankyrin, which in turn is bound to the cytoplasmic domains of integral membrane protein band 3. Other proteins, such as protein 4.2 and glycophorin A have been shown to be associated with the band 3–ankyrin complex, but their roles remain unclear. Additional membrane connections are provided at the junctional point by a ternary complex involving glycophorin C, protein p55, protein 4.1 and membrane-associated guanylate kinase (MAGUK).

Together, this intricate cytoskeleton network is responsible for the high degree of cellular deformability of the RBC (Bennett, 1983).

1.7.5.2 Parasite proteins and the RBC cytoskeleton

Previous studies have clearly demonstrated that the deformability of intact pRBC is profoundly reduced compared to unparasitized RBC (Cranston et al., 1984, Nash et al., 1989). Using the micropipette aspiration technique, ring-stage pRBC were shown to have a slightly impaired deformability, whereas mature-stage pRBC displayed no deformation at all under the same conditions (Nash et al., 1989). Although some of the increased rigidity of pRBCs can be explained by the presence of the growing intracellular parasite, it is generally believed to be the result of parasite-derived proteins that are exported into the RBC cytoplasm where they then associate with the host cell cytoskeleton. By engaging parasite proteins in such interactions, *P. falciparum* can prevent premature egress and merozoite invasion into an already parasitized cell, increase thermal stability of the host cell membrane, anchor the PfEMP1 cytoadhesion complex in the RBC PM, and destabilize the RBC PM at completion of the IDC. Many of the exported and cytoskeleton-interacting proteins are large (ranging in size from 100 to 700 kDa) and they generally contain extensive regions of low complexity sequence, often occurring in tandem repeats (Cooke et al., 2001). Furthermore, the repeats are typically highly charged, either positively or negatively.

Already after invasion, *P. falciparum* targets protein to the cytoskeleton and ring parasite–infected erythrocyte surface antigen (Pf155/RESA) is one of the first proteins detectable in the host cell cytosol where it binds spectrin (Culvenor et al., 1991, Coppel et al., 1984, Foley et al., 1991, Ruangjirachuporn et al., 1991). RESA contains two blocks of glutamic acid-rich repeats (5′ and 3′), which are degenerate along the molecule and highly conserved between different parasite isolates (Cowman et al., 1984, Favaloro et al., 1986, Coppel et al., 1984). Between the two repeat regions is a segment of 70 residues with similarity to the J domain of *Escherichia coli* and human DnaJ chaperone proteins, suggesting that RESA may have some chaperone-like properties, perhaps while bound to the RBC cytoskeleton (Bork et al., 1992). Biochemical studies using recombinant RESA fragments have demonstrated that the interaction with spectrin leads to a degree of protection against heat-induced denaturation of spectrin, thus implying that RESA protects the RBC cytoskeleton from heat-induced damage during febrile episodes (Da Silva et al., 1994). This hypothesis was supported by two independent reports where transgenic parasites with a disrupted *RESA* gene were used (Mills et al., 2007, Silva et al., 2005). Mature parasite-erythrocyte surface antigen (MESA, also known as PfEMP2) has been reported to compete with host protein p55 for binding to protein 4.1 in trophozoite-stage pRBC, and in turn modulate the 4.1-glycophorin C-p55-MAGUK ternary complex resulting in a more rigid host cell (Bennett et al., 1997, Waller et al., 2003). Using recombinant proteins, the spectrin-binding region of MESA was mapped to an N-terminal 19-residue region (Bennett et al., 1997). Interestingly, both RESA and MESA bind the host cytoskeleton via non-repetitive regions. By micropipette aspiration, the membrane rigidity of transgenic parasites with a *kahrp* or a *pfemp3* deletion was shown to be significantly reduced compared to the corresponding wild-type parasite (Glenister et al., 2002). Current evidence suggests that KAHRP binds spectrin, actin and ankyrin (Magowan et al., 2000, Pei et al., 2005, Kilejian et al., 1991), as well as the negatively charged ATS-region of PfEMP1 (Waller et al., 1999). The positively charged histidine-rich region and the 5′ repeats appear to be important for cross-linking to the host cytoskeleton and is required for knob formation (Rug et al., 2006, Waller et al., 1999). Using recombinant fragments and inside-out-vesicles, PfEMP3 was

demonstrated to bind spectrin via a non- repetitive 14-residue region in the N-terminus of the protein (Waller et al., 2007). As mentioned in Section 1.7.3., also MC resident protein SBP1 has been shown to interact with the cytoskeleton in mature-stage pRBC.

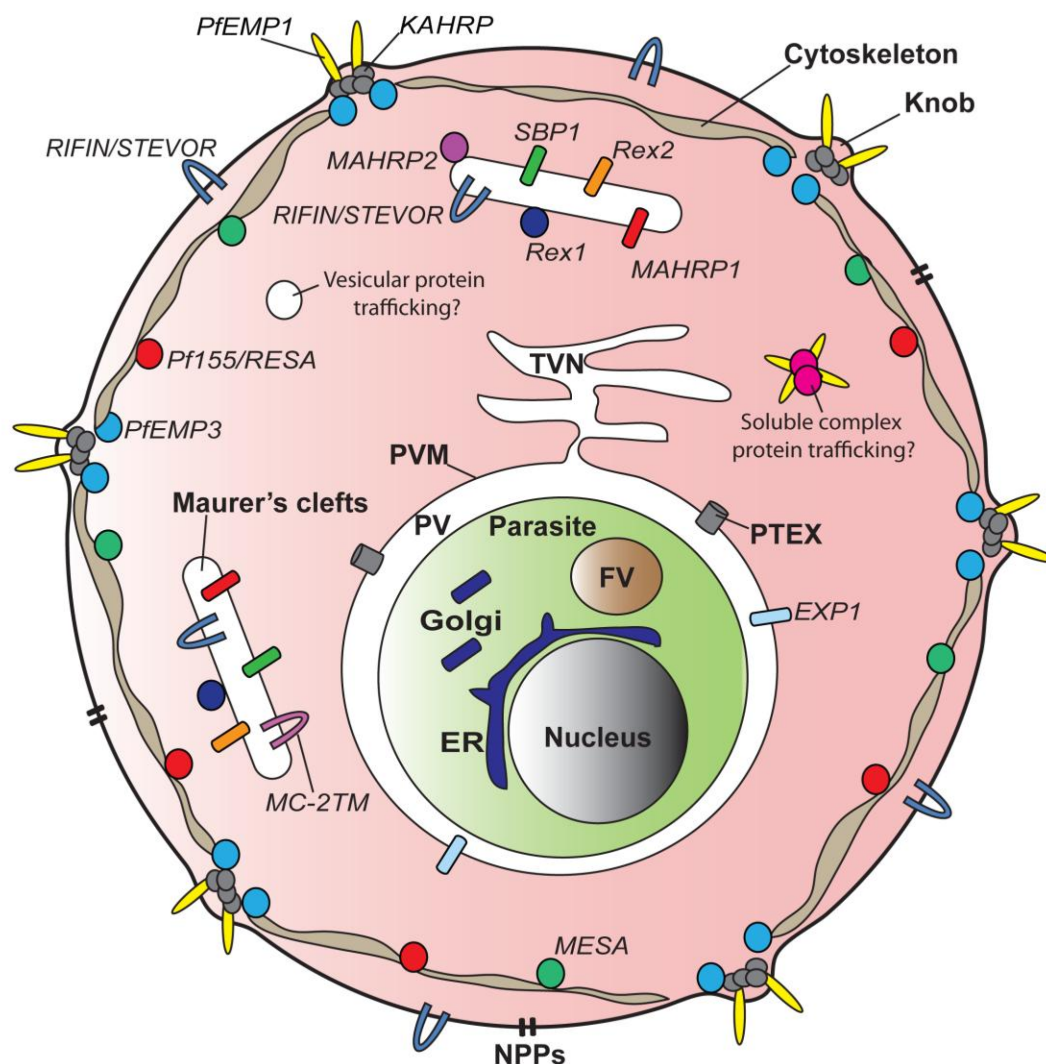

Figure 4. Summary of host cell modifications induced by *P. falciparum* parasites. (TVN; tubulovesicular network, PV; parasitophorous vacuole, PVM; parasitophorous vacuole membrane, PTEX; *Plasmodium* translocon of exported proteins, NPPs; new permeation pathways, FV; food vacuole, ER; endoplasmatic reticulum). Note that Pf332 has been excluded from the illustration, since its subcellular localization will be discussed in Paper IV.

1.8 PROTEIN SECRETION AND EXPORT

Trafficking of parasite proteins into the host cell is a multi-step process involving entry into the secretory pathway of the parasite and trafficking to the PV, followed by translocation across the PVM into the host cell cytosol. Some parasite-derived proteins then associate with MCs present in the host cell cytosol for further transport to either the cytoplasmic side of the RBC PM or translocation onto the RBC surface.

1.8.1 General features of the secretory pathway in *P. falciparum*

The secretory and endocytic pathways in eukaryotic cells serve as major routes for protein transport out of and into the cell. These are very selective pathways and only a subset of proteins and lipids are given access to the machinery. A classical protein secretory pathway required some key components, such as an endoplasmatic reticulum

(ER) and a Golgi apparatus. Proteins destined for secretion are typically co-translationally inserted into the ER via a hydrophobic N-terminal SP, and by the anterograde secretory pathway proteins are directed from the ER to the Golgi, endosomes, lysosomes, or to the PM for secretion into the extracellular milieu, which in mammalian cells is the default pathway. Proteins generally move between compartments by budding and fusion of COPI, COPII and clathrin-coated vesicles. The outward secretory pathway is counteracted by an inward (retrograde) endocytic pathway originating from the PM. The two pathways interconnect at various steps and together they form a complex intracellular trafficking system.

There are several lines of evidence for a classical secretory pathway in *P. falciparum*. Firstly, several conserved characteristics of the secretory pathway have been identified in the parasite, including homologs of BiP, ERD2, Sec61 components, as well as various trafficking-associated Rab GTPases (for review see (Przyborski and Lanzer, 2005, Foley and Tilley, 1998)). Secondly, many *P. falciparum* proteins contain a classical SP of approximately 15 hydrophobic amino acids commencing 3-17 amino acids from the N-terminus, similar to the SP that target proteins to the secretory pathway in mammalian cells. Thirdly, the fungal metabolite Brefeldin A (BFA), which in mammalian cells inhibits the anterograde transport between ER and Golgi resulting in redistribution of Golgi proteins back to the ER (Lippincott Schwartz *et al.*, 1989), blocks secretion of numerous parasite proteins (Hinterberg et al., 1994b, Wickham et al., 2001). However, although the secretory pathway seems to be present in *P. falciparum*, it is clearly unusual in several aspects. Firstly, it has been difficult to identify an obvious Golgi apparatus, suggesting that this organelle is either absent or highly rudimentary. It now appears as if an "unstacked" apparatus is present, where the *cis* Golgi is spatially separated from the *trans* Golgi (Struck et al., 2005, Van Wye et al., 1996). Secondly, not all secreted *P. falciparum* proteins contain a classical SP. Thirdly, the *P. falciparum* parasite has a range of unique intracellular organelles fed by the secretory pathway. These include a food vacuole, an apicoplast and three different types of secretory granules used by the merozoite during RBC invasion (rhoptries, dense granules and micronemes). Protein trafficking in malaria pRBC also has an added level of complexity in that the parasite exports proteins beyond the confines of its own PM. The secretory system of *P. falciparum* must therefore differentially target proteins to a wide array of diverse subcellular organelles and compartments.

1.8.2 Export of *P. falciparum* proteins into the host cell cytosol

In contrast to proteins that remain within the parasite, parasite proteins destined for export typically have a longer (up to 30 amino acids) hydrophobic region that can be recessed by up to 80 amino acids, and this non-canonical SP is required to traffic proteins to the PV (for review see (Lingelbach, 1993)). The parasite's secretory machinery appears to be able to recognize both the classical and the recessed SP, and in absence of any additional signaling information, the proteins follow the default pathway, which in *P. falciparum* pRBC leads to the PV (Wickham et al., 2001). Since malaria proteins destined for export into the host cell cytosol must first pass the PV, this compartment may be conceptually viewed as an additional station for protein sorting.

1.8.2.1 Translocation across the PVM

A major advance in our understanding of parasite protein export came with the discovery that exported *Plasmodium* proteins possess a conserved amino acid motif located 15-20 amino acids downstream of the N-terminal hydrophobic SP. This motif, called the *Plasmodium* export element (PEXEL) (Marti et al., 2004) or vacuolar

transport signal (VTS) motif (Hiller et al., 2004), consists of a pentameric sequence with the consensus R/KxLxE/Q/D, where x is any non-charged amino acid. Arginine in position 1 and leucine in position 3 are the most conserved residues, and alanine replacement of the two abrogates export into the host cell (Hiller et al., 2004, Marti et al., 2004). PEXEL proteins are not unique to *P. falciparum*; they are also predicted in the exportome of many other *Plasmodium* spp. (Hiller et al., 2004, Marti et al., 2004, Sargeant et al., 2006, van Ooij et al., 2008).

A motif similar to the PEXEL has been identified in the plant pathogen *Phytophora infestans*, within proteins that enter the plant cell (Whisson et al., 2007). The N-terminal motif consists of a highly conserved core, RxLR, positioned within 60 amino acids of the ER-type SP. Intriguingly, the RxLR motif and an E/D rich domain further downstream could efficiently export *P. falciparum* fusion proteins out of the PV into the host RBC cytosol (Bhattacharjee et al., 2006). Similarly, the PEXEL motif could efficiently substitute for the *P. infestans* export motif in driving protein translocation into the host plant cell (Grouffaud et al., 2008). This is of particular interest, as it suggests that deep branching eukaryotes belonging to distinct groups share conserved secretion strategies to access host cells. However, recent data have demonstrated that the machinery for delivering *P. infestans* proteins into the plant cell is host cell derived, casting some doubts on the close resemblance of the export pathways used by *P. infestans* and *Plasmodium* spp. (Dou et al., 2008).

The discovery of the *Plasmodium* PEXEL motif has allowed for an *in silico* prediction of the *P. falciparum* "exportome". The term "secretome" has also been used, although the term exportome is to prefer as it describes a subset of secreted proteins. Depending on the algoritm used, 5-8% of the *P. falciparum* genome is predicted to be exported (Hiller et al., 2004, Marti et al., 2004, Sargeant et al., 2006, van Ooij et al., 2008). The majority of these genes are located in subtelomeric regions, and apart from the *var*, *rif*, and *stevors,* are genes predicted to encode proteins involved in host cell remodeling overrepresented (Maier *et al.*, 2008, Sargeant *et al.*, 2006).

Surprisingly, the fate of proteins destined for export is determined much earlier along the trafficking pathway than originally believed. By the time PEXEL-containing proteins reach the PV, the PEXEL motif has already been cleaved in the ER after the leucine residue, generating a new N-terminus, xE/Q/D, which becomes N-acetylated (Chang et al., 2008, Boddey et al., 2010). An aspartic protease, Plasmepsin V, has in two separate studies been demonstrated to be responsible for PEXEL cleavage (Boddey et al., 2010, Russo et al., 2010). Attempts to disrupt the gene in *P. berhei* and genetically alter the active site in *P. falciparum* have both been unsuccessful, thus the gene appears to have an essential function in the parasite. The critical role of Plasmepsin V in protein export provides an important target for development of novel antimalarials. It is currently unknown how the N-acetylated xE/Q/D-proteins destined for export reach the PV. They may be transported via bulk flow or chaperone recruitment in the ER. Alternatively, proteins may be transported via distinct trafficking pathways in the ER that ultimately lead to specialized regions of the parasite PM closely connected to the PVM (Crabb et al., 2010). The machinery responsible for protein translocation into the host cell was until recently unknown. By combining proteomic analysis with strict prediction criteria, de Koning-Ward and colleagues identified a translocon of parasitic origin residing on the cytosolic side of the PVM (de Koning-Ward et al., 2009). This machinery, termed the "*Plasmodium* translocon of exported proteins" or PTEX, is an ATP-powered complex comprised of a heat shock protein (Hsp 101), a known integral membrane protein of the PVM (EXP2), thioredoxin 2 and two novel proteins (PTEX150 and PTEX88). Apart from thioredoxin 2, the PTEX components are absent from any other organism, including other apicomplexans, which is in accordance with the lack of PEXEL motifs in other

organisms. This is indicative of a unique requirement for *Plasmodium* spp. to have an efficient export machinery capable of translocating proteins into the host cytosol.

1.8.2.2 Do multiple pathways exist?

Several well-documented exported proteins such as SBP1, MAHRP1, MAHRP2, REX1, and REX2 lack the PEXEL motif and these proteins have been collectively termed PEXEL-negative exported proteins, or PNEPs (Spielmann and Gilberger, 2010). Interestingly, all currently identified PNEPs localize to MC. However, there may be many more hidden in the *Plasmodium* genome, but since no systematic approach to search for additional PNEPs is available, this remains speculative. PNEPs lack a classical SP, but are in any case believed to be trafficked into the host cytosol via the classical secretory pathway, involving translocation into the ER. Although several studies have attempted to address the sequence requirements for PNEP export (Dixon et al., 2008a, Haase et al., 2009, Saridaki et al., 2009, Spycher et al., 2008), these proteins do not appear to share an obvious motif that promotes their export. However, the TM and sequences in the N-terminus of the protein have been shown to be involved. SURFINs and Pf332 could represent additional PNEPs, although both proteins have sequences that resemble PEXEL motifs (Spielmann and Gilberger, 2010). Interestingly, $SURFIN_{4.2}$ was recently shown to be trafficked into the host cell cytosol in a PEXEL-independent manner involving the TM but not sequences in the N-terminus of the protein (Alexandre et al., 2011). The presence of PNEPs raises the important question of whether *Plasmodium* parasites have more than one export pathway into the host cell?

1.8.2.3 Trafficking beyond the PVM

How exported proteins are trafficked within the host cell cytosol is a matter of debate (Przyborski and Lanzer, 2005). Some studies have suggested that vesicles budding from the PVM bridge the gap between the PVM to MC, and possible onwards to the RBC PM (Trelka et al., 2000, Taraschi et al., 2003, Wickham et al., 2001). *P. falciparum* homologs of COPII secretory proteins have been shown to be exported and to associate with MCs, consistent with a vesicular model (Albano et al., 1999, Adisa et al., 2001, Wickert et al., 2003a) although this view was challenged in a more recent report (Adisa et al., 2007). So far have no COPI vesicle coat proteins been detected in the RBC cytosol, which may indicate that trafficking of parasite proteins to the RBC PM or MCs is unidirectional, i.e. with no retrograde pathway; however, additional work is required to confirm this (Cooke et al., 2004). Others have proposed a model where proteins move by lateral diffusion along a continuous membrane network that encompasses MC and connects the PVM to the RBC PM (Wickert et al., 2003b). Soluble proteins are most likely trafficked across the host cell cytosol by diffusion or as part of a soluble protein complex, as has been shown for KAHRP, PfEMP3 and MESA (Wickham *et al.*, 2001, Howard *et al.*, 1987, Knuepfer *et al.*, 2005) (Figure 4).

That the PTEX acts as a common gateway for both soluble and membrane proteins, is supported by both classes of proteins harboring PEXEL motifs (Hiller et al., 2004, Marti et al., 2004). It has been postulated that membrane proteins may arrest during their translocation across the PVM via their hydrophobic TM, after which they are loaded into nascent MCs (Spielmann et al., 2006, Spycher et al., 2006) or vesicles budding from the PVM. For PfEMP1, which also harbors a TM/hydrophobic region, the situation appears to be somewhat different. Evidence obtained from studies employing either a FRAP (fluorescence recovery after photobleaching) GFP-chimera approach or biochemical methods suggests that PfEMP1 passes through the PVM translocon in a soluble state after which it is transported in a multimeric protein

complex (possible involving chaperones) to MCs before reaching the RBC PM (Knuepfer *et al.*, 2005, Papakrivos *et al.*, 2005). Whether other TM proteins can be trafficked in a similar manner remains elusive.

1.9 GLUTAMIC-ACID RICH PROTEINS IN *PLASMODIUM*

Several *P. falciparum* blood-stage antigens were originally identified by screening recombinant DNA libraries with immune sera from individuals residing in malaria endemic areas. Many of these antigens contain low complexity sequences with blocks of tandem repeats rich in glutamic acid (Glu), giving the proteins a negative net charge. While these Glu-rich repeats are highly immunogenic, they do not necessarily induce protective antibodies. Indeed, it has been proposed that proteins containing tandem repeats may serve as smokescreens to divert the immune response away from other more important epitopes (Anders, 1986). Consistent with this hypothesis, many Glu-rich proteins are exported into the host cell cytosol, are highly abundant and can typically be found in close proximity to the RBC PM. Presumably, they are released and exposed to the immune system at time of schizont rupture. Furthermore, many of the Glu-rich proteins induce cross-reactive antibodies (Mattei *et al.*, 1989, Ahlborg *et al.*, 1991, Wåhlin *et al.*, 1990), and the frequently occurring pairs of Glu found in most of these antigens are considered responsible for this. The Glu-rich proteins may additonally be important in host cell remodeling, since some of them interact with the host cell cytoskeleton. While in association with the RBC PM, many Glu-rich antigens become phosphorylated (Wiser et al., 1983), and this may influence protein-protein associations, induce relaxation of the cytoskeleton, or alter the structure and mechanical properties of the RBC PM. Examples of Glu-rich antigens include *P. falciparum* antigens; Pf155/RESA, Pf332 (also known as antigen 332) (Mattei et al., 1989, Mattei and Scherf, 1992a), GLURP (Borre et al., 1991), MESA (Coppel, 1992), Pf11-1 (Scherf et al., 1992), and D260 (Barnes et al., 1995), *P. chabaudi*; Pc(em)93 (Giraldo et al., 1999) and *P. berghei*; Pb(em)65 and Pb(em46) (Wiser and Plitt, 1987).

1.9.1 Pf332

Although identified more than two decades ago, the function of Pf332 still remains elusive. Studies on Pf332 have been hampered by the cross-reactive nature of antibodies generated against the molecule due to its high content of Glu-rich repeats. Pf332 was originally identified from a genomic expression library using human immune sera, which led to the characterization of a single exon gene (Mattei and Scherf, 1992a). One of the most striking characteristics of Pf332 is its extremely high content of negatively charged repeats that are not identical but with the consensus $(X)_3$-EE-$(X)_2$-EE-$(X)_{2\text{-}3}$, where X is any hydrophobic amino acid and E is Glu (Mattei and Scherf, 1992b). Together, the repeats make up more than 90% of the protein and the total Glu-content of the antigen is 28%. The *Pf332* gene is located in the subtelomeric region on chromosome 11 and has been detected in all clinical parasite isolates surveyed to date. Subtelomeric genes are prone to frequent gene recombination event, and the *Pf332* gene displays a marked sequence variation as demonstrated by RFLP. *Pf332* is present as a single copy gene in all parasite strains analyzed so far, with the exception being the HB3 parasite, in which the gene is duplicated and the second copy is present in the subtelomeric region on chromosome 13 (Hinterberg et al., 1994a)(Paper V).

Pf332 was early on considered to be a malaria vaccine candidate. This was based on the observation that a Pf332-reactive human monoclonal antibody (mAb 33G2) was able to inhibit both parasite growth and cytoadherence of pRBC to melanoma cells *in vitro* (Udomsangpetch et al., 1986). However, detailed analysis of

the specificity of the antibody reveled that it was cross-reactive with other Glu-rich antigens including Pf155/RESA and Pf11-1 (Udomsangpetch et al., 1989a, Ahlborg et al., 1991, Mattei et al., 1989, Iqbal et al., 1993a). However, an invasion inhibitory effect of anti-Pf332 antibodies has been supported in several studies where both human affinity-purified and polyclonal animal antibodies targeting different regions of Pf332 were used (Wåhlin *et al.*, 1992, Ahlborg *et al.*, 1993, Ahlborg *et al.*, 1995, Balogun *et al.*, 2009)(Paper I). A detailed morphological analysis of pRBC grown in the presence of anti-Pf332 antibodies, revealed an abundance of abnormal schizonts with tightly clumped merozoites and dispersed hemozoin, suggesting that the antibodies induced parasite growth arrest (Ahlborg et al., 1996). Collectively, these findings led to the suggestion that Pf332 may play a role in parasite invasion/growth. The interpretation of these results is, however, complicated by the extensive serological cross-reactivity of Pf332, since most studies have used a highly Glu-rich and repetitive fragment of the molecule, denoted EB200 (Mattei and Scherf, 1992b), for both antibody production and affinity purification. In 2009, two transgenic parasite lines were described in which the *Pf332* gene had been disrupted (Glenister et al., 2009, Hodder et al., 2009). Interestingly, complete ablation of Pf332 expression had no effect on parasite growth or replication.

Pf332 is highly immunogenic and antibodies reactive with the antigen have frequently been identified in sera from malaria exposed individuals (Iqbal et al., 1993b, Warsame et al., 1997, Israelsson et al., 2008, Balogun et al., 2009, Kulane et al., 1999). In areas of intense malaria transmission, antibodies appear to be acquired at an early age (Paper II) and increased titers of Pf332-reactive IgG antibodies in humans have been associated with a decreased number of malaria incidents (Ahlborg et al., 2002, Giha et al., 2010). Experimental animal immunizations using the EB200 fragment of Pf332, have further been shown to induce opsonizing antibodies in *Samiri* monkeys (Gysin et al., 1993).

1.9.1.1 Subcelllular localization

The 700 kDa Pf332 protein is the largest known *P. falciparum* protein exported to the RBC PM. Export of the antigen is sensitive to treatment with BFA (Hinterberg et al., 1994b)(Paper IV), indicating that Pf332 is trafficked via the classical secretory pathway. Pf332 is a mature-stage antigen that is first detectable within the parasite at 18-24 h p.i. after which it can be visualized in MCs together with PfEMP1 and RIFINs en route to the RBC PM (Haeggström *et al.*, 2004). Interestingly, both *Pf332* knockout- and truncation mutant parasites (missing the C-terminus) display larger and less numerous MCs that tend to aggregate and form multilamellar stacks rather than individual lamellae (Glenister et al., 2009). This was particularly evident in schizonts where over 60% of pRBC displayed this abnormal phenotype. A deletion of *Pf332* has also been reported to affect PfEMP1 export, although this appears to be parasite strain dependent. While CS2 parasites with a *Pf332* deletion expressed less PfEMP1 on the surface (Glenister et al., 2009), 3D7 parasites with a *Pf332* deletion expressed PfEMP1 at similar levels as the wild-type parasite (Hodder et al., 2009). Immunofluorescence microscopy assays (IFA) of mature-stage pRBC using anti-Pf332 antibodies have revealed a rim-like fluorescence staining along the RBC PM and Pf332 was therefore suggested to be exposed on the surface of mature-stage pRBC (Hinterberg *et al.*, 1994b). A surface location of Pf332 was further supported by IFA of live mature-stage pRBC using anti-Pf332 antibodies (Hinterberg *et al.*, 1994b, Mattei and Scherf, 1992a). Based on IFA observations where the Pf332 fluorescence signal disappeared shortly before merozoite egress, Wiesner et al. have suggested that Pf332 is proteolytically cleaved and that this may activate a putative membrane destabilizing and host cell

rupture function of the antigen (Wiesner et al., 1998).

1.9.1.2 Cytoskeleton binding properties of Pf332

Parasites with a *Pf332* deletion (Glenister et al., 2009, Hodder et al., 2009) or truncation (Glenister et al., 2009) appear to be more rigid, indicating that Pf332 interacts with the host cell cytoskeleton. Intriguingly, whereas most cytoskeleton binding proteins make the pRBC *more* rigid, Pf332 apparently do the opposite. Using a series of recombinant Pf332 fusion proteins in combination with purified actin or inside-out vesicles of human RBCs, an actin-binding region was identified in the C-terminus of the protein (a Glu-rich sequence encompassing residues 5155-5201) (Waller et al., 2010). Biochemical analyses of pRBC have; however, brought about contradicting results concerning the solubility of Pf332 within the pRBC. Mattei et al. observed the protein mainly in the Triton X-100 (TX-100) soluble fraction of lysed pRBC (Mattei and Scherf, 1992a), which implies that Pf332 is associated with membranous structure within the pRBC, but speaks against an association with the RBC cytoskeleton. In contrast, Glenister et al. found Pf332 to be largely TX-100 insoluble but SDS soluble (Glenister et al., 2009), which supports a direct association between Pf332 and the RBC cytoskeleton. Thus, although data obtained from knockout studies and recombinant protein binding assays support an interaction between Pf332 and the cytoskeleton, a biochemical confirmation using endogenous protein is missing.

2 SCOPE OF THE THESIS

The overall objective of the investigations presented in this thesis was to further the understanding of the massive Pf332 antigen of the malaria parasite *Plasmodium falciparum*. The Pf332 gene structure, immunogenicity, subcellular localization, host cell remodeling properties, and transcriptional activity were particular targets in this context.

Specific aims:

The specific objectives of the presented papers were as follows:

I. To characterize the structure of the gene encoding Pf332 and the Duffy binding-like (DBL)-domain encoded by exon I.

II. To describe the DBL-domain of Pf332 in terms of three-dimensional structure, naturally acquired immunity and antibody specificity.

III. To evaluate the immune response in different animals immunized with the DBL-domain of Pf332 in combination with a set of different adjuvants.

IV. To investigate the subcellular localization and cytoskeleton interacting properties of the endogenous Pf332 protein.

V. To elucidate transcriptional activity of duplicated *P. falciparum* genes, including *Pf332* and *var2csa*, by allelic discrimination.

3 EXPERIMENTAL PROCEDURES

Material and methods are detailed in each respective study included in this thesis (Paper I-V). This chapter will highlight some of the more important methods from each of the studies included.

3.1 PARASITE IN VITRO CULTURE CONDITIONS

In vitro adapted parasites used in these studies were the *Plasmodium falciparum* laboratory strains FCR3, FCR3S1.2, HB3, 3D7AHI, NF54, and 7G8. Parasites were kept in continuous culture according to standard procedures with red blood cells (O+) at 5% hematocrit and 10% A+ serum in buffered malaria culture medium. Parasites were synchronized with 5% sorbitol for 10 min and kept at static conditions for all the described experiments (Moll et al., 2008).

3.2 RBC BINDING ASSAYS

In order to investigate the RBC-binding properties of the Pf332-DBL domain, recombinant proteins (tagged with Glutathione-S-Transferease; GST) corresponding to the DBL-domain, a region downstream of the DBL-domain referred to as nonDBL of Pf332 (both regions encoded by exon I) and an unrelated protein, were generated in *E. coli*. Recombinant protein (200 pmol) was incubated with 5 µl of washed RBCs in RPMI-1640 for 2.5 h at 4ºC and thereafter washed in PBS. RBCs were subsequently collected by centrifugation, and bound protein was visualized by Western blot using anti-GST monoclonal antibodies. To further illustrate the binding properties of Pf332-DBL, Chinese hamster ovary (CHO) cells were transfected with the DBL-domain of Pf332 or the nonDBL-region using the FuGENE 6 transfection reagents. Surface expression of the domains was confirmed by IFA 48 h later. For detection of RBC-binding, transfected CHO-cells were detached and stained with PKH67 (green) and human RBC were stained with PKH26 (red). CHO-cells were incubated with RBCs at a ratio of 1:5 for 1 h at room temperature and RBC-binding was evaluated by fluorescence microscopy.

3.3 INVASION ASSAYS

Invasion inhibition assays were performed with laboratory adapted strains FCR3S1.2, 3D7AH1 and 7G8. Trophozoite-stage pRBC were synchronized and the hematocrit was set to 2.5% and the starting parasitemia to 1%. Assays were performed in 96-well U-bottom culture plates and 3/4 of the total volume constituted pRBC in suspension, whereas the remaining 1/4 corresponded to the added antibody/PBS control. Parasites were cultivated until reinvasion of merozoites was completed, after which pRBC were stained with acridine orange and counted by flow cytometry. Investigated antibodies included purified IgG from Pf332-DBL/nonDBL or GST immunized rabbits. PBS was included in all plates as a non-inhibitory control and an anti-AMA1/MSP-1 antibody was included as an inhibitory control. Antibodies were titrated and used at a final concentration of 1, 0.5 and 0.25 mg/ml. Results are presented as percentage of invasion and calculated as follows: 100 x (mean parasitemia of culture grown with test antibodies/mean parasitemia of culture grown with PBS). Experiments were performed in duplicates in three separate assays.

3.4 STRUCTURE MODELING OF PF332-DBL

In order to assess structural similarities of Pf332-DBL with the EBL-DBLs, a homology model of 3D7 Pf332-DBL (PF11_0506 amino acids 1-255) was constructed using the HHpred server with default settings. The crystal structure of the EBA-175 F2-domain was used as a template (Protein Data Bank code 1ZRO, chain A). The alignment was manually adjusted at positions 180 and 254 to align two cysteines that were in close proximity, to allow for the modeling of two disulphide bridges conserved in the DBL-domains of PfEBA-175 and PkαDBP. MODELLER 9v3 and the MPI-toolkit were used to create the model and structural visualizations were made in PyMol. The model was validated by using the PROCHECK program available in the SWISS-MODEL Workspace and Verify3D, available in the HHpred server toolkit.

3.5 IMMUNIZATION REGIMEN AND ANTIBODY DETECTION

Immunogenicity and antibody responses to most malaria antigens are tested in either rodents or rabbits. BALB/c mice, C57BL/6 mice, New Zealand white rabbits and Sprague-Dawley rats were immunized with *E. coli* expressed recombinant Pf332-DBL protein carrying a His-tag in combination with any of the following adjuvants; Montanide ISA 720, aluminum hydroxide, levamisole or complete Freund's adjuvant/incomplete Freund's adjuvant. Animals were immunized intramuscularly with the antigen-adjuvant formulation on week 0, 3, 6 and 9. The amount of recombinant protein in the immunizations was 10 μg/mouse, 50 μg/rat or 100 μg/rabbit. Blood was collected prior to the first immunization and two weeks after each immunization, as well as on week 13, 15 and 17. Antibody responses were subsequently measured by ELISA, and IgG1/Ig2a ratios were determined as an indication of the type of immune response that was elicited.

3.6 DIFFERENTIAL PROTEIN EXTRACTION

Peripheral membrane proteins are extracted from the membrane by treatment with alkaline sodium carbonate and urea, whereas these solutions leave the lipid bilayer and integral membrane proteins intact. The latter proteins are only extracted by treatment with a detergent, such as the non-ionic detergent TX-100. Cytoskeleton-interacting proteins are typically insoluble in TX-100, but soluble in the ionic detergent SDS, and this property is commonly used as a biochemical definition of a cytoskeleton association. Trophozoite or schizont-stage pRBC were enriched by magnetic cell sorting, which yielded a parasitemia of approximately 90%. Parasitized cells were subsequently resuspended in a hypotonic solution (7.5 mM Tris-HCl pH 8.0) and freeze thawn. Following an ultracentrifugation step (100 000 x g for 60 min), aqueous proteins (soluble in the hypotonic solution) were separated from membrane proteins (pellet proteins). The latter were extracted by one of the following solutions; 100 mM sodium carbonate pH 11.5, 6 M urea, 1% TX-100 or 2% SDS plus 1% TX-100, followed by centrifugation to separate soluble and insoluble proteins. Equal amount of parasite extracts ($2.5x10^6$ – $3.3x10^6$ pRBC/lane) were subsequently separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blot analysis.

3.7 SELECTIVE PERMEABILIZATION AND TRYPSIN DIGESTION

To verify the subcellular localization and extraction profile of Pf332, we used a biochemical approach based on the accessibility of the protein to trypsin digestion after selectively permeabilizing membranes using detergents Equinatoxin II (EqtII) and saponin. EqtII is a eukaryotic pore-forming toxin from the venom of the sea anemone

Actinia equin (Schon *et al.*, 2008), which selectively forms pores (up to 100 nm) in the sphingomyelin-containing RBC PM, whilst leaving the membranes of MCs and PV intact. Saponin on the other hand, is a plant-derived glycoside that interacts with cholesterol and forms pores (up to 30 nm) in the RBC PM, MCs and PVM, but leaves the parasite PM intact. By adding trypsin to selectively permeabilized cells, the subcellular localization of a protein can be determined. For example, in EqtII-treated cells, proteins/parts of proteins present in the lumen of MC will be protected from digestion by trypsin as a result of the intact MC membrane, whereas proteins/parts of proteins located in the RBC cytosol will be digested. In EqtII/saponin-treated cells, proteins will be completely digested by trypsin regardless of a luminal or a cytosolic location, as saponin also disrupts the MC and PV membrane. Subsequently, a TM protein with the N-terminus facing the MC lumen and the C-terminus facing the host cell cytosol will be truncated in EqtII-lysed cells, whereas it will be completely digested in EqtII/saponin-lysed cells. By using antibodies targeting both the N- and the C-termini, the topology of a protein can thus be determined. A simplified outline of the method (with a focus on MCs) is depicted in Figure 5.

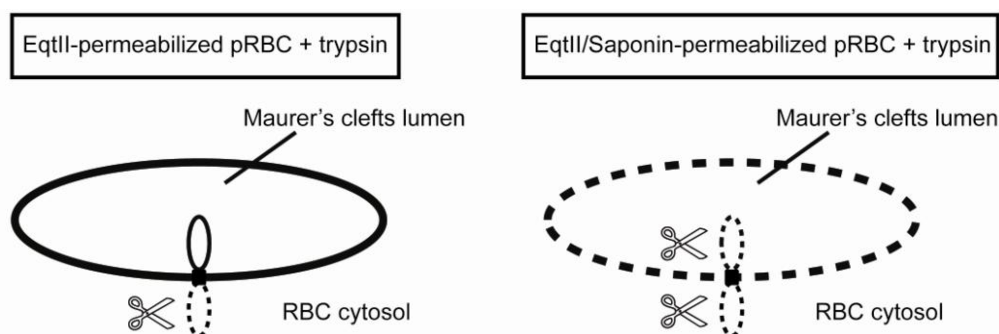

Figure 5. Selective permeabilization of membranes followed by trypsin digestion. In EqtII-permeabilized pRBCs, the RBC PM is permeabilized, but the MC membrane is left intact (left). Accordingly, when adding trypsin, proteins/parts of proteins located in the lumen of MCs are protected (full line) from digestion, whereas proteins/parts of proteins located in the RBC cytosol are sensitive (dashed line). A membrane spanning protein will subsequently be truncated in EqtII-lysed cells. In EqtII/saponin-permeabilized pRBCs, the RBC PM and the MC membrane are permeabilized (right). Accordingly, a membrane spanning protein will be completely digested (dashed lines) when adding trypsin. Note that the illustration only shows MC.

3.8 ALLELIC DISCRIMINATION

Real-time PCR allelic discrimination primers and probes (MGB probes labeled with either FAM or VIC) were manually designed using Primer Express 3.0. The risk of primer dimerization and secondary structure formation of all primers and probes were assessed using ΔG estimations in NetPrimer. Allelic discrimination assays were designed based on the sequenced genome of 3D7, FCR3, Dd2 and HB3 parasites and validated with amplification of dilution series of HB3 gDNA and mixtures of FCR3 and NF54 gDNA. Allelic discrimination was conducted on both gDNA and cDNA from whole parasite cultures and single cells, using the above described primers and discriminative probes. Amplifications were performed in at least triplicates in an ABI 7500 real-time PCR system, starting with a pre-read (for background fluorescence measurements), followed by 40 cycles of amplification, and a final post-read (for total fluorescence emission measurement after amplification). The relative allele frequency was evaluated from fluorescence ratios from the allele specific probes.

4 RESULTS AND DISCUSSION

Results are detailed and discussed in each respective study included in this thesis (Paper I-V). The original results and interpretations are also summarized here.

4.1 PAPER I

"The gene encoding Pf332 is comprised of two exons, one of which is encoding a DBL-domain"

Previous to this investigation, the gene encoding Pf332 was believed to have a single exon structure. During a bioinformatical search for open reading frames (ORFs) predicted to encode RBC-binding domains, the ORF PF11_0506, located only 280 base pairs (bp) upstream of the start codon of adjacent gene encoding Pf332 (PF11_0507), was identified. The length of the intergenic region was very short compared to the mean length in *P. falciparum* (1694 bp) (Gardner *et al.*, 2002), thus we hypothesized that the region was an intron rather than an intergenic region. To test our hypothesis, we designed primers towards the 3′ end of PF11_0506 and the 5′ end of PF11_0507. The size of the reverse-transcriptase PCR product from cDNA was 350 bp, whereas the PCR product from gDNA was 600 bp, indicative of a splicing event. Comparing the sequences of the PCR and RT-PCR products further revealed an intron sequence possessing the typical gt-ag splicing site at its ends. Northern blot hybridizations using a probe located at the 5′ end of PF11_0506 and a probe covering the splicing site, confirmed that PF11_0506 and PF11_0507 constitute a single mRNA. Thus, Pf332 is encoded by a two-exon gene composed of a 5′ exon with a size of 1704 bp (PF11_0506) and a 3′ exon with a size of 16 578 bp (PF11_0507), separated by a short intron of 236 bp (Figure 6). The 3′ exon of Pf332 was initially identified from a gDNA expression library containing only a region of repetitive sequence without additional 5′-RACE sequencing to identify the 5′-end of the transcript. The existence of the additional upstream exon has therefore previously been overlooked and the 5′ exon of Pf332 was as a consequence annotated in the *P. falciparum* genome as a separate gene encoding a hypothetical protein.

Exon I did not encode an N-terminal SP or a classical PEXEL motif, but encode a PEXEL-like motif (RSLAD) commencing 78 amino acids downstream of the N-terminus. When performing a BLASTP search and an alignment of the amino acid sequence encoded by exon I, the exon was found to encode a region homologous to the Duffy binding-like (DBL)-domains of the Erythrocyte binding-like (EBL)-family of invasion proteins. In order to investigate the RBC-binding properties of the newly identified DBL-domain of Pf332, we generated *E. coli* recombinant proteins corresponding to both the DBL-domain and a region downstream of the DBL-domain. By Western blot, it was evident that only the DBL-domain and not the downstream region was able to bind RBCs, and this was subsequently verified when using CHO-cells transiently expressing the DBL-domain or the downstream region. Sequencing of 11 laboratory strains and field isolates revealed that the DBL-domain is conserved as only a few point mutations were found.

Although having a homologous DBL-domain in common, Pf332 differs considerably from the EBL-DBLs in three important aspects. Firstly, Pf332 lacks the N-terminal segment (NTS) containing a SP, two tandem DBL-domains, the C-terminal c-cys region and TM. The revised structure of Pf332 contains an N-terminal DBL-domain followed by an adjacent predicted TM and a large number of negatively charged Glu-repeats, which make up the major part of the molecule. A tryptophan-rich

domain (WRD) with similarities to WRDs found in SURFINs, PkSICA*var* and PfEMP1 (Winter et al., 2005) is also present in the C-terminus of the protein (Figure 6). Secondly, by real-time PCR, it was evident that the *Pf332* gene was activated at approximately 16 h p.i., reaching a maximum transcription at 24 h p.i.. In contrast, most EBL-members are activated at approximately 38 h p.i., in line with their role in merozoite invasion. Thirdly, the EBL-members are localized to the micronemes, whereas Pf332 is exported into the host cell cytosol where it associates with MCs and the RBC PM. The interaction of Pf332 with the host cell PM appeared to be very close, as anti-Pf332 antibodies were able to detect Pf332 on unfixed live schizont-stage pRBC. Collectively, these observations led us to hypothesize about the DBL-domain of Pf332 being involved in binding to RBCs and thereby causing rosetting of mature-stage pRBCs, potentially as a mean to provide close proximity of new host cells and thus indirectly facilitating merozoite invasion. To test this hypothesis, we carried out invasion inhibition assays where three different laboratory strains were cultivated for a complete IDC in the presence of anti-Pf332-DBL antibodies. Indeed, antibodies to Pf332-DBL were found to reduce the invasion efficiency when used at a concentration of 1 mg/ml and this effect was concentration dependent.

Taking into account more recent data (Paper IV), do the findings in Paper I support a surface expression and a role for Pf332 in invasion/parasite growth? Localization studies of mature-stage pRBC are notoriously difficult due to the increased permeability of the RBC PM, which can allow antibodies to gain access to parasite proteins on the cytoplasmic face of the membrane. This can result in a staining resembling surface reactivity, leading to the conclusion of surface exposure of a molecule that is really located in close proximity to the inner leaflet of the RBC PM. A cytoplasmic location of Pf332 is further supported by the observed lack of variation in the DBL-domain, which indicates that the protein is not under any selective pressure. In contrast, nonsynonomous SNPs are frequently detected in the EBL-DBLs, in line with the antigens being under diversifying selection from the human immune system (Baum et al., 2003, Ozwara et al., 2001). Moreover, compared to antibodies targeting proteins critical for the invasion process, the anti-Pf332-DBL antibodies investigated in the present study had to be used at a relatively high concentration in order to give a significant effect.

The comparison of the Pf332-DBL and EBL-DBLs raised the issue of their structural similarities and furthermore as to what extent antibodies towards Pf332-DBL and members of the EBL-family cross-react; questions that were addressed in Paper II.

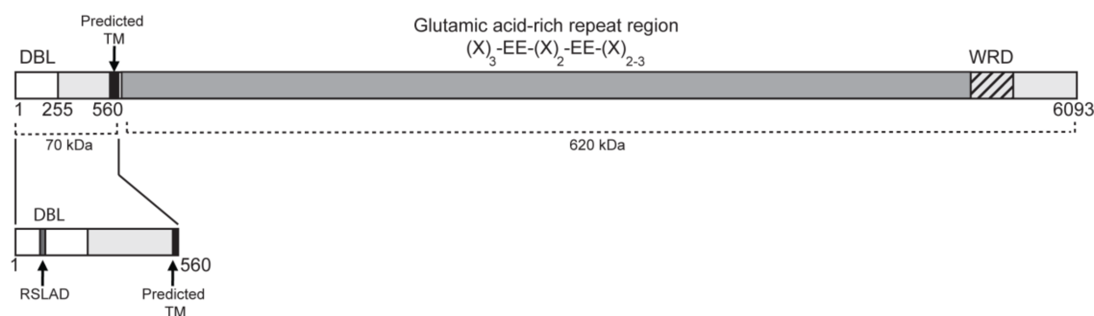

Figure 6. Schematic representation of the revised structure of the Pf332 protein. Residues 1-570 are encoded by the first exon, which contains the DBL-domain (white), a PEXEL-like sequence RSLAD and the predicted TM (black). The second exon encodes an extensive Glu-rich repeat region (dark grey) with the consensus $(X)_3$-EE$(X)_2$-EE-$(X)_{2\text{-}3}$ and the tryptophan-rich region (WRD; striped). (X=hydrophobic amino acid, E=Glu).

4.2 PAPER II

"The DBL-domain of Pf332 generates antigen specific antibodies, which do not cross-react with DBL-domains of other malaria antigens"

EB200, a 157 amino acid long sequence of Pf332, was the first region of the molecule to be cloned. To this date, EB200 is the most extensively used region for both antibody production and immunorecognition studies of Pf332. However, EB200 contains arrays of tandemly repeated 11-mers having a high frequency of Glu residues, often occurring in pairs. Due to the presence of Glu-rich repeats in a wide array of malaria antigens, there is a significant risk of cross-reactivity when using the EB200 region as a Pf332 marker. We therefore wanted to evaluate the usefulness of the conserved DBL-domain (Paper I), as a specific marker for Pf332. Moreover, we were interested in investigating any potential cross-reactivity of antibodies towards Pf332-DBL with other EBL-DBLs.

Firstly, we assessed the structural similarities between the DBL-domain of Pf332 and the homologous PkαDBL and the tandem DBL-domains of EBA-175 (F1 and F2), which structures have recently been solved. Using the crystal structure of F2 as a template, we constructed a 3D model of Pf332-DBL by comparative modeling on the basis of the 3D7 sequence (PF11_0506, residues 1-255). According to our 3D model, Pf332 has a scaffold of α-helices surrounded by flexible loops. Thus Pf332 shares the basic DBL fold despite having only 24-29% sequence identity with the PkαDBL and EBA-175 DBLs. The high content of α-helices observed in our 3D model was in accordance with the secondary structure content of a recombinant Pf332-DBL protein expressed in *E. coli*, as determined by circular dichroism. Sequence alignment revealed that of the twelve cysteine residues present in Pf332-DBL, eight corresponded in location to cysteines found in F1, F2 and PkαDBL. Two cysteines were found to be unique to Pf332-DBL, whereas the remaining two cysteines were shared only with EBA-175 F2. Interestingly, one of the latter is shared with all *var* DBL-types. All cysteines except for the two that are unique for Pf332-DBL were predicted to form conserved disulphide bridges, illustrating the importance of the cysteines in maintaining the DBL fold. Although the overall fold of Pf332-DBL was similar to that of the EBL-DBLs, structural differences were observed, implying that Pf332-DBL could have a different dynamical behavior leading to differences in its receptor specificity or binding preferences. The presence of a DBL-domain in Pf332 is intriguing. The F1-domain of EBA-175 is related to the single DBL-domain of *P. vivax* and *P. knowlesi*, and the domain is common to all EBLs. In contrast, the F2-domain of EBA-175 has so far only been found in EBLs in *P. falciparum* and *P. reichenowi* and the domain has been suggested to be the progenitor of *var* DBLs. It is therefore interesting to note that the DBL-domain of Pf332 has more similarities to F2 than to F1, as the DBL-domain of Pf332 so far only has been identified in *P. falciparum*, although Pf332-like proteins are present in species such as *P. berghei* and *P. yoelii.*

Secondly, we analyzed the presence of naturally acquired antibodies to Pf332-DBL in individuals residing in distinct malaria endemic regions (Uganda, Burkina Faso and Mali). Antibodies were highly prevalent in adults and they were frequently of high titer. In an area of intense malaria transmission (Apac, Uganda), antibodies were acquired early in life, but there was no difference in prevalence or titer when comparing plasma from children suffering from either mild or severe malaria. Similarly, there was no difference in prevalence and antibody titer when comparing plasma from individuals belonging to distinctive sympatric tribes having different susceptibility to malaria. This shows that Pf332-DBL is highly immunogenic and is in accordance with the domain having a conserved sequence (Paper I). As Pf332 appears to be an intracellular protein

(Paper IV), we hypothesize that antibodies towards the antigen are generated upon schizont rupture, at which time the protein becomes accessible to the immune system. The assumption is then that the high levels of anti-Pf332 antibodies are a reflection of parasite exposure rather than malaria protection, as has been suggested for other Glu-rich antigens.

Finally, we set out to evaluate the antigen-specificity of naturally acquired antibodies in order to exclude the possibility of cross-reactivity between Pf332-DBL and EBL-DBLs. Following affinity purification using the recombinant Pf332 DBL-domain, the human antibodies were probed on a peptide microarray of overlapping 15-mers, where they were found to only react with peptides present in Pf332-DBL but not in the EBL-DBLs, here represented by the tandem DBL-domains of EBA-175. The peptide array only takes liner epitopes into consideration; however, it gives a strong indication that there is little or no cross-reactivity between the DBL-domains of Pf332 and the EBL-family. To confirm that this was true also for native and conformational protein, we carried out IFA. The anti-Pf332-DBL antibodies gave a typical Pf332 fluorescence pattern as demonstrated by the co-localization of the antibodies with MC marker MAHRP1. There was no cross-reactivity with native EBA-175, as the anti-Pf332-DBL and anti-EBA-175 antibodies gave very distinct immunofluorescence pattern. Thus, we conclude that the conservation and distinct sequence of the Pf332 DBL-domain (Paper I) and the antigen specificity of generated antibodies, make Pf332-DBL an attractive region for future studies on the molecule Pf332.

4.3 PAPER III

“Immunizations with Pf332-DBL in combination with adjuvant Montanide ISA 720 generate significant levels of Pf332 specific antibodies in mice”

The first recombinant subunit vaccine approved for human use was the hepatitis B vaccine, which was expressed in yeast cells. Advantages of recombinant subunit vaccines are numerous; however, they are often poor immunogens and proper adjuvants are therefore essential. The main targets for a malaria blood-stage vaccine are invasion ligands and surface expressed antigens, but these often undergo antigenic variation or are polymorphic. Increased titers of anti-Pf332 antibodies have been associated with fewer clinical malaria attacks in individuals residing in malaria endemic regions, as well as a reduced parasite growth *in vitro*. Based on these previous observations, together with the findings from Paper I, we set up to assess the immunogenicity of Pf332-DBL in different animals. Additionally, in order to find a suitable adjuvant for malaria antigen immunizations, we compared the effect of different adjuvants in combination with the Pf332-DBL antigen.

To get a more complete view of the immunogenicity of Pf332-DBL, we included four different animal species; BALB/c mice (Th2-prone), C57BL/6 mice (Th1-prone), New Zealand white rabbits and Sprague-Dawley rats. Groups of eight (mice) or four (rabbits and rats) were immunized on four consecutive occasions with a His-tagged Pf332-DBL recombinant protein expressed in *E. coli* in combination with the human compatible adjuvants Montanide ISA 720 (M-ISA 720), aluminum or levamisol. Control groups received the antigen either alone (negative control) or in combination with Freund’s adjuvant (positive control).

Specific antibodies towards Pf332-DBL were generated in all animal species investigated, reaching a maximum after the final immunization. Antibody levels were in general of highest magnitude in BALB/c mice. Formulations of Pf332-DBL in combinations with Freund’s adjuvant, M-ISA 720 or aluminum generated significantly higher antibody levels compared to when the antigen was used alone, and M-ISA 720 and aluminum generated even significantly higher antibody level than did Freund’s adjuvant. Eight weeks after the final immunization, BALB/c mice still had a significantly higher IgG level in groups that had received Freund’s adjuvant, M-ISA 720 or aluminum compared to mice immunized with protein alone. Particularly M-ISA 720 stood out, since IgG levels were more stable over time. Moreover, M-ISA 720 and aluminum induced a Th2-biased immune response, whereas Freund’s adjuvant generated a mixed Th2/Th1 response.

In C57BL/6 mice, Pf332-DBL formulations containing Freund’s adjuvant or M-ISA 720 generated significantly higher antibody levels compared to when the antigen was used alone. Eight weeks following the final immunization, the groups which had received Freund’s adjuvant and M-ISA 720 had significantly higher antibody levels compared to when the antigen was used alone; however, IgG levels were gradually declining after the final immunization. Similar to in BALB/c mice, M-ISA 720 and aluminum induced a Th2 response, whereas Freund’s adjuvant generated a mixed Th2/Th1 response.

After the final immunization, rabbits that had received antigen in combination with Freund’s adjuvant gave a more prominent antibody response compared to rabbits that had received antigen alone; however, this was not significant. In general, rabbits generated lower antibody responses towards the Pf332-DBL adjuvant formulations than did any of the other animals.

In rats, IgG levels gradually increased with each immunization; however, IgG levels quickly declined after the final immunization. Nevertheless, the rat group that had received the antigen/M-ISA 720 formulation had significantly elevated antibody

levels compared to the group that had received the antigen alone. In general, rats displayed more variation within the group than did any of the other animals. Levamisole did not show any obvious adjuvant effect in any of the immunized animals.

Montanide adjuvants (including M-ISA 720) are formulated as water-in-oil emulsions and are similar to incomplete Freund's adjuvant in physical characteristics, but biodegradable. Formulations containing M-ISA 720 have been shown to be safe, well tolerated and capable of eliciting high antibody responses in combination with several malarial antigens in both human vaccination trials and animal immunization studies. In the present study, M-ISA 720 proved to be the best-suited adjuvant of the three different types investigated, as it induced a significant antibody response in both BALB/c mice and C57BL/6 mice with IgG1 as the prominent isotype produced. Moreover, elicited antibodies were antigen specific as determined by their ability to recognize native Pf332 from the FCR3S1.2 parasite by Western blot analysis. Thus, our findings support the use of Montanides as an adjuvant in additional immunization studies of malaria antigens. Importantly, this study illustrates that there may be a marked variation in response to the same antigen/adjuvant formulation by different animal species, which should be taken into consideration when designing animal immunization studies.

4.4 PAPER IV

"Pf332 associates with the cytosolic side of Maurer's clefts via protein-protein interactions and interacts with the host cytoskeleton in mature parasite stages"

The solubility characteristics and subcellular localization are two highly informative clues as to what function a certain protein may exert. We therefore set up to examine these properties in the context of Pf332, using biochemical, IFA and flow cytometry based methods.

To elucidate if or when Pf332 interacts with the cytoskeleton, we conducted a time-course where enriched pRBC were collected and extracted with TX-100 at three different time-points. At 26-30 and 32-36 h p.i., Pf332 was mainly found in the TX-100 soluble fraction as examined by Western blot. In contrast, at 38-42 h p.i. the entire Pf332 population was found in the TX-100 insoluble fraction, thus Pf332 appears to be interacting with the cytoskeleton only in schizont-stage pRBC. To be able to correlate the observed biochemical profile of Pf332 with the subcellular localization of the antigen, samples were collected for IFA at each of the above described time-points. Pf332 co-localized with the MC marker MAHRP1 at all three time-points, indicating that the association with MC is not transient, but permanent. At 26-30 and 32-36 h p.i., MCs were located throughout the RBC cytosol, whereas at 38-42 h p.i. (and Pf332 had shifted to become TX-100 insoluble), MCs were closely associated with the RBC PM. This is in accordance with Pf332 interacting with the cytoskeleton in mature parasite stages. The insolubility of Pf332 in TX-100 in mature-stage pRBCs could not be explained by a surface-expressed population, as no live schizont-stage pRBC stained positively for Pf332 by flow cytometry. These findings led us to conclude that Pf332 is closely associated with MCs throughout trophozoite maturation and schizogony, and that the interaction of Pf332 with the cytoskeleton increases as the parasite matures. The obtained results provide an explanation for the conflicting findings presented by Mattei et al. and Glenister et al. regarding TX-100 solubility of Pf332 and highlight the importance of assaying parasite-lysates collected from more than one time-point.

Because Pf332 is expressed in very mature pRBCs and appears to make the host cell less rigid, it is tempting to speculate that Pf332 is involved in cytoskeleton destabilization upon completion of the IDC, possible as a mechanism to prepare the host cell for merozoite egress. By interacting with the cytoskeleton, Pf332 may reduce the cytoskeletal affinity to junctional complex components or parasite proteins. Alternatively, the massive size of Pf332 may sterically hinder other cytoskeleton-interacting proteins from binding. Pf332 may also become the target of specific proteases activated at schizont-stage, whose actions ultimately lead to pRBC rupture. The Western blot observation of an increasing number of smaller sized Pf332 polypeptides as the parasite matures, supports this view. Furthermore, when using the PEST finder program, which identifies proline (P), glutamic acid (E), serine (S) and threonine (T) rich regions present in proteins targeted for proteolysis (Rogers *et al.*, 1986), 17 predicted PEST-domains were identified throughout the Pf332 molecule.

Due to the cysteine-rich nature of the DBL-domain and the proposed TM region (Paper I), we hypothesized that Pf332 is a membrane spanning protein with the N-terminal DBL-domain located in the lumen of MC and the C-terminal repeat region facing the RBC cytosol. Intriguingly, Pf332 was completely extractable by urea and alkaline sodium carbonate, which is indicative of a peripheral membrane association, but speaks against an integral membrane association. This was further verified when using a biochemical approach based on the accessibility of the protein to trypsin digestion after selectively permeabilizing membranes of enriched trophozoite/schizont-stage pRBC using the detergents saponin and/or EqtII. If the DBL-domain was located in the lumen of MCs, it would be protected from digestion by trypsin in EqtII-

permeabilized cells, whereas the C-terminus would be accessible, resulting in a truncated protein. However, by Western blot it was evident that the Pf332-DBL signal was lost completely, demonstrating that the entire antigen must be present in the host cell cytosol. Hence, Pf332 appears to be a peripheral membrane protein of MCs attaching via protein-protein interactions (Figure 7). On the basis of the above mentioned findings, the predicted TM of Pf332 does not appear to be a membrane spanning region, but rather a hydrophobic stretch/recessed SP, possibly involved in protein trafficking and/or MC association, although this needs to be experimentally confirmed.

A peripheral membrane location of Pf332 implies that the antigen is synthesized and trafficked in a water-soluble state. However, in the presence of BFA, Pf332 resisted extraction by a hypotonic solution, but was readily solubilized by alkaline sodium carbonate. This led us to conclude that Pf332 was rather trafficked as a peripheral membrane protein, possibly interacting in a multimeric protein-complex. Given the extremely large size of Pf332, it seems reasonable to assume that correct trafficking of this massive antigen requires additional help from chaperones. Indeed, Pf332 was recently proposed to interact with two putative co-chaperones in a yeast two-hybrid screen (Pavithra *et al.*, 2007). Comparable protein solubility results have been observed also for PfEMP1 (Papakrivos et al., 2005), implying that Pf332 and PfEMP1 may be synthesized and trafficked in a similar manner, possibly involving chaperones.

The present study establishes Pf332 as an intracellular antigen, resident at MCs. The cytoskeleton interacting property of Pf332 only near completion of the IDC may have important implications in host cell remodeling exerted by the *P. falciparum* parasite.

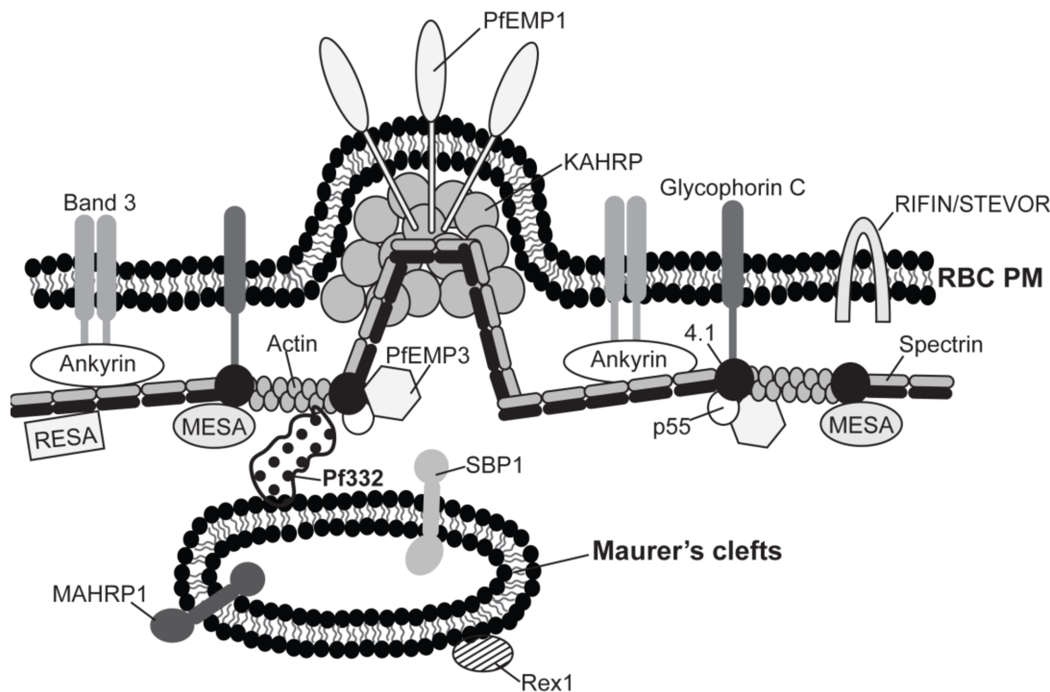

Figure 7. Proposed model of the subcellular localization of Pf332 in *P. falciparum* pRBC. Pf332 (dotty) is located on the cytosolic side of MC and interacts with the cytoskeleton in very mature parasite stages.

4.5 PAPER V

“Duplicated *var2csa* genes are simultaneously transcribed in individual parasites”

Gene duplications are frequent in *P. falciparum* parasites. Previous findings of copy number polymorphisms (CNPs) in genes related to drug resistance and parasite invasion, suggest that parasites employ gene duplications as a strategy to enhance their survival, thus gene duplications may play an important role in malaria pathogenesis. Single nucleotide polymorphisms (SNPs) are often introduced into the duplicated gene, either during the duplication event or through subsequent mutations. These modifications can lead to the formation of either pseudogenes or functional genes, and in case of the latter the gene product may retain its original function or display an altered function (loss of function, gain of function or dominant-negative effect). Hence, one cannot assume that there is a linear correlation between gene transcription and gene dosage or biological function. The *Pf332* and *var2csa* genes have been found duplicated in the genome of the HB3 parasite; however, it is currently unknown whether both gene copies are transcriptionally active or not.

In an attempt to study transcriptional activity of duplicated genes, we developed an allele discriminative real-time PCR assay based on slight sequence variations in the duplicated *Pf332* and *var2csa* genes in HB3. Laboratory parasite strains FCR3, 3D7 and Dd2 were used as negative controls for the assay, as they contain singly copy genes of both *var2csa* and *Pf332*. To be able to discriminate between the duplicated *var2csa* genes in HB3, we designed allele-specific primers towards conserved regions and FAM and VIC labeled TaqMan MGB probes towards variable regions, resulting in two assays (towards DBL2x and DBL4ε). A third assay was similarly designed to identify different *Pf332* variants in NF54 and HB3, and the discriminative probes were designed towards a nonsynonomous SNP (S326P) present in exon I. Using the allelic discriminating assay, we first analyzed the presence of the sequence-variable alleles in gDNA from the different laboratory strains, resulting in the amplification of both alleles in HB3 and the single alleles in FCR3, NF54 and Dd2 parasites. *var2csa* transcripts were subsequently analyzed in HB3, FCR3 and NF54 both before and after CSA-selection, and both *var2csa* alleles were actively transcribed in HB3. Transcripts of both the wild-type and the mutant *Pf332* genes were similarly present and actively transcribed in HB3. Taken together, the allelic-discrimination assay proved to be specific, efficient and straightforward. This approach can be extended to study other issues related to genetic polymorphisms, thus providing a useful tool for further investigations regarding the impact of gene duplications on *P. falciparum* biology.

The transcription of *var* genes at trophozoite-stage is presumed to be mutually exclusive, with a single expressed *var* gene at a time. Thus the finding of simultaneously transcribed *var2csa* genes on a population level was of particular interest. However, this did not provide information about whether both genes copies are expressed in single cells. In an effort to elucidate transcriptional activity in individual cells, single HB3CSA parasites were collected by micromanipulation and analyzed in a nested PCR/real-time PCR approach. Interestingly and somewhat surprisingly, both alleles of *var2csa* were transcribed in individual parasites collected at 24±4 h p.i.. These results were confirmed by RNA-FISH using probes designed towards one of the most sequence-variable regions of the *var2csa* paralogs in order to discriminate between them. This also enabled us to use NF54CSA and FCR3CSA as controls for one of the *var2csa* sequences. Indeed, most HB3CSA parasites displayed a high abundance of *var2csa* transcripts from both paralogs, whereas the control parasites displayed only transcripts from their single allele types. The RNA-FISH further

revealed exclusive nuclear co-localization of the two transcripts, despite being located on different chromosomes. Also the active genes were found to co-localize in the majority of cells by DNA-FISH. The co-localization of *var2csa* genes and the corresponding transcripts supports a previously suggested specific site for *var* gene expression that can accommodate more than one active *var* gene at a time. Whether both transcripts are translated into protein that will be surface expressed remains to be elucidated; however, both alleles were detected using only oligo(dT) primers in the reverse transcription, suggesting that the transcripts were destined for translation.

The simultaneously transcribed *var2csa* genes challenge the dogma of mutually exclusive expression of *var* genes, at least in respect to the duplicated *var2csa* genes. In should be noted that this may represent a special case since the sequence similarity among different *var2csa* variants is high compared to that of other *var* genes. Interestingly, whereas one of the *var2csa* genes is found on chromosome 12, the other copy is found on chromosome 1, which could suggest the presence of *var2csa* specific transcription factors with preserved DNA-binding regions in the duplicated gene copies. Indeed, the upstream regions of the *var2csa* paralogs are highly similar. Further studies on potential *var2csa* specific transcription factors will be of great interest in this context.

These findings were supported by a recent study in where Joergensen and colleagues reported simultaneously transcribed *var* genes in a single cell using limiting dilution real-time PCR and RNA-FISH (Joergensen et al., 2010). Interestingly, by using confocal immunofluorescence microscopy they could detect two different PfEMP1 molecules simultaneously expressed on a single cell, illustrating that not only can two *var* genes be simultaneously transcribed, these can also be translated into protein and become surface expressed. Whether this is an artifact of *in vitro* adapted parasite lines, or a reflection of the selection process employed by Joergensen et al. remains elusive. It would be of great interest to see if also clinical parasite isolates can express more than a single *var* gene at a time, also with regard to *var2csa*.

5 CONCLUDING REMARKS AND FUTURE ASPECTS

Based on the papers included in this thesis, the following conclusion can be drawn and future aspects proposed:

I. The gene encoding Pf332 consists of two exons, where exon I encodes a Duffy binding-like (DBL)-domain homologous to the Erythrocyte binding-like (EBL) family of invasion proteins. Pf332 lacks a canonical SP and a classical PEXEL motif. Although the DBL-domain appears capable of binding RBCs *in vitro*, the function of the domain still remains elusive. Hence, additional studies are needed in order to get a more complete picture of what role the Pf332 DBL-domain plays in *P. falciparum* biology.

II. Antibodies towards the DBL-domain of Pf332 are readily acquired in individuals residing in malaria endemic areas and do not cross-react with the DBL-domains of the EBL-family of invasion proteins. Previous studies of Pf332 have been hampered by the cross-reactive nature of the antigen. Thus, the conserved DBL-domain of Pf332 is an attractive marker to use in future studies of the antigen, and we expect this to facilitate more antigen-specific investigations of the molecule. It would be interesting to see whether the DBL-domain of Pf332 can form the characteristic EBL-DBLs disulphide bridges, since the endogenous antigen appear to be present in a reducing cytosolic environment (Paper IV). The structural and sequence similarities of Pf332-DBL and the EBL-DBLs is from an evolutionary perspective of significant interest.

III. The Pf332 DBL-domain is immunogenic in combination with different adjuvants currently used in human vaccination studies. Of the different adjuvants evaluated, Montanide ISA 720 appears to be the best suited adjuvant for immunization studies using recombinant proteins. Importantly, there may be a marked variation in response to the same antigen/adjuvant formulation by different animal species, which should be taken into consideration when designing animal immunization studies.

IV. Pf332 is a host cytoskeleton interacting protein that is synthesized as a peripheral membrane protein and associates with the cytosolic side of MCs via protein-protein interactions throughout trophozoite maturation and schizogony. The antigen is not exposed on the host cell surface. Taken together, this implies that the TM is not a membrane spanning region. Instead, this region may represent a recessed SP and/or an MC attachment domain. It would be interesting to investigate if GFP-Pf332 chimeras containing the TM/hydrophobic stretch alone or in combination with the DBL-domain are exported and correctly targeted to the MC. This would provide some much-needed additional insight into the trafficking mechanism employed by PEXEL-negative exported proteins. The interaction of Pf332 with the submembrane cytoskeleton only in mature-stage pRBC implies that the protein participates in host cell modifications at completion of the IDC, possibly to destabilize the submembrane skeleton and the RBC PM. It would be of great interest to

investigate the molecular nature and importance of such an interaction in *P. falciparum* biology.

V. Real time allelic discrimination and discriminative RNA-FISH can be used to distinguish between highly similar gene copies in *P. falciparum,* including *Pf332* and *var2csa*. The assay provides a robust and straightforward tool to study the impact of gene duplications on the biology of *P. falciparum* and this versatile approach can be extended to study other issues related to polymorphisms and gene regulation. In this context, it is of interest to study regulation of *var* genes, which play a pivotal role in host cell remodeling and malaria pathogenesis.

Protein export and host cell remodeling is central to the malaria pathogenesis. Although much information has been gained during the past decades, there are currently many aspects that remain obscure. Pf332, the largest exported antigen in malaria, is certainly of interest in this context due to its characteristic protein structure, immunogenicity, and host cell remodeling properties. Taken together, the papers presented in this thesis has opened up for more detailed analyses of Pf332. Future studies of this massive antigen are expected to provide important information regarding evolution of DBL-domains in Plasmodia, protein trafficking pathways of PEXEL-negative exported proteins, and molecular processes that underlie parasite-induced host cell modifications. Ultimately, this may lead to an improved understanding of malaria pathogenesis, and perhaps, suggest new approaches how to combat the disease.

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