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Characterization of the Novel Maurer's Clefts Protein MAHRP1 in *Plasmodium falciparum*

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Anna Cornelia Spycher
aus Köniz (BE)

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Dekan

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Zusammenfassung

Plasmodium falciparum verursacht die schlimmste Form der menschlichen Malaria und ist verantwortlich für 1-2 Millionen Todesfälle jährlich. Ein Impfstoff ist nicht vorhanden, und Resistenz gegen Medikamente ist weit verbreitet. Die Identifizierung neuer Interventionsziele für die medikamentöse Behandlung wäre eine Möglichkeit, diese Probleme anzugehen. Dieser Ansatz wird jedoch durch ein limitiertes Verständnis vieler Aspekte der *P. falciparum*-Biologie behindert.

Morbidität und Mortalität zufolge Malaria werden durch die asexuellen Stadien des Erregers in den menschlichen roten Blutkörperchen (RB) verursacht. Während die meisten intrazellulären Parasiten mit aktiven Wirtszellen interagieren, entwickelt sich der Malaria-Erreger in einer terminal differentierten Zelle ohne Organellen und ohne Transportmaschinerie für Proteine. Der Parasit residiert in einer parasitophoren Vakuole (PV), die von einer parasitophoren Vakuolenmembran (PVM) umgeben ist. Der Parasit modifiziert die Wirtszelle durch Errichtung von membranösen Strukturen im Zytosol des RB. Diese Modifikationen beinhalten unter anderem diskoide Strukturen mit unbekannter Funktion in der Peripherie des RB, genannt „Maurer's clefts" (MC). Zusätzlich verändert der Parasit die Wirtszelle durch den Export von eigenen Proteinen an die Oberfläche der des RB. Diese Exportprodukte tragen massgeblich zur Virulenz des Parasiten bei, wobei das variante Oberflächen-Antigen „*Plasmodium falciparum* erythrocyte membrane protein 1" (PfEMP1) ein Hauptakteur ist, der die Adhärenz von infizierten Erythrozyten an die Endothelzellen bewirkt und dadurch, so vermutet man, die Eliminierung des infizierten RB durch die Milz verhindert. Der Parasit entgeht dem Immunsystem zusätzlich durch Antigenvariation, d.h. durch die wechselnde Expression verschiedener Mitglieder der PfEMP1-Proteinfamilie.

Da reife RB keine zelluläre Transportmaschinerie mehr haben, etabliert der Parasit sein eigenes Sekretionssystem, um Proteine über die Parasitenmembran (PM) hinaus durch die PVM und durch das Erythrozytenzytosol hindurch zu den MCs und der Wirtszellenmembran zu exportieren. Die Mechanismen und molekularen Komponenten, welche an diesem Prozess beteiligt sind, sind noch nicht vollständig geklärt. Eine klassische Signalsequenz ist ausreichend, um Proteine in das endoplasmatische Retikulum (ER) zu lenken mit vorgegebener Weiterleitung in die PV. Weitertransport durch die PVM benötigt eine

zusätzliche Signalsequenz, genannt „*Plasmodium* Export Element“ (PEXEL) oder „Vacuolar Targeting Signal“ (VTS).

Ziel dieser Arbeit war die Charakterisierung eines neuen Transmembran (TM)-Proteins, des sogenannten „membrane associated histidine-rich protein 1“ (MAHRP1). Es wird ausschliesslich in jungen Stadien transkribiert, hat eine C-Domäne mit rund 30% aus DHGH-Wiederholungen bestehenden Histidinen und ist an den Maurer’s Clefts lokalisiert. MAHRP1 hat keine klassische Signalsequenz und keine PEXEL/VTS-Signale, und es ist somit unklar, wie das Protein zu den MCs gelangt.

Histidinreiche Proteine sind bekannt als gute Kationen-Binder. Der histidinreiche Teil von MAHRP1 wurde rekombinant exprimiert und verwendet für Interaktionsstudien mit Ferriprotoporphyrin (FP), einem toxischen Abfallprodukt, welches bei der Zersetzung von Hämoglobin anfällt, einer Hauptnahrungsquelle des Parasiten. MAHRP1 interagiert spezifisch mit FP und die Bindungsstöchiometrie korreliert mit der Anzahl vorhandener DHGH-Wiederholungen. Gebundenes FP weist eine erhöhte Peroxidase-ähnliche Aktivität auf, und seine Anfälligkeit für H_20_2-induzierte Degradation ist zehnmal höher als diejenige von ungebundenem FP. Diese Eigenschaften von MAHRP1 lassen vermuten, dass es bei den MCs eine protektive Rolle gegen oxidative Belastung spielt.

Für die Untersuchung der Aminosäuren, die für den korrekten Transport von MAHRP1 zustaendig sind, wurden Plasmide generiert, welche verschiedene MAHRP1-Domänen enthalten, die an grün fluoreszierendes Protein (GFP) gekoppelt sind. Parasiten wurden anschliessend mit diesen Plasmiden transfiziert. Analysen der verschiedenen Transfektanten ergaben, dass das ganze MAHRP1-GFP erfolgreich an die MCs gelangt, hingegen die TM- oder TM-C-Domänen im ER zurückgehalten werden. Weitere Zergliederungen der N-Domäne deckten eine 18 Aminosäuren grosse Region auf, welche ein Signal mit limitierter Aehnlichkeit zum PEXEL/VTS-Signal aufweist und welche notwendig ist für den Export von MAHRP1 zu den MCs. „Fluorescence recovery after photobleaching“ (FRAP) und Zeitreihenphotographie-Anwendungen weisen darauf hin, dass MAHRP1-GFP zuerst zu isolierten Subdomänen der PV/PVM transportiert wird, die entstehende MCs darzustellen scheinen. Die gewonnenen Daten lassen vermuten, dass die MCs in der PVM knospen, anschliessend durchs Zytosol der RB diffundieren und sich schliesslich an der Zellperipherie ansiedeln.

Zur weiteren Klärung der Funktion von MAHRP1 wurde eine Mutante mit einer *mahrp1* Disruption und einer MAHRP1 Ablation generiert. MCs werden auch in Abwesenheit von MAHRP1 immer noch gebildet, aber der Export von PfEMP1 wird im PV/PVM-Bereich angehalten. Dementsprechend ist kein PfEMP1 an der Wirtszellen-Oberfläche zu finden. Der Export von anderen ausgewählten Proteinen scheint jedoch unverändert zu funktionieren. Dies deutet darauf hin, dass MAHRP1 eine essentielle Rolle beim Export des bedeutenden Virulenzfaktors PfEMP1 spielt und somit ein attraktives Interventionsziel im Kampf gegen Malaria darstellt.

Summary

Plasmodium falciparum causes the worst form of human malaria and is responsible for 1-2 million deaths annually. A vaccine is not available and resistance to drugs is widespread. One approach to tackle these problems is to identify new intervention targets. However, this is hampered by a limited understanding of many aspects of *P. falciparum* biology.

The morbidity and mortality associated with malaria is due to the asexual erythrocyte stages of *P. falciparum*. While most intracellular pathogens interact with an active host cell, the malaria parasite develops within the red blood cell (RBC) that is devoid of all organelles and any protein trafficking machinery. The parasite resides in a parasitophorous vacuole (PV), which is encircled by a parasitophorous vacuolar membrane (PVM). The parasite modifies its host cell by establishing membranous structures in the RBC cytoplasm. These comprise disc-shaped structures at the RBC periphery called Maurer's clefts (MC) with an elusive function. In addition the parasite modifies the surface of the infected RBC by exporting own proteins that contribute to the virulence of *P. falciparum*. A key protein in this process is the variant surface antigen, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates adherence of infected RBCs to host endothelial cells. This is thought to prevent the infected RBC of being cleared by the spleen. Furthermore the parasite evades the immune system by a process called antigenic variation, the switching of the expression between different members of PfEMP1.

As mature RBCs lack a cellular trafficking machinery, the parasite establishes its own secretory system for exporting proteins beyond the parasite membrane (PM) through the PVM and across the host cell cytoplasm to the MCs and the RBC membrane. The mechanisms and molecular apparatus involved in this process are not completely understood. A classical signal sequence is sufficient to direct proteins into the endoplasmatic reticulum (ER) with default release into the PV. Onward transport across the PVM requires an additional signature termed *Plasmodium* export element (PEXEL) or vacuolar targeting signal (VTS).

The aim of this thesis was to characterize a novel transmembrane (TM) protein termed membrane-associated histidine-rich protein 1 (MAHRP1). It is transcribed exclusively in early stages, has a C-terminus with approximately 30% histidines present as DHGH repeats and localizes to the Maurer's clefts. MAHRP1 has no classical signal sequence and no PEXEL/VTS motif and it is thus unclear how it is directed to the MCs.

Histidine-rich proteins have been shown to be effective cation binders. The histidine-rich region of MAHRP1 was recombinantly expressed and used for interaction studies with the toxic waste product ferriprotoporphyrine (FP), which accumulates upon degradation of hemoglobin -a main nutrient source of the parasite. MAHRP1 specifically interacts with FP and binding stoichiometry correlates with the amount of DHGH repeats. The bound FP has increased peroxidase-like activity and is 10-fold more susceptible to H_2O_2-induced degradation compared with unbound FP. These properties of MAHRP1 suggest it may play a protective role against oxidative stress at the MCs.

To investigate the amino acids responsible for correct trafficking of MAHRP1 to the MCs, plasmids were generated encoding different green fluorescent protein (GFP)-tagged domains of MAHRP1 and parasites were subsequently transfected with these plasmids. Analysis of transfectants showed that the full length MAHRP1- GFP is successfully trafficked to the MCs, whereas the domains TM or TM-C-terminus were retained at the ER. Dissection of the N-terminus revealed a segment of 18 amino acids containing a motif with limited similarity to the PEXEL/VTS motif, and which is needed for export of MAHRP1 to the MCs. Fluorescence photobleaching and time-lapse imaging techniques indicate that MAHRP1-GFP is initially trafficked to isolated subdomains in the PV/PVM that appear to represent nascent MCs. The data suggest that the MCs bud from the PVM and diffuse within the RBC cytoplasm before taking up residence at the cell periphery.

To understand the function of MAHRP1, a mutant with a *mahrp1* disruption and MAHRP1 ablation was generated. MCs are still formed in the absence of MAHRP1 but the export of PfEMP1 is interrupted at the PM/PVM interface. As a consequence, no PfEMP1 is detected on the surface of infected RBCs. By contrast, export of other selected proteins appears to be uninhibited. This indicates that MAHRP1 plays an essential role in the export of major virulence factor PfEMP1 and thus represents an interesting intervention target in the battle against malaria.

Abbreviations

EBA	erythrocyte-binding antigen
EM	electron microscopy
ER	endoplasmatic reticulum
ETRAMP	early transcribed membrane protein
EXP1	exported protein 1
FP	ferriprotoporphyrine
FRAP	fluorescence recovery after photobleaching
FV	food vacuole
GFP	green fluorescent protein
HPI	hours post invasion
HRP	histidine-rich protein
HRP2	histidine-rich protein 2
HTS	host targeting signal, used equivalent of VTS
IC_{50}	half maximal inhibitory concentration
iRBC	infected red blood cell
KAHRP	knob-associated histidine-rich protein
MAHRP1	membrane-associated histidine-rich protein 1
MC	Maurer's clefts
PEXEL	*Plasmodium* export element
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein 1
PM	parasite membrane
PV	parasitophorous vacuole
PVM	parasitophorous vacuolar membrane
RBC	red blood cell
RBCM	red blood cell membrane
REX1	ring expressed protein 1
SBP1	skeleton binding protein 1
TVN	tubovesicular network
VTS	vacuolar targeting signal, used equivalent of HTS

Introduction

Malaria is caused by protozoans of the genus *Plasmodium*. It belongs to the phylum of the *Apicomplexa* that contains other human and animal pathogens such as *Toxoplasma*, *Cryptosporidium*, *Eimeria*, *Babesia* and *Theileria*. *Apicomplexa* are characterized by the presence of an apical complex in juxtaposition to the nucleus. The genus *Plasmodium* contains more than 100 species of which four infect humans: *Plasmodium falciparum*, the most important pathogen for humans, *P. vivax*, *P. ovale*, and *P. malariae*. Other species that infect monkeys (eg. *P. knowlesi*, *P. cynomolgi*) or rodents (*P. yoelii*, *P. berghei*, *P. chabaudi*, *P. vinckei*) are studied and used as animal models.

Today approximately 40% of the world's population, mostly living in the world's poorest countries, is at risk of malaria. Each year, 300-500 million people become infected and 1 to 2 million will die (WHO, 2005). Clinical manifestations of severe malaria include cerebral malaria, severe anaemia, hypoglycaemia, renal failure, non-cardiac pulmonary oedema, and respiratory failure (WHO, 2000). Once in the blood, the processes associated with the multiplication of the parasite in the red blood cells (RBCs) are responsible for most of the clinical symptoms of malaria and its associated morbidity and mortality.

No vaccine is available and resistance to most antimalarial drugs occurs in many parts of the world. Research is mainly focussing on *P. falciparum* because of its clinical importance. In addition, an *in vitro* culturing system is available (Trager and Jensen, 1976) and the genome has been sequenced for the culture strain 3D7 (www.plasmodb.org).The nuclear genome of *P. falciparum* (3D7) contains 22.8 million base pairs distributed on 14 chromosomes (Gardner et al., 2002). About 5300 coding genes have been identified of which more than 60% have no assigned function as they lack homology to any known protein based on their primary sequence.

Chapter 1

Plasmodium falciparum Biology

1.1 *Plasmodium falciparum* life cycle

Plasmodium falciparum maintains a complex life cycle. It alternates between the vertebrate human host and the invertebrate female mosquito of the genus *Anopheles* (several species). The cycle begins when haploid sporozoites are injected into the human host by an infected mosquito during a blood meal. Once in the bloodstream, sporozoites migrate to the liver where they invade hepatocytes, multiply asexually and after 5-20 days release thousands of merozoites into the blood stream. These now invade a second type of host cells, namely human erythrocytes, where they undergo a 48 h cycle of asexual replication. Encompassed in a parasitophorous vacuolar membrane (PVM), the parasites develop from ring stages or early trophozoites (0–22 h post invasion [hpi]) to trophozoites (22–36 hpi) and finally to schizonts (36–48 hpi). Rupture of schizonts releases up to 32 merozoites into the bloodstream, which initiate a new round of schizogony. Some of the intraerythrocytic parasites develop into sexual blood stages (gametocytes). These gametocytes must be taken up by an anopheline mosquito during another blood meal. Once in the mosquito gut, gametocytes develop into gametes and sexual reproduction takes place whereby 8 flagellated microgametes are released from a male gametocyte fertilizing a female macrogamete to form a diploid zygote. The zygote develops into an ookinete capable of penetrating the gut wall and maturing to an oocyst in about 10 days. The oocyst finally ruptures to release a large number of haploid sporozoites into the haemolymph. The sporozoites migrate to the salivary gland from where they are injected into the human host during the next blood meal to complete the cycle.

1.2 Parasite cell biology

Our work focuses on specific molecular processes that take place in the intraerythrocytic part of the *P. falciparum* life cycle. For this reason this chapter will focus on aspects of this part of the parasite's complex life cycle only. Figure 1 summarizes and exemplifies key points described below.

Rapid growth of the parasite within the host erythrocyte is achieved, in part, by digesting hemoglobin within a lysosome-like compartment that is referred to as the **food vacuole** (FV). The food vacuole becomes evident at the transition between ring and subsequent trophozoite stages (Francis et al., 1997). Hemoglobin proteolysis takes place by a series of parasite-derived proteases. Digestion of hemoglobin by the parasite first requires the engulfment of portions of host cytoplasm across the parasitophorous vacuole (PV) and the plasma membrane

(PM) and into the FV. This process has been morphologically characterised and involves a specialized endosome-like organelle, referred to as the **cytostome**. The molecular events mediating this process, however, are completely unknown.

Approximately 35 hours after invasion, when most of the hemoglobin has been consumed, the parasite initiates simultaneous synthesis of up to 32 daughter merozoites, each of which is capable of invading a new red blood cell. As a part of merozoite formation, the apical organelles comprising of the rhoptries, micronemes and dense granules are formed *de novo* by the secretory pathway (Tonkin et al., 2006).

Micronemes are storage organelles for adhesive proteins such as members of the EBL-family, EBA-175, EBA-140/BAEBL, and EBA-181/JSEBL (Sim et al., 1994; Thompson et al., 2001; Gilberger et al., 2003). They release their contents onto the parasite surface upon host cell recognition. **Rhoptries** are larger club-shaped organelles that are found at the apex of the polarized merozoite. They are approximately 400 nm long (Bannister, 2001) and they contribute to the invasion process as well as to the synthesis of the PV (Kats et al., 2006). The third set of apical organelles are dense, rounded vesicles termed **dense granules** which probably expel their contents into the PV upon entry into the host cell and may contribute to the expansion of the PVM and also cause other modifications of the RBC.

As a member of the Apicomplexans, *P. falciparum* contains a plastid called the **apicoplast** that is essential for parasite survival. The apicoplast is surrounded by four membranes and is a remnant of a secondary endosymbiontic event. It allows the parasite to synthesize various vitamins and aromatic precursor molecules *de novo* which cannot be obtained from its host (Kohler et al., 1997; Roos et al., 2002; Waller et al., 2003).

Little is known about the molecular organization of the secretory pathway of *P. falciparum*. The **endoplasmatic reticulum** (ER) resembles a simple perinuclear structure with two protruding ‘horns’, and several eukaryotic ER homologues have been found, such as the endoplasmic reticulum calcium-binding protein (La Greca et al., 1997) and a homologue of the heat shock protein 70 (Bianco et al., 1986). A typically stacked **Golgi apparatus** has not been identified in the parasite, but a discoid cisterna close to the nucleus has been described that was provisionally specified as a minimal Golgi apparatus (Bannister et al., 2000; Bannister et al., 2003; Struck et al., 2005). Different Golgi homologues have been localized in perinuclear area such as the GTPase Rab6 (Van Wye et al., 1996), the KDEL receptor (Elmendorf and Haldar, 1993b) and the Golgi re-assembly stacking protein GRASP (Struck et al., 2005).

Electron microscopy (EM) studies have identified a single **mitochondrion** as a crescent-shaped organelle in merozoites, which branches out in trophozoites, before segregating along with the nucleus into daughter merozoites (Slomianny and Prensier, 1986; Akompong et al., 2002; van Dooren et al., 2005). Interestingly, EM studies revealed a close association between the apicoplast and the mitochondrion at various stages during the intraerythrocytic cycle (Hopkins et al., 1999). This mitochondrion/apicoplast association has been postulated to occur to allow transfer of metabolites between the two organelles (Hopkins et al., 1999; Ralph et al., 2004).

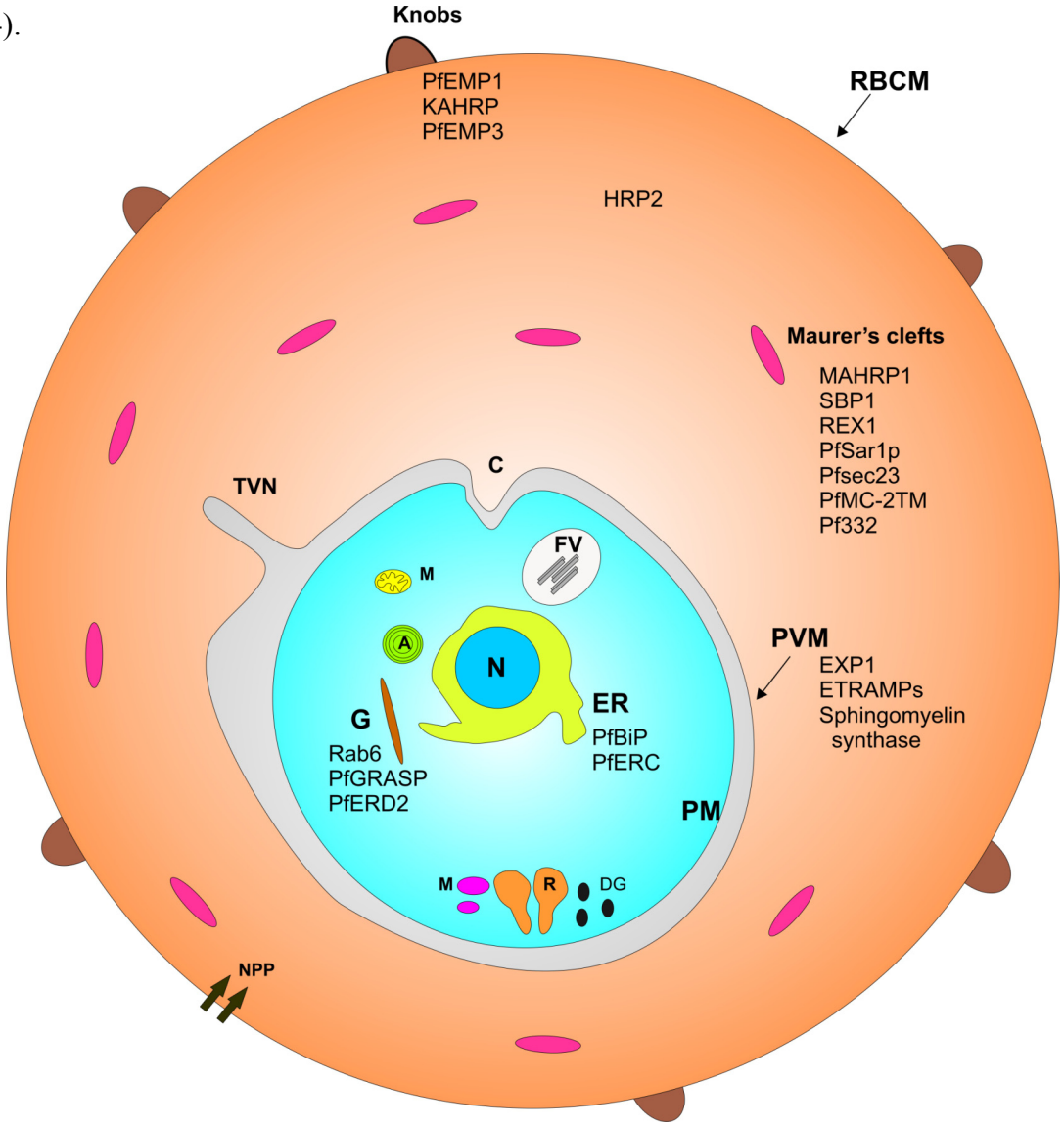

Figure 1. Schematic representation of a *Plasmodium falciparum*-infected red blood cell. Selected proteins are shown at their final destinations.
Abbreviations: RBCM, red blood cell membrane; PVM, parasitophorous vacuolar membrane; PM, parasite membrane; TVN, tubovesicular network; N, nucleus; ER, endoplasmatic reticulum, G, Golgi apparatus; R, rhoptries; DG, dense granules, M, micronemes; FV, food vacuole; A, apicoplast; M, mitochondrion; C, cytostome; NPP, new permeation pathways.

1.3 Refurbishing the host cell

Human erythrocytes are highly specialized cells, denucleated, devoid of internal organelles, lacking a full repertoire of nutrient transporters and a functional protein trafficking system, and also lacking major histocompatibility complex (MHC) molecules. This metabolically inert cell allows the parasite to hide from the immune system. As a trade-off, the parasite needs to refurbish the host cell to import nutrients, dispose of waste products, and export proteins across its plasma membrane (PM), the surrounding PVM, the erythrocyte cytosol and red blood cell membrane (RBCM). The parasite radically modifies the properties of its host cell. A few are listed in the following:

- A **tubovesicular network** (TVN) extends from the PVM into the cytoplasm of trophozoite infected RBCs. This network harbours the Golgi marker sphingomyelin synthase and was therefore thought to possess secretory properties (Elmendorf and Haldar, 1993a). However, other results provide evidence for involvement of the TVN in nutrient import that may happen at a TVN-RBC membrane junction (Lauer et al., 1997).

- **New permeation pathways** are established at 15 hpi in the host cell membrane (Staines et al., 2001), and several parasite proteins become associated with the RBC cytoskeleton (Cooke et al., 2001). However, not only parasite proteins are suspected to be the molecular basis for these new permeation pathways. There is evidence that parasite-induced membrane oxidation is also responsible for activation of an endogenous RBC membrane channel (Huber et al., 2002). Appearance of these modifications at the late ring to early trophozoite stage coincides with onset of rapid parasite growth and sequestration in postcapillary venules.

- Flattened vesicular structures called **Maurer's clefts (MC)** are parasite-derived structures positioned in the red blood cell cytosol. Maurer's clefts are characterized as flattened lamellar, slender membrane structures with a translucent lumen and an electron-dense coat (Langreth et al., 1978; Atkinson et al., 1988; Elford et al., 1997; Kriek et al., 2003). These compartments appear near the parasitophorous vacuole, but they gradually relocate closer to the RBC membrane and appear to be tethered by fibrous connections to the RBC membrane skeleton (Atkinson and Aikawa, 1990;

Waterkeyn et al., 2000). There is currently some debate as to whether the Maurer's clefts are independent structures, or sub-domains of the tubovesicular network. A recent study used serial electron micrograph sections to examine these structures in the *P. falciparum* iRBC cytosol (Wickert et al., 2004). These authors postulate that Maurer's clefts and the tubovesicular network form part of a continuous meshwork. Other reports analysed the dynamics of GFP chimeras of Maurer's cleft–associated cargo and suggest that Maurer's clefts are distinct entities (Wickham et al., 2001; Knuepfer et al., 2005; Spycher et al., 2006).

To present it is not fully understood what the role of the Maurer's clefts is and different functions have been proposed. Two independent studies have localized the hypoxanthine phosphorybosyl transferase to the MCs (Shahabuddin et al., 1992; Vincensini et al., 2005). In addition, a fatty acyl-Co synthase 1 (Matesanz et al., 1999) and a phosphoethanolamin-N-methyl transferase (Vincensini et al., 2005) have been proposed to be MC-associated. These findings suggest that Maurer's clefts house metabolic enzymes. Other studies have identified several kinases including *P. falciparum* exported serine-threonine kinase (FEST ,Kun et al., 1997), *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1, Moskes et al., 2004) and *P. falciparum* glycogen synthase kinase 3 ortholog (PfGSK3, Droucheau et al., 2004) to be at MCs and thus suggest them to act as a signalling platform. The most frequently discussed hypothesis suggests that Maurer's clefts are in some way involved in transport of parasite proteins to the RBC plasma membrane (Barnwell, 1990). The fact that most of the parasite-derived surface-exposed proteins are trafficked via Maurer's clefts, independent of their solubility, underlines this hypothesis. In addition, *P. falciparum* homologs of proteins involved in vesicle transport such as Pfsec23 and Pfsec31 (Albano et al., 1999; Adisa et al., 2001) have been localized to the MCs. Furthermore, Maurer's clefts have been shown to be involved in transport and possibly the assembly of proteins forming knobs (Wickham et al., 2001), and a Maurer's clefts resident, skeleton-binding protein-1 has been shown to be essential for the export of the major virulence factor *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) to the RBC surface (Cooke et al., 2006).

Bringing together the different ideas on connectivity of MCs with the suggested functional involvement of Maurer's clefts as a "secretory" compartment has led to 4 different models as exemplified in figure 2 below.

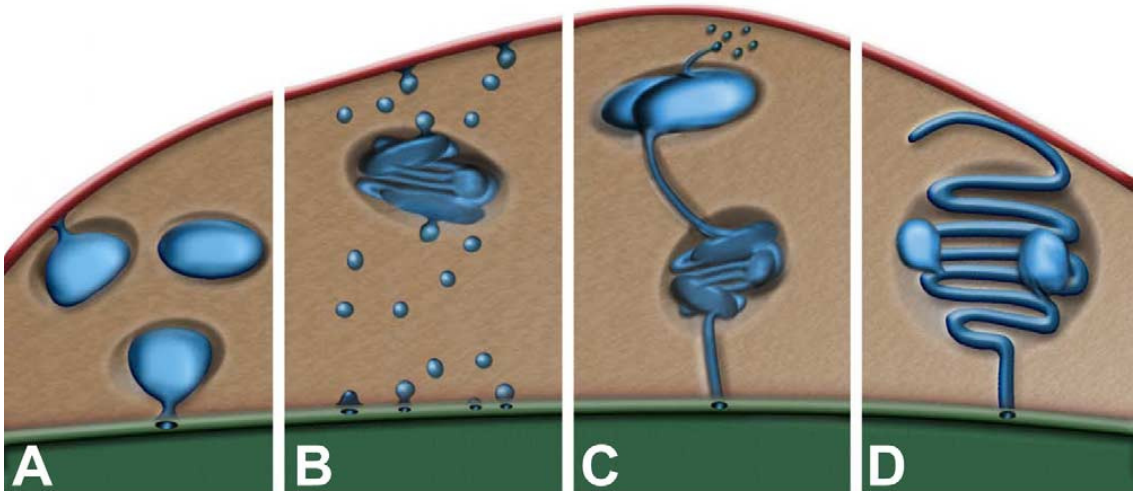

Figure 2. Putative protein trafficking pathways from the parasite (in green) to the erythrocyte plasma membrane (in red). (A) Giant vesicle. Parasite proteins are transported by a conglomerate of Maurer's clefts membranes that pinch off the parasitophorous vacuolar membrane (PVM) and finally fuse with the host cell plasma membrane. **(B) Vesicular transport model**. Parasite proteins are transported by small vesicles that bud off the PVM and fuse with Maurer's clefts residing as isolated entities within the cytosol of the infected red blood cell. In a second step, target vesicles bud off from the Maurer's clefts to fuse with the plasma membrane of the infected erythrocyte. **(C) Lateral diffusion model**. Proteins of parasite origin are routed by an interconnected Maurer's cleft membrane network bridging the distance between the PVM and the plasma membrane of the infected red blood cell. Small vesicles pinch off at the very edges of the Maurer's cleft membrane network just beneath the membrane of the infected red blood cell, thereby shuttling parasitic proteins to the cell surface. **(D) Lateral diffusion model without vesicles**. In this model, parasitic proteins are transported by lateral diffusion from the PVM to the surface of the infected erythrocyte along the Maurer's cleft membrane network without the need of small vesicles. Instead, local fusion of Maurer's cleft membranes with the plasma membrane of the erythrocyte would allow proteins to reach the surface (Lanzer et al., 2006).

1.4 Early events upon invasion

To date, little is known about the molecular events taking place in the earliest steps upon invasion: the ring stage. Compared to later stage parasites, ring stages are characterized by low metabolic and biosynthetic activity (Zolg et al., 1984; de Rojas and Wasserman, 1985), and little change in size and morphology. However, it is unlikely that the parasite lies dormant during half of its asexual development in RBCs, and it is assumed that this "lag"-phase serves the parasite to induce the elaborate host cell modifications apparent in later stages and crucial for establishment in the host cell. This initial host cell refurbishment is necessary for growth and survival and must include a protein-trafficking system to deliver the required components beyond the parasite's boundaries into different locations of the host cell. Apart from the intriguing cell biological aspects, this situation most probably demands unusual processes essential for parasite survival. Deciphering these early events does not only contribute to the understanding of this parasite's "establishment strategies" but also represents an interesting target for drug intervention.

To address the question of early events upon invasion, Spielmann and Beck have used suppression subtractive hybridization to clone genes exclusively transcribed during the *P. falciparum* ring stage (Spielmann and Beck, 2000). In contrast to genes originating from a trophozoite-specific library, few of the identified ring-specific genes showed homologies to known genes of other organisms, which is in accordance with the unique nature of the molecular events in early stages. One of these genes, *skeleton binding protein 1* (*sbp1)* has previously been shown to code for a protein located in Maurer's clefts and was proposed to bind the erythrocyte scaffold (Blisnick et al., 2000). Furthermore, their work identified three members of a new gene family coding for highly charged putative membrane proteins referred to as early transcribed membrane proteins (Spielmann and Beck, 2000). Among the other ring stage-specific genes, two histidine-rich proteins were found. They were named membrane-associated-histidine-rich protein 1 (Spycher et al., 2003) and 2 (MAHRP2, unpublished). In an attempt to better understand early events upon invasion, we characterized MAHRP1 and addressed the question of its locations and possible function. These findings are summarized in Chapter 2.

2. Histidine-rich proteins

Histidine is one of the 20 most common natural amino acids present in proteins. The imidazole side chains and the relatively neutral pK of histidine (ca 6.0) mean that relatively small shifts in cellular pH will change its charge. For this reason, this amino acid side chain finds its way into considerable use as a co-ordinating ligand in metalloproteins, and also as a catalytic site in certain enzymes.

There are numerous studies on histidine-rich proteins (HRPs) in prokaryotic and eukaryotic organisms. Human saliva, for instance, contains a family of low molecular weight cationic, histidine-rich polypeptides known as histatins. Reports describe their potent bactericidal and fungicidal properties (MacKay et al., 1984; Pollock et al., 1984). These peptides form part of the innate immune system and play an important role in maintaining oral health by limiting infections in the oral cavity.

Histidine-rich glycoprotein (HRG) is found in the plasma of many vertebrates and has an overall histidine content of 13%. Metal and heme binding to HRG is mediated via the numerous histidine residues and it appears that the metal binding function of HRG is to modify the conformation of the molecule and thereby regulate subsequent biological activities such as the modulation of angiogenesis regulation of cell adhesion and migration (Jones et al., 2005).

A third example is the histidine-rich protein Hpn in *Heliobacter pylori*. The gram-negative bacterium colonizes the stomach and causes gastritis and ulcerations. Its adaptation to this hostile environment is linked to the production of abundant urease, a nickel containing metalloenzyme catalyzing the hydrolysis of urea and carbamate for neutralization of gastric acid, which is critical for survival under low pH conditions. Therefore, a constant supply of nickel ions is required for the synthesis and activity of ureases by *H. pylori.* Hpn has been proposed to play a role in nickel storage (Ge et al., 2006).

In *Plasmodium*, first publications on histidine-rich proteins date back to 1975. In the meantime there is a small number of well characterized HRPs. HRPs seem to play a key role in the parasite survival strategy. One day into the two-day intraerythrocytic cycle, parasites begin to ingest the abundant hemoglobin of their host erythrocyte by an endocytic process. The parasite degrades about 75% of the hemoglobin (Loria et al., 1999). The released amino acids are incorporated into parasite proteins and also appear to be available for energy metabolism. Proteolysis is thought to be essential for parasite survival because *P. falciparum*

has a limited capacity for *de novo* amino acid synthesis (Sherman, 1977). However, the proteolytic degradation of hemoglobin also produces heme (ferriprotoporphyrin IX). Free intracellular ferriprotoporphyrin (FP) is toxic due to its detergent-like (Chou and Fitch, 1981), and redox properties (Tappel, 1953). The parasite appears to dispose of FP largely by sequestration in the parasite's food vacuole as a granular pigment known as hemozoin (β-hematin (Slater and Cerami, 1992; Egan et al., 2002). It has been shown previously that this crystallization process is initiated and accelerated *in vitro* by parasite-derived histidine-rich proteins (Sullivan et al., 1996; Papalexis et al., 2001), and the inhibition of hemozoin formation is believed to underlie the mode of action of chloroquine *in vivo* (Ziegler et al., 2001). It has also been suggested that non-enzymatic degradation, including reactions with H_2O_2 and glutathione may contribute to heme detoxification (Ginsburg et al., 1998; Loria et al., 1999) and that histidine-rich proteins may modulate these processes (Ginsburg et al., 1998). Several parasite-encoded histidine-rich proteins have been described, of which three are discussed in more detail in the following:

- ***P. falciparum*** **histidine-rich protein 2** (HRP2, MAL13P1.480) is one of the best characterized. This soluble protein is found in the food vacuole but also in the erythrocyte cytosol (Howard et al., 1986; Papalexis et al., 2001). Earlier studies suggested that HRP2 is ingested by the parasite's cytostome from the red cell together with hemoglobin and thereby delivered to the food vacuole by bulk endocytosis (Sullivan et al., 1996). However, recent studies show that although 75% of the hemoglobin is ingested by the parasite, more than 90% of HRP2 remains in the erythrocyte cytosol (Akompong et al., 2002). Cytosolic HRP2 seems to concentrate mainly near the periphery of the erythrocyte, suggesting that it may not diffuse freely through the erythrocyte cytoplasm and could thereby avoid its ingestion. These recent findings suggest that HRP2 could play a role in the detoxification of the by-products of hemoglobin degradation in the host cytoplasm. Recently, it has been shown that HRP2 modulates the redox activity of FP and that the HRP2-FP complex may have antioxidant properties (Mashima et al., 2002). Hence, the location of HRP2 near the erythrocyte membrane may function to protect the host membrane from oxidative stress and thus prevent premature host cell lysis.

  HRP2 is nowadays an established marker in malaria rapid diagnostic tests such as ParaSight®-F-Test (Becton Dickinson) and ICT MalariaPf® Test (ICT diagnostics, reviewed in (Moody, 2002).

- The ***P. falciparum*** **knob-associated histidine-rich protein** (KAHRP, PFB0100c) is another well-characterized HRP. KAHRP is a soluble protein and essential for the formation of knob-like protuberances on the surface of infected erythrocytes (Crabb et al., 1997; Waller et al., 1999; Rug et al., 2006). KAHRP interacts with various cytoskeletal components of the erythrocyte including spectrin, actin and actrin-spectrin-band 4.1 complexes (Kilejian et al., 1991; Oh et al., 2000; Pei et al., 2005). Knobs act as platforms for the presentation of *P. falciparum* erythrocyte membrane protein-1 (Waller et al., 1999), which is responsible for adherence of parasitized erythrocytes to vascular endothelial cells (Su et al., 1995). This adhesion and subsequent accumulation of infected erythrocytes in the microvasculature are pivotal events in the pathogenesis of *P. falciparum* malaria, and are considered to represent major virulence mediators. It has been shown, that targeted *kahrp* disruption leads to a reduction of cytoadherence under shear stress (Crabb et al., 1997).

- The most recently discovered HRP in *P. falciparum* is the **membrane-associated histidine-rich protein 1** (MAHRP1, MAL13P1.413). We have characterized this novel transmembrane protein, which is expressed in early stages upon invasion of RBCs and localizes to the Maurer's clefts (MCs). Unlike KAHRP and HRP2, it is not a soluble protein and is thought to reside permanently in association with the Maurer's clefts. To elucidate if MAHRP1 plays a role in metal-binding and putative "microprotection" of MCs from deleterious effects caused by free FP, we recombinantly expressed the C-terminus, which consist of nearly 30% histidines, and used it in binding studies with ferriprotoporphyrin FP IX. At the MCs, the histidine-rich C-terminus is predicted to face the cytosolic side and this may point to a role in local detoxification processes at the MCs. Findings were published and are shown and discussed in chapter 2.

3. Cellular transport pathways

In eukaryotic cells, there are two main protein transport pathways. (I) The **secretory pathway**, which is used to transport not only proteins that are secreted from the cell but also resident proteins in the lumen of the endoplasmic reticulum, Golgi, and lysosomes as well as integral proteins of the membrane of these organelles and the plasma membrane. (II) The **endocytic pathway**, which transports ingested molecules in vesicles generated at the plasma

membrane to early endosomes and then via late endosomes to lysosomes. Furthermore, there are transport pathways directing proteins to mitochondria or chloroplasts, peroxisomes and the nucleus.

Transport of proteins and lipids along the endocytic or secretory pathways is a hallmark of eukaryotic cells. The secretory system allows cells to regulate delivery of newly synthesized proteins, carbohydrates, and lipids to the cell surface - a necessity for growth and homeostasis. Intracellular traffic is very selective. Only a subset of the proteins and lipids in the donor membrane are given access into the transport vesicle, effectively preventing the homogenization of membrane components and permitting membranous organelles to maintain distinct identities throughout the life of the cell.

3.1 General features of the secretory pathway

All nuclear-encoded mRNAs are translated on cytosolic ribosomes. Nascent proteins destined for the secretory pathway often contain an ER signal sequence, which directs them to the ER. The protein therefore enters or crosses the ER membrane by translocation during its synthesis. At the ER exit sites, proteins destined to further compartments in the secretory pathway, leave the ER packaged into transport vesicles and are directed towards the Golgi where they fuse to form and maintain the *cis*-Golgi compartment. Upon processing and maturation, they progress to the *trans*-Golgi compartment and thus form the trans-Golgi network (TGN). Proteins destined for secretion are sorted into one of two types of vesicles according to the secretion pattern of the protein. Some proteins are secreted continuously and these vesicles immediately move to the plasma membrane, fuse and release their contents by exocytosis. Other proteins are exported by regulated secretion. They accumulate in secretory vesicles awaiting a stimulus for controlled exocytosis. This directional membrane flow is balanced by retrieval pathways, which bring membrane and selected proteins back to the compartment of origin.

3.2 General mechanisms of vesicular transport

The principal element of vesicular traffic is that vesicles bud from a donor compartment and fuse with an acceptor compartment. Cargo proteins that are loaded into the forming vesicle are thereby delivered to the acceptor compartment. To provide specificity to the transport mechanism, several proteins on the donor compartment, the vesicle membrane and the

acceptor compartment interact under carefully regulated conditions. The biogenesis of transport vesicles is initiated through the recruitment of large multi-subunit protein complexes termed coats. The coat is thought to perform two principal functions. It integrates specific membrane proteins into the vesicle membrane that help to select the cargo protein and it curves and deforms the membrane, eventually leading to the release of coated transport vesicles from the donor compartment (Bonifacino and Lippincott-Schwartz, 2003). Dependent on the type of coats the vesicles are recruited to a specific membrane compartment within the cell.

The best studied trafficking pathways are those that use carrier vesicles clearly identifiable by their coats, made of the coatmer COPI, COPII, or clathrin and its partners (see Figure 3). During the formation of a vesicle, a limited set of coat proteins carries out a programmed set of sequential interactions that lead to budding from the parent membrane, uncoating, fusion with a target membrane, and recycling of the coat components. There are clear similarities and differences between the ways that COPI, COPII and clathrin coats handle these steps. COPI and COPII vesicles traffic between the endoplasmic reticulum and the Golgi complex - COPI primarily retrograde from the Golgi to the ER and between Golgi cisternae, and COPII anterograde from the ER to the Golgi. The clathrin pathway has two major routes, from the plasma membrane to the early endosome and from the Golgi to the endosome. Other structures have been observed in the cell, which do not have COP or clathrin coats. Internalization from the plasma membrane can also occur via macropinocytosis and phagocytosis.

Before the vesicle fuses with the acceptor membrane the coat is discarded. The next steps in the fate of a transport vesicle involve proteins of the Rab GTPase family. Rabs interact with different proteins, loosely termed Rab effectors. Rab effectors perform diverse functions from vesicle budding to vesicle transport and vesicle docking at the target membrane (Bock et al., 2001). Different Rabs are localised on distinct vesicles and organelles. Due to their specific localisation and to their ability to regulate their activity, Rabs can recruit and/or activate their various effectors at the correct time and to the correct place, providing an element of regulation to the vesicle-trafficking machinery (Bock et al., 2001). The final step in vesicle trafficking is the fusion of a vesicle with its target membrane, believed to be mediated by a family of proteins termed soluble NSF-attachment receptors (Bonifacino and Glick, 2004). SNAREs are integral membrane proteins present on both vesicle and target membranes, and

can form very stable complexes, termed SNAREPIN complexes. The formation of a SNAREPIN complex pulls the vesicle and target membrane together and may provide the energy to provoke fusion of the lipid bilayers (Bonifacino and Glick, 2004).

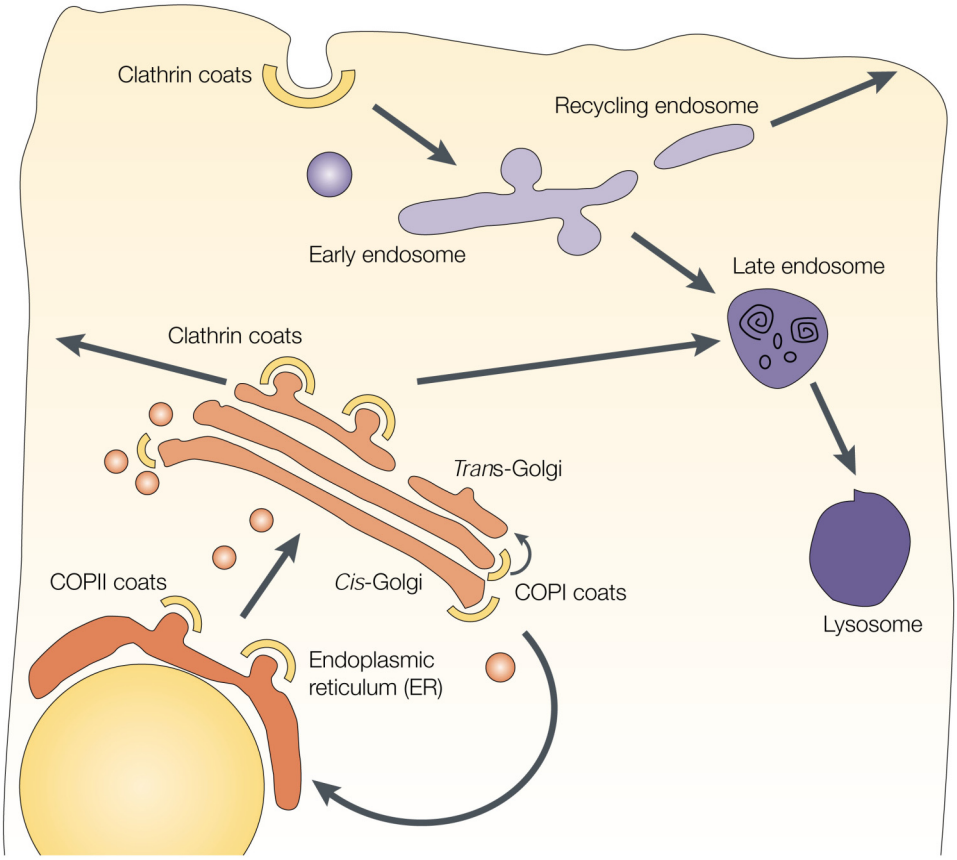

Figure 3: The major membrane trafficking pathways that use carrier vesicles with COPI, COPII and clathrin in eukaryotic cells.
In the biosynthetic pathway, newly synthesized molecules are transported from the endoplasmic reticulum to the Golgi and from one cisterna of the Golgi to the next until they reach the *trans* Golgi network. There, sorting occurs, directing traffic to the plasma membrane or to endosomes. In the endocytic pathway, macromolecules are internalized at the plasma membrane and forwarded to early endosomes, from where they are either recycled to the plasma membrane through recycling endosomes or forwarded towards degradation in late endosomes and lysosomes (Kirchhausen, 2000).

3.3 Trafficking signals

The discovery of the "Signal Hypothesis'' led Gunter Blobel to win the 1999 Nobel Prize for Medicine. Blobel discovered that protein "zip codes'' exist for directing proteins to subcellular compartments such as the nucleus, cytoplasm, mitochondria, endoplasmic reticulum, lysosomes and endosomes, peroxisomes, Golgi, and nucleolus. Proteins need to be

directed to their proper cellular compartments in order to perform their functions. For example, most transcription factors need to be in the nucleus to promote gene expression. Some proteins, such as the glucocorticoid receptor, may start in one compartment (cytoplasm), and move to another compartment (nucleus) in response to a stimulus (ligand). It is astonishing, that a signature, which is only few amino acids long, can be precisely recognized by effector proteins and leads to error-free delivery to the assigned location. The table below summarizes targeting motifs of some subcellular compartments and exemplifies the simplicity and beauty of this trafficking system.

Targeted subcellular compartment	Signal(s)
Nucleus	Monopartite PKKKRKV Bipartite KRPAATKKAGQAKKKKLDK
Cytoplasm (export from nucleus)	$LX_{(1-3)}$ $LX_{(2-3)}$ LXJ (L = Leu X = spacer J = Leu, Val or Ile)
Mitochondria	*M*LSL*R*QSI*R*FF*K*PAT*R*TL (amphipathic alpha helix) + charged residues on one side (italics); non-polar residues on the other side (bold)
Endoplasmic reticulum (return to ER)	KDEL at C-terminus
Lysosomes and endosomes	Tyrosine-based sorting signals: NPXY or YXX Di-leucine-based [DE]XXXL[LI] and DXXLL consensus motifs
Peroxisomes	SKL at C-terminus
Trans-golgi network	Di-leucine motif followed by two acidic clusters: LL*EDDSDEEED* (acidic clusters italicized)
Nucleolus	Basic stretches of aa's such as RRRANNRRR KKKMKKHKNKSEAKKRKI

Table 1. Signal sequences for subcellular compartments in eukaryotic cells (Davis et al., 2006).

3.4 Trafficking in *Plasmodium falciparum*

The malaria parasite *P. falciparum* has a range of unique organelles all fed by the protein secretory pathway. These protein transport pathways are fundamental to the biology and pathology of *P. falciparum* infection, especially during its intracellular existence in the red blood cell. PfEMP1, a key player in the parasite's immune evasion strategies and major virulence factor, is trafficked via a complex export route, traversing two membranes (PM and PVM) and the Maurer's clefts, and finally being inserted at its terminal destination at the host

cell surface. There it mediates cytoadherence to the endothelium and thus prevents clearance by the spleen. It represents products of antigenic variation - a sophisticated way of immune evasion. Understanding processes such as the trafficking of PfEMP1 are crucial for the understanding of the parasite biology and also have great potential as targets for intervention.

A classical protein secretory pathway requires some key components such as an endoplasmatic reticulum and a Golgi apparatus. However, little is known about the molecular organization of the secretory pathway of *P. falciparum*. The ER resembles a simple perinuclear structure with two protruding 'horns' (van Dooren et al., 2005), whereas the Golgi is rudimentary with a so-called 'unstacked' conformation (Elmendorf and Haldar, 1993b; Van Wye et al., 1996; Struck et al., 2005). Like all eukaryotic cells, *P. falciparum* must rely on a huge supply of molecular machinery to shuffle proteins through membranes and in and out of vesicles in order to target them to their correct destination (Bock et al., 2001). The genome of *P. falciparum* has many homologues of core secretory proteins such as the COP components and GTPases (Gardner et al., 2002) but there is very little experimental evidence for the specific role of any of these components.

Like in other eukaryotes, soluble and membrane proteins destined for secretion are directed into the ER by recognition of a classical signal sequence or a recessed signal sequence (Cooke et al., 2004). Proteins most probably pass through a rudimentary Golgi where additional protein-targeting motifs provide essential information for subsequent targeting to secretory destinations (Tonkin et al., 2006). In absence of other signals, the parasitophorous vacuole appears to represent the default destination for secreted proteins in *P. falciparum* (Waller et al., 2000; Wickham et al., 2001; Adisa et al., 2003; Marti et al., 2004). There are at least 11 secretory destinations in *P. falciparum* and some of the signals and pathways of proteins travelling to such destinations have recently been revealed and are summarized below.

-**Trafficking to the host red blood cell**: Recently, two independent groups published the PEXEL/VTS (*Plasmodium* export element/vacuolar targeting signal) targeting motif that is responsible for the translocation across the PVM (Hiller et al., 2004; Marti et al., 2004). It is located 15-20 amino acids downstream of the NH_2-terminal hydrophobic sequence and mediates export of proteins from the PV across the PVM. The consensus sequence is R/KxLxQ/E. Interestingly, this motif is found in both soluble and membrane proteins which are exported into the RBC cytosol. This implies that there is a common machinery responsible

for protein export in the PVM. The nature of this **translocon**- the complex of molecules associated with the translocation of proteins across lipid bilayers-however is still unknown. Preliminary bioinformatics analysis of the *Plasmodium* genome indicates that it has genes encoding sec61 translocation, mitochondrial and possibly chloroplast machineries (Marti et al., 2005).

-**Trafficking to the food vacuole**: Hemoglobin proteolysis is accomplished by a series of parasite-derived proteases (Klemba et al., 2004b). All of the known FV proteases bear a putative N–terminal signal peptide, signifying that they are transported through the endoplasmic reticulum, and a prodomain that might have a role in targeting from the ER to the FV. Recently, the targeting of two FV-targeted proteases has been studied in detail and they have been shown to traffic to the FV by two different routes: Plasmepsin II travels via ER to the food vacuole (Klemba et al., 2004a), whereas the dipeptide aminopeptidase1 does not traffic directly to the food vacuole but instead accumulates in the parasitophorous vacuole before moving to the food vacuole (Klemba et al., 2004a; Klemba et al., 2004b).

-**Protein trafficking to the apicoplast**: The apicoplast is surrounded by four membranes and most apicoplast proteins are nuclear-encoded and require a bipartite N–terminal extension made up of a signal peptide and a transit peptide (Waller et al., 2000). Earlier work has shown that the signal peptide resembles a classical eukaryotic signal peptide and mediates targeting into the ER whereas the transit peptide mediates all remaining steps. Interestingly, it has been shown, that apicoplast-targeted proteins do not traverse the Golgi but rather divert straight from the ER to the apicoplast (van Dooren et al., 2001; DeRocher et al., 2005).

Recent advances in plasmodial trafficking mechanisms have substantially contributed to the understanding of cellular processes of the parasite. The discovery of the PEXEL/VTS motif has enabled the prediction of the whole "exportome". This will help to decipher new parasite proteins that are on the host cell surface and may contribute to new vaccine targets. However, the whole translocation process across the PVM is not understood and a set of established exported markers do not fit into the predicted exportome. MAHRP1 is an exported protein, which has been confirmed to be associated at the Maurer's clefts by immunofluorescence and transfection technology. However, it does not contain an ER signal sequence and it does not bear a PEXEL motif. Is there an alternative trafficking pathway for exported proteins that do not contain a PEXEL/VTS motif? Is there maybe a timing-dependant alternative export

pathway, which overrules the PEXEL/VTS-dependant pathway? We have tackled this question in two different studies shown in chapter 3 and 4.

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Chapter 2

MAHRP-1, a Novel *Plasmodium falciparum* Histidine-rich Protein, Binds Ferriprotoporphyrin IX and Localizes to the Maurer's Clefts

Cornelia Spycher[1], Nectarios Klonis[2], Tobias Spielmann[1,3], Erwin Kump[1,4] , Sylvia Steiger[1], Leann Tilley[2], and Hans-Peter Beck[1*]

Affiliation of authors:

[1] Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 51, CH-4051 Basel, Switzerland.

[2] Department of Biochemistry, La Trobe University, Bundoora, 3086 Victoria, Australia.

[3] Present address: Queensland Institute of Medical Research, Brisbane, P. O. Royal Brisbane Hospital, Brisbane, QLD 4029, Australia.

[4] Present address: Institute of Medical Microbiology, Petersplatz 10,4003 Basel, Switzerland.

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MAHRP-1, a Novel *Plasmodium falciparum* Histidine-rich Protein, Binds Ferriprotoporphyrin IX and Localizes to the Maurer's Clefts*

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Cornelia Spycher‡, Nectarios Klonis§, Tobias Spielmann‡¶, Erwin Kump‡‖, Sylvia Steiger‡, Leann Tilley§, and Hans-Peter Beck‡**

From the ‡Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 51, CH-4051 Basel, Switzerland and §Department of Biochemistry, La Trobe University, Bundoora, 3086 Victoria, Australia

Using a stage-specific cDNA library from *Plasmodium falciparum* we have identified a gene coding for a novel histidine-rich protein (MAHRP-1). The gene is exclusively transcribed during early erythrocyte stages and codes for a small transmembrane protein. The C-terminal region contains a polymorphic stretch of histidine-rich repeats. Fluorescence microscopy studies using polyclonal mouse sera revealed that MAHRP-1 is located at the Maurer's clefts, which represent parasite-induced structures within the cytosol of infected erythrocytes. Biochemical studies showed that recombinant MAHRP-1 binds the toxic hemoglobin degradation product, ferriprotoporphyrin (FP) with a submicromolar dissociation constant and a stoichiometry determined by the number of DHGH motifs. The bound FP has increased peroxidase-like activity and is 10-fold more susceptible to H_2O_2-induced degradation compared with unbound FP. These properties of MAHRP-1 suggest it may play a protective role against oxidative stress, and its location at the Maurer's clefts suggests a function in promoting the correct trafficking of exported proteins, such as *P. falciparum* erythrocyte membrane protein-1.

Plasmodium falciparum causes one of the most life-threatening infectious diseases of humans. Malaria is estimated to be responsible for up to 2 million deaths per year. The pathogenesis of the disease is associated with the intracellular erythrocytic cycle of the parasite involving repeated rounds of invasion, growth, and schizogony. *P. falciparum* invades enucleated (mature) erythrocytes, which are devoid of organelles and lack a biosynthetic and secretory machinery and are, thus, metabolically relatively inert. The parasite feeds on the host cell cytosol and degrades about 75% of the hemoglobin (1). The released amino acids are incorporated into parasite proteins and also appear to be available for energy metabolism. Proteolysis is thought to be essential for parasite survival because *P. falciparum* has a limited capacity for *de novo* amino acid synthesis (2). However, the proteolytic degradation of hemoglobin also produces heme (ferriprotoporphyrin IX (FP)[1]). Free intracellular FP is toxic due to its detergent-like (3) and redox properties (4). The parasite appears to dispose of FP largely by sequestration in the parasite's food vacuole as a granular pigment known as hemozoin (β-hematin) (5, 6). It has been shown previously that this crystallization process is initiated and accelerated *in vitro* by parasite-derived histidine-rich proteins (7, 8), and the inhibition of hemozoin formation is believed to underlie the mode of action of chloroquine *in vivo* (9). It has also been suggested that non-enzymatic degradation, including reactions with H_2O_2 and glutathione may contribute to heme detoxification (1, 10) and that histidine-rich proteins may modulate these processes (8).

Several parasite-encoded histidine-rich proteins (HRP) have been described, of which *P. falciparum* histidine-rich protein 2 (HRP2) is one of the best characterized. This soluble protein is found in the food vacuole but also in the erythrocyte cytosol (8, 11). Earlier studies suggested that HRP2 is ingested by the parasite's cytostome from the red cell together with hemoglobin and thereby delivered to the food vacuole by bulk endocytosis (7). However, recent studies show that although ~75% of the hemoglobin is ingested by the parasite, more than 90% of HRP2 remains in the erythrocyte cytosol (12). Cytosolic HRP2 seems to concentrate mainly near the periphery of the erythrocyte, suggesting that it may not diffuse freely through the erythrocyte cytoplasm and could thereby avoid ingestion. These recent findings suggest the possibility that HRP2 could play a role in the detoxification of the by-products of hemoglobin degradation in the host cytoplasm. Recently, it has been shown that HRP2 modulates the redox activity of FP and that the HRP2·FP complex may have antioxidant properties (13). Hence, the location of HRP2 near the erythrocyte membrane may function to protect the host membrane from oxidative stress and, thus, prevent premature host cell lysis.

The *P. falciparum* knob-associated histidine-rich protein (KAHRP) is another well characterized HRP. KAHRP is essential for the formation of knob-like protuberances on the surface of infected erythrocytes. These knobs act as platforms for the presentation of *P. falciparum* erythrocyte membrane protein-1

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY355312–AY355318.

¶ Present address: Queensland Institute of Medical Research, Brisbane, P. O. Royal Brisbane Hospital, Brisbane, QLD 4029, Australia.

‖ Present address: Institute of Medical Microbiology, Petersplatz 10, CH-4003 Basel, Switzerland.

** To whom correspondence should be addressed. Tel.: 41-61-284-81-16; Fax: 41-61-271-86-54; E-mail: Hans-Peter.Beck@unibas.ch.

[1] The abbreviations used are: FP, ferriprotoporphyrin IX; BSA, bovine serum albumin; EXP-1, *P. falciparum* exported protein 1; GST, glutathione *S*-transferase; HRP, histidine-rich protein; HRP2, *P. falciparum* histidine-rich protein 2; KAHRP, *P. falciparum* knob-associated histidine-rich protein; MAHRP-1, *P. falciparum* membrane-associated histidine-rich protein 1; OPD, *ortho*-phenylene diamine; PfSBP1, *P. falciparum* skeleton-binding protein 1; PfEMP1, *P. falciparum* erythrocyte membrane protein 1; PBS, phosphate-buffered saline; HTPBS, human tonicity phosphate-buffered saline.

(PfEMP1), which is responsible for adherence of parasitized erythrocytes to vascular endothelial cells (14). This adhesion and subsequent accumulation of infected erythrocytes in the microvasculature are pivotal events in the pathogenesis of *P. falciparum* malaria, and these processes are considered to represent major virulence factors.

On route to the erythrocyte membrane, KAHRP is transiently located at the Maurer's clefts, a parasite-derived vesicle-like structure in the erythrocyte cytosol (15). Other proteins have also been shown to be associated with Maurer's clefts, including PfEMP3 (15, 16), Pf322 (17), and *P. falciparum* skeleton-binding protein 1 (PfSBP1) (18). It has been suggested that these cleft structures may act as an intermediate trafficking compartment and may play a critical role in the organization and sorting of parasite proteins. In a host cell such as the erythrocyte, which is devoid of any organelles or trafficking machinery, such parasite-derived structures represent an interesting target for possible intervention.

Here we describe a novel *P. falciparum* membrane-associated histidine-rich protein, MAHRP-1, which localizes to the Maurer's clefts. We identified the sequence encoding MAHRP-1 in a ring stage-specific cDNA library (19) and show that MAHRP-1 is exclusively expressed in the early erythrocytic stages but is present throughout the cycle. MAHRP-1 contains a predicted transmembrane domain and a polymorphic cluster of histidine-rich repeats. MAHRP-1 binds FP *in vitro* in a specific manner, promoting the peroxidase-like activity of FP and enhancing the degradation of FP by H_2O_2. These data suggest that MAHRP-1 might play an important role in generating the Maurer's clefts or in protecting proteins within these structures against the noxious effects of FP and reactive oxygen species.

MATERIALS AND METHODS

FP (porcine), bovine serum albumin (essentially fatty acid free (BSA)), saponin, and *ortho*-phenylenediamine (OPD) were obtained from Sigma. Albumax was provided by Invitrogen. All primers were manufactured by Invitrogen. *Escherichia coli* BL21 strain and plasmid vector pGEX-6P-2 were obtained from Amersham Biosciences. *E. coli* TunerDE3pLacI was obtained from Novagen. The sequencing vector pGEM-T was provided by Promega, Wallisellen, CH. Glutathione *S*-transferase (GST) was expressed from the pGEX-6P-vector and purified as described for the recombinant MAHRP-1c. Secondary antibodies for immunoblot analyses were goat anti-mouse IgG (γ-chain specific) alkaline phosphatase-labeled (Sigma) and goat anti-rabbit IgG (heavy and light chain)-alkaline phosphatase-labeled (Jackson ImmunoResearch Laboratories, West Grove, PA). Secondary antibodies for immunofluorescence labeling were goat anti-mouse IgG conjugated to Alexa 568 (Molecular Probes) and goat anti-rabbit IgG conjugated to fluorescein isothiocyanate (Sigma or Dako). Rabbit antisera recognizing KAHRP, PfEMP3, *P. falciparum* exported protein-1 (EXP-1), and PfEMP1 were kindly donated by Kathy Davern and Dr. Jacqui Waterkeyn, Walter and Eliza Hall Institute of Medical Research, and Prof. Klaus Lingelbach, Philipps-University, Marburg, Germany, and Professor Chris Newbold, John Radcliffe Hospital, Oxford, UK.

Methods

In Silico Analyses—One of the ring stage-specific gene fragments reported in Spielmann and Beck (19) coded for a histidine-rich protein (AJ290925). Query of the malaria sequence data base (www.plasmodb.org) returned the complete gene, now referred to as *mahrp1*. The predicted intron sequence was removed from the complete gene sequence to obtain the open reading frame. This was translated into amino acid sequence using ExPASy Molecular Biology Server (www.expasy.ch). Protein primary structure analysis was performed using www.psort.nibb.ac.uk.

Amplification and Sequencing of Full-length MAHRP-1—The sequence coding for MAHRP-1 was amplified from a collection of DNA derived from the following *P. falciparum* strains: 3D7, K1, SN250, ITG2F6, IFA18, Mad20. Primers used for amplification by PCR were: forward primer, 5′-GTTCCTACTGTTGGAACAGTTCC-3′, and reverse primer, 5′-CTAATTTTGCTTTTTGGCAATCACATGG-3′. The PCR products were cloned into the sequencing vector pGEM-T. The commercially available SP6 and T7 primers were used for sequencing reactions.

Expression of Recombinant MAHRP-1c—The sequence coding for the C-terminal domain of MAHRP-1 was PCR-amplified from both 3D7 and ITG2F6 genomic DNA using primers 5′-CAGATCAAGAGTGAACATAAAGCC-3′ and 5′-CTAATTTTGCTTTTTGGCAATCACATGG-3′. The PCR product was cloned into the *Sma*I restriction site 3′ of GST in pGEX-6P-2 by cycle restriction ligation to yield the plasmid pMAHRP-1c. *E. coli* BL21strain (for the 3D7 fusion protein) and *E. coli* Tuner DE3pLacI (for the ITG2F6 fusion protein) were transformed with plasmid pMAHRP-1c and used for expression of recombinant MAHRP-1c. Luria broth was inoculated with *E. coli* harboring pMAHRP-1c and cultured overnight at 37 °C containing one (for 3D7) or both (ITG2F6) of the following antibiotics: ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml). Fresh Luria broth containing antibiotics was inoculated with the overnight culture at a dilution of 1:10 and cultured at 37 °C to an A_{600} of 1.0. Expression of MAHRP-1c was induced by adding isopropyl-1-thio-β-galactopyranoside to a final concentration of 1 mM. Induced cultures were grown for 3–6 h at 37 °C.

Purification of Recombinant MAHRP-1c—Induced cultures were harvested by centrifugation and resuspended in lysis buffer (2 mg/ml lysozyme, 2 mM phenylmethylsulfonyl fluoride, 2 mg/ml DNase) in 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4 (phosphate-buffered saline (PBS)) and lysed by sonication. The supernatant containing the soluble GST fusion protein was purified using the Bulk GST Purification Module (Amersham Biosciences) as recommended by the manufacturer. Purity and integrity of the protein was checked on a 12.5% SDS-PAGE. 500 ml of culture yielded ~2–4 mg of recombinant protein. To test whether the histidine residues could also be used for further purification, an aliquot of the protein preparation was run over a Ni^{2+} chelate column (Qiagen) according to the supplier's protocol (data not shown).

Production of Specific Sera—C57BL6 mice were immunized subcutaneously with 10 μg of recombinant MAHRP-1c excised from polyacrylamide gels. Mice were boosted three times within 7 weeks and bled 1 week after the last immunization. Specificity of sera was tested on Western blots with recombinant MAHRP-1c, recombinant GST, and parasite protein extracts. Four different mice antisera were generated, each of which gave equivalent reactivity profiles.

Western Blot Analyses—Samples were subjected to electrophoresis on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose (Hybond-C extra, Amersham Biosciences) for 1.5 h using a Trans-Blot semi-dry electroblotter (Bio-Rad). The membrane was washed twice for 5 min in TNT (0.1 M Tris/HCl, 1.5 M NaCl, 0.05% Tween 20, pH 8.0) and blocked in TNT and 5% milk powder overnight at 4 °C. The primary antibody was diluted in 15 ml of TNT and 1% milk powder, and the secondary antibody was diluted in 15 ml of TNT and 0.1% milk powder. The membrane was washed 4 times in TNT and twice in Tris buffer (0.1 M Tris/HCl, 0.5 mM $MgCl_2$, pH 9.5). The membrane was stained in 20 ml of Tris buffer containing 200 μl of 5-bromo-4-chloro-3-indolyl phosphate (15 mg/ml) and 200 μl of nitro blue tetrazolium (30 mg/ml 70% *N*,*N*-dimethylformamide) for 15 min and then washed in distilled H_2O. Antibodies for immunoblot analyses were used at following dilutions: mouse anti-MAHRP-1c, 1/1000; rabbit anti-KAHRP, 1/200; rabbit anti-HRP2, 1/200; goat anti-mouse, 1:2000; goat anti-rabbit 1/30000.

Parasite Culture and Preparation of Parasite Protein Extracts—*P. falciparum* 3D7 strains were cultured at 5% hematocrit as described (20) using RPMI medium supplemented with 5% human serum or 0.5% Albumax (21). Parasites were synchronized with 5% sorbitol (22).

For total parasite extracts infected erythrocytes from a 10-ml culture (5% hematocrit, 10% parasitemia) were lysed with 0.03% saponin for 20 min on ice, washed in PBS, and resuspended in Laemmli sample buffer. Triton-X-114 phase separation was performed as previously described (23). Briefly, 10 ml of parasite culture (15–20% parasitemia) was saponin-treated, and the pellet was washed with PBS and then resuspended in 500 μl of human tonicity phosphate-buffered saline (HTPBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM sodium phosphate, pH 7.2) containing 0.5% Triton-X-114 and 1 mM phenylmethylsulfonyl fluoride and stored at −20 °C until use. After thawing, the extract was kept on ice for 30 min with intermittent mixing. The extract was spun at 15,000 × *g* for 20 min to pellet insoluble material. The supernatant, containing the detergent-soluble proteins, was layered over a 0.4-ml ice-cold sucrose cushion (6% sucrose, 0.06% Triton-X-114 in HTPBS), incubated at 37 °C for 5 min, and centrifuged at 500 × *g* for 5 min at room temperature. The resulting three phases were treated as follows. (i) The upper layer (detergent-depleted fraction) was collected; (ii) the sucrose cushion was discarded; (iii) the detergent pellet, containing the membrane proteins, was resuspended in 0.5 ml of HTPBS, the purification over a sucrose

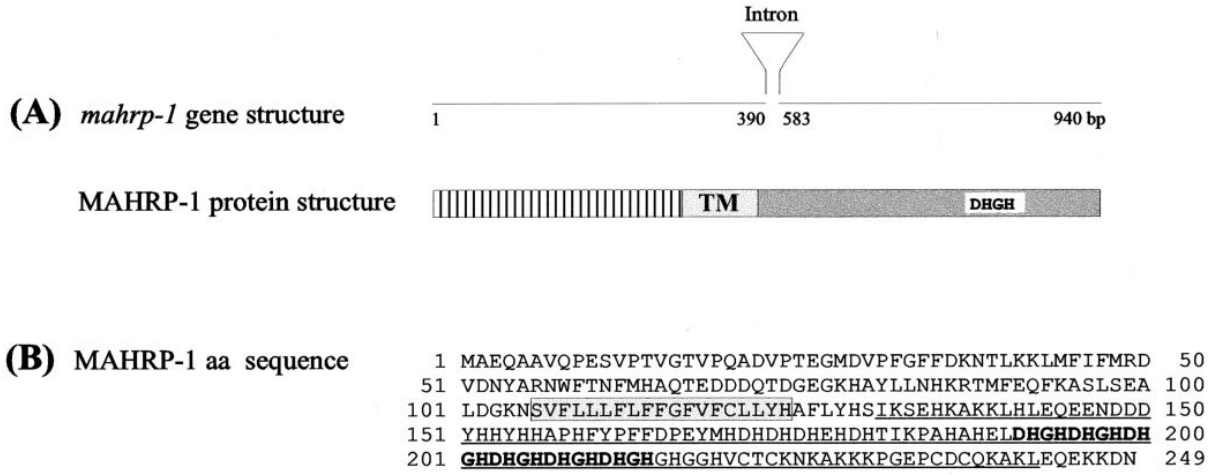

FIG. 1. **Primary structure of *P. falciparum* 3D7 MAHRP-1.** *A*, schematic representation of the *mahrp1* gene and deduced MAHRP-1 protein. The N-terminal domain is shown *hatched*, followed by the transmembrane domain (*TM*), shown in *light gray*. The C-terminal histidine-rich domain is shown in *dark gray*, with DHGH indicating the characteristic repeat motif. *B*, *P. falciparum* 3D7 MAHRP-1 amino acid (*aa*) sequence. The 18-amino acid long transmembrane domain is *highlighted* in *gray*, and the C-terminal repeats are indicated in *bold*. The C-terminal sequence cloned as GST fusion protein and referred to as recombinant MAHRP-1c is *underlined*.

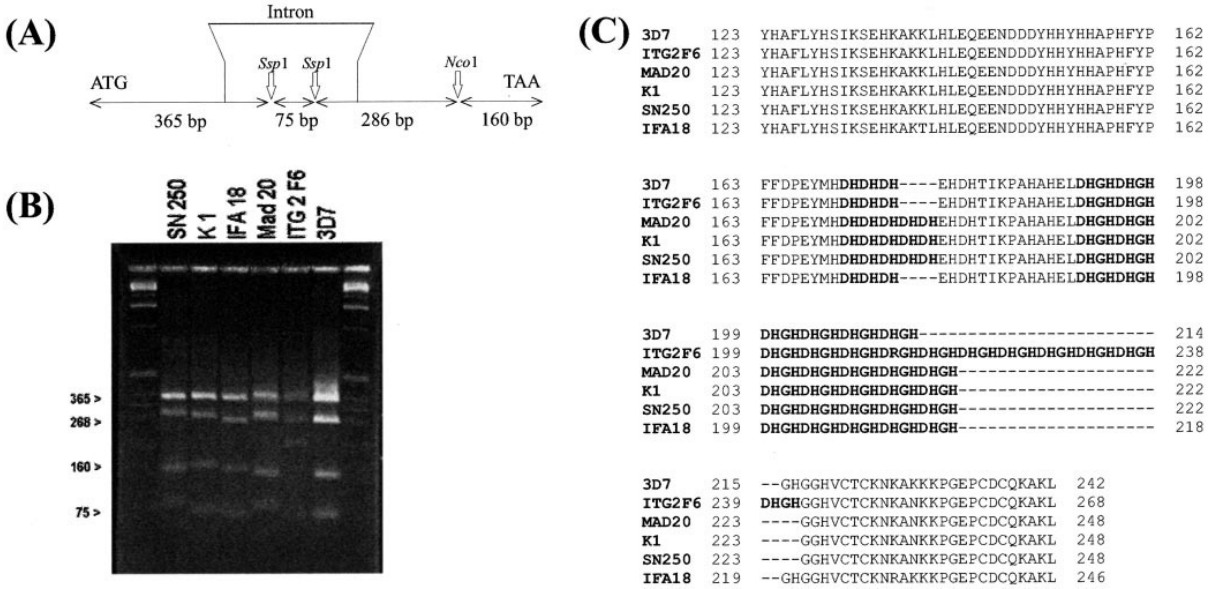

FIG. 2. **Length polymorphisms in the histidine-rich regions of *mahrp1* among different strains of *P. falciparum*.** *A*, schematic representation of *Ssp*I and *Nco*I restriction enzyme recognition sites in *mahrp1*. *B*, RFLP pattern of *Ssp*I- and *Nco*I-digested *mahrp1* alleles of six different strains, visualized by agarose gel electrophoresis. *C*, amino acid sequence alignments of MAHRP-1 from 6 different *P. falciparum* strains. Repeats are indicated in *bold*. Amino acid numbering according to the 3D7-coding sequence.

cushion was repeated, and the pellet was resuspended in 0.5 ml of HTPBS. Both the detergent-depleted fraction (i) and the final detergent fraction (iii) were precipitated with tricarboxylic acid and analyzed by SDS-PAGE.

Localization Studies Using Indirect Immunofluorescence Microscopy—Infected erythrocytes were smeared onto glass slides and fixed in acetone:methanol (1:1 v/v) at −20 °C for 10 min. Slides were partitioned into wells using a hydrophobic pen and incubated for 1 h in a humid chamber with one or both of the following primary antibodies: mouse anti-MAHRP-1c (1:1000), rabbit anti-PfEMP3 (1:50), rabbit anti PfEMP1 (1:200). The primary antibody was washed off with PBS or 1% Tween 20 in Tris-buffered saline, and the slides were then incubated with one or both secondary antibodies: fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:250) and Alexa 568-conjugated anti-mouse IgG (1:50). The slides were mounted in 90% glycerol containing 0.05% *N*-propyl gallate (Sigma) to reduce bleaching. Samples were viewed using an Olympus BX50 light microscope fitted with a fluorescence attachment and a SPOT RT digital camera or with a Leica TCS-NT laser-scanning confocal microscope at the Confocal Microscopy Facility, Monash University.

FP Binding Studies—The interaction between FP and the recombinant proteins was examined spectrophotometrically in PBS, pH 7.4. Concentrated stock solutions of FP (10 mM) were freshly prepared in 0.1 M NaOH and diluted in water as required. Absorption spectra were recorded using a Cary 1E spectrophotometer. The absorption spectrum of FP exhibits a time-dependent change due to gradual self-association events (24). Therefore, spectroscopic measurements of FP in the presence of protein were recorded 2 min after mixing the FP stock into the protein solution.

Redox Activity of FP—The H_2O_2-mediated decomposition of FP at pH

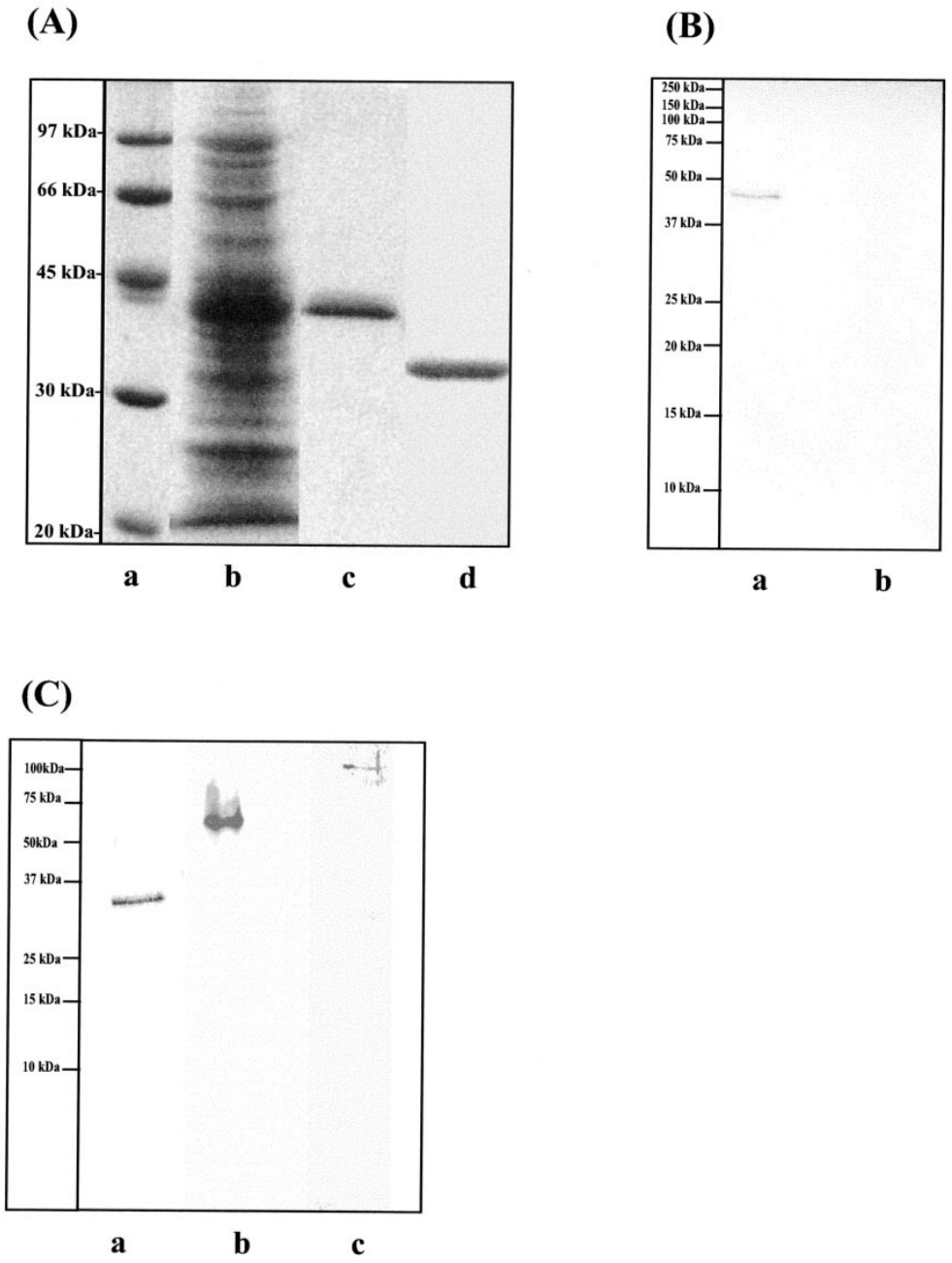

FIG. 3. **Production of recombinant 3D7 MAHRP-1c and immunoblot analysis of anti-MAHRP-1c serum.** *A*, recombinant protein samples were subjected to SDS-PAGE (12.5% acrylamide) and visualized by Coomassie Blue staining. Shown are the molecular weight marker (*lane a*), total protein lysate of *E. coli* expressing MAHRP-1c (*lane b*), purified recombinant MAHRP-1c (*lane c*), and purified recombinant GST (*lane d*). *B*, immunoblot analysis of equal amounts of recombinant proteins (~8 μg/lane), MAHRP-1c (*lane a*) and GST (*lane b*), probed with anti-MAHRP-1c serum. *C*, immunoblot of saponin-lysed parasite extract probed with anti-MAHRP-1c serum (*lane a*), anti-HRP2 serum (*lane b*), and anti-KAHRP serum (*lane c*) to test for reactivity with three histidine-rich proteins.

7.4 was monitored by measuring the decrease in absorbance of FP (5 μM) in the presence or absence of either MAHRP-1c, BSA. or GST (all 2 μM). The reaction (in a final volume of 950 μl) was initiated by the addition of 50 μl of H_2O_2 (final concentration 2 mM) and monitored at 20 °C using a Carry 1E spectrophotometer. The initial measured value for the FP concentration was set as 100%.

To monitor the peroxidase-like activity of FP at pH 7.4, samples (200 μl) containing 5 mM OPD and 5μM FP with or without protein (MAHRP-1c, BSA, or GST; 0.1–2.0 μM) were added to the wells of a 96-well plate. The reaction was initiated by the addition of 10 μl of 40 mM H_2O_2, and plates were incubated at 20 °C for 20 min. The oxidation of OPD was measured at 490 nm after the addition of 50 μl of 3 M HCl.

RESULTS

Identification and Primary Structure of MAHRP-1—Using suppression subtractive hybridization, we identified a set of genes that are exclusively transcribed in the ring stage of *P. falciparum* 3D7 strain, as reported in Spielmann and Beck (19). Among the 13 ring stage-specific gene fragments obtained,

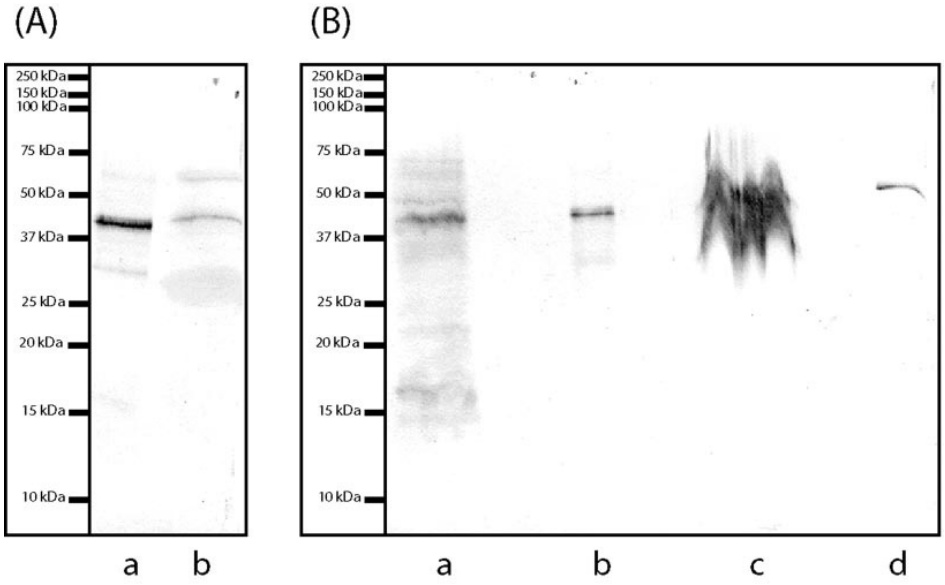

FIG. 4. **Solubility studies of MAHRP-1 in 3D7 parasitized erythrocytes.** Immunoblot (using α-MAHRP-1c mouse polyclonal antibodies, visualized with α-mouse-IgG alkaline phosphatase-labeled antibodies). *A*, parasite pellet (*lane a*) and supernatant (*lane b*) after saponin lysis of infected erythrocytes. *B*, the pellet was further processed and partitioned into an insoluble fraction (*lane a*), Triton X-114-depleted fraction, *i.e.* the non-membrane fraction (*lane b*), and Triton X-114-soluble fraction, *i.e.* membrane fraction (*lane c*). *Lane d* contained 0.04 μg of recombinant MAHRP-1c. All lanes contain protein preparations from a 10-ml culture with 5% hematocrit and 10–20% parasitemia.

one coded for a histidine-rich protein (AJ290925). Using the gene sequence to query the malaria sequence data base revealed that *mahrp1* is located on chromosome 13. The deduced protein and the gene organization are presented in Fig. 1. The predicted intron sequence was removed from the complete gene to obtain the open reading frame. The translated sequence comprises 249 amino acids, with a predicted molecular weight of 28.9 kDa and a pI of 5.92. MAHRP-1 contains a predicted transmembrane domain (amino acids 106–124) but no predicted N-terminal signal sequence. The region C terminus to the transmembrane domain has a histidine content of nearly 30% and contains 6 tandem repeats of the amino acid sequence, DHGH, with additional preceding DH repeats.

We examined the degree of conservation of MAHRP-1 among different strains of *P. falciparum*. Digestion of six strains with restriction enzymes *Ssp*I and *Nco*I resulted in four distinguishable fragments (Fig. 2). The major length polymorphism was found in the 160-bp fragment in the histidine-rich region of the C-terminal domain, between amino acids 195 and 249. Sequence analysis revealed that alleles differed in the number of DHGH repeats, whereby 3D7 has 6 and ITG2F6 has13 repeats.

Immunochemical Characterization of MAHRP-1 in Parasitized Erythrocytes—The C-terminal domain of MAHRP-1 (3D7 strain, amino acids 131–242), referred to as MAHRP-1c, was expressed as a 41-kDa GST fusion protein in *E. coli* (Fig. 3*A*), and the purified protein MAHRP-1c was used to raise antibodies in mice. The serum recognized the recombinant MAHRP-1c-GST fusion protein but not recombinant GST on a Western blot (Fig. 3*B*). The antiserum recognized a single band of the predicted size at ~30 kDa in an extract of parasitized erythrocytes (Fig. 3*C*, *lane a*). The antiserum does not appear to cross-react with other proteins. In particular, it does not recognize bands corresponding to the ~60-kDa protein recognized by an antiserum against HRP2; Fig. 3*C* (*lane b*) or the ~90-kDa band obtained with serum against the KAHRP (Fig. 3*C*, *lane c*). In addition, the anti-MAHRP-1c serum gave no signal on protein preparations from uninfected erythrocytes (data not shown).

Infected erythrocytes were treated with detergents to elucidate the physical organization of MAHRP-1. Most of the MAHRP-1 signal was associated with the saponin-insoluble pellet (Fig. 4*A*, *lane a*). Further partitioning of the pelleted fraction using the detergent Triton X-114 yielded three fractions, *i.e.* a detergent-soluble fraction, an aqueous (detergent-depleted) fraction, and an insoluble fraction. MAHRP-1 appeared to be largely associated with the detergent-soluble fraction, which is consistent with it being a membrane protein (Fig. 4*B*, *lane c*).

Subcellular Location of MAHRP-1—We have used indirect immunofluorescence microscopy to examine the subcellular location of MAHRP-1 in *P. falciparum*-infected erythrocytes. MAHRP-1c antibodies reacted with punctuate structures within the cytoplasm of the host erythrocytes (Fig. 5*A*). The pattern obtained resembles the pattern obtained for Pf322 (17), PfSBP1 (18), and PfEMP3 (14, 16), which has been shown to be located in parasite-derived membranous structures in the erythrocyte cytosol, referred to as Maurer's clefts.

MAHRP-1 was first observed in vesicle-like structures within the erythrocyte cytosol approximately ~10 h post-invasion. As the parasite matured, the labeled structures increased in number. In the schizont stage, a diffuse staining of the entire erythrocyte appeared to underlie the punctuate foci.

To confirm the Maurer's cleft location, we performed dual labeling studies using an antiserum against PfEMP3 (16), which has previously been described to be Maurer's cleft-located (Fig. 5*B*). A high degree of colocalization between PfEMP3 and MAHRP-1 was observed. Dual labeling with EXP-1, a parasite protein that is exported into the parasitophorous vacuolar membrane, indicated that MAHRP-1 is mainly located outside the parasite confines and within the confines of the erythrocyte membrane. We also used antisera against PfEMP1, a protein also transiently located in Maurer's clefts (15). As shown in Fig. 5*C*, there is a tight colocalization of the erythrocyte cytosol population of PfEMP1 with MAHRP-1 in early trophozoite stages. In mature stages, PfEMP1 is partly

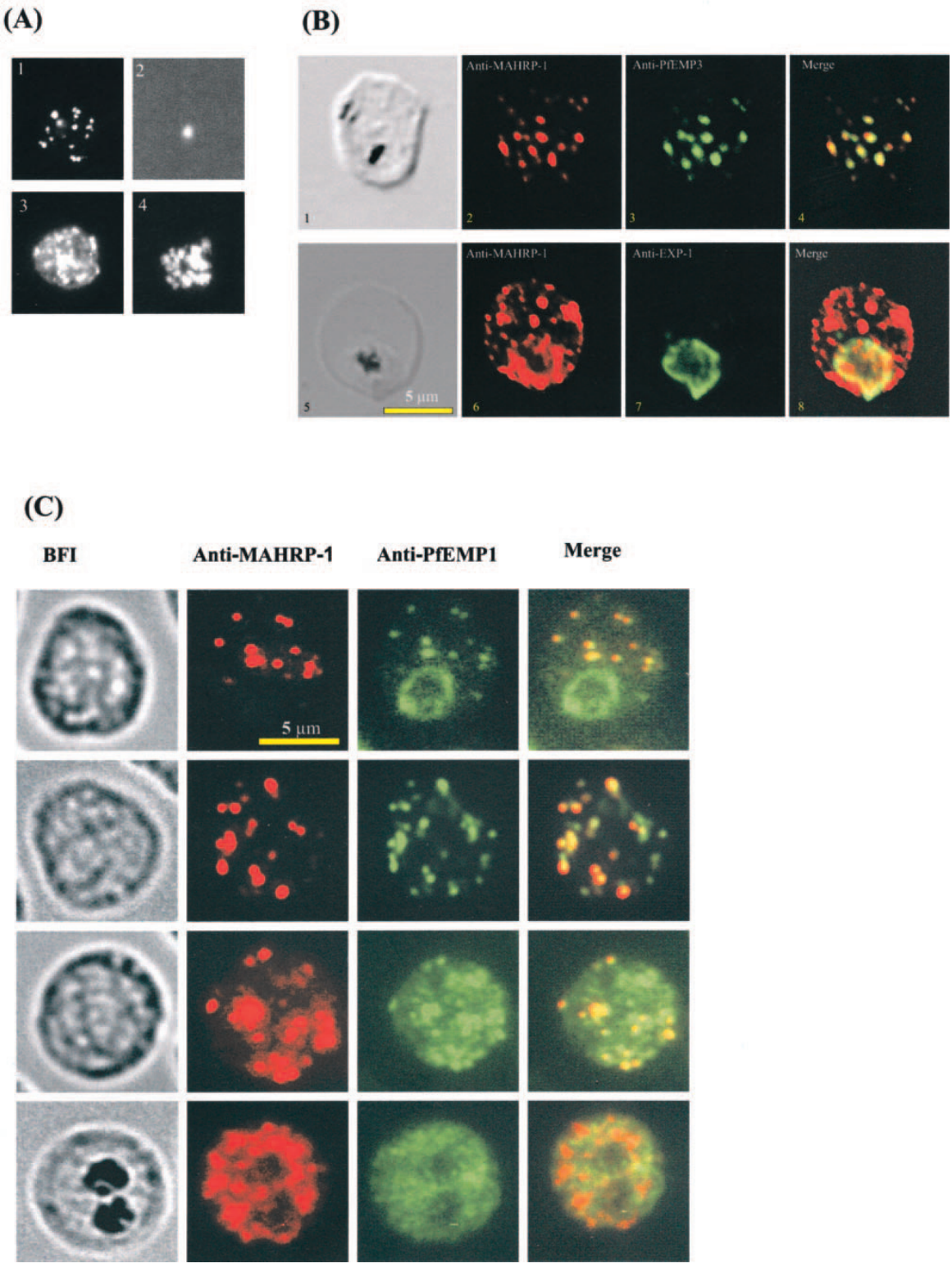

FIG. 5. **Immunofluorescence analysis of MAHRP-1 in *P. falciparum* (3D7) infected erythrocytes.** *A*, a young trophozoite stage-infected erythrocyte showing distinct punctuate staining within the erythrocyte cytosol (*panel 1*) and a mature, multinucleated schizont showing punctuate staining overlaying a more generalized staining (*panel 3*). The same infected erythrocytes were 4,6-diamidino-2-phenylindole-stained to visualize the nuclei (*2* and *4*). *B*, dual labeling studies with PfEMP3 and EXP-1. *Panels 1–4*, a young trophozoite-infected erythrocyte examined by bright field imaging (*panel 1*) or labeled with MAHRP-1c antiserum (*panel 2*, *red*) and PfEMP3 antiserum (*panel 3*, *green*). *Panel 5–8*, a young trophozoite-infected erythrocyte examined by bright field imaging (*panel 5*) or for labeling with MAHRP-1c antiserum (*panel 6*, *red*) and EXP-1

redistributed to the erythrocyte membrane, whereas MAHRP-1 remains largely associated with the Maurer's clefts.

MAHRP-1 Interacts with FP—Because of the high histidine content of MAHRP-1, we examined the ability of recombinant 3D7 MAHRP-1c to bind to an immobilized metal column. We found that MAHRP-1c-GST fusion protein bound strongly to a Ni^{2+}-chelate column and was eluted with imidazole, thus providing potentially an additional step for purification of the protein (data not shown).

Given the ability of MAHRP-1 to bind to metal ions and the proposed function of HRPs in FP detoxification, we examined the interaction between the protein and FP. Use was made of the visible absorption spectrum of FP to examine its interaction with protein. As shown in Fig. 6*A*, FP exhibits a characteristic absorption peak known as the Soret band with a maximum at 390 nm and a major shoulder at 350 nm and smaller (α and β) bands at wavelengths greater than 500 nm. Quantitative spectroscopic measurements of FP in aqueous solution are complicated by the presence of an equilibrium between the monomer and μ-oxo dimeric form of the FP, which is both pH- and concentration-dependent (24, 25). The FP dimer is favored under basic conditions and at high concentrations. Under acidic conditions, the monomer is favored; however, FP also tends to aggregate and to form β-hematin crystals (6).

The maximum wavelengths and extinction coefficients for the FP absorption bands are sensitive to the presence of bound ligand and can be used to examine an interaction between FP and FP binding molecules (26). In this work, we examined the interaction of recombinant MAHRP-1c with FP. We performed FP binding assays at pH 7.4 in an effort to resemble the physiological environment of the erythrocyte cytosol. Under these conditions, FP exhibits a broad Soret band of relatively low intensity and another much lower intensity band at ~620 nm. These features are characteristic of the μ-oxo dimer under basic conditions and indicate that the FP is largely present as a dimer, probably in equilibrium with some monomeric and aggregated species. This absorption spectrum is dramatically altered in the presence of 3D7 MAHRP-1c. There is a sharpening of the Soret band and a 25-nm blue shift to a wavelength maximum at 410 nm. In addition, the 620-nm peak disappears, and two peaks become apparent at 530 and 570 nm. These results indicate that the FP is present in a monomeric form.

The interaction between the 3D7 MAHRP-1c and FP was examined in greater detail by titrating 2.5 μM 3D7 MAHRP-1c with FP. Fig. 6*B* shows the normalized absorption spectra of the Soret and α and β bands as a function of the FP:protein ratio. There is little change in the FP spectrum when it is present at up to a 4 M excess relative to MAHRP-1c. However, the spectra exhibit a blue shift and a broadening of the Soret band and the appearance of the 620 nm band at the higher ratios examined, indicating the presence of unbound FP. An examination of the Soret absorbance as a function of added FP shows an increased absorbance compared with that for the addition of FP to PBS, with an inflection point corresponding to a FP:MAHRP-1c ratio of 5 (Fig. 6*C*, *open circles*). This indicates that the stoichiometry is about 5 FP molecules per 3D7 MAHRP-1c, with an affinity in the submicromolar range. This stoichiometry is roughly consistent with the number of DHGH motifs in the primary sequence. We also examined binding of MAHRP-1c from the ITG2F6 strain to FP. Similar spectra were obtained (not shown); however, the stoichiometric point occurred at a FP:protein ratio of ~11:1 (Fig. 6*C*, *open squares*). This is consistent with the increased number of DHGH motifs present in this protein and supports our hypothesis that DHGH

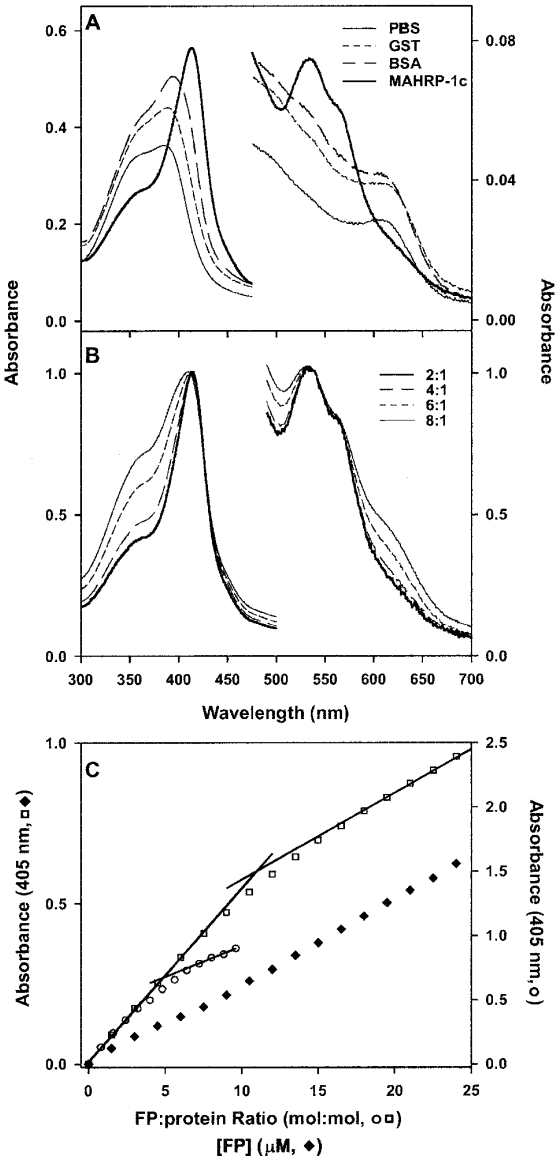

FIG. 6. **Interaction of MAHRP-1c with FP.** *A*, the absorption spectrum of FP (10 μM) in PBS was measured in the absence and presence of 3D7 MAHRP-1c (2.5 μM), BSA (2.5 μM), or GST (2.5 μM). *B*, normalized absorption spectra of the Soret and α and β bands of FP at different molar ratios of 3D7 MAHRP-1c. The 3D7 MAHRP-1c concentration was constant (2.5 μM), and increasing amounts of FP were added. *C*, Soret absorption of FP in the absence and presence of MAHRP-1c. Aliquots of FP were added to PBS in the absence (*filled diamonds*) or presence of 3D7 MAHRP-1c (2.5 μM) (*open rings*) or ITG2F6 MAHRP-1c (1.0 μM) (*open diamonds*). The lines are regression lines using the points before and after each stoichiometric point. The two absorbance scales in this panel differ by a factor of 2.5 to permit a direct comparison of the two MAHRP-1c proteins.

antiserum (*panel 7*, *green*). Merges of the red and green channels are shown in *panels 4* and *8*. *C*, dual labeling studies with PfEMP1. Erythrocytes infected with parasites of increasing maturity (*top* to *bottom*) were examined by bright field imaging (*left-hand panels*) or for labeling with MAHRP-1c antiserum (*second column*, *red*) and PfEMP1 antiserum (*third column*, *green*). Merges of the red and green channels are shown in the *right-hand panels*. *Yellow* areas represent regions of co-localization. *Bars* represent 5 μm.

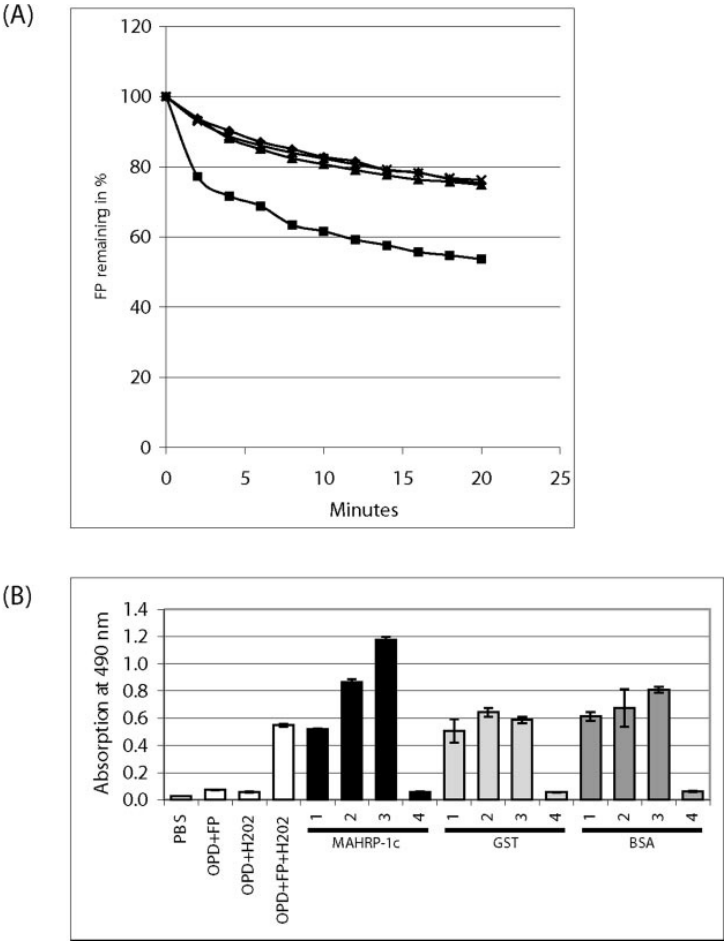

FIG. 7. **Effect of MAHRP-1c on the redox properties of FP.** *A*, effect of 3D7 MAHRP-1c on H_2O_2-mediated decomposition of FP. H_2O_2 (2 mM) was added to FP (5 μM) at pH 7.4 either in absence of protein (*crosses*) or in the presence of 2 μM MAHRP-1c (*squares*) or BSA (*diamonds*) or GST (*triangles*). The FP concentration was monitored at 400 nm. *B*, effect of MAHRP-1c on the peroxidase-like activity of FP. The ability of MAHRP-1c to enhance the FP-catalyzed oxidation of OPD was examined in triplicate at pH 7.4. Oxidation of OPD required the presence of both FP and H_2O_2 (*open columns*). Proteins were added at increasing concentrations of 0.1 μM (*1*), 0.5 μM (*2*), 2.0 μM (*3*) in the presence of FP and at 2.0 μM protein in the absence of FP (4).

represents the FP binding motif.

We also examined the effects of BSA, a known FP-binding protein, and GST, the fusion moiety present in MAHRP-1c, on the spectral properties of FP. Fig. 6*A* shows that both GST and BSA cause an increase in the absorption of the Soret band, with only a small red shift and slight sharpening of the band. Similarly, the α and β bands have an increased absorption, but their relative positions are not affected. The different effects on the spectrum suggest a different mode of FP binding for these proteins compared with MAHRP-1c. It is likely that the FP binds to BSA (27) and GST via nonspecific hydrophobic interactions. The increase in absorbance may therefore reflect the increased solubility or decreased aggregation of the FP in the presence of these proteins.

Effect of MAHRP-1c on the Redox Properties of FP—FP can react with H_2O_2 to form a ferryl (Fe(IV)) intermediate that can participate in a number of enzyme-like reactions. These reactions can destroy FP and convert H_2O_2 to the innocuous compounds water and oxygen (1, 28). In earlier studies, we have shown that the antimalarial drug chloroquine inhibits H_2O_2-mediated FP destruction under conditions designed to mimic the food vacuole (1). We have also shown that HRP2 can modulate the redox properties of FP (8, 13). In this work, we have examined the interaction of FP with H_2O_2 at pH 7.4, the presumed pH of the erythrocyte cytosol. We find that about 20% of FP is degraded by H_2O_2 within 20 min at pH 7.4 (Fig. 7*A*). Interestingly, we found that MAHRP-1c (2 μM) enhances the initial rate of destruction of 5 μM FP by reaction with H_2O_2 by 10-fold (Fig. 7*A*). By contrast, equivalent concentrations of GST or BSA had no effect on the rate of degradation of FP by H_2O_2.

We also examined the ability of FP to catalyze H_2O_2-mediated oxidation of OPD. At pH 7.4, FP (5 μM) greatly increases the level of oxidation of OPD (5 mM) by H_2O_2 (2 mM) (Fig. 7*B*, *open columns*). Varying protein concentrations (0.1–2 μM) were added to determine the effect of protein on this oxidation process. MAHRP-1c substantially increased the level of oxidation of OPD. It is noteworthy that the 2.2-fold increase in OPD oxidation with 2 μM MAHRP-1c is comparable with the 1.8-fold increase in OPD oxidization observed with the same amount of HRP2 at pH 7.4 (8). BSA or GST had relatively little effect on the level of oxidation of OPD (Fig. 7*B*).

DISCUSSION

Invasion of mature human erythrocytes, which are devoid of all subcellular organelles, presents a major challenge to *P. falciparum*. To survive and to multiply the parasite needs to modify the properties of the host membrane. To do this it needs to establish the machinery for trafficking of proteins beyond its own boundaries. Shortly after invasion, the parasite begins the process of exporting proteins to the erythrocyte cytosol. The initial set of exported proteins may represent the machinery for the subsequent trafficking of important cargo molecules. Indeed maturation of the ring stage parasite is associated with the development of membranous extensions of the parasitophorous vacuole and characteristic Maurer's clefts with electron-

dense coats that can be observed by electron microscopy in the host cytosol. By the late ring stage, a number of parasite-encoded proteins are present in the erythrocyte cytosol, bound to the cytoskeleton, or inserted into the erythrocyte membrane (29–31). The Maurer's clefts are thought to be an important intermediate compartment in the trafficking of proteins to the red blood cell membrane (15); however, very little is known about the molecular components of the Maurer's clefts or the molecular basis of the function of these parasite-derived structures.

In an attempt to identify crucial processes and possibly targets for intervention, we have generated a stage-specific cDNA library from early erythrocytic stages (19). Among a small set of genes exclusively expressed during the ring stage, we have identified MAHRP-1. Its tightly regulated early expression, a putative transmembrane domain, and the presence of a histidine-rich domain made it a prime candidate for further investigation. Several histidine-rich proteins have been described in *P. falciparum* with interesting structural and functional properties (7, 8, 13, 32–34), but MAHRP-1 is the first membrane-associated HRP.

MAHRP-1 is a small (28.9 kDa) protein with no significant homology to any known protein. It has no predicted N-terminal signal sequence but has a 16-amino acid long putative transmembrane domain (Fig. 1). Its C-terminal domain contains a histidine-rich region comprising a variable number of DHGH repeats. We have shown that polymorphism exists between different *P. falciparum* strains, which is confined to the histidine-rich domain, and in particular to the number of DHGH repeats.

Specific antisera were generated against the MAHRP-1 C-terminal domain. These antisera recognized a 29-kDa protein in parasite extracts and did not cross-react with two other parasite-derived histidine-rich proteins, KAHRP and HRP2. The antisera were used to study the location and organization of MAHRP-1. Immunofluorescence microscopy studies indicated that MAHRP-1 is located in the erythrocyte cytosol as clearly defined foci in a similar pattern to that observed for PfSBP1, Pf332, and PfEMP3 (16–18). Indeed, in dual-labeling studies we found a high degree of colocalization of MAHRP-1 with PfEMP3. Maurer's clefts have been visualized by electron microscopy as long, slender structures coated with electron-dense material (35, 36). It is likely that MAHRP-1 is anchored via its transmembrane domain in the bilayer that delineates these structures. Very little MAHRP-1 is detected inside the parasite's cytosol or in the parasitophorous vacuole, indicating that the protein is rapidly translocated into the erythrocyte cytosol upon expression.

The Maurer's clefts appear to be a transit depot in the export of PfEMP1 to the erythrocyte membrane (15). PfEMP1 is a family of proteins with a variable extracellular segment containing cysteine-rich binding domains that are responsible for cytoadherence to the vascular endothelium. PfEMP1 is produced in ring stage parasites and trafficked to the erythrocyte surface (via the Maurer's clefts) about 20 h after invasion (15, 16, 37, 38). In this work, dual labeling studies were used to confirm that PfEMP1 is located in the same compartment as MAHRP-1 at least for part of the intraerythrocytic cycle. Thus, MAHRP-1 might play a direct or indirect role in the trafficking of this critical virulence factor.

Although KAHRP and PfEMP1 are transiently associated with the Maurer's clefts (16), MAHRP-1 appears to reside in these organelles throughout the parasite's development. Upon maturation of the parasite, the Maurer's clefts increase in number and are often observed in close apposition to the periphery of the host cell; however, they do not appear to fuse with the erythrocyte membrane. It is possible that MAHRP-1 has a structural role in the Maurer's clefts. Indeed it is possible that the electron-dense appearance of the Maurer's clefts in electron microscopy studies is due to binding of the heavy metal electron microscopy stain to the histidine moieties in MAHRP-1. However, it is also possible that MAHRP-1 plays an active role in protein trafficking, such as in the sorting or chaperoning of proteins such as PfEMP1.

It is interesting to note that MAHRP-1 is slightly acidic (pI = 5.92). This could allow an interaction with basic residues within KAHRP (pI = 9.2). In this regard, it is useful to note that PfEMP1 has a highly acidic cytoplasmic domain (pI = 4.50), which has been shown to interact with domains of KAHRP (39).

We also performed detergent solubility studies that are consistent with MAHRP-1 being located in Maurer's cleft membranes. Saponin is a cholesterol binding detergent that perforates the erythrocyte membrane, releasing soluble, cytosolic proteins such as hemoglobin. MAHRP-1 was found in the pellet fraction after saponin treatment, which is consistent with previous studies suggesting that the Maurer's clefts remain associated with the erythrocyte membrane upon host cell lysis (18, 35). Further fractionation of the parasite samples using two phase-forming Triton X-114/water mixtures revealed that the protein is largely soluble in Triton-X-114. This is consistent with MAHRP-1 being a membrane-embedded protein.

It is interesting to speculate on the possible sequence signals for trafficking of proteins to the Maurer's clefts. Apart from PfEMP1, only one Mauer's cleft-associated membrane protein (PfSBP1) has been described previously (18). Like MAHRP-1, PfSBP1 and PfEMP1 have a single transmembrane domain and lack a classical N-terminal secretory signal (18, 40). For membrane proteins lacking an N-terminal signal, the transmembrane domain can act as a start transfer signal for translocation into the ER membrane. The transmembrane domain can also contain information for trafficking to particular compartments such as the Golgi (41). Thus, the transmembrane segments of MAHRP-1, PfSBP1, and PfEMP1 may contain the signal for export to the Maurer's clefts. By contrast, other parasite-encoded membrane proteins, such as EXP-1, EXP-2, and AMA-1, which are not exported past the parasitophorous vacuole membrane, have classical N-terminal signal sequences (42–44).

The mode of insertion of membrane-embedded proteins into the ER membrane determines their final orientation. Membrane proteins that lack an N-terminal signal sequence can be inserted into the ER with the N-terminal region facing the ER lumen (type Ib orientation) or the cytoplasm (type II orientation) (for review, see Ref. 45). Analysis of the MAHRP-1 sequence using the Psort algorithms (available at psort.nibb.ac.jp) suggests a type Ib orientation. PfEMP1 and PfSBP1 are also predicted to be type Ib proteins and are known to be exported to the erythrocyte cytosol with their C-terminal regions facing the erythrocyte cytoplasm (18, 40). Thus, it appears likely that the histidine-rich C-terminal domain of MAHRP-1 also faces the host cytoplasm.

Given its location in the Maurer's clefts and the prominent histidine-rich domain, we considered the possibility that MAHRP-1 might play a role in protecting the parasite from the deleterious effects of FP. The parasite degrades hemoglobin as a source for amino acids, and the consequent byproducts such as FP and H_2O_2 are highly toxic (for review, see Ref. 46). Recently it has been reported that ~90% of the ~15 mM heme that is released from hemoglobin is sequestered as hemozoin (6). Nonetheless, it is likely that a fraction of the pool of released FP escapes sequestration and diffuses down the concentration gradient into the parasite cytosol. FP readily crosses membranes and would be expected to rapidly equilibrate throughout the cell. Indeed significant levels of free FP (~100 μM) are distributed between the

parasite and host compartments (1, 10).

As a consequence of its toxic waste products, the parasite confers a high level of oxidative stress on the host cell (47). This leads to deposition of hemichromes at the cytoplasmic surface of the erythrocyte membrane and binding of autoantigens to the external surface (48). Indeed it has been suggested that *P. falciparum* alters the permeability of the erythrocyte by applying a controlled oxidative stress to the host cell membrane (49). It appears likely therefore that the parasite would produce proteins to modulate the effect of FP and reactive oxygen species on the host cell. For example, HRP2 has been suggested to play a role in detoxification of the byproducts of hemoglobin digestion (7, 8, 12, 13).

In this work we have examined the ability of the histidine-rich C-terminal domain of MAHRP-1 to interact with FP and to modulate its redox properties. A spectroscopic analysis showed that in the absence of protein, at pH 7.4 FP exhibited a broad Soret peak. This may reflect the dominance of FP μ-oxo dimers and the presence of some aggregates. Upon the addition of proteins such as BSA or GST that interact with FP by hydrophobic interactions, there was an increase in the Soret absorption indicative of increased solubilization of the dimeric form of FP. Upon the addition of MAHRP-1c, there was a sharpening and a red shift of the Soret band of FP as well as changes in the red region of the spectrum. Similar changes have also been reported with FP titrated with imidazole (50) or with HRP2 (8, 51). Thus, the results indicate that in the presence of MAHRP-1c, the FP is present in a monomeric form presumably coordinated to histidines within the protein in a similar fashion to that for HRP2.

Our stoichiometric analysis reveals that 3D7 MAHRP-1c binds FP at a ratio of 1:5, whereas the ITG2F6 MAHRP-1c binds with a stoichiometry of 1:11. This is consistent with the increased number of DHGH motifs in the ITG2F6 isoform and indicates that DHGH is the FP binding motif. Thus, it might be possible that MAHRP-1 isoforms with an increased number of repeats might be able tolerate higher concentrations of FP, although this needs to be tested experimentally. It is interesting to note that HRP2 contains 45 repeats of the tripeptide, HHA, partly contained within 21 copies of a degenerate repeat based on AHHAHHAAD and has been shown to bind FP in a ratio of up to 1:50 (7, 51).

FP has been shown to react with H_2O_2 to form a ferryl (Fe(IV)) intermediate that can participate in a number of enzyme-like reactions, including a peroxidase-like reaction that reduces H_2O_2 at the expense of organic substrates (1). Low concentrations of H_2O_2 and other reactive oxygen species may be present in the host compartment as byproducts of methemoglobin formation or hemoglobin denaturation. In this work we have shown that MAHRP-1 can enhance the peroxidase-like activity of FP at pH 7.4, the presumed pH of the erythrocyte cytosol, with an efficiency similar to that for HRP2. These data suggest that MAHRP-1 could play a role in binding FP and detoxifying reactive oxygen species in the immediate vicinity of the Maurer's clefts.

HRP2 is soluble protein that is mainly located in the erythrocyte cytosol in close proximity to the red blood cell membrane (8, 12). It is possible that HRP2 acts as a roving scavenger of free FP moieties, protecting the erythrocyte membrane and cytoskeletal proteins. The central role of HRP2 in parasite metabolism has, however, been questioned due to the existence of a clone (clone 3B-D5) lacking both HRP2 and the related protein, HRP3. This parasite strain shows no distinct phenotype, at least under the low oxygen tensions used for *in vitro* culture (52). These data suggest substantial redundancy of the role(s) played by histidine-rich proteins in the malaria parasite. With the advent of the malaria genome (53), sequences encoding a number of histidine-rich proteins have appeared in the sequence databases. These proteins may contribute to the scavenging of FP in different compartments. For example, MAHRP-1, which is confined to the Maurer's clefts, may play an important role in protecting the lipids and proteins in this important secretory organelle. In particular, it is possible that MAHRP-1 plays a crucial role in protecting cysteine-rich motifs in PfEMP1 from oxidative damage.

In summary, we have identified and characterized a novel Maurer's cleft-associated protein that may be involved in the formation and/or protection of these important subcellular structures. For the parasite, this might ensure correct trafficking of proteins to the erythrocyte membrane. Understanding the processes whereby the parasite modifies its host cell and protects itself against the stress that derives from hemoglobin degradation might lead to an improved way to control this devastating disease.

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Chapter 3

Genesis of and Trafficking to the Maurer's Clefts of *Plasmodium falciparum*-Infected Erythrocytes

Cornelia Spycher[1], Melanie Rug[2], Nectarios Klonis[3], David J. P. Ferguson[4], Alan F. Cowman[2], Hans-Peter Beck[1] and Leann Tilley[3]

Affiliation of authors:

[1] Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 51, CH-4051 Basel, Switzerland.

[2] The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Melbourne 3050.

[3] Department of Biochemistry and ARC Centre of Excellence for Coherent X-Ray Science, La Trobe University, Melbourne 3086,3 Victoria, Australia.

[4] Nuffield Department of Pathology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom.

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Genesis of and Trafficking to the Maurer's Clefts of *Plasmodium falciparum*-Infected Erythrocytes†

Cornelia Spycher,[1] Melanie Rug,[2] Nectarios Klonis,[3] David J. P. Ferguson,[4] Alan F. Cowman,[2] Hans-Peter Beck,[1] and Leann Tilley[3]*

Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 57, CH 4002 Basel, Switzerland[1]; The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Melbourne 3050,[2] and Department of Biochemistry and ARC Centre of Excellence for Coherent X-Ray Science, La Trobe University, Melbourne 3086,[3] Victoria, Australia; and Nuffield Department of Pathology, John Radcliffe Hospital, Oxford OX3 9DU, United Kingdom[4]

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Malaria parasites export proteins beyond their own plasma membrane to locations in the red blood cells in which they reside. Maurer's clefts are parasite-derived structures within the host cell cytoplasm that are thought to function as a sorting compartment between the parasite and the erythrocyte membrane. However, the genesis of this compartment and the signals directing proteins to the Maurer's clefts are not known. We have generated *Plasmodium falciparum*-infected erythrocytes expressing green fluorescent protein (GFP) chimeras of a Maurer's cleft resident protein, the membrane-associated histidine-rich protein 1 (MAHRP1). Chimeras of full-length MAHRP1 or fragments containing part of the N-terminal domain and the transmembrane domain are successfully delivered to Maurer's clefts. Other fragments remain trapped within the parasite. Fluorescence photobleaching and time-lapse imaging techniques indicate that MAHRP1-GFP is initially trafficked to isolated subdomains in the parasitophorous vacuole membrane that appear to represent nascent Maurer's clefts. The data suggest that the Maurer's clefts bud from the parasitophorous vacuole membrane and diffuse within the erythrocyte cytoplasm before taking up residence at the cell periphery.

Plasmodium falciparum causes one of the most life-threatening infectious diseases of humans. Malaria is estimated to be responsible for up to 2 million deaths per year (34). The pathogenesis of the disease is associated with the intraerythrocytic cycle of the parasite, involving repeated rounds of invasion, growth, and schizogony. The erythrocyte provides a ready source of protein building blocks, but this quiescent cell provides little in the way of cellular architecture, as it possesses no internal organelles and no protein synthesis or trafficking machinery. As the parasite develops, it effectively remodels its adopted home by generating membranous structures outside its own cell and by implementing a complex and unusual system for transporting proteins across the host cell compartment and to its surface. This has led to particular interest in the membrane-bound compartments that appear in the red blood cell (RBC) cytoplasm as the parasite matures.

Once the parasite has invaded a new host cell, it resides within a parasitophorous vacuole (PV). In the ring stage of intraerythrocytic growth, electron microscopy studies have revealed finger-like extensions of the PV membrane (5, 11, 43). These extensions are thought to remain connected to the PV and to develop to form a tubulovesicular network (TVN). As the parasite matures, disk-like structures appear at the RBC periphery, characterized by a translucent lumen and an electron-dense coat of variable thickness (4, 11, 22). These structures are referred to as Maurer's clefts, which is something of a misnomer, as they do not appear to be formed by invagination of the RBC membrane (4, 11, 22), though they often appear to be tethered to the RBC membrane by fibrous connections with the erythrocyte cytoskeleton (7, 22). The origin of the Maurer's clefts is not known, and there is currently some debate as to whether the peripheral Maurer's clefts are independent structures or subdomains of the TVN (24, 30). Recent studies used fluorescence microscopy of cells labeled with lipid probes and electron microscopy of serial sections to examine the structures in the *P. falciparum*-infected RBC cytoplasm (43, 45). These authors postulated that Maurer's clefts and the TVN form part of a continuous meshwork. However, other reports using green fluorescent protein (GFP) chimeras of Maurer's cleft-associated cargo (21, 46) suggest that Maurer's clefts are distinct, independent entities.

An important virulence molecule that is exported to the RBC membrane, via the Maurer's clefts, is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (9, 24). PfEMP1 is a transmembrane protein that is presented at the RBC surface, where it mediates adhesion of parasitized RBCs to endothelial cells in various organs (12, 33). The subsequent accumulation of infected RBCs in the microvasculature is a pivotal event in the pathogenesis of falciparum malaria (23). Given the proposed role of Maurer's clefts in PfEMP1 trafficking, it is of some interest that components of the plasmodial protein trafficking machinery have been reported to be exported to these structures (1, 3, 16, 38, 44). Moreover, some Maurer's cleft-associated proteins, such as the ring exported protein 1 (REX1, (15) and *P. falciparum* erythrocyte membrane protein 3 (PfEMP3) (42), show structural similarity to vesicle-tethering proteins. This suggests that the Maurer's clefts may function as a protein-sorting compartment that is exported into the RBC cytoplasm.

* Corresponding author. Mailing address: Department of Biochemistry, La Trobe University, Melbourne 3086, Australia. Phone: 61-3-94791375. Fax: 61-3-94792467. E-mail: L.Tilley@latrobe.edu.au.

† Supplemental material for this article may be found at http://mcb.asm.org/.

A series of integral membrane proteins have been shown to be resident at the Maurer's clefts (24). These include *P. falciparum* skeleton binding protein 1 (PfSBP1) (7), the Maurer's cleft two-transmembrane domain proteins (Pfmc2-tm) (32), and the subtelomeric variable open reading frame (STEVOR) proteins (18). Several novel proteins identified by proteomics analysis (39) are also thought to be Maurer's cleft associated. We recently characterized a Maurer's cleft resident protein referred to as the membrane-associated histidine-rich protein 1 (MAHRP1) (37). MAHRP1 is a protein with a predicted molecular mass of 28.9 kDa. The C-terminal domain of MAHRP1 in 3D7 has a histidine content of almost 30% and contains six tandem repeats of the amino acid sequence DHGH, with additional preceding DH repeats.

Recent work (17, 26) has identified a pentameric vacuolar transit sequence/protein export element (VTS/PEXEL motif) in the N-terminal region of exported proteins that is required for transport across the PV membrane. The knob-associated histidine-rich protein, KAHRP, and many other exported proteins possess a highly conserved VTS/PEXEL motif, while a related translocation motif appears to be important for trafficking of PfEMP1 across the PV membrane. MAHRP1 does not contain a sequence that conforms to the criteria used to define the VTS/PEXEL motif, indicating that this resident protein may be trafficked to the RBC cytoplasm via a different route.

Despite the importance of the Maurer's clefts as an intermediate compartment in the delivery of PfEMP1, little is known about the molecular processes that generate these structures and the signals that direct resident proteins to this compartment. In this study, we have generated *P. falciparum* transfectants expressing GFP chimeras of MAHRP1 to dissect the domains needed for correct trafficking. The data suggest that MAHRP1 is trafficked through the parasite ER to the PV membrane. MAHRP1 is then incorporated into small foci (presumably nascent Maurer's clefts) that extend from the PV/TVN prior to transfer to the cell periphery.

MATERIALS AND METHODS

Materials. Rabbit antiserum recognizing GFP and REX1 was kindly donated by Mike Ryan, La Trobe University, Australia, and Paula Hawthorne, Queensland Institute of Medical Research, Australia. Preparation of an antiserum recognizing the PfEMP1 cytoplasmic domain will be described elsewhere (13). Mouse anti-MAHRP1 serum was generated as described previously (37). Mouse antibodies recognizing GFP were obtained from Roche. BODIPY-TR-ceramide was obtained from Molecular Probes Pty Ltd. The pARL1a vector was generated as described previously (10).

Plasmid constructs and transfection of *P. falciparum*. *MAHRP1*$_{1\text{-}249}$, *MAHRP1*$_{1\text{-}105}$, *MAHRP1*$_{106\text{-}124}$, *MAHRP1*$_{125\text{-}249}$, and *MAHRP1*$_{106\text{-}249}$ were cloned into vector pHH2 (31) and then subcloned into pARL1a (10), which had been modified to contain the *GFP* coding region and additional cloning sites (pARL1mGFPmT; kindly generated by A. Adisa, La Trobe University). Briefly, constructs were PCR amplified using primers (see Table I at http://www.latrobe.edu.au/biochemistry/labs/tilley/SuppS1T1T2.html) incorporating ApaI and AflII sites and cloned into the vector pARL1mGFPmT. *P. falciparum*-infected RBCs (ring stage, 3D7 strain) were transfected with 100 μg plasmid DNA and cultured in the presence of 10 nM WR99210, a plasmodial dihydrofolate reductase inhibitor (31). Parasites expressing the GFP chimeric proteins were obtained 20 to 35 days after transfection and thereafter remained close to 100% positive for GFP expression when maintained in the presence of 10 nM WR99210.

Western blot analysis of transfectants. Asynchronous *P. falciparum*-infected RBCs were purified by magnetic sorting (Miltenyi Biotec). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% acrylamide), transferred to a nitrocellulose membrane (Hybond-C extra; Amersham Biosciences) for 1.5 h using a Trans-Blot semidry electroblotter (Bio-Rad), and probed with antiserum followed by alkaline phosphatase-conjugated secondary antibodies (Sigma). The membrane was developed in 20 ml of Tris buffer containing 200 μl of 5-bromo-4-chloro-3-indolyl phosphate (15 mg/ml) and 200 μl of nitroblue tetrazolium (30 mg/ml in 70% dimethylformamide). Antibodies for immunoblot analyses were used at the following dilutions: mouse anti-MAHRP1, 1:500; mouse anti-GFP, 1:2,000; goat anti-mouse immunoglobulin G (IgG), 1:20,000.

Fluorescence microscopy and localization studies using indirect immunofluorescence. Fluorescence microscopy was performed using either an Olympus BX50 epifluorescence microscope with 4′,6′-diamidino-2-phenylindole, fluorescein, and rhodamine filter cubes or an inverted Leica TCS-SP2 confocal microscope using ×100 oil immersion objectives (1.4 numerical aperture). In the latter case, argon ion (488 nm) and helium-neon (543 nm) laser lines were employed with the appropriate dichroic filters, and emission wavelengths were selected as previously described (2). Parasitized RBCs expressing MAHRP1-GFP were mounted wet on a glass slide, covered by a glass coverslip, sealed, and imaged within 20 min at ambient temperature (maintained at 20°C).

BODIPY-ceramide was used to label parasitized RBCs as described previously (2). Briefly, parasitized RBCs were resuspended in complete medium (5% parasitemia, 10% hematocrit) and incubated in the presence of 1 μM BODIPY-TR-ceramide at 37°C for 60 min, and then they were washed three times in complete medium and examined by fluorescence microscopy. Serial optical sections (~0.1 to 0.2 μm per section) of GFP- and/or BODIPY-labeled cells were used to generate average maximum-projection three-dimensional (3-D) reconstructions using either the Leica SP2 imaging software or NIH ImageJ (http://rsb.info.nih.gov/ij).

For indirect immunofluorescence microscopy, infected RBCs were smeared onto glass slides and fixed in ice-cold acetone:methanol (1:1, vol/vol) for 10 min. Slides were probed with one of the following primary and secondary antibodies: mouse anti-MAHRP-1c (1:100), rabbit anti-PfEMP1 (1:200), rabbit anti-REX1 (1:1,000), rabbit anti-GFP (1:100), fluorescein isothiocyanate-conjugated anti-rabbit immunoglobin G (IgG; 1:50), and Alexa 568-conjugated anti-mouse IgG (1:200). The slides were mounted in 90% glycerol containing 0.05% *N*-propyl gallate (Sigma) to reduce bleaching.

Electron microscopy. Isolated A4 strain-parasitized RBCs were permeabilized with saponin (36) and incubated with rotation at 37°C with a 1:100 dilution of anti-MAHRP1 serum in RPMI medium with protease inhibitors, followed by 5 nm gold-labeled goat anti-mouse IgG (BioCell). Samples were postfixed with 4% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2, treated with osmium tetroxide, dehydrated, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate before examination in a Joel 1200EX transmission electron microscope.

Fluorescence recovery after photobleaching. The general theory and practice of photobleaching measurements has been described elsewhere (19). RBC suspensions (~30% hematocrit) were placed under number 1.5 coverslips, sealed with petroleum jelly, and viewed on an inverted Leica TCS SP2 confocal microscope with a 100× oil immersion objective (1.4 numerical aperture). Images (8 or 12 bit, typically 256 by 256 pixels) were obtained using the 488-nm line of an argon ion laser, and the fluorescence collected through a long-pass 495-nm filter before wavelengths of 500 to 560 nm was selected using the instrument's software. To minimize photobleaching during image acquisition and to maximize the fluorescence signal, the confocal pinhole was opened (3.3 Airy units), the photomultiplier gain was set to high levels, and the laser intensity was minimized to achieve less than 2% bleaching per image. A typical photobleaching measurement comprised two prebleach images to assess the degree of bleaching during image acquisition, a 100- to 1,000-ms spot irradiation of a defined point with the unattenuated laser, and a series of postbleach images. The first postbleach image was obtained immediately after the bleach pulse and is defined as postbleach time zero. Image processing, including background correction, smoothing, and image analysis were performed as described previously (2).

RESULTS

Full-length MAHRP1-GFP is expressed in transfected *P. falciparum*. The MAHRP1 protein sequence (3D7 strain) is shown in Fig. 1a. Exon 1 of the *MAHRP1* gene encodes the first 130 amino acids of the protein, including the 19-amino-acid transmembrane domain and the beginning of the carboxy-terminal domain. Residues 125 to 249 comprise the histidine-rich C-terminal domain. We have generated a chimera comprising

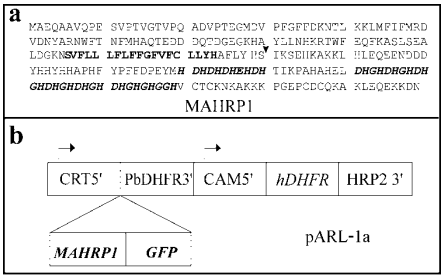


FIG. 1. Organization of the MAHRP1 sequence and transfection construct. (a) *P. falciparum* 3D7 MAHRP1 amino acid sequence (accession no. AAQ63567; MAL 13P1.413). The transmembrane domain and C-terminal histidine-rich repeats are highlighted in boldface and boldface italics, respectively. The exon boundary site is shown with an arrowhead. (b) Schematic diagram of the pARL1a vector showing insertion of the *MAHRP1* sequence upstream of the *GFP* coding region. The plasmid contains a human dihydrofolate reductase (hDHFR) selection cassette. CRT, chloroquine resistance transporter; CAM, calmodulin; HRP2, histidine-rich protein 2.

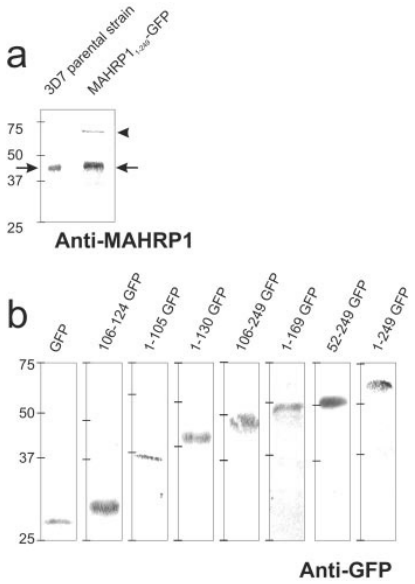


FIG. 2. Western blot analysis of parasite proteins showing expression of MAHRP1-GFP chimeras. (a) *P. falciparum* parental 3D7-infected RBCs ($\sim 5 \times 10^7$ parasites) and $MAHRP1_{1\text{-}249}$-GFP transfectants ($\sim 10^8$ parasites) were subjected to Western blot analysis and probed with anti-MAHRP1 mouse serum. Endogenous MAHRP1 is indicated with an arrow. $MAHRP1_{1\text{-}249}$-GFP is indicated with an arrowhead. (b) Total protein extracts from the different transfectants were subjected to Western blot analysis and probed with anti-GFP antibodies. The predicted sizes of the fusion proteins are given in Table II at http://www.latrobe.edu.au/biochemistry/labs/tilley/SuppS1T1T2.html.

the sequence coding for full-length MAHRP1 linked to the 5′ end of sequence encoding GFP. The full-length *MAHRP1*$_{1\text{-}249}$*-GFP* chimera was inserted into the transfection vector pARL1a (10) under the control of the *PfCRT 5′* region (Fig. 1b). *PfCRT 5′* is a moderate promoter that drives maximal expression in the late ring stages (8), which is similar to the expression profile for MAHRP1 (35).

Asynchronous parasite cultures of parent and transfected lines were subjected to Western blotting and probed with a mouse anti-MAHRP1 antiserum (37) and a rabbit antiserum against GFP. Anti-MAHRP1 recognized a band of approximately 40 kDa (Fig. 2a) in lysates of parental 3D7-infected erythrocytes, as reported previously (37), and an additional band of approximately 70 kDa in the $MAHRP1_{1\text{-}249}$-GFP transfectant (Fig. 2a, arrowhead). The relative intensity of the upper band indicates that MAHRP1-GFP is expressed at lower levels than endogenous MAHRP1. When the blotted lysates were probed with antibodies recognizing GFP, no reactivity was observed in uninfected RBCs or in the 3D7 parent parasites (data not shown), while an approximately 70-kDa protein was observed in $MAHRP1_{1\text{-}249}$-GFP transfectants (Fig. 2b, right lane). Both endogenous MAHRP1 and MAHRP1-GFP migrate with slightly higher apparent molecular masses than predicted (28.9 kDa and 55.9 kDa, respectively). The higher apparent molecular mass is probably due to charged residues in the repeat region of the protein which can cause anomalous migration. There is no evidence for proteolytic processing of either endogenous MAHRP1 or the $MAHRP1_{1\text{-}249}$-GFP chimera.

The $MAHRP1_{1\text{-}249}$-GFP chimera is trafficked to Maurer's clefts via subdomains of the PV membrane. Fluorescence microscopy of live cells has been used to examine the location of $MAHRP1_{1\text{-}249}$-GFP at different stages of growth (Fig. 3). In early-ring-stage parasites (Fig. 3a and b), the MAHRP1-GFP chimera is already present in structures in the host cell cytoplasm that appear to be Maurer's clefts (white arrows). In late ring and early trophozoite stages, some cells showed an accumulation of GFP fluorescence in what appear to be subcompartments associated with the PV or PV membrane (Fig. 3b to d, yellow arrows). In schizont-stage parasites, the structures in the host cell cytoplasm containing MAHRP1-GFP appear to be pushed against the host cell membrane (Fig. 3e), and upon lysis of the host cell membrane (visualized by loss of edge effects in the differential interference contrast [DIC] image) during schizont rupture they remain associated with the lysed RBC membrane (Fig. 3f).

To obtain additional information regarding the organization of the GFP chimera, we generated a 3-D reconstruction from a series of confocal optical slices of the transfectants (see Fig. S2 in the supplemental material for a rotatable image). Distinct compartments containing the GFP chimera are observed close to the periphery of the infected RBC and may be physically tethered to the RBC cytoskeleton (7). In addition, we observed GFP-containing structures located near the surface of the parasite that are visualized as bright puncta and more extended worm-like structures (see Fig. S2 in the supplemental material). We suggest that these may be protein sorting sites or subdomains of the PV, or they may represent nascent Maurer's clefts forming at the PV membrane.

The relationship between the PV-associated (presumably nascent) Maurer's clefts and various membranous structures in the host cell cytoplasm was examined by colabeling the $MAHRP1_{1\text{-}249}$-GFP transfectants with BODIPY-ceramide. This lipid label has previously been used to examine the organization of the PV membrane and TVN (2, 14). Single optical slices through two dual-labeled trophozoite-stage-infected

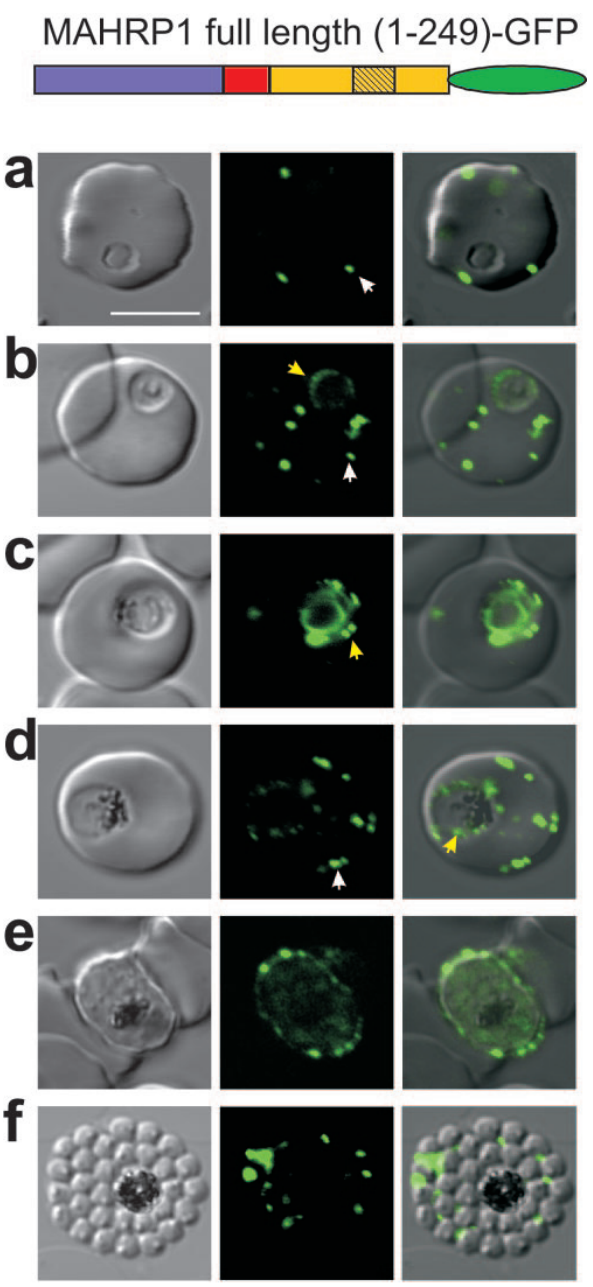


FIG. 3. Expression of MAHRP1$_{1-249}$-GFP at different stages of the intraerythrocytic cycle of *P. falciparum*. The images represent a DIC image, the GFP fluorescence signal, and an overlay of these images. Ring and trophozoite stage parasites (a to d) show puncta of fluorescence in the RBC cytoplasm which appear to represent peripheral Maurer's clefts (white arrows). Some cells (see, e.g., panel c) show a ring of "beads" of fluorescence around the PV. These foci may represent nascent Maurer's clefts (yellow arrows). Mature schizont-stage parasites (e) show flattening of the peripheral Maurer's clefts against the RBC membrane. In a burst schizont (f), the remnant Maurer's clefts remain associated with the lysed host cell membrane. Bar, 5 μm. The intensities of the images were adjusted to optimize the fluorescence signal at each parasite stage.

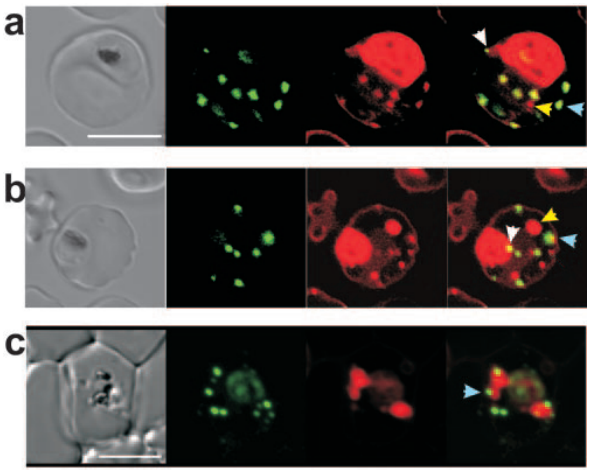


FIG. 4. Dual labeling of MAHRP1$_{1-249}$-GFP transfectants with BODIPY-ceramide. (a to c) The images represent (from left to right) a DIC image, GFP fluorescence, BODIPY-ceramide fluorescence, and an overlay of the GFP (green) and BODIPY-ceramide (red) images. The parasite membranes are intensely labeled with the lipid probe. Some extensions of the PV membrane are dotted with foci of MAHRP1-GFP (white arrows). Some of the BODIPY-labeled structures (probably TVN extensions and buds) are not labeled with GFP (yellow arrows), while others (presumably Maurer's clefts) are labeled with GFP (blue arrows). The fluorescence images in panel c correspond to an average projection generated from a series of optical slices. See Fig. S3 in the supplemental material for a video showing a 3-D rotation. Bar, 5 μm.

RBCs are shown in Fig. 4a and b. BODIPY-ceramide labels the membrane-rich parasite cytoplasm very heavily as well as punctate structures in the RBC cytoplasm. Most of these structures are labeled with both BODIPY-ceramide and GFP (Fig. 4a and b, white and blue arrows) and presumably represent nascent and mature Maurer's clefts. The labeling with BODIPY-ceramide is fairly weak, which may indicate a relatively low lipid content. Some BODIPY-ceramide-labeled structures do not contain MAHRP1$_{1-249}$-GFP (Fig. 4a and b, yellow arrows). These appear to represent a second population of TVN buds and extensions that are distinct from the Maurer's clefts. We have previously reported the budding of regions of the TVN (2). Figure 4c shows an average projection of a cell generated from a series of optical slices. A video showing rotatable images can be found in Fig. S3 in the supplemental material. Rotation of this image reveals what appear to be nascent Maurer's clefts associated with extensions of the PV membrane or TVN (Fig. 4c, blue arrow). Again, the data suggest that MAHRP1$_{1-249}$-GFP collects into subdomains on the PV membrane or TVN.

Immunofluorescence analysis confirms the Maurer's cleft location of MAHRP1-GFP. We have used indirect immunofluorescence microscopy to confirm the subcellular location of MAHRP1$_{1-249}$-GFP in the transfectants. In cells fixed with acetone:methanol (which destroys the endogenous GFP fluorescence), antibodies against GFP gave a pattern of distribution similar to that observed in live cells. The chimera was present in punctate structures in the host cell cytoplasm (Fig. 5a). Dual labeling with an antiserum recognizing endogenous

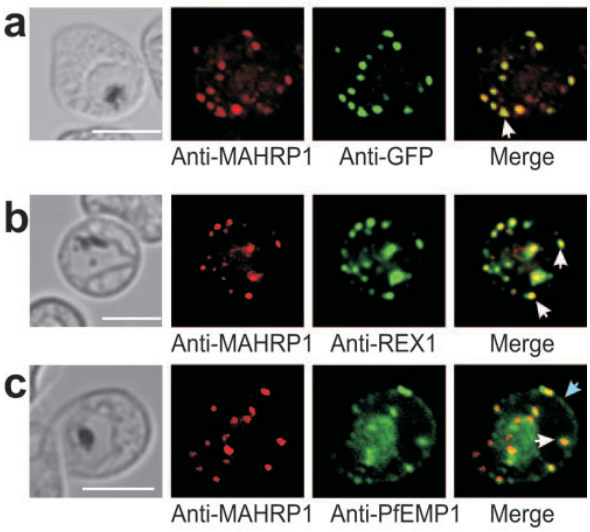


FIG. 5. Immunofluorescence microscopy of MAHRP1$_{1-249}$-GFP. (a) Trophozoite-stage-infected RBC labeled with mouse anti-MAHRP1 antiserum (red) and rabbit anti-GFP antiserum (green). A dual-labeled Maurer's cleft (white arrow) is indicated. (b) Trophozoite stage infected RBC labeled with mouse anti-MAHRP1 (red) and rabbit anti-REX1 (green) antisera. (c) Trophozoite stage infected RBCs labeled with mouse anti-MAHRP1 (red) and rabbit anti-PfEMP1 (green). Overlap of these signals confirms the Maurer's clefts location (white arrow). PfEMP1 is also partly located at the RBC membrane (blue arrow). The bars represent 5 μm.

MAHRP1 showed a very similar pattern, with almost complete overlap of GFP and MAHRP1 in the punctate structures in the RBC cytoplasm (Fig. 5a, white arrow).

To confirm the identity of the punctate compartments in the host cell cytoplasm, we undertook dual labeling with a known Maurer's cleft marker, the ring-stage-exported protein REX1 (Fig. 5b). The complete overlap of these signals (Fig. 5b, white arrows) provides further evidence that these structures are Maurer's clefts. We also examined the profiles of cells labeled with an antiserum against the cytoadherence antigen, PfEMP1. This protein is exported to peripheral Maurer's clefts (Fig. 5c) prior to redistribution to the RBC membrane (Fig. 5c, blue arrow). MAHRP1 is colocalized with PfEMP1 when it is associated with the Maurer's clefts (Fig. 5c, white arrow) and may play an important role in transit of this protein through the Maurer's cleft compartment.

Ultrastructural analysis reveals MAHRP1 organization and orientation at the Maurer's clefts. To examine the exact location of endogenous MAHRP1, we have introduced antibodies against the C-terminal domain of MAHRP1 into saponin-permeabilized infected RBCs using methods developed previously (36). The permeabilized cells were incubated with 5 nm gold-labeled anti-mouse IgG prior to processing for transmission electron microscopy. This allowed us to probe the population of endogenous MAHRP1 in the RBC cytoplasm compartment. Maurer's clefts with electron-dense coats were frequently observed in sections of early trophozoite-stage parasitized RBCs (Fig. 6). The Maurer's clefts were decorated with gold particles that appeared to be concentrated in the central region of the organelle. The fact that the MAHRP1 antibody (which is raised against the C-terminal domain) is able to access its binding epitope in these permeabilized cells and the observation that the particles are largely arrayed at the cytoplasmic surface of the Maurer's clefts indicates that the C terminus (His-rich region) of MAHRP1 is facing the RBC cytoplasm. In a few (<10%) of the labeled cells, it was possible to observe gold particles associated with areas of the PV membrane (Fig. 6b and c, arrowheads). These may represent areas of the PV membrane where MAHRP1 has accumulated prior to the formation of Maurer's clefts.

Part of the N-terminal domain and the transmembrane domain of MAHRP1 are needed for correct trafficking to the Maurer's clefts. MAHRP1 does not contain a sequence that conforms to the criteria used to define the VTS/PEXEL motifs (17, 26), indicating that this resident protein may be trafficked to the Maurer's clefts via a different route to other exported proteins. Alternatively, MAHRP1 may contain an unusual VTS/PEXEL motif or a different export signal. In an effort to determine the regions of the MAHRP1 sequence that are needed for correct trafficking, we have generated transfectants expressing a range of fragments of MAHRP1 appended to GFP and examined their intracellular locations.

When GFP alone is expressed in transfected *P. falciparum*, it is present in the parasite cytoplasm (Fig. 7a; see Fig. S1a at http://www.latrobe.edu.au/biochemistry/labs/tilley/SuppS1T1T2.html). When the N-terminal domain of MAHRP1 was fused to GFP, the chimeric protein appears to remain in the parasite cytoplasm (Fig. 7b; see Fig. S1b at the website listed earlier in this paragraph). By contrast, a GFP fusion protein containing the transmembrane domain of MAHRP1 appeared to enter the endoplasmic reticulum (ER) and to remain in this compartment (Fig. 7c; see Fig. S1c at the website listed earlier in this paragraph). The ER location is indicated by the fact that the protein is located in a compartment that surrounds the nucleus (see Fig. S1c at the website listed earlier in this paragraph). A construct comprising the transmembrane and C-terminal domains also appears to remain trapped in the ER (Fig. 7d; see Fig. S1d at the website listed earlier in this paragraph).

Thus, it appears that the transmembrane domain is needed for the chimera to enter the ER, the first step of the export pathway, but that additional sequence is needed for subsequent steps. Therefore, we generated a chimera with the first 169 amino acids of MAHRP1 (comprising the N-terminal and transmembrane domains and the first 45 amino acids of the C-terminal region) fused to GFP. The fusion protein was successfully exported to the RBC cytoplasm (Fig. 7f) and became associated with the Maurer's clefts. This indicates that the first 169 amino acids are sufficient for correct trafficking and that the His-rich repeats (amino acids 170 to 214) are not needed. A further construct was generated comprising the first 130 amino acids fused to GFP; this is equivalent to the sequence encoded by exon 1 (i.e., up to the sixth amino acid of the C-terminal domain). This fusion protein was partly exported to the RBC cytoplasm and became associated with the Maurer's clefts (Fig. 7e, second row); however, the export process appeared to be less efficient. In this transfectant, a build-up of the fluorescent chimera in what appears to be the ER and structures associated with the PV membrane was frequently ob-

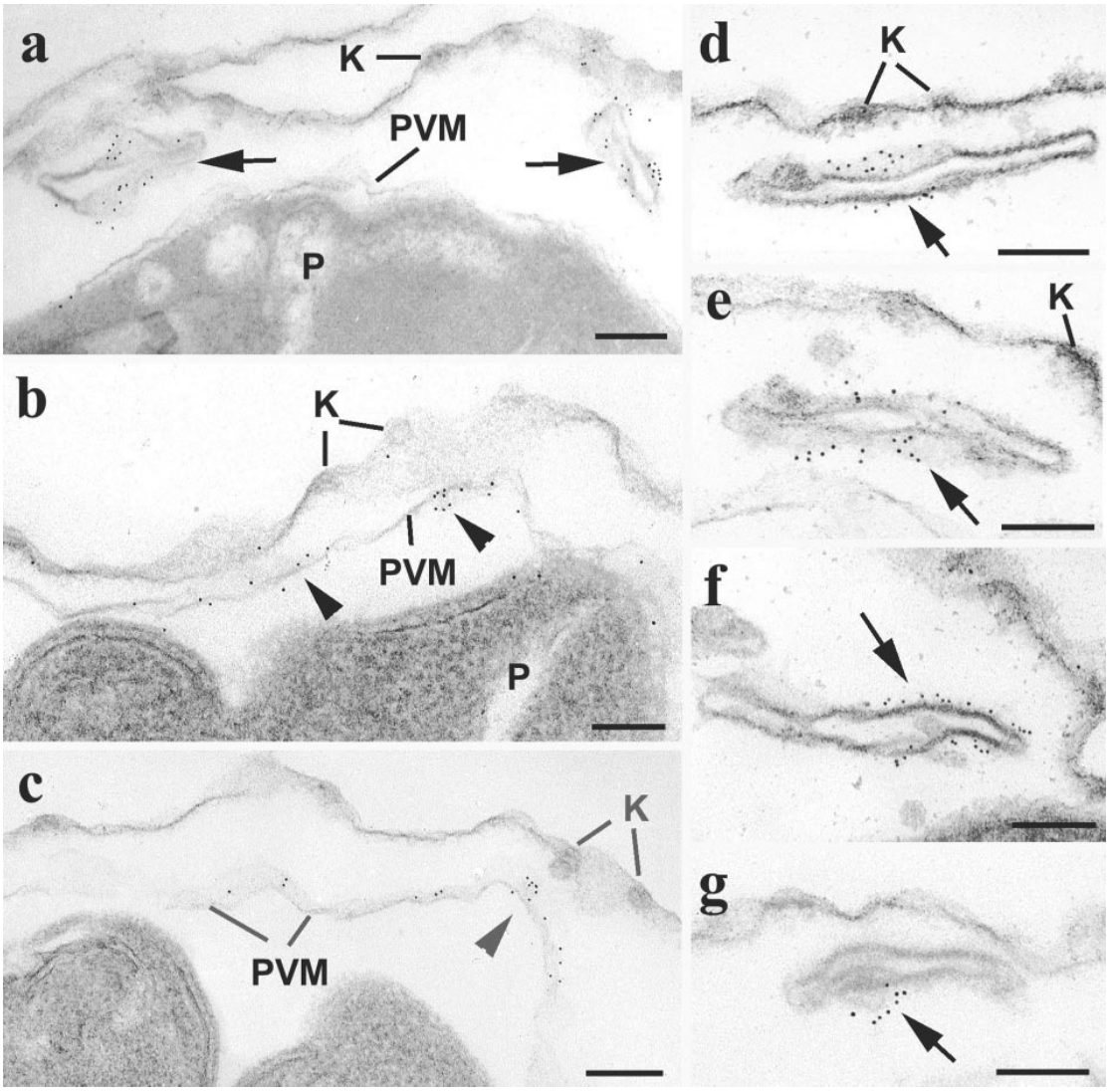
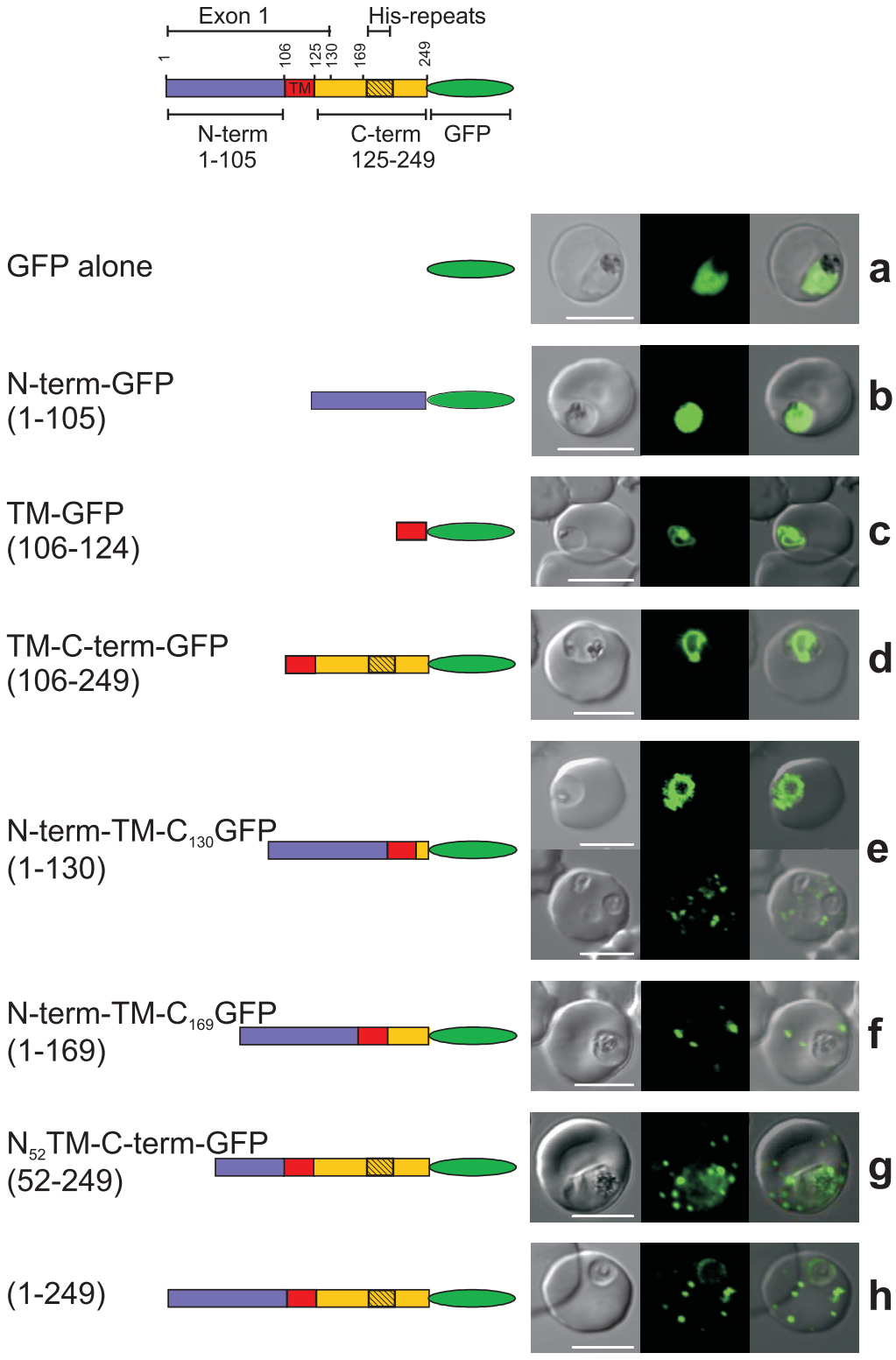


FIG. 6. Transmission electron microscopy of immunogold labeling of MAHRP1 in saponin-permeabilized parasitized RBCs. (a to c) Sections through the periphery of infected RBCs showing labeling of the Maurer's clefts (arrows) with variable labeling of the PV membrane (PVM; arrowheads). In most sections (a), the PVM is unlabeled; however, in a few sections (b and c), there is focal labeling. (d to g) Details of Maurer's clefts showing that the gold particles are mainly located in the central region (arrows). K, knobs; P, parasite. Bars, 100 nm.

served (Fig. 7e, first row). This indicates that information in the first half of the C-terminal domain (amino acids 131 to 169), while not necessary for export, may facilitate delivery to the Maurer's clefts. To assess whether an export signal is present within the sequence close to the N terminus, we generated a further chimera with a truncation of the first 51 amino acids. As shown in Fig. 7g, this chimera is successfully exported to the Maurer's clefts with efficiency similar to that of full-length MAHRP1-GFP (Fig. 7h). This indicates that the second half of the N-terminal domain contains the signal for transfer from the parasite's endomembrane system to the Maurer's clefts. It is important to note that endogenous MAHRP1 was expressed and correctly trafficked to peripheral Maurer's clefts in all transfectants (data not shown).

In some (5 to 10% of cells) of the young trophozoite-stage transfectants expressing the $MAHRP_{1\text{-}130}$-GFP and $MAHRP1_{52\text{-}249}$-GFP constructs, structures were observed that appeared to be Maurer's cleft compartments that had budded from the PV membrane but had not yet become attached to the RBC membrane. Stills of one of these cells taken at different time points are presented in Fig. 8a. Movies showing the movement of untethered clefts in this cell and in some other examples can be found in Fig. S4 of the supplemental material. The occasional observation of these mobile clefts supports the


Exon 1
His-repeats
1
106
125
130
169
249
TM
N-term
1-105
C-term
125-249
GFP
GFP alone
a
N-term-GFP
(1-105)
b
TM-GFP
(106-124)
c
TM-C-term-GFP
(106-249)
d
N-term-TM-C130GFP
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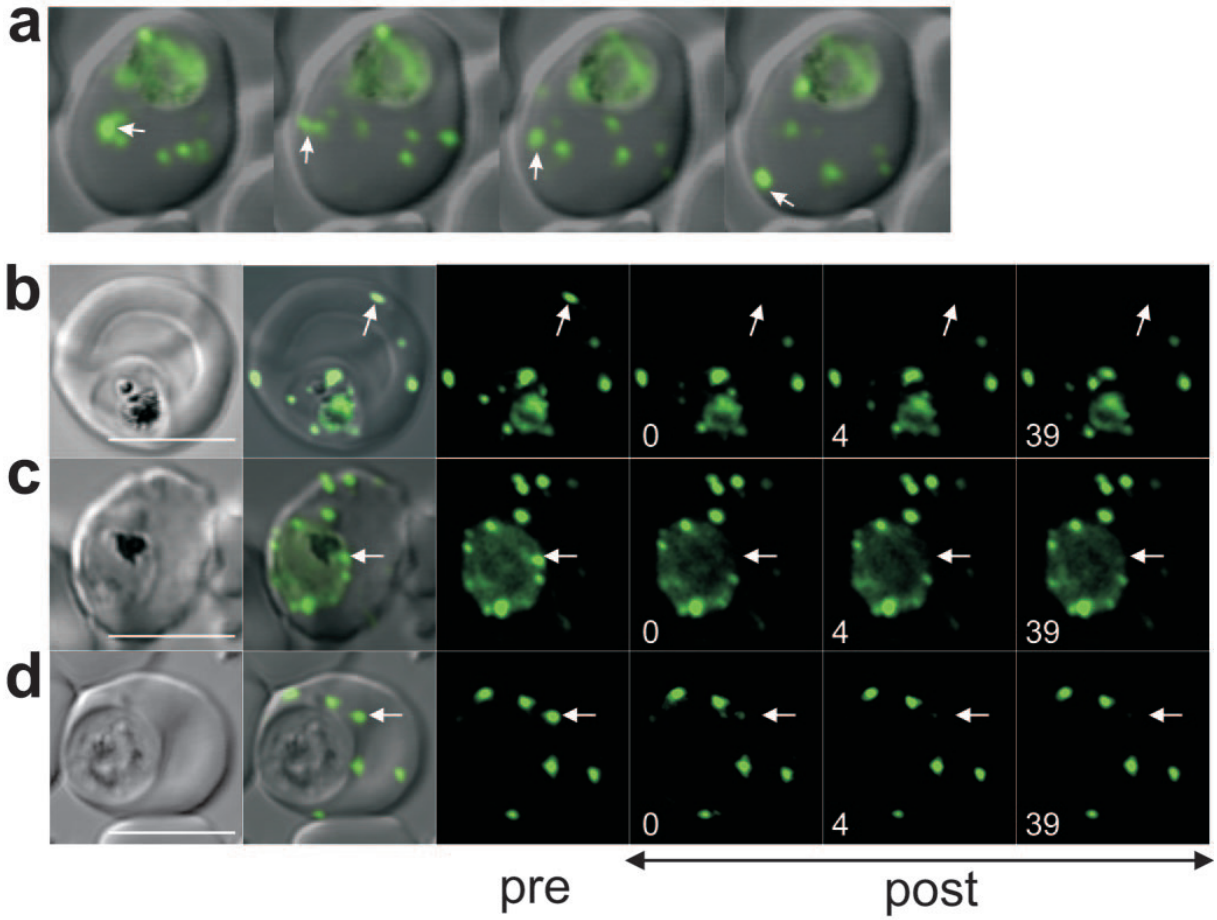

FIG. 8. Molecular organization of GFP chimeras in different compartments. (a) Overlay images of a MAHRP1$_{1\text{-}130}$-GFP transfectant displaying mobile Maurer's clefts taken at consecutive time points. One moving cleft observed over time is indicated with white arrows. A movie of this cell and other examples can be viewed in Fig. S4. The bar represents 5 μm. (b to d) Photobleach analysis. The panels show the DIC images and the prebleach (pre) and postbleach (post) fluorescence images at the times (in seconds) indicated. The position of the bleach pulse is indicated by white arrows. (b) Bleaching of MAHRP1$_{52\text{-}249}$-GFP in a tethered peripheral Maurer's cleft with a 300-ms bleach pulse. There is no recovery of the signal after photobleaching, indicating a physically separate structure. Bleaching of a PV membrane-associated MAHRP1$_{52\text{-}249}$-GFP focal region with 300-ms bleach pulses (c) and a structure in close proximity to the PV membrane with a 200-ms bleach pulse (d). There is no recovery of fluorescence into the bleached areas. Scale bars, 5 μm.

suggestion that Maurer's clefts bud from the PV membrane as preformed structures and then take up residence at the cell periphery.

To determine whether the correct GFP-MAHRP1 fragment chimeras were expressed, we performed Western blot analyses of each of the different transfectants. Total parasite extracts were blotted and probed with anti-GFP (Fig. 2b). The immunoreactive bands have molecular weights that are close to or (for parasite lines expressing products incorporating the C-terminal repeats) slightly higher than the predicted molecular

FIG. 7. Dissecting the domains required for correct trafficking of MAHRP1 to the Maurer's clefts. At the top is a schematic representation of the MAHRP1 full-length protein with its three domains (N terminal [N-term], transmembrane [TM], and C terminal [C-term]), with numbering of key amino acids and the GFP. Transfected *P. falciparum*-expressing GFP linked to different domains of MAHRP1 were examined by confocal fluorescence microscopy. (a) GFP alone is evenly distributed throughout the parasite cytoplasm. A chimera of the N-terminal region (amino acids 1 to 105) (b) also appears to be restricted to the parasite cytoplasm. Chimeras of the transmembrane domain of MAHRP1 (106 to 124) (c) and of the transmembrane domain and C-terminal region of MAHRP1 (amino acids 106 to 249) (d) appear to be located in the parasite's endomembrane system. A chimera of the N-terminal region and transmembrane domain of MAHRP1 (amino acids 1 to 130) (e) is partly exported to the RBC cytoplasm but in some cells accumulates within the parasite. A chimera containing amino acids 1 to 169 (f), a deletion of the first 51 amino acids (g), and full-length MAHRP1-GFP (h) are efficiently exported to the RBC cytoplasm. Bars, 5 μm.

weights. Predictions of the expected sizes for each of the fusion proteins are given in Table II at http://www.latrobe.edu.au/biochemistry/labs/tilley/SuppS1T1T2.html. In each case a single band was observed, indicating that there is no proteolytic processing of the chimeras.

Exported MAHRP1-GFP becomes tightly associated with the Maurer's clefts. We performed photobleaching studies (19) of Maurer's cleft-associated full-length MAHRP1-GFP ($MAHRP1_{1-249}$-GFP) and of the chimera with the first 51 amino acids deleted ($MAHRP1_{52-249}$-GFP) to examine the physical organization of the fusion proteins and the connectivities of the compartments in which they are located. We first examined whether the population of the fusion proteins associated with the Maurer's cleft is tightly associated with these structures or rapidly exchanging with a cytoplasmic pool which, although not readily visible, may be present. Selective bleaching of $MAHRP1_{52-249}$-GFP associated with a tethered cleft at the periphery of the RBC was achieved with a 0.3-s exposure to a laser pulse (Fig. 8b, white arrow). There was no recovery of fluorescence onto the Maurer's clefts within the time scale examined. The same result was obtained for full-length $MAHRP_{1-249}$-GFP (data not shown). This indicates that the MAHRP1-GFP fusion proteins are tightly bound at the Maurer's clefts and that there is no connectivity between different Maurer's clefts, or at least no exchange of the chimeras. The data are consistent with the suggestion that the fusion proteins are embedded in the Maurer's cleft membrane and that the Maurer's clefts lack a direct physical connection to other membrane-bound structures.

We also examined the physical organization of the MAHRP1-GFP fusion proteins within the fluorescent puncta that are associated with the PV membrane. We bleached compartments that were close to the parasite surface (Fig. 8c, arrow) or seemed to be in the process of budding from the PV membrane (Fig. 8d, arrow). In both cases the bleach profile was restricted to the region to which the laser pulse was applied, and there was no fluorescence recovery into the bleached area. This indicates that even at the PV membrane, the MAHRP1-GFP-containing compartments are physically separate structures. The lateral segregation of MAHRP1-GFP molecules into non-exchanging subdomains of the PV membrane is consistent with the suggestion that these domains are sites of formation of Maurer's clefts.

Taken together with the BODIPY-ceramide dual-labeling experiments, the movies of untethered clefts, and the fact that we did not observe any small MAHRP1-containing vesicles in the RBC cytoplasm, the data for bleaching of the proximal (presumably nascent) and peripheral (presumably mature) Maurer's clefts suggest that these compartments are assembled at the PV membrane and trafficked as "ready-made" structures to the periphery of the RBC. However, it remains formally possible that MAHRP1 is packaged into small vesicles (that are transient and difficult to observe) that subsequently fuse with mature clefts.

DISCUSSION

The malaria parasite establishes a novel and unusual secretory pathway in the cytoplasm of its erythrocytic host. Unable to commandeer any trafficking machinery from its host cell, the parasite needs to establish both rolling stock and transport routes for transfer of material across the host cell cytoplasm. A major feature of the infected RBC cytoplasm is the appearance of densely coated, saucer-shaped structures, known as Maurer's clefts, that are proposed to function as a transit station or sorting depot for proteins en route to the RBC membrane (9, 24). Because of their presumed importance in the trafficking of PfEMP1 and other exported proteins, the Maurer's clefts are currently being studied in some detail.

MAHRP1 is a Maurer's cleft resident protein that may be involved in the formation of this important organelle or in the transit of PfEMP1 through this compartment (37). It is a small protein with no significant homology to any known sequence. It has no predicted N-terminal signal sequence but has a 19-amino-acid Phe-rich transmembrane domain. In this work, we have generated transfected parasites expressing fusions of either full-length MAHRP1 or a series of MAHRP1 fragments in an effort to dissect the pathway for trafficking to the Maurer's clefts. Indeed, full-length MAHRP1-GFP is trafficked to the host cell cytoplasm, where it occupies the same compartments as endogenous MAHRP1.

We have used saponin permeabilization of infected RBCs as a means of introducing gold-labeled antibodies that recognize the C-terminal domain of endogenous MAHRP1. Transmission electron micrographs of these labeled cells revealed Maurer's clefts decorated with gold particles on their cytoplasmic surface. This indicates that the C-terminal domain of MAHRP1 is exposed to the RBC cytoplasm. This is in agreement with the prediction of the PSORT algorithm (27), which suggests a type Ib orientation for MAHRP1. Another Maurer's cleft resident, PfSBP1, is also predicted to be a type Ib protein and is known to be oriented with its C-terminal region facing the RBC cytoplasm (7).

MAHRP1 appears to be concentrated in the central region of the Maurer's clefts. Permeabilization of parasitized RBCs has previously been used to introduce antibodies against the cytoplasmic domain of PfEMP1 into the host cell cytoplasm. A similar distribution was observed for PfEMP1, although PfEMP1 was also observed associated with fibrous extensions of the Maurer's clefts (22). It is possible that MAHRP1 plays a role in docking PfEMP1 complexes onto the Maurer's cleft membrane or helping to insert PfEMP1 into a membrane environment. In contrast, electron microscopy analysis of PfSBP1 revealed preferential labeling of the rims of the Maurer's clefts (7). These data suggest that the Maurer's clefts could contain subcompartments that perform different functions.

The availability of transfectants expressing MAHRP1-GFP offers the possibility of following the pathway for trafficking of this protein and potentially for observing the mode of formation of Maurer's clefts. In some transfected cells, we observed an accumulation of MAHRP1-GFP in foci associated with the PV membrane, presumably en route to the host cell cytoplasm. We have used colabeling with BODIPY-ceramide and 3-D image reconstruction to examine the organization of MAHRP1-GFP within the PV membrane. The lipid probe labels the endomembrane system of the parasite as well as the PV membrane/TVN extensions and small discrete structures in the host cell cytoplasm. Some of the membranous structures in the RBC cytoplasm (presumably the Maurer's clefts) are labeled with MAHRP1-GFP, while some are not; these could represent

regions of the TVN that have budded into the RBC cytoplasm but are distinct from Maurer's clefts, as has been reported previously (2).

We were particularly interested in the foci of MAHRP1-GFP that appear to accumulate in regions of the PV membrane and in tubulovesicular extensions. These foci are reminiscent of ER "exit complexes" (28), and we propose that this accumulation event represents the genesis of the Maurer's clefts. The tendency of MAHRP1-GFP to concentrate into particular subdomains of the PV/TVN membranes may be due to self association or to association with other Maurer's cleft resident proteins. In some of the transfectants expressing truncated MAHRP1-GFP, we occasionally observed an accumulation of mobile clefts that appeared to have budded from the PV membrane. These data suggest that clefts that are released from the PV membrane diffuse within the RBC cytoplasm until they become tethered to the RBC membrane.

Florescence recovery after photobleaching can be used to provide information on the organization and dynamics of GFP chimeras in transfected malaria parasites (2, 19, 46). We have used confocal microscope-based photobleaching protocols to probe the physical organization of MAHRP1-GFP en route to the Maurer's clefts. MAHRP1 chimeras that are present in the peripheral Maurer's clefts or in foci at the PV membrane appear to represent distinct nonconnected structures. Bleaching of one focal aggregate did not deplete the fluorescence from other regions, indicating that the proteins are not able to move laterally within the membrane or that the separate foci are not connected by a fluid continuum. We have previously reported that soluble GFP-labeled proteins in the PV lumen can be restricted to subcompartments within the PV, forming a so-called necklace of beads around the parasite surface (2, 46). The integral membrane protein, exported protein-1 (Exp1), and early transcribed membrane protein, ETRAMP, have also been reported to undergo lateral segregation into different regions of the PV membrane (36). Thus, our current findings reinforce the idea that the PV contains separate subcompartments.

A recent study postulated that Maurer's clefts and the TVN form part of a continuous network (45). An earlier immunofluorescence microscopy study employing an antibody against protein Ag45 also led to the conclusion that Maurer's clefts are subcompartments of the TVN (6). Our results indicate that the peripherally located Maurer's clefts are not interconnected, or at least that a physical barrier prevents the diffusion of GFP chimeras between adjacent Maurer's clefts. Similarly, we find no evidence for continuity of the peripheral Maurer's cleft structures with the PV membrane. However, the nascent Maurer's clefts do appear to form subdomains of the PV/TVN membrane, which is consistent with the interconnected structures observed by other workers (6, 43). This work therefore serves to clarify the apparent differences between these different studies.

In an effort to determine the domains that are responsible for correct trafficking of MAHRP1 to the Maurer's clefts, we generated transfectants expressing subdomains of MAHRP1 linked to GFP. When the N-terminal domain was used alone, the chimera remained trapped in the parasite cytoplasm. Constructs containing the transmembrane domain, either alone or combined with the C-terminal domain, entered the ER but were unable to progress beyond this compartment. By contrast, a construct comprising the first 130 amino acids of MAHRP1 (N-terminal and transmembrane domains and the first 6 amino acids of the C-terminal region) was trafficked to the Maurer's clefts, albeit with lower efficiency, while a construct with the first 169 amino acids was trafficked with similar efficiency to the full-length (amino acids 1 to 249) construct. Similarly, a construct comprising the second half of the N-terminal domain plus the transmembrane and C-terminal domains (amino acids 52 to 249) was efficiently trafficked to the Maurer's clefts. Taken together, these data indicate that the information required for directing MAHRP1 to its final destination is contained within the second half of the N-terminal domain and that the transmembrane domain is required for entry into the ER.

The sequence information directing MAHRP1 trafficking appears to be different from that needed for correct export of STEVOR, another Maurer's cleft-associated protein (29). Most exported plasmodial proteins, including the STEVOR family, are encoded by 2-exon genes and possess both a hydrophobic signal sequence, encoded by the first exon, and an RxLxE/Q sequence, close to the start of the second exon (17, 26). PfEMP1 represents a second class of exported proteins. It lacks an N-terminal hydrophobic signal sequence, and its export is thought to be determined by a related sequence, AKHLLDRLG (26) or FFRWFSEWSE (17), in the N-terminal region of the protein. MAHRP1 has a sequence (comprising amino acids 87 to 99) which has a charge distribution similar to that of the PfEMP1 VTS/PEXEL. It is possible that this region represents the signal directing forward transit of this protein.

Charge differences between the N- and C-terminal regions are important in determining the topology of integral membrane proteins (40). It is possible that charge differences contribute to the signal information needed for correct MAHRP1 trafficking. The N-terminal domain of MAHRP1 has a theoretical pI of 4.73, while the C-terminal domain has a pI of 6.19. This charge difference is responsible for the type Ib prediction (C terminus cytoplasmic) using the PSORT algorithm (27). This charge difference is lower in some of the constructs generated in this study, but for each of the exported chimeras it is still sufficient to predict a type Ib orientation. Indeed, a lesser charge difference may explain why $MAHRP1_{1\text{-}130}$-GFP is exported somewhat less efficiently. Interestingly, PfSBP1, another integral membrane protein that is directed to the Maurer's cleft, has a similar charge differential between the N- and C-terminal domains.

PfEMP1 is thought to insert into a membrane environment when it reaches the Maurer's clefts prior to transit to the RBC membrane (21). It is interesting to note that while the cytoplasmic domains of MAHRP1 and PfSBP1 are relatively basic, the cytoplasmic domain of PfEMP1 is highly acidic. KAHRP and PfEMP3 are also transiently associated with the Maurer's clefts before redistribution to the cytoplasmic face of the RBC membrane (42, 46). The regions responsible for Maurer's cleft binding in KAHRP and PfEMP3 have been defined (20, 25, 46). For PfEMP3, residues 120 to 500 are sufficient for tight Maurer's cleft binding (20); this region has a pI of 9.21. KAHRP residues 60 to 120, with a pI of 7.07, are sufficient for weak binding to the Maurer's clefts (25, 46). However, stronger

interaction with the cytoplasmic domain of PfEMP1 requires the 5′ and 3′ repeat regions (41); the pI for this region of the protein is 9.64. Positively charged regions in the cytoplasmic domains of MAHRP1 and PfSBP1 may interact with the highly acidic cytoplasmic domain of PfEMP1 and facilitate the process of insertion of PfEMP1 into the Maurer's cleft membrane. It is possible that subsequent charge interactions with proteins such as KAHRP and PfEMP3 may assist in separating PfEMP1 into a separate compartment within the Maurer's cleft and facilitate forward transit of this protein.

Taken together with data from other studies, our work suggests a possible model for trafficking of proteins to the RBC membrane. Maurer's cleft resident proteins, such as MAHRP1, are expressed in the ring stage and are likely to be needed for the genesis of the parasite's extracellular secretory pathway. Our current data provide evidence that MAHRP1 is directed into the ER and, somehow, transferred from the parasite plasma membrane to the PV/TVN membrane. Here, MAHRP1 appears to coalesce into small foci that may represent nascent Maurer's clefts. The Maurer's clefts appear to bud from the PV/TVN membrane before docking at sites near the RBC periphery, although it remains possible that small vesicle-mediated trafficking is also involved. The insertion of PfEMP1 may take place at either nascent or peripheral Maurer's clefts. Electrostatic interactions may facilitate the docking and sorting functions within this compartment.

In summary, we have provided the first insights into the formation of Maurer's clefts and determined regions of MAHRP that are needed for trafficking to these important subcellular structures. Strategies that inhibit the formation of the Maurer's clefts or the insertion of proteins into this compartment could provide a means of interfering with the display of virulence epitopes at the surface of infected RBCs.

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Chapter 4

The Maurer's clefts protein MAHRP1 is essential for trafficking of PfEMP1 to the surface of *Plasmodium falciparum* infected erythrocytes

Cornelia Spycher[1*], Melanie Rug[2*], Alan F. Cowman[2], Leann Tilley[3], Hans-Peter Beck[1§]

Affiliation of authors:

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 57, CH 4002 Basel, Switzerland.
[2]The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Melbourne 3050.
[3]Department of Biochemistry and ARC Centre of Excellence for Coherent X-Ray Science, La Trobe University, Melbourne 3086,3 Victoria, Australia.

*These authors contributed equally to this work.

Manuscript to be submitted.

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Cornelia Spycher[1*], Melanie Rug[2*], Alan F. Cowman[2], Leann Tilley[3], Hans-Peter Beck[1§]

[1]Department of Medical Parasitology and Infection Biology, Swiss Tropical Institute, Socinstrasse 57, CH 4002 Basel, Switzerland.
[2]The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Melbourne 3050.
[3]Department of Biochemistry and ARC Centre of Excellence for Coherent X-Ray Science, La Trobe University, Melbourne 3086,3 Victoria, Australia.

*These authors contributed equally to this work.

[§]Corresponding author: Hans-Peter Beck, Swiss Tropical Institute, Socinstrasse 57, 4051 Basel, Switzerland. Telefone: +41 61 284 8116, Fax: +41 61 284 8101. Email: hans-peter.beck@unibas.ch

ABSTRACT

During the intraerythrocytic development of *Plasmodium falciparum*, the parasite modifies the host cell surface by exporting proteins that interact with or insert into the erythrocyte membrane. These proteins include the principal mediator of cytoadherence, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). To implement these changes, the parasite establishes a protein trafficking system beyond its own confines. Membrane-bound structures called Maurer's clefts (MC) are an intermediate trafficking compartment for proteins that are destined for the host cell membrane. We have used transfection technology to disrupt the gene for the MC protein membrane-associated histidine-rich protein 1 (MAHRP1). MAHRP1 is not essential for parasite viability or Maurer's cleft formation. In the absence of MAHRP1, PfEMP1 accumulates in the parasite and is no longer detectable at the Maurer's clefts or on the red blood cell surface. Maurer's cleft resident proteins, such as skeleton-binding protein-1 and ring exported protein and transit cargo such as the knob-associated histidine-rich protein are exported normally. In an effort to understand how MAHRP1 is trafficked to the Maurer's clefts, we have dissected the N-terminal region and identified a segment of 18 amino acids, containing a motif with some similarity to a protein export element, which occurs to be necessary for export.

INTRODUCTION

The apicomplexan parasite *Plasmodium falciparum* causes the most severe form of human malaria. The blood stages are responsible for most of the clinical symptoms of the disease and the associated mortality. While most intracellular pathogens interact with an active host cell, the malaria parasite develops within a cell that is devoid of all organelles and protein trafficking machinery. The parasite resides in a parasitophorous vacuole (PV) encircled by a limiting membrane. The parasite modifies its host cell by establishing membranous structures in the red blood cell (RBC) cytoplasm. These comprise a tubovesicular network (TVN) extending from of the PV membrane (Behari and Haldar, 1994; Elmendorf and Haldar, 1994) and disc-shaped structures called Maurer's clefts (MC) at the RBC periphery (Atkinson et al., 1988; Elford et al., 1997; Lanzer et al., 2006). In addition the parasite modifies the surface of the infected RBC with exported proteins that contribute to the virulence of *P. falciparum* (Kyes et al., 2001). A key protein in this process is the variant surface antigen, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which mediates adherence of infected RBCs to the endothelial cells of the host. PfEMP1 is presented at knob-like structures on the RBC surface. Its C-terminal domain faces the host cell cytoplasm and interacts with the knob-associated histidine rich protein (KAHRP, Pologe et al., 1987).

As mature RBCs lack cellular trafficking machinery, the parasite establishes its own secretory system for exporting proteins through the PV membrane and across the host cell cytoplasm. The mechanisms and molecular apparatus involved in this process are not completely understood. A classical signal sequence is sufficient to direct proteins into the parasite's endoplasmic reticulum (ER) with default release into the PV (Waller et al., 1998; Adisa et al., 2003). Onward transport across the PV membrane requires an additional signature that was recently identified and termed the *Plasmodium* export element (PEXEL, Marti et al., 2004) or host targeting signal (HTS, Hiller et al., 2004).

A number of exported soluble proteins, such as KAHRP and the *P. falciparum* erythrocyte membrane protein 3 (PfEMP3), transiently associate with the cytosolic surface of the Maurer's clefts before redistribution to their final destinations at the RBC membrane (Wickham et al., 2001; Knuepfer et al., 2005b). The integral membrane protein, PfEMP1, also accumulates in Maurer's clefts before transfer to the RBC membrane (Kriek et al., 2003;

Knuepfer et al., 2005a). These data suggests that the Maurer's clefts perform a role as an intermediate compartment for proteins *en route* to the RBC membrane.

The Maurer's clefts have a number of resident integral membrane proteins (reviewed in Lanzer et al., 2006). These include the membrane-associated histidine-rich protein-1 (MAHRP1, Spycher et al., 2006), skeleton binding protein-1 (SBP1, Blisnick et al., 2000a; Cooke et al., 2006; Maier et al., 2006), the Maurer's cleft 2 transmembrane proteins (PfMC-2TM, Sam-Yellowe et al., 2004), the STEVORs (Kaviratne et al., 2002; Przyborski et al., 2005) and the ring exported proteins REX1 and REX2 (Hawthorne et al., 2004; Spielmann et al., 2006). Knock-out of SBP1 prevents export of PfEMP1 to the surface of infected RBCs revealing the potential importance of the Maurer's cleft-resident proteins (Cooke et al., 2006; Maier et al., 2006).

The STEVOR, Rifin and the PfMC-2TM families have a hydrophobic ER entry sequence and a HTS/PEXEL motif (Hiller et al., 2004; Marti et al., 2004; Khattab and Klinkert, 2006) and presumably transit through the PV membrane by interacting with the same transporter that passages soluble cargo. The transmembrane domain of STEVOR-GFP is also needed for final sorting to the Maurer's clefts (Przyborski et al., 2005).

We have previously described the Maurer's clefts resident protein MAHRP1, which is exclusively transcribed in ring-stages (Spycher et al., 2003; Spycher et al., 2006). It is a transmembrane protein with a histidine-rich C-terminal domain facing the RBC cytoplasm, that may be involved in detoxification of haemoglobin break down products (Spycher et al., 2003). MAHRP1 contains neither a classical signal sequence nor a PEXEL motif. Analysis of a series of green fluorescent protein (GFP)- fusions of MAHRP1 fragments showed that the transmembrane domain (TM) directs the protein into the endoplasmatic reticulum (ER), while additional as yet unidentified motifs within the N-terminal region are required for translocation of the protein to the Maurer's clefts (Spycher et al., 2006).

To understand the function of MAHRP1, we have generated deletion mutants of the *mahrp1* gene in *Plasmodium falciparum* (3D7 strain). Maurer's clefts are still formed in the absence of MAHRP1 but the export of PfEMP1 is interrupted at the plasma membrane/PV membrane interface. As a consequence, no PfEMP1 is detected on the surface of the infected RBC. By contrast, other exported proteins such as KAHRP and PfEMP3 are still correctly trafficked.

These data suggest that MAHRP1 plays an essential role in the export of PfEMP1 from the parasite to the Maurer's clefts. In an effort to understand how MAHRP1 is trafficked to the Maurer's clefts, we have dissected the N-terminal region and found a region that harbours a motif with some similarity to the PEXEL/HTS motif.

MATERIAL AND METHODS

Parasites

P. falciparum parasites (3D7 strain) were cultured *in vitro* (Trager and Jenson, 1978) in RPMI medium supplemented with 0.5% Albumax (Invitrogen) or 4% human serum plus 0.25% Albumax. Growth synchronization at the ring stage was achieved by sorbitol lysis (Lambros and Vanderberg, 1979).

Plasmid constructs

To disrupt the *mahrp1* gene in 3D7 parasites, the untranslated regions (UTR) flanking the *mahrp1* gene were cloned as potential recombination sites into the *P. falciparum* transfection plasmid pHHT-TK (Duraisingh et al., 2002) to get pTK_M1. The 5'UTR of *mahrp1* was PCR amplified using primers, 5-atatgtatttttttactttgtgaag-3 and 5-aacataaaaagaaaaaaaaatattccacg-3, and cloned into pHHT-TK upstream of the human dihydrofolate reductase coding sequence (*hdhfr*) using Sac2 and Hpa1 restriction sites. The 3'UTR region of mahrp1 was PCR amplified using the primers, 5-aggagaaccatgtgaattccaaaaagc and 5-gccaaacacttcatgaggagataatg-3, and cloned into pHHT-TK downstream of *hdhfr* using EcoR1 and Sfo1 restriction sites.

To generate MAHRP1-GFP chimeras, the sequence encoding MAHRP1 amino acids 74-169 or 92-169 was PCR amplified and cloned into pARLmGFPmT (Marti et al., 2004; Spycher et al., 2006) upstream of GFP using Afl2 and Apa1 restriction sites to yield plasmids pARLM74_169GFP and pARLM92_169GFP. PCR primers used for the 74-169 amino acid region were 5-gcccttaagatgcaatttaaggcttctctttctg-3 and 5-cgagggccccatatattctggatcaaaaaatgg-3. Primers used for the 92-169 amino acid sequence were 5-gcccttaagatgcaatttaaggcttctctttctg-3 and 5-cgagggccccatatattctggatcaaaaaatgg-3.

P. falciparum transfection

P. falciparum infected red blood cells (ring stage) were transfected by electroporation with 100 μg of plasmid DNA. Transfected parasites were cultured in the presence of 10 nM WR99210, a plasmodial DHFR inhibitor, for 20-30 days until viable parasites were observed in Giemsa-stained smears (Crabb and Cowman, 1996; Fidock and Wellems, 1997). For the establishment of a MAHRP1-deficient strain, parasites were further selected as described (Duraisingh et al., 2002).

DNA extraction and Southern blotting

Genomic DNA was prepared by phenol/chloroform extraction of saponin-lysed, proteinase K-treated parasites as described previously (Beck, 2002). Precipitated genomic DNA was resuspended in TE buffer. Genomic DNA (from 3D7 and 3D7ΔM1 strains) and the pTK-M1 plasmid were digested with Mlu1 and TspR1, separated on a 0.7% agarose gel and transferred to a nylon membrane. Southern blot hybridization was performed using standard procedures and hybridization detected by ECL (Amersham). The probe, corresponding to the 3'UTR of *mahrp1,* was PCR amplified from pTK_M1 using the primers 5-aggagaaccatgtgaattccaaaaagc and 5-gccaaacacttcatgaggagataatg-3.

Trypsin cleavage assay

For trypsin cleavage parasites were synchronized by sorbitol, grown to trophozoite stage, harvested by magnet cell sorting (CS columns; Mitenyi Biotec), and treated with either TPCK-treated trypsin (Sigma) (1 mg/ml in PBS) or incubated in PBS for 1 h at 37°C. After incubation, soy bean trypsin inhibitor (Sigma)(10mg/ml in PBS) was added followed by incubation at room temperature for 15 min. Cell pellets were extracted with Triton X-100 (1%) and subsequently with sodiumdodecylsulfate (SDS, 2%) as described (Baruch et al., 1996).

Western Blotting

Cultures were harvested by magnet cell sorting, washed three times in PBS and taken up in SDS sample buffer (Invitrogen). Proteins were separated on a 12.5% acrylamide gel and blotting to nitrocellulose was performed according to standard protocols. Antibodies used were mouse anti-GFP (1:2000) and rabbit anti-ATS preabsorbed on erythrocyte ghosts (1:1000).

Immunofluorescence analyses and microscopy

Thin smears of infected RBCs on glass slides were fixed with acetone: methanol for 10 minutes at -20°C and optionally permeabilized with 0.1% Triton X-100 for 10-30 minutes followed by a washing step. Cells were incubated for 1 hour at room temperature with mouse anti-MAHRP1-1c (1: 200), mouse anti-SBP1 (1:200), rabbit anti-REX1 (1:500), mouse anti-KAHRP (1:100) or rabbit anti-MAHRP2 (1:250). Slides were washed twice and subsequently incubated with goat anti-mouse FITC (1:500, Kierkegaard KPL), goat anti-mouse cy3 (1:500, Jackson Immunological), goat anti-rabbit cy3 (1:500, Jackson Immunological), all in the presence of Hoechst 33256 (1 μg/ml).

RESULTS

Disruption of the *MAHRP1* locus

MAHRP1 (plasmoDB MAL13P1.413) is a transmembrane protein (see supplementary data S1A) that is expressed during ring stages and exported beyond the parasite's confines into parasite-derived structures known as Maurer's clefts. To determine the function of MAHRP1 and to better understand the role of Maurer's clefts in protein export, we disrupted the expression of endogenous *mahrp1* in *P. falciparum* 3D7 (Supplementary data S1B). We generated the plasmid pTK_M1 containing the *hdhfr* cassette flanked by *mahrp1* 5' and 3' UTRs. Integration of the drug resistance cassette into the *mahrp1* locus is expected to disrupt the coding region.

Southern blot analysis of genomic DNA of both wild type (Pf3D7) and transfected parasites (Pf3D7ΔM1) with a labelled 3'UTR PCR product revealed single crossover recombination into the *mahrp1* locus at the 5'UTR as shown in Figure 1. Western blot analysis using a polyclonal mouse serum revealed a band at approximately 29 kDa (corresponding to the correct size of MAHRP1) in the parent line (Figure 2, left hand lane). However MAHRP1 was not be detected in Pf3D7ΔM1 transfectants (Figure 2, right hand lane), showing that disruption of the *mahrp1* locus ablated MAHRP1 expression.

Maurer's clefts are formed in the absence of MAHRP1

To confirm the absence of MAHRP1 at the cellular level, we performed immunofluorescence microscopy on acetone-methanol fixed infected RBCs as shown in Figure 3A. When the parent line was probed with mouse anti-MAHRP1 serum, a punctuated pattern was observed in the RBC cytoplasm consistent with Maurer's cleft labelling. No labelling of Maurer's clefts was observed in Pf3D7ΔM1, confirming the knockdown of the MAHRP1 protein (Figure 3A, lower row).

To determine whether the formation or composition of the Maurer's clefts was altered in the absence of MAHRP1, we looked at three different resident Maurer's clefts proteins. Antibodies against SBP1 (Blisnick et al., 2000b) (Figure 3B) label punctuate structure in both the parent line and the Pf3D7ΔM transfectants showing that both strains still express and export SBP1 to the Maurer's clefts. Similarly, anti-REX 1 (Hawthorne et al., 2004) and antiserum against another resident Maurer's clefts protein, termed MAHRP2 (PF13_0276, unpublished data) label Maurer's clefts in both parasite lines (Figure 3 C,D). These data show that the absence of MAHRP1 does not impair export of other selected Maurer's clefts resident proteins and that MAHRP1 is not essential for the formation of Maurer's clefts.

In the absence of MAHRP1 the export of PfEMP1 is arrested at the PV or PV membrane

PfEMP1 is trafficked via the Maurer's clefts to the red blood cell membrane where it is inserted, with its variable N-terminal domain exposed at the surface and able to interact with host cell ligands. *En route* PfEMP1 is trafficked through the ER, the plasma membrane, the PV membrane and the Maurer's clefts, however details of mode of trafficking are currently debated (Knuepfer et al., 2005a; Marti et al., 2005; Lanzer et al., 2006). To assess whether MAHRP1 plays a role in the export of PfEMP1 we compared the immunofluorescence pattern obtained using anti-PfEMP1 for the parent line and the MAHRP1 knock-out transfectants (Figure 4A). Wild type parasites showed characteristic Maurer's clefts staining, however PfEMP1 was not exported beyond the PV or PV membrane in Pf3D7ΔM1-infected RBCs. This is strong evidence for an important function of MAHRP1 in the trafficking of PfEMP1 beyond the parasite's border.

We also examined other proteins that transiently associate with Maurer's clefts, such as KAHRP and PfEMP3. KAHRP is present in a rim like fluorescence at the red blood cell

membrane in both wild type and Pf3D7ΔM1 parasites (Figure 4B), consistent with trafficking to knob structures under the red blood cell membrane (Pologe et al., 1987). PfEMP3 shows a heterogeneous distribution at the host cell membrane (Figure 4C) in both lines. This is consistent with previous reports (Pasloske et al., 1993) and shows that this protein is also correctly exported to the host cell cytoskeleton. Thus, both KAHRP and PfEMP3 are exported independently of MAHRP1.

We have used trypsin digestion of intact infected RBCs to confirm the effect of MAHRP1 deletion on PfEMP1 trafficking. In wild type parasites a portion of the PfEMP1 population is transported to the RBC surface and can be cleaved with trypsin (Kriek et al., 2003; Maier et al., 2006). Western analysis of the trypsin-treated parent line with an antibody recognising the conserved C-terminal domain revealed two cleavage products (Figure 5, right hand lane) that are not present in untreated cells. By contrast these bands were not present in uninfected RBCs (Figure 5, left hand lane) or in RBCs infected with the MAHRP1 knock-out line (Figure 5, middle lane). The dramatic effect of MAHRP1 deletion on the export of PfEMP1 implies that MAHRP1 plays an important role in the translocation of PfEMP1 beyond the PV membrane.

MAHRP1 export sequence

MAHRP1 has no hydrophobic ER entry signal sequence and no motif that meets the criteria specified for a PEXEL/HTS sequence (Marti et al., 2005; Hiller et al., 2004; Sargeant et al., 2006). We previously generated GFP chimeras of a series of MAHRP1 fragments and used them to follow trafficking of this protein to the Maurer's clefts (Spycher et al., 2006). A construct comprising full length MAHRP1 fused to GFP is successfully trafficked to the Maurer's clefts. We showed that information in the second half of the N-terminal domain is needed for correct trafficking to the Maurer's clefts (Spycher et al., 2006).

Here we have further dissected the N-terminal domain of MAHRP1 by generating transfectants expressing amino acids 74-169 and 92-169 of MAHRP1 fused to GFP. These transgenes are expressed in the presence of endogenous full-length MAHRP1. Transfected *P. falciparum* were analysed for presence of the GFP-fusion proteins by probing saponin pellets with anti-GFP. Chimeric proteins of 39 and 37 kDa, respectively were detected (Figure 6A). Fluorescence microscopy of live cells was used to examine the locations of the MAHRP1 chimeras at the young trophozoite stage and to compare these transfectants with parasites

expressing full length MAHRP1-GFP (Figure 7B. top row). Full length MAHRP1-GFP is efficiently trafficked to the Maurer's clefts, however a small amount of the fusion protein is dotted around the parasitophorous vacuole (Figure 6B, top row). MAHRP1$_{74\text{-}169}$-GFP is partly exported to the Maurer's clefts (Figure 6B, second row), however the export process appeared to be less efficient than for the full-length construct. In this transfectant, a build-up of the fluorescent chimera in what appears to be the ER and structures associated with the PV membrane was frequently observed. Interestingly removal of a further 18 amino acids from the N-terminal domain (MAHRP1$_{92\text{-}169}$-GFP) resulted in trapping of the fusion protein with the parasite confines (Figure 6B, third row). It is excluded from the nucleus (as judged by dual labelling with DAPI, data not shown) and the digestive vacuole, which is consistent with an ER location. This suggests that amino acids within the residues 74-92 of MAHRP1 contain the information for transfer of MAHRP1 from the endomembrane system to the PV and on to the Maurer's clefts. Analyses of the amino acid sequence in this region revealed a pentameric sequence motif RxWxE which shows some similarity to the PEXEL/HTS motif R/KxLxE/D.

DISCUSSION

P. falciparum replicates asexually in terminally differentiated RBCs that are devoid of any organelles. The parasite resides within a parasitophorous vacuole, feeding on haemoglobin, and subverting the physiology of the RBC by remodelling the host compartment. To do this the parasite establishes an export system for proteins that are involved in nutrient uptake or in mediating cytoadherence and antigenic variation.

A number of proteins destined for the RBC membrane, such as PfEMP1, PfEMP3 and KAHRP, are trafficked via the Maurer's clefts, however the precise role of these organelles as an intermediate in the trafficking process is not yet completely understood. MAHRP1 is a Maurer's cleft resident protein and is transcribed exclusively during early parasite stages although the protein persists throughout the asexual lifecycle (Spycher et al., 2003). To test the functional importance of MAHRP1 we generated a *P. falciparum* 3D7 strain which is deficient in this protein (Pf3D7ΔM1). We show that the endogenous locus has been disrupted by a 5' single crossover integration of the plasmid (Figure 1). This leads to alteration of the endogenous promoter and to an ablation of the protein as judged by Western blot analysis (Figure 2).

We tested whether the absence of MAHRP1 had an effect on the formation of Maurer's clefts or on the presence of other Maurer's cleft resident proteins. Immunofluorescence assays showed that SBP1, MAHRP2 and REX1 were still present at the Maurer's clefts. The data indicate that the Maurer's clefts can be assembled in the absence of MAHRP1.

It is important to note that the four Maurer's cleft resident proteins, MAHRP1, MAHRP2, REX1 and SBP1 have several features in common. They are all expressed very early in the intraerythrocytic cycle and none of them has a homolog in other plasmodial species or in other organisms. They each possess a single predicted transmembrane domain and no predicted N-terminal ER entry signal sequence. It is interesting to note that while the C-terminal domains of MAHRP1 and SBP1 (which have been shown to face the cytoplasm) are quite basic, the luminal domains are more acidic. A similar dichotomy is apparent between the N- and C-terminal domains of REX1. By contrast the cytosolic domain of PfEMP1 is highly acidic. Finally none of these Maurer's cleft resident proteins has a recognisable PEXEL/HTS motif suggesting that they are trafficked across the PV membrane via a different route to proteins such as KAHRP and PfEMP1. Indeed analysis of MAHRP1-GFP transfectants suggest that this protein is inserted into nascent Maurer's clefts as they form (Spycher et al., 2006).

We used immunofluorescence assays to determine whether parasite proteins are still exported in the absence of MAHRP1. We found that while the trafficking of proteins that are exported to the RBC cytoskeleton, namely KAHRP and PfEMP3, was not affected, PfEMP1 export was prevented. In the parent line, PfEMP1 is present in the Maurer's clefts from the early ring stage. However, in the MAHRP1 deficient strain, PfEMP1 was not exported beyond the PV membrane. Proteolysis of surface-exposed PfEMP1 confirmed the loss of export of this protein in Pf3D7ΔM1 infected RBCs. These data support the suggestion that different modes of transport are employed for soluble cargo and integral membrane proteins.

It is possible that MAHRP1 acts as chaperone for PfEMP1. RAP1 has been shown to act as an escort for the rhoptry protein, RAP2 (Baldi et al., 2000). Alternatively, MAHRP1 may be responsible for correct insertion of PfEMP1 into the Maurer's clefts, for example by recognition of the PEXEL/HTS signal motif. Recently, deletion mutants of another Maurer's cleft resident protein, SBP1, have been generated in two separate parasite strains. In both

cases the Maurer's clefts were still formed however the two strains displayed different phenotypes with respect to PfEMP1 trafficking. When SBP1 was knocked out in the 3D7 strain (Cooke et al., 2006) PfEMP1 was still exported to the Maurer's clefts but was not transferred to the infected RBC surface. The ability of infected RBCs to adhere to endothelial cell receptors was reduced in these knock-out parasites but was restored by complementation with SBP1. By contrast, when SBP1 was knocked out in the CS2 strain (Maier et al., 2006) PfEMP1 trafficking was arrested at the PV membrane (Maier et al., 2006). This is similar to the phenotype that we report here with the MAHRP1 knockout parasites.

Taken together these studies indicate that resident Maurer's cleft proteins play a crucial role in the export of PfEMP1 to the membrane of the infected RBC. They may perform important structural roles or they may interact with cargo *en route* to the RBC membrane. It is still a matter of some debate how cargo proteins, such as KAHRP and PfEMP1, are trafficked to the Maurer's clefts and then transferred onward to the RBC membrane. Based on 3D reconstructions of EM sections, Wickert and colleagues postulated that Maurer's clefts and the PV membrane form a continuous network (Wickert et al., 2004). By contrast, we have reported that nascent Maurer's clefts are formed as sub-compartments of the PV membrane but that peripheral Maurer's clefts are independent structures that have lost their connection to the PV membrane (Spycher et al., 2006).

These conflicting views of the architecture of the Maurer's clefts lead to different, though not necessarily exclusive, models for trafficking of proteins to the Maurer's clefts. If the Maurer's clefts remain connected to the PV membrane, soluble proteins could diffuse from the PV lumen along connecting strands to the Maurer's cleft lumen and then translocate through protein transporters into the RBC cytoplasm. Integral membrane proteins, such as PfEMP1, could transfer by lateral diffusion along the connected membrane network from the PV membrane to the Maurer's clefts. If on the other hand the Maurer's clefts are independent structures, soluble proteins could reach the RBC cytoplasm by transiting through exporters located in the PV membrane. These proteins would then diffuse across the RBC cytoplasm before interacting with the cytoplasmic surface of the Maurer's clefts and eventually the RBC membrane. PfEMP1 could incorporate into nascent Maurer's clefts before they bud from the PV membrane. Alternatively, PfEMP1 could be trafficked across the RBC cytoplasm in vesicles or as protein complexes and assemble at the peripheral Maurer's clefts (for reviews see Cooke et al., 2004; Marti et al., 2005; Lanzer et al., 2006). While additional work is

needed to distinguish between these different possibilities, it is clear that MAHRP1 and SBP1 could play roles as accessory proteins at various steps in these processes.

The signals that direct MAHRP1 and SBP1 to the Maurer's clefts are also intriguing. Neither of these proteins has an N-terminal signal sequence nor a PEXEL/HTS motif (as defined by the ExportPred algorithms, Hiller et al., 2004; Marti et al., 2005; Sargeant et al., 2006). We previously showed that the transmembrane domain of MAHRP1 is required for entry into the ER and that information in the second half of the N-terminal domain is needed for correct trafficking to the Maurer's clefts (Spycher et al., 2006). In an effort to further dissect this signal we generated transfectants expressing GFP fused to MAHRP1 fragment commencing at either residue 74 or residue 92. MAHRP1$_{74\text{-}169}$-GFP was still trafficked to the Maurer's clefts albeit somewhat less efficiently than the full length MAHRP1-GFP chimera. This shows that the first 73 amino acids are not essential for export to the Maurer's clefts. By contrast, MAHRP1$_{92\text{-}169}$-GFP was trapped within the ER. Therefore, residues 74-91 must contain a region that allows trafficking of MAHRP1 to the Maurer's clefts. Intriguingly a pentameric sequence, RxWxE, commencing at residue 87, shows some similarity to the PEXEL motif, RxLxE. A large hydrophobic tryptophan replaces the highly conserved smaller leucine residue, however the PEXEL recognition machinery appears to be relatively promiscuous (Bhattacharjee et al., 2006).

Whether this motif is indeed responsible for MAHRP1 trafficking to the Maurer's clefts is the subject of ongoing research, although it is interesting to note the presence of a related RxFxE motif in the N-terminal domain of SBP1. If these motifs indeed prove to be the PEXEL/HTS equivalents for Maurer's cleft resident proteins, this raises the question: How can the same motif direct the export of soluble proteins through a protein exporter and direct a set of integral membrane proteins into nascent Maurer's clefts?

In conclusion, we have defined a region of 18 amino acids that contains the information for transfer of MAHRP1 into nascent Maurer's clefts, thereby pointing to a common mechanism for export of Maurer's cleft machinery and both soluble and membrane-embedded cargo. We have shown that loss of MAHRP1 prevents transfer of PfEMP1 to the Maurer's clefts indicating that it may also perform a chaperone function. Taken together with other recent reports, the work presented here shows that Maurer's cleft resident proteins play a very important role in trafficking of PfEMP1 to the RBC surface. An increased understanding of

factors that interfere with the formation or function of this compartment may lead to the development of novel antimalarial strategies.

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Figure Legends

Figure 1. Southern blot analysis of the *mahrp1* locus in parent 3D7 and transgenic Pf3D7△M1 lines. Genomic DNA and the transfection plasmid pTK_M1 were digested with TspR1 and Mlu1 restriction enzymes and probed with a labelled 3' UTR PCR product. The predicted sizes are 5137 bp (plasmid pTK_M1), 3792 bp (wild type 3D7), 5333 bp and 3590 bp (5' single crossover integrant Pf3D7△M1). The schematic representation depicts the disruption of the endogenous promoter by a 5' single crossover event.

Figure 2. Western blot analysis of 3D7 and Pf3D7△M1 infected red blood cells. Saponin insoluble material from the wild type and Pf3D7△M1 parasites were subjected to SDS-PAGE, transferred to nitrocellulose and probed with mouse anti-MAHRP1 antiserum.

Figure 3. Immunofluorescence microscopy of Maurer's cleft resident proteins. Smears of infected RBCs were fixed with acetone methanol and probed with a series of antibodies recognising Maurer's cleft resident proteins. Each row represents a phase (or DIC) image, a DAPI stain of the parasite nuclei, the immunofluorescence signal and an overlay of this image with the transmission image. The top row of each panel is wild type 3D7; the second row is Pf3D7△M1. **A**. Membrane associated histidine-rich protein 1 (MAHRP1) **B**. Skeleton binding protein 1 (SBP1). **C**. Ring-expressed protein 1 (REX1). **D**. Membrane-associated histidine-rich protein 2 (MAHRP2). Scale bar = 5 μm.

Figure 4. Immunofluorescence microscopy of exported proteins. Smears of RBCs infected with wild type 3D7 (top row) and Pf3D7△M1 (bottom row) were probed with antibodies recognising proteins exported to the RBC membrane via the Maurer's clefts. **A**. *P. falciparum* erythrocyte membrane protein (PfEMP1). **B**. knob-associated histidine-rich protein (KAHRP). **C**. *P. falciparum* erythrocyte membrane protein 3 (PfEMP3). Scale bar = 5 μm.

Figure 5. PfEMP1 is not exposed on the surface of Pf3D7△M1 infected red blood cells. Intact erythrocytes infected with parental 3D7, Pf3D7△M1 and uninfected erythrocytes were treated with (+) or without (-) trypsin and extracts were analysed by Western blotting with rabbit anti-PfEMP1 antibody. Surface exposed PfEMP1 is cleaved.

Figure 6. MAHRP1-GFP chimeras revealing the export-mediating sequence of MAHRP1. A. Western analysis *P. falciparum* transfectants expressing MAHRP1$_{74-169}$-GFP or MAHRP1$_{92-169}$-GFP, compared with the parent parasite line. Mouse anti-GFP was used to probe blots of saponin-lysed infected RBCs. Recombinant GFP is shown for comparison. The MAHRP1 amino acid sequence is shown with residues D74 to Q92 underlined, the putative PEXEL-like motif in red, the transmembrane domain in a grey box, and the histidine-rich C-terminal domain indicated in yellow. **B.** Confocal fluorescence microscopy images of *P. falciparum* transfectants GFP linked to different MAHRP1 sequences. Upper panel. Full length MAHRP1-GFP chimera is efficiently exported to the Maurer's clefts. Middle panel. MAHRP1$_{74-169}$-GFP is partly exported to the Maurer's clefts and partly retained within the parasite. Lower panel. MAHRP1$_{92-169}$-GFP is located in a compartment that surrounds the nucleus consistent with the ER. Scale bar 5 μm.

Figure S1. Schematic representation of the endogenous *mahrp1* locus and disruption by single crossover recombination. A. Organisation of the *P. falciparum* 3D7 *mahrp1* gene and MAHRP1 protein. Exon 1 (grey) codes for the first 130 amino acids including the predicted transmembrane domain. Exon 2 codes for the C-terminal domain (aa 131-249) containing the histidine-rich region. **B**. Protein sequence of MAHRP1. The transmembrane domain (residues 105-124) is boxed. The 18 amino acid region containing the export signal is marked with italics. The putative export signal (RxWxE) is in red and underlined. The intron site is denoted by ▲. **C.** Schematic representation of the crossover recombination event that incorporated the transfection plasmid pTK_M1 into the MAHRP1 locus. TK, thymidine kinase; UTR, untranslated region; hDHFR, human dihydrofolate-reductase.

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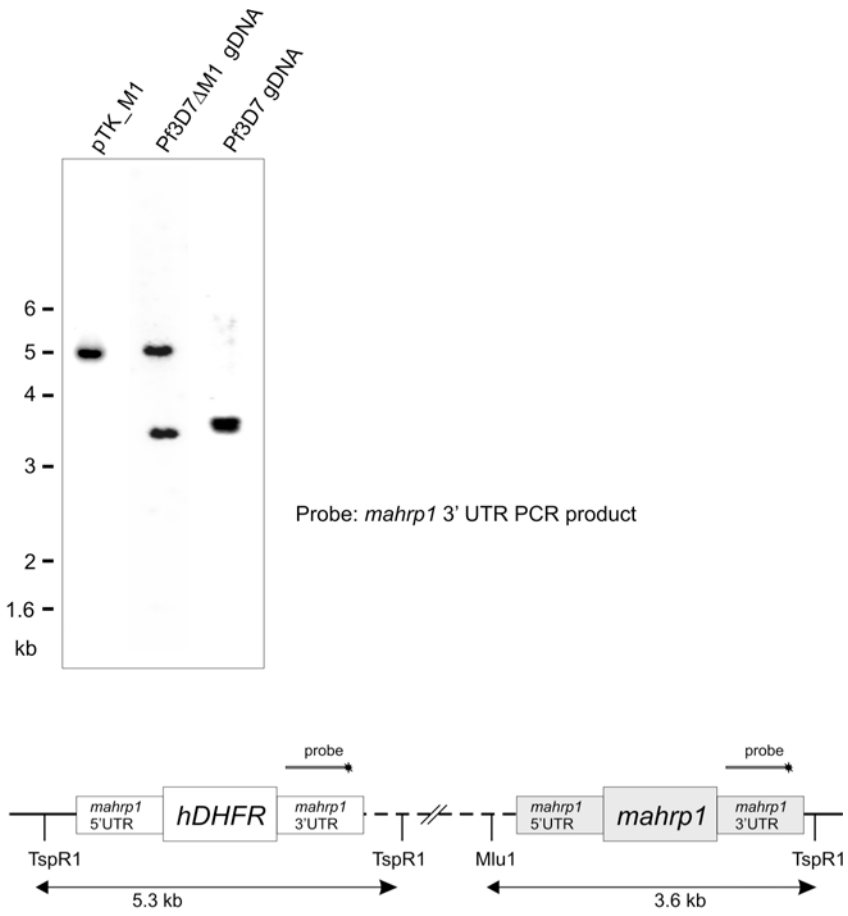
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Figure 1

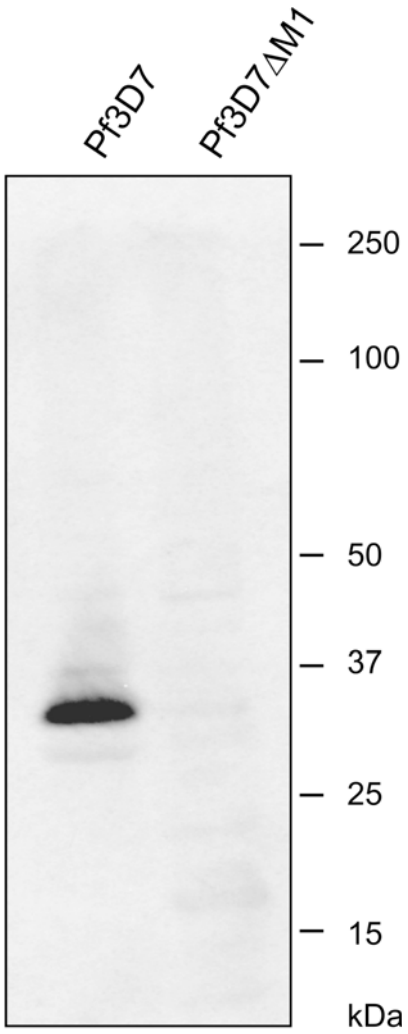
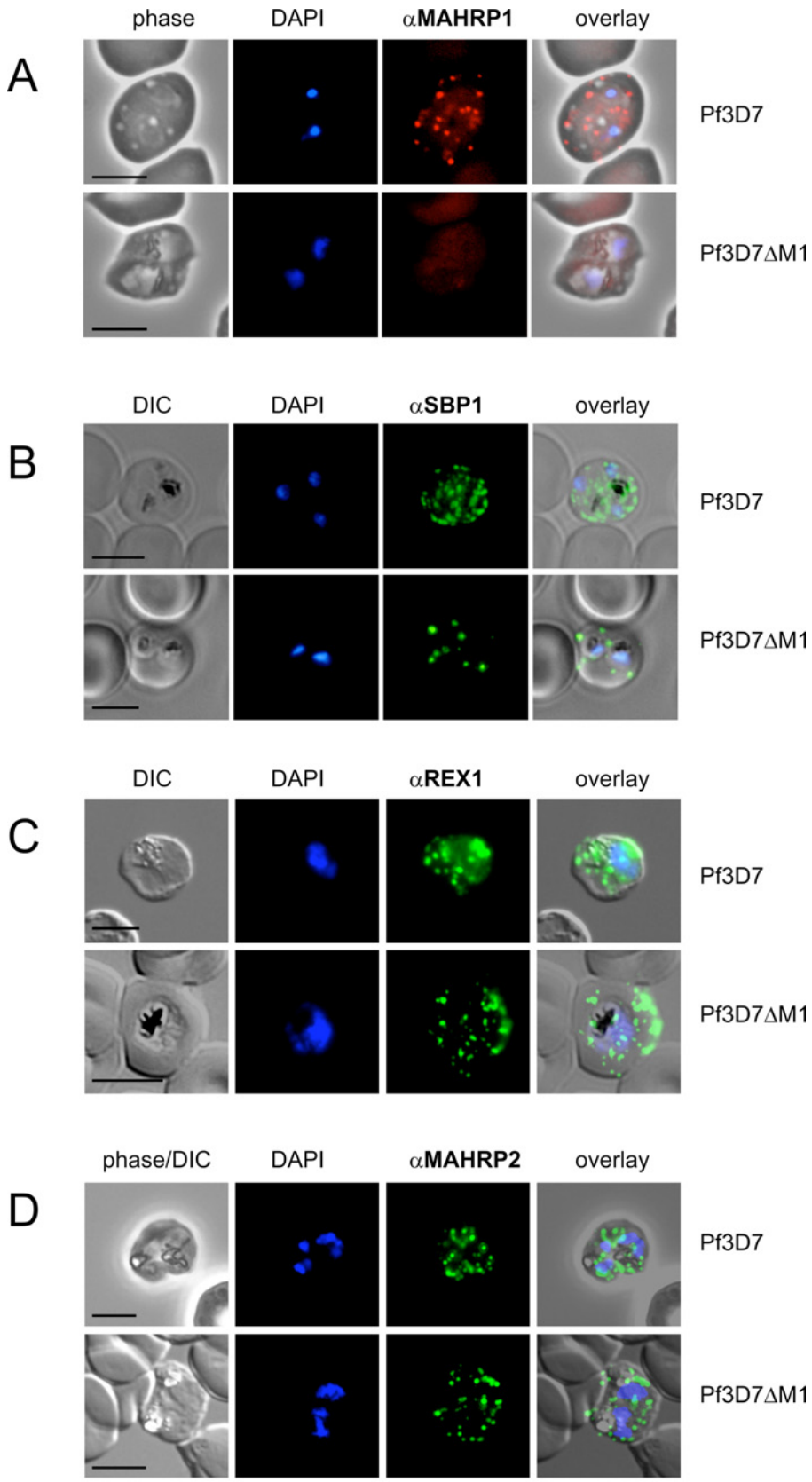


Figure 2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C
DIC
DAPI
αREX1
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Figure 3

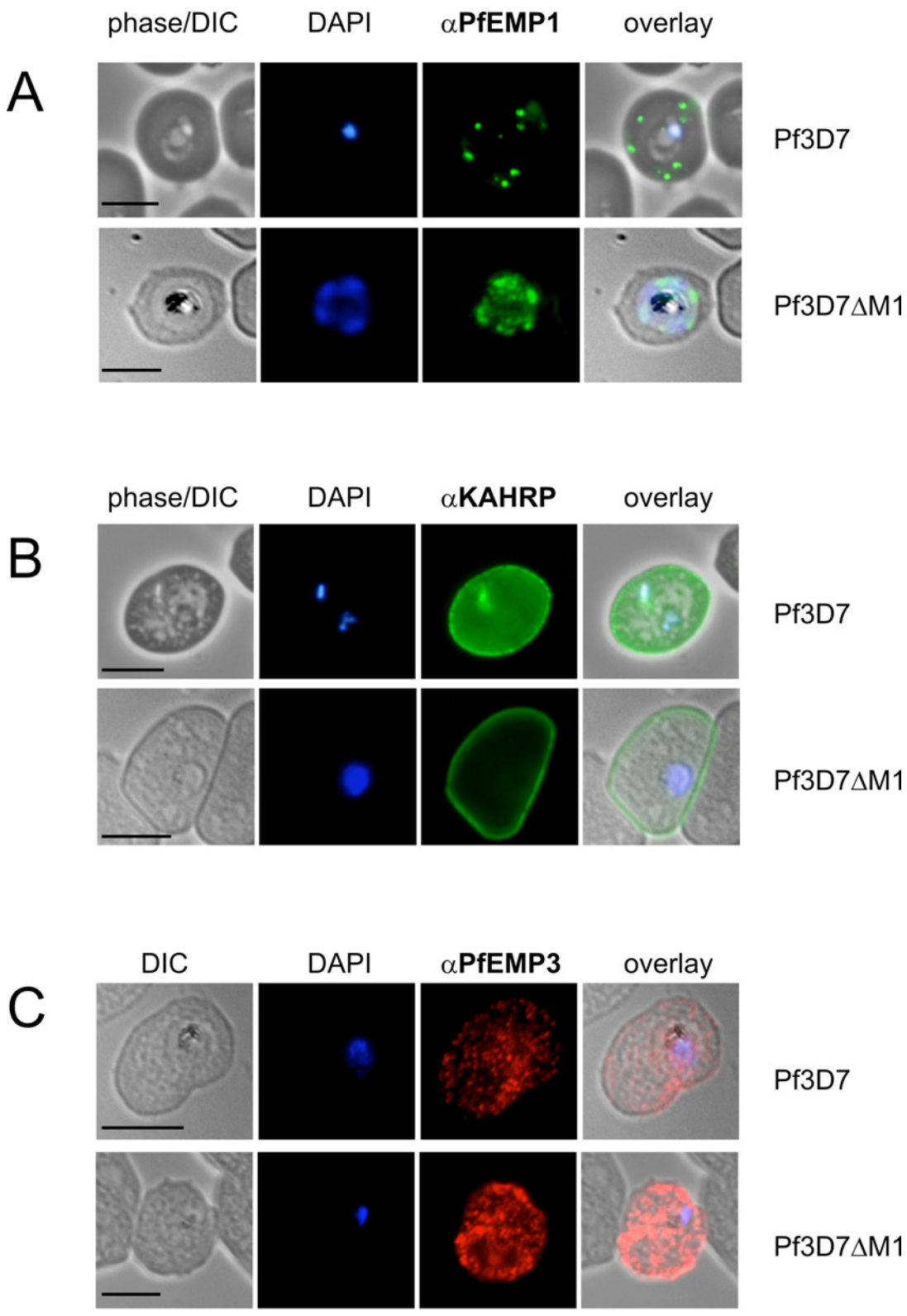
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Figure 4

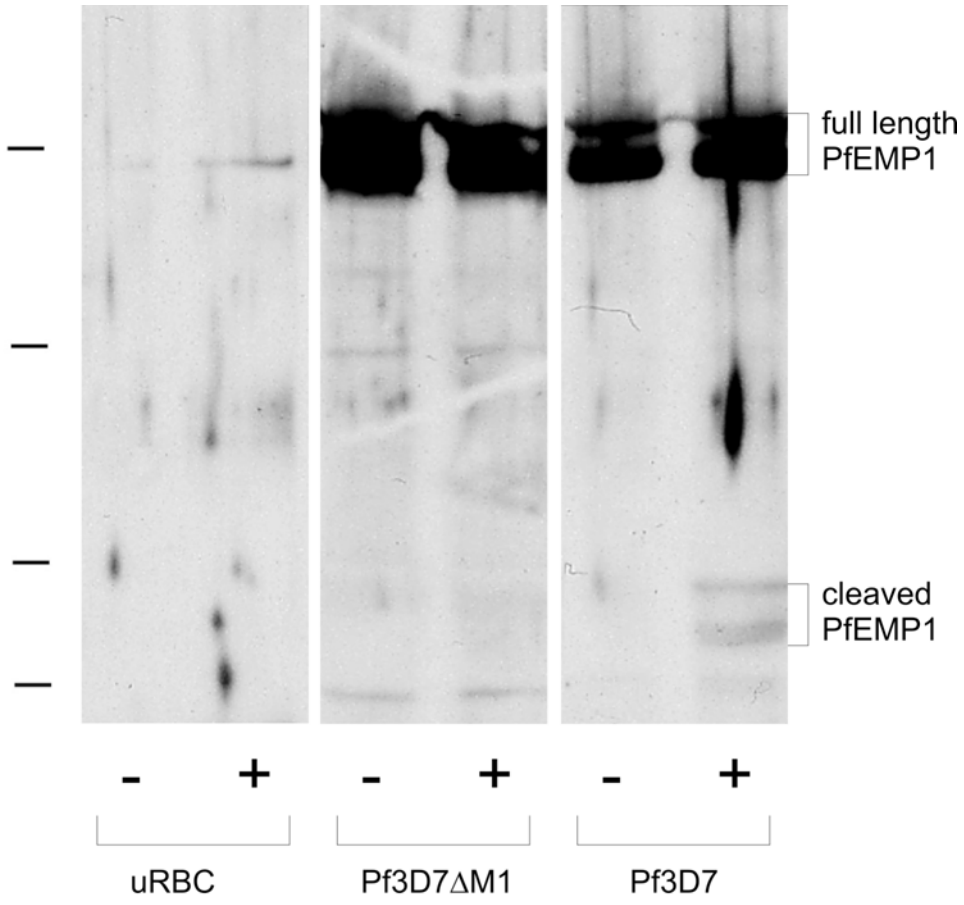

Figure 5

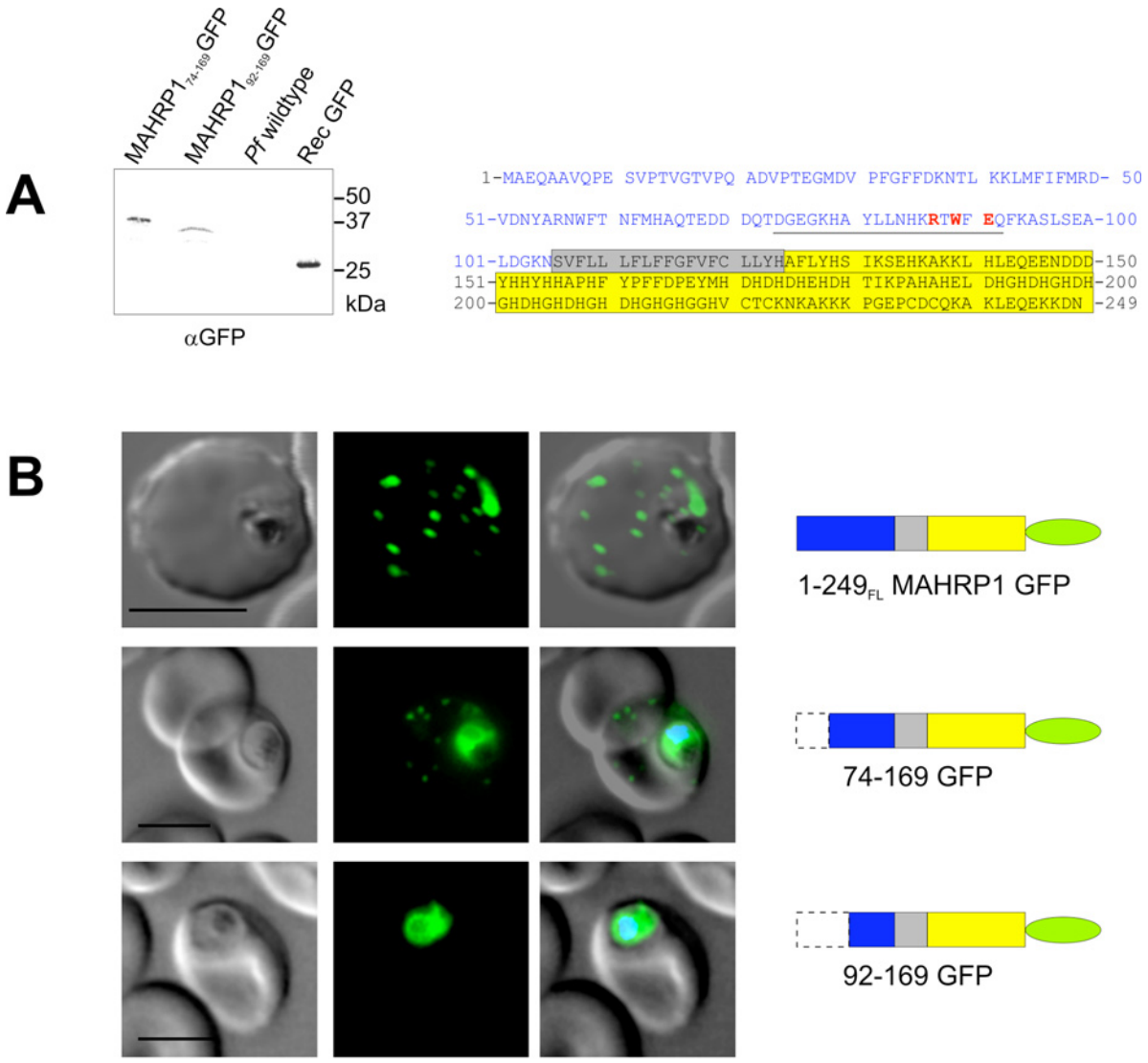
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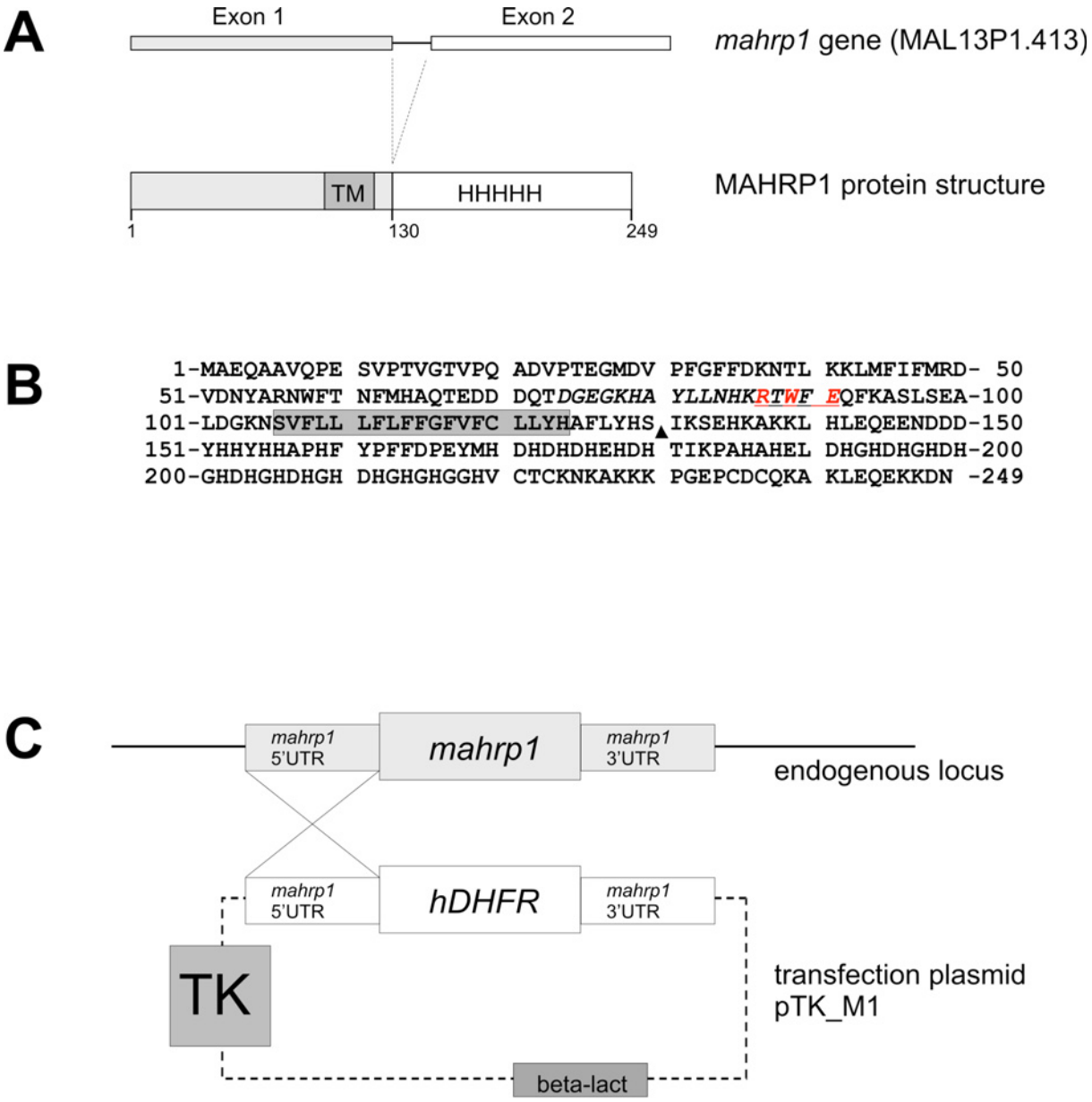
Figure 6



Supp Figure 1

Chapter 5

Discussion

Malaria is one of the top ranking causes of morbidity and death in the world. The causative parasite has a complex life cycle with two different hosts and both intra- and extracellular existence. The blood stages of *Plasmodium falciparum* within the vertebrate host are responsible for disease symptoms and pathologies. By exporting proteins to the host cell surface, it allows the parasite to cytoadhere to the host endothelium and thus avoids clearance by the spleen (Rogerson et al., 1995; Baruch et al., 1997). In addition, it undergoes antigenic variation (Su et al., 1995) and alters its surface-exposed antigens in a manner that the immune system is subrogated. It is therefore important to understand the mechanisms underlying these processes to eventually use the knowledge for innovative interventions. I would like to start this section with a summary of my work and then discuss key findings in the context of specific aspects.

Parasites undergo asexual replication in red blood cells (RBC). The red blood cell represents a unique choice of host cell. It is a terminally differentiated cell devoid of any eukaryotic structures or organelles except for the remaining cell membrane. It has to be assumed that on cellular level the parasite undergoes a parasitic relationship in where the host cell has not much to offer “except” a hiding niche and a high concentration of hemoglobin. In real-estate terms this is comparable with the model that the parasite invades/acquires an empty building devoid of any walls (compartmentalization and organelles) and lacking any functional infrastructure (biosynthetic machinery and trafficking components). During its processes of maturation however, the parasite completely remodels its host cell, and if the real-estate

business was to purchase the original building back prior to its lysis, it would encounter a totally different interior of the building, where a complete process of refurbishment has happened and where the exterior has undergone a total remake.

These remodelling processes within the host cell are essential for the parasite's survival and mark the beginning of our studies. Our work has its origin in a stage specific cDNA library, which was generated by Spielmann and Beck (2000). Their work focused on finding genes, which are exclusively transcribed in ring stages and are then switched off in later stages. The rational behind this work was the hypothesis that genes, which are switched on early upon invasion of the parasite in the RBC are crucial for the successful establishment of the parasite in its host cell. The authors found 13 ring-stage specific genes and only 2 had significant homology to known genes (Spielmann and Beck, 2000). Among the hypothetical genes two histidine-rich proteins were found and termed MAHRP1 and MAHRP2. In our work we have aimed at the characterization of MAHRP1 (Spycher et al., 2003; Spycher et al., 2006).

We investigated the localization of MAHRP1 and found it situated at parasite-induced structures termed Maurer's clefts (MC). These to date still enigmatic vesicle-like structures are exported from the parasite into the host cell cytosol and persist until the lysis of the cell. In accordance with the hypothesis that genes, switched on early upon invasion are products of parasite-induced host modifications, MAHRP1 seems to be part of the early remodelling component called Maurer's clefts (reviewed in Lanzer et al., 2006).

In our work we had set two main objectives being 1) what is the possible function of MAHRP1 and 2) how is it trafficked to the Maurer's clefts. To investigate the role of the abundant histidine-rich repeats, we recombinantly expressed the C-terminus and undertook spectrophotometric analyses to study its interaction with ferriprotoporphyrine IX (FP) *in vitro*. We found a clear correlation between abundance of repeats and the binding stoichiometry to FP. Furthermore we observed an effect on redox properties of FP in its bound state to MAHRP1c (Spycher et al., 2003). The functional question was also tackled by generating a MAHRP1-deficient strain Pf3D7ΔM1. We used this transgenic strain to assess the effect of MAHRP1 on other cellular markers. We found that the major virulence factor PfEMP1 is no more exported beyond the parasite's confines, whereas other selected proteins at both Maurer's clefts and in the knobs where not affected (see chapter 4).

MAHRP1 does not possess any of the known trafficking motifs of *P. falciparum*. It is thus unclear, which part of the protein directs MAHRP1 to the Maurer's clefts. To address the second objective we transfected parasites with truncated, GFP-tagged MAHRP1 fragments. Transfectants were analyzed by fluorescence microscopy. We broke the protein down into its different domains and combinations thereof, and traced the chimera in the infected erythrocytes. We found that the transmembrane domain (TM) is sufficient to guide the protein to the secretory pathway i.e. the endoplasmatic reticulum (ER). Additional amino acids from the C-terminus did not alter its localisation to the ER, whereas amino acids from the N-terminus added to the transmembrane domain did support the export of the protein to the MCs (Spycher et al., 2006). Within the N-terminus there is an region, which together with the TM is sufficient for translocation to the Maurer's clefts. This N-terminal part bears a motif with striking similarity to the PVM translocation motif termed VTS or PEXEL (Hiller et al., 2004; Marti et al., 2004).

Looking at MAHRP1 from the histidine perspective

In the beginning, prior to any evidence on localisation or function of MAHRP1, the most striking feature was its histidine-rich C-terminus containing 6 tandem repeats of the amino acid sequence DHGH, with additional preceding DH repeats. We found length polymorphisms in the histidine-rich domain within different *Plasmodium falciparum* strains and they varied in the number of DHGH repeats (see chapter 2, figure 2). This, together with the fact that the presence of MAHRP1 is required for the export of PfEMP1 to the host cell cytosol, allows to speculate that these repeats serve to interact with PfEMP1 and that increasing repeat numbers would allow interaction with more PfEMP1 molecules. Consequently, strains with more DHGH repeats would export more PfEMP1 and thus cytoadhere better.

According to algorithms generated by The Institute of Environmental Modelling (www.tiem.utk.edu/~gross/bioed/webmodules/aminoacid.htm), the average frequency of histidine in vertebrate proteins is 2.9% whereas MAHRP1 has an overall histidine content of 13.7% and thus is nearly 5 times higher. However, taking into consideration that it is a transmembrane protein, the histidine rich C-terminus is exposed to and faces a different

compartment than the N-terminus, and therefore will exhibit a domain, which has nearly 30% histidines and thus a histidine content ten times higher than average.

The above average frequency of histidines in MAHRP1 can be assumed to be beneficial to the parasite. Histidine-rich proteins have been described in other organisms, too, some of which have been mentioned in more detail in the introduction. They have in common a role as co-ordinating ligand in metalloproteins. In some cases this serves to protect the organisms from deleterious effects, whereas in other cases these cations are essentially needed for enzyme activity.

In *Plasmodium falciparum* several histidine-rich proteins have been described, of which *P. falciparum* histidine-rich protein 2 (HRP2) is one of the best characterized. Two different studies have investigated the role of HRP2 using recombinant protein or synthetic peptides and reproducing possible cellular reactions *in vitro* (Papalexis et al., 2001; Huy et al., 2003). Both studies show that HRP2 can specifically bind heme (FP). FP is released as a result of hemoglobin breakdown within the food vacuole and in the RBC cytosol and is toxic due to its detergent-like and redox properties. Once FP has been polymerized to β-hematin it is no longer toxic. The interaction of FP with HRP2 promotes the formation of β-hematin and it is thought that HRP2 acts as a platform for this polymerization. Hemoglobin degradation is an important amino acid nutrient source for the parasite once it is in the RBC. A red blood cell contains around 350 mg/ml hemoglobin, which equates to a concentration of FP of about 20 mM (Hellerstein et al., 1970). *P. falciparum* degrades at least 75% of the hemoglobin (Loria et al., 1999) and therefore needs to have a mechanisms to reduce subsequent FP levels. HRP2 is a soluble protein found in both red blood cell cytosol and the food vacuole, where most of the β-hematin is stored as "malaria pigment". HRP2 seems to be a rather widely distributed protein, whereas KAHRP, another plasmodial histidine-rich protein appears to have a more defined localisation. It is a soluble protein exported to the cytosolic side of the red blood cell membrane where it is located at the knobs (Culvenor et al., 1987; Pologe et al., 1987) and is essential for knob formation (Crabb et al., 1997). There it acts as part of the "anchoring platform" and has been shown to interact with spectrin and PfEMP1 (Kilejian et al., 1991; Waller et al., 1999). To our knowledge no FP-binding studies have been undertaken with histidine-rich parts of the KAHRP protein sequence. However, it is likely that histidines from KAHRP can also interact with heme and might also have a detoxifiying role. Given its location at the knobs, where the major virulence factor is anchored, such a protective role becomes even more apparent.

We have recombinantly expressed the histidine-rich domain of MAHRP1 (MAHRP1c) and shown that it specifically interacts with FP *in vitro* (Spycher et al., 2003). Furthermore we have analyzed properties of FP in its bound state with MAHRP1c and found it to be 10-fold more susceptible to degradation by H_2O_2 than in its unbound state. This fast degradation in a bound state seems beneficial to the parasite as it reduces innocuous effects of FP. Transmission electron micrographs with antibodies against MAHRP1c revealed MCs decorated with gold particles on their cytoplasmic side. This is in agreement with the prediction of the PSORT algorithm that suggests a type 1b orientation i.e. the histidine-rich domain facing the erythrocyte cytoplasm. Given its location and orientation at the Maurer's clefts and its detoxifying effects on FP, we consider the possibility that MAHRP1 plays a role in protecting Maurer's clefts-associated proteins from deleterious effects of FP. Our *in vitro* experiments however, represent a very limited part of highly complex set of metabolic and chemical reactions taking part *in vivo*. This certainly has to be taken into consideration when interpreting these obtained *in vitro* data.

The detoxifying role of MAHRP1 could be tested by other means. As we have demonstrated, the presence of MAHRP1c significantly enhances the oxidative destruction of FP by H_2O_2. In contrast, chloroquine is an efficient inhibitor of the oxidative destruction of FP by reaction with H_2O_2 (Loria et al., 1999). It would be interesting to compare the IC_{50} of chloroquine in Pf3D7ΔM1 and wildtype Pf3D7 parasites. We hypothesize a higher IC_{50} for the wildtype than for the Pf3D7ΔM1, because of the absence of the degradation enhancing effect of MAHRP1 on FP.

Because these two strains only differ within the *mahrp1* locus, any difference in drug toleration observed could be attributed to the absence of MAHRP1. To confirm that the effect is due to absence of MAHRP1, the Pf3D7ΔM1 could then be complemented with a plasmid carrying *mahrp1* driven by the endogenous promotor, which should reconstitute the wildtype IC_{50}.

If no difference in IC_{50} would be observed, then it would be worthwhile to undertake a transcriptional analysis to test if any other histidine-rich protein, including MAHRP2, is upregulated to compensate for the missing MAHRP1. However, it is also possible that the observed FP binding of MAHRP1 is an insignificant side effect of its yet unknown function.

Shedding light "into" Maurer's clefts

We have transfected Pf3D7 with a MAHRP1 full-length protein fused to GFP (Spycher et al., 2006). This fusion protein was successfully exported to the Maurer's clefts. To our knowledge this is the first transgenic strain expressing an exported full length transmembrane protein fused to GFP. We used fluorescent microscopy of live cells to examine the location of $MAHRP1_{FL}$-GFP at different stages of the intraerythrocytic cycle. We found MAHRP1 in the erythrocyte cytosol in a punctuated pattern consistent with being Maurer's clefts (MC), in young parasites already. As the parasite grows, these dots are less spread out as the host cell's cytosolic space becomes more narrow and the Maurer's clefts move closer to the RBC plasma membrane. Some cells showed accumulation of GFP fluorescence in what appeared to be subcompartments associated with the parasitophorous vacuole (PV) or PV membrane. This "necklace of beads" was not observed in immunofluorescence assays when probing with anti-MAHRP1 and might represent a decelerated export due to over expression of the chimeric protein.

We used fluorescence recovery after photobleaching (FRAP) to investigate if these subcompartments are independent structures. We monitored the fluorescence over minutes and found no diffusion of fluorescence into the bleached area. This finding indicates that these subcompartments are independent entities as there is no (lateral) diffusion of fluorescent molecules back into the bleached area. Our interpretation of these data is that the Maurer's clefts proteins assemble in what appear to be subcompartments in the PV/PVM prior to exit into the erythrocyte cytosol. These findings are consistent with previous reports showing that there is no random distribution of integral membrane proteins within the PVM (Spielmann et al., 2006). These authors showed that different PVM proteins such as ETRAMPs and EXP1 define distinct areas and form a mosaic of different microdomains. Such microdomains could represent areas that compartmentalize different processes or serve as seeds to initiate structures such as the tubovesicular network, the cytostome, or nascent Maurer's clefts. Furthermore, they could serve to generate local polarization in this compartment, which could be needed for selection and sorting of proteins during the trafficking process.

There is an ongoing debate on whether MCs are independent structures or extensions of the tubovesicular network and thus in connection with the PVM (Lanzer et al., 2006). This debate gave rise to different models on how proteins are trafficked to the erythrocyte membrane. We

used FRAP to asses the connectivity of Maurer's clefts. Our data suggest that MCs are independent structures and are not connected to the PVM. This is in contrast to a previous study by Wickert et al., in which they three dimensionally reconstructed EM sections of Maurer's clefts (Wickert et al., 2004) and postulated that all membrane profiles observed within the infected erythrocyte form a continuous membrane network extending from the PVM. They used the strains HB3 and Dd2 whereas we have used 3D7 parasites, but it is unlikely that different plasmodium strains would possess two completely different trafficking mechanisms. Therefore, further evidence is needed to fully clarify this point.

We have used MAHRP1$_{FL}$-GFP parasites to do three-dimensional reconstructions of live-imaged infected red blood cells (iRBC). By pre-incubating iRBCS with Bodipy ceramide, a lipid stain, we were able to get a better spatial representation of compartments. We observed two different populations of Maurer's clefts: some were located in close proximity to the PVM, whereas others were situated towards the periphery of the erythrocyte cytosol. It still remains to be shown, if these two populations represent early and late MCs, or if these are two different populations persisting at two different locations. These experiments should be followed up using additional cellular markers and doing single cell time course analyses. With further technical advances, more information will be obtained on vicinity, connectivity, and timing of these structures.
It is important to get a more refined understanding of the Maurer's clefts. These organelles seem to be unique to *P. falciparum* and as an extracellular trafficking intermediate represent a new paradigm in cell biology. They act as trafficking compartment for major virulence factors and might represent the heel of Achilles and thus an intervention target.

Tackling the trafficking of MAHRP1

P. falciparum modifies its host cell by exporting own proteins into the cytoplasm and to the plasma membrane of the erythrocyte. Thereby it establishes immune evasion mechanisms and creates new permeation pathways for nutrient uptake. To transfer these proteins, the parasite must establish a protein trafficking system beyond the bounds of its own plasma membrane, because the erythrocyte lacks a secretory apparatus and hence cannot contribute to it. Because the parasite-induced secretory pathway is of great importance for the parasite survival, it represents an interesting target for drug intervention. In our work we have investigated the

export of MAHRP1 to the Maurer's clefts. Findings, which are relevant for our work, and our own findings are summarized schematically in figure 1 of this chapter.

In **eukaryotic cells**, soluble and membrane proteins destined for export are directed into the endoplasmatic reticulum by recognition of a classical signal sequence. In the ER proteins undergo a "checkup" by the quality control machinery consisting of chaperones and associated factors. Additional protein targeting motifs then provide the information for subsequent targeting via the Golgi to secretory destinations. The ER thus represents the gateway to the secretory pathway.

In ***P. falciparum*** not much is known about the export mechanism and it appears unique because the parasite not only exports proteins beyond its plasma membrane into the PV (which would be equivalent with secretion in secretory cells), but it also directs and controls trafficking through the PVM, into the host cell cytosol, as far as the distal erythrocyte membrane. Recent identification of a host cell-targeting signal (PEXEL/VTS) in exported *P. falciparum* proteins has contributed to the understanding of extracellular trafficking (Hiller et al., 2004; Marti et al., 2004). It consists of a pentameric sequence, R/KxLxQ/E, and is thought to act as PVM translocation signal (Hiller et al., 2004; Marti et al., 2004). When the motif is partially or completely mutated, the protein is retained in the PV (Figure 1A). The presence of a PEXEL motif encoded close to the start of exon 2 and a first exon encoding a signal sequence were used as parameters to predict the plasmodial exportome *in silico*. Approximately 400 plasmodial proteins were found (~8% of its proteome) and they appear to be unique to *Plasmodium* because most show no sequence homology to known protein domains (Hiller et al., 2004; Marti et al., 2004). Two findings in these two studies are particularly noteworthy:

(i) The translocation motif is present in both soluble and in membrane bound proteins. If exported proteins first transit through the secretory pathway of the parasite, soluble proteins will be released from the parasite cell surface into the lumen of the PV, whereas transmembrane proteins will end up on the parasite plasma membrane. If the same signal is used to target both types of protein into the erythrocyte, it must be present on the extracellular domain of all exported membrane proteins in order to be recognized by a putative receptor on the PV membrane. This raises the possibility that transmembrane proteins might be

present as protein complexes in the PV instead of being inserted in the plasma membrane. The nature of the translocon/channel in the PVM is however not known.

(ii) For PfEMP1, two different export motifs where found by two independent groups. Marti et al. describe it to be KxLxD in the N-terminus preceding the first DBL domain, whereas Hiller et al. describe it to be QFFRWFSEWSE within the DBL1 domain. Interestingly, PfEMP1 is a transmembrane protein and does not contain a signal sequence. Marti et al show that the TM directs it to the PV.

MAHRP1 has no predicted N-terminal signal peptide, which directs it to the ER and lacks a PEXEL motif, for translocation through the PVM. Nevertheless, we have shown by immunofluorescence experiments that MAHRP1 is present at Maurer's clefts. With the current knowledge it is thus unclear how MAHRP1 is exported. To address this question of MAHRP1 trafficking, we have transfected parasites with plasmids containing fusions of different MAHRP1 domains with GFP.

We found that the transmembrane domain (TM) in presence or absence of the C-terminus directs MAHRP1 to the ER where it is exclusively visible as a perinuclear fluorescence pattern. No fluorescence is seen downstream of the export pathway indicating that the TM acts as an ER entry signal of the protein, which is then retained. This is in contrast to previous findings where it was shown that ER signal sequence solely fused to GFP directs the protein via the ER to the PV, which would be the default secretory destination for plasmodial proteins containing only a signal sequence (Adisa et al., 2003). Transmembrane domains and ER-entry signal sequences have in common an approximately 20 amino acid hydrophobic core and this could explain its direction to the ER, as thought for PfEMP1, but does not explain the retention of this construct. Further constructs comprising partial N-terminal deletions fused to the GFP-tagged TM were investigated. We found that the truncation of 18 amino acids from residues 74 to 92 led to ablation of export and retention of the chimera in the ER. This stretch is located 13 amino acids upstream of the TM and contains a motif RxWxE with limited similarity to the PEXEL motif. If this PEXEL-like motif would be responsible for the translocation from PV through the PVM it is still unclear which information directs the chimera from the ER into the PV. It is conceivable that this short stretch of 18 amino acids not only contains a PEXEL-like translocation motif but also the information to direct MAHRP1 from the ER to the PV, therefore harbouring a bi-partite signal sequence.

Alternatively, the 18 amino acid region contains one signal, which directs it all the way from the ER to the Maurer's clefts.
However, it seems more likely that as suggested for PfEMP1, the transmembrane domain acts as a secretion signal and that our previous constructs, which are retained in the ER might be a result of retention by the quality control machinery due to some unusual conformation of the fusion protein.

Additional experiments need to be done to consolidate our evidence. In order to prove that the 18 amino acids are responsible for export and PVM translocation, it would be worthwhile to fuse them to a non-exported transmembrane protein. We have planned now to use ETRAMP4 (PFD1120c), which would be an ideal candidate as it is an integral membrane protein of the PVM (Spielmann et al., 2006). If this chimera is successfully exported, then a further narrowing of the sequence should be attempted. Once the minimal sequence requirements are identified, site-directed mutagenesis could provide further evidence for abrogation of its functionality. If these experiments reveal a novel motif, though PEXEL-like, this would raise questions to its function or need. It is thinkable that *P. falciparum* possesses several different export pathways.
MAHRP1 is not the only exported protein without a signal sequence and without a PEXEL motif. Interestingly, there are at least 3 other Maurer's clefts residents which share these similar features with MAHRP1 and are all ring-stage expressed: SBP1 (Blisnick et al., 2000), REX1 (Hawthorne et al., 2004) and MAHRP2 (unpublished, PF13_0276). Unfortunately none of them has been further investigated in trafficking studies.

We have generated a MAHRP1-deficient parasite strain to investigate its phenotype. We found that in the absence of MAHRP1 Maurer's clefts are still formed, as selected Maurer's clefts markers MAHRP2, REX1, and SBP1 are still present at the clefts. We further tested for the presence of PEXEL-containing exported proteins KAHRP and PfEMP3 and found that their trafficking to the RBC membrane was not impaired. In contrast, PfEMP1 is no longer exported but is thus retained in what seems to be the PV/PVM. These findings indicate that MAHRP1 plays a role in the translocation of PfEMP1 through the PVM (Figure 1B/C). It is conceivable that MAHRP1 and PfEMP1 associate in microdomains of the PV/PVM and that MAHRP1 acts as an escorter protein for PfEMP1. In a recent study, Maier et al. (2006) have generated a SBP1 knockout and made a similar finding. One can speculate that both Maurer's clefts proteins gather in the same microdomains and that both SBP1 and MAHRP1 play an

essential role in the translocation of PfEMP1 through the PVM. It would be interesting to see if this dependency between PfEMP1 and MAHRP1 is mutual. This could be tested by performing immunofluorescence assays with MAHRP1 antibodies on PfEMP1 knockdown parasites (Voss et al., 2006). Furthermore, the presence of additional exported proteins should be investigated to see if also soluble proteins are translocated MAHRP1-dependently.

The inability of PfEMP1 to be exported in the Pf3D7ΔM1 raises questions as to the nature of the interaction of PfEMP1 with MAHRP1. Pf3D7ΔM1 represents a good platform tool to address this question. The strain could be transfected with plasmids containing partial domains of MAHRP1 in order to test which reconstitution enables export of PfEMP1. Crosslinking experiments and co-precipitation might identify other interacting proteins.

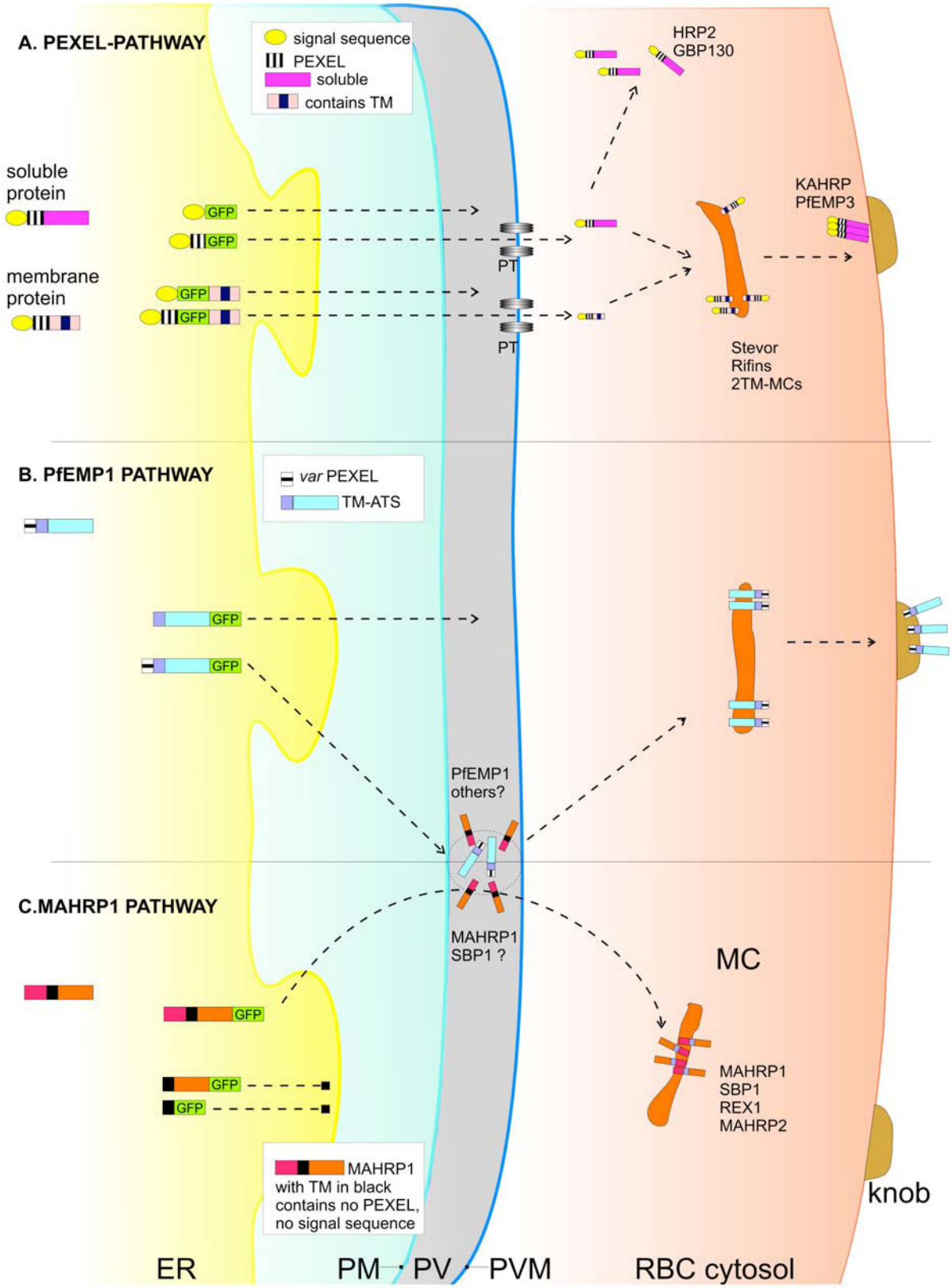
In this work we have attempted to characterize MAHRP1. By deciphering its amino acid sequence in biochemical and protein trafficking aspects we have obtained important information for MAHRP1, which represents in many aspects a unique protein.

Figure 1. Schematic representation of 3 different export-pathway models and their targeting sequences in *Plasmodium falciparum*. This figure summarizes findings of two recent publications (Hiller et al., 2004; Marti et al., 2004) in A and B, and outlines findings on the export pathway for MAHRP1 in C. The figure places emphasis on trafficking determinants, which have been investigated using green fluorescent protein (GFP)-tagged chimera and proposes a novel interaction between the export pathway of PfEMP1 and MAHRP1.

(A) Soluble and membrane proteins destined for export are directed to the endoplasmatic reticulum (ER) by recognition of a classical signal sequence or recessed N-terminal signal sequence. Proteins most probably pass through a rudimentary Golgi (not shown) en route to the parasitophorous vacuole (PV), while recognition of a translocation motif (PEXEL) downstream of the signal sequence results in the translocation of proteins such as KAHRP, HRP2, and PfEMP3 across the PV membrane via a putative transporter (PT). The exported proteins diffuse across the erythrocyte and may interact with the Maurer's clefts (MC) and with the erythrocyte cytoskeleton. Some proteins are diverted from the ER or Golgi to intracellular organelles such as the food vacuole and the apicoplast or to regulated secretory compartments such as the rhoptries (all not shown).

(B) The exported integral membrane protein PfEMP1 has a PEXEL motif but does not have a signal sequence. The transmembrane domain appears to act as a start transfer signal for insertion into the ER and the protein is then transferred to the PV. It is then translocated across the PVM in a MAHRP1 dependant fashion, shown as a dotted circle. It is then transferred to the MC, which represents an intermediate compartment prior to insertion into the red blood cell membrane at the knobs.

(C) The transmembrane domain of MAHRP1 directs it to the ER. Additional amino acids from the N-terminus are required for its export to the Maurer's clefts via the PV and through the PVM. We propose that MAHRP1 and PfEMP1 interact in the PV/PVM and that MAHRP1 is essential for translocation of PfEMP1 through the PVM.


A. PEXEL-PATHWAY
signal sequence
PEXEL
soluble
contains TM
soluble protein
membrane protein
GFP
PT
HRP2
GBP130
KAHRP
PfEMP3
Stevor
Rifins
2TM-MCs
B. PfEMP1 PATHWAY
var PEXEL
TM-ATS
PfEMP1
others?
C.MAHRP1 PATHWAY
MAHRP1
SBP1 ?
MC
MAHRP1
SBP1
REX1
MAHRP2
knob
MAHRP1
with TM in black
contains no PEXEL,
no signal sequence
ER
PM
PV
PVM
RBC cytosol

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Curriculum vitae

Personal data

Name	Anna Cornelia Spycher
Date of birth	06.11.1974
Place of Birth	Zürich, Switzerland
Nationality	Swiss
Address (work)	Swiss Tropical Institute Socinstrasse 57, P.O. Box, 4002 Basel, Switzerland email: cornelia.spycher@unibas.ch
	Tel. Work : +41 (0)61 2848 237/ 120 Mobile: +41 (0)78 9144 951 Fax : +41 (0)61 2848 101
Languages	German, English, Spanish, French

Education/Professional training

1983 - 1989	Elvel School, Tegucigalpa, Honduras
1990 - 1995	Wirtschaftsgymnasium Kirchenfeld, Bern, Switzerland
1995 - 1998	University of Bern, Faculty of Medicin, preclinical years of medicin
1998 – 2002	University of Bern, Master of Science with a major in medical microbiology.
2002	Master thesis in molecular parasitology at the Swiss Tropical Institute in Basel, Switzerland. Supervisor: Prof. H-P. Beck "Analysis of a novel histidine-rich protein of *P.falciparum*"
2003-2006	PhD in molecular parasitology at La Trobe University (LTU), Melbourne, Australia and the Swiss Tropical Institute in Basel (STI). Supervisors: Prof. HP. Beck (STI), Prof. Leann (LTU) *"Characterization of the novel Maurer's Clefts protein MAHRP1 in Plasmodium falciparum*."

Publications

Spycher C, Klonis N, Spielmann T, Kump E, Steiger S, Tilley L, Beck HP. MAHRP-1, a novel Plasmodium falciparum histidine-rich protein, binds ferriprotoporphyrin IX and localizes to the Maurer's clefts.
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Mol Cell Biol. 2006 Jun; 26(11):4074-85.

Meetings

2002	Swiss Parasitology Conference, Bern Talk: "MAHRP-1, a novel membrane-associated histidine-rich protein in *Plasmodium falciparum*."
2003	PhD student meeting of the Swiss Society of Tropical Medicine and Parasitology, Münchenwiler. Talk: "Characterization of a novel, early transcribed protein in the malaria parasite."
2004	Molecular Approaches in Malaria Conference (MAM), Lorne, AU Poster:"MAHRP-1, a novel resident at the Maurer's Clefts of *Plasmodium falciparum*."
2004	Queensland Institute of Medial Research QIMR, Brisbane,AU Talk: "A set of genes exclusively transcribed in ring-stage reveals a novel histidine-rich protein in *Plasmodium falciparum*."
2004	Australian Society of Parasitology, Annual Conference, Perth, AU Poster: "Unique organelles at the host-malaria parasite interzone: the Maurer's Clefts."
2005	Molecular Parasitology Meeting IX, Woods Hole, USA Poster: "Trafficking of a Maurer's Clefts resident protein MAHRP-1 in the malaria parasite."
2005	Swiss Society of Tropical Medicine and Parasitology, Annual Meeting, Ascona, Switzerland Talk: „Trafficking of the Maurer's Clefts protein MAHRP-1 in the malaria parasite."

Work related internships

University of Bern, Institute of Cell Biology, Prof. T. Seebeck, 1999
University of Wales, Cardiff, UK, Biosciences, Prof. A. Morby, 2000

During my studies I attended lectures by the following lecturers:

D. Schümperli, C. Wedekind, W. Nentwig, M. Milinski, B. Lanzrein, H. Riedwyl, H. Balsiger, G. Calzaferri, T. Stocker, B. Erni, C. Rothen, A. Boschetti, G. Pfander, A. Azzi, C. Kuhlemeier, K. Amman, D. Newbery, J. Kohli, P. Munz, T. Seebeck, M. Schweinegruber, I. Roditi, O. Fleck, B. Stadler, T. Jungi, B. Gottstein, N. Müller, J. Frei, U. Baumann
R. Braun, N. Weiss, I. Felger, H.P. Beck, G. Pluschke, N. Hogenraad, M. Ryan, L. Tilley, M. Foley, R. Anders, D. Philips.