Characterization of novel malaria vaccine candidates representing alpha-helical coiled coil domains

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Summary

The future vision in the battle against malaria goes beyond controlling the disease. Envisioned is the world-wide eradication of malaria. A substantial contribution to reach this goal is the development of an effective vaccine. Today's most advanced and most effective malaria vaccine, RTS,S/AS01, showed efficacy of 30 to 66% against all clinical episodes. There is a great need to increase efficacy by the next generation malaria vaccines. A strategy for increasing RTS,S efficacy could be to combine it with an effective blood stage vaccine. The disappointing outcomes of clinical trials conducted for most current blood stage vaccines demands the identification of novel promising candidates.

Under persistent exposure individuals develop immunity that protects against clinical disease but not parasitemia. This natural acquired immunity develops slowly and is reached in adolescence. In contrast, immunity against severe disease develops already after few infections. The mechanisms that underlie naturally acquired immunity or severe disease immunity remain poorly understood. Antibodies were demonstrated to play a critical role for controlling blood stage infection. It remains unclear which proteins elicit the production of protective antibodies and through which antibody effector function protection is provided. The relevance of antibodies in blood stage protection has the consequence that the immunogen correctly mimic the three-dimensional structure of the native protein. This PhD thesis has its major focus on immunogens that adopt a stable tertiary structure in aqueous environment.

The availability of the *P. falciparum* genome sequences, transcriptome and proteome data has opened the avenue for the identification of novel targets for vaccine development. However, blood stage vaccine development has focused on only a few candidates. Previously our collaborators in this project have identified promising candidates by genome-wide screening for alpha-helical coiled coil domains in proteins expressed in the erythrocytic parasite stages. The

segments with high probability score for coiled coil formation where selected. The 166 coiled coil segments derived from 131 proteins representing 4% of the blood stage proteome. 95 coiled coil fragments of a length of 30-40 amino acids were synthesized and analyzed systematically in a pre-clinical evaluation pathway.

The aim of this thesis was to fill the gaps in the preclinical evaluation pathway of novel synthetic peptide vaccine candidates.

The extensive polymorphism found in most parasite antigens represents a major obstacle for the development of efficacious blood stage vaccines. The genetic diversity of the identified coiled coil protein segments was studied in great detail. We found that coiled coil segments are well conserved, 82% of all selected 166 segments showed complete sequence conservation. Polymorphism was found predominantly in segments containing almost perfect tandem repeats. Based on these findings an optimized bioinformatic selection strategy was formulated proposing to exclude coiled coil segments consisting of almost perfect tandem repeats.

The availability of basic knowledge about vaccine candidates is a prerequisite for vaccine development and is essential to attract further funding for continued clinical development. A detailed cell biological characterization was undertaken for the most promising candidate, PFF0165c (newly termed Trophozoite exported protein 1 (Tex1)) Transcript and protein levels were analyzed throughout the intra-erytrocytic development cycle. *Tex1* transcripts were found up-regulated in the early trophozoite stage. This was supported by Tex1 protein levels. Tex1 abundance persisted until parasite egress. Immunofluorescence experiments revealed that Tex1 is exported and associates to parasite-derived structures, termed Maurer's clefts. Before parasite egress Tex1 resides in close proximity to the red blood cell membrane. In the search of sequence motifs responsible for Tex1 export we found that the actual translational start site is positioned 43 amino acids upstream of the start site previously predicted. The additional 43

amino acids function as signal peptide, directing the protein into the classical secretory pathway.

This thesis contributed to the immunological characterization of the intrinsically unstructured region (P27A) of Tex1. P27A was evaluated for vaccine potential and met the principal requirements to be downselected for a phase 1 trial. P27A was recognized by a majority of naturally exposed individuals, highly immunogenic, highly conserved and P27A-specific human and mouse sera were effective in *in vitro* parasite killing by Antibody-dependent cellular inhibition assay. High P27A-specifc antibody titers were found to positively correlate with protection. Clinical grade P27A peptide is currently produced.

In order to validate synthetic peptides as antigens the recognition by sera of adults from endemic region was compared to the recognition of the antigen recombinant expressed in *E. coli*. Comparable recognition of both types of antigens was observed.

This thesis provides evidence that the approach initiated by our collaborators is invaluable. This strategy, if proven successful in clinical trials, could be applied for vaccine development against many other pathogens from which genome data is available.

Abbreviations

RBC	red blood cell
iRBC	infected red blood cell
uRBC	uninfected red blood cell
PfEMP1	*Plasmodium falciparum* erythrocyte protein 1
Abs	antibodies
IDC	intra-erythrocytic development cycle
PE	pre-erythrocytic stage
RAS	radiation-attenuated sporozoites
CSP	circumsporozoite protein
PV	parasitophorous vacuole
PVM	parasitophorous vacuole membrane
MC	Maurer's clefts
PEXEL	*Plasmodium* export element
VTS	vacuole transport signal
ER	endoplasmic reticulum
RAS	radiation-attenuated sporozoites
MSP1, 2, 3	merozoite surface protein 1, 2, 3
AMA1	apical membrane antigen 1
EBA175, 140, 181	erythrocyte binding antigen 175, 140, 181
SERA	serine-rich antigen
EXP1	exported protein 1
GLURP	glutamate-rich protein
NK	natural killer cell
DC	dendritic cell
IFNγ	interferon-gamma
CC	alpha-helical coiled-coil
ADCI	antibody dependent cellular inhibition
Tex1	Trophozoite exported protein 1 (PFF0165c)
IUR	intrinsically unstructured region

P27A	intrinsically unstructured region of Tex1
P27	alpha-helical coiled-coil motif of Tex1
P90	alpha-helical coiled-coil motif of PFD0520c
ELISA	Enzyme -linked immunosorbent assay
recPFD0520c	PFD0520c recombinantly expressed in *E. coli*
CD	Circular dichroism
MAHRP1	membrane-associated histidine-rich protein-1
MAHRP2	membrane-associated histidine-rich protein-2
SBP1	skeleton-binding protein-1
Rex1, 2	Ring exported protein 1, 2
KAHRP	knob-associated histidine-rich protein
PNEPs	PEXEL-negative exported proteins
MS	mass spectrometry
MHC	major histocompatibility complex molecules

Chapter 1: Introduction

1.1 Malaria disease

Malaria is caused by the parasitic protozoan *Plasmodium*. *P. falciparum* is responsible for the most common and serious form of the disease. Approximately one-third of the world's population is at risk of malaria infection. Per year, malaria is estimated to affect 300-500 million individuals and lead to 1-3 million deaths, the majority being young children [1]. There is a striking correlation between malaria and poverty, and malaria-endemic countries have lower rates of economic growth [2]. Sub-Saharan Africa has the largest burden of malaria and accounts for 70% of all malaria cases worldwide.

The pathogenesis of human *P. falciparum* infection is a complex interplay of parasite-induced red blood cell (RBC) alteration, microcirculatory abnormalities, accompanied by local and systemic immune reactions resulting in multiple clinical forms of variable severity [3]. Severe malaria includes cerebral malaria and/or severe malarial anemia combined with complications of hypoglycemia and/or acidosis.

Cerebral malaria stems from the ability of later stage (trophozoite or schizont) infected RBCs (iRBC) to sequester in narrow blood vessels thus preventing clearance from the spleen [4]. Sequestration is mediated by adherence to endothelial cells, uninfected RBCs, platelets and other blood cells [4]. A prominent parasite molecule involved in cytoadherence is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). Due to its role in RBC sequestration, PfEMP1 is regarded as a major virulence factor. PfEMP1 is encoded by a multi-gene family consisting of 60 *var* genes that are expressed in a mutually exclusive manner in that only one variant is expressed at a single time point per parasite. The generation of antibodies against frequent PfEMP1 variants has been shown to contribute to protection against severe disease [5,6].

Severe malarial anemia results from massive RBC loss and/or impaired erythropoiesis **[7]**. Rupture of iRBC upon parasite egress as well as destruction of uninfected RBCs (uRBC) contribute to dramatically diminished cell counts. uRBCs have been shown to be modified by parasite molecules released during invasion. These modifications lead to spleen-mediated clearance, phagocytosis or complement-mediated lysis in the presence of specific antibodies [8,9,10].

The mechanisms underlying **hypoglycemia** and **acidosis** in malaria are not fully understood. These are a common complications of severe malaria frequently seen in children in combination with cerebral malaria [11,12].

1.2 Life cycle

P. falciparum undergoes a complex life cycle during which it goes through morphologically distinct stages. Infection of the human host occurs by the bite of a female *Anopheles* mosquito. *Plasmodium* sporozoites are harbored in the salivary glands and transferred during the blood meal into the human host. The sporozoites immediately find their way into the liver via the blood stream and invade hepatocytes. Within the liver cells, the parasite multiplies asexually to produce 10’000 to 30’000 merozoites which are capable of invading RBCs. All clinical symptoms are associated exclusively with the asexual multiplication in RBCs (completed every 48 hours). During the intra-erythrocytic development cycle (IDC) some merozoites enter a sexual differentiation program and develop into gametocytes. The mature gametocytes are eventually taken up by the mosquito during a blood meal. The sudden change in environmental conditions triggers male gametocytes to develop into motile microgametes and female gametocytes into round gametes [13]. Fusion of a male microgamete with a female gamete gives rise to a zygote that initiates meiosis and further develops into a motile ookinete. The ookinete traverses the mid-gut epithelium and develops into an oocyst. Sporozoites develop within the oocysts. Upon release sporozoites migrate to the salivary gland of the mosquito.

1.3 *P. falciparum* intra-erythrocytic development cycle

The IDC is responsible for all the clinical symptoms associated with malaria disease. Up to 30'000 merozoites are formed per infected hepatocyte. These merozoites are released in the blood stream and are capable of invading RBCs. *Plasmodium* parasites belong to the phylum Apicomplexa. All apicomplexan parasites share various features, including the presence of a specialized **apical complex**, which is central to the invasion process. During the IDC the parasites develop from the ring stage (hours 0 to 22 post invasion) to the trophozoite (hours 22 to 36 post invasion) to the schizont stage (hours 36 to 48 post invasion). During the schizont stage the parasite develops into 16-32 infective merozoites that are released upon RBC rupture.

1.3.1 Merozoite invasion

The whole process of merozoite invasion occurs very rapidly within 10-60 seconds [14,15]. Since the RBC is non-endocytic, merozoite invasion is an entirely active process. After the initial reversible interaction with the erythrocyte, the merozoite re-orients to bring its apical end into direct contact with the erythrocyte plasma membrane. To enter the cell, an irreversible interaction, the "tight junction", is formed between the parasite and the host membrane. The tight junction then migrates towards the posterior end of the merozoite. As the parasite pushes its way into the host cell, it creates a parasitophorous vacuole (PV) to seal itself off from the host-cell cytoplasm and form an environment hospitable for its development.

1.3.2 Remodeling of the infected erythrocyte

After parasite invasion, fundamental structural and morphological changes occur in the iRBC. These changes include the generation of an elaborate membrane system in the RBC cytosol known as the tubulovesicular network, which emanates from the parasitophorous vacuole membrane (PVM) [16], and compartments known as Maurer's clefts (MC) [17,18,19]. The parasite modifies

the RBC surface leading to knob-like protrusions which anchor proteins involved in cytoadherence [20].

The MC are flat and elongated membrane-bound cisterna with an electron-dense coat and electron-lucent lumen. MC are intermediate compartments for the transport of many parasite-derived proteins to various destinations within the RBC cytosol, cytoskeleton or membrane. These exported proteins function in the generation of new permeation pathways for nutrient uptake or act as virulence factors. The major virulence factor, the erythrocyte membrane protein-1 (PfEMP1), is trafficked via the MC to the RBC surface [21]. PfEMP1 localizes to the knobs and mediates adherence of the trophozoite and schizont infected RBCs to the host endothelium [22].

Recently, novel parasite-derived structures in the RBC cytosol have been identified. In close proximity to the MC, electron-dense tubular structures - so called “tethers” - were identified that were shown to connect the MC to the RBC membrane [16] [23].Vesicle-like structures of various sizes have been observed in the RBC cytosol [19,24,25,26,27].

1.3.3 Protein export in *P. falciparum*

A large number of parasite proteins are involved in the remodeling of the host cell. These proteins are exported beyond the plasma membrane of the parasite, across the PVM into the RBC cytosol, to the RBC membrane and beyond. In addition to cytoadherence and immune evasion, many exported proteins are involved in nutrient uptake and solvent exchange and therefore are crucial for parasite survival and development [4,28].

The *Plasmodium* export element sequence (PEXEL), or vacuole transport signal [29,30], located downstream of the hydrophobic N-terminal signal sequence functions as a signal for translocation across the PVM. Recently it was shown that the PEXEL motif is cleaved by a novel endoplasmic reticulum (ER)

peptidase, followed by acetylation [31,32,33]. The new N-terminus is thought to be recognized by a specific transporter located in the PVM [34]. There are several exported proteins that lack a PEXEL motif and/or a classical N-terminal signal sequence [23,35,36,37,38,39], suggesting the existence of an alternative translocation mechanism across the PVM.

1.4 Malaria vaccine development

Intervention strategies to fight against malaria include drug treatment of infected individuals, preventive drug treatment of populations at high risk of infection as well as insecticide-treated bed nets and indoor insecticide spraying for mosquito control. However, the spread of drug-resistant parasites and insecticide-resistant vectors is increasingly reducing the success of these interventions. Vaccination provides one of history's most cost effective public-health tools. The development of a malaria vaccine has been identified as a key component in an integrated approach to malaria control and an important step toward sustainable elimination of malaria.

An ideal malaria vaccine should be safe, cheap, easy to manufacture, easy to administer and confer long-lived immunity. The life cycle of *Plasmodium* parasites is highly complex, involving several developmental stages in both the insect (*Anopheles* species) and human host. Vaccine development strategies aim at inducing immunity against all stages of the life cycle.

1.4.1 Vaccine development against the pre-erythrocytic stage

Vaccines against the **pre-erythrocytic stage (PE)** target sporozoites transmitted by *Anopheles* mosquitoes as well as the liver stage of the parasite. It is well established that immunization with radiation-attenuated sporozoites (RAS) induces sterile protective immunity against malaria infection in humans [40,41] and rodents [42]. Gamma irradiation attenuated the parasite such that it could invade the host hepatocyte but failed to differentiate into erythrocytic stages [43].

The fact that immunization with dead sporozoites did not confer protection [42,44] indicated that live sporozoites in the skin, infection of hepatocytes and the development to liver stages are crucial for inducing sterile protective immunity. The major obstacle for PE vaccine development is that the vaccine must provide 100% protection. The development of one single liver stage parasite into merozoites entering the IDC would result in a blood stage infection. The development of RAS for vaccine development remains difficult. The safety and efficacy of RAS is dependent on a precise irradiation dose; too little irradiation allows the parasite to complete liver stage development and causes blood stage infection, too much irradiation inactivates the sporozoites and inactivated sporozoites do not induce significant protection [45]. The development of RAS for vaccine development meets great technical challenges and comprises many manufacturing-related safety risks. In addition to the correct irradiation dose, the contact of the RAS with human blood and potential contamination with salivary gland proteins creates great safety concerns [46]. The disadvantages accompanying the development of whole organism vaccines call for the development of recombinantly produced or synthetic vaccines, ideally composed of several subunits.

The currently most advanced subunit vaccine directed against PE is RTS,S. RTS,S is based on the hepatitis B surface antigen virus-like particle platform, genetically engineered to include the carboxy terminus of the *P. falciparum* circumsporozoite protein (CSP) [47] [48]; [49]; [50]; [51]. The RTS,S vaccine has demonstrated efficacy against both infection and clinical malaria in adults and children in different endemic settings (reviewed in Casares et al., 2010). In a phase IIb trial conducted in Mozambique, RTS,S formulation (RTS,S/AS02) resulted in 65% protection against infection in infants however protection persisted only 3 month and completely waned after a 6 month period [52]. RTS,S formulated as RTS,S/AS01 showed equal efficacy against infection in infants however protection was shown to pesists longer (60% for 10 month) [53]. A

multicenter, mutlicountry Phase III clinical trials of RTS,S/AS01 is currently ongoing.

The mechanism by which protection of around 50% of volunteers is attained remains unknown. An only weak association between CSP-specific antibody titer and protection was observed [54]. Vaccine development demands the identification of predictive correlates of protection after vaccination [55,56,57]. For the phase IIa RTS,S trials conducted in adults, an up-regulation of genes involved in the immunoproteasome pathway was detected in protected individuals after vaccination, when compared to unprotected individuals [58]. Immuno-proteasomes are absolutely essential for generating ligands bound by major histocompatibility complex molecules (MHC). This indicates that adaptive immune responses are involved in RTS,S-induced protection.

1.4.2 Vaccine development against the gametocyte stage

Another malarial life cycle stage which vaccines target is the **gametocyte stage** (mosquito, gamete, or sexual stage). It has been shown that antibodies against gametocytes generated in the infected or immunized host can contribute to killing of the parasite once the mosquito has taken a blood meal and parasites have emerged from the host erythrocyte [59,60]. The approach to kill parasites within the vector through immune factors taken up with the blood meal can prevent development of infective mosquito. The vaccine development strategy is termed transmission blocking vaccines [61]. These vaccines provide no direct benefit to individuals but might fight the disease at the population level. In the vision to eradicate malaria completely the inclusion of targets that efficiently block transmission are central [62].

1.4.3 Vaccine development against the erythrocytic stage

A third vaccine target is the **erythrocytic stage**, which is the focus of this thesis. Vaccines against the erythrocytic stage of the parasite do not aim at preventing

infection, but at reducing the morbidity and mortality, particularly among children. Targeting blood stage antigens is essential in combination with PE-vaccines. If no 100% of protection by a PE-vaccine can be guarantied, individuals in endemic areas as well as travelers may be left without any substantial protection against the parasite blood stage and may then soon develop severe disease. In endemic settings protection against severe disease is acquired rapidly following one or two malaria infections. Vaccines that target blood stage parasite could reliably prevent severe clinical disease. After a history of disappointing results, the current strategies of malaria vaccine development aim at reducing severe disease by 50% or more. This level of efficacy is considered to be worth a large scale implementation of such a vaccine in malaria control programs [63].

1.4.4 Blood stage vaccine candidates

Until now vaccine development against the erythrocytic stages of the parasite has focused on proteins mainly localized to the surface of the merozoite, such as members of the merozoite surface protein family (MSP1, MSP2, MSP3), to the organelles of the apical complex, such as the apical membrane antigen 1 (AMA1), the proteins of the erythrocyte binding antigens family (EBA175, EBA140, EBA181) and rhoptry proteins or to proteins exported by the intracellular parasite to the parasitophorous vacuole, the red blood cell or beyond (SERA, PfEMP1, EXP1 and GLURP) [63,64,65]. There are currently many blood-stage candidates approaching clinical evaluation.

One of the few blood-stage vaccines that had undergone a phase IIb field trial is Combination B vaccine [66], a subunit vaccine of three recombinant proteins MSP1 (C-terminus), MSP2 and RESA. The results of the clinical trial showed that the vaccine had significantly reduced the prevalence of parasites with a 3D7-type MSP2 and thus corresponding to the vaccine component, but not the prevalence of MSP2 forms belonging to the alternative FC27 allelic family [66].

MSP3 has been developed as a long synthetic peptide vaccine (MSP3-LSP with alum). A phase-Ib trial conducted in Mali revealed that the vaccine is safe and immunogenic. Sera from immunized individuals inhibited *in vitro* parasite growth by the Antibody-dependant cellular inhibition assay (ADCI), [67,68,69].

Another potential blood-stage vaccine is a combination of the rather conserved fragments of AMA1 and MSP1 [70]. A phase I trial in malaria-naive healthy adults induced high titer of antibodies. However, biological function of these antibodies was not reflected by the *in vitro* inhibition of parasite growth, and there was limited recognition of fixed parasites in an immunofluorescence assay.

Great hopes for an imminent effective malaria vaccine was raised by the first SPf66 vaccine trials in the 1990s. The SPf66 vaccine was composed of a 45-amino-acid long synthetic peptide composed of fragments of three blood-stage antigens, of which only one represented a known antigen, linked by the amino acids APNANP and PNANP [71]. Early vaccine trials showed vaccine efficacy of 31% [72], but later trials in Africa and Thailand showed no efficacy and therefore the development of this vaccine was suspended.

1.4.5 Major challenges for blood stage vaccine development

There are many obstacles to the development of efficacious vaccines against the blood stage. One is the high degree of polymorphic variability associated with the blood stage antigens. It is thought that high levels of polymorphism in malaria antigens are part of the parasite's strategy to escape the host's immune defense. However, by including polymorphic sequences in a malaria vaccine, variant-specific immune responses will be elicited. As a consequence, alleles distinct from the vaccine molecule will be favored by selective advantage giving rise to escape variants [66] [73] [74] [75]). In some approaches the frequency of SNPs as a signature of selection was used to identify new vaccine targets in known antigens [76] or in the entire *P. falciparum* genome [77].

If a vaccine is designed to induce antibody secretion by B cells, the vaccine candidate must mimic the conformation of the parent protein in order to elicit an effective antibody response. That creates another obstacle because the production of pure and correctly folded recombinant proteins are difficult and the antigen structure is often not known. In addition blood stage proteins show a high level of functional redundancy.

1.5 Immunity to malaria

Immune responses against malaria are highly complex and only poorly understood. Described immune effector mechanisms include the CD8+ T cell response to pre-erythrocytic stages and both the antibody-mediated and CD4+ T cell response to the blood stage.

1.5.1 Natural acquired immunity

Individuals living in malaria endemic regions develop natural acquired immunity through repeated infection. This immunity is defined by an age-dependent acquisition of non-sterilizing immunity that protects against clinical disease but not parasitemia [78]. Natural acquired immunity develops slowly. This is evident from the fact that in endemic settings the burden of disease falls on young children. The onset of clinical immunity requires 10 to 15 years of roughly five infections per year [79]. Distinct hypothesis exist as to the slow onset of clinically immunity with direct consequences for malaria vaccine development.

(i) The most widely accepted hypothesis implies parasite diversity as the major factor and views natural acquired immunity as the cumulative product of exposure to multiple parasite infections over time, resulting in a sufficiently diverse repertoire of strain-specific immune responses [79]. A successful vaccine should ideally overcome the inadequate response to antigenic diversity.

(ii) Another hypothesis suggests that the appropriate immune responses leading to natural acquired immunity are governed predominantly by intrinsic characteristics that change with age [79]. A successful vaccine should therefore overcome an age-dependent inappropriate immune response.

(iii) There is also evidence for the induction of antibodies towards conserved epitopes which recognize a broad array of *P. falciparum* strains [79]. The acquisition of these cross-reactive antibodies increases with repeated infections and age. In general it was found that conserved antigens are less immunogenic than polymorphic antigens [75,80,81,82]. However, it remains to be demonstrated that Abs against conserved epitopes are more relevant for protection than Abs directed against polymorphic antigens. If that were to hold true, then the late onset of clinical immunity could be explained by the poor immunogenicity to conserved but relevant antigens. An effective vaccine formulation should therefore increase the immunogenicity of these relevant antigens.

1.5.2 Importance of innate immunity in malaria infection

Innate immunity acts as an early line of defense against pathogens. It contributes to the control of acute infection by the invading pathogens before the onset of T and B cell-mediated immunity. Innate responses are crucial for the stimulation and modulation of adaptive immune responses. Natural killer cells (NK) appear to play an important role in the early immune response to *P. falciparum* infection. NK activation depends on cytokines released by macrophages and dendritic cells (DC) following parasite phagocytosis, but NK are also activated by direct interaction with iRBCs, as [83]. NK cells are the first cells that produce interferon gamma (IFN-γ) in response to *P. falciparum* infected RBCs. IFN–γ is the crucial link between innate immunity to the adaptive immune system. IFN–γ induces further DC maturation and the differentiation of T helper 1 cells.

1.5.3 Antibody-mediated responses against blood stage antigens

The important role of humoral immunity in protection was confirmed by passive transfer studies showing that immunoglobulins from clinically immune individuals can confer clinical immunity to individuals exposed to geographically diverse parasite strains [84,85,86]. Similarly, passively transferred maternal antibodies are one factor among others that provide effective protection from clinical malaria in newborns. However, opinions diverge on how those antibodies achieve protection. The current understanding is that antibodies can mediate protection through various mechanisms:

(1) Antibodies block invasion of merozoites into erythrocytes.
(2) Antibodies prevent sequestration of iRBC by preventing binding to adhesion molecules on the vascular endothelium.
(3) Antibodies trigger the release of parasitostatic and parasitocidal substances by monocytes.
(4) Antibodies neutralize parasite glycosylphosphatidylinositol and inhibit induction of the inflammatory cytokine cascade.
(5) Opsonization and destruction of free merozoites and iRBC by phagocytic cells.

1.5.4 T-cell responses against blood stage antigens

The observation that human CD4+ T cells can inhibit parasite growth *in vitro* has led to the hypothesis that CD4+ T cells acting in an antibody-independent manner can control parasite density very effectively. Immunization with low dose iRBC followed by drug treatment induced protective immunity against blood stage challenge [87]. The protective immunity was characterized by the absence of detectable antibodies but by the presence of a T-cell response, involving both CD4+ T cells and CD8+ T cells [87]. However, in vivo infection was shown to inhibit T cell responses due to overwhelming antigen abundance [88,89].

1.6 Alpha-helical coiled-coil motif

Alpha-helical coiled-coils (CC) share a heptad motif (**abcdefg**)$_n$ containing hydrophobic residues at positions **a** and **d** and generally polar residues at the remaining positions. Chemically synthesized short peptides consisting of such a motif can fold into their native structure in aqueous environment. A further advantage of CC is that they are recognized by conformation dependent antibodies [90]. This is an appealing characteristic and represents a new approach to malaria vaccine development. The use of synthetic peptides representing conformational epitopes over entire recombinantly expressed proteins in vaccines is advantageous because no elaborate expression and purification systems or refolding is required, making the development process much less time consuming [91]. CC are highly abundant in the eukaryotic cell. They are found in about 10% of all protein sequences [92]. This widespread occurrence in nature is explained by the broad range of function pertaining to the specific design of CC [93]. The crucial biological function of this domain has been investigated in numerous proteins. Generally, CC serve as oligomerization motifs in proteins.

1.7 Approach and rational of current thesis

New malaria blood-stage vaccine candidates were selected in a genome-wide approach by screening for alpha-helical coiled-coil domains (CC) using generalized sequence profile. Proteins expressed in the erythrocytic stage of the parasite were selected based on publicly available proteome and transcriptome data. These analyses led to the identification of 166 protein segments present in 131 proteins that are associated with the erythrocyte stage and displayed putative CC motifs with a high probability score. 95 were chemically synthesized and HPLC purified.

The rationale for targeting a coiled coil structural motif lay in the stable tertiary structure adopted by these peptides in an aqueous environment. Thus, the synthetic peptides corresponding to a coiled coil domain were expected to mimic

structurally “native” epitopes. Indeed, the majority of the chemically synthesized peptides were specifically recognized by human immune sera obtained from adult donors from Burkina Faso, Tanzania and Colombia, respectively, although with varying prevalence.

Preceding studies had shown that affinity chromatography purified antibodies specific for the 18 most recognized peptides reacted with native parasite proteins in infected erythrocytes. 12/18 antibody preparations were active in inhibiting *in vitro* parasite growth in antibody dependent cellular inhibition (ADCI) assays [94]. In addition, immunizing mice with these peptides induced antisera reacting with native proteins. The chosen approach of bioinformatic selection combined with chemical synthesis has led to the rapid identification of molecules that elicit functional antibodies, thus identifying suitable vaccine candidates. This represents a new strategy to identify novel malaria vaccine candidates.

The research topics of the present thesis are embedded in the frame work of an ongoing collaborative project initiated by Prof. G. Corradin from University of Lausanne, involving researchers from different fields of vaccinology, such as biochemistry (Giampietro Corradin, University of Lausanne, Switzerland), immunology (Pierre Druilhe, Pasteur Institute, Paris, France), bioinformatics (Andrey Kajava, Centre de Recherches de Biochimie Macromoleculaire, Montpellier, France) and clinical trials of malaria vaccines (Socrates Herrera, Immunology Institute, Universidad del Valle, Cali, Colombia.

The present thesis is a continuation of previous work of Villard and coworkers (2007). The genetic diversity of the CC sequences identified in the course of this earlier work became a focus of the current investigations. High levels of polymorphism in malaria antigens are thought to be part of the parasite’s strategy to evade host’s immune responses and this creates the major difficulty for blood stage vaccine development. There is therefore a great demand to identify new antigens that are both, immunogenic and conserved.

Availability of basic knowledge about vaccine candidates is essential to advocate continued preclinical and clinical development and to attract further funding. Therefore we investigated in greater detail the most promising candidate, PFF0165c (termed Trophozoite exported protein 1 (Tex1)). We performed detailed cell biological characterization of Tex1 with respect to subcellular localization, expression profile and solubility characteristics during the IDC.

Work performed during this PhD thesis contributed to the immunological characterization of the intrinsically unstructured region (P27A) of Tex1 published by Olugbile et al. 2009. P27A was meets the principal requirements expected for clinical development of a malaria vaccine candidate which is currently under way with funding from European Malaria Vaccine initiative (EMVI) (Phase I clinical trial).

The ability of peptides to act as potent immunogens has been questioned in the past. The wide application as vaccines or drugs now steadily reduces the skepticism [95]. To contribute to the validation of vaccines consisting of synthetic peptides one of the candidates identified previously (PFD0520c) was now recombinant expressed and its serological performance was compared to the CC of this protein (P90).

1.8 Objectives

1.8.1 General objectives

Today's battle against malaria goes beyond controlling the disease. Envisioned is a malaria-free world. A substantial contribution to reach this goal is the development of an effective vaccine. Vaccine efficacy can be increased by targeting all stages of the *P. falciparum* life cycle. Targeting the blood stages of the parasite is essential for aiding populations in malaria endemic areas because protective responses against these stages could prevent severe clinical disease. This thesis aimed at filling the gaps in the preclinical evaluation pathway of novel synthetic peptide vaccine candidates.

1.8.2 Specific objectives

A. Assessment of sequence conservation of 166 alpha-helical coiled coil domains present in proteins expressed in the blood stage of *P. falciparum* based on single nucleotide polymorphism data available in PlasmoDB 5.4 database (http://PlasmoDB.org).

B. Assessment of sequence conservation of 14 most promising peptides based on pre-clinical evaluation data in additional 13 culture strains.

C. Assessment of sequence conservation of 5 of these 14 peptides in 63 malaria positive field samples from Tanzania and 19 positive field samples from Papua New Guinea.

D. Analysis of the subcellular localization of the proteins corresponding to the selected peptides by Immunofluorescence assay (IFA).

E. In depth cell biological characterization of one of the vaccine candidates.

F. Validation of the synthetic peptide approach by recombinant expression of a protein harboring a coiled coil motif. Comparison of serological assays performed using synthetic peptides and with those using the corresponding recombinant antigen.

1.9 References

1. Kamau EM (2006) Roll Back Malaria and the new partnership for Africa's development: is there potential for synergistic collaboration in partnerships? Afr J Health Sci 13: 22-27.
2. Sachs J, Malaney P (2002) The economic and social burden of malaria. Nature 415: 680-685.
3. Buffet PA, Safeukui I, Deplaine G, Brousse V, Prendki V, et al. The pathogenesis of Plasmodium falciparum malaria in humans: insights from splenic physiology. Blood.
4. Maier AG, Cooke BM, Cowman AF, Tilley L (2009) Malaria parasite proteins that remodel the host erythrocyte. Nat Rev Microbiol 7: 341-354.
5. Bull PC, Lowe BS, Kaleli N, Njuga F, Kortok M, et al. (2002) Plasmodium falciparum infections are associated with agglutinating antibodies to parasite-infected erythrocyte surface antigens among healthy Kenyan children. J Infect Dis 185: 1688-1691.
6. Bull PC, Marsh K (2002) The role of antibodies to Plasmodium falciparum-infected-erythrocyte surface antigens in naturally acquired immunity to malaria. Trends Microbiol 10: 55-58.
7. Chang KH, Stevenson MM (2004) Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. Int J Parasitol 34: 1501-1516.
8. Layez C, Nogueira P, Combes V, Costa FT, Juhan-Vague I, et al. (2005) Plasmodium falciparum rhoptry protein RSP2 triggers destruction of the erythroid lineage. Blood 106: 3632-3638.
9. Awah NW, Troye-Blomberg M, Berzins K, Gysin J (2009) Mechanisms of malarial anaemia: potential involvement of the Plasmodium falciparum low molecular weight rhoptry-associated proteins. Acta Trop 112: 295-302.
10. Pouvelle B, Buffet PA, Lepolard C, Scherf A, Gysin J (2000) Cytoadhesion of Plasmodium falciparum ring-stage-infected erythrocytes. Nat Med 6: 1264-1268.

11. Thien HV, Kager PA, Sauerwein HP (2006) Hypoglycemia in falciparum malaria: is fasting an unrecognized and insufficiently emphasized risk factor? Trends Parasitol 22: 410-415.
12. Planche T, Krishna S (2006) Severe malaria: metabolic complications. Curr Mol Med 6: 141-153.
13. Alano P (2007) Plasmodium falciparum gametocytes: still many secrets of a hidden life. Mol Microbiol 66: 291-302.
14. Dvorak JA, Miller LH, Whitehouse WC, Shiroishi T (1975) Invasion of erythrocytes by malaria merozoites. Science 187: 748-750.
15. Mitchell GH, Bannister LH (1988) Malaria parasite invasion: interactions with the red cell membrane. Crit Rev Oncol Hematol 8: 225-310.
16. Hanssen E, Carlton P, Deed S, Klonis N, Sedat J, et al. Whole cell imaging reveals novel modular features of the exomembrane system of the malaria parasite, Plasmodium falciparum. Int J Parasitol 40: 123-134.
17. Lanzer M, Wickert H, Krohne G, Vincensini L, Braun Breton C (2006) Maurer's clefts: a novel multi-functional organelle in the cytoplasm of Plasmodium falciparum-infected erythrocytes. Int J Parasitol 36: 23-36.
18. Wickert H, Krohne G (2007) The complex morphology of Maurer's clefts: from discovery to three-dimensional reconstructions. Trends Parasitol 23: 502-509.
19. Hanssen E, Sougrat R, Frankland S, Deed S, Klonis N, et al. (2008) Electron tomography of the Maurer's cleft organelles of Plasmodium falciparum-infected erythrocytes reveals novel structural features. Mol Microbiol 67: 703-718.
20. Luse SA, Miller LH (1971) Plasmodium falciparum malaria. Ultrastructure of parasitized erythrocytes in cardiac vessels. Am J Trop Med Hyg 20: 655-660.
21. Bhattacharjee S, van Ooij C, Balu B, Adams JH, Haldar K (2008) Maurer's clefts of Plasmodium falciparum are secretory organelles that concentrate virulence protein reporters for delivery to the host erythrocyte. Blood 111: 2418-2426.

22. Kyes S, Horrocks P, Newbold C (2001) Antigenic variation at the infected red cell surface in malaria. Annu Rev Microbiol 55: 673-707.

23. Pachlatko E, Rusch S, Muller A, Hemphill A, Tilley L, et al. MAHRP2, an exported protein of Plasmodium falciparum, is an essential component of Maurer's cleft tethers. Mol Microbiol.

24. Kriek N, Tilley L, Horrocks P, Pinches R, Elford BC, et al. (2003) Characterization of the pathway for transport of the cytoadherence-mediating protein, PfEMP1, to the host cell surface in malaria parasite-infected erythrocytes. Mol Microbiol 50: 1215-1227.

25. Taraschi TF, O'Donnell M, Martinez S, Schneider T, Trelka D, et al. (2003) Generation of an erythrocyte vesicle transport system by Plasmodium falciparum malaria parasites. Blood 102: 3420-3426.

26. Przyborski JM, Wickert H, Krohne G, Lanzer M (2003) Maurer's clefts--a novel secretory organelle? Mol Biochem Parasitol 132: 17-26.

27. Kulzer S, Rug M, Brinkmann K, Cannon P, Cowman A, et al. Parasite-encoded Hsp40 proteins define novel mobile structures in the cytosol of the P. falciparum-infected erythrocyte. Cell Microbiol.

28. Haldar K, Murphy SC, Milner DA, Taylor TE (2007) Malaria: mechanisms of erythrocytic infection and pathological correlates of severe disease. Annu Rev Pathol 2: 217-249.

29. Marti M, Good RT, Rug M, Knuepfer E, Cowman AF (2004) Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science 306: 1930-1933.

30. Hiller NL, Bhattacharjee S, van Ooij C, Liolios K, Harrison T, et al. (2004) A host-targeting signal in virulence proteins reveals a secretome in malarial infection. Science 306: 1934-1937.

31. Chang HH, Falick AM, Carlton PM, Sedat JW, DeRisi JL, et al. (2008) N-terminal processing of proteins exported by malaria parasites. Mol Biochem Parasitol 160: 107-115.

32. Boddey JA, Moritz RL, Simpson RJ, Cowman AF (2009) Role of the Plasmodium export element in trafficking parasite proteins to the infected erythrocyte. Traffic 10: 285-299.

33. Russo I, Babbitt S, Muralidharan V, Butler T, Oksman A, et al. Plasmepsin V licenses Plasmodium proteins for export into the host erythrocyte. Nature 463: 632-636.

34. de Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, et al. (2009) A newly discovered protein export machine in malaria parasites. Nature 459: 945-949.

35. Spycher C, Klonis N, Spielmann T, Kump E, Steiger S, et al. (2003) MAHRP-1, a novel Plasmodium falciparum histidine-rich protein, binds ferriprotoporphyrin IX and localizes to the Maurer's clefts. J Biol Chem 278: 35373-35383.

36. Spielmann T, Hawthorne PL, Dixon MW, Hannemann M, Klotz K, et al. (2006) A cluster of ring stage-specific genes linked to a locus implicated in cytoadherence in Plasmodium falciparum codes for PEXEL-negative and PEXEL-positive proteins exported into the host cell. Mol Biol Cell 17: 3613-3624.

37. Dixon MW, Hawthorne PL, Spielmann T, Anderson KL, Trenholme KR, et al. (2008) Targeting of the ring exported protein 1 to the Maurer's clefts is mediated by a two-phase process. Traffic 9: 1316-1326.

38. Saridaki T, Frohlich KS, Braun-Breton C, Lanzer M (2009) Export of PfSBP1 to the Plasmodium falciparum Maurer's clefts. Traffic 10: 137-152.

39. Haase S, Herrmann S, Gruring C, Heiber A, Jansen PW, et al. (2009) Sequence requirements for the export of the Plasmodium falciparum Maurer's clefts protein REX2. Mol Microbiol 71: 1003-1017.

40. Clyde DF (1975) Immunization of man against falciparum and vivax malaria by use of attenuated sporozoites. Am J Trop Med Hyg 24: 397-401.

41. Hoffman SL, Goh LM, Luke TC, Schneider I, Le TP, et al. (2002) Protection of humans against malaria by immunization with radiation-attenuated Plasmodium falciparum sporozoites. J Infect Dis 185: 1155-1164.

42. Nussenzweig RS, Vanderberg J, Most H, Orton C (1967) Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei. Nature 216: 160-162.

43. Sigler CI, Leland P, Hollingdale MR (1984) In vitro infectivity of irradiated Plasmodium berghei sporozoites to cultured hepatoma cells. Am J Trop Med Hyg 33: 544-547.

44. Alger NE, Harant J (1976) Plasmodium berghei: heat-treated sporozoite vaccination of mice. Exp Parasitol 40: 261-268.

45. Vaughan AM, Wang R, Kappe SH Genetically engineered, attenuated whole-cell vaccine approaches for malaria. Hum Vaccin 6: 107-113.

46. Ripley Ballou W (2007) Obstacles to the development of a safe and effective attenuated pre-erythrocytic stage malaria vaccine. Microbes Infect 9: 761-766.

47. Pichyangkul S, Kum-Arb U, Yongvanitchit K, Limsalakpetch A, Gettayacamin M, et al. (2008) Preclinical evaluation of the safety and immunogenicity of a vaccine consisting of Plasmodium falciparum liver-stage antigen 1 with adjuvant AS01B administered alone or concurrently with the RTS,S/AS01B vaccine in rhesus primates. Infect Immun 76: 229-238.

48. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, et al. (2004) Efficacy of the RTS,S/AS02A vaccine against Plasmodium falciparum infection and disease in young African children: randomised controlled trial. Lancet 364: 1411-1420.

49. Alonso PL, Sacarlal J, Aponte JJ, Leach A, Macete E, et al. (2005) Duration of protection with RTS,S/AS02A malaria vaccine in prevention of Plasmodium falciparum disease in Mozambican children: single-blind extended follow-up of a randomised controlled trial. Lancet 366: 2012-2018.

50. Aponte JJ, Aide P, Renom M, Mandomando I, Bassat Q, et al. (2007) Safety of the RTS,S/AS02D candidate malaria vaccine in infants living in a highly endemic area of Mozambique: a double blind randomised controlled phase I/IIb trial. Lancet 370: 1543-1551.

51. Sacarlal J, Aponte JJ, Aide P, Mandomando I, Bassat Q, et al. (2008) Safety of the RTS,S/AS02A malaria vaccine in Mozambican children during a Phase IIb trial. Vaccine 26: 174-184.

52. Macete E, Aponte JJ, Guinovart C, Sacarlal J, Ofori-Anyinam O, et al. (2007) Safety and immunogenicity of the RTS,S/AS02A candidate malaria vaccine in children aged 1-4 in Mozambique. Trop Med Int Health 12: 37-46.

53. Pool R, Munguambe K, Macete E, Aide P, Juma G, et al. (2006) Community response to intermittent preventive treatment delivered to infants (IPTi) through the EPI system in Manhica, Mozambique. Trop Med Int Health 11: 1670-1678.

54. Kester KE, Cummings JF, Ofori-Anyinam O, Ockenhouse CF, Krzych U, et al. (2009) Randomized, double-blind, phase 2a trial of falciparum malaria vaccines RTS,S/AS01B and RTS,S/AS02A in malaria-naive adults: safety, efficacy, and immunologic associates of protection. J Infect Dis 200: 337-346.

55. Daubenberger CA Gene-expression analysis for prediction of RTS,S-induced protection in humans. Expert Rev Vaccines 9: 465-469.

56. Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, et al. A prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray. Proc Natl Acad Sci U S A 107: 6958-6963.

57. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, et al. (2008) Profiling humoral immune responses to P. falciparum infection with protein microarrays. Proteomics 8: 4680-4694.

58. Vahey MT, Wang Z, Kester KE, Cummings J, Heppner DG, Jr., et al. Expression of genes associated with immunoproteasome processing of major histocompatibility complex peptides is indicative of protection with adjuvanted RTS,S malaria vaccine. J Infect Dis 201: 580-589.

59. Bousema T, Sutherland CJ, Churcher TS, Mulder B, Gouagna LC, et al. Human immune responses that reduce the transmission of Plasmodium falciparum in African populations. Int J Parasitol.

60. Mlambo G, Kumar N, Yoshida S Functional immunogenicity of baculovirus expressing Pfs25, a human malaria transmission-blocking vaccine candidate antigen. Vaccine 28: 7025-7029.

61. Saul A (2007) Mosquito stage, transmission blocking vaccines for malaria. Curr Opin Infect Dis 20: 476-481.

62. Butler D (2009) Initiative targets malaria eradication. Nature 462: 19.

63. Vekemans J, Ballou WR (2008) Plasmodium falciparum malaria vaccines in development. Expert Rev Vaccines 7: 223-240.

64. Goodman AL, Epp C, Moss D, Holder AA, Wilson JM, et al. New candidate vaccines against blood-stage Plasmodium falciparum malaria: prime-boost immunization regimens incorporating human and simian adenoviral vectors and poxviral vectors expressing an optimized antigen based on merozoite surface protein 1. Infect Immun 78: 4601-4612.

65. Richards JS, Stanisic DI, Fowkes FJ, Tavul L, Dabod E, et al. Association between naturally acquired antibodies to erythrocyte-binding antigens of Plasmodium falciparum and protection from malaria and high-density parasitemia. Clin Infect Dis 51: e50-60.

66. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, et al. (2002) A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J Infect Dis 185: 820-827.

67. Sirima SB, Tiono AB, Ouedraogo A, Diarra A, Ouedraogo AL, et al. (2009) Safety and immunogenicity of the malaria vaccine candidate MSP3 long synthetic peptide in 12-24 months-old Burkinabe children. PLoS One 4: e7549.

68. Nebie I, Diarra A, Ouedraogo A, Tiono AB, Konate AT, et al. (2009) Humoral and cell-mediated immunity to MSP3 peptides in adults immunized with

MSP3 in malaria endemic area, Burkina Faso. Parasite Immunol 31: 474-480.

69. Lusingu JP, Gesase S, Msham S, Francis F, Lemnge M, et al. (2009) Satisfactory safety and immunogenicity of MSP3 malaria vaccine candidate in Tanzanian children aged 12-24 months. Malar J 8: 163.
70. Ballou WR, Arevalo-Herrera M, Carucci D, Richie TL, Corradin G, et al. (2004) Update on the clinical development of candidate malaria vaccines. Am J Trop Med Hyg 71: 239-247.
71. Lopez MC, Silva Y, Thomas MC, Garcia A, Faus MJ, et al. (1994) Characterization of SPf(66)n: a chimeric molecule used as a malaria vaccine. Vaccine 12: 585-591.
72. Alonso PL, Tanner M, Smith T, Hayes RJ, Schellenberg JA, et al. (1994) A trial of the synthetic malaria vaccine SPf66 in Tanzania: rationale and design. Vaccine 12: 181-186.
73. Fluck C, Schopflin S, Smith T, Genton B, Alpers MP, et al. (2007) Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity. Infect Genet Evol 7: 44-51.
74. Lyon JA, Angov E, Fay MP, Sullivan JS, Girourd AS, et al. (2008) Protection induced by Plasmodium falciparum MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. PLoS ONE 3: e2830.
75. Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, et al. (2007) Plasmodium falciparum merozoite surface protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection. J Infect Dis 195: 279-287.
76. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, et al. (2000) A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. Nat Med 6: 689-692.
77. Mu J, Awadalla P, Duan J, McGee KM, Keebler J, et al. (2007) Genome-wide variation and identification of vaccine targets in the Plasmodium falciparum genome. Nat Genet 39: 126-130.

78. Doolan DL Plasmodium immunomics. Int J Parasitol.

79. Doolan DL, Dobano C, Baird JK (2009) Acquired immunity to malaria. Clin Microbiol Rev 22: 13-36, Table of Contents.

80. Franks S, Baton L, Tetteh K, Tongren E, Dewin D, et al. (2003) Genetic diversity and antigenic polymorphism in Plasmodium falciparum: extensive serological cross-reactivity between allelic variants of merozoite surface protein 2. Infect Immun 71: 3485-3495.

81. Fluck C, Smith T, Beck HP, Irion A, Betuela I, et al. (2004) Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun 72: 6300-6305.

82. Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, et al. (2007) Naturally acquired antibodies to polymorphic and conserved epitopes of Plasmodium falciparum merozoite surface protein 3. Parasite Immunol 29: 387-394.

83. Korbel DS, Finney OC, Riley EM (2004) Natural killer cells and innate immunity to protozoan pathogens. Int J Parasitol 34: 1517-1528.

84. Cohen S, Mc GI, Carrington S (1961) Gamma-globulin and acquired immunity to human malaria. Nature 192: 733-737.

85. Sabchareon A, Burnouf T, Ouattara D, Attanath P, Bouharoun-Tayoun H, et al. (1991) Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg 45: 297-308.

86. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P (1990) Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J Exp Med 172: 1633-1641.

87. Pombo DJ, Lawrence G, Hirunpetcharat C, Rzepczyk C, Bryden M, et al. (2002) Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. Lancet 360: 610-617.

88. Xu H, Wipasa J, Yan H, Zeng M, Makobongo MO, et al. (2002) The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. J Exp Med 195: 881-892.

89. Good MF (2001) Towards a blood-stage vaccine for malaria: are we following all the leads? Nat Rev Immunol 1: 117-125.

90. Sedegah M, Brice GT, Rogers WO, Doolan DL, Charoenvit Y, et al. (2002) Persistence of protective immunity to malaria induced by DNA priming and poxvirus boosting: characterization of effector and memory CD8(+)-T-cell populations. Infect Immun 70: 3493-3499.

91. Lu SM, Hodges RS (2002) A de novo designed template for generating conformation-specific antibodies that recognize alpha-helices in proteins. J Biol Chem 277: 23515-23524.

92. Walshaw J, Woolfson DN (2001) Open-and-shut cases in coiled-coil assembly: alpha-sheets and alpha-cylinders. Protein Sci 10: 668-673.

93. Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol 11: 82-88.

94. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, et al. (2007) Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One 2: e645.

95. Corradin G, Kajava AV, Verdini A Long synthetic peptides for the production of vaccines and drugs: a technological platform coming of age. Sci Transl Med 2: 50rv53.

CHAPTER 2

Sequence conservation in *Plasmodium falciparum* alpha-helical coiled coil domains proposed for vaccine development

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Sequence Conservation in *Plasmodium falciparum* α-Helical Coiled Coil Domains Proposed for Vaccine Development

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Abstract

Background: The availability of the *P. falciparum* genome has led to novel ways to identify potential vaccine candidates. A new approach for antigen discovery based on the bioinformatic selection of heptad repeat motifs corresponding to α-helical coiled coil structures yielded promising results. To elucidate the question about the relationship between the coiled coil motifs and their sequence conservation, we have assessed the extent of polymorphism in putative α-helical coiled coil domains in culture strains, in natural populations and in the single nucleotide polymorphism data available at PlasmoDB.

Methodology/Principal Findings: 14 α-helical coiled coil domains were selected based on preclinical experimental evaluation. They were tested by PCR amplification and sequencing of different *P. falciparum* culture strains and field isolates. We found that only 3 out of 14 α-helical coiled coils showed point mutations and/or length polymorphisms. Based on promising immunological results 5 of these peptides were selected for further analysis. Direct sequencing of field samples from Papua New Guinea and Tanzania showed that 3 out of these 5 peptides were completely conserved. An *in silico* analysis of polymorphism was performed for all 166 putative α-helical coiled coil domains originally identified in the *P. falciparum* genome. We found that 82% (137/166) of these peptides were conserved, and for one peptide only the detected SNPs decreased substantially the probability score for α-helical coiled coil formation. More SNPs were found in arrays of almost perfect tandem repeats. In summary, the coiled coil structure prediction was rarely modified by SNPs. The analysis revealed a number of peptides with strictly conserved α-helical coiled coil motifs.

Conclusion/Significance: We conclude that the selection of α-helical coiled coil structural motifs is a valuable approach to identify potential vaccine targets showing a high degree of conservation.

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Introduction

The majority of known malaria antigens are highly polymorphic [1]. Tandem repeats are found in central domains of many antigens giving rise to extensive length polymorphism (LP) [2]. In addition, single nucleotide polymorphisms (SNPs) are abundant in antigenic genes, with 65% of SNPs on a genome-wide scale being non-synonymous (i.e the nucleotide substitution results in an amino acid change) [3]. The genetic diversity of new vaccine candidates is generally determined in the preclinical characterization of the candidate. High levels of polymorphism in malaria antigens are thought to be part of the parasite's strategy to avoid destruction by the host's immune defense. By including polymorphic sequences in a malaria vaccine, variant-specific immune responses will be elicited. As a consequence, alleles distinct form the vaccine molecule will be favored by selective advantage giving rise to escape variants. This situation was observed by molecular and immunological monitoring in the Phase I/IIb trial of the malaria vaccine Combination B that, in addition to two other components, contained almost the full length of merozoite surface protein 2 (MSP2) allele of the 3D7 cloned parasite line [4]. In vaccine recipients, a lower prevalence of the 3D7-type genotype was observed and genotypes belonging to the alternative allelic family were responsible for a higher incidence of malaria episodes [5]. A significant strain-specific humoral response was directed against the repetitive and family-specific MSP2 domains, whereas only low antibody titres were observed against conserved domains of MSP2 [6]. Similarly, a strain-specific response was observed in a challenge trial in Aotus monkeys with the two alleles of $MSP1_{42}$ [7]. There are also contrasting findings from clinical trial of RTS,S where no selection was observed in break-through infections for SNPs in the circumsporozoite protein T-cell-epitope regions [8]. The question remains whether the inclusion of more than one allelic form of an antigen can compensate substantial polymorphism [9]. As for MSP2, the inclusion of two variants into a vaccine has been proposed for MSP3 [6,10]. So far there is little

experimental evidence that multi-allele vaccines actually reduce morbidity in contrast to single antigen vaccines [4]. An other interesting aspect in immune evasion is that naturally occurring variants of the same epitope can prevent memory T cells effector functions referred as "altered peptide ligand" antagonism [11,12]. The above examples highlight a major obstacle for vaccine development posed by polymorphism in vaccine candidates. By using non-polymorphic domains of antigens, selection of vaccine escape variants could be avoided. A further important consideration in vaccine development is the complexity of candidate molecules in the vaccine formulation. If more variants are required in order to cover the major alleles found world-wide, highly complex mixtures, particularly for multi-component vaccines, would result; thus risking high costs and potential antagonistic effects [4].

Our approach to discover novel vaccine candidates is based on the selection of protein segments with defined structural motifs, with emphasis on identifying conserved domains of antigens. A genome-wide bioinformatic approach was taken to identify potential candidates that contain an α-helical coiled coil motif [13]. The α-helical coiled coils share a $(\mathbf{abcdefg})_n$ motif containing hydrophobic residues at positions **a** and **d** and generally polar in the remaining positions. Chemically synthesized short peptides consisting of this motif can fold into their native structure. This is an appealing characteristic and represents a new approach to malaria vaccine development. The use of synthetic peptides over recombinantly expressed proteins in vaccines is advantageous because no expression or elaborate purification system is required, making the development process much less tedious and time consuming [14]. A further advantage of α-helical coiled coil motifs is that they are recognized by conformational dependent antibodies [15]. These coiled coil motifs are highly abundant in the eukaryotic cell. They are found in about 10% of all protein sequences [16]. This widespread occurrence in nature is explained by the broad range of function pertaining to the specific design of their coiled coil domains [17]. The crucial biological function of this domain has been investigated in numerous proteins. Generally, α-helical coiled coil domains serve as oligomerization motifs in proteins.

The rationale for our focus on peptides with little or no polymorphism was that these coiled coil regions were immunogenic in mice and well recognized by naturally occurring antibodies [13] (Olugbile et al., manuscript in preparation). In addition, affinity purified antibodies against these peptides killed parasites *in vitro* as shown by an assay involving antibody-dependent cellular inhibition [13,18,19]. Presence of hydrophobic residues in **a** and **d** positions is important for formation of the critical interhelical interactions while mostly hydrophilic residues in the remaining positions are exposed on the surface of the α-helical coiled coil motif and assumed to function as sites for protein interaction. Such structural and functional constraints associated with coiled coil domains likely signify these motifs are under purifying selection and led us to expect and investigate sequence conservation.

In an attempt to elucidate the relationship between coiled coil structure and sequence conservation, we analyzed the polymorphism in 166 synthetic peptides, previously identified in a genome-wide selection process [13]. Many of these molecules have undergone immunological testing [13] and some have successfully entered the vaccine development pipeline. 14 peptides included in the analysis were further assessed in 13 culture strains. The sequence diversity of 5 of these 14 peptides was also investigated in parasite populations from endemic countries.

Materials and Methods

Ethics Statement

Research clearance for blood sampling and genetic analysis of parasites was granted by the Tanzanian Commission for Science and Technology and by the Medical Research and Advisory Committee of the Ministry of Health in Papua New Guinea.

Parasite culture

The culture strains were grown in 10 cm Petri dishes and cultured by standard methods in an atmosphere of 93% N_2, 4% CO_2, 3% O_2 at 37°C as described previously [20]. The culture medium was RPMI 1640 10.44 g/L, supplemented with Hepes 5.94 g/L, Albumax II 5 g/L, hypoxanthine 50 mg/L, sodium bicarbonate 2.1 g/L and neomycin 100 mg/L.

Polymorphism study in culture strains

Genetic diversity of 14 peptides spanning the α-helical coiled coil region of 10 hypothetical proteins was assessed in 13 *in vitro* culture strains (3D7, W2mef, HB3, ITG2F6, IFA18, FVO, 7G8, K1, RO33, MAD20, FCR3, RFCR3 and FC27). The geographical origin of each strain is listed in **Table 1**. Genomic DNA was isolated with phenol/chloroform extraction. PCR primers used to amplify the α-helical coiled-coil region are listed in **Table S1**. 63 blood samples derived from 1–5 year old children from Ifakara, Tanzania with uncomplicated acute malaria. These samples were collected in the course of an antimalarial drug trial [21]. 19 samples were asymptomatic community samples from Mugil village, Papua New Guinea [22]. Genomic DNA was isolated with phenol/chloroform extraction or the QIAamp DNA Blood Mini Kit 250 (Qiagen). PCR conditions consisted of denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 1 min), annealing (50°C for 1 min) and extension (72°C for 1 min). The reaction products were incubated at 72°C for 10 min to ensure complete DNA extension. The PCR products were directly sequenced and aligned using Auto Assembler software to screen for SNPs and LP within the sequences corresponding to the peptides.

Table 1. Origin of culture strains.

culture strain	origin
3D7	Airport malaria (Amsterdam)
FC27	Madang, PNG
MAD20	PNG
RFCR3	Gambia
FCR3	Gambia
W2mef	Indochina
K1	Thailand
Hb3	Honduras
IFA18	Ifakara, Tanzania
RO-33	Ghana
ITG2F6	Brazil
FVO	Vietnam
7G8	Brazil

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RNA isolation and cDNA synthesis

Due to large introns in the genomic DNA corresponding to P38 and P77 cDNA was produced for sequencing of the 13 culture strains (3D7, W2mef, HB3, ITG2F6, IFA18, FVO, 7G8, K1, RO33, MAD20, FCR3, RFCR3 and FC27). 10 ml of parasite culture of 5% mixed stage parasites were lysed in 3 ml of Trizol (Invitrogen) and RNA was extracted with 0.2 volumes of chloroform and precipitated with 0.8 volumes of isopropanol. The extraction was repeated in half of the original Trizol volume to reduce contamination with gDNA. Residual gDNA was digested twice with RQ1 Dnase (Promega) according to the manufacturer's protocol in a total volume of 50 µl. RNA was dissolved in 25 µl of 5 mM Tris/0.5 mM EDTA and 9.5 µl RNA was used for the reverse transcription by AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) with random primers (Invitrogen) as described by the manufacturer. P38 and P77 sequences were amplified from cDNA with Advantage cDNA polymerase (BD Biosciences) using the primers listed in **Table S1**. To determine gDNA contamination the corresponding peptide sequences were amplified with Advantage Taq from RNA processed simultaneously without the addition of reverse transcriptase.

Identification of α-helical coiled coil motif

For the original genome-wide selection of coiled coil domains, we generated 25 residues-long<-helical coiled coil profiles [13] by using *pftools* package [23]. This profile was constructed by using a multiple alignment of amino acid sequences corresponding to the known α-helical coiled coil domains found in the Protein Data Bank (PDB) [24], release 2006. In this work, the profile was updated by adding new sequences of the known coiled coil structures from PDB (release 2008). The score of this profile reflects the level of similarity of an analyzed amino acid sequence to the typical coiled coil motif which was deduced from the alignment of the known coiled coils. Tests of this profile against a sequence database of proteins with the known 3D structures showed that (1) the scores above 3.0 corresponded exclusively to coiled coil structures; (2) some coiled coil structures may have scores above 2.1. The 2.1 cut-off level was chosen for the first stage of the identification procedure to include most of the putative coiled coils. Subsequently, the selected coiled coil regions were tested manually for the presence of the characteristic heptad repeats. Although all putative α-helical coiled coil domains identified in the *P. falciparum* genome share the heptad repeat sequence motif, they can be distinguished by fidelity of the heptad repetitions. We subdivided the analyzed coiled coil regions into two groups. The first group contains peptides with perfect, or, in case of one or more SNPs, almost perfect tandem repetition of a certain sequence motif. The length of the perfect repeat either coincides with the length of the 7 residue coiled coil repeat or is divisible by 7. The second group of imperfect repeats is characterized by the repetition of amino acid residues with similar physico-chemical properties rather than by repeat units of exactly the same amino acid residues. Both types of repeats contain hydrophobic residues at positions **a** and **d** of the heptads and polar residues in the remaining positions. In this work, we assed the extend of polymorphism in the identified α-helical coiled coil domains and examined the polymorphism in perfect or almost perfect repeats as opposed to that in imperfect repeats.

Results

The extensive genetic diversity of blood stage antigens is one of the key challenges in vaccine development against malaria. After the selection of 166 novel blood stage vaccine candidates, all harbouring α-helical coiled coil motif, we undertook a comprehensive *in silico* analysis of these domains. In addition, we performed an in depth molecular epidemiological analysis on selected peptides that proved to be the most promising vaccine candidates according to an immunological evaluation process [13].

SNP data for a maximum of 15 *P. falciparum* culture strains are currently available in the PlasmoDB 5.4 database (http://PlasmoDB.org), the official database of the *P. falciparum* genome sequencing consortium. Unfortunately, PlasmoDB 5.4 does not incorporate information on insertions or deletions. Nevertheless, insertions and deletions are thought to provide as much diversity as SNPs in *P. falciparum* [25]. In order to determine the full extent of diversity in recently identified vaccine candidates [13], we have analyzed the polymorphism of the selected α-helical coiled coil regions in 13 different culture strains and in cross sectional field samples from malaria endemic regions. Direct sequencing made it possible to detect both, SNP and LP.

Genetic Diversity in *in vitro* culture strains of *P. falciparum*

Table 1 lists the 13 strains that were analyzed for SNPs and LP by PCR and direct sequencing of the corresponding 14 most promising peptides selected from the preclinical evaluations. Primers used for PCR amplification and sequencing are listed in **Table S1**. Polymorphism results are presented in **Table 2**. In addition we have included all information on polymorphism that is publicly available at PlasmoDB 5.4. Peptides P1, P14 and P83 showed either LP alone (P1, **Figure 1**) or both types of polymorphism, SNP plus LP (P14, **Figure 2** and P83 **Figure 3**). Peptides showing LP revealed tandem repeats and differed from each other by 1 to 3 heptad repeat units. Therefore, these mutations do not introduce a frame shift into the coiled coil motif. For example, P83 corresponding to the α-helical coiled coil domain of the gene product of PFC0345w shows a duplication of the heptamer DMNIKEN between amino acids N276 and D277, and was detected with a frequency of 0.31 (4/13) in 4 culture strains (**Table 2**).

Some SNPs were prevalent in our culture strains. For P83 the SNP at nt 863 T→A occurred with a frequency of 0.38 (5/13). This SNP was also described in PlasmoDB 5.4 and was observed with a frequency of 0.43 (6/14). Two additional SNPs were found for P83. A G→A substitution at nt position 927 resulted in a non-synonymous change from methionine (M) to isoleucine (I) at amino acid 309. This SNP was only found once and only in our culture strains. An additional SNP at nt position 999, arising from an A→C conversion, results in an amino acid change from glutamic acid (E) to aspartic acid (D), and was also found only once. These SNPs were also reported in PlasmoDB. With respect to the underlying heptad repeat positions labeled **a** *to* **g**, all SNPs detected in P83 were at hydrophobic positions **a** and **d**, however, these SNPs do not affect the coiled coil formation significantly and the antibody epitopes on the surface of the coiled coil likely remain conserved. It is worth mentioning that, in principle, one point mutation can change the oligomerization state of the coiled coil. However, the general rules governing the stoichiometry of the coiled coil structures are still largely unknown. Due to the fact that most of the observed non-synonymous SNPs represent change to the residues with similar physico-chemical properties (e.g. hydrophobic to hydrophobic), we assume that the oligomerization states of the coiled coils also remain conserved. One SNP of P83 leads to a change of a hydrophobic methionine (M288) to a charged lysine (K) in **d**-position of the heptamer unit underlying coiled coil structural motifs (**Table 2**). The charged residue in **d**-position favors a coiled coil dimer. However, we do not know the

Table 2. Genetic diversity in the 14 peptides sequences.

peptide[a]	Gene ID PlasmoDB 5.4	SNP data extracted from PlasmoDB 5.4			SNPs detected by sequencing of culture strains[b]		
		position	type of mutation[c-f]	frequency	position	type of mutation[c-f]	frequency
P1 VNNLDSTVNYMN**S**TGNN INNI VNNLDSTVNYMNSTGNN **a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f**a**bc**d** I NNI VNNLDST VNYMNSTGNNI NNI **a**bc**d**e f g**a**bc**d**e f g a b c**d**e f g**a**bc**d**	PFA0170c	nt 1525 A→T nt 1518 A→G nt 1581 G→A	ns (S509G) s s	1/15 1/15 1/15	nt 1599 nt 1662	Del Del	2/13 10/13
P2 T I S SL SNK I VNYESK I E ELEKE LKEVK g**a**bc**d**e f g**a**bc **d**e f g**a**bc**d**e f g**a**bc**d**e	PFB0145c	conserved			conserved		
P5 I I DI KKHLEKL KIE I KEKKEDLE NL **a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f g**a**bc **d**		conserved			conserved		
P8 I KTMNTQI S TL KNDVHLLNEQDKLNNEKGTLN SKI S EL **a**bc **d** e f g**a**bc**d**e f g a bc**d**e f g a bc**d**e f g a bc**d**e f**a**bc**d** NVQ I MDL e f g**a**b c **d**		conserved			conserved		
P9 L LS KDKE I E EKNKK I KEL NNDI KKL **a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f g**a**bc**d**		conserved			conserved		
P13 LDENEDN I K KMKSK I DDMEKE I KYR **a**bc**d**e f g**a**bc **d** e f g**a**b c **d** e f g**a**bc**d**		conserved			conserved		
P14 GMNNMN**G**DI NNI NGDI NNMNGDI NNMNGDI NNMNGDI N g **a** b c **d** e f g**a**b c**d**e f g**a**b c **d** e f g**a**bc**d** e f g**a**b c **d** e f g**a**b NMN c **d** e	PFC0245c	nt 6662 C→T nt 6726 T→A	ns (G2221E) s	1/10 1/8	nt 2227	Del (GDINNMN)	7/13
P27 KKRNVEEEL HSL RKNYN I I NEE I E E I T g**a**bc **d**e f g**a**bc**d**e f g **a**bc**d**e f g**a**bc**d**e	PFF0165c	conserved			conserved		
P35 ARDD I QKDI NKMESE L I NVSNE I NRL D g**a** b c**d**e f g**a**b c**d** e f g**a**bc**d**e f g**a**bc**d**e	PF14_0045	conserved			conserved		
P38 N I T NI NKN I E N I K NDMSNL NNMNDSNQ g**a**bc**d**e f g**a**bc**d**e f g **a** bc**d**e f g **a** b c**d**e	PF14_0089	conserved			conserved		
P77 EKL KKYNNE I S SL KKELD I L NEKMGKC T f g**a**bc**d**e f g**a**bc**d**e f g**a**bc**d**e f g **a** b c **d**e	PF08_0048	conserved			conserved		
P79 NEMNKEVNKMNEEVNKMNEEVNKMNEEVNKMNKEVNKM bc**d** e f g**a**bc **d** e f g**a**bc **d** e f g**a**bc **d** e f g**a**bc**d** e f**a**bc **d**e DEEVNKMNKEVNKMNK f g**a**bc**d** e f g**a**bc**d** e f g	PFB0315w	nt 4845 G→A	s	1/12	conserved		

Table 2. cont.

peptide[a]	Gene ID PlasmoDB 5.4	SNP data extracted from PlasmoDB 5.4			SNPs detected by sequencing of culture strains[b]		
		position	type of mutation[c-f]	frequency	position	type of mutation[c-f]	frequency
P83 QNKMENDMN I I KNDMN I**M**ENDMN I MENDMNI I KNDMNI **a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bcd**e**fg**a**bcd**e**fab**c**d	PFC0345w	nt 863 T→A	ns (M288K)	4/9	nt 863 T→A	ns (M288K)	5/13
		nt 927 G→A	ns (M309I)	1/10	nt 927 G→A	ns (M309I)	1/13
		nt 999 A→C	ns (E333D)	1/10	nt 999 A→C	ns (E333D)	1/13
MEKDMNI I KNDMNI I KNNMNI I KN**E**MNI I KNV **a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bc**d**					nt 827	dup (DMNIKEN)	4/13
P90 TKKLNKELSEGNKELEKLE KN I KELE ETNNTLE NDI KV g**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**bc**d**efg**a**b	PFD0520c	nt 195 A→G	s	1/9	conserved		

[a]Peptide sequences are aligned with the coiled coil heptad repeat (abcdefg); lines with arrows over some sequences indicate tandem repeats.
[b]*P. falciparum* in vitro cultre strains 3D7, FC27, K1, IFA18, FV0, 7G8, HB3, ITG2F6, RO-33, W2mef, FCR3S1.2, RFCR3, MAD20
[c]non synonymous (ns);
[d]synonymous (s);
[e]deletion (del);
[f]duplication (dup).
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oligomerization state of the original P83 coiled coil, thus it is impossible to conclude whether the M288K mutant indeed changes the oligomerization state. Further experimental studies are required to clarify the oligomerization state of P83 wildtype and mutant.

Diversity in parasite populations

Five vaccine candidates, prioritized according to their performance in immunological and functional assays, were further analyzed in a small-scale molecular epidemiological survey. The basis for selection and results of the preclinical evaluation process were published previously [13,14]. The five candidates were peptides P8, P27, P77, P83 and P90. In addition to showing promising results, all were conserved or showed limited polymorphism in culture strains (**Table 2**). The extent of sequence conservation was determined in field samples from two malaria endemic areas: Tanzania (TZ) and Papua New Guinea (PNG) (**Table 3**). P27 and P77 were found to be completely conserved. P90 is also conserved on the amino acid level in 23 samples from TZ and 31 samples from PNG, with only a synonymous SNP 195 A→G in samples from TZ with a frequency of 0.17 (4/23). This SNP was also reported in PlasmoDB 5.4 to occur in the GHANA1 strain (**Table 2**). The P8 sequence was conserved in culture strains, but in the field samples 2 SNPs were detected. 4 SNPs (SNP1-4) and 2 LP (LPT2, LPT26) were detected for P83 in both populations examined (**Figure 4**). Thus, in field samples 3/5 peptides showed complete conservation on the amino acid level and only minor polymorphism was observed for the remaining 2 candidates. This is in line with sequence diversity detected in

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                    abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
Reference (3D7) GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNYLNRKNNNHLSYTN
3D7             GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNYLNRKNNNHLSYTN
ITG2F6          GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
RO-33           GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
IFA18           GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
FCR3            GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
RFCR3           GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
Fc27            GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
FVO             GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
Hb3             GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
MAD20           GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
W2mef           GTGNNINNIVNNLDSTVNYMTSTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------YLNRKNNNHLSYTN
7G8             GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------------------------------YLNRKNNNHLSYTN
K1              GTGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN----------------------------------YLNRKNNNHLSYTN
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Figure 1. Alignment of P1 sequences amplified from 13 culture strains. Two types of deletions were detected in the PFA0170c gene: a longer deletion (del1) between T1599 and T1726 and a shorter deletion (del2) T1662 and T1726. On the protein level del1 results in the loss of NINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGN amino acid motif between N533 and Y576 and del1 in the loss of NINNIVNNLDSTV-NYMNSTGN between N554 and Y576 of P1. The peptide sequences P1 is indicated with bold letters. The heptad reapets are marked on top of the alignment sequence as a repeated abcdefg motif in which the hydrophobic position **a** (bold) and **d** (bold) are highlighted.
doi:10.1371/journal.pone.0005419.g001

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                  gabcdefgabcdefgabcdefgabcdefgabcdefgabcde
Reference (3D7)   GMNNMNGDINNINGDINNMNGDINNMNGDINNMNGDINNMN
3D7               GMNNMNGDINNINGDINNMNGDINNMNGDINNMNGDINNMN
7fA18             GMNNMNGDINNINGDINNMNGDINNMNGDINNMNGDINNMN
RO-33             GMNNMNEDINNINGDINNMNGDINNMNGDINNMNGDINNMN
FCR3              GMNNMNGDINNINGDINNINGDINNMNGDINNMNGDINNMN
MAD20             GMNNMNGDINNINGDINNINGDINNMNGDINNMNGDINNMN
Fc27              GMNNMNGDINNINGDINNINGDINNMNGDINNMNGDINNMN
Hb3               GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
RFCR              GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
K1                GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
ITG               GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
W2mef             GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
7G8               GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
FVO               GMNNMNGDINNIN-------GDINNMNGDINNMNGDINNMN
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Figure 2. Alignment pf P14 sequences amplified from 13 culture strains. A deletion of the GDINNMN amino acid motif was observed with a frequency of 0.54 (7/13). In addition an imperfect repeat (GDINNIN) of this motif was detected with a frequency of 0.23 (3/13).
doi:10.1371/journal.pone.0005419.g002

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                  abcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefgabcdefg
Reference (3D7)   QNKMEN-------DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
3D7               QNKMEN-------DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
RO-33             QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIIEKDMNIIKNDMNIIKNNMNIIKNDMNIIKNV
Hb3               QNKMEN-------DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
IfA18             QNKMEN-------DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
K1                QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
Fc27              QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
MAD20             QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
7G8               QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
FCR3              QNKMEN-------DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
FV0               QNKMENDMNIKENDMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
W2mef             QNKMENDMNIKENDMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
ITG2F6            QNKMENDMNIKENDMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
RFCR              QNKMENDMNIKENDMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
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Figure 3. Alignment of P83 sequences amplified from 13 culture strains. SNP in P83 at nt position 863 T→A results in an amino acid change from Methionine (M) to a Lysine (K) at position 288 in PFC0345w gene product (red). This SNP was observed with a frequency of 0.38 (5/13). An insertion of DMNIKEN amino acid motif (bold and underlined) was detected with a frequency of 0.31 (4/13).
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culture strains where 3/14 peptides had shown LP or SNP plus LP.

Effects of polymorphism on the probability of α-helical coiled coil structure formation

A comprehensive *in silico* analysis was performed on 166 selected coiled coil sequences to determine the effects of SNP and LP on the probability to form an α-helical coiled coil. Our approach allowed the prediction of structure modifications caused by the known SNPs within our peptides, which are recorded in the SNP database in PlasmoDB

Overall we detected a high degree of sequence conservation in 166 predicted α-helical coiled coil domains. Only 29/166 peptides showed limited polymorphism. In one of the 29 polymorphic peptides the score fell below the cut-off (altered score bolded in **Table 4**). In contrast to the above result on P83 that had shown SNPs exclusively at hydrophobic residues, the majority of the SNPs in the 166 peptides were found at the surface positions **b**, **c**, **e**, **f** and **g** within the heptad repeat and are unlikely to destabilize the coiled coil structures.

For peptides P17 and P23 -wild type (in culture strain 3D7; **Table 4**), P17-mutant (in culture strain GHANA1), and P23-mutant (in culture strain 7G8, FCB, K1, GHANA1; **Table 4**) the length of the synthesized peptide was too short to be analyzed by the 25 residue-long profile. These short peptides were then analyzed manually and it was shown that mutations do not affect the heptad pattern and therefore do not prevent α-helical coiled coil formation.

Discussion

One of the hurdles in vaccine development against erythrocytic stages of the parasite is the extensive polymorphism observed in blood stage antigens. A function of polymorphic epitopes may be to divert the effective response. In natural and artificially induced humoral responses, the polymorphic regions of antigens were found to be immunodominant [2,10,26], but it is not known whether polymorphic regions are better or worse than conserved regions as targets of protective immunity.

The fact that polymorphism is maintained in populations lead to the question whether this is due to immune selection through allele-specific protective responses. For several polymorphic antigens immune selection has been confirmed [10,27]. Similarly, SNPs were demonstrated to be under balancing selection, and the

Table 3. Genetic diversity of P27, P90, P83, P8 and P77 in *P. falciparum* positive field samples from Tanzania and Papua New Guinea.

peptide	Gene ID	SNPs detected by sequencing of field samples		
		nt position	frequency	type of mutation[a–d]
P27	PFF0165c	conserved 46/46 (TZ)		
		conserved 17/17 (PNG)		
P90	PFD0520c	nt 195 T→C	4/23 (PNG)	s
			conserved 31/31 (TZ)	
P77	PF08_0048	conserved 14/14 (TZ)		
		conserved 10/10 (PNG)		
P8	PFB0145c	nt 1817 C→T	1/33 (TZ)	ns (T606I)
		nt 1856 T→A	1/29 (PNG)	ns (M619K)
P83	PFC0345w	nt 863 T→A	4/8 (PNG)	ns (M288K)
		nt 999 A→C	1/27 (TZ)	ns (E333D)
		nt 976 A→G	1/27 (TZ)	ns (N326D)
		nt 937 A→C	1/27 (TZ)	ns (M313L)
		nt 843 A→C	1/27 (TZ)	ns (K282Q)
		nt 844 A→C	1/27 (TZ)	ns (K282Q)
		nt 823	1/27 (TZ)	dup (DIIKN)
		nt 885	1/27 (TZ)	del (DMNIIKN)

[a]non synonymous (ns).
[b]synonymous (s).
[c]deletion (del).
[d]duplication (dup).
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frequency of SNPs as a signature of selection was used to identify new vaccine targets in known antigens [27] or in the entire *Plasmodium falciparum* genome [3].

Conserved regions were found to be less antigenic and immunogenic than polymorphic regions [6,10,26,28]. To investigate whether conserved regions can elicit adequate protection, the effect of antibody responses to both the conserved and polymorphic regions of MSP3 was measured [10]. Antibodies against both regions were associated with a reduced risk to develop clinical malaria [10]. Moreover, antibodies against the conserved epitopes elicited in humans inhibited parasites growth *in vitro* as shown by the antibody-dependent cellular inhibition assay [29] and lead to rapid parasite clearance after injection to humanized mice [29,30].

During preclinical evaluation of new vaccine candidates, both antigenicity and sequence conservation are generally determined. We have shown that the majority of our candidates were both conserved and antigenic. In sero-epidemiological surveys the most peptides were found to be recognized by sera of adults from malaria endemic countries [13] (own unpublished results). Immunogenicity of most of the peptides investigated in more detail was confirmed in mice or rabbits [13] (Olugbile, unpublished results). It remains to be shown whether our described strategy to select non-polymorphic epitopes for inclusion in a vaccine will lead to greater efficacy in a field trial.

We analyzed the degree of conservation in predicted α-helical coiled coil regions of all proteins expressed in the blood stages of the parasite. Sequencing revealed that SNPs observed in field samples did not seem to disturb the heptad motif and thus do not destabilize the coiled coil structure. SNPs mostly occurred at hydrophilic surface positions of the coil except for P83. It is likely that SNPs located at surface positions result in a decreased antibody response to the variant epitope and may lead to immune evasion. However, the extent of polymorphism detected in our candidates was very limited and thus might not create a major limitation for vaccine efficiency.

Our sequence analysis revealed that both SNPs and LP were preferentially observed in the α-helical coiled coil motifs containing almost perfect tandem repeats. The perfect repeat units either coincide with the 7-residue coiled coil repeat (e.g. P14, P45, P50, P51, P64, P81, P83, P144, P166) or covering two or more heptad repeats (e.g. P1, P94). This correlation between the repeats perfection and polymorphism leads to a practical recommendation for selection of vaccine candidates: when searching for α-helical coiled coil regions with a reduced level of polymorphism, one should avoid regions with almost perfect tandem repeats.

α-helical coiled coil domains were found to be crucial for the biological function of various proteins. Coiled coils have been shown to be involved in oligomerization, protein-protein interaction and complex formation. These features support many cellular processes such as membrane fusion, protein transport and cell motility [17]. But for the proteins investigated by it is not known whether the putative coiled coil domains are of any functional importance. If these domains play a role in protein function, purifying selection might counteract diversification and polymorphism. Sequence conservation due to functional constraints was reported from viral envelope proteins where the conserved region was found at those positions of the coiled coil that are responsible for protein-protein interaction [31].

Extensive polymorphism has been an issue for the most promising blood stage vaccine candidates, such as apical membrane antigen 1, MSP1 and 2 [6,32–35]. In the past, vaccine research was focused on a limited number of vaccine candidates. Due to recent disappointing results from clinical trials where a number of vaccine candidates were not found to be immunogenic, safe, or protective against artificial challenge [36–38], new emphasis is laid upon the discovery of novel target antigens. If more of the current candidates fail, additional antigens are

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3D7             QNKMEN-----DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
SNP3            QNKMEN-----DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNDMNIIKNEMNIIKNV
SNP4            QNKMEN-----DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDLNIIKNDMNIIKNNMNIIKNEMNIIKNV
SNP2            QNKMEN-----DMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNDMNIIKNV
SNP1            QNKMEN-----DMNIIKNDMNIKENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
LPT2            QNKMENDIIKNDMNIIQNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
LPT26           QNKMEN------------DMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKNDMNIIKNNMNIIKNEMNIIKNV
```

Figure 4. SNPs and LP of P83 sequences amplified in field samples from PNG and Tanzania. In PNG samples essentially only one SNP (SNP1) at nt position 863 T→A results in an amino acid change from Methionine (M) to a Lysine (K) at position 288 in PFC0345w gene product. This SNP was detected with a high frequency of 0.5 (4/8) and was also observed in culture strains. In Tanzanian samples 3 SNPs (SNP2, SNP3, SNP4; bolded) and 2 LP (LPT2, LPT26; insertion indicated in bold) occurring with low frequencies of 0.04 (1/27) were detected.
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Table 4. SNPs detected in peptide sequences based on the SNP data published in PlasmoDB 5.4

Peptide	Protein	Sequence	Frequency	Score [a]
P1	PFA0170c	3D7:		
		VNNLDSTVNYMN**S**TGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNI		2.570
		Hb3:		
		VNNLDSTVNYMN**G**TGNNINNIVNNLDSTVNYMNSTGNNINNIVNNLDSTVNYMNSTGNNINNI	1/15	2.570
P14	PFC0245c	3D7:		
		GMNNMN**G**DINNINGDINNMNGDINNMNGDINNMNGDINNMN		3.100
		RO-33:		
		GMNNMN**E**DINNINGDINNMNGDINNMNGDINNMNGDINNMN	1/10	3.450
P17	PFD0110w	3D7:		
		EKKLDILK**V**NISNINNSLDKLK		manual[b]
		GHANA1:		
		EKKLDILK**A**NISNINNSLDKLK	1/11	manual[b]
P23		3D7:		
		FQK**V**KEKAE**I**QKENIEKIKQEINTL		manual[b]
		7G8;FCB;K1:		
		FQKVKEKAE**N**QKENIEKIKQEINTL	4/10	manual[b]
		GHANA1:		
		FQK**I**KEKAE**N**QKENIEKIKQEINTL	1/10	manual[b]
P33	PF13_0277	3D7:		
		YIDDV**D**RDVENYDKGIANVDHHLNDVH		3.020
		106_1;D6;Dd2;FCC-2;FCR3;GHANA1;Hb3;IT;K1;RO-33		
		YIDDV**N**RDVENYDKGIANVDHHLNDVH	10/12	3.080
P34	MAL13P1.336	3D7:		
		NMNNMNNNMNNMNNNMNNNMNNMN**N**MN		3.570
		D6:		
		NMNNMNNNMNNMNNNMNNNMNNMN**S**MN	1/5	3.550
P36	PF14_0093	3D7:		
		SSNNLSDQINILNNNIQHINS**T**FNNLR		2.920
		Senegal3404:		
		SSNNLSDQINILNNNIQHINS**I**FNNLR	1/8	2.730
P45	PF11_0207	3D7:		
		EE**I**KEEIKEVKEEIKEVKEEIKEVKEEIKEVKEEIKE		4.550
		GHANA1:		
		EE**V**KEEIKEVKEEIKEVKEEIKEVKEEIKEVKEEIKE	1/2	4.550
P47	PFA0635c	3D7:		
		NHDTRINDYNKRLTEYNKRLT**E**YNKRLTEYTKRLNE		3.520
		Hb3:		
		NHDTRINDYNKRLTEYNKRLT**A**YNKRLTEYTKRLNE	1/9	3.320
P50	PFL1605w	3D7:		
		KNDINVQLDDINVQLDDIN**V**QLDDIN**I**QLDEINLN		3.290
		7G8:		
		KNDINVQLDDINVQLDDIN**I**QLDDINIQLDEINLN	1/10	3.270
		D10:		
		KNDINVQLDDINVQLDDINVQLDDIN**V**QLDEINLN	1/13	3.310
P51	PFD0985w	3D7:		
		DNNVNN**M**DNNVNNVDNNVNNVDNNLNNVDNNVNN		3.950
		Dd2:		
		DNNVNN**V**DNNVNNVDNNVNNVDNNLNNVDNNVNN	1/11	3.900
P54	MAL6P1.147	3D7:		
		DSMNNHKDDMNNYNDNINNYVESMNNYDDIMNK		2.470

Table 4. cont.

Peptide	Protein	Sequence	Frequency	Score [a]
		FCC-2:		
		D**N**MNNHKDDMNNYNDNINNYVESMNNYDDIMNK	1/9	2.470
		GHANA1:		
		NS**N**NNHKDDMNNYNDNINNYVESMNNYDDIMNK	1/9	**1.920**
P64	MAL6P1.131	3D7:		
		NNFVNNKMNNMNNMKNNMNNMNNIMNN**I**MN		3.100
		FCC-2:		
		NNFVNNKMNNMNNMKNNMNNMNNIMNN**N**MN	1/8	2.650
P81	PF07_0086	3D7:		
		NEMNKEVNKMNEEVNKMNEEVNKMN**E**EVNKM**NK**EVNKMDEEVNKMN		4.580
		D10;D6;GHANA1;Santa Lucia:		
		NEMNKEVNKMNEEVNKMNEEVNKMNEEVNKMN**E**EVNKMDEEVNKMN	4/9	4.580
		GHANA1:		
		NEMNKEVNKMNEEVNKMNEEVNKMN**K**EVNKM**D**KEVNKMDEEVNKMN	1/7	4.580
P83	PFC0345w	3D7:		
		QNKMENDMNIIKNDMNI**M**ENDMNIMENDMNIIKNDMNI**M**E		3.250
		KDMNIIKNDMNIIKNNMNIIKN**E**MNIIKNV		
		D10;FCC-2;V1_S:		
		QNKMENDMNIIKNDMNI**K**ENDMNIMENDMNIIKNDMNIME	4/9	3.220
		KDMNIIKNDMNIIKNNMNIIKNEMNIIKNV		
		RO-33:		
		QNKMENDMNIIKNDMNI**K**ENDMNIMENDMNIIKNDMNI**I**E	1/10	3.200
		KDMNIIKNDMNIIKNNMNIIKN**D**MNIIKNV		
P86	PFL1930w	3D7:		
		NFIKELELQIKNLNNEI**N**TLNDMLKDSEEEIRMLNHTLEEK		3.710
		7G8:		
		NFIKELELQIKNLNNEI**K**TLNDMLKDSEEEIRMLNHTLEEK	1/11	3.760
P94	PFD0970c	3D7:		
		ENINNMDEK**I**N**N**VDEQNNN**M**DEKINNVDEK**K**		3.460
		FCC-2:		
		ENINNMDEKINNVDEQNNNMDEKINNVDEK**I**	1/6	3.460
		D10;K1:		
		ENINNMDEK**K**N**K**VDEQNNNMDEKINNVDEKK	4/7	2.990
		D6;FCC-2:		
		ENINNMDEKIN**K**VDEQNNNMDEKINNVDEKK	3/5	2.970
		RO-33:		
		ENINNMDEKIN**K**VDEQNNN**I**DEKINNVDEKK	3/6	3.480
P97	PFL0650c	3D7:		
		DTANNVSEMQKIIHTFSLDIKDFKILFEALTKSIQLLNDN		3.390
		IENINKEIEL**L**KKKIST		
		FCC-2:		
		DTANNVSEMQKIIHTFSLDIKDFKILFEALTKSIQLLNDN	1/8	3.140
		IENINKEIEL**F**KKKIST		
P103	PFE0840c	3D7:		
		NNMNNNININAYN**I**LKDINKYRNIVNNLDH		2.310
		Dd2;FCC-2		
		NNMNNNININAYN**V**LKDINKYRNIVNNLDH	2/7	2.310
P116	PF13_0182	3D7:		
		RISNILFMLNNIKKEIENVKENILKYID**K**		2.570
		106_1;7G8;GHANA1;IT;Senegal3404;V1_S		

Table 4. cont.

Peptide	Protein	Sequence	Frequency	Score [a]
		RISNILFMLNNIKKEIENVKENILKYID**R**	6/11	2.570
P119	PF11_0240	3D7:		
		DNVEELNKDIE**N**LNNEIHEIEKMWLFIKK		3.010
		GHANA1:		
		DNVEELNKDI**E**ILNNEIHEIEKMWLFIKK	1/8	2.770
P122	PFI1475w	3D7:		
		Y**DLS**IYNKQLEEAHNLISVLEKRIDTLKK		2.930
		D10;Dd2;FCC-2		
		Y**N**LSIYNKQLEEAHNLISVLEKRIDTLKK	3/16	2.640
		7G8;D10;Dd2;FCC-2;K1;RO-33;V1_S		
		YDL**F**IYNKQLEEAHNLISVLEKRIDTLKK	7/16	2.640
P134	PF14_0535	3D7:		
		KQIDLNINNIDD**NK**NNIDDHINNIDD		2.750
		7G8:		
		KQIDLNINNIDD**HI**NNIDDHINNIDD	1/7	3.060
P144	PF13_0210	3D7:		
		HDVNNYQHDVNNYQQDVNNYQHDVNNYQHDVNNY**N**H		2.470
		Dd2;Hb3;RO-33;Senegal3403;V1_S		
		HDVNNYQHDVNNYQQDVNNYQHDVNNYQHDVNNY**HH**	4/5	2.470
P149	MAL13P1.252	3D7:		
		NLALNNLRNKLKLLQEDYNDLEKEYLYLKK**S**LSK		3.350
		Dd2:		
		NLALNNLRNKLKLLQEDYNDLEKEYLYLKK**G**LSK	1/8	3.350
P154	PFL1205c	3D7:		
		QFIILMVKLLSNVYEI**V**KKINSLNNNYTYLYIN		2.680
		7G8;D10;FCB;FCC2;GHANA1;Hb3;K1;Senegal3404		
		QFIILMVKLLSNVYEI**E**KKINSLNNNYTYLYIN	7/8	3.030
P156	PFD1030c	3D7:		
		KINSLKENIENMFDRIDNYIH**E**VISLKNFVNQ		2.820
		K1:		
		KINSLKENIENMFDRIDNYIH**V**VISLKNFVNQ	1/8	2.460
P160	PF11_0268	3D7:		
		MNISEFETKITELKEDINKINFMMK**D**FESKF		3.170
		7G8;D10;D6;Dd2;FCC-2;FCR3;GHANA1;Hb3;IT;K1;RO-33;Senegal3404;V1_S:		
		MNISEFETKITELKEDINKINFMMK**A**FESKF	13/13	3.020
P166	PFI1180w	3D7:		
		Y**Y**NNFDNYNNNFDNYNNNFDNYNNNFDNYN		2.420
		GHANA1:		
		Y**N**NNFDNYNNNFDNYNNNFDNYNNNFDNYN	1/9	2.420

[a]profile score for α-helical coiled coil formation;
[b]profile score was manually analyzed.
Positions of SNPs are indicated in bold.
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required for supplementing the vaccine pipeline. There is a great demand to identify new antigens that are both, immunogenic and conserved. It remains to be shown whether the strategy to include non-polymorphic antigens in a vaccine formulation will increase protection.

Supporting Information

Table S1
Found at: doi:10.1371/journal.pone.0005419.s001 (0.04 MB DOC)

Author Contributions

Conceived and designed the experiments: GC IF. Performed the experiments: CK. Analyzed the data: CK AVK GC IF. Contributed reagents/materials/analysis tools: AVK IF. Wrote the paper: CK AVK GC IF.

References

1. Good MF (2005) Vaccine-induced immunity to malaria parasites and the need for novel strategies. Trends Parasitol 21: 29–34.
2. Kemp DJ, Coppel RL, Anders RF (1987) Repetitive proteins and genes of malaria. Annu Rev Microbiol 41: 181–208.
3. Mu J, Awadalla P, Duan J, McGee KM, Keebler J, et al. (2007) Genome-wide variation and identification of vaccine targets in the Plasmodium falciparum genome. Nat Genet 39: 126–130.
4. Saul A, Fay MP (2007) Human immunity and the design of multi-component, single target vaccines. PLoS ONE 2: e850.
5. Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, et al. (2002) A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J Infect Dis 185: 820–827.
6. Fluck C, Smith T, Beck HP, Irion A, Betuela I, et al. (2004) Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun 72: 6300–6305.
7. Lyon JA, Angov E, Fay MP, Sullivan JS, Girourd AS, et al. (2008) Protection induced by Plasmodium falciparum MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. PLoS ONE 3: e2830.
8. Enosse S, Dobano C, Quelhas D, Aponte JJ, Lievens M, et al. (2006) RTS,S/AS02A malaria vaccine does not induce parasite CSP T cell epitope selection and reduces multiplicity of infection. PLoS Clin Trials 1: e5.
9. Saul A, Lawrence G, Smillie A, Rzepczyk CM, Reed C, et al. (1999) Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. Vaccine 17: 3145–3159.
10. Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, et al. (2007) Plasmodium falciparum merozoite surface protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection. J Infect Dis 195: 279–287.
11. Plebanski M, Lee EA, Hill AV (1997) Immune evasion in malaria: altered peptide ligands of the circumsporozoite protein. Parasitology 115 Suppl: S55–66.
12. Pouniotis DS, Proudfoot O, Minigo G, Hanley JL, Plebanski M (2004) Malaria parasite interactions with the human host. J Postgrad Med 50: 30–34.
13. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, et al. (2007) Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS ONE 2: e645.
14. Corradin G, Villard V, Kajava AV (2007) Protein structure based strategies for antigen discovery and vaccine development against malaria and other pathogens. Endocr Metab Immune Disord Drug Targets 7: 259–265.
15. Lu SM, Hodges RS (2002) A de novo designed template for generating conformation-specific antibodies that recognize alpha-helices in proteins. J Biol Chem 277: 23515–23524.
16. Walshaw J, Woolfson DN (2001) Open-and-shut cases in coiled-coil assembly: alpha-sheets and alpha-cylinders. Protein Sci 10: 668–673.
17. Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol 11: 82–88.
18. Jafarshad A, Dziegiel MH, Lundquist R, Nielsen LK, Singh S, et al. (2007) A novel antibody-dependent cellular cytotoxicity mechanism involved in defense against malaria requires costimulation of monocytes FcgammaRII and FcgammaRIII. J Immunol 178: 3099–3106.
19. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P (1995) Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages. J Exp Med 182: 409–418.
20. Trager W, Jensen JB (1997) Continuous culture of Plasmodium falciparum: its impact on malaria research. Int J Parasitol 27: 989–1006.
21. Irion A, Felger I, Abdulla S, Smith T, Mull R, et al. (1998) Distinction of recrudescences from new infections by PCR-RFLP analysis in a comparative trial of CGP 56 697 and chloroquine in Tanzanian children. Trop Med Int Health 3: 490–497.
22. Marfurt J, Mueller I, Sie A, Maku P, Goroti M, et al. (2007) Low efficacy of amodiaquine or chloroquine plus sulfadoxine-pyrimethamine against Plasmodium falciparum and P. vivax malaria in Papua New Guinea. Am J Trop Med Hyg 77: 947–954.
23. Bucher P, Karplus K, Moeri N, Hofmann K (1996) A flexible motif search technique based on generalized profiles. Comput Chem 20: 3–23.
24. Berman H, Henrick K, Nakamura H (2003) Announcing the worldwide Protein Data Bank. Nat Struct Biol 10: 980.
25. Volkman SK, Sabeti PC, DeCaprio D, Neafsey DE, Schaffner SF, et al. (2007) A genome-wide map of diversity in Plasmodium falciparum. Nat Genet 39: 113–119.
26. Franks S, Baton L, Tetteh K, Tongren E, Dewin D, et al. (2003) Genetic diversity and antigenic polymorphism in Plasmodium falciparum: extensive serological cross-reactivity between allelic variants of merozoite surface protein 2. Infect Immun 71: 3485–3495.
27. Conway DJ, Cavanagh DR, Tanabe K, Roper C, Mikes ZS, et al. (2000) A principal target of human immunity to malaria identified by molecular population genetic and immunological analyses. Nat Med 6: 689–692.
28. Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, et al. (2007) Naturally acquired antibodies to polymorphic and conserved epitopes of Plasmodium falciparum merozoite surface protein 3. Parasite Immunol 29: 387–394.
29. Druilhe P, Spertini F, Soesoe D, Corradin G, Mejia P, et al. (2005) A malaria vaccine that elicits in humans antibodies able to kill Plasmodium falciparum. PLoS Med 2: e344.
30. Singh S, Soe S, Mejia JP, Roussilhon C, Theisen M, et al. (2004) Identification of a conserved region of Plasmodium falciparum MSP3 targeted by biologically active antibodies to improve vaccine design. J Infect Dis 190: 1010–1018.
31. Lamb D, Schuttelkopf AW, van Aalten DM, Brighty DW (2008) Highly specific inhibition of leukaemia virus membrane fusion by interaction of peptide antagonists with a conserved region of the coiled coil of envelope. Retrovirology 5: 70.
32. Bai T, Becker M, Gupta A, Strike P, Murphy VJ, et al. (2005) Structure of AMA1 from Plasmodium falciparum reveals a clustering of polymorphisms that surround a conserved hydrophobic pocket. Proc Natl Acad Sci U S A 102: 12736–12741.
33. Crewther PE, Matthew ML, Flegg RH, Anders RF (1996) Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strain-specific epitopes. Infect Immun 64: 3310–3317.
34. Felger I, Irion A, Steiger S, Beck HP (1999) Genotypes of merozoite surface protein 2 of Plasmodium falciparum in Tanzania. Trans R Soc Trop Med Hyg 93 Suppl 1: 3–9.
35. Tetteh KK, Cavanagh DR, Corran P, Musonda R, McBride JS, et al. (2005) Extensive antigenic polymorphism within the repeat sequence of the Plasmodium falciparum merozoite surface protein 1 block 2 is incorporated in a minimal polyvalent immunogen. Infect Immun 73: 5928–5935.
36. Vekemans J, Ballou WR (2008) Plasmodium falciparum malaria vaccines in development. Expert Rev Vaccines 7: 223–240.
37. Genton B (2008) Malaria vaccines: a toy for travelers or a tool for eradication? Expert Rev Vaccines 7: 597–611.
38. Bejon P, Mwacharo J, Kai OK, Todryk S, Keating S, et al. (2006) Immunogenicity of the candidate malaria vaccines FP9 and modified vaccinia virus Ankara encoding the pre-erythrocytic antigen ME-TRAP in 1–6 year old children in a malaria endemic area. Vaccine 24: 4709–4715.

CHAPTER 3

Vaccine potentials of an intrinsically unstructured fragment derived from the blood stage-associated Plasmodium falciparum protein PFF0165c.

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Vaccine Potentials of an Intrinsically Unstructured Fragment Derived from the Blood Stage-Associated *Plasmodium falciparum* Protein PFF0165c▽

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We have identified new malaria vaccine candidates through the combination of bioinformatics prediction of stable protein domains in the *Plasmodium falciparum* genome, chemical synthesis of polypeptides, in vitro biological functional assays, and association of an antigen-specific antibody response with protection against clinical malaria. Within the predicted open reading frame of *P. falciparum* hypothetical protein PFF0165c, several segments with low hydrophobic amino acid content, which are likely to be intrinsically unstructured, were identified. The synthetic peptide corresponding to one such segment (P27A) was well recognized by sera and peripheral blood mononuclear cells of adults living in different regions where malaria is endemic. High antibody titers were induced in different strains of mice and in rabbits immunized with the polypeptide formulated with different adjuvants. These antibodies recognized native epitopes in *P. falciparum*-infected erythrocytes, formed distinct bands in Western blots, and were inhibitory in an in vitro antibody-dependent cellular inhibition parasite-growth assay. The immunological properties of P27A, together with its low polymorphism and association with clinical protection from malaria in humans, warrant its further development as a malaria vaccine candidate.

Attempts to develop an effective malaria vaccine have focused on only a few malaria proteins out of the possible 5,400 predicted in the *Plasmodium falciparum* genome (36). The publication of the genome (16) is, however, expected to stimulate the development of new vaccines, drugs, and other interventions for the control of malaria infection.

In our earlier published work (34), we described a new approach for the identification of novel malaria vaccine candidates through a genome-wide search for proteins with predicted α-helical coiled-coil motifs. These protein segments are known to be stable as isolated fragments, and some of the malaria vaccine candidates under development contain such segments (34). The synthetic polypeptides were recognized by sera of adults living in different regions where malaria is endemic, and affinity-purified human antibodies specific for some of them were found to be active in antibody-dependent cellular inhibition (ADCI) assays. Some of them also induced high antibody titers in mice, and both the peptide-specific purified human antibodies and mouse sera recognized native epitopes present in *P. falciparum* 3D7-infected erythrocytes. One of the most promising peptides identified was peptide 27 (P27), which is derived from the hypothetical protein PFF0165c that consists of 1,103 amino acids (16). Since P27 contains only 27 amino acids and activated only 30% of peripheral blood mononuclear cells (PBMC) from human semi-immune donors (unpublished data), it is probable that coverage of the general population will not be obtained when it is used as a vaccine. Thus, we proceeded to screen the entire PFF0165c protein for intrinsically unstructured protein domains which could be easily mimicked and rapidly obtained through peptide synthesis for evaluation of their vaccine potential.

It was previously assumed that the functions of proteins are closely linked to their three-dimensional structure. However, in the post-genome era, many gene sequences appear to code for proteins or long stretches of amino acids that are likely to be unfolded (nonglobular) in solution (14, 37). They are identified by their low content of bulky hydrophobic amino acids, which normally form the core of folded globular proteins. Two malaria vaccine candidates currently being evaluated (MSP2 and MSP3) have been found to contain such segments (26, 38, 39), and they have both been shown to be safe and immunogenic in human trials (4, 17).

In the case of the PFF165c protein, it was found that it contains at least three segments that are low in hydrophobic amino acid content and high in hydrophilic amino acid content and thus are likely to be intrinsically unstructured. The work presented here focuses on one of these unstructured domains, the 104-residue-long peptide P27A, which was the largest one and did not contain long stretches of identical amino acids (poly-Glu or poly-Asn; Fig. 1).

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MATERIALS AND METHODS

Peptide synthesis. The synthetic P27A polypeptide sequence with 104 amino acids was produced by solid-phase 9-fluorenylmethoxycarbonyl chemistry accord-

Protein PFF0165c

MSNKKRSKNENDESTSLPLENSELLIEYIHNLKSCLNVYRREIQEKNKYISIIKNDLSFHEC
ILTNVNVVWSVFNNDLLNLLCNNEQKEEGEEIIKQRNIGDEINEYNNLTKLQNDENIKNNNM
IKEDLEDDANQNILMKSPYYNIENFLQVFLKYINKKKKKVKVKVKDEGKKEKIEDKKYEQDD
EEENEEEEEEEEEEEGEEENKEDEEFFKTFVSFNLYHNNNEKNISYDKNLVKQENDNKDEAR
GNDNMCGNYDIHNERGEMLDKGKSYSGDEKINTSDNAKSCSGDEKVITSDNGKSYDYVKNES
EEQEEKENMLNNKKRSLECNPNEAKKICFSLEEKIGTVQSVKLKEYNELSKENIEKNKHDDN
NICNYLSHNEGENVIEREDKLFNKLNNKNYRNEEEKKKNQINFDYLKKKIKNNQDVFEETIQ
KCFLINLKKTLNLINKIMYLKNVEFRKYNLDYIRKINYEKCFYYKNYIDIKKKISELQKDNE
SLKIQVDRLEKKKATLIYKLNNDNIRKHILDNNIKDYQNGIDNSKVSYFDEGENPYNRNNKN
YRTDNKNSDDNNNNNNYYYNNYNSDDNYNSEDNEYNNGNYRFRNNYKKDSLNEDDVKKNPLK
VCHKINSDSNIFVNFENIITKQNIIHSEPFRNLLKESNELYITLKEKEKENIILKNEILKME
NKKDEEYEHLLNNTIEDKKELTRSIKELEINMMTCNMEKDKISNKVNTLEYEINVLKNIDKN
QTMQLQQKENDILKMKLYIEKLKLSEKNLKDKIILLENEKDKMLSGIHIKDNSFNEESKSEE
GKIQLRDIQNDNDEKYDDEKKRFKELFIENQKLKEELNKKRNVEEELHSLRKNYNIINEEIE
EITKEFEKKQEQVDEMILQIKNKELELLDKFNNKMNKAYVEEKLKELKNTYEEKMKHINNIY
KKHDDFVNIYLNLFFQARKNAILSDSQREEQMNLFIKLKDKYDIIFQKKIELTDILKNVYDC
NKKLIGHCQDLEKENSTLQNKLSNEIKNSKMLSKNLSKNSDDHLLIEENNELRRRLICSVCM
ENFRNYIIIKCGHIYCNNCIFNNLKTRNRKCPQCKVPFDKKDLQKIFLD

Schematic Representation

FIG. 1. Protein sequence and schematic representation of the PFF0165c protein. The protein sequence is 1,103 amino acids in length. Amino acids in blue form the unstructured region that was synthesized, shown by the blue block in the schematic representation. Residue 297, where a n E/G mutation occurs, is underlined. Amino acids in red form the α-helical coiled-coil region, shown in red in the schematic representation.

ing to the method of Atherton and Sheppard (3) as modified by Verdini et al. (33), using Applied Biosystems 431A and 433A synthesizers (Foster City, CA). The final product was cleaved from *p*-alkoxybenzylalcohol resin and then reverse-phase high-pressure liquid chromatography (HPLC) purified. The purity was assessed by analytic C_{18} HPLC and matrix-assisted laser desorption ionization–time of flight mass spectrometry (Applied Biosystem) and was found to be higher than 85%. All the reagents used were purchased from Fluka (Buchs, Switzerland) and Novabiochem (Laufelfingen, Switzerland).

A pilot industrial synthesis of the construct was later prepared by Almac Sciences, Craigavon, Northern Ireland. The purity of the product was 98% as judged by analytical HPLC (Fig. 2A and B).

Circular dichroism (CD) spectroscopy. The CD spectrum of P27A was evaluated on a Jasco J-810 CD spectrometer. The measurements were taken over a wavelength range of 190 to 250 nm at 20°C and pH 7.3 with a 1-mm-path-length cuvette. The polypeptide was dissolved in water or phosphate-buffered saline (PBS) at a concentration of 150 μg/ml.

Human sera. Serum samples were collected from the regions of Burkina Faso and Tanzania, where malaria is endemic, and were the same samples used in our previous study (34). Briefly, the samples from Burkina Faso were collected from donors living in the village of Goundry in the province of Oubritenga ($n = 37$). Ethical approval was obtained from the Ministry of Health, Burkina Faso. The Tanzanian samples were obtained from donors in Kikwawila village in the Morogoro region ($n = 42$). Ethical approval was obtained from the Tanzanian Commission for Science and Technology. Negative control samples were Swiss adult donors with no history of malaria and no previous travel to areas where malaria is endemic ($n = 6$). Additional serum samples obtained from Papua New Guinea were collected in the Maprik District of the East Sepik Province during a cross-sectional survey in July 1992 within the framework of the Malaria Vaccine Epidemiology and Evaluation Project (MVEEP), supported by the United States Agency for International Development. Malaria is highly endemic in the area. Ethical clearance for MVEEP was obtained from the Papua New Guinea Medical Research Advisory Committee. Blood samples were obtained by venipuncture into tubes containing EDTA. Sera of 20 of these subjects were pooled and used as the positive control for enzyme-linked immunosorbent assays (ELISAs).

Human PBMC. PBMC were obtained from adult donors living in Lagos, southwest Nigeria, where malaria transmission is high all year, with seasonal peaks during the rainy season ($n = 17$). Ethical approval was obtained from the Lagos State University Teaching Hospital (LASUTH) ethical review committee.

Affinity-purified human antibodies. For the antigen-Sepharose conjugate preparation, 2 to 5 mg of antigen was dissolved in 1 ml of coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.0). The CNBr-Sepharose 4B (Amersham Bioscience AB, Uppsala, Sweden) was swollen in 1 mM HCl and then washed with coupling buffer. The antigen solution was added to the gel, and the mixture was stirred for 1 h at room temperature (RT). After the coupling reaction, excess antigen was washed away with coupling buffer. The remaining activated groups were blocked by treatment with ethanolamine (0.25 M, pH 8.0) for 30 min at RT. The gel was then washed with sodium acetate buffer (0.1 M, pH 4.0), followed by coupling buffer. The antigen-Sepharose beads were either used immediately or stored at 4°C in PBS (1×) containing 1 mM azide.

For the isolation of specific antibody, pooled human or rabbit sera were diluted five times with PBS (1×) containing 0.5 M sodium chloride and mixed with the antigen-Sepharose conjugate. This mixture was then stirred gently on a wheel overnight at 4°C. After centrifugation, the supernatant was collected and stored at −20°C for further use. The antigen-Sepharose beads were washed first with 5 ml of Trizma base Tris (20 mM containing 0.5 M NaCl, pH 8.0) and then with 5 ml of Tris (20 mM, pH 8.0). The elution of bound antibody was achieved with glycine (0.1 M, pH 2.5). The fractions obtained were instantly neutralized with Tris (1 M, pH 8.0) and dialyzed against phosphate buffer (0.1 M, pH 7.0), and the antibody concentration was determined by the absorbance of the solution at 280 nm.

ELISA. Antigenic recognition of P27A by human, mouse, and rabbit sera was assessed as previously described (34). Briefly, each 96-well microtiter plate (Maxisorb F96; Nunc, Roskilde, Denmark) was coated with 1 μg/ml of polypeptide solution and incubated overnight in a humid chamber at 4°C. Serum solutions (50 μl/well) were added (1:200 dilution for human samples; 1 to 3 serial dilutions starting from 1:100 for mouse/rabbit sera) and incubated for 1 h. The secondary antibodies used were anti-human immunoglobulin G (IgG), anti-mouse polyclonal Ig, or anti-rabbit polyclonal Ig conjugated to alkaline phosphatase (Sigma, St. Louis, MO). Optical density (OD) was measured at 405 nm with a Thermo Labsystems Multiskan Ascent. A sample was considered positive when the OD was higher than the mean OD of the negative controls plus 3 standard deviations (SD). Negative controls were sera obtained from the naïve animals prior to

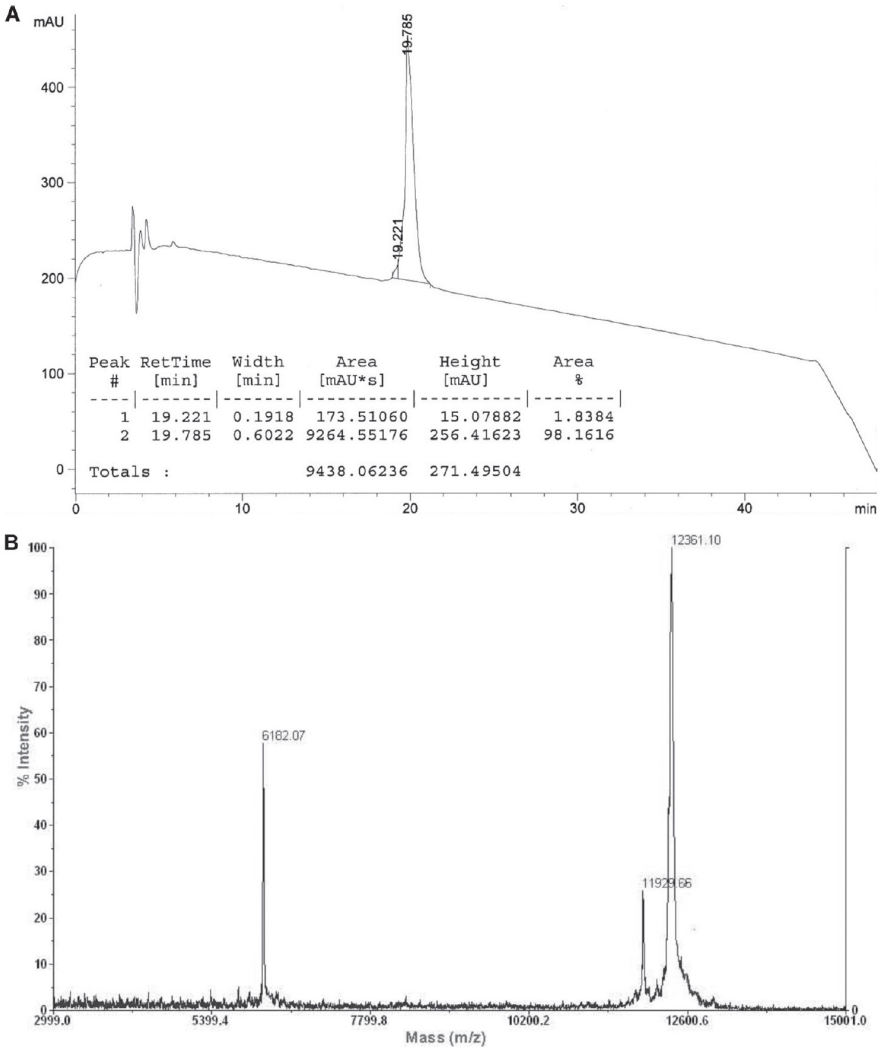

FIG. 2. (A) Analytical HPLC of P27A. mAU, milli-absorbance unit, #, number. (B) Mass spectrum of P27A. Peaks at 12,361.10 and 6,182.07 represent the cytochrome *c* mass plus 1 or 2 hydrogen ions, respectively, taken as the standard. The peak at 11,929.66 represents the mass of P27A.

immunization. For human IgG1 and IgG3 isotypes, anti-human Ig1 (Skybio) and IgG3 (Immunotech) were used at dilutions of 1:2,000 and 1:10,000, respectively. A third horseradish peroxidase-labeled goat anti-mouse antibody was used at 1:4,000, and the OD was read at 492 nm after color development. For mouse IgG isotypes, secondary antibodies were used at dilutions of 1:1,000 (IgG1), 1:5,000 (IgG2a), and 1:2,000 (IgG2b). The color development was stopped with 1 M H_2SO_4, and the OD was read at 492 nm.

T-cell proliferation. PBMC were isolated from venous blood of 17 healthy adult Nigerians by Ficoll gradient centrifugation and stored in liquid nitrogen until use. Five replicates containing 2×10^5 cells per well were put in culture with 3 μM of P27A solution. The complete culture medium used consisted of RPMI medium (Sigma) supplemented with glutamine, 100 IU/ml penicillin-streptomycin, 100 μM nonessential amino acids, 100 μg/ml kanamycin (Invitrogen, Paisley, United Kingdom), 2 mM sodium pyruvate (Invitrogen), and 8% human AB serum (Blutspendendienst SRK, Bern, Switzerland). The positive control was a mixture of tetanus toxoid (Pasteur Merieux, Lyon, France), purified protein derivative (SSI, Copenhagen, Denmark), and *Candida albicans* extract (NIBSC, London, United Kingdom).

On day 5, the cultures were pulsed with 1 μCi of [^{3}H]thymidine dissolved in culture medium. The cells were harvested 24 h later, and the tracer incorporation rate was measured by beta counting. The stimulation index (SI) was calculated with the formula (average count/minutes in culture with antigen)/(average count/minutes in culture without antigens). An SI value of ≥2 was considered positive.

Immunizations of mice and rabbits. ICR (outbred) and C3H ($H\text{-}2^k$) mice were injected three times with P27A (20 μg/mouse) formulated with Alhydrogel (500 μg/mouse) or EM005 (20 μg/mouse), a newly developed adjuvant (IDRI, Seattle, WA), on days 0, 21, and 42. Each mouse was injected with 500 μl of the formulation intraperitoneally (Alhydrogel) or 100 μl subcutaneously (EM005). Blood samples were collected from the base of the tail 10 days after second and third injections. Rabbits (four per group) were immunized intramuscularly with P27A (100 μg) with Alhydrogel or EM005 three times at 3-week intervals and bled at baseline and 10 days after each injection. The rabbit experiments were carried out by Eurogentec, Seraing, Belgium. Negative control animals used for ELISA analysis were unimmunized animals.

Indirect immunofluorescence antibody test. For indirect immunofluorescence microscopy, infected red blood cells (iRBCs) were fixed in paraformaldehyde-glutaraldehyde as described previously (30) or smeared onto glass slides and fixed in ice-cold acetone/methanol (1:1 [vol/vol]) for 10 min. Slides were blocked for 1 h with 3% bovine serum albumin–PBS and probed with one of the following primary or secondary antibodies: mouse anti-P27A (1:200), rabbit anti-P27A (1:1,000), human affinity-purified antibodies specific for P27A (1:1,000), Cy3-labeled anti-mouse IgG (1:500), fluorescein isothiocyanate-labeled anti-human IgG (1:500; Jackson ImmunoResearch, Suffolk, United Kingdom), and Alexa Fluor 488-conjugated anti-rabbit IgG (1:200; Invitrogen). The slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) supplemented with 4′,6′-diamidino-2-phenylindole (DAPI) for nucleus staining. Fluorescence microscopy was performed on a Leica DM-5000B using a 60× oil immersion objective lens and documented with a Leica DC200 digital camera system.

ADCI assay. The inhibition of *P. falciparum* (3D7 strain) growth in vitro in the presence of human monocytes (MN) and antigen-specific antibodies was carried out using methods described elsewhere (7). In brief, PBMC isolated from healthy (malaria naïve) blood donors were separated by Ficoll-Hypaque gradient, and MN were further separated by adherence to plastic surfaces as described previously (1 h at 37°C). Mature schizonts from a synchronized parasite culture were diluted at a starting parasitemia of 0.5% in human type AB^+ RBCs from healthy donors, and the hematocrit was adjusted to 2% with RPMI 1640 culture medium. Duplicate assays were set up in preheated 96-well flat-bottom sterile plastic plates (TPP, Trasadingen, Switzerland) containing 2×10^5 MN/well with the addition of 50 μl of parasite culture mixed with 50 μl of RPMI and various dilutions of each pool of sera at final concentrations of 0.5% to 10%. Control wells with parasite culture and RPMI medium were done in parallel. The plates were incubated in a candle jar at 37°C in a 5% CO_2 incubator for 96 h. Thin blood smears for each well were fixed in methanol and stained in eosin and methylene blue. The parasitemia was determined by microscopic examination and counting of at least 5,000 RBCs in duplicate. The specific growth inhibitory index (SGI), which estimates the parasite growth inhibition due to the effect of test antibodies cooperating with MN, was calculated as follows: SGI = 100 × [1 − (% parasitemia with MN and test antibodies/% parasitemia with test antibodies)/(% parasitemia with MN and IgGs from healthy donors/% parasitemia with IgGs from healthy donors)].

For each antibody tested, duplicate wells included the following controls: (i) for nonspecific monocytic inhibition, both MN plus parasite and MN plus IgGs from healthy donors plus parasites, and (ii) for direct inhibition by control or test IgGs, both IgGs from healthy donors plus parasites and test antibodies plus parasites. A pool of IgGs from sera of hyperimmune African donors and IgGs from healthy donors were used at a final concentration of 1 mg/ml as positive and negative controls, respectively. Immunopurified test human antibodies were used at 15 μg/ml, while mouse sera were used at different dilutions.

Parasite culture. The culture strains were grown in 10-cm petri dishes and cultured by standard methods in an atmosphere of 93% N_2, 4% CO_2, 3% O_2 at 37°C as described previously (31). The culture medium was 10.44 g/liter RPMI 1640 supplemented with 5.94 g/liter HEPES, 5 g/liter Albumax II, 50 mg/liter hypoxanthine, 2.1 g/liter sodium bicarbonate, and 100 mg/liter neomycin.

Preparation of parasite protein extracts. The protein extracts of late-stage parasites, trophozoites and schizonts, were obtained from *P. falciparum* 3D7-infected erythrocytes in a 30-ml petri dish (5% hematocrit, 6% parasitemia) which was enriched using a magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched infected erythrocytes were lysed in a 200-ml volume of PBS, 0.03% saponin (Fluka) in the presence of proteinase inhibitors (Roche Diagnostics, Basel, Switzerland) for 5 min at 4°C. The parasites were pelleted by centrifugation at 4,000 × *g* for 10 min, the supernatant was removed, and an equal volume of 2× Laemmli sample buffer was added. The parasite pellet was resuspended in 0.1 M Tris, pH 6.8, and an equal volume of 2× Laemmli sample buffer.

Western blot analysis. Protein extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose (0.2 mm; Whatman Schleicher + Schuell, Florham Park, NJ) under cooling conditions for 1 h at 80 V and an additional hour at 100 V. The membrane was blocked for 1 h in PBS–3% milk powder at RT, and the primary antibody was diluted 1:500 in PBS and 1.5% milk powder. The membrane was incubated in the primary antibody solution overnight at 4°C. It was then washed 8× in PBS, 0.05% Tween 20 and incubated with a peroxidase-conjugated goat anti-mouse IgG antibody (1:3,000, Sigma). Bound secondary antibodies were visualized using Western Lightning (PerkinElmer Life Sciences, Schwerzenbach, Switzerland).

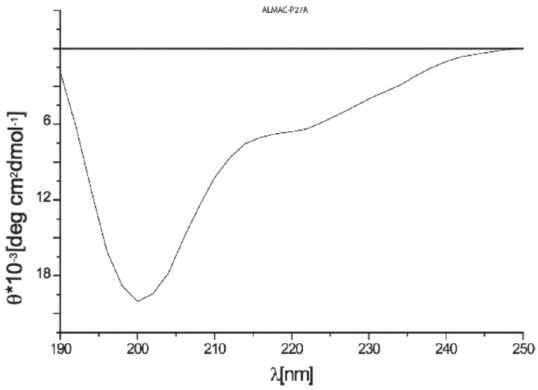

FIG. 3. The CD spectrum of P27A was assessed over a 190- to 250-nm-wavelength range at 20°C, pH 7.3, in a 150-μg/ml solution, using a Jasco J-810 spectrometer. There is a single dip at around 200 nm, which is indicative of random coil conformation.

Polymorphism study of *P. falciparum* in vitro culture strains and field samples. The genetic diversity of the P27A sequence was assessed in 13 *P. falciparum* in vitro culture strains (3D7, W2mef, HB3, ITG2F6, IFA18, FVO, 7G8, K1, RO33, MAD20, FCR3, RFCR3, and FC27) and malaria-positive field samples from Tanzania and Papua New Guinea. Sixty-three blood samples were obtained from 1- to 5-year-old children from Ifakara, Tanzania, with uncomplicated malaria (18). Nineteen samples were from asymptomatic donors living in Papua New Guinea (29). Genomic DNA was isolated by phenol-chloroform extraction or with a QIAamp DNA blood mini kit 250 (Qiagen, Hombrechtikon, Switzerland). Forward primer 5′-ACACTTTGCACAGTTCCTATCTTCTCTTCTA-3′ and reverse primer 5′-AGAAGGAGAAGAAGAAAATAAAGAGGATGAAG-3′ were used to amplify the P27A region from genomic parasite DNA. PCR conditions consisted of denaturation at 94°C for 5 min followed by 35 cycles of denaturation (94°C for 30 s), annealing (58°C for 1 min), and extension (72°C for 1 min). The reaction products were incubated at 72°C for 10 min to ensure complete DNA extension. The PCR products were directly sequenced on both strands and aligned with Auto Assembler software to screen for single nucleotide polymorphisms (SNPs) and length polymorphisms within the sequence corresponding to P27A.

RESULTS AND DISCUSSION

Only a few malaria antigens are currently being evaluated as candidate vaccines (23, 25). The published *P. falciparum* genomic data (16) provide ample opportunities for the discovery of new malaria antigens. Our group previously explored this publicly available data to identify novel malaria antigens through the combination of chemical peptide synthesis, natural human immunity, and biological functional assays (10, 34). Exploration of the structural domains present within one of the hypothetical proteins, PFF0165c, identified by the α-helical coiled-coil P27 (34) revealed several segments with low hydrophobic amino acid content. Such segments have been found to be functional and to fold on binding to their targets (13). The *P. falciparum* genome is predicted to contain numerous such

TABLE 1. Prevalence of total IgG and cytophilic IgG1/IgG3 among adult donors living in regions where malaria is endemic

Ig class	No. (%) of donors with indicated Ig[a] from:		
	Burkina Faso (n = 37)	Tanzania (n = 42)	Papua New Guinea (n = 56)
Total IgG	>28 (76)	32 (76)	53 (95)
IgG1	25 (68)	30 (71)	50 (89)
IgG3	28 (76)	31 (73)	52 (93)

[a] Positive samples were those with OD values greater than the average of the OD values of negative controls (Swiss adults with no history of malaria) plus 3 SD.

unstructured proteins and segments (15). Similar predicted unstructured segments (or full-length proteins) have been found in the genomes of other organisms (37), and the frequency likely increases with increasing complexity of organisms.

We chemically synthesized one such segment (P27A) that consists of amino acid residues 222 to 326 of PFF0165c and explored its malaria vaccine potential. This choice was dictated by several factors which took into account the feasibility of chemical synthesis of the fragment; the necessity to contain a maximum number of T- and B-cell epitopes in order to be immunogenic in the majority of the target population; and the lack of stretches containing poly-glutamic acid or poly-asparagine residues, potentially dangerous when present in a vaccine (Fig. 1). The advantage of choosing unstructured regions of proteins is that no folding procedure is required to mimic the native conformation. As expected, the CD pattern of P27A solution in water or PBS was indicative of random coil structure (Fig. 3), with a single deep minimum at around 200 nm, thus confirming the bioinformatics prediction (32, 39).

Antigenic recognition by human sera and PBMC. The antigenic recognition of P27A was assessed with serum samples from adults living in the regions of Burkina Faso, Tanzania, and Papua New Guinea, where malaria is endemic. The sera of the majority of donors living in these geographically diverse regions recognized P27A (Table 1 and Fig. 4A). The donor samples were obtained from adults living in Burkina Faso and Tanzania and from children living in Papua New Guinea. The prevalence of anti-P27A antibodies among these populations ranged from 76% (Burkina Faso and Tanzania) to 95% (Papua New Guinea). The average OD values for all donor samples were generally high (0.67, 0.68, and 0.54, respectively), while those for positive samples were 1.29, 1.18, and 0.56, respectively (Fig. 4B). The average OD was lowest among samples obtained from children living in Papua New Guinea, as expected. This may suggest that there is age-related (i.e., exposure related) acquisition of anti-P27A antibodies, as is observed with other malaria antigens. The majority of these donors also have P27A-specific antibodies of the IgG1 and IgG3 cytophilic classes (Table 1), which have been shown to be necessary for protection against intracellular pathogens, espe-

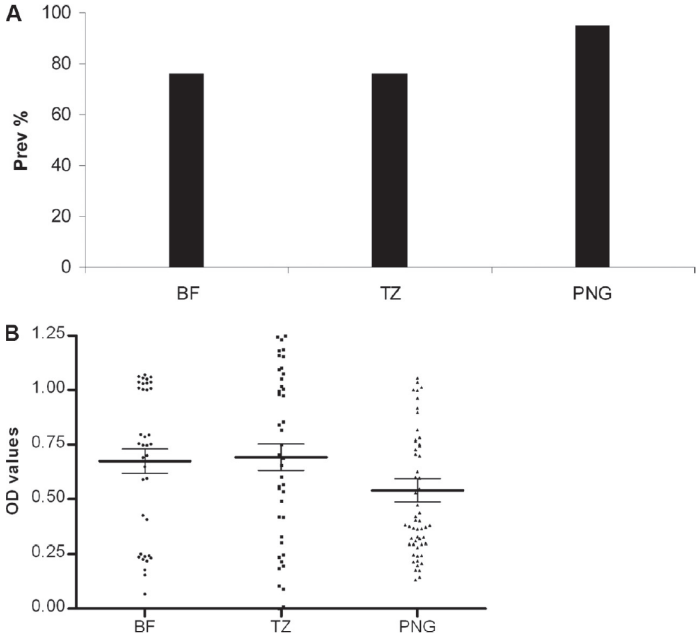

FIG. 4. (A) Prevalence of recognition of P27A by sera of adults living in regions of Burkina Faso (BF, n = 37), Tanzania (TZ, n = 42), and Papua New Guinea (PNG, n = 56) where malaria is endemic. A serum sample was considered positive when the OD is greater than the average OD of naïve sera plus 3 SD. (B) Average OD values of donor samples with significant anti-P27A antibodies (i.e., serum samples with OD values greater than the average OD of negative controls plus 3 SD). BF, Burkina Faso (n = 28/37); TZ, Tanzania (n = 32/42); PNG, Papua New Guinea (n = 53/56). Bars represent mean OD values, while whikers indicate ±SD.

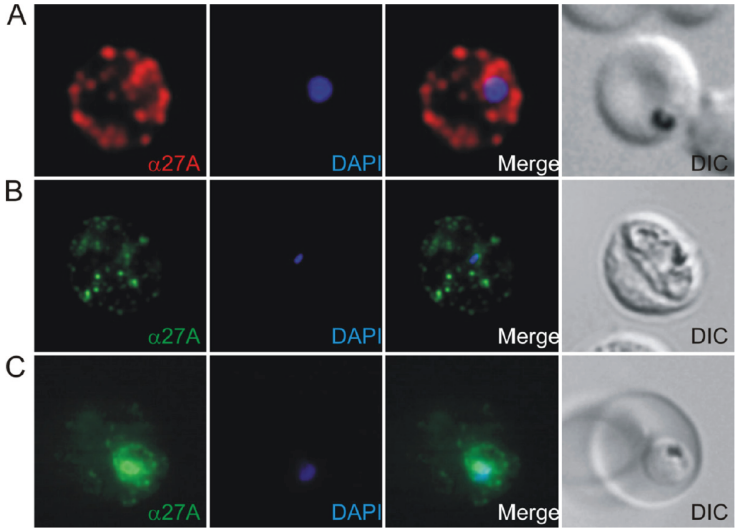

FIG. 5. Immunofluorescence staining of malaria-infected erythrocytes. (A) Trophozoite-stage parasites were stained with mouse antibodies against P27A (red). Mouse antibodies recognize the parasite and structures of the parasite in the iRBC cytosol. (B, C) Polyclonal rabbit sera (B) and human affinity-purified sera (C) specific to P27A recognize identical structures, suggesting that all the sera recognize the same target (green), the parasite-derived PFF0165c protein. Nuclei are stained with DAPI (in blue). Right panels, transmission light microscopy images of the iRBC (differential interference contrast). α, anti.

cially malaria (8, 9, 28). Such a distribution of IgG isotypes specific for P27A may suggest that it is an important part of the natural protection against malaria infection (or disease) that is observed in adults living in regions where malaria is endemic.

Affinity-purified human antibodies specific for P27A recognized *P. falciparum* 3D7-infected erythrocytes (Fig. 5 and 6), as did mouse and rabbit antibodies (details below).

Prevalence of recognition by PBMC and sera of Nigerian adults. Antigenic recognition of P27A by PBMC of adults exposed to malaria was assessed by T-cell proliferation assay, and SI values equal to or higher than 2 were considered positive. Over half (9/17, 53%) of the PBMC samples from adult donors from Nigeria proliferated when stimulated with a 3 μM solution of P27A. The average SI of the positive samples was 3.6, with a range of 2.1 to 5.3. Recognition of P27A by sera was assessed by ELISA, and a test sample was considered positive when the OD value was greater than the average of the OD values of naïve controls (adult Europeans with no prior exposure to malaria) plus 3 SD. Of sera of the same 17 donors, 14 (82%) recognized P27A, and the average OD value was 0.31 ($P = 0.0668$); these results are comparable to the SI results. All donor PBMC proliferated in the presence of tetanus toxoid, purified protein derivative, and *C. albicans* extract (positive control).

The high prevalence of recognition of P27A by both sera and T cells of semi-immune adults, together with its immunogenicity in several strains of mice and rabbits (see below), suggests that P27A contains multiple B- and T-cell epitopes and that the T-cell epitopes are recognized by a wide range of major histocompatibility complex molecules present in the general population. These are important characteristics to be considered for a potential malaria vaccine.

Immunogenicity in mice and rabbits. The immunogenicity of P27A formulated with Alhydrogel was studied in several

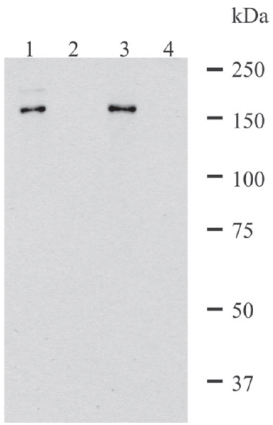

FIG. 6. Western blot analysis of 3D7 parasite lysate with mouse sera specific for P27A and affinity-purified rabbit sera specific for P27A. Late stages (trophozoites/schizonts) were enriched using a magnetic cell sorter. The enriched infected erythrocytes were fractionated into pellet fraction (lanes 1 and 3) and supernatant fraction (lanes 2 and 4) by saponin lysis in a 200-μl volume. P27A-specific rabbit sera (lanes 1 and 2) and P27A-specific mouse sera (lanes 3 and 4) recognize the protein at 160 kDa in the pellet fraction.

TABLE 2. Geometric mean antibody titers of sera of mice and rabbits after three injections with P27A formulated with alum or EM005

Type of animal	Ig class[b]	Alum[a]			EM005[a]		
		GMT (log_{10})	SD (log_{10})	No. of responders	GMT (log_{10})	SD (log_{10})	No. of responders
Rabbits		3.74	1.62	3/4	4.32	0.87	4/4
ICR mice		2.2	0.48	1/8	3.73	1.03	7/8
C3H mice		5.23	0.4	5/5	5.9	0.4	5/5
	IgG1	5.04	0.43	5/5	5.57	0.40	5/5
	IgG2a	4.31	1.37	4/5	5.97	0.87	5/5
	IgG2b	3.64	0.80	4/5	5.52	0.72	5/5

[a] GMT, geometric mean antibody titer. Antibody titer is the serum dilution at which the OD is greater than the average OD of sera of naïve mice/rabbits plus 3 SD. Responders are mice/rabbits with titers of >1:1,000.
[b] Values obtained from C3H mice.

strains of mice. All the C3H ($H\text{-}2^k$) mice immunized with this formulation had antibody titers of over 1:1,000 after the third injection, with an average of 160×10^3 (Table 2). The prevalent IgG isotypes were the cytophilic IgG1 and IgG2a. However, lower titers were obtained in outbred ICR mice. Similar immunogenicity results were also obtained with the Montanide ISA 720 formulation (data not shown). This prompted us to assess P27A immunogenicity with the recently developed adjuvant EM005 (IDRI, Seattle, WA).

EM005 is an oil-in-water stable emulsion containing a lipid A-like synthetic compound (2, 6). Lipid A is a component of lipopolysaccharide, which is responsible for the toxicity associated with gram-negative bacteria. Lipid A is known to be a potent Toll-like receptor 4 agonist that activates antigen-presenting cells, leading to the induction of high antibody titers and Th1 response (1, 5, 11, 19, 21, 24, 27, 35). Unlike the currently available lipid A-like adjuvant MPL, which is naturally derived and thus heterogeneous in composition, with a predisposition to batch-to-batch variations, EM005 is a homogeneous synthetic product with potentially higher potency and a better batch-to-batch consistency. It has been found to be safe in several animal models, including nonhuman primates (S. Bertholet, personal communication).

In outbred ICR mice, seven of the eight mice injected with the EM005 formulation had antibody titers of over 1:1,000, with an average of 57×10^3, compared to only one out of eight with the Alhydrogel formulation (Table 2). The average antibody titers induced in C3H mice were also higher with the EM005 formulation than with the Alhydrogel formulation ($1{,}094 \times 10^3$ and 160×10^3, respectively). Very high titers of the cytophilic IgG1 and IgG2a were induced (Table 2). These isotypes are functionally similar to the predominant, naturally

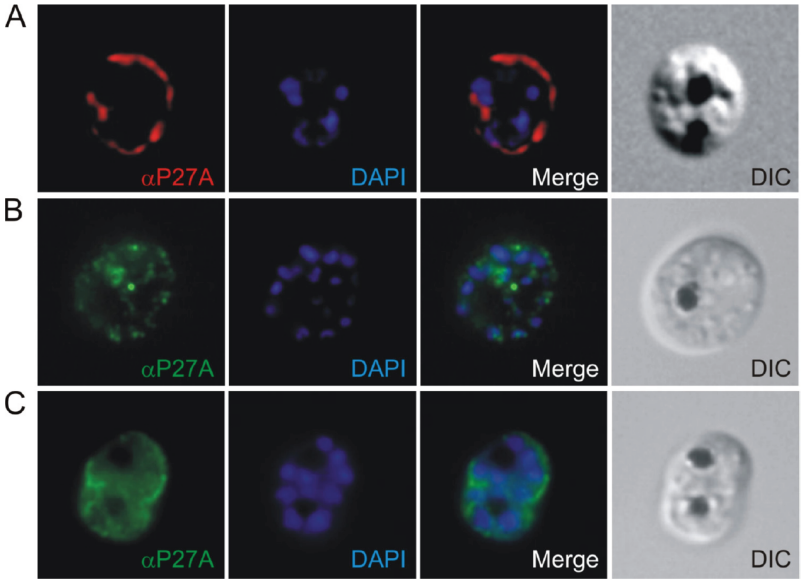

FIG. 7. Immunofluorescence staining of malaria-infected erythrocytes. (A) Schizont-stage parasites were stained with mouse antibodies against P27A (red). Polyclonal mouse antibodies recognize structures in very close proximity to or at the RBC membrane. (B, C) Affinity-purified rabbit sera (B) and human affinity-purified sera (C) specific to P27A recognize identical structures (green). Nuclei are stained with DAPI (blue). Right panels, transmission light microscopy images of the iRBC (differential interference contrast). α, anti.

induced cytophilic antibodies found in human sera (IgG1 and IgG3).

The mouse antibodies recognized native proteins in erythrocytes infected with different strains of *P. falciparum* (Fig. 5 shows results for 3D7; results for others are not shown) at dilutions of up to 1:8,000. This is consistent with the finding that the sequence of P27A is conserved, with only a single mutation among the 90 *P. falciparum* field and laboratory isolates tested (see below).

High antibody titers were also induced in rabbits (four per group) injected with P27A formulated with EM005 and, to a lower degree, Alhydrogel.

The antigen specificity of the antibodies obtained with the formulations used was determined by using sera from mice and rabbits immunized with two MSP2 long synthetic fragments in Alhydrogel and EM005. These sera did not recognize P27A (data not shown).

Mouse polyclonal antibodies and affinity-purified rabbit antibodies specific for P27A detected the corresponding protein in Western blot analysis of lysate of late-stage parasites (trophozoites/schizonts) (Fig. 7). Affinity-purified rabbit antibodies specific for P27A mainly recognized a protein at 160 kDa, exclusively in the pellet fraction (Fig. 6, lane 1). This suggests that the protein associates with membranes (Maurer's clefts, RBC membrane) after export out of the parasite. Similar to the affinity-purified rabbit sera, the polyclonal mouse sera recognized a protein at 160 kDa (lane 3), suggesting identical specificities of mouse and rabbit sera. The full-length PFF0165c protein has an expected size of 132 kDa, which apparently runs at a higher molecular mass, probably due to a low content of hydrophobic amino acids.

Genetic diversity of the sequence of P27A. The genetic diversity of P27A was assessed by PCR amplification and direct sequencing in order to detect SNPs and length polymorphisms. P27A shows limited polymorphism with essentially a single SNP at nucleotide position 875 (T→C) that results in an amino acid change from glutamic acid to glycine at position 292 of the protein (E292G) (Fig. 1). The prevalence varies, with allelic frequencies of 0.31 (6/19) in samples from Papua New Guinea, 0.6 (38/63) in samples from Tanzania, and 0.44 (4/11) in worldwide isolates published in the SNP database available at PlasmoDB. Two additional SNPs at nucleotide positions 880 (C→T; V294I) and 798 (C→T; M266I) were only observed in one isolate from PlasmoDB by microarray, a technique prone to false positives (22).

Cytological localization of P27A. In indirect immunofluorescence assays, *P. falciparum*-infected erythrocytes (3D7 strain) were stained with murine antibodies specific to P27A and analyzed by immunofluorescence microscopy. With trophozoites, P27A mouse sera recognized the parasites in the iRBC and parasite-derived structures in the cytoplasm of the iRBC (Fig. 5A). The polyclonal rabbit sera (Fig. 5B) and the human affinity-purified sera specific for P27A (Fig. 5C) localized the PFF0165c protein to identical structures. With schizonts, the polyclonal mouse sera recognized structures in close proximity to the RBC membrane or the corresponding protein at the RBC membrane. Similar structures are recognized by the affinity-purified rabbit (Fig. 6B) and human sera (Fig. 6C), suggesting identical specificities of the antibodies generated.

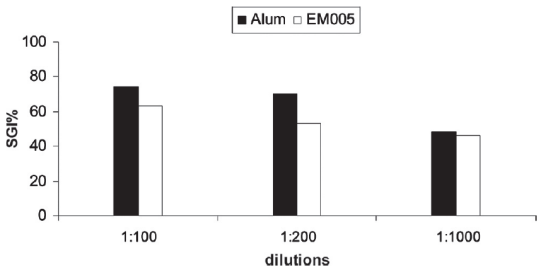

FIG. 8. Results of ADCI assays with murine antibodies. Pooled sera of C3H ($H\text{-}2^k$) mice immunized with P27A formulated with Alhydrogel and EM005. The percent SGI is calculated with the formula $100 \times [1 - (\%$ parasitemia with MN and test antibodies/% parasitemia with test antibodies)/(% parasitemia with MN and IgGs from healthy donors/parasitemia with IgGs from healthy donors)].

Biological activity in ADCI assay of mouse and human purified antibodies. One of the principal mechanisms that mediates clinical malaria in humans has been shown to be the cooperation between cytophilic antibodies and MN (7, 20). The in vitro ADCI assay, based on this mechanism, has been used to assess the capacity of purified human and murine antibodies to inhibit malaria parasite growth (Fig. 8). This mechanism has also been shown to be induced following immunization with a malaria vaccine candidate (12).

The ability of the human Fc receptors to bind cytophilic murine isotypes (IgG2a and IgG2b) enables the ADCI mechanism. Significant levels of inhibition of parasite growth were obtained with pooled sera of C3H mice immunized with P27A formulated with alum and EM005 (Fig. 8). This was as high as 74% SGI at 1:100 dilutions and still significant at 46% at 1:1,000 dilutions. The degree of inhibition obtained with affinity-purified human antibodies was also very high and comparable to that of pooled Igs of adults living in areas of Africa where malaria is endemic (7, 12, 20). These results, obtained with two different adjuvants in animal models and with naturally induced antibodies from humans, suggest that the primary mechanism of protection employed by P27A antibodies is likely to be in cooperation with MN.

Concluding remarks. P27A, a synthetic polypeptide that is derived from a recently identified unstructured segment present in the malaria protein PFF0165c (2), meets the principal requirements that are vital for a malaria vaccine candidate. In fact, it is recognized by sera of a majority of naturally exposed individuals, is highly immunogenic with different adjuvants, and has an extremely well conserved sequence. Both human-specific antibodies and antibodies induced in mice actively inhibit parasite growth and recognize native proteins in immunofluorescence assays. In addition, human P27A-specific antibodies are associated with clinical protection against malaria in humans in two cohorts of semi-immune adults (P. Druilhe, C. Roussilhon, A. Jafarshad, A. Kajava, S. Olugbile, A. Tall, C. Sokhna, C. Kulangara, I. Felger, and G. Corradin, unpublished data). Taken together, these data support further preclinical and clinical development of P27A, which is scheduled to begin soon.

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REFERENCES

1. **Alderson, M. R., P. McGowan, J. R. Baldridge, and P. Probst.** 2006. TLR4 agonists as immunomodulatory agents. J. Endotoxin Res. **12:**313–319.
2. **Anderson, R. C., C. B. Fox, T. S. Dutill, N. Shaverdian, T. Evers, G. R. Poshusta, et al.** Physicochemical characterization and biological activity of synthetic TLR4 agonist formulations. Colloids Surf. B, in press.
3. **Atherton, E., and R. C. Sheppard.** 1989. Solid phase synthesis; a practical approach. Oxford University Press, Oxford, United Kingdom.
4. **Audran, R., M. Cachat, F. Lurati, S. Soe, O. Leroy, G. Corradin, P. Druilhe, and F. Spertini.** 2005. Phase 1 malaria vaccine trial with a long synthetic peptide derived from merozoite surface protein 3. Infect. Immun. **3:**8017–8026.
5. **Baldridge, J. R., P. McGowan, J. T. Evans, C. Cluff, S. Mossman, D. Johnson, and D. Persing.** 2004. Taking a toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and immunotherapeutic agents. Expert Opin. Biol. Ther. **4:**1129–1138.
6. **Baldwin, S., S. Bertholet, M. Kahn, I. Zharkikh, G. Ireton, T. Vedvick, S. Reed, and R. Coler.** 2009. Intradermal immunization improves protective efficacy of a novel TB vaccine candidate. Vaccine **27:**3063–3071.
7. **Bouharoun-Tayoun, H., P. Attanath, A. Sabchareon, T. Chongsuphajaisiddhi, and P. Druilhe.** 1990. Antibodies that protect humans against *Plasmodium falciparum* blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. **172:**1633–1641.
8. **Bouharoun-Tayoun, H., and P. Druilhe.** 1992. *Plasmodium falciparum* malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infect. Immun. **60:**1473–1481.
9. **Bouharoun-Tayoun, H., and P. Druilhe.** 1992. Antibodies in *falciparum* malaria: what matters most, quantity or quality? Mem. Inst. Oswaldo Cruz **87:**229–234.
10. **Corradin, G., V. Villard, and A. Kajava.** 2007. Protein structure based strategies for antigen discovery and vaccine development against malaria and other pathogens. Endocr. Metab. Immune Disord. Drug Targets **7:**259–265.
11. **De Becker, G., V. Moulin, B. Pajak, C. Bruck, M. Francotte, C. Thiriart, J. Urbain, and M. Moser.** 2000. The adjuvant monophosphoryl lipid A increases the function of antigen-presenting cells. Int. Immunol. **12:**807–815.
12. **Druilhe, P., F. Spertini, D. Soesoe, G. Corradin, P. Mejia, S. Singh, R. Audran, A. Bouzidi, C. Oeuvray, and C. Roussilhon.** 2005. A malaria vaccine that elicits in humans antibodies able to kill *Plasmodium falciparum*. PLoS Med. **2:**e344.
13. **Dyson, H. J., and P. E. Wright.** 2002. Coupling of folding and binding for unstructured proteins. Curr. Opin. Struct. Biol. **12:**54–60.
14. **Dyson, H. J., and P. E. Wright.** 2005. Intrinsically unstructured proteins and their functions. Nat. Rev. Mol. Cell Biol. **6:**197–208.
15. **Feng, Z. P., X. Zhang, P. Han, N. Arora, R. F. Anders, and R. S. Norton.** 2006. Abundance of intrinsically unstructured proteins in *P. falciparum* and other apicomplexan parasite proteomes. Mol. Biochem. Parasitol. **150:**256–267.
16. **Gardner, M. J., N. Hall, E. Fung, O. White, M. Berriman, R. W. Hyman, J. M. Carlton, A. Pain, K. E. Nelson, et al.** 2002. Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **419:**498–511.
17. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, et al.** 2002. A recombinant blood-stage malaria vaccine reduces *Plasmodium falciparum* density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J. Infect. Dis. **185:**820–827.
18. **Irion, A., I. Felger, S. Abdulla, T. Smith, R. Mull, M. Tanner, C. Hatz, and H. P. Beck.** 1998. Distinction of recrudescences from new infections by PCR-RFLP analysis in a comparative trial of CGP 56 697 and chloroquine in Tanzanian children. Trop. Med. Int. Health. **3:**490–497.
19. **Ismaili, J., J. Rennesson, E. Aksoy, J. Vekemans, B. Vincart, Z. Amraoui, F. Van Laethem, M. Goldman, and P. M. Dubois.** 2002. Monophosphoryl lipid A activates both human dendritic cells and T cells. J. Immunol. **168:**926–932.
20. **Jafarshad, A., M. H. Dziegiel, R. Lundquist, L. K. Nielsen, S. Singh, and P. L. Druilhe.** 2007. A novel antibody-dependent cellular cytotoxicity mechanism involved in defence against malaria requires costimulation of monocytes FcγRII and FcγRIII. J. Immunol. **78:**3099–3106.
21. **Johnson, A. G., and M. A. Tomai.** 1990. A study of the cellular and molecular mediators of the adjuvant action of a non-toxic monophosphoryl lipid A. Adv. Exp. Med. Biol. **256:**567–579.
22. **Kidgell, C., S. K. Volkman, J. Daily, J. O. Borevitz, D. Plouffe, Y. Zhou, J. R. Johnson, K. Le Roch, O. Sarr, O. Ndir, et al.** 2006. A systematic map of genetic variation in *Plasmodium falciparum*. PLoS Pathog. **2:**e57.
23. **Malkin, E., F. Dubovsky, and M. Moree.** 2006. Progress towards the development of malaria vaccines. Trends Parasitol. **22:**292–295.
24. **Martin, M., S. M. Michalek, and J. Katz.** 2003. Role of innate immune factors in the adjuvant activity of monophosphoryl lipid A. Infect. Immun. **71:**2498–2507.
25. **Matuschewski, K.** 2006. Vaccine development against malaria. Curr. Opin. Immunol. **18:**449–457.
26. **McColl, D. J., and R. S. Anders.** 1997. Conservation of structural motifs and antigenic diversity in the *Plasmodium falciparum* merozoite surface protein-3 (MSP3). Mol. Biochem. Parasitol. **90:**21–31.
27. **Puggioni, F., S. R. Durham, and J. N. Francis.** 2005. Monophosphoryl lipid A (MPL) promotes allergen-induced immune deviation in favour of Th1 responses. Allergy **60:**678–684.
28. **Roussilhon, C., C. Oeuvray, C. Müller-Graf, A. Tall, C. Rogier, J. F. Trape, M. Theisen, A. Balde, J. L. Pérignon, and P. Druilhe.** 2007. Long-term clinical protection from falciparum malaria is strongly associated with IgG3 antibodies to merozoite surface protein 3. PLoS Med. **4:**e320.
29. **Schoepflin, S., J. Marfurt, M. Goroti, M. Baisor, I. Mueller, and I. Felger.** 2008. Heterogeneous distribution of *Plasmodium falciparum* drug resistance haplotypes in subsets of the host population. Malar. J. **7:**78.
30. **Tonkin, C. J., G. G. van Dooren, T. P. Spurk, N. S. Struck, R. T. Good, E. Handman, A. F. Cowman, and G. I. McFadden.** 2004. Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol. Biochem. Parasitol. **137:**13–21.
31. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673–675.
32. **Uversky, V. N.** 2002. What does it mean to be natively unfolded? Eur. J. Biochem. **269:**2–12.
33. **Verdini, A., S. Terenzi, V. Brossard, M. Roggero, and G. Corradin.** 2008. Oxidative folding of synthetic polypeptides S-protected as tert-butylthio derivatives. J. Pept. Sci. **14:**1271–1282.
34. **Villard, V., G. W. Agak, G. Frank, A. Jafarshad, C. Servis, I. Nébié, S. B. Sirima, I. Felger, M. Arevalo-Herrera, S. Herrera, F. Heitz, et al.** 2007. Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS ONE **2:**e645.r
35. **Wheeler, A. W., J. S. Marshall, and J. T. Ulrich.** 2001. A Th1 adjuvant, MPL, enhances antibody profiles in experimental animals, suggesting it has the potential to improve the efficacy of allergy vaccines. Int. Arch. Allergy Immunol. **126:**135–139.
36. **World Health Organization.** April 2005. New vaccines against infectious diseases: research and development status, updated February 2006. Initiative for Vaccine Research, WHO, Geneva, Switzerland. http://www.who.int/vaccine_research/documents/en/Status_Table.pdf.
37. **Wright, P. E., and J. H. Dyson.** 1999. Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. J. Mol. Biol. **293:**321–331.
38. **Yang, X., C. G. Adda, D. W. Keizer, V. J. Murphy, M. M. Rizkalla, M. A. Perugini, D. C. Jackson, R. F. Anders, and R. S. Norton.** 2007. A partially structured region of a largely unstructured protein, Plasmodium falciparum merozoite surface protein 2 (MSP2), forms amyloid-like fibrils. J. Pept. Sci. **13:**839–848.
39. **Zhang, X., M. A. Perugini, S. Yao, C. G. Adda, V. J. Murphy, A. Low, R. F. Anders, and R. S. Norton.** 2008. Solution conformation, backbone dynamics and lipid interactions of the intrinsically unstructured malaria surface protein MSP2. J. Mol. Biol. **379:**105–121.

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Cell biological characterization of the malaria vaccine candidate Trophozoite exported protein 1

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Abstract

In a genome-wide screen for alpha-helical coiled coil motifs aiming at structurally defined vaccine candidates we identified PFF0165c. This protein is exported in the trophozoite stage and was named accordingly Trophozoite exported protein 1 (Tex1). In an extensive preclinical evaluation of its coiled coil peptides Tex1 was identified as promising novel malaria vaccine candidate providing the rational for a comprehensive cell biological characterization of Tex1. Antibodies generated against an intrinsically unstructured N-terminal region of Tex1 and against a coiled coil domain were used to investigate cytological localization, solubility and expression profile. Co-localization experiments revealed that Tex1 is exported across the parasitophorous vacuole membrane and located to Maurer's clefts. Change in location is accompanied by a change in solubility: from a soluble state within the parasite to a membrane-associated state after export to Maurer's clefts. No classical export motifs such as PEXEL, signal sequence / anchor or transmembrane domain was identified for Tex1.

Keywords

Alpha-helical coiled coil, malaria vaccine, Maurer's clefts, PFF0165c

Introduction

In the past few years Tex1 encoded by PFF0165c was characterized as a novel malaria vaccine candidate. According to PlasmoDB version 6.5 (http://plasmodb.org) *tex1* spans nucleotide positions 133'147 to 136'458 on chromosome 6. Tex1 had been identified originally in a genome-wide screen of alpha-helical coiled coil domains in a search for novel vaccine candidates against the blood stage of *P. falciparum* [1,2]. Chemically synthesized short peptides consisting of such a motif can fold into their native structure in aqueous environment and therefore mimic structurally native epitopes. Two regions of Tex1 were chemically synthesized. One of the synthetic peptides, P27, is spanning the coiled coil domain (K845 to T871), the other, P27A, corresponds to N-terminal intrinsically unstructured region (H223 to S326). Both peptides were tested in an extensive preclinical evaluation protocol to analyze the properties of anti-P27 and anti-P27A antibodies regarding *in vitro* parasite killing in presence of monocytes [1,3], correlation with protection in adults and children [3,4], prevalence of peptide recognition by sera from semi-immune adults from different endemic region throughout the world [1,3] and sequence conservation in different culture strains and field isolates [3,5]. Both fragments of Tex1, peptides P27A and P27, are considered promising novel malaria blood stage vaccine candidates. A phase 1 clinical study of P27A is scheduled in 2011.

In view of the promising outcome of preclinical evaluation and the imminent phase 1 clinical trial, a comprehensive biological characterization of Tex1 was called for. Here we present results of a cell biological analysis characterizing Tex1 in relation to other known exported parasite proteins. We show that Tex1 associates to Maurer's clefts (MC) membrane facing the cytosol of the RBC. Tex1 export depends on the classical secretory pathway. But it seems to lack a classical signal sequence as well as a PEXEL motif, suggesting the presence of alternative sequences involved in protein export to the PV and across the PVM to the RBC cytosol.

Material and Methods

Ethical Treatment of Animals

The animal work has been carried out according to relevant national and international guidelines. The immunization experiments in CB6F1 mice and the immunization protocol was approved by the Canton de Vaud (Permit number: 805.6). Immunization of rabbits were performed by the commercial company Eurogentec, 4102 Seraing, Belgium.

Cell culture and protein extracts.

P. falciparum 3D7 strain was cultured at 5% haematocrit as described [6], using RPMI medium supplemented with 0.5% Albumax [7]. Parasites were synchronized with 5% sorbitol [8]. To obtain protein extract of mixed stage infected erythrocytes parasites (10 ml petri-dish) were grown to 5% to 10% parasitemia, lysed on ice in 0.03% saponin in phosphate-buffered saline (PBS, pH 7.4) for 10 min, washed with ice cold PBS for complete removal of hemoglobin, and resuspended in Laemmli sample buffer. The protein extracts of late-stage parasites (trophozoites and schizonts) were obtained from *P. falciparum* 3D7-infected erythrocytes in a 30-ml petri dish (5% hematocrit, 6% parasitemia) which was enriched using a magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). The enriched infected erythrocytes were lysed in a 200 μl volume of PBS, 0.03% saponin (Fluka) in the presence of protease inhibitors (Roche Diagnostics, Basel, Switzerland) for 5 min at 4°C. The parasites were pelleted by centrifugation at 4,000 x *g* for 10 min, the supernatant was collected and mixed with sample buffer. The parasite pellet was resuspended in 0.1 M Tris, pH 6.8, and an equal volume of 2x Laemmli sample buffer. For protein expression profiling 5 ml of tightly synchronized culture (2h time frame; 8% parasitemia) was harvested in a 4 hours interval, parasites were lysed on ice in 0.03% saponin in PBS for 10 min and wash 3 times in ice-cold PBS. Parasite pellet was resuspended in cold 0.1 M Tris, pH 6.8, and an equal volume of 2x Laemmli sample buffer.

Recombinant expression and purification of recP27 fragment.

The C-terminal fragment of Tex1 containing the coiled coil motif P27 (Figure 1A, M681 to E910) was amplified from 3D7 genomic DNA by PCR and cloned into the pQE60 plasmid via the NcoI and BamHI restriction sites (primers used are listed in **Table S1**). Recombinant expression was performed following the manufacturer's protocol (Qiagen Inc.).

Generation of anti-P27, anti-P27A and anti-recPf27 polyclonal rabbit sera and anti-P27 polyclonal mouse sera.

Rabbit sera were produced by Eurogentec, Seraing, Belgium. In short, the recPf27 protein (250 μg) was used for immunization with Freud's adjuvant into two New Zealand white rabbits. Sera samples (20 ml) were affinity purified using recPf27-6xHis protein or the P27 coiled coil peptide coupled to HiTrap NHS-activated HP columns (GE-Healthcare, 1 ml). After antibody binding columns were washed with 50 ml PBS, bound IgG was eluted with 0.1 M glycine, pH 2.5, and the buffer was subsequently changed to PBS using HiTrap Desalting Columns (GE Healthcare). Purified antibodies were stored at −80°C until further use. Polyclonal mouse sera was obtained by immunization of CB6F1 mice. CB6F1 mice were injected 3 times with 20 μg of the P27 peptide in Montanide ISA 720 at the base of the tail on day 1, 22 and 78. Bleeding was performed 10 days after the second and third immunization. Affinity purification of P27A-specific rabbit has previously been described [1,3].

Western blot analysis.

Protein extracts were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose (Hybond-C extra; GE Healthcare) at 4°C for 1 h at 80 V and an additional hour at 100 V. The membrane was blocked for 1 h in 5% skim milk, 0.1% Tween in Tris-buffer. Antibodies used were: Polyclonal rabbit anti-P27A (1:5000); anti-P27 (1:2500) and anti-Pf27rec (1:2500); anti-MAHRP1 (1:5000), monoclonal mouse anti-MSP1 ([9], 1:1000); anti-SERA5 ([10], 1:2000); horseradish peroxidase-conjugated goat anti-mouse (Pierce, 1:20 000), goat anti-rabbit (Acris, 1:10000).

Solubility analysis.

P. falciparum 3D7-infected erythrocytes (30-ml petri dish; 6% parasitemia) were enriched using a magnetic cell sorter (Miltenyi Biotec, Bergisch Gladbach, Germany). Purified mature stages were resuspended in 200 µl 5mM Tris pH 8 in the presence of protease inhibitors (Roche Diagnostics, Basel, Switzerland) and lysed by 3 freezing-thawing cycles. Soluble protein fraction was separated by centrifugation 30 min at 20 000xg at 4°C. The membrane-containing pellet was resuspended in 200 µl 0.1 M Na_2CO_3 and incubated for 30 min on ice to extract peripheral membrane proteins. Supernatant containing peripheral proteins was separated by centrifugation (30 min at 20 000xg at 4°C). Integral membrane proteins were extracted from the pellet with 1% Triton X-100 on ice for 30 min. Supernatant containing integral proteins was separated by centrifugation (30 min at 20 000xg at 4°C) The remaining proteins were extracted with 4% SDS, 0.5% TritonX-114 in 0.5x PBS for 30 min at room temperature and separated from the pellet by centrifugation. The supernatant was analyzed as insoluble protein fraction. 10 µl of each fraction was analyzed by Western Blot.

RNA isolation, cDNA synthesis and real time PCR

3D7-infected erythrocytes were tightly synchronized using 5% D-sorbitol [8]. Three rounds of 5% D-sorbitol treatment was applied (2^{nd} and 3^{rd} treatment was applied 8 hours and 14 hours after the 1^{st} D-sorbitol treatment). Parasites were grown to 8-10% parasitemia (5% heamatocrit). 1.5 ml culture was taken in 4h intervals. In brief, parasite RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. TRIzol extraction was repeated. Residual gDNA was digested twice with RQ1 DNase (Promega) according to the manufacturer's protocol. Reverse transcription was done by AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) with random primers (Invitrogen) as described by the manufacturer. To control for gDNA contamination, the target sequence was amplified from the RNA solution prior to reverse transcription. Absolute transcript quantification was performed at final primer concentrations of 0.4 µM using SYBR® Green Master Mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied

Biosystems) in a reaction volume of 12 µl. All reactions were performed in triplicate yielding virtually identical Ct values. A serial dilution of gDNA was used as standard for absolute quantification. Relative transcript profiles were calculated by normalization against transcript levels of the house-keeping gene PF13_0170 (glutaminyl-tRNA synthetase). Validation of synchronization procedure was obtained by analyzing transcript levels of the merozoite surface protein 8 (*msp8*). The primers used for qPCR of *tex1*, PF13_0170 and *msp8* are shown in **Table S2**. The time points of harvest (1-14), the corresponding age of parasites (in hours post infection) and the corresponding parasite stages are illustrated in **Table 1** and were confirmed by Giemsa staining before RNA and Protein extraction.

Indirect Immunofluorescence Assay (IFA).

Infected erythrocytes were fixed with 4% paraformaldehyde (Polyscience) and 0.0075% glutaraldehyde (Polyscience) for 30 min under constant agitation, permeabilized using 0.1% Triton X-100/PBS for 10 min and blocked with sodium borohydride ($NaBH_4$)/PBS for 10 min followed by an additional blocking step using 3% BSA in PBS for 1 h as described in [11]. Primary antibodies were used with the following concentrations: polyclonal rabbit sera anti-P27A (1:2000); anti-P27 (1:1000), anti-MAHRP2 ([12], 1:100); anti-REX1 (kind gift from Prof. Don Gardiner, 1:500); mouse polyclonal antibodies anti-P27 (1:200); anti-SBP1 N-terminus specific (kind gift from Prof. Catherine Braun-Breton, 1:200); anti-MAHRP1 (1:200). Secondary antibodies used: Alexa Fluor 488 (Invitrogen; 1:400); Texas Red (Invitrogen; 1:400). Cells were mounted in Vectashield Hard Set supplemented with DAPI (Vector Laboratories) for staining of the nuclear DNA. For the Equinatoxin II assay infected erythrocytes were lightly fixed with 2% paraformaldehyde in RPMI medium (10 min), permeabilized with Equinatoxin II [13], and re-fixed with 4% formaldehyde and 0.00075% glutaraldehyde in PBS (pH 7.4, Gibbco). Cells were blocked with 3% BSA (Sigma) in PBS. Cells were split and one half was additionally treated with 0.1% triton (Merck) for complete permeabilization. Synchronized Ring stage parasites (aged 4h to 8h post invasion) were treated with Brefeldin A solved in 100% ethanol (Fluka) to a final concentration of 5 µg ml^{-1}. Control cultures were incubated in the presence of equivalent

amounts of ethanol. After 18 h parasites were fixed for IFA. BFA was removed from the remaining parasites which were further cultured to ensure viability after treatment. Images were obtained by the Zeiss confocal microscope LSM 700 or Leica DM 5000B fluorescence microscope. Images were processed by ImageJ software or the Huygens Essential Software (Scientific Volume Imaging, The Netherlands). Quantitative analysis of co-localization was done with Huygens Essential Software (Scientific Volume Imaging, The Netherlands).

Results

According to PlasmoDB version 6.5 (http://plasmodb.org), the predicted protein has a length of 1103 amino acids (aa) and contains 3 predicted coiled coil domains. Alpha-helical coiled coils share the heptad motif (**abcdefg**)$_n$ with positions **a** and **d** representing hydrophobic residues, whereas the remaining positions are generally polar. Depending on slight variations in their sequences the coiled coil bundles consist of 2 to 7 alpha-helices that spontaneously self assemble in aqueous solutions. One of the 3 coiled coil domains in Tex1 is the P27 region from position K845 to T871 (in black**, Figure 1A**.), which has been identified as potential malaria vaccine candidate previously [1]. The C-terminus of Tex1 consists of a predicted RING (Really Interesting New Gene) domain, spanning amino acids K1025 to L1102 (in grey, **Figure 1A**). Furthermore, a large portion of the C-terminal half of this protein (650-1040) has sequence similarity to several proteins with known 3D structure which have elongated alpha-helical domains capped by the RING-domains (e.g. [14]. This supports correctness of our previous prediction of alpha-helical coiled coil regions in Tex1 (Villard et al., 2007). A long intrinsically unstructured region (IUR) named P27A, ranging from position H223 to S326 (black dotted, **Figure 1A**), corresponds to the second identified potential vaccine candidate within Tex1 [3]. P27A is currently under clinical development and a phase 1 clinical trial is scheduled for 2011.

Tex1 is expressed in intraerythrocytic blood stage parasites and its transcription is up-regulated in the early trophozoite stage.

In order to characterize the protein by IFA and Western Blot, rabbit antibodies were generated against P27A and a 240 aa long (including linker and His-tag) recombinant protein (recPf27) encompassing amino acid M681 to E910 (**Figure 1A**) in the C-terminal part of Tex1 and including the P27 coiled coil domain (K845 to T871). Both polyclonal rabbit sera were affinity purified on the respective immunogens. In addition, recPf27 rabbit serum was alternatively affinity purified on the P27 peptide. Thus, three polyclonal rabbit sera were available with specificities to P27A, recPf27 and P27.

Previously, we showed that P27A specific mouse and rabbit sera both detected a protein with the mass of 160 kDa in Western Blot broadly consistent with the predicted mass of 132 kDa [3]. When using several sera raised against different parts of Tex1, all sera recognized a band at about 160kDa both, in the pellet fraction of mixed parasite stages and in late stage parasites (**Figure 1B**).

The transcription profile was analyzed by quantitative real-time PCR on RNA from tightly synchronized cultures harvested in 4 h intervals covering the 48h intra-erythrocytic developmental cycle. The collected time points, the corresponding age of the parasites (in hours post invasion) and the respective parasite stages are listed in table 1. The transcription level of *tex1* was analyzed in relation to that of a constitutively transcribed gene, *glutaminyl-tRNA synthetase* (PF13_0170). *Tex1*-specific transcripts were detected throughout the intra-erythrocytic development cycle, but an up-regulation of transcript abundance was detected in early trophozoites (gray bars**, Figure 1C**). To validate the synchronization procedure, transcript levels of the merozoite surface protein 8 (*msp8*) were analyzed at each time point. The *msp8* profile obtained (white bars, **Figure 1C**) showed an up-regulation in ring stages and in very late schizont stages as shown in PlasmoDB. The RNA levels of *tex1* were compared with a time course of Tex1 protein abundance analyzed by Western Blot during the intra-erythrocytic cycle. Tex1 protein levels detected in 4 h intervals were highest during early trophozoite stage at

time point 8 (**Figure 1D**). The protein persisted until egress, reflected. in the presence of the full length merozoite surface protein 1 (MSP1) [15].

Tex1 is exported to the host cell cytosol and localizes to Maurer's clefts. Previously we reported that Tex1 was exported and accumulated at structures in the cytosol of the infected RBC [3]. To study the exact subcellular localization of Tex1 during the intra-erythrocytic cycle, synchronized 3D7 parasites were analyzed by IFA. In early ring stages (0-6 hours post invasion) the protein was absent (data not shown), whereas in late ring stages (12-16 hours post invasion) Tex1 was detected in punctuated structures within the parasite (**Figure 2A**). In trophozoite stages, Tex1 is exported to the host cell cytosol and associates with elongated structures in the cytosol of the infected RBC (**Figure 2B**) suggestive of Maurer's clefts (MC) staining [16]. In schizont stages the protein was much less focused and seemed to associate to the periphery of the host cell in vicinity to the host cell membrane (**Figure 2C**).

To prove the localization to MC, co-localization experiments were performed using antibodies against known MC markers. In late ring stages the ring exported protein 1 (Rex1) (**Figure 3A**), SBP1 (**Figure 4A**) and MAHRP1 (**Figure 5A**) associated with MC, whereas Tex1 still remained within the parasite. In trophozoite, schizont and late schizont stages, Tex1 appeared to associate with MC as demonstrated by co-localization with Rex1 (**Figure 3B, 3C, 3D**), SBP1 (**Figure 4B, 4C**) and MAHRP1 (**Figure 5B and 5C**. In schizont stages Tex1 signal was detected similar to Rex1 adjacent to the RBC membrane. Tex1 was also detected in close proximity to new structures called tethers **(Figure 6A)** that are characterized by the membrane-associated histidine rich protein 2 (MAHRP2**,** [12]. However, Tex1 is not found anymore in close proximity to MAHRP2 in schizont stage parasites (**Figure 6B**). Antibodies directed against Tex1 failed to detect the protein at the surface of infected RBCs in unpermeabilized cells (**Figure S1**) suggesting that in schizonts the protein resides inside of the infected cell in close proximity to the RBC membrane.

Tex1 occurs in two conditions: as soluble protein and in association with membrane structures.

Late parasite stages were purified by magnetic cell sorting and were lysed by repeated freeze thaw cycles to release all soluble proteins of the parasite and the RBC. The peripheral, membrane-associated proteins were extracted from the pellet fraction containing the membranes by sodium carbonate buffer (pH 11). The remaining integral membrane proteins were extracted with Triton X-100. This fractionation revealed that Tex1 was partly found soluble but equal amounts of the protein could only be extracted by carbonate buffer indicating that Tex1 associated with membranes (**Figure 7**). As control for the integrity of our fractions we used monoclonal antibodies against serine-rich antigen 5 (SERA5), a soluble protein found in the PV [17,18,19]; MAHRP1 was used as control representing an integral membrane protein [20,21]; MSP1 served as control representing a glycosylphosphatidylinositol lipid anchored protein on the merozoite surface and also as marker for the integral membrane fraction [22]

In order to analyze the localization of Tex1 at the MC, infected RBCs were lysed with Equinatoxin II (EqtII), a pore-forming toxin binding preferentially to sphingomyelin-containing membranes [13]. It lyses the RBC membrane ensuring integrity of PVM and MC membranes [23]. SBP1 is an integral membrane protein localizing to MCs. The C-terminus of SBP1 is directed to the RBC cytosol, whereas the N-terminus is directed to the lumen of MCs. The upper panel of **Figure 8** shows Tex1 localization in EqtII lyzed parasite infected RBC. In these EqtII treated parasites the N-terminus of SBP1 is not detected because antibodies specific to this part cannot access their target due to intact MC membranes.

SBP1 staining was performed to demonstrate the integrity of the MC membrane. P27-specific antibodies detected Tex1 in cells treated with EqtII at the MC. This demonstrated the localization of Tex1 at the surface of MC facing the RBC cytosol (**Figure 8**).

In the lower panel of **Figure 8,** the parasites were further lyzed with Triton X-100, which permeabilized also the MCs membrane, therefore SBP1 can be detected with antibodies directed against the N-terminus of the protein (**Figure 8**).

Export of Tex1 is Brefeldin A sensitive.

Protein secretion pathways in the eukaryotic cell are classified into the classical and nonclassical secretory pathway as reviewed by [24]. Whereas the classical secretory pathway involves co-translational translocation of proteins into the ER or posttranslational insertion into the ER followed by vesicular transport from the ER via Golgi to the cell surface or the extracellular space reviewed in [25], the molecular mechanisms involved in the nonclassical protein secretion are independent of the ER/Golgi system [26,27]. To test by which route Tex1 is exported, infected RBCs were treated with Brefeldin A (BFA), a fungal metabolite shown to block the classical protein secretion pathway [28]. BFA treatment blocked Tex1 export (**Figure 9)** suggesting that Tex1 export depends on components of the classical secretory pathway.

Discussion

Extensive preclinical evaluation of the annotated hypothetical protein Tex1 revealed that two regions, the intrinsically unstructured region P27A and the coiled coil domain P27, show great potential as new malaria vaccine candidates [1,3,5]. Its clinical development, currently in phase 1, called for an in depth analysis of the cytological characteristics of Tex1, which was named “Trophozoite exported protein 1” due to its localization to MC at the trophozoite stage. Association to MC was confirmed by co-localization with Rex1 and other MC proteins. Tex1 associated with the MC membranes facing the cytosol of the RBC. This was demonstrated by EqtII lysis of infected RBCs, which in contrast to Triton X-100 permeabilizes exclusively the RBC membrane. While antibodies detected Tex1, other antibodies, directed against

the luminal N-terminus of SBP1, could not access the lumen of MCs and thus gave no signal.

Exported proteins in *P. falciparum* are classified based on the presence or absence of the PEXEL motif which is mostly located downstream of a hydrophobic stretch. Recently, an increasing number of PEXEL-negative exported proteins (PNEPs) were identified [12,16,29,30]. Tex1 also is a PEXEL negative exported protein. To date it is only poorly understood how PNEPs are trafficked across the PVM, and sequence signatures responsible for export across the PVM and to the MC remain to be identified, if these exist at all.

A common characteristic of PNEPs seems to be the presence of either N-terminal signal sequence or a transmembrane domain [31]. For Tex1 no classical signal sequence, nor PEXEL motif, could be identified. The Tex1 expression pattern varies from that of PNEPs. Whereas Tex1 is expressed in trophozoites, PNEPs are expressed early in the intra-erythrocytic developmental cycle. We identified a potential alternative start site at position - 43 in respect to the predicted translational start site (PlasmoDB, **Figure S2**). This stretch of 43 aa was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/) to function as signal anchor and is unique for *P. falciparum* Tex1. No such preceding sequence stretch was detected in the orthologues of *P. vivax* (PVX_113335) and *P. knowlesi* (PKH_114650, **Figure S2**). Constructs of Tex1 including a GFP tag at the C-terminus were generated with or without the 43 aa hydrophobic stretch and episomally expressed. However, the GFP signals of both variants remained inside the parasite. More experimental data is needed to further investigate sequences responsible for Tex1 export. GFP-tagging of Tex1 might have interfered with the function of the RING domain at its very C-terminus. This would suggest that the RING domain plays an important role in Tex1 export. Brefeldin A treatment resulted in the accumulation of Tex1 at close proximity to the nucleus suggestive for ER or ER exit sites, indicating the involvement of the classical secretory pathway in the export of Tex1.

Tex1 exhibited a differential solubility pattern, whereby a portion of the protein was found in the soluble fraction, while the rest was present as peripheral membrane protein. No soluble Tex1 was detected in the RBC cytosol or PV, as demonstrated in the fractionation experiment using saponin lysed infected RBCs (**Figure 1B**), suggesting that the soluble pool of Tex1 is present exclusively within the parasite. This finding suggests that Tex1 changes its solubility during export: Tex1 is exported as a soluble protein, but associates with MC membranes after export. Our solubility assay showed equal amounts of soluble Tex1 and membrane-associated Tex1. However, the soluble portion likely is overrated due to freeze/thaw-mediated release of Tex1 from its MC's association.

Also for other proteins a solubility change after export has been reported, e.g. for Rex1 [32]. Similar to Tex1, Rex1 was found to associate with MCs via protein-protein interaction [32]. Rex 1 has a predicted transmembrane domain and its alpha-coiled coil region (amino acids 160-370) seems to be responsible for MC association [32]. Tex1 contains three putative coiled coil domains (**Table S3**). The alpha-helical coiled coil motif is a very abundant protein motif present in around 10% of all proteins [33]. Coiled coils have been shown to function as protein-protein interaction sites and to be involved in oligomerization and complex formation [34]. Thus, coiled coils participate in many cellular processes, such as membrane fusion, vesicular trafficking and cell motility. Further experiments are needed to elucidate the function of Tex1 coiled coil domains for MC membrane association.

Also for PfEMP1 a change in solubility during export had been reported [35]. Despite the presence of a transmembrane domain, PfEMP1 seems to be synthesized as a carbonate extractable protein. After export PfEMP1 becomes increasingly insoluble [35].

Noteworthy was the observation of very good co-localization of both peripheral membrane proteins Tex1 and Rex1, in contrast to the incomplete/partial co-localization of Tex1 with the integral membrane proteins

MAHRP1 and SBP1 at MC's. This provides further evidence for a peripheral membrane association of Tex1.

We investigated, whether the export of Tex1 is influenced by other exported proteins. Tex1 export was not altered in D10 parasites (data not shown), which have a partial deletion of chromosome 9 and a truncation of chromosome 2, eliminating 22 genes, including Rex1, 2, 3, 4 and KAHRP, and resulting in loss of cytoadherence [36,37,38,39] and alteration of the MC structure [40,41]. Similarly, in MAHRP1 knock out parasites, where PfEMP1 trafficking to the RBC membrane is blocked [16], Tex1 was correctly exported and its association with the MC remained intact (data not shown).

Tex1 orthologues were found in *P. vivax* and *P. knowlesi* as well as in *P. berghei*, *P. chabaudi or P. yoelii*. P27 was highly conserved among *Plasmodium* species (**Figure S3A**). Interestingly, the unstructured region was present exclusively in *P. falciparum* (**Figure S3B**). Many of the other ring stage exported proteins of *P. falciparum,* such as MAHRP1 and 2, SBP1 and Rex1, 2, 3, and 4, as well as the *resa*-multi gene family, do not have orthologues in *P.vivax*. Discrepancies were found also in a comparison of *P. falciparum* and *P. vivax* transcription profiles [42]. Eleven percent of syntenic genes of *P. vivax and P. falciparum* differed in gene expression during the intra-erythrocytic developmental cycle [42]. Similar results were obtained for *tex1* transcripts. According to PlasmoDB the *P. vivax* orthologue showed a completely different transcriptional profile with transcripts up-regulated in ring stage parasites suggesting a divergent evolution of Tex1 function.

Antibodies directed against P27 and P27A of Tex1 were effective in *in vitro* parasite killing in the presence of monocytes [1,3] and both P27 and P27A were recognized by serum from semi-immune adults from various endemic settings [1,3]. These results suggested that Tex1 holds a crucial immunological function. However, we found that Tex1 was absent on the surface of the infected RBC. The effector function of Tex1-specific antibodies excludes therefore blocking of cytoadherence or opsonization and destruction of iRBC by phagocytic cells, but involves monocytes. We conclude that the

activation of monocytes by P27/P27A-specific antibodies may occur after parasite egress.

The persistence of Tex1 until egress could indicate functional activity at the end of the 48 h blood stage cycle. To elucidate the biological function of Tex1, we attempted to knock-out *tex1*. These attempts failed indicating that the *tex1* locus resists recombination events due to an essential role of Tex1 for parasite survival.

Conclusion

Tex1 was identified based of extensive preclinical evaluation as promising novel vaccine candidate against *P. falciparum* blood stage infection. In the past, malaria blood-stage vaccine development has focused on antigens located on the surfaces of iRBC or free merozoites. This approach assumed that protective antibodies would opsonize, block invasion or prevent sequestration. Tex1 was not found to be surface exposed, but instead localized to the surface of MC. Upon egress, Tex1 gets exposed to the host immune system. A Tex 1-specific antibody effector function remains to be elucidated, but likely involves the presence of monocytes.

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References

1. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, et al. (2007) Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One 2: e645.
2. Corradin G, Villard V, Kajava AV (2007) Protein structure based strategies for antigen discovery and vaccine development against malaria and other pathogens. Endocr Metab Immune Disord Drug Targets 7: 259-265.
3. Olugbile S, Kulangara C, Bang G, Bertholet S, Suzarte E, et al. (2009) Vaccine potentials of an intrinsically unstructured fragment derived from the blood stage-associated Plasmodium falciparum protein PFF0165c. Infect Immun 77: 5701-5709.
4. Agak GW, Bejon P, Fegan G, Gicheru N, Villard V, et al. (2008) Longitudinal analyses of immune responses to Plasmodium falciparum derived peptides corresponding to novel blood stage antigens in coastal Kenya. Vaccine 26: 1963-1971.
5. Kulangara C, Kajava AV, Corradin G, Felger I (2009) Sequence conservation in Plasmodium falciparum alpha-helical coiled coil domains proposed for vaccine development. PLoS One 4: e5419.
6. Jensen JB, Trager W (1978) Plasmodium falciparum in culture: establishment of additional strains. Am J Trop Med Hyg 27: 743-746.
7. Dorn A, Stoffel R, Matile H, Bubendorf A, Ridley RG (1995) Malarial haemozoin/beta-haematin supports haem polymerization in the absence of protein. Nature 374: 269-271.
8. Lambros C, Vanderberg JP (1979) Synchronization of Plasmodium falciparum erythrocytic stages in culture. J Parasitol 65: 418-420.
9. Helg A, Mueller MS, Joss A, Poltl-Frank F, Stuart F, et al. (2003) Comparison of analytical methods for the evaluation of antibody responses against epitopes of polymorphic protein antigens. J Immunol Methods 276: 19-31.
10. Okitsu SL, Boato F, Mueller MS, Li DB, Vogel D, et al. (2007) Antibodies elicited by a virosomally formulated Plasmodium falciparum serine repeat antigen-5 derived peptide detect the processed 47 kDa fragment both in sporozoites and merozoites. Peptides 28: 2051-2060.
11. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, et al. (2004) Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 137: 13-21.
12. Pachlatko E, Rusch S, Muller A, Hemphill A, Tilley L, et al. MAHRP2, an exported protein of Plasmodium falciparum, is an essential component of Maurer's cleft tethers. Mol Microbiol.
13. Anderluh G, Pungercar J, Strukelj B, Macek P, Gubensek F (1996) Cloning, sequencing, and expression of equinatoxin II. Biochem Biophys Res Commun 220: 437-442.
14. Caly DL, O'Toole PW, Moore SA The 2.2-A structure of the HP0958 protein from Helicobacter pylori reveals a kinked anti-parallel coiled-coil hairpin domain and a highly conserved ZN-ribbon domain. J Mol Biol 403: 405-419.

15. Child MA, Epp C, Bujard H, Blackman MJ Regulated maturation of malaria merozoite surface protein-1 is essential for parasite growth. Mol Microbiol 78: 187-202.
16. Spycher C, Rug M, Pachlatko E, Hanssen E, Ferguson D, et al. (2008) The Maurer's cleft protein MAHRP1 is essential for trafficking of PfEMP1 to the surface of Plasmodium falciparum-infected erythrocytes. Mol Microbiol 68: 1300-1314.
17. Knapp B, Hundt E, Kupper HA (1989) A new blood stage antigen of Plasmodium falciparum transported to the erythrocyte surface. Mol Biochem Parasitol 37: 47-56.
18. Yeoh S, O'Donnell RA, Koussis K, Dluzewski AR, Ansell KH, et al. (2007) Subcellular discharge of a serine protease mediates release of invasive malaria parasites from host erythrocytes. Cell 131: 1072-1083.
19. Blackman MJ (2008) Malarial proteases and host cell egress: an 'emerging' cascade. Cell Microbiol 10: 1925-1934.
20. Spycher C, Klonis N, Spielmann T, Kump E, Steiger S, et al. (2003) MAHRP-1, a novel Plasmodium falciparum histidine-rich protein, binds ferriprotoporphyrin IX and localizes to the Maurer's clefts. J Biol Chem 278: 35373-35383.
21. Spycher C, Rug M, Klonis N, Ferguson DJ, Cowman AF, et al. (2006) Genesis of and trafficking to the Maurer's clefts of Plasmodium falciparum-infected erythrocytes. Mol Cell Biol 26: 4074-4085.
22. Gerold P, Schofield L, Blackman MJ, Holder AA, Schwarz RT (1996) Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of Plasmodium falciparum. Mol Biochem Parasitol 75: 131-143.
23. Jackson KE, Spielmann T, Hanssen E, Adisa A, Separovic F, et al. (2007) Selective permeabilization of the host cell membrane of Plasmodium falciparum-infected red blood cells with streptolysin O and equinatoxin II. Biochem J 403: 167-175.
24. Nickel W (2003) The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. Eur J Biochem 270: 2109-2119.
25. Mellman I, Warren G (2000) The road taken: past and future foundations of membrane traffic. Cell 100: 99-112.
26. Rubartelli A, Cozzolino F, Talio M, Sitia R (1990) A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. Embo J 9: 1503-1510.
27. Cooper DN, Barondes SH (1990) Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism. J Cell Biol 110: 1681-1691.
28. Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD (1989) Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell 56: 801-813.
29. Haase S, Herrmann S, Gruring C, Heiber A, Jansen PW, et al. (2009) Sequence requirements for the export of the Plasmodium falciparum Maurer's clefts protein REX2. Mol Microbiol 71: 1003-1017.
30. Saridaki T, Frohlich KS, Braun-Breton C, Lanzer M (2009) Export of PfSBP1 to the Plasmodium falciparum Maurer's clefts. Traffic 10: 137-152.

31. Spielmann T, Gilberger TW Protein export in malaria parasites: do multiple export motifs add up to multiple export pathways? Trends Parasitol 26: 6-10.
32. Dixon MW, Hawthorne PL, Spielmann T, Anderson KL, Trenholme KR, et al. (2008) Targeting of the ring exported protein 1 to the Maurer's clefts is mediated by a two-phase process. Traffic 9: 1316-1326.
33. Walshaw J, Woolfson DN (2001) Open-and-shut cases in coiled-coil assembly: alpha-sheets and alpha-cylinders. Protein Sci 10: 668-673.
34. Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol 11: 82-88.
35. Papakrivos J, Newbold CI, Lingelbach K (2005) A potential novel mechanism for the insertion of a membrane protein revealed by a biochemical analysis of the Plasmodium falciparum cytoadherence molecule PfEMP-1. Mol Microbiol 55: 1272-1284.
36. Day KP, Karamalis F, Thompson J, Barnes DA, Peterson C, et al. (1993) Genes necessary for expression of a virulence determinant and for transmission of Plasmodium falciparum are located on a 0.3-megabase region of chromosome 9. Proc Natl Acad Sci U S A 90: 8292-8296.
37. Barnes DA, Thompson J, Triglia T, Day K, Kemp DJ (1994) Mapping the genetic locus implicated in cytoadherence of Plasmodium falciparum to melanoma cells. Mol Biochem Parasitol 66: 21-29.
38. Bourke PF, Holt DC, Sutherland CJ, Kemp DJ (1996) Disruption of a novel open reading frame of Plasmodium falciparum chromosome 9 by subtelomeric and internal deletions can lead to loss or maintenance of cytoadherence. Mol Biochem Parasitol 82: 25-36.
39. Spielmann T, Hawthorne PL, Dixon MW, Hannemann M, Klotz K, et al. (2006) A cluster of ring stage-specific genes linked to a locus implicated in cytoadherence in Plasmodium falciparum codes for PEXEL-negative and PEXEL-positive proteins exported into the host cell. Mol Biol Cell 17: 3613-3624.
40. Culvenor JG, Langford CJ, Crewther PE, Saint RB, Coppel RL, et al. (1987) Plasmodium falciparum: identification and localization of a knob protein antigen expressed by a cDNA clone. Exp Parasitol 63: 58-67.
41. Hanssen E, Hawthorne P, Dixon MW, Trenholme KR, McMillan PJ, et al. (2008) Targeted mutagenesis of the ring-exported protein-1 of Plasmodium falciparum disrupts the architecture of Maurer's cleft organelles. Mol Microbiol 69: 938-953.
42. Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, et al. (2008) The transcriptome of Plasmodium vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. Proc Natl Acad Sci U S A 105: 16290-16295.

Figures:

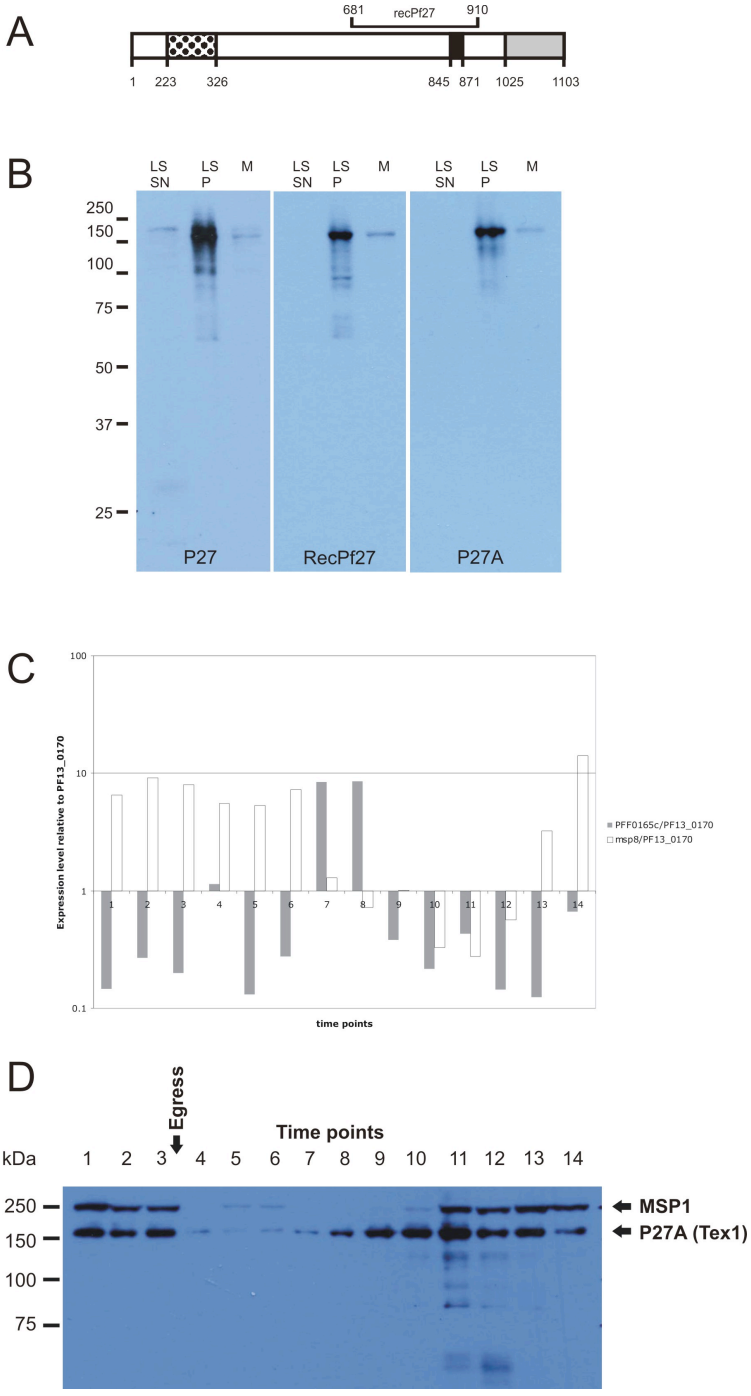

Figure1: Tex1 structure and expression dynamics on transcriptional and protein level. A) Schematic representation of Tex1. Black dotted: intrinsically unstructured region P27A; Black: coiled coil domain P27; grey: RING motif. B) Western Blot analysis of antibodies specific for P27A and P27 on the pellet fraction after saponin lysis of mixed stage parasite (M) and late stage parasite (LS). The late stage parasites were fractionated into a supernatant (SN) and a pellet (P) fraction after saponin lysis. M: marker C) Abundance of *tex1* transcripts by gRT-PCR. RNA was isolated from tightly synchronized culture in a 4h interval (table 1). *Tex1* transcript levels (grey bars) were normalized to the transcript abundance of the constitutively expressed glutaminyl-tRNA

synthetase (PF13_0170). As a control for the level of synchronization *msp8* transcripts were measured and compared to the PF13_0170 transcript level (white bars). D) Protein level was analyzed throughout the intraerythrocytic development cycle in a 4h interval by Western Blot analysis using antibodies against P27A and compared to protein abundance of MSP1. The parasite age (in hours post infection) and the parasite stages (confirmed by Giemsa staining) corresponding to the time points of harvest are illustrated in table 1.

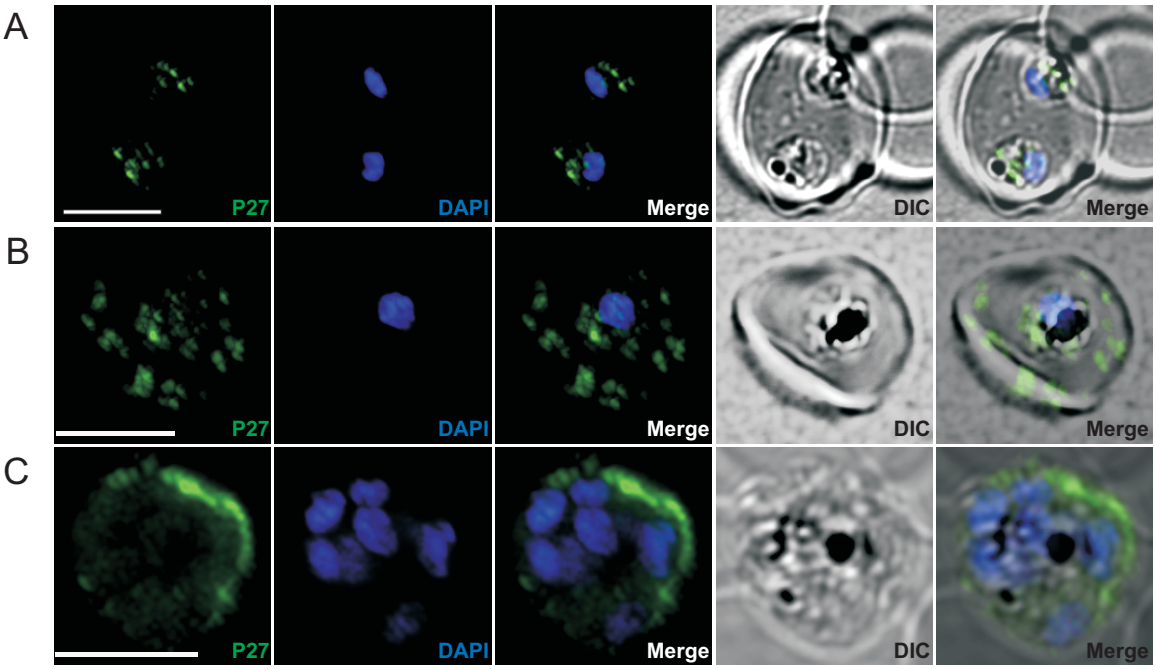

Figure 2: Immunofluorescence staining of erythrocytes infected by *P. falciparum* (ring, trophozoites and schizont stages) using P27-specific polyclonal rabbit sera. P27-specific polyclonal rabbit sera was used to detect Tex1 (green) A) in late ring stages B) in trophozoite stages C) in schizont stages. Nucleus stained with DAPI (blue), transmission picture of the infected red blood cell (DIC) and merged picture of the two signals or the signals merged with transmission picture (merge), Scale bar: 5 μm.

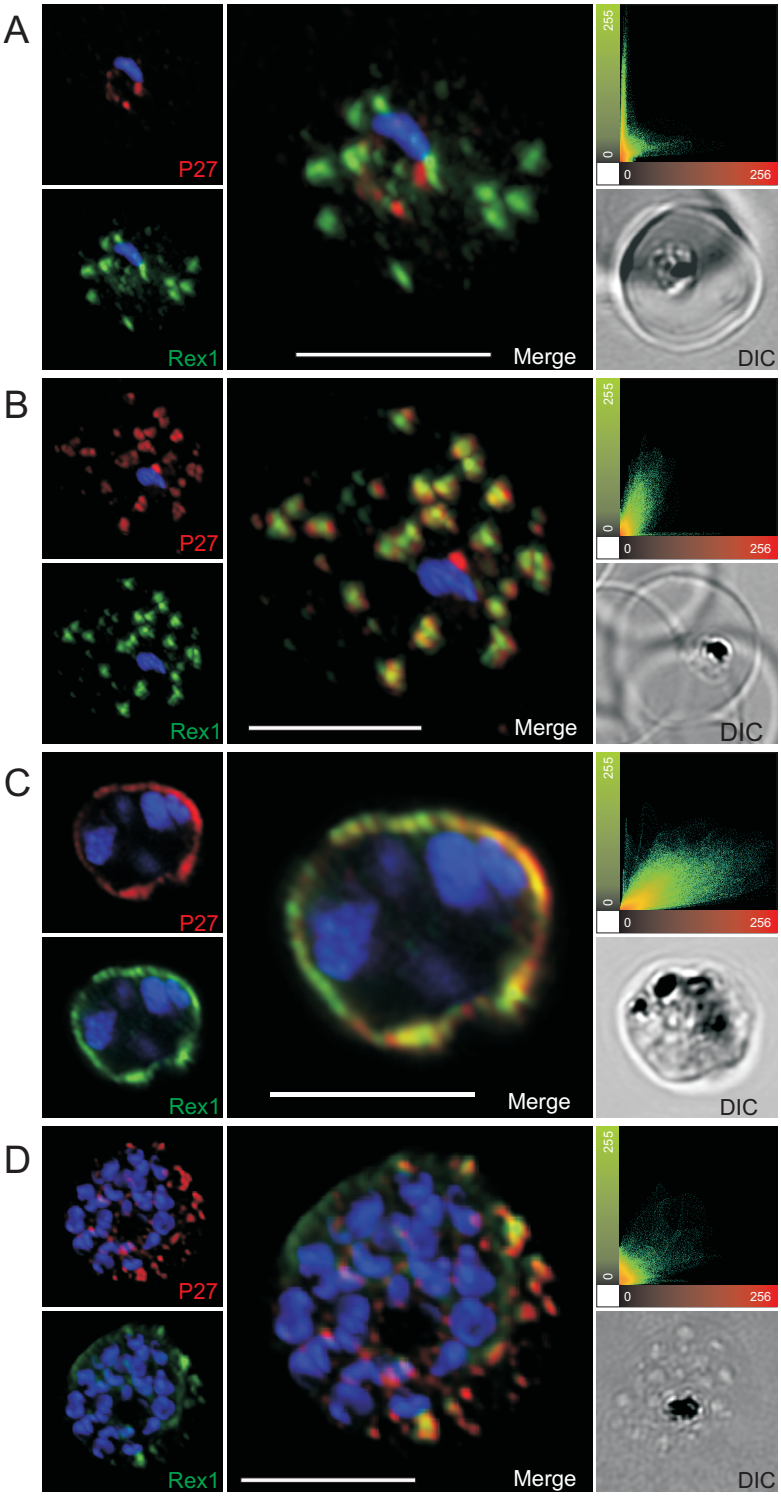

Figure 3: Co-localization of Tex1 with Rex1. P27-specific polyclonal mouse sera (in red) was used to detect TEX1. Rex1 polyclonal rabbit sera (in green). (A) Ring stage parasites; (B) trophozoite stages; (C) schizont stages. Scatter plots show the degree of co-localization of the Tex1 with Rex1 signal. Nuclear DNA was stained with DAPI (blue), Transmission image (DIC), Scale bar: 5 μm.

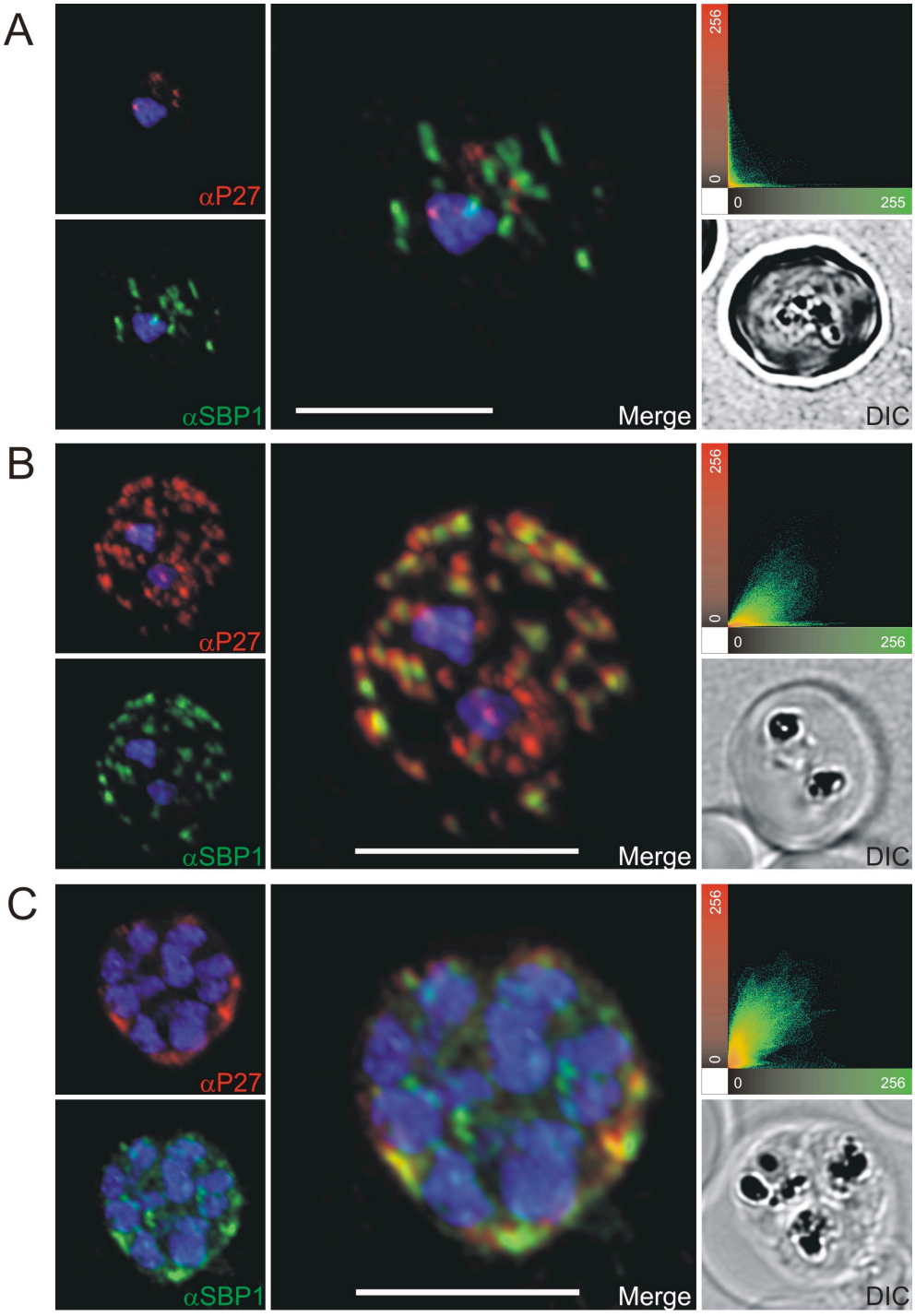

Figure 4: Co-localization of Tex1 with SBP1. P27-specific polyclonal rabbit sera was used to detect Tex1 (red). Co-localization was performed using SBP1 polyclonal mouse sera (green). Co-localization was performed in ring (A) trophozoite (B) and schizont stage (C) infected RBCs. Nuclear DNA was stained with DAPI (blue), Transmission image (DIC), Scale bar: 5 μm.

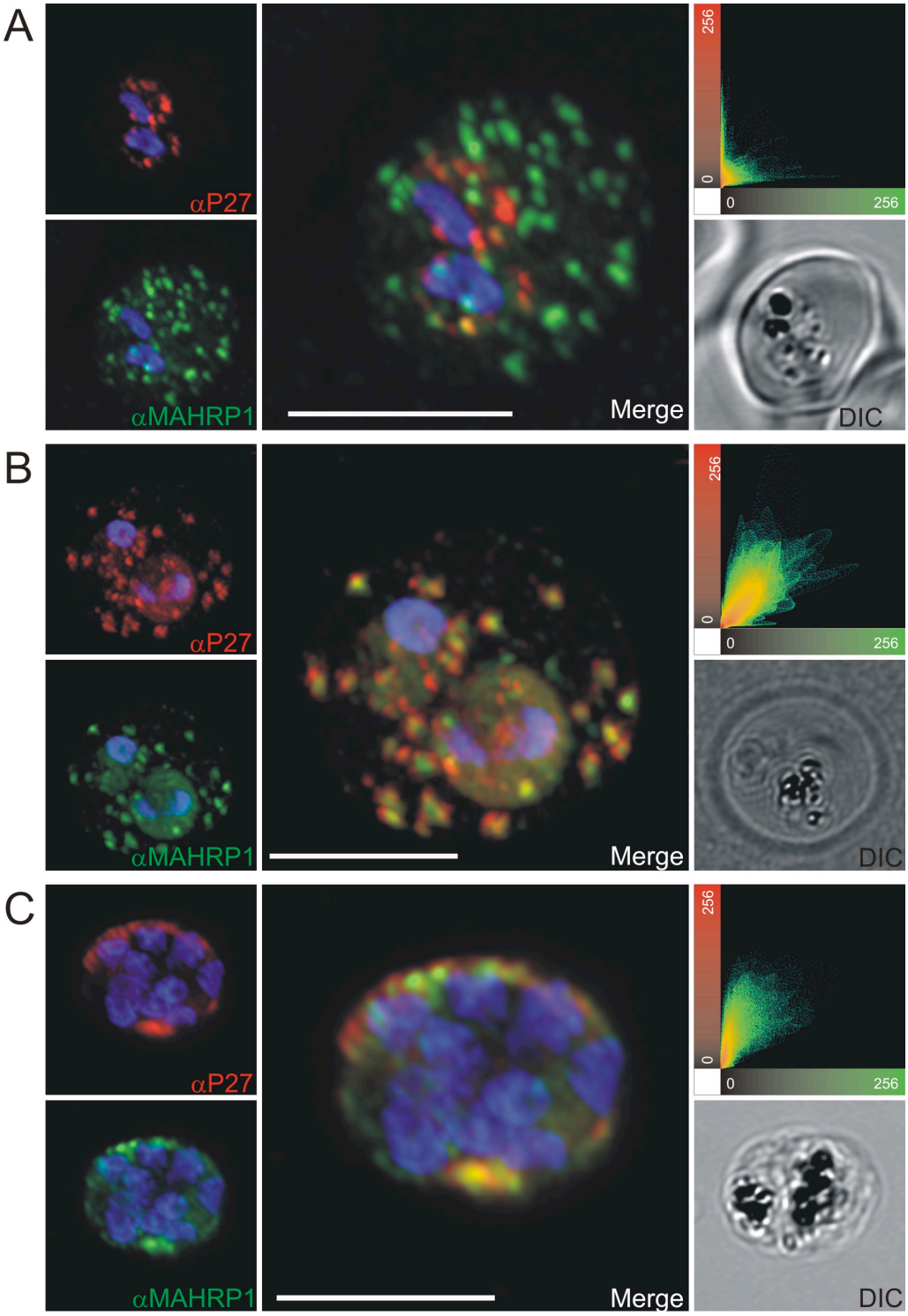

Figure 5: Co-localization of Tex1 with MAHRP1. P27-specific polyclonal rabbit sera was used to detect Tex1 (red). Co-localization was performed using MAHRP1 polyclonal mouse sera (green). Co-localization was performed in ring stage (A) trophozoite (B) and schizont stage (C) infected RBC. Nuclear DNA was stained with DAPI (blue), Transmission image (DIC), Scale bar: 5 μm.

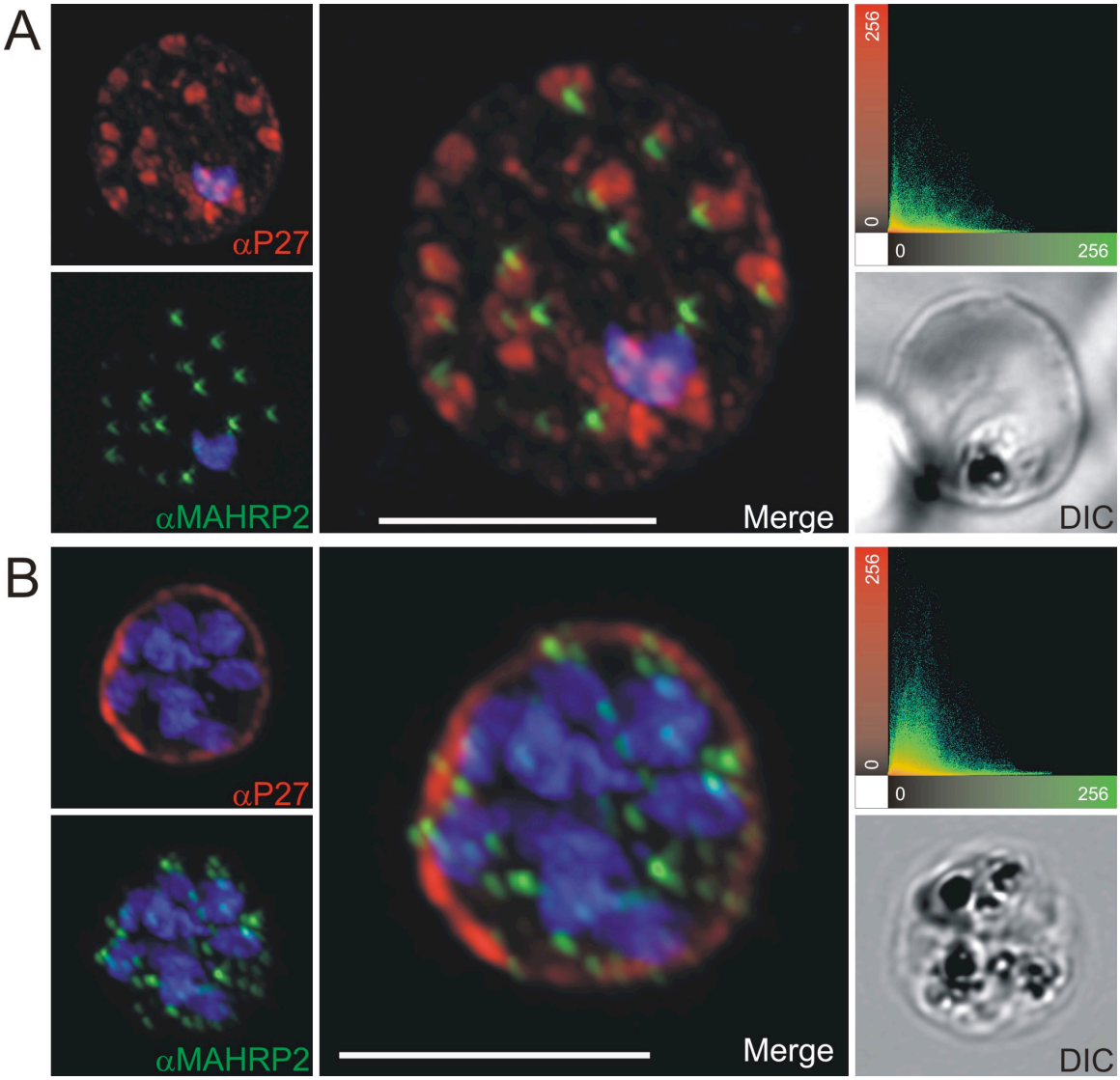

Figure 6: Tex1 localization in trophozoite and schizont stages with respect to newly described structures called tethers. Co-localization of Tex1 (red) with MAHRP2 (green) A) in trophozoite stages and B) in schizont stages. Nuclear DNA was stained with DAPI (blue), Transmission image (DIC), Scale bar: 5 μm.

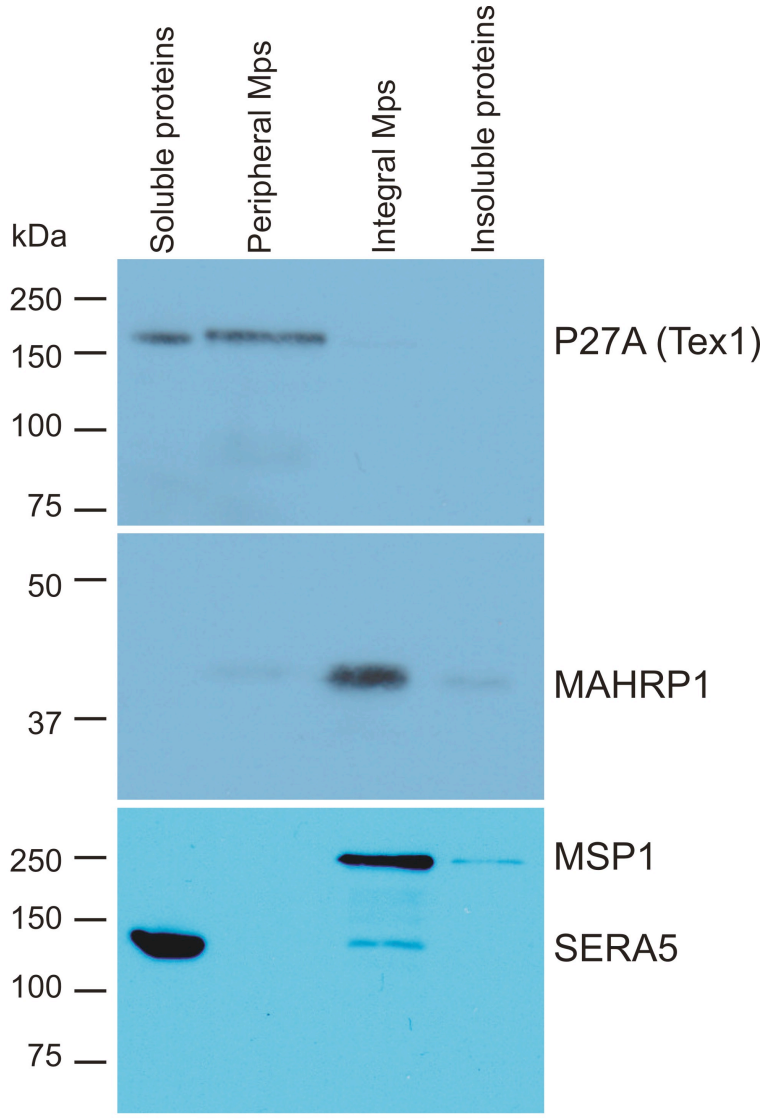

Figure 7: Dual solubility pattern of Tex1 shown by Western blot analysis of membrane fractionation assay of late stage parasites. Soluble proteins from membranes of RBCs infected with late stage parasites lysed by freezing thawing cycles (lane 1). Peripheral membrane proteins extracted by sodium carbonate buffer, (lane 2). Integral membrane proteins obtained by additional 1% Triton X-100 extraction (lane 3). Insoluble proteins (remaining membrane proteins after Triton X-100 extraction (lane 4). Blot was probed with P27A-specific polyclonal rabbit sera (panel 1), anti-MAHRP1 polyclonal rabbit sera (panel 2) and SERA5 and MSP1 mouse monoclonal antibodies (panel 3).

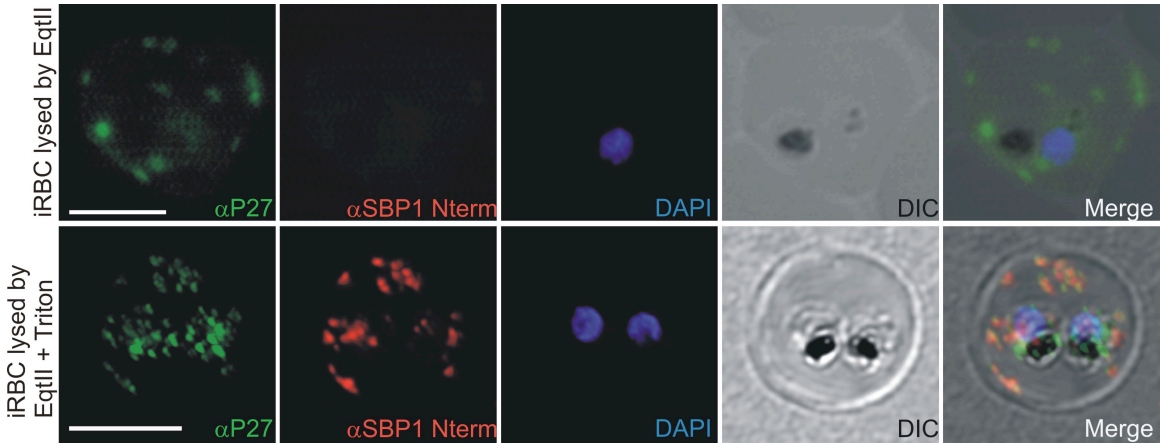

Figure 8: Equinatoxin II assay. A) 3D7 infected RBC lysed with equinatoxin II. Integrity of MCs is demonstrated by the absence of the SBP1 signal after using SBP1 N-terminus specific polyclonal mouse sera (note: N-terminus of SBP1 faces the lumen of MCs). Tex1 signal on the MC surface was obtained with P27-specific polyclonal rabbit sera (in green). B) 3D7 infected RBC lysed with equinatoxin followed by Triton lysis. MC lumen is now accessible for antibodies as shown by the SBP1 signal (in red). Nuclear DNA stained with DAPI (blue), Transmission image (DIC). Scale bar: 5 μm.

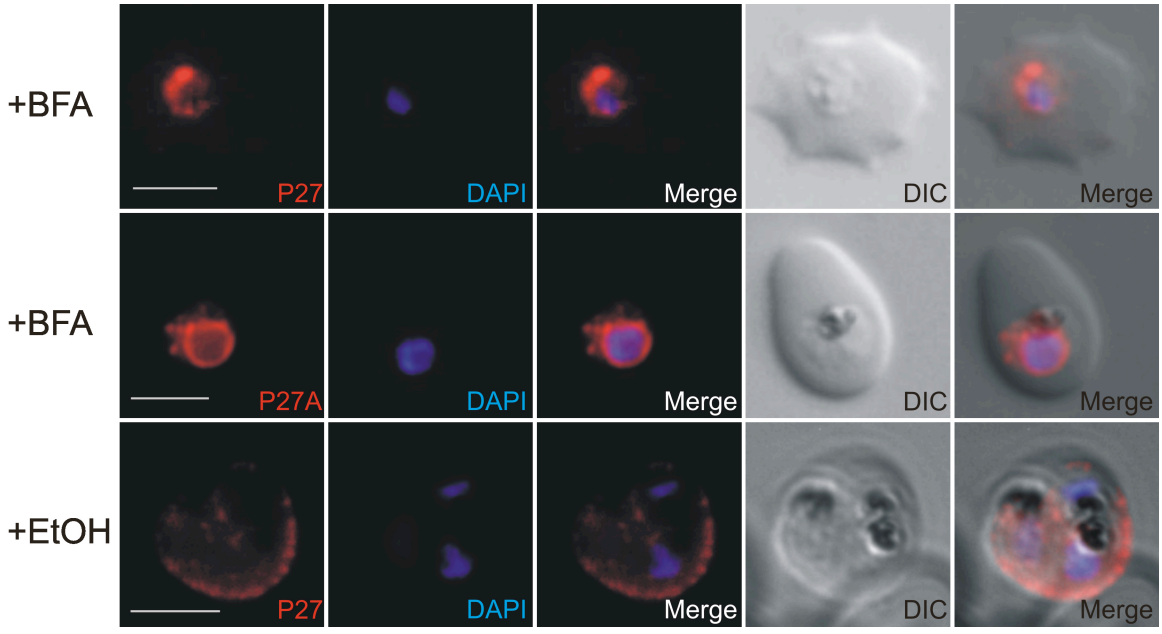

Figure 9: Brefeldin A sensitivity of Tex1 export. 3D7 infected RBC were treated with BFA and fixed (+BFA). Tex1 was stained using P27 or P27A-specific mouse antibodies (in red, upper panel: early trophozoite, middle panel: trophozoite). Tex1 visible inside the parasite in close proximity to the nucleus. A control culture (+ETOH) was incubated with equivalent concentration of ethanol, the solvent of Brefeldin A. In the control culture Tex1 was correctly exported and associated to MC (in red). The nucleus was stained with DAPI (in blue). Transmission image (DIC). scale bar: 5 μm

Tables

Table 1: Time points of harvest of synchronized 3D7 *in vitro* culture.

Time points	Hours post infection	Parasite stage
1	46-48	Schizont / Ring
2	0-2	Schizont / Ring
3	4-6	Ring
4	8-10	
5	12-14	
6	16-18	Late Ring
7	20-22	Early Trophozoite
8	24-26	
9	28-30	Trophozoite
10	32-34	
11	36-38	Late Trophozoite
12	40-42	Late Trophozoite / Schizont
13	44-46	Schizont
14	48-50	Schizont / Ring

Synchronized *P. falciparum* 3D7 parasite culture were harvested in 4 hours interval. Time points of harvest 1-14 (column 1), the corresponding age of synchronized parasites at each time point of harvest (in hours post invasion, column 2) and the corresponding parasite stage (column 3).

Supporting Information:

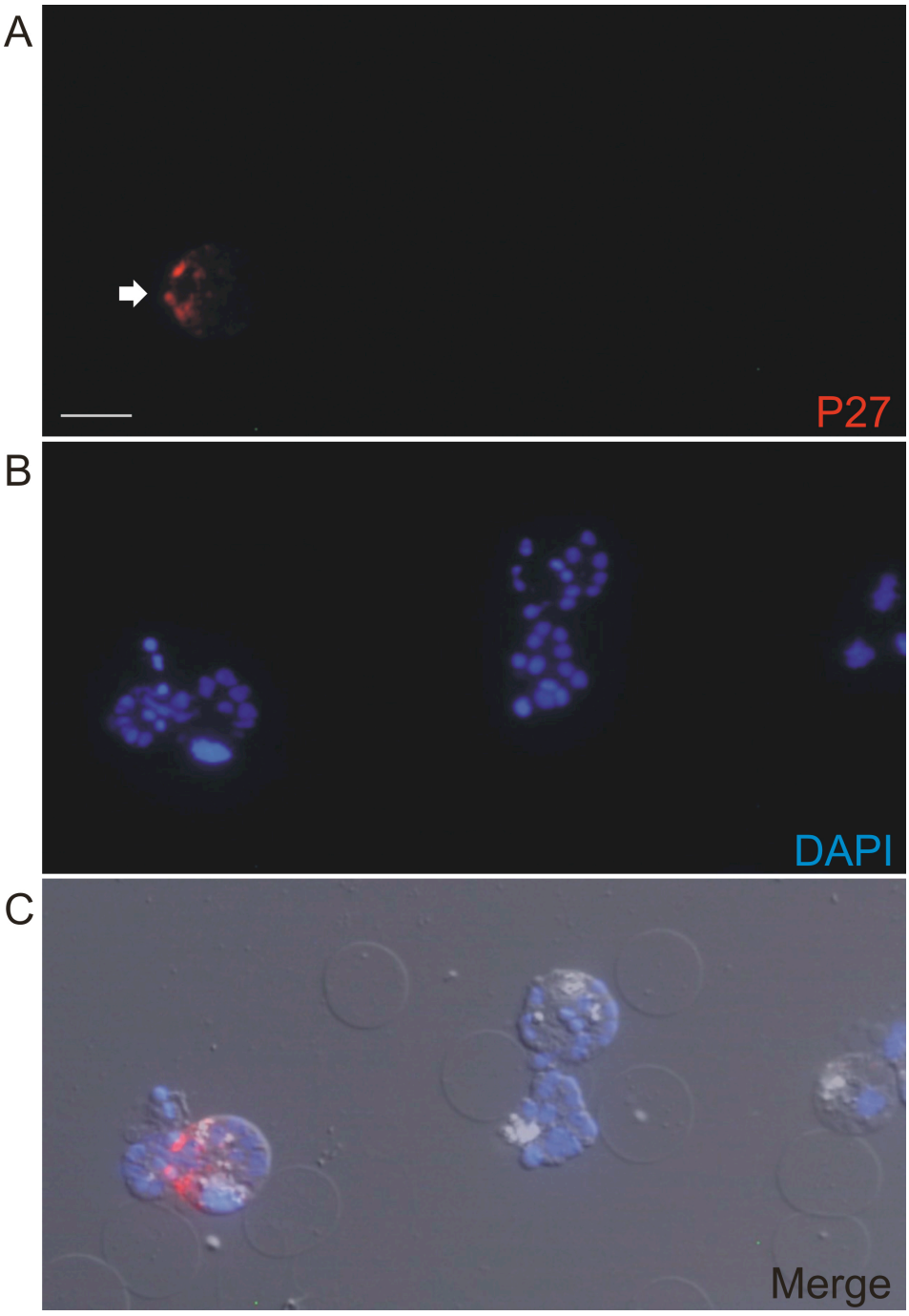

Figure S1: **Absence of surface exposure of Tex1.** The absence of Tex1 from the surface of infected RBCs was shown by incubating live cells with P27-specific polyclonal mouse sera directed against Tex1 (panel A). Tex1 signal was detected only in a lysed cell (panel A, white arrow). Nucleus stained with DAPI (panel B). Merged pictures of both signals and the transmission image (panel C). Scale bar: 5 μm

PFF0165c (*P. falciparum*, 3D7)

```
ATGCCTAATTTTTATATACATGATATAATAACATCTTTTTGGTTTTCCATATCTAACATATATATATAT
ATATATATATGTACATATTTTATAATATCTTTTTTTTGTTTGTTTTCGATTTATAAAATAATGAGTAAT
AAGAAAAGAAGTAAAAATGAAAATGACGAATCAACATCATTACCTTTAGAAAATTCCGAGTTATTA

M P N F Y I H D I I T S F W F S I S N I Y I Y I Y I C T Y F I I S F F
C L F S I Y K I M S N K K R S K N E N D E S T S L P L E N S E L L
```

PVX_113335 (*P. vivax*, Sal-1)

```
AAAAGGGAATAGTATATAGCACCCTTCAAGAGACGTAAGCGAAAGCAGTCACGGAAGTGACTCCCCCCA
TATATGTCCTATTGAGAGTCATCAACACCACCGCTCCCCCGCCAGCAAAAGTCAAAAGAAATGAATAGC
AAGAAAAGGAACAGAAGTGGAAAAAGCGAATGCATAACTTTGCCACTAGAAAAAACGGAGCTCCTA

K R E * Y I A P F K R R K R K Q S R K * L P P Y M S Y * E S S T P P L
P R Q Q K S K E M N S K K R N R S G K S E C I T L P L E K T E L L
```

PKH_114650 (*P. knowlesi*, H strain)

```
TTTTTTTTTTCAAAAAAAAAGAAAAGCGTCGTTAGCGTGAAGTAAAGAGAATAGTCGAAGGAAGTAATT
CCATCCGATGTGTCCAATAAAGACCCACCCCTGCAATTTTCTCTACACAACTCAGAAGAAATGAATAGT
AAGAAAAGGAGTAGGAGTGGAAAAAGCGAATGTATTACTTTGCCACTTGAGAAAACTGAACTTTTG

F F F S K K K K S V V S V K * R E * S K E V I P S D V S N K D P P L Q
F S L H N S E E M N S K K R S R S G K S E C I T L P L E K T E L L
```

Figure S2: **Upstream region of Tex1 and its orthologues in *P. vivax (PVX_113335)* and *P. knowlesi (PKH_114650)*.** Sequence highlighted in gray represents the region upstream of the of the predicted start Methionine. Stars (*) represent stop codons.

A

```
P.falciparum    FNEESKSEEGKIQLRDIQNDNDEKYDDEKKRFKELFIENQKLKEELNKKRNVEEELHSLR
P.vivax         CDELVAAPGADLHS-------------------ELLVENKQLKRALEKKQNAEKELSVLK
                 :*   :  ..::                    **::**::**. *:**:*.*:**  *:

P.falciparum    KNYNIINEEIEEITKEFEKKQEQVDEMILQIKNKELELLDKFNNKMNKAYVEEKLKELKN
P.vivax         KNYDAMSEEIEEITKEFEKKQEQVDEMIIQIKNKEFESLEKYKNAMNKICVEENIQQLER
                ***: :.********************:******:* *:*::* ***  ***::::*:.

P.falciparum    TYEEKMKHINNIYKKHDDFVNIYLNLFFQARKNAILSDSQREEQMNLFIKLKDKYDIIFQ
P.vivax         SYQEKVSSINRLYEKYESFAHVYLTLFYYARRSAVLSDSAREEQMSLFIKMKDKCESIFQ
                :*:**:. **.:*:*::.*.::**.**: **:.*:**** *****.****:*** : ***

P.falciparum    KKIELTDILKNVYDCNKKLIGHCQDLEKENSTLQNKLS----------------------
P.vivax         RKNELAEILQTVYGSNRKLMDKCNALYRENQHLERVATPTGGKGRRDPKDASCKGEAKRA
                :* **::**:.**..*:**:.:*: * :**. *:.  :

P.falciparum    ---------NEIKNSKMLSKNLSKNSDDHLLIEENNELRRRLICSVCMENFRNYIIIKCG
P.vivax         GAEEDADEADEADEADQADQGEEELEERQLLIEENNELRRRLMCSVCMENFKNYIIVKCG
                         :* .::.  .:. .: .: :**************:********:****:***

P.falciparum    HIYCNNCIFNNLKTRNRKCPQCKVPFDKKDLQKIFLD
P.vivax         HIYCESCIFSNLKTRNRKCPQCKIPFDKKDLQKIFLD
                ****:.***.*************:*************
```

B

```
P.falciparum    MPNFYIHDIITSFWFSISNIYIYIYICTYFIISFFCLFSIYKIMSNKKRSKNENDESTSL
P.vivax         ---------------------------------------------MNSKKRNRSGKSECITL
                                                             *..***.:. :.*. :*

P.falciparum    PLENSELLIEYIHNLKSCLNVYRREIQEKNKYISIIKNDLSFHECILTNVNVVWSVFNND
P.vivax         PLEKTELLIENIYNLRNAISLYRKELQEKNKCISDVKGELTFYECLLSNVSVVWGVFNDD
                ***::***** *:**:..:.:**:*:***** ** :*.:*:*:**:*:**.***.***:*

P.falciparum    LLNLLCNNEQKEEGEEIIKQRNIGDEINEYNNLTKLQNDENIKNNNMIKEDLEDDANQNI
P.vivax         LLRLAGEEGAIGDG------RCDGGGVGAVGGDCGDCGDPRCDG----AGQAQPSTGHCK
                **.*  ::    :*      *  *. :.  ..     .* . ..      : : .:.:

P.falciparum    LMKSPYYNIENFLQVFLKYINKKKKKVKVKVKDEGKKEKIEDKKYEQDDEEENEEEEEEE
P.vivax         GAAAPYYDVEQFKRIFLKYVSKNN---------------------QESDEEEDSQFSEAP
                   :***::*:* ::****:.*::                     ::.****:.: .*

P.falciparum    EEEEGEEENKEDEEFFKTFVSFNLY HNNNEKNISYDKNLVKQENDNKDEARGNDNMCGNY
P.vivax         SEDDACGEGKG-------------------------------------------------
                .*::.  *.*

P.falciparum    DIHNERGEMLDKGKSYSGDEKINTSDNAKSCSGDEKVITSDNGKSYDYVKNESEEQEEKE
P.vivax         ------------------------------------------------------EVEERG
                                                                      * **:

P.falciparum    NMLNNKKRSLECNPNEAKKICFSLEEKIGTVQSVKLKEYNELSKENIEKNKHDDNNICNY
P.vivax         GLPNGGAIKGEPLPGDLKKSQKSKEAVCGKDKERSRKQRNEFSSSKEE--THLEGEAAQK
                .: *.   . *  *.: **   * *   *. :. . *: **:*..: *  .* :.: .:
```

Figure S3: Sequence alignment of the *P. falciparum* Tex1 with the *P. vivax* orthologue.
A) Sequence alignment of the Tex1 C-terminus, P27 highlighted in grey. B) Sequence alignment of the Tex1 N-terminus, predicted signal sequence highlighted in light grey; P27A highlighted in bold.

Table S1: Oligonucleotide sequences used for cloning (restriction sites in bold)

Fragment	Forward primer 5'-3'	Reverse primer 5'-3'
recPf27	GATC**CCATGG**ATGGAAAATAAAAAGG ATGAAG	GATC**GGATCC**CTCTTCTACATACGCTTTA

Table S2: Oligonucleotide sequences used for qRT-PCR

gene	Forward primer 5'-3'	Reverse primer 5'-3'
tex1	GATCAGATCTTGTAGCGGCTTATGTT CACCAAAA	GATCAGATCTATTTTATAAATCGAAAACAAAC AAAAAA
msp8	AGTGCTGTAACTTCTAATGTAGGGGA TACAAAT	ATCATCATCACCATTATCATCATTATCATCAC G
PF13_0170	TGGCTAGGATATGATTGGAAAGAACA	TACGGTTCTATTTCTATATGGTGAATCA

Table S3: alpha-helical coiled coil domains in Tex1 (P27 in bold)

Position	Sequence
E23-L82	ELLIEYIHNLKSCLNVYRREIQEKNKYISIIKNDLSFHECILTNVNVVWSV FNNDLLNLL
Y477-N582	YYKNYIDIKKKISELQKDNESLKIQVDRLEKKKATLIYKLNNDNIRKHILD NNIKDYQNGIDN
N652- N1016	NLLKESNELYITLKEKEKENIILKNEILKMENKKDEEYEHLLNNTIEDKKE LTRSIKELEINMMTCNMEKDKISNKVNTLEYEINVLKNIDKNQTMQLQQKE NDILKMKLYIEKLKLSEKNLKDKIILLENEKDKMLSGIHIKDNSFNEESKS EEGKIQLRDIQNDNDEKYDDEKKRFKELFIENQKLKEELN**KKRNVEEELHS LRKNYNIINEEIEEIT**KEFEKKQEQVDEMILQIKNKELELLDKFNNKMNKA YVEEKLKELKNTYEEKMKHINNIYKKHDDFVNIYLNLFFQARKNAILSDSQ REEQMNLFIKLKDKYDIIFQKKIELTDILKNVYDCNKKLIGHCQDLEKENS TLQNKLSN

CHAPTER 5

Antigenicity and *in vitro* activity of recombinant malaria vaccine candidate in comparison to synthetic peptide with defined tertiary structure

Manuscript draft

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Abstract

The use of synthetic peptides in vaccine development offers many advantages over recombinantly expressed proteins. However, the ability of short peptides to fold into their native structure and act as potent immunogens remains in question. Using a genome-wide bioinformatic approach, potential candidates were identified that contain alpha-helical coiled coil motif to overcome these impediments. The full length, hypothetical *Plasmodium falciparum* protein PFD0520c, which contains the alpha-helical coiled coil segment P90, was recombinantly expressed and purified. To validate the use of P90, a comparative analysis was performed against recPFD0520c in an antibody dependent cellular inhibition assay, and an enzyme-linked immunosorbent assay to determine antigenic recognition by adult sera from Burkina Faso. Both P90 and recPFD0520c showed similar effectivity in *in vitro* parasite killing. Antigenic recognition was greater for P90. Structural studies revealed that the recombinant protein adopted an alpha-helical conformation and formed oligomers. Low transcript levels of PFD0520c found throughout the intra-erythrocytic development cycle suggest a low abundance of PFD0520c in the blood stage. Indirect immunofluorescence assays were performed to determine the cellular localization of native PFD0520c using antibodies raised against recPFD0520 in mice. An accumulation of the protein in punctuated structures within the parasite was detected. A tagged version of PFD0520c was recognized by the recPFD0520c-specific antibodies, but in Western blots performed on parasite lysate the same antiserum raised against recPFD0520c detected a protein at molecular weight twice the expected size, the nature of which remains to be determined.

Introduction

Malaria caused by the protozoan parasite *Plasmodium falciparum* is the most common and serious form of the disease, estimated to account for about 1 million deaths each year, the majority being young children (WHO, 2006). Efforts to control this devastating disease include the development of a vaccine. Obtaining protective immunity is a foreseeable goal and illustrated by the fact that individuals living in endemic areas continuously exposed to the parasite, acquire natural immunity over time [1,2].

Recent advances in technology and the availability of the *P. falciparum* genome have led to novel ways to rapidly identify potential vaccine candidates compared to traditional empirical approaches. Villard *et al.* (2007) took a genome-wide bioinformatic approach to identify potential candidates that contain an alpha-helical coiled coil motif (CC). The rationale behind this approach is that candidates can be quickly identified based on the characteristic heptad repeat structure of the CC, in which the first and fourth residues of the heptamer are hydrophobic while the other residues are generally hydrophilic. Secondly, CC are recognized by conformational dependent antibodies. Some of these peptides have undergone extensive validation as malaria vaccine candidates, illustrating their immunogenicity [3]. Lastly, CC are, in general, stable and, when chemically synthesized, they readily fold in to their native structure in aqueous environment.

CC segments, identified by Villard *et al.* (2007) are generally 30-40 amino acids in length and derive from proteins expressed in *P. falciparum* blood stages. 95 of these bioinformatically selected peptides were chemically synthesized and purified, and were subject to preclinical evaluations as vaccine candidates [3,4,5].

Despite the advantages of synthetic peptides, controversy exists towards short- to medium-length peptides and their ability to fold into their native structure and act as potent immunogens. Although the approach taken by Villard *et al.* expects

to overcome these issues by synthesizing stable structures, it is of importance to compare the preclinical evaluations of the peptides with corresponding results obtained with the full length protein from which the peptide is derived. Such comparison will further validate the use of CC peptides in vaccinology. Here we further investigate the CC segment P90 deriving from the unknown predicted protein, PFD0520c. P90 was found to be a promising candidate in previous preclinical evaluation [3]. The recombinant protein and synthetic peptide are compared with respect to their performance in two assays: (i) an Antibody-dependent Cellular Inhibition assay (ADCI) for demonstrating functional activity of anti-P90 antibodies, and (ii) Enzyme-linked Immunosorbent Assay (ELISA) for determining the proportion of responders to both antigens among clinically immune adults from a malaria endemic population.

Further characterization of this vaccine candidate was undertaken, including investigation of structural properties of PFD0520c and P90, their ability to form a CC and their oligomeric state, as well as the expression profile of PFD0520c throughout the intra-erythrocytic development cycle (IDC) and the subcellular localization of the protein in infected red blood cells.

Methods

P. falciparum culture

P. falciparum 3D7 strain was continuously cultured with fresh human erythrocytes and RPMI 1640 10.44 g/l, supplemented with Hepes 5.94 g/l, Albumax II 5 g/l, hypoxanthine 50 mg/l, sodium bicarbonate 2.1 g/l and neomycin 100 mg/l as described previously [18].

Preparation of recombinant His6-PFD0520c (recP)

The ORF encoding the predicted full length sequence of PFD0520c (retrieved from PlasmoDB, version 5.3) was amplified from genomic *P. falciparum* 3D7 DNA using primers listed in **Table S1** The PCR amplified product was digested with NcoI and BamHI and inserted into the pQE-60 vector (Qiagen) to generate C-terminal His-tagged PFD0520c (His6-PFD0520c) expressing plasmid (pQE-60-His6-PFD0520c).

Expression of recombinant His6-PFD0520c

pQE-60-His6-PFD0520c plasmid was transformed into *E. coli* M15 cells (Qiagen). Recombinant expression was performed following the manufacturer's protocol (Qiagen Inc.). Subsequent preparation was performed using Qiagen kit (Qiaexpressionist) for non-native conditions. The resulting pellet containing insoluble proteins was resuspended in binding buffer suitable for fast protein liquid chromatography (FPLC) His-tag affinity purification.

Recombinant protein purification

Insoluble protein lysate was passed through a 0.45 µm filter. Purification of His6-PFD0520c was performed by affinity chromatography using a HisTrap column (GE Healthcare) equilibrated with extraction buffer on a ACTA Prime Plus (GE

healthcare) and the manufacturer's pre-programmed method template named "on-column refold". To remove the high salt content of the elution buffer, a further

FPLC purification step was performed using a HiPrep Desalting column. The program was input manually (15 ml equilibration step, 5 ml sample application, 5 ml sample elution). All steps were carried out at a 1ml/min flow rate). Exchanged buffers were 1 X PBS, 20 mM Tris/150 mM NaCl/0.1% Tween-20 pH 7.5, or 0.1 M ammonium acetate pH 7.5 depending on the subsequent experiment.

Determination of protein purity and identity

To determine the purity of the protein, a Coomassie staining was performed. Purified protein was diluted to a 1:100 dilution before loading onto SDS-PAGE. His-tagged purified protein was also visualized in a Western blot using monoclonal mouse anti-6xHIS antibodies (1:2000, R&D Systems) and secondary anti-mouse IgG antibodies conjugated with horse radish peroxidase (1:20'000, Pierce).

Peptide synthesis

Peptides were produced by solid-phase Fmoc chemistry according to Atherton and Sheppard (3) and as modified by [6] using Applied Biosystems 431A and 433A synthesizers (Foster City, CA). The final product was cleaved from p-alkoxybenzylalcohol resin and later RP-HPLC purified. The purity was assessed by analytic C18 HPLC and mass spectrometry (MALDI-TOF, Applied Biosystem) and was found to be higher than 85%.

Circular dichroism (CD)

The CD spectra of His6-PFD0520c were recorded on a JASCO J-810 spectrometer (JASCO corporation, Tokyo, Japan) equipped with a temperature controller and a 0.1 cm path length cuvette. The measurements were made in 1 X PBS at pH 7.3 and 22°C and at a protein concentration of 0.2 mg/ml.

Mass Spectrometry

Mass spectrometry was performed using Applied Biosystems Voyager-DE. 0.5 ul recPFD0520c in 20mM Tris-Hcl, 150 mM Nacl, 0.1% Tween, pH 7.4 buffer (0.2 mg/ml) and 0.5 of sinapinic acid matrix solution were spotted on plate. A spectrum was obtained in the mass range 21 997.0 to 150 000.3. Data were analyzed with Data Explorer version 4.

Gel filtration FPLC

Gel filtration (Äkta FPLC, Amersham Biosciences) of 1 ml recPFD0520c (0.2ug/mL) in 20 mM Tris-Hcl, 150 mM Nacl, 0.1% Tween, pH 7.4 buffer was performed using a Superdex 200 (GE Healthcare) column following the manufacturer`s protocol.

Affinity Purification of PFD0520c-specific antibodies from pooled sera of adults from Burkina Faso.

Coupling of cyanogen bromide-activated Sepharose 4B beads (Amersham Biosciences) to 5 mg of recombinant protein and the isolation of specific antibodies was performed as described [3] using a serum pool of adult individuals living in Burkina Faso.

ADCI

The inhibition of P. *falciparum* (3D7 strain) growth *in vitro* in the presence of human monocytes (MN) and antigen specific antibodies was carried out by the method described previously [7].

ELISA

ELISA's were performed to determine recPFD0520c recognition of 39 clinically immune patients from Burkina Faso (BF). 96-well polysterene plates (NUNC^{TM} Maxicorp cat # 468667, Nalge Nunc International Corp., Naperville, Il, USA) were coated with with 1 µg/ml of recPFD0520c or 5 µg/ml of peptide 90 (p90) and incubated overnight in a humid chamber at 4˚C. Plates were washed 4 times with

washing solution (1 X PBS, 0.05% Tween-20), and blocked with 1 X PBS, 0.05% Tween-20 containing 5% milk for 1 hour at room temperature in humid chamber. Sample sera were diluted 1:200 in 1 X PBS, 0.05% Tween-20, and 2.5% milk solution and added to the wells (50µl/well). Following 1 hr incubation at room temperature in humid chamber, plates were washed (as above). Antibody-Antigen complexes were detected by the use of goat-anti-human IgG polyvalent antibodies conjugated with alkaline phosphatase (Sigma Chemical Co. cat # A9544). The binding of goat antibodies was detected using an enzyme substrate buffer containing para-nitrophenol phosphate (Sigma Chemical co. cat # N-2770). The plates were incubated at room temperature in dark conditions for 15 minutes and then the reaction was stopped by the addition of 50 µl of 1 M NaOH. The resultant yellow colour was quantified measuring the absorbance at 405 nm.

The sera from 40 Swiss, non-exposed to malaria donors were used to generate the cut off value for data analysis. The cut-off value was calculated using the equation: cut-off = (mean OD_{405} of Swiss donors) + (3*standard deviation (SD)). The percentage of BF adults who had a greater OD_{405} value compared to the cut-off is expressed as percent recognition in (**Table 1).** The ratio of positive adults expressed as a percentage was also calculated by considering an individual positive if their sample OD_{405} was 2 times greater then the mean OD_{405} of the non-exposed controls.

Immunogenicity in mice

Two inbred strains of mice were injected with 20 µg of antigen formulated in Montanide ISA 720 (35 µg/ mouse), alum or EM005 [8]. Each mouse was injected either subcutaneously at baseline of the tail (Montanide, 50 µl/ mouse or EM005 100 µl/ mouse) or intraperitoneally (Alum, 500 µl/ mouse) at 0, 3 and 8 weeks. The induced antibody responses were assessed by ELISA 10 days following second and third immunizations.

RNA isolation, cDNA synthesis and real time PCR

3D7-infected erythrocytes were tightly synchronized to a theoretical time frame of 2 hours and grown to 8-10% parasitemia (5% heamatocrit). 1.5 ml culture was taken in 4h intervals. In brief, parasite RNA was extracted using TRIzol (invitrogen) according to the manufacturer's instructions. TRIzol extraction was repeated. Residual gDNA was digested twice with RQ1 DNase (Promega) according to the manufacturer's protocol. Reverse transcription was done by AffinityScript Multiple Temperature Reverse Transcriptase (Stratagene) with random primers (Invitrogen) as described by the manufacturer. To control for gDNA contamination, the target sequence was amplified from the RNA solution prior to reverse transcription. The primers used for qPCR of *PFD0520c*, *PF13_0170* and *msp8* are shown in supplementary material Table S1. Absolute transcript quantification was performed at a final primer concentrations of 0.4mM using SYBR® Green Master Mix (Applied Biosystems) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems) in a reaction volume of 12 µl. All reactions were performed in triplicate yielding virtually identical Ct values. A serial dilution of gDNA was used as standard for absolute quantification. Relative transcript profiles were calculated by normalization against transcript levels of the house-keeping gene *PF13_0170* (glutaminyl-tRNA synthetase).

Plasmid construct and parasite transfection

The PFD0520c ORF was amplified from 3D7 genomic DNA using the primers listed in Table S1. The fragment was cloned into the pBcam-3xHA vector [9] via the BamHI and NheI restriction sites. Transfections were performed as described in [10] and selected on 5µg/ml blasticidin-S-HCl.

Indirect Immunofluorescence Assay (IFA).

Infected erythrocytes were fixed, permeabilized and blocked as described [11]. Primary antibodies were used with the following concentrations: polyclonal rabbit sera anti-SERP (1:400); polyclonal mouse antibodies anti-recPFD0520c (1:500); anti-mpPFD0520c (1:500); monoclonal anti-rat antibodies: anti-HA (Roche

Diagnostics, 1:200). Secondary antibodies used: anti-rabbit IgG (Alexa Fluor 488, Invitrogen; 1:400); anti-mouse-IgG (Texas Red, Invitrogen; 1:400) and anti-rat IgG (Alexa-Fluor® 568 Molecular Probes, 1:400). Cells were mounted in Vectashield Hard Set supplemented with DAPI (Vector Laboratories) for staining of the nuclear DNA. Images were obtained by the Zeiss confocal microscope LSM 700. Images were processed by ImageJ software or the Huygens Essential Software (Scientific Volume Imaging, The Netherlands).

Lysate preperation and Western Blot

To obtain protein extract of mixed stage infected erythrocytes, 10 ml of 3D7 parasite culture was grown in a petri-dish to 5% to 10% parasitemia, lysed on ice in 0.03% saponin in phosphate-buffered saline (PBS, pH 7.4) for 10 min, washed with ice-cold PBS for complete removal of haemoglobin, and resuspended in Laemmli sample buffer.

For Western blot analysis protein extracts were separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose (Hybond-C extra; GE Healthcare) at 4°C for 1 h at 80 V and an additional hour at 100 V. The membrane was blocked for 1 h in 5% skim milk, 0.1% Tween in Tris-buffer. The following antibodies were used: polyclonal mouse sera: anti-recPFD0520c (raised in C3H mice, 1:2000); anti-recPFD0520c (raised in CB6F1 mice, 1:2000); anti-mpPFD0520c; (1:2000); anti-p90 (1:200); monoclonal anti-rat antibodies: anti-HA (Roche Diagnostics, 1:500); horseradish peroxidase-conjugated goat anti-mouse (Pierce, 1:20 000), goat anti-rat (Acris, 1:5000).

Two-dimensional gel electrophoresis (2-DE)

Protein extraction was done using 50 ml of mixed stage infected erythrocytes grown to 5% parasitemia. Cells were lysed on ice in 0.03% saponin in phosphate-buffered saline (PBS, pH 7.4) for 10 min, washed with ice-cold PBS for complete removel of haemoglobin and further extracted by methanol/chloroform.

Isoelectric focusing (IEF) was carried out in 7 cm IPG strips (ReadyStrip™, Bio-Rad) with nonlinear pH gradient of 3-10. The process was performed using Protean® IEF System (Bio-Rad) with a surface temperature of 20°C and a maximum current of 50 µA/strip. Protein pellet was reconstructed in sample buffer (7M urea, 2M thiourea, 4% CHAPS, 5mM TBP, 0.25% SDS, 2% Biolyte). An aliquot containing 100 µg of protein was mixed with appropriate amount of rehydration buffer (8M urea, 1% CHAPS, 2mM TBP, 0. 25% Biolyte) so that 130 µl of sample were applied to the IPG strip by in-gel rehydration at 20°C for 12h. After the rehydration step the IEF was run for a total of 62 250 Vh. Following the IEF, the strip was reduced in equilibration buffer (6M urea, 30% glycerol, 5% SDS, 0.05 M Tris, pH 8.8) containing 1% DTT for 10 minutes and then alkylated in equilibration buffer containing 5% iodoacetamide for 10 minutes. The second dimension was performed on a 12% SDS-polyacrylamide gel. Western blot was performed as described above.

Results

The predicted coding sequence for PFD0520c spans nucleotide positions 482'841 to 483'482 on chromosome 4. According to PlasmoDB version 6.5 (http://plasmodb.org), the PFD0520c has a length of 213 amino acids (aa) and a predicted molecular weight of 25 377 Da. PFD0520c contains 1 predicted coiled coil domain (P90) encompassing region from T61 to V98. On protein sequence level P90 was found completely conserved in 14 different culture strains and in malaria positive field samples from Tanzania and Papua New Guinea. One synonymous SNP was detected at nucleotide position 195 (T to C) in 1/14 culture strains and in 4/23 samples from Papua New Guinea [5].

Recombinant PFD0520c (recPFD0520c) expression and purification

The molecular weight of the recombinantly expressed PFD0520c with the additional 6xHIS tag was calculated to be 26 433 Da. The protein was successfully expressed in *E.coli* from which it was retrieved predominantly as insoluble protein **(Figure 1A/1B)**. RecPFD0520c was purified from the insoluble protein fraction using FPLC **(Figure 1C)**. The purity of the protein was confirmed with mass spectrometry (MS, data not shown). CD data indicate the presence of alpha-helical structures in the recombinant protein **(Figure 1D)**.

Specificity of polyclonal antibodies raised in mice against recPFD0520c

Polyclonal antibodies purified from C3H and CB6F1 mice strain immunized with recPFD0520c were analysed in Western blot to confirm recombinant protein recognition. The recombinantly expressed protein was recognized by the polyclonal mouse sera. At a dilution of 1:10'000, a clean and sharp band was obtained at the expected protein MW **(Figure 2)**.

ADCI assay results using human purified anti-recPFD0520c antibodies were comparable to those using human purified anti-P90.

The antibody-dependent cellular inhibition assay investigates the function of antibodies for *in vitro* parasite killing in the presence of monocytes [7,12]. Purified IgG from semi-immune adults was shown to inhibit parasite growth *in vitro* in cooperation with monocytes [7]. When using human purified antibodies against recPFD0520c a specific growth index (SGI) of 75% was measured. Two independent ADCI assays were performed using human purified antibodies against P90 and the SGI values were 70% and 43%.

Adult immune sera showed similar recognition of P90 and overlapping protein fragments compared to recPf90.

The sera from 39 clinically immune adults from Burkina Faso were tested in ELISA for reactivity with recPFD0520c and P90 (**Table 1**). The percent recognition of sera tested was 59% and 43% for p90 and recPFD0520c respectively. The percentage of positive individuals when a secondary method of determining reactivity based on ratio is used was 41% for both antigens.

PFD0520c was expressed in intraerythrocytic blood stage parasites but at very low level

The transcription profile was analyzed by RNA isolation and quantification of reverse transcribed cDNA from tightly synchronized cultures harvested in 4 h intervals covering the 48h intra-erythrocytic development cycle. In these experiments the developmental stage of synchronized parasites of each time point were expected not to vary for more than 2 h. The transcript abundance was quantified by gRT-PCR at all time points. The transcription level of *PFD0520c* was normalized to a constitutive transcribed gene, *glutaminyl-tRNA synthetase* (PF13_0170). *PFD0520c*-specific transcripts were detected throughout the intra-erythrocytic development cycle, however, the transcript abundance was very low (**Figure 4)**. The low level of PFD0520c expression in blood stage parasite was in agreement with the expression data available at PlasmoDB. To validate the

synchronization procedure, transcript levels of the merozoite surface protein 8 (*msp8*) were analyzed at each time point. The *msp8* profile obtained was in good agreement with the previously shown up-regulation of *msp8* in ring stages and in very late schizont stages (data retrieved from PlasmoDB, http://plasmodb.org).

Polyclonal mouse sera did not recognize the endogenous protein at the expected MW in parasite lysate

Anti-recPF0520c specific polyclonal mouse sera raised in C3H and CB6F1 mice were used to characterize by Western Blot the native protein from 3D7 parasite lysate. Both mouse sera recognized a single band of the approximate molecular weight of 50 kDa **(Figure 5A)**. However, the predicted molecular weight of PFD0520c is 25 377 Da (PlasmoDB). In order to analyze PFD0520c migration and possible protein dimerization, a transgenic parasite line was generated that expressed the predicted full-length PFD0520c protein fused to a Hemagglutinin Tag (HA-tag). HA-specific antibodies as well as the recPFD0520c-specific polyclonal mouse sera detected the HA-tagged version at the expected MW of around 30 kDa **(Figure 5A and 5B)**. Dimer formation of the HA-tagged protein was not detected **(Figure 5A and 5B)**. Migration of the HA-tagged version did not differ between reduced versus non-reduced condition for the protein lysate of the transgenic parasite line **(Figure 5A and 5B)**.

The entire predicted PFD0520c protein had previously been synthesized as five overlapping peptide fragments **(Figure 6).** Mouse serum was generated against this peptide mixture (anti-mpPFD0520c). Equally to the anti-recPFD0520c mouse sera, the anti-mpPFD0520c sera recognized the HA-tagged version and the 50 kDa protein **(Figure 5B)**. Sera raised against P90 failed to detect the HA-tagged protein or additional bands **(Figure 5B)**.

PFD0520c localized to punctuated structure within the parasite

To study the subcellular localization of PFD0520c, infected RBCs were analyzed by immunofluorescence analysis (IFA). The endogenous protein was detected in

punctuated structures within the parasite **(Figure 7)**. The parasite boundary is illustrated by staining of the parasitophorous vacuole (PV) by polyclonal rabbit sera specific for SERP (serine-rich protein/antigen 5). SERP was shown to be exported and to accumulate in the PV until parasite egress [13]. To compare the localization pattern of the native protein with the HA-tagged PFD0520c version, the transgenic parasite line was analyzed in IFA. HA-tag specific antibody detected the fusion protein at similar punctuated structure within the parasite **(Figure 7)**.

Proteomic analysis of parasite lysate

To determine whether the recPFD0520c-specific mouse serum cross-reacted with a protein of an approximate size of 50 kDa 3D7 lysate of mixed parasite stages was separated in two dimensions (Figure 8A). A single signal was obtained after Western blot of the 2D gel with recPFD0520c polyclonal mouse antibody.

Discussion

RecPFD0520c was successfully expressed. Western blots analysis suggest that contaminant proteins were still present in the purified protein solution. Polyclonal mouse recPFD0520c-specific antibodies indeed recognized the recombinant protein, but also additional bands both below and above the expected weight of recPFD0520c appeared when a higher antibody concentration was used. These additional bands, however, were not recognized with anti-HIS monoclonal antibodies and therefore it was concluded that contaminant proteins were present in the purified protein solution.

In order to investigate the structural properties of recPFD0520c a CD spectra was obtained. The protein's secondary structure is, in part, alpha helical. Reference spectra are available from a set of CD spectra with known secondary

structure determined by x-ray crystallography [14]. The consensus reference CD spectra for an α-helix consists of a positive peak at about 190-195 nm with a magnitude of about 60 000-80 000 deg cm^2/decimole, and two negative peaks at 207 nm and 222 nm with a magnitude of around -36 000 $\pm$ 3 000 deg cm^2/decimole at 222 nm [14]. Comparison of the standard α-helix spectra with that of recPFD0520c confirmed that all characteristic peaks occur at the same wavelengths. The magnitude of the 222 nm peak of recPFD0520c protein is above 50% of the reference value, indicating that the purified full-length recPFD0520c protein exhibits an alpha-helical content of at least 50%.

Purified recPFD0520c protein was analyzed by gel filtration. The peak height represents the protein concentration. For RecPFD0520c three protein elution peaks were observed, with increasing peak heights and thus lower concentration for the highmolecular elution peaks and higher concentration of the low molecular weight (monomeric) peak. **(Figure S1)**. This suggested that the recPFD0520c protein solution contained monomers, dimers, and trimers of the full length protein.

Further indication of recPFD0520c dimer formation was provided by SDS page followed by Coomassie staining of unreduced and reduced conditions. The strong band at the expected MW of the protein (27 kDa) represents the monomer. An additional band of about 54 kDa representing the dimer was detected **(Figure S2)**. Difference in band intensity suggested that the monomeric form was more abundant and dimer formation could be completely resolved in reducing conditions.

MS analysis also infers the presence of monomers, dimers and to a slight extent trimers due to the peaks found at approximately each expected oligomeric molecular weight. Discrepancies were found between the molecular weight of the monomeric form of recPFD0520c calculated from the sequence data (26 443

Da) and that calculated by MS (26 746 Da) (data not shown). This discrepancy remains to be resolved by recalculation and repeating the MS experiments.

The results of the ADCI assay performed with human purified antibodies, specific to recPFD0520c and P90, suggested that these antibodies cooperated with human monocytes to inhibit malaria erythrocytic growth *in vitro*. The SGI values obtained for purified human-recPFD0520c antibodies were comparable to the P90-specific human antibodies with SGIs of 75% and 45, 73% respectively. The structural analysis of recPFD0520c suggested that the CC region was correctly folded. However no structural information is available from the remaining parts of the protein and if the recPFD0520c mimics entirely the parent protein remains unknown. The slightly increased effect measured for the recPFD0520c-specific antibodies in ADCI might indicate that additional epitopes are recognized by these antibodies. However, there is the possibility of protein-unrelated antibodies due to the presence of *E. coli* proteins in the recPFD0520c preparation resulted in an increased effect measured by ADCI.

ELISA results demonstrated a good recognition of the coiled coil fragment P90 by 59% of hyperimmune sera tested. In contrast, only 43% of these sera gave positive results for recPFD0520c. Insufficient recombinant protein purification might explain these differences. This highlights one of the greatest advantages of chemical peptide synthesis. The background in ELISA assays is considerably reduced using pure synthetic peptide. As negative controls were the same in both experiments, the cut off calculated for plates coated in P90 was much lower than that for the recombinant protein. This led to a higher number of responders for P90. It is unlikely that differences in coating concentration would account for the observed discrepancy. The concentration of P90 used was 5µg/ml, and the concentration of recPFD0520 was 1 µg/ml. However, when an ELISA was repeated using 5 µg/ml of recPFD0520c, the same results were observed.

The impurities present likely consist of *E. coli* proteins not removed during the purification process. Humans are confronted constantly with *E. coli* antigens as *E. coli* is a natural symbiont of the intestinal tract. Humans likely mount humoral responses against *E. coli* proteins that could interfere with serological testing.
Another factor may be a greater representation of conformational epitopes associated with the CC structure in the P90 ELISA. In the absence of other competing epitopes on the plate surfaces coated, the CC fragments might reach an optimal packaging concentration.

Mouse sera detected a protein at 50 kDa. We initially hypothesized that the shift of molecular weight was due to strong dimer formation also observed for the recombinant protein expressed in *E. coli* .To test this hypothesis we generated a transgenic parasite line expressing PFD0520c fused to an HA-tag. The Western blot analysis on transgenic lines did not confirm our hypothesis of dimer formation. In reduced and non-reduced condition the HA-tagged PFD0520c protein was detected as monomer. The HA-fusion protein might be prevented from adopting dimer conformation due to steric hindrance caused by the HA-tag. Moreover, the possibility exists that the endogenous protein contains modifications favouring dimer formation or constituting other causes for the observed shift in migration.

Alternatively, the mouse sera could cross-react with another protein expressed in blood stages. Expression profile revealed that PFD0520c is expressed at low level. Detection of low abundant proteins by Western blot is difficult. This open question could be addressed by MS analysis to identify the protein content of the single spot in the 2D gel that was detected by the polyclonal mouse sera.

Subcellular localization of PFD0520c using different mouse sera and the localization of the HA-tagged version revealed similar localization pattern. The protein accumulated in punctuated structures within the parasite. However,

further co-localization experiments are needed to determine the identity of these structures.

Conclusion

The goal of this study was the comparative analysis of the synthetically produced P90 and the recombinantly expressed PFD0520c, in order to validate the continued development of P90 towards a long synthetic peptide based malaria vaccine. All comparisons performed indicated similar performance of both antigens. The two most important parameters relevant for evaluation of vaccine candidates are reactivity with sera from endemic areas, and a high SGI value in the functional assay. Both criteria were met equally well by the synthetic peptide and by the purified recombinant protein.

Acknowledgment

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Tables

Table 1: **Summary of ELISA results of BF clinically immune individual's recognition of P90 and recPFD0520c.**

Peptide/Protein	MW (Da)	% recognition	Ratio (%)	Mean OD_{405}	Cut off
P90	4 475	59	41	0.401	0.140
recPFD0520c	26 443	43	41	0.257	0.218

Figures

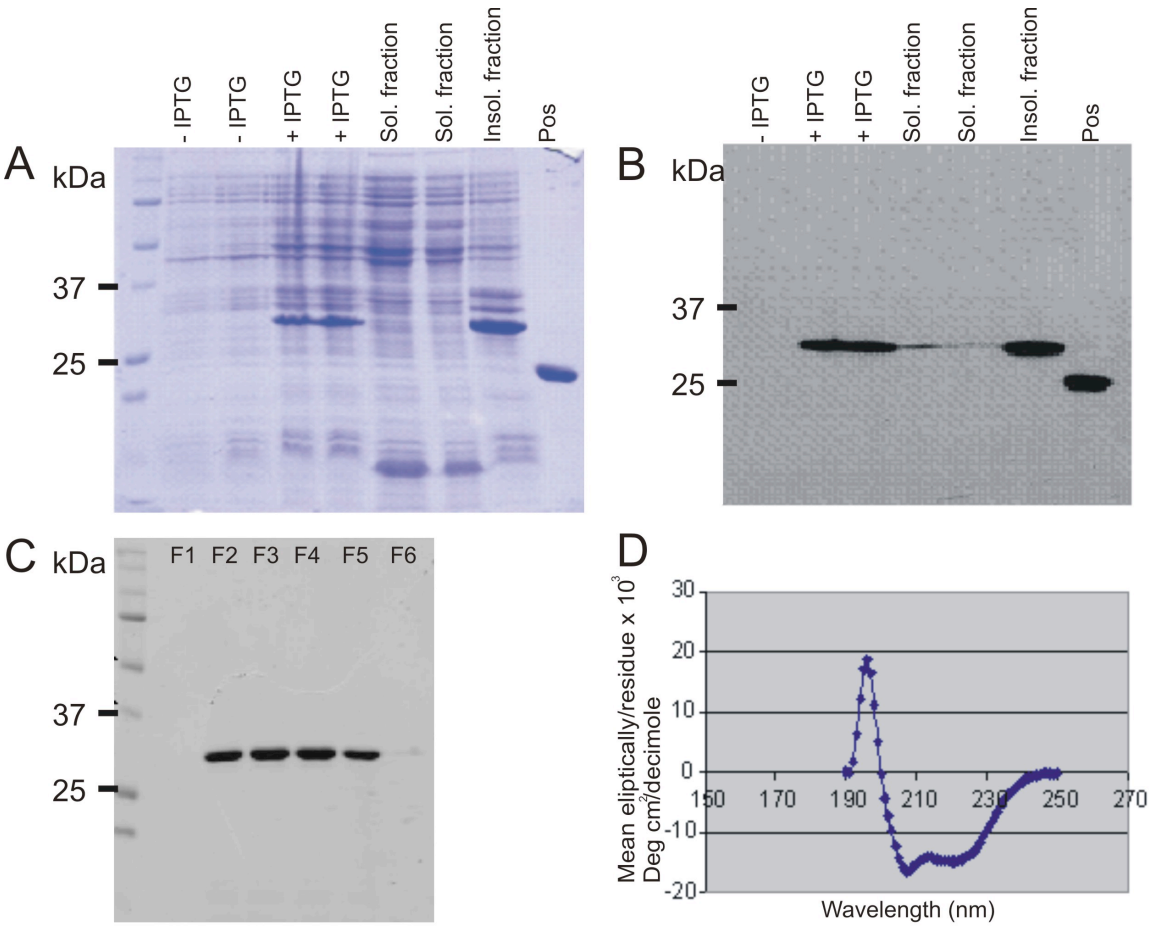

Figure 1: Expression, purification and structural analysis of recPFD0520c. Coomasie gel of *E. coli* protein extracts of parallel 25 ml cultures (A & B) of M15 cells containing pQE-60 with PFD0520c insert. Protein extract of uninduced culture (-IPTG) and induced culture (+IPTG). Soluble protein fraction after *E. coli* lysis and insoluble protein fraction after lysis. Western blot analysis of the same fractions. Antibody used for recPFD0520c detection: mouse monoclonal antibody: anti-6xHis (R&D Systems). Positive control recDHFR fused to 6xHis-tag (pos).

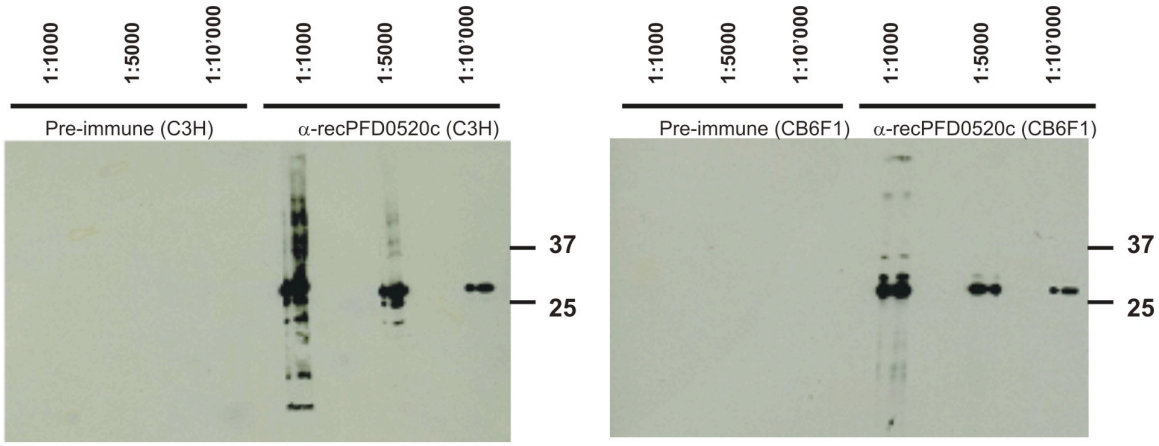

Figure 2: recPFD0520c-specific mouse recognized recPFD0520c. Sera raised against recPFD0520c in C3H mice (left) and in CB6F1 mice (right) recognize the recombinant protein. Different dilution of the polyclonal mouse antibodies were used (1:1000; 1:5000; 1:10'000). The pre-immune of both mice did not recognize the recombinant protein.

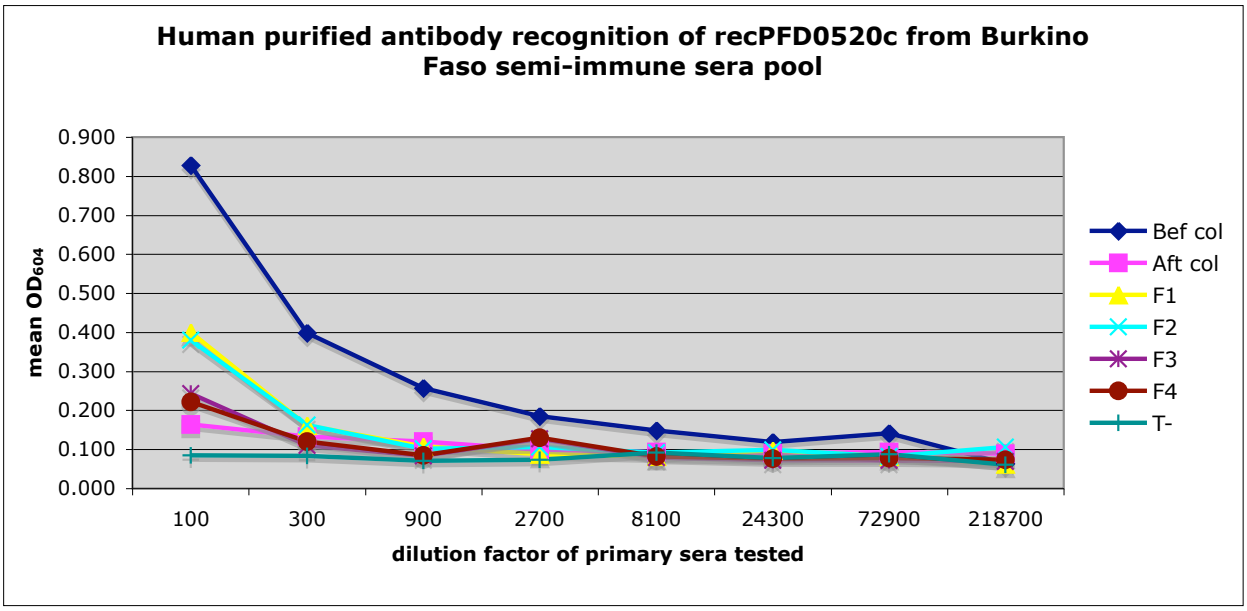

Figure 3: anti-recPFD0520c affinity purified antibodies from a pool of adult sera from Burkina Faso.

T- stands for negative test sera from a Swiss donor, non-exposed to malaria (negative control); Bef col (before purification, dark blue graph); Aft col (after purification, pink squared graph)

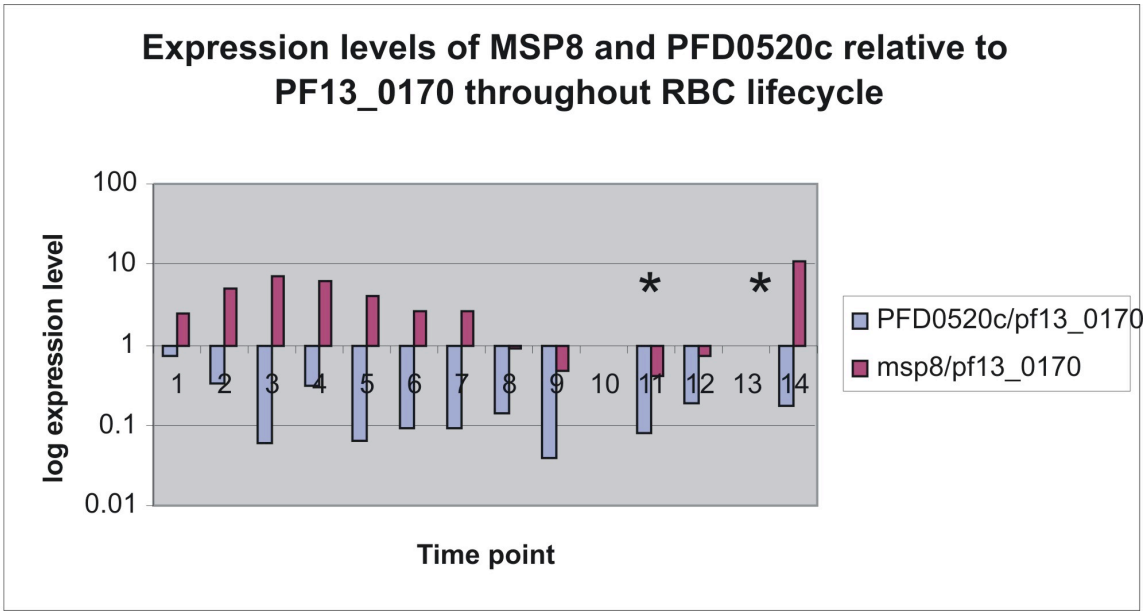

Figure 4: **Abundance of PFD0520c transcripts by gRT-PCR.** RNA was isolated from tightly synchronized culture in a 4h interval. Transcript levels were normalized to the transcript abundance of the constitutively expressed glutaminyl-tRNA synthetase (PF13_0170). As a control for complete synchronization *msp8* transcripts were measured and compared to the PF13_0170 transcript level (green curve)

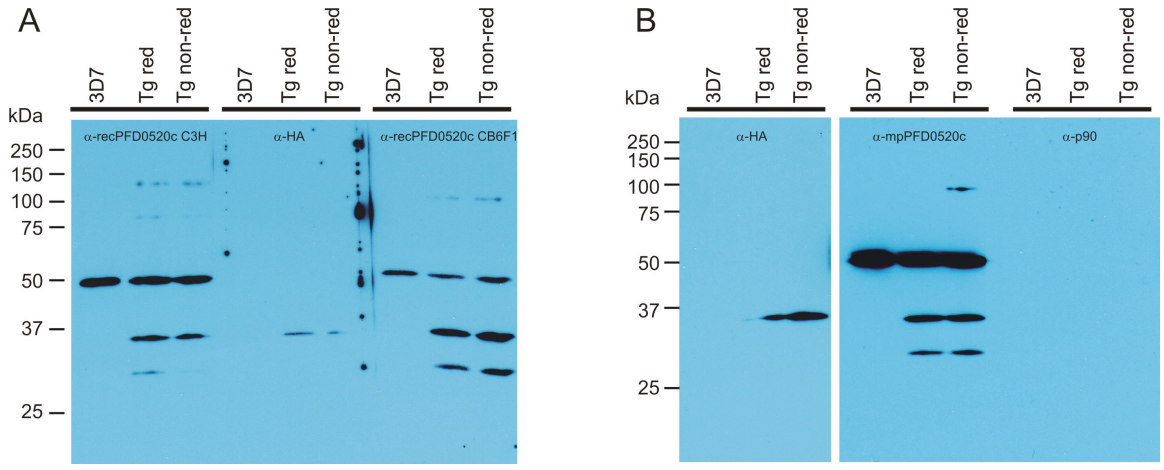

Figure 5: Serum specificity tested on parasite lysate by Western blot. Protein lysate of mixed stage 3D7 parasites (3D7) and protein lysate of the transgenic parasite line (Tg) expressing the PFD0520c fused to HA-tag was separate by SDS-PAGE. Tg lysate was either reduced (Tg red) or non-reduced (Tg non-red).

Figure 6: Predicted PFD0520c protein sequence. The localization of synthetic peptides (P1, P2, P3, P4, P5) within the protein are indicated with coloured bars. P90, the coiled coil region of PFD0520c is highlighted in grey.

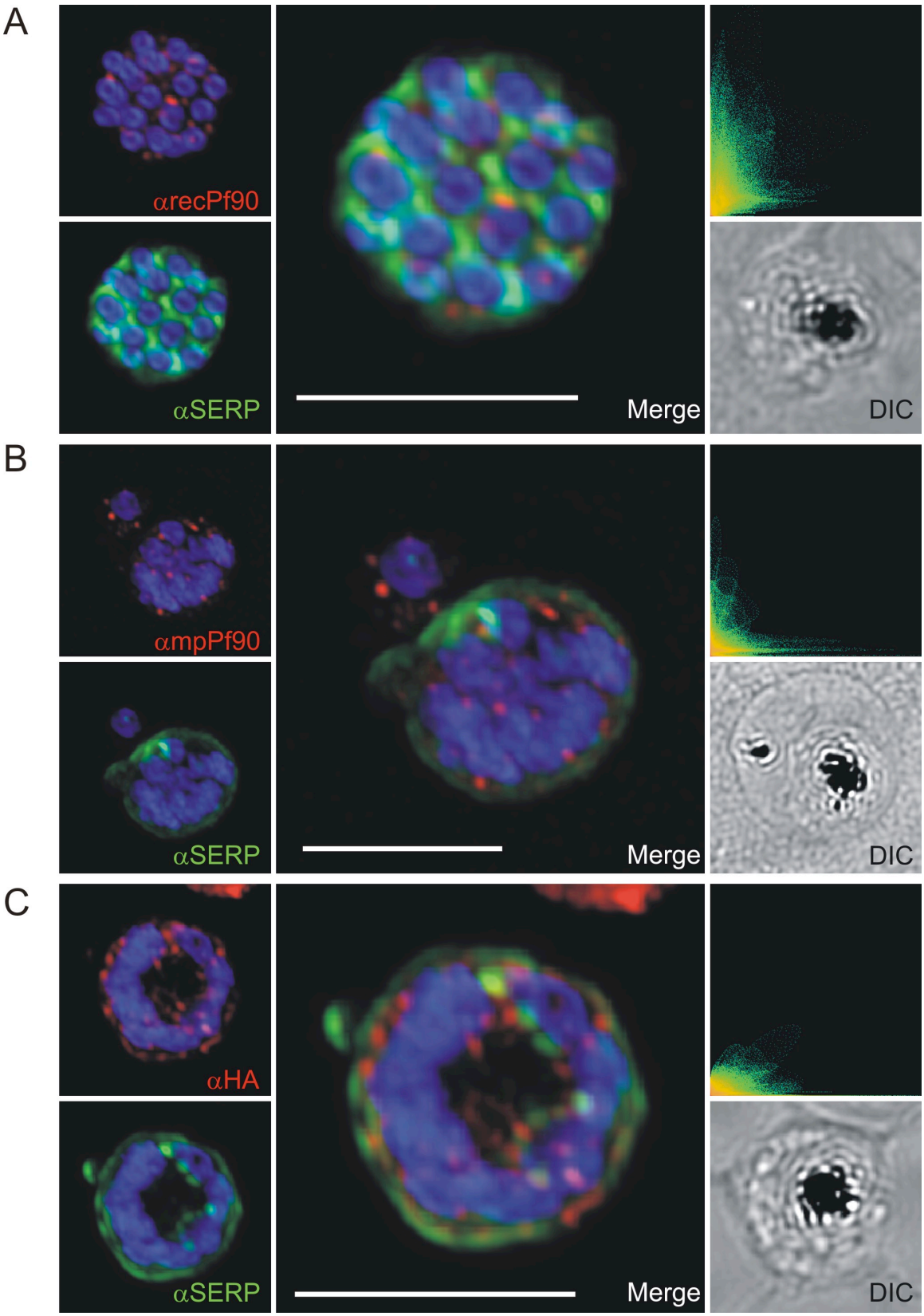
A
αrecPf90
αSERP
Merge
DIC
B
αmpPf90
αSERP
Merge
DIC
C
αHA
αSERP
Merge
DIC

Figure 7: Localization of PFD0520c in late stage parasites. Anti-recPFD0520c-specific polyclonal mouse sera (A, in red) or anti-mpPFD0520c polyclonal mouse sera (B, in red) was used to detect endogenous PFD0520c in fixed 3D7 infected RBC. PV staining was obtained by SERP-specific polyclonal rabbit sera (in green). Anti-HA antibodies were used to detect the HA-tagged PFD0520c in transgenic cells (C, in red). Scatter plots show the result of the co-localization of the PFD0520c and SERP signal. Nuclear DNA was stained with DAPI (blue), Transmission image (DIC), scale bar: 5 mm.

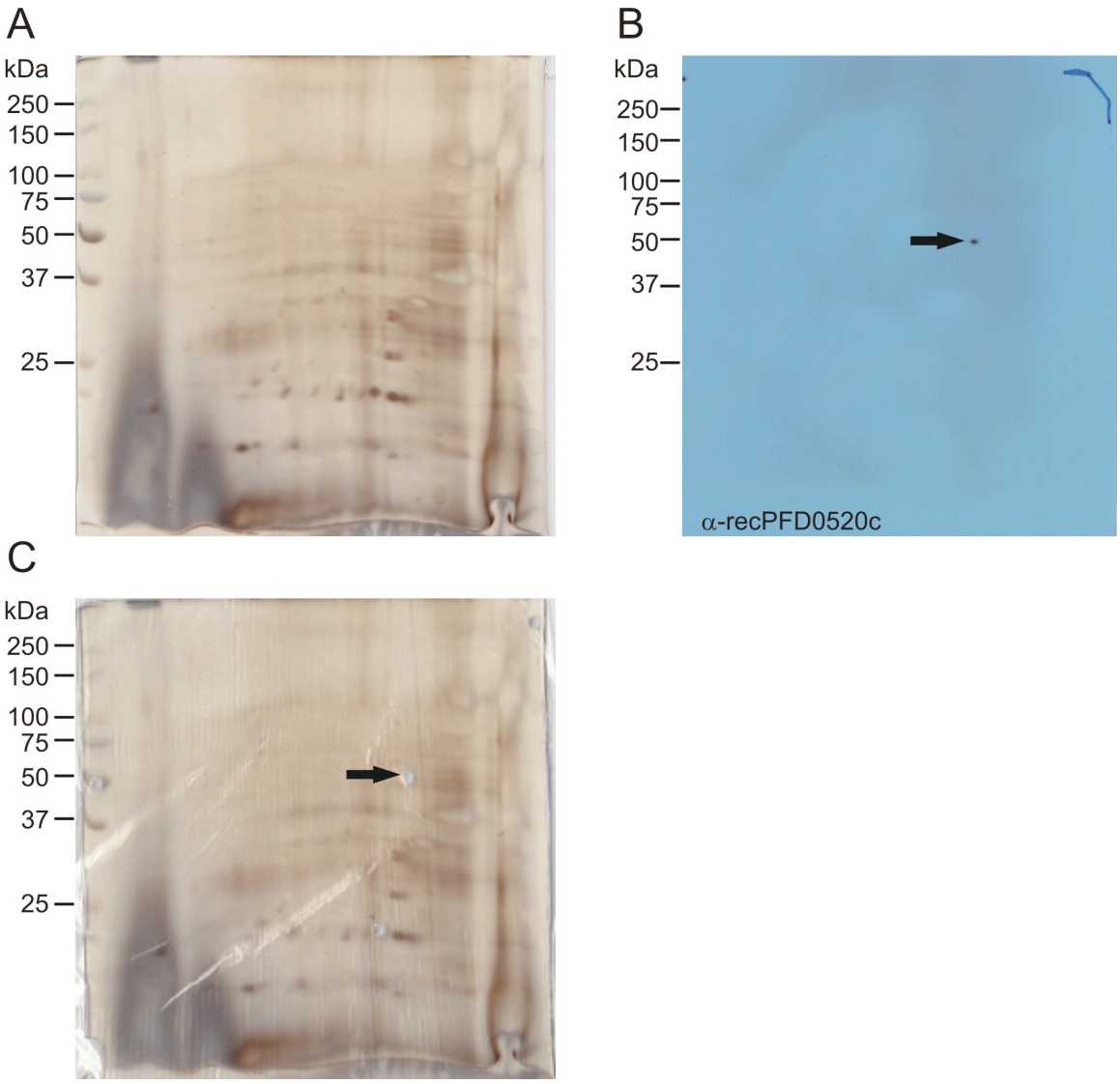

Figure 8: 2D separation and protein for protein identification by mass spectrometry. Mixed stage 3D7 parasite lysate was separated in two dimensions. recPFD0520c-specific polyclonal mouse sera (CB6F1 mice) was used to detect the supposed endogenous PFD0520c protein (B, black arrow). This area was cut out of the protein gel (C, black arrow).

Supplementary material

Table S1: Oligonucleotide sequences used for cloning (restriction sites in bold) or qRT-PCR

Primers for cloning HA-tagged fusion protein		
Fragment	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
PFD0520c	GCGT**GGATCC**CCATGAGACATAAAATTTCCG	GCGT**CCATG**GATATTGGAAGCGCGCTTATCA
Primers for cloning E. coli expression construct		
	GCGT**CCATGG**CCATGAGACATAAAATTTCCG	GTCG**AGATCT**ATATTGGAAGCGCGCTTATCA
Primers for qRT-PCR		
PFD0520c	GACGCCTCCGCCTGTATGAATAAT	CTTGAGCTTTAGAAATTCGTACTTTGTT
Msp8	AGTGCTGTAACTTCTAATGTAGGGGATACAAAT	ATCATCATCACCATTATCATCATTATCATCACG
PF13_0170	TGGCTAGGATATGATTGGAAAGAACA	TACGGTTCTATTTCTATATGGTGAATCA

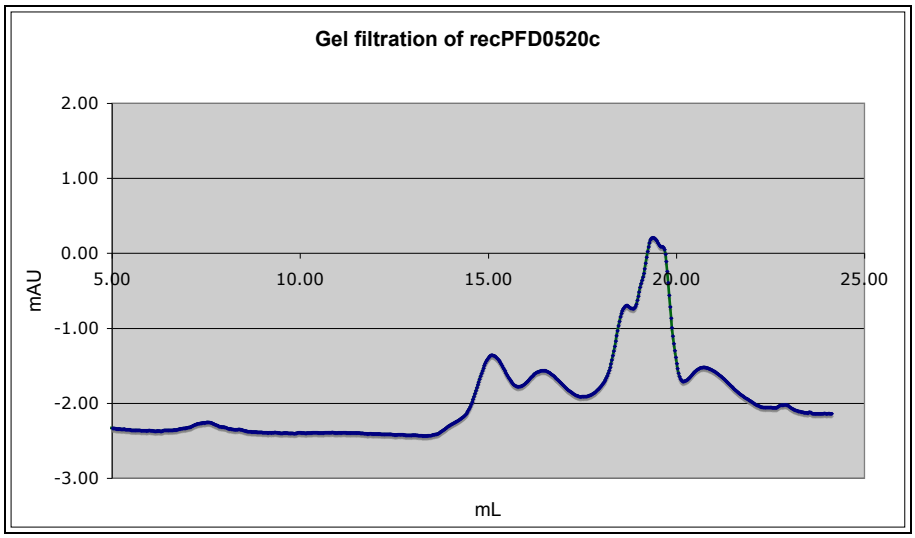

Figure S1: Gel filtration chromatography elution profile of recPFD0520c.

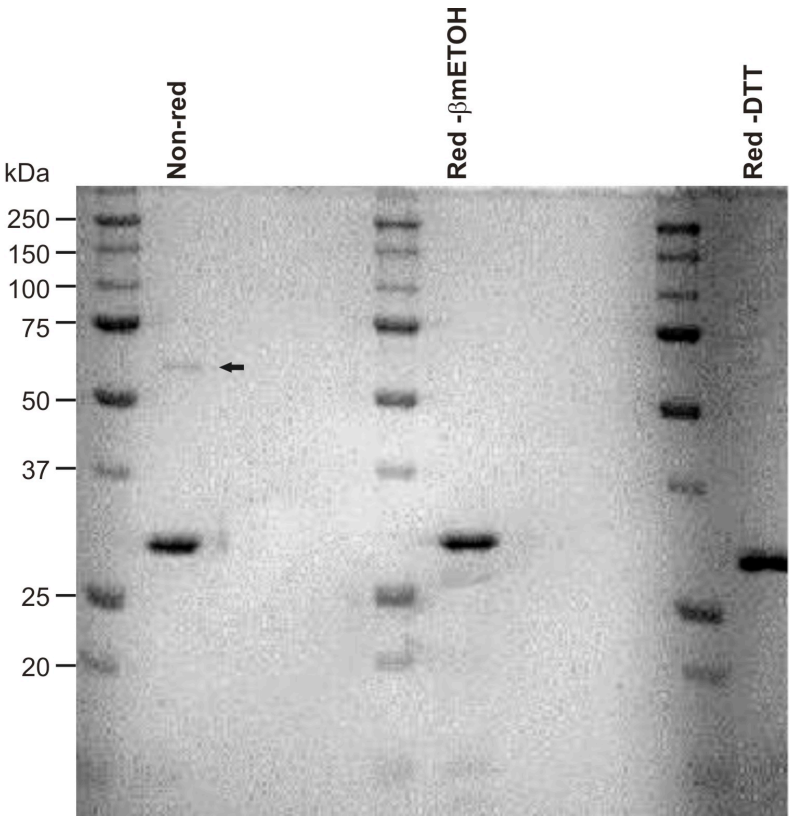

Figure S2: Dimer formation of recPFD0520c in non-reducing conditions. Non-red (non-reduced), dimer band indicated with a black arrow. Reduced conditions: addition of β-mercaptoethanol (red-bmETOH) and DTT (red-DTT).

References

1. Matuschewski K, Mueller AK (2007) Vaccines against malaria - an update. Febs J 274: 4680-4687.
2. Vekemans J, Ballou WR (2008) Plasmodium falciparum malaria vaccines in development. Expert Rev Vaccines 7: 223-240.
3. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, et al. (2007) Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One 2: e645.
4. Agak GW, Bejon P, Fegan G, Gicheru N, Villard V, et al. (2008) Longitudinal analyses of immune responses to Plasmodium falciparum derived peptides corresponding to novel blood stage antigens in coastal Kenya. Vaccine 26: 1963-1971.
5. Kulangara C, Kajava AV, Corradin G, Felger I (2009) Sequence conservation in Plasmodium falciparum alpha-helical coiled coil domains proposed for vaccine development. PLoS One 4: e5419.
6. Verdini A, Terenzi S, Brossard V, Roggero M, Corradin G (2008) Oxidative folding of synthetic polypeptides S-protected as tert-butylthio derivatives. J Pept Sci 14: 1271-1282.
7. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P (1990) Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J Exp Med 172: 1633-1641.
8. Anderson RC, Fox CB, Dutill TS, Shaverdian N, Evers TL, et al. Physicochemical characterization and biological activity of synthetic TLR4 agonist formulations. Colloids Surf B Biointerfaces 75: 123-132.
9. Flueck C, Bartfai R, Volz J, Niederwieser I, Salcedo-Amaya AM, et al. (2009) Plasmodium falciparum heterochromatin protein 1 marks genomic loci linked to phenotypic variation of exported virulence factors. PLoS Pathog 5: e1000569.

10. Voss TS, Tonkin CJ, Marty AJ, Thompson JK, Healer J, et al. (2007) Alterations in local chromatin environment are involved in silencing and activation of subtelomeric var genes in Plasmodium falciparum. Mol Microbiol 66: 139-150.
11. Tonkin CJ, van Dooren GG, Spurck TP, Struck NS, Good RT, et al. (2004) Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. Mol Biochem Parasitol 137: 13-21.
12. Jafarshad A, Dziegiel MH, Lundquist R, Nielsen LK, Singh S, et al. (2007) A novel antibody-dependent cellular cytotoxicity mechanism involved in defense against malaria requires costimulation of monocytes FcgammaRII and FcgammaRIII. J Immunol 178: 3099-3106.
13. Knapp B, Hundt E, Kupper HA (1989) A new blood stage antigen of Plasmodium falciparum transported to the erythrocyte surface. Mol Biochem Parasitol 37: 47-56.
14. Park K, Perczel A, Fasman GD (1992) Differentiation between transmembrane helices and peripheral helices by the deconvolution of circular dichroism spectra of membrane proteins. Protein Sci 1: 1032-1049.

General discussion

The importance of research for vaccines development against malaria cannot be overstated. Today's most advanced vaccine formulation against malaria is RTS,S/AS01. This vaccine targets the pre-erythrocytic parasite stage but has shown to provide only 30-66% protection against clinical episodes [1]. The new generation malaria vaccine should provide at least 80% protection. This might be only achieved by targeting all stages of the parasite. To target the blood stage of parasite is essential because this stage is associated with all the symptoms of the disease. In contrast to the pre-erythrocytic vaccines, vaccines targeting the parasite blood stage could without showing 100% efficacy provide reliable protection against severe disease.

Children from malaria endemic regions develop immunity against severe disease quite rapidly, in general already after the first or the second infection [2]. Under constant exposure individuals develop clinical immunity. The mechanisms that underlie this immunity remains only poorly understood but antibodies were demonstrated to play a critical role in controlling blood stage infection. The transfer of purified IgG from malaria-immune adults to children suffering from high parasitemia and severe symptoms were shown to rapidly reduce parasitemia and symptoms [3]. Maternal antibodies are among the factors protecting infants in their first few month of life against [2] providing another evidence for the central role of antibodies in malaria immunity. However, which proteins elicit the production of protective antibodies and what is the nature of the antibody effector function remains unclear.

The availability of the *P. falciparum* genome [4] has opened the path to identifying, evaluating and characterizing new targets for malaria vaccine development. The *P. falciparum* genome encodes for 5400 putative proteins the majority of which is uncharacterized and therefore annotated as hypothetical proteins. The expression profile of these proteins varies due to the complex

multi-stage life cycle of this parasite. Despite the completion of the genomic sequence of *P. falciparum* [4] and availability of the protein expression data [5,6,7], vaccine development has focused only on 0.5% of all proteins of the entire *P. falciparum* proteome [8].

The approach described in this thesis was based on genome-wide screens for alpha-helical coiled coil domains present in proteins expressed in the blood stage of the parasite. Villard and coworkers predicted 166 coiled coil domains in 131 blood stage expressed proteins [9]. Analysis of the transcriptome of the complete blood stage cycle revealed that at least 60% of the genome is transcriptionally active during this stage [10,11,12]. This corresponds to 3240 expressed proteins. Our selection therefore represents 4% of the proteome expressed in the blood stage.

The rationale behind choosing this structural motif was that (1) candidates can be quickly predicted based on the characteristic heptad repeat structure of the alpha-helical coiled coil motif; (2) alpha-helical coiled coil motifs are recognized by conformational-dependent antibodies; (3) alpha-helical coiled coils are stable and, when chemically synthesized, peptides with this motif readily fold into their native structure; (4) synthetic peptides are advantageous over recombinantly expressed proteins in that no expression or elaborate purification system is required, making the vaccine development process much less time consuming.

Alpha-helical coiled coils are highly abundant structural protein motifs [13]. Coiled coil domains mediate oligomerization and were found to be a requirement for the biological function of many investigated proteins [14,15,16]. In the ring exported protein 1 (Rex1) the coiled coil domain was found to be crucial for the association to MC [17]. This led us to hypothesize that these motifs might exhibit very limited sequence diversity. We therefore studied sequence conservation of alpha-helical coiled coil domains present in *P. falciparum* proteins and found a high degree of conservation of this structural motif. In culture strains only 3 out of 14 alpha-

helical coiled coils showed point mutations and/or length polymorphisms. In field isolates 3 out of 5 investigated peptides were completely conserved. All 166 putative alpha-helical coiled coil domains, which had been originally identified in the *P. falciparum* genome by the bioinformatics selection procedure described by Villard and coworkers (2007), were analyzed *in silico* for the presence of singles nucleotide polymorphism. By querying the SNP data base at PlasmoDB, which contains the genomes of 15 different isolates, we revealed that 82% (137/166) of these peptides were conserved. This important finding supports the choice of the coiled coil structural motif for vaccine development because such an immunogen should ideally be derived from conserved epitopes. This represents a potential advantage in vaccine development since extensive polymorphism had previously posed a major obstacle.

Polymorphic epitopes may divert the effective response. Detailed investigations of blood stage antigens revealed that the polymorphic regions are generally immunodominant [18,19,20]. There is evidence that naturally exposed individuals develop both, strain-specific immune responses as well as cross-reactive responses recognizing a broad array of *P. falciparum* strains [2]. However, it remains unknown whether the antibodies against polymorphic regions or those against conserved regions are more relevant for protection. Conserved regions were found to be less antigenic and immunogenic than polymorphic regions [19,20,21,22]. In contrast to these observations we showed that the majority of our candidates were both conserved [23] and antigenic [9]. Constraints on the secondary structure of coiled coils likely contribute to sequence conservation.

Peptide recognition by semi-immune adults from different endemic areas was initially studied by ELISA in order to demonstrate antigenicity of these fragments in humans [9]. However, a high prevalence of responders is no evidence of a B-cell mediated protective response. There is no direct link between antibody recognition and protection in the absence of further functional information.

In vitro assays that correlate with *in vivo* protection would facilitate the selection of effective vaccine candidates. *In vitro* growth inhibition assay measures the function of antibodies to block RBC invasion and/or intra-RBC growth. Purified IgG from semi-immune adults was shown to inhibit parasite growth *in vitro.* A positive correlation was found between high degree of growth-inhibitory activity of sera collected prior to the rainy season and a decreased probability of experiencing clinical malaria during malaria season [24]. However, there were also individuals with a relatively high level of growth-inhibitory activity who got malaria and individuals with a relatively low level who did not get malaria [24]. This suggests that antibodies that block merozoite invasion or and/or inhibit parasite growth may contribute, but are not sufficient for protection. The primary method for measuring antibody-mediated effector function in the course of the current collaborative project, as well as for selecting the candidates investigated in this PhD thesis, was the antibody-mediated cellular inhibition assay (ADCI) [25,26,27,28]. This assay investigates the function of peptide-specific antibodies for *in vitro* parasite killing in the presence of monocytes. Monocytes are activated when two distinct Fcγ-receptors are simultaneously engaged by a minimum of two cytophilic IgG molecules bound to an antigen containing at least two B cell epitopes [27]. However, it remains to be determined whether the extent of *in vitro* parasite killing by ADCI can serve as correlate for protection. This assay depends on the use of fresh monocytes and is therefore difficult to standardize. There are efforts to adapt this assay using a monocyte-like cell line. However, these efforts have so far been unsuccessful, possibly due the lack of the full complement of human Fc-receptors that are essential for mediating immunity [29]. Taken into account that various antibody-mediated effector functions (blocking of cytoadherence, opsonization and destruction of free merozoites and iRBC by phagocytic cells, neutralization of toxic molecules and ADCI) could be involved in protection seen in semi-immune individuals, it seems unlikely that a single assay measuring one specific effector function could predict reliably *in vivo* protection.

In recent years peptides exposing stable structures including alpha-helical coiled coil motif and intrinsically unstructured regions (IUR) were increasingly used for vaccine development [30]. The skepticism towards synthetic peptides for vaccine or drug development has almost vanished due to many successful vaccine developments in the fields of cancer or against allergies [30]. IURs are highly abundant in the *P. falciparum* genome and are present in approximately 40 % of all proteins [31]. These segments are longer than 50 aa. It would be promising to evaluate them for vaccine potential because they may contain more epitopes than the our selected alpha-helical coiled coil segments. The IUR of Tex1 (PFF0165c) was investigated in more detail [32]. The principal findings that P27A contains multiple T- cell and B-cell epitopes, is recognized by sera of a majority of naturally exposed individuals, is highly immunogenic with different adjuvants, is well conserved and that P27A-specific human and mouse sera inhibit parasite growth *in vitro* support further preclinical and clinical development of P27A. A phase I clinical trial was approved by the European Malaria Vaccine Initiative (EMVI) and currently the GLP-grade immunogen production is ongoing.

Interestingly, a sequence analysis of the Tex1 orthologues in *P. vivax* (PVX_113335) and other *Plasmodium* species (*P. knowlesi* (PKH_114650), *P. berghei* (PB001170.02.0), *P. chabaudi* (PCAS_010290), and *P. yoelii* (PY02581)) revealed that the IUP region as well as the signal sequence is exclusively present in *P. falciparum* Tex1. We showed that the *P. falciparum* Tex1 expression was upregulated in early trophozoites. However, the *P. vivax* orthologues is expressed mostly in ring stage parasites (PlasmoDB). Transcriptome analysis of *P. falciparum* and *P. vivax* throughout the RBC cycle revealed that 11% of the syntenic genes show dramatically altered expression between the two species [33]. For many proteins diversion of the amino acid sequence is coupled with alteration of the transcriptional profile. This may reflect functional evolution of these proteins between the two *Plasmodium* species.

In the traditional approach of considering only surface-located antigens as potential vaccines, it was assumed that blocking antibodies were elicited. The detailed analysis of Tex1 subcellular localization revealed no surface exposure. The protein was exported in trophozoites and associates to MC membrane facing the RBC cytosol. In late schizont Tex1 was found in very close proximity to the RBC membrane, probably still associated to MC. To determine the exact localization of Tex1 in this stage would go beyond the resolution of fluorescence microscopy, and would require electron microscopy images. Both P27A-specific as well as P27-specific antibodies did not detect Tex1 at the surface of unpermeabilized late schizont stage-infected RBCs. Upon parasite rupture, Tex1 is freed and exposed to the human immune system. Antibody-mediated effector mechanisms providing protection via Tex1-specific antibodies therefore exclude processes like invasion inhibition or blocking of cytoadherence. Previously it was shown that soluble antigens are just as efficient to triggering ADCI as antigens present at the merozoite surface [27]. This indicates that antigens released upon schizont rupture as well as antigens released by lyzed parasites are capable of inducing *in vitro* parasite killing in combination with monocytes. The previous work of our collaborators showed that P27A- and P27 specific human antibodies were equally effective in triggering ADCI as a pool of purified IgG from semi-immune adults [9,32]. we have additionally shown that this effect of *in vitro* parasite killing can be induced also by P27A-specific mouse serum [32]. Intracellular proteins of other parasites have been shown to induce protective antibodies. For example, immunization with heavy chain *Brugia malayi* myosin induced protection after challenge in rodent models [34]. It is therefore not justified to dismiss immunogenic proteins categorically as potential vaccine targets based solely on their lack of surface exposure.

This thesis contributed to the characterization of novel blood stage vaccine candidates that were identified by bioinformatic tools and high through-put peptide synthesis followed by stringent experimental selection. The results of this thesis provide evidence that the approach initiated by our collaborators indeed is

valuable. Provided the outcome of clinical trials further supports this strategy, the same approach could be applied for vaccine development against many other pathogens from which genome data is available.

References

1. Bejon P, Lusingu J, Olotu A, Leach A, Lievens M, et al. (2008) Efficacy of RTS,S/AS01E vaccine against malaria in children 5 to 17 months of age. N Engl J Med 359: 2521-2532.
2. Doolan DL, Dobano C, Baird JK (2009) Acquired immunity to malaria. Clin Microbiol Rev 22: 13-36, Table of Contents.
3. Cohen S, Mc GI, Carrington S (1961) Gamma-globulin and acquired immunity to human malaria. Nature 192: 733-737.
4. Gardner MJ, Hall N, Fung E, White O, Berriman M, et al. (2002) Genome sequence of the human malaria parasite Plasmodium falciparum. Nature 419: 498-511.
5. Florens L, Washburn MP, Raine JD, Anthony RM, Grainger M, et al. (2002) A proteomic view of the Plasmodium falciparum life cycle. Nature 419: 520-526.
6. Lasonder E, Ishihama Y, Andersen JS, Vermunt AM, Pain A, et al. (2002) Analysis of the Plasmodium falciparum proteome by high-accuracy mass spectrometry. Nature 419: 537-542.
7. Lasonder E, Janse CJ, van Gemert GJ, Mair GR, Vermunt AM, et al. (2008) Proteomic profiling of Plasmodium sporozoite maturation identifies new proteins essential for parasite development and infectivity. PLoS Pathog 4: e1000195.
8. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, et al. (2008) Profiling humoral immune responses to P. falciparum infection with protein microarrays. Proteomics 8: 4680-4694.
9. Villard V, Agak GW, Frank G, Jafarshad A, Servis C, et al. (2007) Rapid identification of malaria vaccine candidates based on alpha-helical coiled coil protein motif. PLoS One 2: e645.
10. Bozdech Z, Llinas M, Pulliam BL, Wong ED, Zhu J, et al. (2003) The transcriptome of the intraerythrocytic developmental cycle of Plasmodium falciparum. PLoS Biol 1: E5.

11. Wilson RJ (2004) The transcriptome: malariologists ride the wave. Bioessays 26: 339-342.
12. Bischoff E, Vaquero C In silico and biological survey of transcription-associated proteins implicated in the transcriptional machinery during the erythrocytic development of Plasmodium falciparum. BMC Genomics 11: 34.
13. Burkhard P, Stetefeld J, Strelkov SV (2001) Coiled coils: a highly versatile protein folding motif. Trends Cell Biol 11: 82-88.
14. Frenal K, Polonais V, Marq JB, Stratmann R, Limenitakis J, et al. Functional dissection of the apicomplexan glideosome molecular architecture. Cell Host Microbe 8: 343-357.
15. Waterhouse RM, Povelones M, Christophides GK Sequence-structure-function relations of the mosquito leucine-rich repeat immune proteins. BMC Genomics 11: 531.
16. Nelson JD, Kinkead H, Brunel FM, Leaman D, Jensen R, et al. (2008) Antibody elicited against the gp41 N-heptad repeat (NHR) coiled-coil can neutralize HIV-1 with modest potency but non-neutralizing antibodies also bind to NHR mimetics. Virology 377: 170-183.
17. Dixon MW, Hawthorne PL, Spielmann T, Anderson KL, Trenholme KR, et al. (2008) Targeting of the ring exported protein 1 to the Maurer's clefts is mediated by a two-phase process. Traffic 9: 1316-1326.
18. Kemp DJ, Coppel RL, Anders RF (1987) Repetitive proteins and genes of malaria. Annu Rev Microbiol 41: 181-208.
19. Polley SD, Tetteh KK, Lloyd JM, Akpogheneta OJ, Greenwood BM, et al. (2007) Plasmodium falciparum merozoite surface protein 3 is a target of allele-specific immunity and alleles are maintained by natural selection. J Infect Dis 195: 279-287.
20. Franks S, Baton L, Tetteh K, Tongren E, Dewin D, et al. (2003) Genetic diversity and antigenic polymorphism in Plasmodium falciparum: extensive serological cross-reactivity between allelic variants of merozoite surface protein 2. Infect Immun 71: 3485-3495.

21. Fluck C, Smith T, Beck HP, Irion A, Betuela I, et al. (2004) Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun 72: 6300-6305.
22. Osier FH, Polley SD, Mwangi T, Lowe B, Conway DJ, et al. (2007) Naturally acquired antibodies to polymorphic and conserved epitopes of Plasmodium falciparum merozoite surface protein 3. Parasite Immunol 29: 387-394.
23. Kulangara C, Kajava AV, Corradin G, Felger I (2009) Sequence conservation in Plasmodium falciparum alpha-helical coiled coil domains proposed for vaccine development. PLoS One 4: e5419.
24. Crompton PD, Miura K, Traore B, Kayentao K, Ongoiba A, et al. In vitro growth-inhibitory activity and malaria risk in a cohort study in mali. Infect Immun 78: 737-745.
25. Bouharoun-Tayoun H, Attanath P, Sabchareon A, Chongsuphajaisiddhi T, Druilhe P (1990) Antibodies that protect humans against Plasmodium falciparum blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J Exp Med 172: 1633-1641.
26. Bouharoun-Tayoun H, Oeuvray C, Lunel F, Druilhe P (1995) Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages. J Exp Med 182: 409-418.
27. Jafarshad A, Dziegiel MH, Lundquist R, Nielsen LK, Singh S, et al. (2007) A novel antibody-dependent cellular cytotoxicity mechanism involved in defense against malaria requires costimulation of monocytes FcgammaRII and FcgammaRIII. J Immunol 178: 3099-3106.
28. Tebo AE, Kremsner PG, Luty AJ (2001) Plasmodium falciparum: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. Exp Parasitol 98: 20-28.
29. Pleass RJ (2009) Fc-receptors and immunity to malaria: from models to vaccines. Parasite Immunol 31: 529-538.

30. Corradin G, Kajava AV, Verdini A Long synthetic peptides for the production of vaccines and drugs: a technological platform coming of age. Sci Transl Med 2: 50rv53.
31. Feng ZP, Zhang X, Han P, Arora N, Anders RF, et al. (2006) Abundance of intrinsically unstructured proteins in P. falciparum and other apicomplexan parasite proteomes. Mol Biochem Parasitol 150: 256-267.
32. Olugbile S, Kulangara C, Bang G, Bertholet S, Suzarte E, et al. (2009) Vaccine potentials of an intrinsically unstructured fragment derived from the blood stage-associated Plasmodium falciparum protein PFF0165c. Infect Immun 77: 5701-5709.
33. Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, et al. (2008) The transcriptome of Plasmodium vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. Proc Natl Acad Sci U S A 105: 16290-16295.
34. Vedi S, Dangi A, Hajela K, Misra-Bhattacharya S (2008) Vaccination with 73kDa recombinant heavy chain myosin generates high level of protection against Brugia malayi challenge in jird and mastomys models. Vaccine 26: 5997-6005.

Appendix

Table 1: Sequences and Nomenclature of peptides (1 to 95) used in the study.

Peptides	Proteins	Sequences (Plasmodb.org)
1	PFA0170c	(VNNLDSTVNYMNSTGNNINNI)3
2	PFB0145c	TISSLSNKIVNYESKIEELEKELKEVK
3		NIKTMNTQISTLKNDVHLLNEQIDKLN
4		EIEKLNKQLTKCNKQIDELNEEVEKLN
5		IIDIKKHLEKLKIEIKEKKEDLENL
6		INNLNEKLEETNKEYTNLQNNYTNE
7		IDKLNNEKGTLNSKISELNVQIMDL
8		IKTMNTQISTLKNDVHLLNEQIDKLNNEKGTLNSKISELNVQIMDL
9		LLSKDKEIEEKNKKIKELNNDIKKL
10		LNLVDQGKKKLKKDVEKQKKEIEKL
11		ICSLTTEVMELNNKKNELIEENNKLNLVDQGKKKLKKDVEKQKKEIEKL
12	PFB0145c	VDKIEEHILDYDEEINKSRSNLFQLKNEICSLTTEVMELNNKKNELIEENN KLNLVDQGKKKLKKDVEKQKKEIEKL
13		LDENEDNIKKMKSKIDDMEKEIKYR
14	PFC0245c	GMNNMNGDINNIN(GDINNMN)4
15	PFD0110w	LIKYMNERYQNMQQGYNNLTNYINQYE
16		DINSTNNNLDNMLSEINSIQNNIHTYI
17		EKKLDILKVNISNINNSLDKLK
18		DVHNIKEDYNLLQQYLNYMKNEMEQLK
19		DEKINDYLEEIKNEQNKIDKTIDDI
20		MDVVINQLRDIDRQMLDLYKELDEK

21		ISNIFKDIQNIKKQSQDIITNMNDM
22		LEEIIKNLDILDEQIMTYHNSIDEL
23		FQKVKEKAEIQKENIEKIKQEINTL
24		EKKLDILKVNISNINNSLDKLKKYYEEALFQKVKEKAEIQKENIEKIKQEINTL
25	PFD1115c	D(VTHLTND)4VTHLT
26	PFE0570w	NLNKVKININDLNNNIVDVNNSIHNIE
27	PFF0165c	KKRNVEEELHSLRKNYNIINEEIEEIT
28		YIDIKKKISELQKDNESLKIQVDRL
29	MAL7P1.162	QMEGFQKQLDRLSDSLSKIQKALGEYL
30	PF11_0213	LKLNEGLENIKQELHIIDRELKNIL
31	PFL1135c	NINSVNNNINSVDNNINNVDNNINSVN
32	MAL13P1.147	NIIQIKNDIEQCQKSIKKIEDNLNTYE
33	PF13_0277	YIDDVDRDVENYDKGIANVDHHLNDVH
34	MAL13P1.336	NMNNMNNNMNNMNNNMNNNMNNMNNMN
35	PF14_0045	ARDDIQKDINKMESELINVSNEINRLD
36	PF14_0093	SSNNLSDQINILNNNIQHINSTFNNLR
37	PF14_0175	NNNVNNINMNNINSNVNNINNSMNNIN
38	PF14_0089	NITNINKNIENIKNDMSNLNNMNDSNQ
39	PF11_0210	IEINMLTNNLLREMMKIKNKLQKLSNLLNALRSNIEKILKN
40	PFC0235w	NEIKELNNTLNKYKEEMNNYKEEIIVINEKYKLLEIELCK
41	PF11_0455	RLINNIEEIYNSNCEQIQNVRDEFAELKNDLNKIMNLINI
42	PF07_0014	NSLDYYKKVIIKLKNNINNMEEYTNNITNDINVLKAHID
43	PFD0685c	STDINSLNDEVKKLKEELNKIRNEYDDFKNKLELLYQK
44	MAL6P1.61	IPLNQKVLEISKKLNNMNNNINEYKNYLSNFIHMLKE
45	PF11_0207	EEIKEEIKEVKEEIKEVKEEIKEVKEEIKEVKEEIKE
46	MAL13P1.176	TIVQNSYNSFSDINKNINDIDKEMKTLIPMLDELLNE
47	PFA0635c	NHDTRINDYNKRLTEYNKRLTEYNKRLTEYTKRLNE

48	PF11_0240	KGLEEANEKLQIVREKVQSLKAKLSELISQYDHAIY
49	MAL6P1.80	NNINNINNNINNINNNINNINNNINNINNNVNNYY
50	PFL1605w	KNDINVQLDDINVQLDDINVQLDDINIQLDEINLN
51	PFD0985w	DNNVNNMDNNVNNVDNNVNNVDNNLNNVDNNVNN
52	PFL0770w	KIQIEEIKKETNQINKDIDHIEMNIINLKKKIEF
53	PFE0595w	MSQEKISEIIKDISALKTSCEKLNSQLDELITQ
54	MAL6P1.147	DSMNNHKDDMNNYNDNINNYVESMNNYDDIMNK
55	PF13_0065	TSFSKYVRQLEQYFDNFDQDFLSLRQKISDILQ
56	PF14_0013	PYLRRAKHNLNNLQGGINNLYSSVNVVYDNLFN
57	PF13_0198	MEIKTIVQNSYNSFSDINKNINDIDKEMKTLI
58		TISELEQEFNNNNQKLDNILQDINAMNLNINILQT
59	PF14_0397	SLLDTLEKSVKGIDENIEKYNKELNVIKQKIE
60	PFC0810c	KNVIELKEYLEDLKKRMFDMQKRLNDIIITK
61	MAL7P1.13	SNKTFEKLNEKLNDIRNDVTNYKNELEEFKN
62	PFL0150w	SSISSSLTNISSSLTNISSSLTNISSSLSNS
63	PF14_0444	NNEMDETINKLKKDINKLNEKIEKYDNFMKM
64	MAL6P1.131	NNFVNNKMNNMNNMKNNMNNMNNIMNNIMN
65	PFB0460c	NNVIRSKMYNIKKRISKINDELHELSNFFL
66	PFL0250w	MCELNVMENNMNNIHSNNNNISTHMDDVIE
67	PFL1235c	TYTLSKLNNQINELTKKINILRGNLDKARK
68	PFL2310w	NFFLEQMENDMSSTYDKMNRINMDLSKLKR
69	PF13_0088	EKLVKHLDVIDKLIENIYDNINNLNEYINK
70	PFB0765w	EKLNDMQKKLNDVNEKYKNIVECLNNYKT
71		KLEEMKQKNKELINNLNDISDELKNCIEQVNSVSRNMANVEK
72	PFC0760c	EEIYKLNNDIDMLSNNCKKLKESIMMMEK
73		KEIQMLKNQILSLEESIKSLNEFINNLKN
74	PFE0100w	TLIDSFNLNLSYLRESINNKKKHINKIND
75	PFL2520w	EKLYILEKSINKLKKLLNDINNKYQTIKK

76	MAL13P1.304	GGLKNSNHNLNNIEMKYNTLNNNMNSINK
77	PF08_0048	EKLKKYNNEISSLKKELDILNEKMGKCT
78	PF07_0021	KNMDTVYKNIINMSNNMTQMYNSMNNMSHNIINASHDMMDASGNINSH
79	PFB0315w	EKMNMKMEQMDMKMEKIDVNMDQMDVKMEQMDVKMEQMDVKMKR MNK
80	MAL8P1.12	KNKLNKKWEQINDHINNLETNINDYNKKIKEGDSQLNNIQLQCENIEQKIN KIKE
81	PF07_0086	NEMNKEVNKMNEEVNKMNEEVNKMNEEVNKMNKEVNKMDEEVNKMN KEVNKMNK
82	MAL13P1.96	EIINEIEKKIEDIEKNINITKENLKELENKITELQSSFSSYENEMKHVVKKIE DLEK
83	PFC0345w	QNKMENDMNIIKNDMNIMENDMNIMENDMNIIKNDMNIMEKDMNIIKND MNIIKNNMNIIKNEMNIIKNV
84	PFL0115w	DFLDVIYYKLNIKEINKSLTEVKNELTELQKNQEEAKNILAFK
85	PFL0350c	ASIDNINKNINCINNDVDNINSNINNINDNIHKINSNVYGN
86	PFL1930w	NFIKELELQIKNLNNEINTLNDMLKDSEEEIRMLNHTLEEK
87		KYKIEINVLNDEITKLKNEINTYKNDLKNINATLDFYKST
88	PF13_0120	NVLEYAELIIDRQRDKINELEKKLEELRSSSEELQKNVIK
89	PF13_0239	GIFIYNMNLLREILKLMTDNIDTLKDKINEIKCSYAFLK
90	PFD0520c	TKKLNKELSEGNKELEKLEKNIKELEETNNTLENDIKV
91	PF07_0111	NFVNNYINENILNLKSVDNYLEKINNKIKDLDDNINDR
92	MAL6P1.254	PDFDAYNEKLGSISQSIDEIKKKIDNLQKEIKVANK
93	PF11_0424	QLEEKTKQYNDLQNNMKTIKEQNEHLKNKFQSMGK
94	PFD0970c	ENINNMDEKINNVDEQNNNMDEKINNVDEKK
95	PF14_0574	EKGLKSLNEKIKNYDSIIEEQKNQLENLKM

CURRICULUM VITAE

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Education

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05/2004 – 09/2005	**Diploma thesis** *Function of Rab1 in the secretory pathway of Giardia lamblia* University of Zurich, Switzerland (PD Dr. Adrian Hehl)
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PUBLICATIONS

Kulangara C, Kajava AV, Corradin G, Felger I (2009) **Sequence conservation in *Plasmodium falciparum* alpha-helical coiled coil domains proposed for vaccine development.** PLoS One 4: e5419.

Olugbile, S, **Kulangara, C**, Bang, G, Bertholet, S, Suzarte, E, Villard, V, Frank, G, Audran, R, Razaname, A, Nebie, I, Awobusuyi, O, Spertini, F, Kajava, A V, Felger, I, Druilhe, P, Corradin, G (2009). **Vaccine potentials of an intrinsically unstructured fragment derived from the blood stage associated *P. falciparum* protein PFF0165c.** Infect. Immun. 0: IAI.00652-09

Sasa Stefanic, Laura Morf, **Caroline Kulangara**, Attila Regös, Sabrina Sonda, Elisabeth Schraner, Cornelia Spycher, Peter Wild, and Adrian B. Hehl (2009) **Neogenesis and maturation of transient Golgi-like cisternae in a simple eukaryote.** *J. Cell Sci.* 122, 10.1242/jcs.049411

TECHNICAL SKILLS

Molecular biology	PCR, sequence analysis, cloning, transformation (bacteria, giardia, plasmodium), DNA/RNA isolation cDNA synthesis and Real time PCR Immunofluorescence assay and fluorescence microscopy, confocal microscopy
Biochemistry	protein extraction, SDS PAGE, immunoblotting, subcellular fractionation
General	cell culture

CONFERENCES

13.06. – 16.06.2007
1st Three Countries Joint Meeting (French Society of Parasitology, German Society of Parasitology, Swiss Society of Tropical Medicine and Parasitology)
Physiopathlogy of Intracellular Parasitic Diseases, Strasbourg, France

Talk:
Evaluation and characterization of new malaria blood-stage vaccine candidates

14.04. – 16.04.2008
Fourth Annual **BioMalPar Conference** on Biology and Pathology of the Malaria Parasite, EMBL Heidelberg, Germany

Poster title:
Evaluation and characterization of new malaria blood-stage vaccine candidates

08.06. – 13.06.2008
Keystone Symposia
Malaria: Immunology, Pathogenesis and Vaccine Perspectives Alpbach, Austria

Poster title:
Evaluation and characterization of new malaria blood-stage vaccine candidates

15.05. – 18.05.2009
Fifth Annual **BioMalPar Conference**, EMBL Heidelberg, Germany

Poster title:
Sequence conservation in *Plasmodium falciparum* α-helical coiled coil domains proposed for vaccine development

13.09. – 17.09.2009
20th **Molecular Parasitology Meeting**, Woods Hole, USA

Poster title:
Functional characterization of a newly proposed malaria blood-stage vaccine candidate

LANGUAGES

German	mother tongue
English	good written and oral skills
French	good written and oral skills

REFERENCES

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