Functional and Immunological Analysis of the Different Domains of *Plasmodium falciparum* Merozoite Surface Protein 2 (MSP2)

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Christian Flück
aus Brienz (BE)

Basel 2005

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Niklaus Weiss, PD Dr. Ingrid Felger, Prof. Isabel Roditi

Basel, den 25. Oktober 2005

Prof. Dr. Hans-Jakob Wirz

Dekan

Acknowledgments

First of all I would like to thank Ingrid Felger and Hans-Peter Beck for giving me the opportunity to conduct my PhD thesis in their lab.

I am very grateful to my supervisor Ingrid for her continuous support and for always always having time for me, even in times when there was no time. Especially during the writing phase, her support was just excellent!

I would also like to thank Peter, who was not really involved in my thesis, but anyhow, was were very important to me during my time at the STI. I remember, when things were not going too well, he was probably the person who could motivate me most.

I also wish to thank Isabel Roditi for being part of my thesis committee and for her very helpful inputs during our far too sporadic meetings and for her encouragement. Don't give in too easily… I'll keep that in mind. ;-)

I would like to thank Niklaus Weiss for acting as faculty representative in my thesis committee.

I am very thankful to Alan Cowman for giving me the opportunity to do parts of this work in his lab. It was certainly a great experience and I learnt a lot during my time at WEHI.

Very very special thanks go to Till Voss for all his inimtable support, interest, and motivation especially during my time in Melbourne and for always believing in me! Special thanks go also to Melanie Rug for her enthusiasm in the search for fluorescent parasites and for culturing.

I thank all the people of the Cowman lab for their support, fruitful discussions and their friendship: Andrew Pearce, Melanie Rug, Tony Triglia, Jenny Thompson, Marian Cravino, Matthias Marti, Alex Maier, Rob Good, Nicole Struck, Julie Healer, Jake Baum, Dave Richard, Chris Tonkin, and Janine Stubbs. Many thanks also go to Paul Gilson for sharing his results, Paul Sanders for helping out with antibodies, and Brendan Crabb, Kathy Davern, and Nicolette Wark for antibody production, Stella Kyvetos for managing shipments, as well as Viki Milovac and Catherine Tarlington for FACS sorting.

Last but not least of the Melbourne people my special thanks go to Robin Anders, Mr. MSP2, for revealing some MSP2 secrets to me.

Very special thanks go also to all the people of the Molecular Parasitology group at the Swiss Tropical Institute, (listed here more or less in order of their appearance), for their friendship, for many fruitful discussions and BIG help with all sorts of things, not to forget the innumerous, unforgettable Thursday nights:

Tobias Spielmann, Till Voss, Andrea Irion, Jean Burckhardt, Sylvia Steiger, Jutta Marfurt, Mirjam Kästli, Igor Niederwieser, Attila Regös, Alexandra Lüscher, Nicki Tiaden, Richard Burki, Marc Annaheim, Silue Kigbafori, Divine Makia, Cornelia Spycher, Selina Bopp, Sebastian Rusch, Alexander Luginbühl, Andreas Crameri, Joseph Paschal Mugasa, Kefas Mugittu, Sonja Maerky, Nicole Falk, Eric Huber, Dania Müller, Sonja Schöpflin, Marie Ballif, Kathrin Witmer, Anouk Müller, Chichi Maire.

Special thanks go to Alex Luginbühl for invaluable tips in antibody purification and to Sonja Maerky for being an enormous help in pulling through all these ELISAs. I also thank Julia Birk for subclass ELISAs.

Very special thanks go to Cornelia Spycher, for convincing me to persevere even a bit longer and to transfect those plasmids a third time, (which was definitely worth while!) and for her long term cultivation service and support.

All of you guys: It was a real pleasure to meet you and whenever I will look back to my time at the STI I will think it was a great time, and that's because of youse!

I am very grateful to all the immunologists for contributing to the friendly atmosphere in the basement and for helping out generously with all sorts of reagents on many occasions. In particular I would like to thank Diana Diaz for a lot of help with antibody purification and many useful tips and very helpful discussions and for her support and motivation during our common writing phase. Many thanks go also to Jean-Pierre Dangy for help with antibody purification and Shinji Okitsu for introducing me to in vitro growth inhibition assays and immunofluorescence microscopy.

Many thanks go also to Reto Brun and Christian Scheurer for providing blood, malaria culture medium, and consumables, to Nicolas Maire for his ingenious Excel work, and Amanda Ross for Stata help and for sharing the best Wednesday joke I have ever heard.

I would also like to thank our collaborators in Lausanne, Giampietro Corradin and Géraldine Frank for what I would call a fruitful collaboration.

My stay in Melbourne was supported by the Basler Stiftung für experimentelle Zoologie.

Last but really not least I would like to thank my parents for their love and continous support.

Merci viu mau to all of you! (I really hope I didn't forget anyone. I'm sure I did anyway…sorry for that)

Abstract

Malaria caused by *Plasmodium falciparum* is still a major health problem in many tropical countries infecting 500 million people leading to 1 to 2 millions of deaths annually. An effective vaccine is not available but is a major goal to reach as a measure for disease control. The blood stages responsible for the pathology exhibit great antigenic variation and diversity in surface antigens, an immune evasion strategy of the parasite, which hampers the rapid acquisition of protective immunity. Antigenic variation is used for parasite proteins expressed at the surface of infected erythrocytes while antigenic diversity manifests at the surface of merozoites, the invasive form of blood stages. Despite their extensive polymorphism, merozoite surface antigens are among the most promising vaccine candidates since high antibody titres against these proteins are associated with protection from clinical disease and specific antibodies to different merozoite antigens can inhibit parasite growth *in vitro*.

One antigen displayed on merozoites is the merozoite surface protein 2 (MSP2). MSP2 is GPI-anchored and an abundant component of the merozoite surface coat. Its structure shows a central polymorphic part containing extensive tandem amino acid repeats. These repeat regions are flanked by semi-conserved non-repetitive domains defining two allelic families (3D7-like and FC27-like). The repeat sequences and their organisation differ considerably between the two families. The N- and C-terminal parts of MSP2 are conserved among all alleles.

The 3D7 allele of MSP2 was one of three components in the blood stage subunit vaccine Combination B recently tested in an area of Papua New Guinea endemic for *Plasmodium falciparum* malaria. The Combination B trial showed promising results in reducing parasite densities and genotyping of blood samples revealed that MSP2 was a major active component. The vaccine exerted a selective effect on infecting parasite strains favouring those carrying an MSP2 of the FC27-type, not represented by the vaccine.

In this thesis we showed that the antibody response against the MSP2 component of Combination B was exclusively directed against the repetitive and semi-conserved central domains. Together with the observed selective effect on infecting parasite genotypes in the vaccinees, this has implications for future MSP2-based vaccines. It strongly suggests the inclusion of representatives of both allelic families.

We also analyzed the sequences of 3D7-like MSP2 alleles found during the Combinaton B trial and compared the sequences of alleles found in vaccinees with those found in placebo recipients. Phylogenetic analysis showed no clustering of alleles found in breakthrough

infections in vaccinees. This suggests that vaccine-induced antibodies against the family-specific non-repetitive part were protective.

In the search for an improved MSP2-based vaccine we designed and evaluated two long synthetic peptides representing the two allelic families. These peptides contain the family-specific part and the C-terminal conserved part. We show that both peptides are well recognized by immune sera and that recognition matches the one of recombinant proteins corresponding to the family-specific parts. The peptides elicited high antibody titres in mice and monoclonal antibodies raised were shown to react with parasite-derived MSP2 in immunofluorescence assays. We also show that antibodies purified on our peptides react with the merozoite surface and that they have the potential to inhibit parasite growth in cooperation with human monocytes. The results obtained from our evaluation studies encourage the further development of long synthetic peptides as vaccine candidates.

We also asked the question of the functional role of the different domains of MSP2. We hypothezised that the immunodominant repeat region has merely an immunological role in distracting the antibody response to non-protective epitopes and that it is dispensable for *in vitro* growth. Therefore we made transgenic parasites attempting to replace the endogenous *msp2* gene with a gene showing an internal deletion of the repeat region. Our plasmid constructs targeted the *msp2* locus, however, a gene replacement was not achieved. This indicates that the repeat region has an essential function in addition to its proposed importance in immune evasion.

We also addressed the question of the functional role of the conserved domains of MSP2. MSP2 is a GPI-anchored protein and therefore the mechanisms used for its transport to the cell surface are not known. We hypothesised that putative transport signals are likely to lie within the conserved domains. We generated a series of transgenic parasites expressing fluorescent reporter proteins flanked with different portions of terminal MSP2 sequences. We found that a fluorescent reporter flanked by the conserved MSP2 domains was transported to the surface of merozoites while the same reporter only flanked by the MSP2 signal peptide and the GPI anchor signal remained in the ER. This suggests that trafficking signals necessary for targeting of MSP2 to the plasma membrane are located in either of the conserved domains.

Table of contents

1. Literature Review 9

1.1 Introduction 9

1.2 Life cycle 9

1.2.1 The asexual blood stages 10

1.2.2 Merozoite invasion of human erythrocytes 11

1.3 The merozoite surface coat 12

1.3.1 Merozoite Surface Protein 2 (MSP2) 13

1.4. Immunity to malaria 14

1.4.1 Antibody-mediated immunity 15

1.4.1.1 Importance of IgG subclasses 16

1.4.1.2 Effector functions of antibodies against the malaria parasite 17

1.4.1.3 The humoral immune response to MSP2 18

1.4.3 Malaria vaccines 19

1.4.3.1 Prerequisites of a malaria vaccine 20

1.4.3.2 Vaccine candidates 21

1.4.3.3 Erythrocytic stage vaccine candidates 21

2. Strain-specific humoral response to a polymorphic malaria vaccine 31

3. Effect of the malaria vaccine Combination B on subsequent merozoite surface protein 2 diversity 39

4. Evaluation of two long synthetic peptides derived from *Plasmodium falciparum* merozoite surface protein 2 as malaria vaccine candidates 69

5. Replacement of the *Plasmodium falciparum* merozoite surface protein 2 gene by partially deleted coding regions 97

6. Sequence requirements for a GPI-anchored protein to be transported to the surface of *Plasmodium falciparum* merozoites 125

7. General Discussion and Conclusions 157

8. Appendix 169

1. Literature review

1.1 Introduction

Malaria is the most important human parasitosis and is caused by protozoan parasites of the genus *Plasmodium*. Four species can cause human disease: *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae*, and *Plasmodium ovale. Plasmodium falciparum* is responsible for the majority of severe disease and death (Greenwood et al., 1991).

Today approximately 40% of the world's population, mostly those living in the world's poorest countries, is at risk of malaria. Each year, 300-500 million people become infected with this deadly pathogen and 1 to 2 million will die (WHO, 2004). Clinical manifestations of severe malaria include cerebral malaria, severe anaemia, hypoglycaemia, renal failure, non-cardiac pulmonary oedema and respiratory failure (Marsh et al., 1995; Warrell et al., 1990)

Once in the blood, the processes associated with the multiplication of the parasite in red blood cells are responsible for most of the clinical symptoms of malaria and its associated morbitity and mortality. The synchronicity of rupture of infected red blood cells and the concomitant release of large numbers of merozoites leads to the well known malarial fever. The infected red blood cell undergoes dramatic structural and morphological changes during development of the parasite. These changes play a major role in the development of the severe complications accompanied by *falciparum* malaria.

No vaccine is available and resistance to most antimalarial drugs occurs in many parts of the world. Research focuses mainly on *P. falciparum* because of its clinical importance. An *in vitro* culturing system is available (Trager and Jensen, 1978) and the genome has been sequenced for the culture strain 3D7 (www.plasmodb.org).

1.2 Life cycle

P. falciparum is an obligate parasite that cycles between the human host and a mosquito vector. The cycle begins when haploid sporozoites are injected into the human host by an infected female mosquito of the genus *Anopheles* (several species) during a blood meal.

Once in the bloodstream, sporozoites pass the liver, where they penetrate endothelial and Kuppfer cells before invading a hepatocyte, the final host cell, thereby forming a parasitophorous vacuole (PV). Within the hepatocyte, the parasite grows and replicates by

asexual division into a so-called liver schizont carrying thousands of merozoites that are released in the bloodstream after schizont and hepatocyte rupture and are ready to invade erythrocytes to start the intraerythrocytic cycle. After invasion, which does also implicate the formation of a parasitophorous vacuole, the parasite grows, feeding mainly on haemoglobin, and develops into a trophozoite and finally an erythrocytic schizont. The schizont and the host cell rupture 48 hours after invasion, releasing 16 to 32 merozoites to start the next intraerythrocytic cycle. Some of the intraerythrocytic parasites develop into sexual blood stages (gametocytes). These gametocytes must be taken up by an anopheline mosquito during another blood meal. Once in the mosquito gut, gametocyes develop into gametes and sexual reproduction takes place whereby 8 flagellated microgametes are released from a male gametocyte fertilizing the female macrogamete to form a diploid zygote. The zygote develops into an ookinete capable of penetrating the gut wall and maturing to an oocyst in about 10 days. The oocyst finally ruptures to release a large number of haploid sporozoites into the haemolymph. The sporozoites migrate to the salivary gland from where they are injected into the human host during the next blood meal to complete the cycle.

1.2.1 The asexual blood stages

The asexual blood stages of the parasites are fully responsible for the symptoms and the pathology of malaria. Three aspects are particularly noteworthy: i) The rapid exponential growth of the parasites and the synchronicity of schizont and red blood cell rupture accounts for the intermittent malarial fever. ii) The parasite modifies the surface of the infected red blood cell by insertion of a number of parasite-derived proteins into the plasma membrane of the host cell. One of these proteins, *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1), present in knob-like structures, mediates binding of infected red blood cells (iRBCs) to the host microvasculature via endothelial markers like ICAM-1 and CD36. *Pf*EMP1 is a protein family encoded by around 60 highly variable *var* genes per genome of which only one is expressed at a certain time. Sequential expression of different var genes leads to antigenic variation, a way to evade the hosts immune system. iii) Schizont rupture releases around 24 merozoites into the bloodstream. The merozoites are the only stage of the asexual blood stages that are directly exposed to the host's immune system. Therefore, the parasite has to find a way to resist elimination by the host's immune response. It does so by expressing a

large panel of surface antigens with stretches of high sequence diversity to mislead the host's immune system.

1.2.2 Merozoite invasion of human erythrocytes

After asexual division in the hepatocyte, up to 30'000 merozoites are released in the blood stream that have the capability of invading red blood cells. *P. falciparum* merozoites are small oval shaped cells with a diameter of 1-1.5µm and a length of 1.5-2µm. (Aikawa, 1971; Aikawa et al., 1978; Bannister et al., 1975). The conical apical end of the merozoite consists of three proteinaceaous rings, known as the polar rings, attached to subpellicular microtubules. The rhoptries and micronemes that localise to the apical end of the merozoite build the so-called apicomplex. The apicomplex and the dense granules, which are distributed throughout the merozoite, are organelles implicated in erythrocyte invasion (Levine et al., 1980). The merozoite also has a thick bristly coat, composed of a number of glycoproteins, the merozoite surface proteins, implicated in the initial interaction with the erythrocyte.

The whole process of merozoite invasion is believed to occur in about 10-60 seconds (Dvorak et al., 1975; Mitchell and Bannister, 1988) and free merozoites thought to be non-viable after a short period of time (Johnson et al., 1980). It is also believed that a merozoite will die unless the first attempt to invade an erythrocyte was successful. Since the host erythrocyte is non-endocytic, merozoite invasion is an entirely active process. After the initial reversible interaction with the erythrocyte, the merozoite reorients to bring its apical end in direct contact with the erythrocyte plasma membrane. After apical reorientation a closer, irreversible membrane-membrane interaction, the 'tight junction', is formed. When the tight junction moves towards the posterior end of the merozoite, the parasitophorous vacuole is formed and the contents of the rhoptries are released. In the final stage, the tight junction pinches off and the erythrocyte plasma membrane is released to leave the parasite within the host cell, surrounded by a parasitophorous vacuole.

P. falciparum merozoites can invade human erythrocytes using several different pathways, using different receptors on the host erythrocyte. These pathways have been defined using enzyme-treated (neuraminidase, trypsin) and mutant erythrocytes and determining their

susceptibility to merozoite invasion (Dolan et al., 1990; Hadley et al., 1987; Mitchell et al., 1986; Perkins and Holt, 1988).

1.3 The merozoite surface coat

The initial reversible contact of merozoite and erythrocyte seems to involve the merozoite surface coat filaments that consist of 40 nm fibrils and 18-22 nm fibrils in *P. falciparum* and *P.knowlesi* (Bannister et al., 1986b). The interaction occurs over distances between 15-40 nm and is thus larger than those of the tight junction (4nm) that forms later during the invasion process (Aikawa et al., 1978; Bannister et al., 1986a). This reversible adhesion is accompanied by deformations of the erythrocyte plasma membrane that wraps partially around the merozoite and is thought to be important for the apical reorientation, the next step in invasion (Bannister et al. 1986b).

The molecules probably involved in this reversible adhesion are the merozoite surface proteins (MSPs) that are thought to make up the merozoite surface coat, as the GPI-anchored proteins MSP1 (Holder and Freeman, 1982; Holder and Freeman, 1984b), MSP2 (Smythe et al., 1988; Miettinen-Baumann et al., 1988; Clark et al., 1989), MSP4 (Marshall et al., 1997), MSP5 (Marshall et al., 1998), and MSP8 (Black et al., 2001). The alanin-rich heptad repeat-containing MSP3 (McColl et al., 1994; Oeuvray et al., 1994), MSP6, and MSP7 do not show a GPI-anchor signal nor a transmembrane domain, but have a very distinct, acidic C-terminus and a leucine zipper domain. MSP6 and MSP7 form a complex with MSP1 (Heidrich et al., 1983; McBride and Heidrich, 1987; Stafford et al., 1996; Trucco et al., 2001). Recently, MSP10, and MSP11 have been added to the list of proteins associated with the merozoite surface (Black et al., 2003; Pearce et al., 2005). MSP10 contains EGF-like domains, which relates it to MSP1, MSP4, and MSP5, while MSP11 is a paralogue of MSP3/MSP6.

In addition to the MSPs, some other proteins appear to be associated with the merozoite surface. The Apical membrane antigen-1 (AMA-1) is a micronemal transmembrane protein secreted at the apical end and distributed over the merozoite surface during merozoite release (Narum and Thomas, 1994). Acidic basic repeat antigen (ABRA) (Stahl et al., 1986) and the S-antigen (Coppel et al., 1983) are peripheral membrane proteins. ABRA has been shown to interact with Band 3 on the erythrocyte surface (Kushwaha et al., 2002).

Disruption of many merozoite antigens has been attempted in oder to elucidate their functional importance. Only for one merozoite surface protein a successful integration into the chromosomal locus resulting in a truncation of the protein has been reported to date (Mills et al., 2002). The truncated version of MSP3 nor ABRA is trafficked to the surface of the merozoite in these parasites and growth is reduced *in vitro*. 3' replacements, resulting in complementation of the sequence with that from other strains or species have been reported for MSP1, MSP2, MSP3, and AMA1 (reviewed in Cowman et al., 2000). The difficulty to disrupt individual genes coding merozoite surface antigens suggests that there is very limited functional redundancy among merozoite surface porteins. Every member appears to have a specific function that is even essential *in vitro*.

1.3.1 Merozoite Surface Protein 2 (MSP2)

MSP2 has first been identified as a 45 to 51 kDa GPI-anchored protein that localises to the merozoite surface (Clark et al., 1989; Miettinen-Baumann et al., 1988; Smythe et al., 1988) MSP2 is encoded by a single exon on chromosome 2, and analysis of MSP2 sequences from different isolates shows that it is a polymorphic antigen consisting of highly conserved N- and C-terminal regions flanking a variable central region (Anders and Smythe, 1989). The variable region contains a central repeat region, which is flanked by non-repetitive semi-conserved regions. These non-repetitive variable regions have been used to define two allelic families, the 3D7/IC-1 family and the FC27/K1 family (Thomas et al., 1990; Fenton et al., 1991; Felger et al., 1997). The function of MSP2 remains unknown and several attempts of disrupting the *msp2* gene to elucidate the role of MSP2 were not successful (Cowman et al., 2000; Paul Sanders, personal communication).

Interestingly, despite of the obvious essentiality of MSP2 for *P. falciparum*, no homologs or paralogs have been found in any other human malaria parasite species. The only homolog found to date is in the chimpanzee parasite *Plasmodium reichenowi*, which is evolutionary very closely related to *P. falciparum*. The *P.reichenowi* MSP2 basically shares the conserved parts of its *P. falciparum* homolog with only 3 and 9 amino acid substitutions found in the N- and C-terminal regions, respectively (Dubbeld et al., 1998). The repeat region of the only sequenced *P.reichenowi* allele is of the *P. falciparum* 3D7 type. In contrast to *P. falciparum* 3D7-type repeats, Threonine is found as a major component of the repeat but the poly-

Threonin tract always present downstream of *P. falciparum* 3D7-type repeats is missing from the *P.reichenowi* MSP2. The non-repetitive central part is a mosaic of the two *P. falciparum* allelic families and sequences unique to *P.reichenowi*.

Several studies reported that antibodies against the two allelic forms of MSP2 are associated with decreased malaria morbidity (Al-Yaman et al., 1994; Al-Yaman et al., 1995; Taylor et al., 1998).

Two mouse monoclonal antibodies directed against an epitope in the repeat region of the Papua New Guinean isolate FC27 inhibit parasite growth *in vitro*. The epitope comprises the linear amino acid sequence Ser-Asn-Thr-Ser. These antibodies, designated 8G10/48 and 9E3/48 are the only inhibitory MSP2 antibodies described to date (Epping et al., 1988). Monoclonal antibody 8G10/48 has been shown to only marginally inhibit growth of strain Indochina I (IC-I), a parasite carrying a 3D7-type MSP2 (Saul et al., 1989). Other monoclonal antibodies reactive to the family-specific part of MSP2 (8F6/49, Ramasamy 1987) or rabbit sera raised against full-length MSP2 did not inhibit parasite growth *in vitro* (Robin Anders, personal communication). In another study, affinity-purified rabbit sera raised against an octapeptide from the conserved N-terminal region showed inhibitory activity (Lougovskoi et al., 2000). Inhibition experiments were also performed with immune sera from Papua New Guinean adults shown to react with MSP2 in enzyme-linked immuno sorbent assays (ELISA). Using a transgenic parasite line expressing an FC27-type MSP2 allele on a 3D7 background and the parental 3D7 strain, differences in growth inhibition of these two lines were only found for some individual sera (Wickham, 2002). The differential parasite growth inhibition due to MSP2 antibodies is minor compared to that attributable to MSP1-$_{19}$ antibodies as seen in a similar assay using a transgenic *P. falciparum* line expressing a *P.chabaudi* MSP1-19 (O'Donnell et al., 2001a).

In addition to the growth inhibitory MSP2 antibodies, two MSP2 peptides (20mers from the N-terminal conserved region and the 3D7-family-specific non-repetitive part) were shown to inhibit parasite growth directly by binding to red blood cells (Ocampo et al., 2000).

1.4. Immunity to malaria

Sterile immunity to malaria cannot be obtained by natural exposure. However, protective immunity, associated with the absence of clinical symptoms, can be acquired gradually through continuous exposure to infection over several years, depending on the degree of

malaria endemicity (Baird, 1998). The acquisition of immunity is a two-step process. The first step is the development of resistance to the neurological complications induced by *P. falciparum* and is typically acquired by the age of 3 to 5 years. The second step is the development of true anti-parasite immunity allowing the control of high parasite loads. This seems to be acquired only after adolescence. The age at which optimal protection is reached is inversely correlated with the intensity of parasite transmission (reviewed in Baird, 1995). Most of malaria-related morbidity and mortality in endemic regions is found in children below the age of five years, suggesting that malaria antigens inducing protective immune responses are poorly immunogenic.

Malaria antigens are very polymorphic (varying between strains) or variable (changing over time within strains). Both, antigenic diversity and antigenic variation are used by the malaria parasite as immune evasion strategies. Polymorphic antigens are presented at the surface of extracellular stages (merozoites and sporozoites) while antigenic variation is used by intraerythrocytic stages for antigens presented at the surface of the infected red blood cell. Due to these two phenomena, an accumulation of a large and diverse immunological memory is required to reach a state of protective immunity to many different variants.

1.4.1 Antibody-mediated immunity

It is well established that antibodies play a crucial role in immunity to malaria. Passive transfer of gamma-globulin (IgG) fractions of human immune sera to naïve individuals dramatically reduces parasite densities and clinical symptoms (Cohen et al., 1961; Edozien et al., 1962; Cohen and Butcher, 1970). Both, East Africans and Thais could be treated with West African immune sera, suggesting that protective epitopes are not strain-specific (McGregor et al., 1963; Sabchareon et al., 1991). Infants are found to be largely protected against malaria in the first months of life. This protection is thought to involve, together with other protective factors, maternal antibodies transferred through the placenta and ranges from 3 to 6 months depending on the intensity of transmission (Snow et al., 1998).

1.4.1.1 Importance of IgG subclasses

Analysis of antibodies conferring protection in passive transfer experiments showed that specific IgG subclasses are important for the transfer of immunity. According to the structural diffenences in their heavy chains, human IgG can be grouped into four subclasses, IgG1 to IgG4. The major differences lie in sequence length and number of disulfide bonds in the hinge region, resulting in differential rotational flexibilities of the antigen-binding fragments. The structural differences influence the biological properties of the IgG subclasses, especially the effector functions mediated by the constant region of the Fc. Probably the most important effector function of IgG immunoglobulins is the fixation of complement. IgG3 is the strongest complement-fixing subclass, followed by IgG1. IgG2 has a weak potential of complement fixation and IgG4, which does not fix complement, is considered to be protective against the biological effects of complement-fixing antibodies. Another important function of human IgG is to bind to cell surface Fc-receptors. Once it is fixed to the surface of certain cell types, the IgG antibody can complex antigen and facilitate clearance of antigens or immune-complexes by phagocytosis. Three classes of human IgG Fc receptors (FcR) on leukocytes can be found: the FcR-I, FcR-II, and low-affinity receptor (FcR-Io). FcR-I is only expressed on monocytes, FcR-II on monocytes, neutrophils, eosinophils, platelets, and B cells; and FcR-Io on neutrophils, eosinophils, macrophages, and killer T cells. IgG1 and IgG3 strongly bind to all three Fc-receptors while IgG2 and IgG4 bind only weakly or not. Due to their FcR-binding capacities, IgG1 and IgG3 are termed cytophilic subclasses.

The IgG subclasses also differ in their half lives (21–23 days for IgG1, IgG2, and IgG4; 7-8 days for IgG3) and their average serum concentration. IgG1 is by far the most abundant subclass in healthy individuals followed by IgG2, IgG3, and IgG4. All subclasses have been reported to be transferred through the placenta.

The switch from IgM to IgG subclasses occurs by rearrangement of the heavy-chain genes under the influence of cytokine signals. Antigens can influence heavy-chain switching because they can induce different cytokine secretion patterns. The antibody subclass profile to a certain antigen does not only depend on the nature of the antigen, but also on the time that passed since the exposure. IgG1 and IgG3 levels are comparable early after exposure to a protein antigen. Later, switching from IgG3 to IgG1 will occur, leading to a great predominance of IgG1 over IgG3.

Several studies found that antibodies to *Plasmodium falciparum* antigens are predominantly

of the cytophilic subclasses (IgG1 and IgG3) in immune adults. In contrast, in non-protected individuals, i.e. children and adults with a primary malaria attack, anti-parasite antibodies are found to be mainly of non-cytophilic sublasses (IgG2 and IgM) (Bouharoun-Tayoun et al., 1992a, 1992b). Another study suggests that IgG2 antibodies are associated with protection from *P. falciparum* (Aucan et al., 2000), which may be contradictory to the importance of cytophilic subclasses. However, Aucan and co-workers found that 70% of the study subjects possessed the H131 allele of the Fc-RIIa, which is known to bind IgG2. High specific IgG4 levels (to conserved regions of MSP2 and ring-infected erythrocyte membrane protein (RESA)) are associated with enhanced risk of infection (Aucan et al., 2000). This suggests that the non-cytophilic IgG4 blocks cytotoxic effector functions against the parasite by competing with cytophilic subclasses for antigen recognition.

Parasite-specific IgG3 levels as well as IgG3/IgG4 ratios increase with age. It was further found that IgG effective in passive transfer experiments did not inhibit growth of the particular infecting strain on its own *in vitro* (Bouharoun-Tayoun et al., 1990). An inhibitory effect mediated by these antibodies *in vitro* was only seen in cooperation with monocytes (Khusmith and Druilhe, 1983b; Bouharoun-Tayoun et al., 1990).

1.4.1.2 Effector functions of antibodies against the malaria parasite

Taken together, the above mentioned data strongly suggest a crucial role for Fc-receptor-mediated effector functions in immunity to malaria. The inhibitory activity of immune sera was 22-fold increased in the presence of normal human monocytes (Khusmith and Druilhe, 1983b; Bouharoun-Tayoun et al., 1990), indicating that direct inhibition of merozoite invasion by binding to essential invasion molecules is not a major function of parasite-specific antibodies *in vivo*. Nevertheless, *in vitro* inhibition studies have shown that antibodies to merozoite surface proteins and other merozoite stage antigens directly inhibit parasite growth (Epping et al., 1988; Ahlborg et al., 1993; O'Donnell et al., 2001; Moreno et al., 2001; Müller et al., 2003). Immune sera have been found to inhibit merozoite dispersal *in vitro* and it has been proposed that this protective mechanism reflects the immune status of the donor (Green et al., 1981). The specifities of the antibodies present in such immune clusters of merozoites could be assigned to only 8 different antigens (Lyon et al., 1989). Among these antigens MSP1 and MSP2 were present (Lyon et al., 1997; Thomas et al., 1990).

Opsonization and phagocytosis of parasitized erythrocytes (Celada et al., 1982; Celada et al.,

1983) and merozoites (Khusmith and Druilhe, 1983a; Groux and Gysin, 1990) by normal monocytes and polymorphonuclear leukocytes has been demonstrated *in vitro*. Also, the FcRIIa polymorphisms (-Arg/Arg131 and –His/His131) were investigated and it was found that phagocytosis by FcRIIa-His/His131 was highest with immune sera predominantly containing IgG3, while phagocytosis by FcRIIa-Arg/Arg131 was highest with sera containing IgG1 (Tebo et al., 2002). In addition to phagocytosis, an antibody-dependent cellular inhibition (ADCI) mechanism has been proposed that involves soluble factors released from monocytes. An *in vitro* assay has been established, identifying tumor necrosis factor (TNFα) as a crucial, but not the sole factor mediating the effect. It is hypothezised that at the time of schizont rupture, the contact between some components of the merozoite surface and cytophilic antibodies bound to monocytes via Fc-receptors triggers the release of soluble factors. In the *in vitro* assay, these appear to block the division of surrounding intraerythrocytic parasites (Bouharoun-Tayoun et al., 1990; 1992; 1995). Other studies also indicated that soluble mediators are important for parasite killing (Butcher and Clark, 1990; Naotunne et al., 1991). A major role in antibody-dependent cellular inhibition has been assigned to IgG3. IgG fractions containing IgG1, IgG2, and IgG4 but no IgG3 gave negative inhibitions in ADCI assays, while the complete IgG fraction or IgG3 alone showed positive inhibition (Tebo et al., 2001). Specific antibodies to multiple plasmodial antigens have been shown to be associated with protection and to mediate an ADCI effect. The best-studied antigens with regard to their role in ADCI are MSP3 (Oeuvray et al., 1994a; Oeuvray et al., 1994b; Singh et al., 2004) and GLURP (Theisen et al., 2000; Theisen et al., 2001; Theisen et al., 2004)

1.4.1.3 The humoral immune response to MSP2

Antibody responses to MSP2 have been investigated in multiple studies. Aquisition of specific MSP2 antibodies was shown to be age-dependent. Prevalence and titres of MSP2 antibodies are found to be high in semi-immune individuals. This indicates that repeated exposure to infection is required to induce antibody responses. Most semi-immune individuals are positive for antibodies to 3D7- and FC27-type MSP2 but some, and especially children, react only with one form of MSP2, indicating that the major response is directed against the central variable domains, and not the conserved termini (Al-Yaman et al., 1994). As shown in the same study, the presence of antibodies specific for MSP2 was associated with fewer fever

episodes and anaemia. In another, prospective longitudinal study of malaria morbidity the role of the humoral response to MSP2 and other antigens in protection was assessed. When age and post exposure was controlled for, only antibodies to full-length 3D7-MSP2, 3D7-MSP2 with a deleted repeat region, and RESA predicted a reduction of incidence rate of malaria, but not S*Pf*66 nor the FC27 form of MSP2 (Al-Yaman et al., 1995). The reason for the FC27 form of MSP2 not predicting protection may be explained in this case by the low prevalence of parasites with FC27-type MSP2 in the study area (Felger et al., 1994). The qualitative difference observed in the response to FC27 and 3D7 MSP2 indicates that the protective effect is likely to be related to epitopes in the variable, and not the conserved regions (Al-Yaman et al., 1995).

Several studies have looked at the distribution of IgG subclasses in MSP2-specific antibodies. In sera from adults from The Gambia, antibodies to both MSP2 families were predominantly of the cytophilic and complement-fixing IgG3 subclass (Taylor et al., 1995). In a further study it was found that the presence of IgG3 antibodies to 3D7-type MSP2 was negatively associated with the risk of clinical malaria while IgG1 antibodies to the FC27-type were associated with increased risk of clinical malaria (Taylor et al., 1998). Individuals showing IgG3 reactivity to both MSP2 families had the most significantly reduced risk (Metzger et al., 2003). Compared to antibody responses to total schizont lysate the proportion of IgG3 is considerably higher for MSP2-specific antibodies (Rzepczyk et al., 1997; Ferrante and Rzepczyk, 1997).

The conserved N-and C-terminal regions of MSP2 appear to be poorly antigenic (Thomas et al., 1990; Taylor et al., 1995; Saul et al., 1992) indicating that they are not exposed to the immune system. Nevertheless, these conserved parts remain interesting in terms of inclusion in a subunit vaccine since their immunogenicity has been shown on different occasions in mice (Saul et al., 1992; Lougovskoi et al., 2000; Lawrence et al., 2000)

T-cell responses to MSP2 have also been studied and compared between the two allelic forms. T-cell epitopes in both, the N- and C-terminal conserved parts have been identified in mice (Rzepczyk et al., 1992). From this it was concluded that conserved sequences, when used in a vaccine, may induce MSP2-specific memory Th cells that could be boosted by subsequent exposures to all parasite strains.

1.4.3 Malaria vaccines

In 1973 human protection from malaria by vaccination was first reported. The vaccine consisted of attenuated sporozoites delivered by hundreds of X-irradiated mosquitoes on several occasions (Clyde et al., 1973). The protection was also against a heterologous strain but not durable (< 16 weeks after the last immunization with irradiated sporozoites). This study showed that a malaria vaccine should be feasible and that a sterile immunity can be induced by vaccination with pre-erythrocytic stages. In contrast, sterile immunity can hardly be obtained by natural exposure to infection. A whole parasite vaccine is not practical because of difficulties to produce large amounts of irradiated sporozoites. Therefore in the last 30 years, research mainly concentrated on the identification of protective antigens to be used in subunit vaccines against the pre-erythrocytic and blood stages. Vaccines against pre-erythrocytic stages (sporozoites and liver stages) aim at producing sterile immunity or at dramatically reducing parasite numbers reaching the blood stage to attenuate disease. The latter effect would also provide the opportunity for natural immunity to develop.
Vaccines against the blood stages are clearly thought as anti-disease vaccines by reducing the multiplication of the parasite in the blood, the target being free merozoites or intra-erythrocytic stages. An alternative blood stage vaccine strategy is to target malaria toxins to reduce severe disease and death (Schofield et al., 2002). The target population for anti-blood stage vaccines are infants, young children, and pregnant women in endemic areas. Gametocytes present in the blood stream exhibit a target for a transmission-blocking vaccine. Such a vaccine aims at reducing the rate of infected mosquitoes in the population and thus would not give direct protection against disease to the vaccinees.

1.4.3.1 Prerequisites of a malaria vaccine

An effective malaria vaccine must initiate and expand immune responses of the appropriate type and specificity. Depending on the life cycle stage that is targeted, the desirable responses vary considerably. Extracellular invasive stages (sporozoites and merozoites) may be neutralized by antibodies blocking them or eliminating them from the circulation. To achieve this, CD4+ T-helper cells must also be generated because they are required for activation and maturation of B-cells. The elicited antibodies must be sufficiently strong and of a subclass allowing important effector functions (e.g. phagocytosis and complement fixation).

Targeting hepatocytic stages requires a strong T-cell response. Such a response is probably difficult to achieve since the liver is thought to be a relatively immunosuppressive site. Epithelial cells of the liver sinusoid have been shown to induce T-cell tolerance through presentation of local antigens (Knolle et al., 2000). Furthermore it has been found that *P. falciparum*-infected red blood cells can induce apoptosis of parasite-specific T-cells (Xu et al., 2002).

Common to malaria vaccines directed against different life cycle stages is that a long lasting effect is desirable. This is probably most difficult to achieve since natural immunity has been shown to be short-lived (Cavanagh et al., 1998; Giha et al., 1999), which was suggested to involve defects in memory B-cell populations specific for some malarial antigens (Dorfman et al., 2005). Another crucial prerequisite of a subunit vaccine is that it provides strain-transcending protection. This clearly favors conserved and semi-conserved antigens or epitopes over polymorphic ones, however, conserved domains of malarial antigens are generally poorly immunogenic.

1.4.3.2 Vaccine candidates

According to WHO there are currently 21 pre-erythrocytic stage vaccines, 38 blood stage vaccines, 7 combination (multi stage) vaccines, and 3 transmission-blocking vaccines in the pre-clinical developmental phase. (www.who.int/vaccine_research/documents/en/malaria_table.pdf).

1.4.3.3 Erythrocytic stage vaccine candidates

A number of vaccine candidates derived from asexual blood stage antigens have been extensively studied in the past decade. These are MSP1, MSP2, apical membrane antigen 1 (AMA1), rhoptry-associated protein 1 (RAP1), RAP2, ring-infected erythrocyte surface antigen (RESA), erythrocyte-binding antigen 175 kDa (EBA175), and glutamate-rich repeat antigen (GLURP) (reviewed in Anders and Saul, 2000).

The only pure blood stage vaccine, for which a phase IIb trial (efficacy trial in volunteers in a disease endemic country) has been completed, is Combination B (Genton et al., 2002). The

antigens included in Combination B are recombinant proteins corresponding to the N-terminal fragment of MSP1, the full-length MSP2 from strain 3D7, and RESA. The vaccine was formulated in Montanide 720 and injected in Papua New Guinean children aged 5 to 9 years. Combination B showed an efficacy of 62% in reducing parasite density. Vaccinees had a lower prevalence of infection with parasites carrying a 3D7-type MSP2 allele (the one represented by the vaccine), favouring parasites carrying an MSP2 allele of the FC27 type. This suggests that the vaccine effect was at least partially due to the MSP2 component. This was the first occurrence of a selecting effect by a malaria vaccine on the parasite population in the field.

There was no difference in the number of clinical episodes between vaccine and placebo recipients. The vaccine induced a statistically significant antibody response to all three antigens, irrespective of pre-treatment with sulfadoxine-pyrimethamine (SP) (Genton et al., 2003). For MSP2, the pre-vaccination antibodies were considerably higher than post vaccination levels in naïve Australian adults (phase I trial, Saul et al., 1999). Nevertheless, the antibody levels increased substantially post-vaccination. In contrast, MSP1 and RESA antibodies failed to increase to higher levels than that seen in naïve adult volunteers post-vaccination.

In Papua New Guinean adults the antibody titres to the 3 antigens were about one order of magnitude higher than in vaccinated children (Genton et al., 2003). Vaccination following drug treatment (SP) did not increase antibody levels in these adults. Antibody levels even dropped substantially for MSP2 and RESA after vaccination. This suggests that antibodies to MSP2 and RESA are short-lived.

Cellular responses to Combination B were assessed by production of IFN-γ, TNF-α, IL-4, and IL-10). The vaccine induced a significant IFN-γ response to MSP1 only.

References

1. **Ahlborg, N., B. W. Flyg, J. Iqbal, P. Perlmann, and K. Berzins.** 1993. Epitope specificity and capacity to inhibit parasite growth in vitro of human antibodies to repeat sequences of the Plasmodium falciparum antigen Ag332. Parasite Immunol **15:**391-400.
2. **Aikawa, M.** 1971. Parasitological review. Plasmodium: the fine structure of malarial parasites. Exp Parasitol **30:**284-320.
3. **Aikawa, M., L. H. Miller, J. Johnson, and J. Rabbege.** 1978. Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite. J Cell Biol **77:**72-82.
4. **al-Yaman, F., B. Genton, R. Anders, J. Taraika, M. Ginny, S. Mellor, and M. P. Alpers.** 1995. Assessment of the role of the humoral response to Plasmodium falciparum MSP2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria. Parasite Immunol **17:**493-501.
5. **al-Yaman, F., B. Genton, R. F. Anders, M. Falk, T. Triglia, D. Lewis, J. Hii, H. P. Beck, and M. P. Alpers.** 1994. Relationship between humoral response to Plasmodium falciparum merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. Am J Trop Med Hyg **51:**593-602.
6. **Anders, R. F., and A. Saul.** 2000. Malaria vaccines. Parasitol Today **16:**444-7.
7. **Anders, R. F., and J. A. Smythe.** 1989. Polymorphic antigens in Plasmodium falciparum. Blood **74:**1865-75.
8. **Baird, J. K.** 1998. Age-dependent characteristics of protection v. susceptibility to Plasmodium falciparum. Ann Trop Med Parasitol **92:**367-90.
9. **Baird, J. K.** 1995. Host age as a determinant of naturally acquired immunity to Plasmodium falciparum. Parasitol Today **11:**105-11.
10. **Bannister, L. H., G. A. Butcher, E. D. Dennis, and G. H. Mitchell.** 1975. Structure and invasive behaviour of Plasmodium knowlesi merozoites in vitro. Parasitology **71:**483-91.
11. **Bannister, L. H., G. H. Mitchell, G. A. Butcher, and E. D. Dennis.** 1986a. Lamellar membranes associated with rhoptries in erythrocytic merozoites of Plasmodium knowlesi: a clue to the mechanism of invasion. Parasitology **92 (Pt 2):**291-303.
12. **Bannister, L. H., G. H. Mitchell, G. A. Butcher, E. D. Dennis, and S. Cohen.** 1986b. Structure and development of the surface coat of erythrocytic merozoites of Plasmodium knowlesi. Cell Tissue Res **245:**281-90.
13. **Black, C. G., L. Wang, T. Wu, and R. L. Coppel.** 2003. Apical location of a novel EGF-like domain-containing protein of Plasmodium falciparum. Mol Biochem Parasitol **127:**59-68.
14. **Black, C. G., T. Wu, L. Wang, A. R. Hibbs, and R. L. Coppel.** 2001. Merozoite surface protein 8 of Plasmodium falciparum contains two epidermal growth factor-like domains. Mol Biochem Parasitol **114:**217-26.
15. **Blackman, M. J., J. A. Chappel, S. Shai, and A. A. Holder.** 1993. A conserved parasite serine protease processes the Plasmodium falciparum merozoite surface protein-1. Mol Biochem Parasitol **62:**103-14.
16. **Blackman, M. J., H. G. Heidrich, S. Donachie, J. S. McBride, and A. A. Holder.** 1990. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. J Exp Med **172:**379-82.
17. **Blackman, M. J., and A. A. Holder.** 1992. Secondary processing of the Plasmodium falciparum merozoite surface protein-1 (MSP1) by a calcium-dependent membrane-

bound serine protease: shedding of MSP133 as a noncovalently associated complex with other fragments of the MSP1. Mol Biochem Parasitol **50:**307-15.

18. **Blackman, M. J., I. T. Ling, S. C. Nicholls, and A. A. Holder.** 1991. Proteolytic processing of the Plasmodium falciparum merozoite surface protein-1 produces a membrane-bound fragment containing two epidermal growth factor-like domains. Mol Biochem Parasitol **49:**29-33.
19. **Blackman, M. J., T. J. Scott-Finnigan, S. Shai, and A. A. Holder.** 1994. Antibodies inhibit the protease-mediated processing of a malaria merozoite surface protein. J Exp Med **180:**389-93.
20. **Blackman, M. J., H. Whittle, and A. A. Holder.** 1991. Processing of the Plasmodium falciparum major merozoite surface protein-1: identification of a 33-kilodalton secondary processing product which is shed prior to erythrocyte invasion. Mol Biochem Parasitol **49:**35-44.
21. **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:**1633-41.
22. **Bouharoun-Tayoun, H., and P. Druilhe.** 1992b. Antibodies in falciparum malaria: what matters most, quantity or quality? Mem Inst Oswaldo Cruz **87 Suppl 3:**229-34.
23. **Bouharoun-Tayoun, H., and P. Druilhe.** 1992a. Plasmodium falciparum malaria: evidence for an isotype imbalance which may be responsible for delayed acquisition of protective immunity. Infect Immun **60:**1473-81.
24. **Bouharoun-Tayoun, H., C. Oeuvray, F. Lunel, and P. Druilhe.** 1995. Mechanisms underlying the monocyte-mediated antibody-dependent killing of Plasmodium falciparum asexual blood stages. J Exp Med **182:**409-18.
25. **Butcher, G. A., and I. A. Clark.** 1990. The inhibition of Plasmodium falciparum growth in vitro by sera from mice infected with malaria or treated with TNF. Parasitology **101 Pt 3:**321-6.
26. **Cavanagh, D. R., I. M. Elhassan, C. Roper, V. J. Robinson, H. Giha, A. A. Holder, L. Hviid, T. G. Theander, D. E. Arnot, and J. S. McBride.** 1998. A longitudinal study of type-specific antibody responses to Plasmodium falciparum merozoite surface protein-1 in an area of unstable malaria in Sudan. J Immunol **161:**347-59.
27. **Celada, A., A. Cruchaud, and L. H. Perrin.** 1982. Opsonic activity of human immune serum on in vitro phagocytosis of Plasmodium falciparum infected red blood cells by monocytes. Clin Exp Immunol **47:**635-44.
28. **Celada, A., A. Cruchaud, and L. H. Perrin.** 1983. Phagocytosis of Plasmodium falciparum-parasitized erythrocytes by human polymorphonuclear leukocytes. J Parasitol **69:**49-53.
29. **Certa, U., D. Rotmann, H. Matile, and R. Reber-Liske.** 1987. A naturally occurring gene encoding the major surface antigen precursor p190 of Plasmodium falciparum lacks tripeptide repeats. Embo J **6:**4137-42.
30. **Chappel, J. A., and A. A. Holder.** 1993. Monoclonal antibodies that inhibit Plasmodium falciparum invasion in vitro recognise the first growth factor-like domain of merozoite surface protein-1. Mol Biochem Parasitol **60:**303-11.
31. **Clark, J. T., S. Donachie, R. Anand, C. F. Wilson, H. G. Heidrich, and J. S. McBride.** 1989. 46-53 kilodalton glycoprotein from the surface of Plasmodium falciparum merozoites. Mol Biochem Parasitol **32:**15-24.
32. **Clyde, D. F., H. Most, V. C. McCarthy, and J. P. Vanderberg.** 1973. Immunization of man against sporozite-induced falciparum malaria. Am J Med Sci **266:**169-77.
33. **Cohen, S., and G. A. Butcher.** 1970. Properties of protective malarial antibody.

Nature **225:**732-4.
34. **Cohen, S., G. I. Mc, and S. Carrington.** 1961. Gamma-globulin and acquired immunity to human malaria. Nature **192:**733-7.
35. **Coppel, R. L., A. F. Cowman, K. R. Lingelbach, G. V. Brown, R. B. Saint, D. J. Kemp, and R. F. Anders.** 1983. Isolate-specific S-antigen of Plasmodium falciparum contains a repeated sequence of eleven amino acids. Nature **306:**751-6.
36. **Cowman, A. F., D. L. Baldi, J. Healer, K. E. Mills, R. A. O'Donnell, M. B. Reed, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2000. Functional analysis of proteins involved in Plasmodium falciparum merozoite invasion of red blood cells. FEBS Lett **476:**84-8.
37. **Dolan, S. A., L. H. Miller, and T. E. Wellems.** 1990. Evidence for a switching mechanism in the invasion of erythrocytes by Plasmodium falciparum. J Clin Invest **86:**618-24.
38. **Dolan, S. A., J. L. Proctor, D. W. Alling, Y. Okubo, T. E. Wellems, and L. H. Miller.** 1994. Glycophorin B as an EBA-175 independent Plasmodium falciparum receptor of human erythrocytes. Mol Biochem Parasitol **64:**55-63.
39. **Dorfman, J. R., P. Bejon, F. M. Ndungu, J. Langhorne, M. M. Kortok, B. S. Lowe, T. W. Mwangi, T. N. Williams, and K. Marsh.** 2005. B cell memory to 3 Plasmodium falciparum blood-stage antigens in a malaria-endemic area. J Infect Dis **191:**1623-30.
40. **Dubbeld, M. A., C. H. Kocken, and A. W. Thomas.** 1998. Merozoite surface protein 2 of Plasmodium reichenowi is a unique mosaic of Plasmodium falciparum allelic forms and species-specific elements. Mol Biochem Parasitol **92:**187-92.
41. **Dvorak, J. A., L. H. Miller, W. C. Whitehouse, and T. Shiroishi.** 1975. Invasion of erythrocytes by malaria merozoites. Science **187:**748-50.
42. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in Plasmodium falciparum. Mol Biochem Parasitol **28:**1-10.
43. **Felger, I., V. M. Marshal, J. C. Reeder, J. A. Hunt, C. S. Mgone, and H. P. Beck.** 1997. Sequence diversity and molecular evolution of the merozoite surface antigen 2 of Plasmodium falciparum. J Mol Evol **45:**154-60.
44. **Fenton, B., J. T. Clark, C. M. Khan, J. V. Robinson, D. Walliker, R. Ridley, J. G. Scaife, and J. S. McBride.** 1991. Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite Plasmodium falciparum. Mol Cell Biol **11:**963-71.
45. **Ferrante, A., and C. M. Rzepczyk.** 1997. Atypical IgG subclass antibody responses to Plasmodium falciparum asexual stage antigens. Parasitol Today **13:**145-8.
46. **Genton, B., F. Al-Yaman, R. Anders, A. Saul, G. Brown, D. Pye, D. O. Irving, W. R. Briggs, A. Mai, M. Ginny, T. Adiguma, L. Rare, A. Giddy, R. Reber-Liske, D. Stuerchler, and M. P. Alpers.** 2000. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. Vaccine **18:**2504-11.
47. **Genton, B., F. Al-Yaman, I. Betuela, R. F. Anders, A. Saul, K. Baea, M. Mellombo, J. Taraika, G. V. Brown, D. Pye, D. O. Irving, I. Felger, H. P. Beck, T. A. Smith, and M. P. Alpers.** 2003. Safety and immunogenicity of a three-component blood-stage malaria vaccine (MSP1, MSP2, RESA) against Plasmodium falciparum in Papua New Guinean children. Vaccine **22:**30-41.
48. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, D. Pye, D. O. Irving, T. A. Smith, H. P. Beck, and M. P. Alpers.** 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:**820-7.

49. **Giha, H. A., T. Staalsoe, D. Dodoo, I. M. Elhassan, C. Roper, G. M. Satti, D. E. Arnot, T. G. Theander, and L. Hviid.** 1999. Nine-year longitudinal study of antibodies to variant antigens on the surface of Plasmodium falciparum-infected erythrocytes. Infect Immun **67:**4092-8.
50. **Greenwood, B., K. Marsh, and R. Snow.** 1991. Why do some African children develop severe malaria? Parasitol Today **7:**277-81.
51. **Groux, H., and J. Gysin.** 1990. Opsonization as an effector mechanism in human protection against asexual blood stages of Plasmodium falciparum: functional role of IgG subclasses. Res Immunol **141:**529-42.
52. **Hadley, T. J., F. W. Klotz, G. Pasvol, J. D. Haynes, M. H. McGinniss, Y. Okubo, and L. H. Miller.** 1987. Falciparum malaria parasites invade erythrocytes that lack glycophorin A and B (MkMk). Strain differences indicate receptor heterogeneity and two pathways for invasion. J Clin Invest **80:**1190-3.
53. **Heidrich, H. G., W. Strych, and J. E. Mrema.** 1983. Identification of surface and internal antigens from spontaneously released Plasmodium falciparum merozoites by radio-iodination and metabolic labelling. Z Parasitenkd **69:**715-25.
54. **Holder, A. A., and R. R. Freeman.** 1982. Biosynthesis and processing of a Plasmodium falciparum schizont antigen recognized by immune serum and a monoclonal antibody. J Exp Med **156:**1528-38.
55. **Holder, A. A., and R. R. Freeman.** 1984a. Characterization of a high molecular weight protective antigen of Plasmodium yoelii. Parasitology **88 (Pt 2):**211-9.
56. **Holder, A. A., and R. R. Freeman.** 1984b. The three major antigens on the surface of Plasmodium falciparum merozoites are derived from a single high molecular weight precursor. J Exp Med **160:**624-9.
57. **Holder, A. A., M. J. Lockyer, K. G. Odink, J. S. Sandhu, V. Riveros-Moreno, S. C. Nicholls, Y. Hillman, L. S. Davey, M. L. Tizard, R. T. Schwarz, and et al.** 1985b. Primary structure of the precursor to the three major surface antigens of Plasmodium falciparum merozoites. Nature **317:**270-3.
58. **Johnson, J. G., N. Epstein, T. Shiroishi, and L. H. Miller.** 1980. Factors affecting the ability of isolated Plasmodium knowlesi merozoites to attach to and invade erythrocytes. Parasitology **80:**539-50.
59. **Kerr, P. J., L. C. Ranford-Cartwright, and D. Walliker.** 1994. Proof of intragenic recombination in Plasmodium falciparum. Mol Biochem Parasitol **66:**241-8.
60. **Khusmith, S., and P. Druilhe.** 1983. Antibody-dependent ingestion of P. falciparum merozoites by human blood monocytes. Parasite Immunol **5:**357-68.
61. **Khusmith, S., and P. Druilhe.** 1983. Cooperation between antibodies and monocytes that inhibit in vitro proliferation of Plasmodium falciparum. Infect Immun **41:**219-23.
62. **Knolle, P. A., and G. Gerken.** 2000. Local control of the immune response in the liver. Immunol Rev **174:**21-34.
63. **Kushwaha, A., A. Perween, S. Mukund, S. Majumdar, D. Bhardwaj, N. R. Chowdhury, and V. S. Chauhan.** 2002. Amino terminus of Plasmodium falciparum acidic basic repeat antigen interacts with the erythrocyte membrane through band 3 protein. Mol Biochem Parasitol **122:**45-54.
64. **Lawrence, N., A. Stowers, V. Mann, D. Taylor, and A. Saul.** 2000. Recombinant chimeric proteins generated from conserved regions of Plasmodium falciparum merozoite surface protein 2 generate antiparasite humoral responses in mice. Parasite Immunol **22:**211-21.
65. **Levine, N. D., J. O. Corliss, F. E. Cox, G. Deroux, J. Grain, B. M. Honigberg, G. F. Leedale, A. R. Loeblich, 3rd, J. Lom, D. Lynn, E. G. Merinfeld, F. C. Page, G.**

Poljansky, V. Sprague, J. Vavra, and F. G. Wallace. 1980. A newly revised classification of the protozoa. J Protozool **27:**37-58.
66. **Lyon, J. A., J. M. Carter, A. W. Thomas, and J. D. Chulay.** 1997. Merozoite surface protein-1 epitopes recognized by antibodies that inhibit Plasmodium falciparum merozoite dispersal. Mol Biochem Parasitol **90:**223-34.
67. **Lyon, J. A., A. W. Thomas, T. Hall, and J. D. Chulay.** 1989. Specificities of antibodies that inhibit merozoite dispersal from malaria-infected erythrocytes. Mol Biochem Parasitol **36:**77-85.
68. **Marsh, K., D. Forster, C. Waruiru, I. Mwangi, M. Winstanley, V. Marsh, C. Newton, P. Winstanley, P. Warn, N. Peshu, and et al.** 1995. Indicators of life-threatening malaria in African children. N Engl J Med **332:**1399-404.
69. **Marshall, V. M., A. Silva, M. Foley, S. Cranmer, L. Wang, D. J. McColl, D. J. Kemp, and R. L. Coppel.** 1997. A second merozoite surface protein (MSP-4) of Plasmodium falciparum that contains an epidermal growth factor-like domain. Infect Immun **65:**4460-7.
70. **Marshall, V. M., W. Tieqiao, and R. L. Coppel.** 1998. Close linkage of three merozoite surface protein genes on chromosome 2 of Plasmodium falciparum. Mol Biochem Parasitol **94:**13-25.
71. **McBride, J. S., and H. G. Heidrich.** 1987. Fragments of the polymorphic Mr 185,000 glycoprotein from the surface of isolated Plasmodium falciparum merozoites form an antigenic complex. Mol Biochem Parasitol **23:**71-84.
72. **McColl, D. J., A. Silva, M. Foley, J. F. Kun, J. M. Favaloro, J. K. Thompson, V. M. Marshall, R. L. Coppel, D. J. Kemp, and R. F. Anders.** 1994. Molecular variation in a novel polymorphic antigen associated with Plasmodium falciparum merozoites. Mol Biochem Parasitol **68:**53-67.
73. **Metzger, W. G., D. M. Okenu, D. R. Cavanagh, J. V. Robinson, K. A. Bojang, H. A. Weiss, J. S. McBride, B. M. Greenwood, and D. J. Conway.** 2003. Serum IgG3 to the Plasmodium falciparum merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite Immunol **25:**307-12.
74. **Miettinen-Baumann, A., W. Strych, J. McBride, and H. G. Heidrich.** 1988. A 46,000 dalton Plasmodium falciparum merozoite surface glycoprotein not related to the 185,000-195,000 dalton schizont precursor molecule: isolation and characterization. Parasitol Res **74:**317-23.
75. **Mills, K. E., J. A. Pearce, B. S. Crabb, and A. F. Cowman.** 2002. Truncation of merozoite surface protein 3 disrupts its trafficking and that of acidic-basic repeat protein to the surface of Plasmodium falciparum merozoites. Mol Microbiol **43:**1401-11.
76. **Mitchell, G. H., and L. H. Bannister.** 1988. Malaria parasite invasion: interactions with the red cell membrane. Crit Rev Oncol Hematol **8:**225-310.
77. **Mitchell, G. H., T. J. Hadley, M. H. McGinniss, F. W. Klotz, and L. H. Miller.** 1986. Invasion of erythrocytes by Plasmodium falciparum malaria parasites: evidence for receptor heterogeneity and two receptors. Blood **67:**1519-21.
78. **Moreno, R., F. Poltl-Frank, D. Stuber, H. Matile, M. Mutz, N. A. Weiss, and G. Pluschke.** 2001. Rhoptry-associated protein 1-binding monoclonal antibody raised against a heterologous peptide sequence inhibits Plasmodium falciparum growth in vitro. Infect Immun **69:**2558-68.
79. **Mueller, M. S., A. Renard, F. Boato, D. Vogel, M. Naegeli, R. Zurbriggen, J. A. Robinson, and G. Pluschke.** 2003. Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of Plasmodium falciparum apical membrane antigen 1. Infect Immun **71:**4749-58.
80. **Naotunne, T. S., N. D. Karunaweera, G. Del Giudice, M. U. Kularatne, G. E.**

Grau, R. Carter, and K. N. Mendis. 1991. Cytokines kill malaria parasites during infection crisis: extracellular complementary factors are essential. J Exp Med **173:**523-9.

81. **Narum, D. L., and A. W. Thomas.** 1994. Differential localization of full-length and processed forms of PF83/AMA-1 an apical membrane antigen of Plasmodium falciparum merozoites. Mol Biochem Parasitol **67:**59-68.
82. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med **193:**1403-12.
83. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001a. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med **193:**1403-12.
84. **O'Donnell, R. A., A. Saul, A. F. Cowman, and B. S. Crabb.** 2000. Functional conservation of the malaria vaccine antigen MSP-119across distantly related Plasmodium species. Nat Med **6:**91-5.
85. **Ocampo, M., M. Urquiza, F. Guzman, L. E. Rodriguez, J. Suarez, H. Curtidor, J. Rosas, M. Diaz, and M. E. Patarroyo.** 2000. Two MSA 2 peptides that bind to human red blood cells are relevant to Plasmodium falciparum merozoite invasion. J Pept Res **55:**216-23.
86. **Oeuvray, C., H. Bouharoun-Tayoun, H. Gras-Masse, E. Bottius, T. Kaidoh, M. Aikawa, M. C. Filgueira, A. Tartar, and P. Druilhe.** 1994. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. Blood **84:**1594-602.
87. **Oeuvray, C., H. Bouharoun-Tayoun, H. Gras-Masse, E. Bottius, T. Kaidoh, M. Aikawa, M. C. Filgueira, A. Tartar, and P. Druilhe.** 1994b. Merozoite surface protein-3: a malaria protein inducing antibodies that promote Plasmodium falciparum killing by cooperation with blood monocytes. Blood **84:**1594-602.
88. **Oeuvray, C., H. Bouharoun-Tayoun, H. Grass-Masse, J. P. Lepers, L. Ralamboranto, A. Tartar, and P. Druilhe.** 1994a. A novel merozoite surface antigen of Plasmodium falciparum (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. Mem Inst Oswaldo Cruz **89 Suppl 2:**77-80.
89. **Perkins, M. E., and E. H. Holt.** 1988. Erythrocyte receptor recognition varies in Plasmodium falciparum isolates. Mol Biochem Parasitol **27:**23-34.
90. **Perkins, M. E., and L. J. Rocco.** 1988. Sialic acid-dependent binding of Plasmodium falciparum merozoite surface antigen, Pf200, to human erythrocytes. J Immunol **141:**3190-6.
91. **Ramasamy, R.** 1987. Studies on glycoproteins in the human malaria parasite Plasmodium falciparum. Identification of a myristilated 45kDa merozoite membrane glycoprotein. Immunol Cell Biol **65 Pt 5:**419-24.
92. **Rayner, J. C., E. Vargas-Serrato, C. S. Huber, M. R. Galinski, and J. W. Barnwell.** 2001. A Plasmodium falciparum homologue of Plasmodium vivax reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. J Exp Med **194:**1571-81.
93. **Reed, M. B., S. R. Caruana, A. H. Batchelor, J. K. Thompson, B. S. Crabb, and A. F. Cowman.** 2000a. Targeted disruption of an erythrocyte binding antigen in Plasmodium falciparum is associated with a switch toward a sialic acid-independent pathway of invasion. Proc Natl Acad Sci U S A **97:**7509-14.
94. **Reeder, J. C., and G. V. Brown.** 1996. Antigenic variation and immune evasion in

Plasmodium falciparum malaria. Immunol Cell Biol **74:**546-54.
95. **Rzepczyk, C. M., K. Hale, N. Woodroffe, A. Bobogare, P. Csurhes, A. Ishii, and A. Ferrante.** 1997. Humoral immune responses of Solomon Islanders to the merozoite surface antigen 2 of Plasmodium falciparum show pronounced skewing towards antibodies of the immunoglobulin G3 subclass. Infect Immun **65:**1098-100.
96. **Saul, A., G. Lawrence, A. Smillie, C. M. Rzepczyk, C. Reed, D. Taylor, K. Anderson, A. Stowers, R. Kemp, A. Allworth, R. F. Anders, G. V. Brown, D. Pye, P. Schoofs, D. O. Irving, S. L. Dyer, G. C. Woodrow, W. R. Briggs, R. Reber, and D. Sturchler.** 1999. Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. Vaccine **17:**3145-59.
97. **Saul, A., R. Lord, G. Jones, H. M. Geysen, J. Gale, and R. Mollard.** 1989. Cross-reactivity of antibody against an epitope of the Plasmodium falciparum second merozoite surface antigen. Parasite Immunol **11:**593-601.
98. **Schofield, L., M. C. Hewitt, K. Evans, M. A. Siomos, and P. H. Seeberger.** 2002. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. Nature **418:**785-9.
99. **Singh, S., S. Soe, J. P. Mejia, C. Roussilhon, M. Theisen, G. Corradin, and P. Druilhe.** 2004. Identification of a conserved region of Plasmodium falciparum MSP3 targeted by biologically active antibodies to improve vaccine design. J Infect Dis **190:**1010-8.
100. **Smythe, J. A., R. L. Coppel, G. V. Brown, R. Ramasamy, D. J. Kemp, and R. F. Anders.** 1988. Identification of two integral membrane proteins of Plasmodium falciparum. Proc Natl Acad Sci U S A **85:**5195-9.
101. **Snow, R. W., B. Nahlen, A. Palmer, C. A. Donnelly, S. Gupta, and K. Marsh.** 1998. Risk of severe malaria among African infants: direct evidence of clinical protection during early infancy. J Infect Dis **177:**819-22.
102. **Stafford, W. H., B. Gunder, A. Harris, H. G. Heidrich, A. A. Holder, and M. J. Blackman.** 1996. A 22 kDa protein associated with the Plasmodium falciparum merozoite surface protein-1 complex. Mol Biochem Parasitol **80:**159-69.
103. **Stahl, H. D., A. E. Bianco, P. E. Crewther, R. F. Anders, A. P. Kyne, R. L. Coppel, G. F. Mitchell, D. J. Kemp, and G. V. Brown.** 1986. Sorting large numbers of clones expressing Plasmodium falciparum antigens in Escherichia coli by differential antibody screening. Mol Biol Med **3:**351-68.
104. **Stowers, A., D. Taylor, N. Prescott, Q. Cheng, J. Cooper, and A. Saul.** 1997. Assessment of the humoral immune response against Plasmodium falciparum rhoptry-associated proteins 1 and 2. Infect Immun **65:**2329-38.
105. **Tanabe, K., M. Mackay, M. Goman, and J. G. Scaife.** 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite Plasmodium falciparum. J Mol Biol **195:**273-87.
106. **Tebo, A. E., P. G. Kremsner, and A. J. Luty.** 2002. Fcgamma receptor-mediated phagocytosis of Plasmodium falciparum-infected erythrocytes in vitro. Clin Exp Immunol **130:**300-6.
107. **Theisen, M., D. Dodoo, A. Toure-Balde, S. Soe, G. Corradin, K. K. Koram, J. A. Kurtzhals, L. Hviid, T. Theander, B. Akanmori, M. Ndiaye, and P. Druilhe.** 2001. Selection of glutamate-rich protein long synthetic peptides for vaccine development: antigenicity and relationship with clinical protection and immunogenicity. Infect Immun **69:**5223-9.
108. **Thompson, J. K., T. Triglia, M. B. Reed, and A. F. Cowman.** 2001. A novel ligand from Plasmodium falciparum that binds to a sialic acid-containing receptor on the surface of human erythrocytes. Mol Microbiol **41:**47-58.
109. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture.

Science **193:**673-5.
110. **Trucco, C., D. Fernandez-Reyes, S. Howell, W. H. Stafford, T. J. Scott-Finnigan, M. Grainger, S. A. Ogun, W. R. Taylor, and A. A. Holder.** 2001. The merozoite surface protein 6 gene codes for a 36 kDa protein associated with the Plasmodium falciparum merozoite surface protein-1 complex. Mol Biochem Parasitol **112:**91-101.
111. **Warrell, D. A.** 1990. severe and complicated malaria. Trans R soc Trop Med Hyg **84:**1-65.
112. **WHO.** 2004. www.who.int. www.who.int.
113. **Wickham, M. E.** 2002. Molecular analysis of Plasmodium falciparum pathogenesis. PhD Thesis.
114. **Xu, H., J. Wipasa, H. Yan, M. Zeng, M. O. Makobongo, F. D. Finkelman, A. Kelso, and M. F. Good.** 2002. The mechanism and significance of deletion of parasite-specific CD4(+) T cells in malaria infection. J Exp Med **195:**881-92.

2. Strain-specific humoral response to a polymorphic malaria vaccine

INFECTION AND IMMUNITY, Nov. 2004, p. 6300–6305 Vol. 72, No. 11
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.11.6300–6305.2004

Strain-Specific Humoral Response to a Polymorphic Malaria Vaccine

Christian Flück,[1] Tom Smith,[1] Hans-Peter Beck,[1] Andrea Irion,[1] Inoni Betuela,[2] Michael P. Alpers,[2,3] Robin Anders,[4] Allan Saul,[5] Blaise Genton,[1] and Ingrid Felger[1]*

Swiss Tropical Institute, Basel, Switzerland[1]; Papua New Guinea Institute of Medical Research, Goroka, Papua New Guinea[2]; Centre of International Health, Curtin University of Technology, Perth,[3] and Department of Biochemistry, La Trobe University, Victoria,[4] Australia; and Malaria Vaccine Development Unit, National Institutes of Health, Rockville, Maryland[5]

Received 5 April 2004/Returned for modification 9 May 2004/Accepted 1 August 2004

The 3D7 form of the merozoite surface protein 2 (MSP2) of *Plasmodium falciparum* was one of three subunits of the malaria vaccine Combination B that were tested in a phase I/IIb double-blind randomized placebo-controlled trial, which was undertaken with 120 Papua New Guinean children of 5 to 9 years of age. Because only one variant of the highly polymorphic MSP2 was used for vaccination, we examined whether the elicited response was directed against conserved or strain-specific epitopes. Postvaccination (week 12) titers of antibody against recombinantly expressed individual domains of MSP2 were measured by enzyme-linked immunosorbent assay and compared to baseline values. We found that vaccination with the 3D7 form of MSP2 induced a significant strain-specific humoral response directed against the repetitive and semiconserved family-specific part. The conserved N- and C-terminal domains were not immunogenic. Titers of antibody against the alternate FC27 family-specific domain showed a tendency to increase in vaccinated children, but there was no increase in antibodies against FC27-type 32-mer repeats. These results indicate that vaccination with one MSP2 variant mainly induced a strain-specific response, which can explain the selective effect of vaccination with combination B on the genotypes of breakthrough parasites. These findings support the inclusion of both family-specific domains (3D7 and FC27) in an improved vaccine formulation.

The antigenic diversity of *Plasmodium falciparum* represents a significant challenge for the development of a malaria vaccine. The merozoite surface protein 2 (MSP2) has been considered a candidate antigen despite being highly polymorphic. The different *msp2* alleles, which differ in number and sequence of intragenic repeats, can be grouped into two allelic families, FC27 and 3D7, according to the central dimorphic domain (18).

The 3D7 allelic type of MSP2 formed one of three subunits of the malaria vaccine Combination B, which is one of the few malaria vaccines tested in a field trial so far. This subunit vaccine, consisting of the three recombinant proteins MSP1 (190LCS.T3), MSP2, and ring-infected erythrocyte surface antigen, was assessed in a randomized, placebo-controlled, double-blind phase I/IIb trial (natural challenge) using 120 Papua New Guinean (PNG) children of 5 to 9 years of age (12). Montanide ISA720 was used as an adjuvant. The placebo doses consisted of adjuvant alone. Vaccination reduced parasite densities (the primary outcome) by 62%. It remains unclear which of the three vaccine components is responsible for this protection, but molecular monitoring provided evidence that this effect was at least partly due to the efficacy of the MSP2 component (6). The parasites in all blood samples collected during the trial in fortnightly intervals over 18 weeks were genotyped at the *msp2* locus. When the effect of vaccination on parasite prevalence was assessed, the prevalence of parasites with a 3D7-type *msp2* genotype was found to be significantly reduced, while the vaccine made no difference in the prevalence of parasites with an FC27-type *msp2* genotype (12). Also, the vaccine effects on preventing new infections were significantly different for the two allelic families. Genotyping blood samples, collected from these 120 children over a period of 1 year following the trial during morbid episodes, revealed that vaccination led to an increase in incidence of morbid episodes with FC27-type MSP2 alleles. This was the first report of a selective effect exerted by vaccination with a polymorphic malaria vaccine (6, 12). The demonstration of a specific effect of the vaccine against the development of infections of the 3D7 type indicated that the activity of Combination B is due, at least in part, to the MSP2 component, which seems to protect children against homologous parasites.

This finding of vaccine-induced selection targeted at MSP2 prompted us to analyze the anti-MSP2 humoral immune response elicited by vaccination. By analyzing in great detail the effect of Combination B on (i) *msp2* genotypes detected in trial participants and (ii) the strain-specific anti-MSP2 response, we hope to elucidate the effects observed in this field trial in PNG and gather important information for a future MSP2-based vaccine or for other polymorphic vaccines in general.

Of particular interest was the immunogenicity of the conserved N- and C-terminal domains, which could potentially confer immunity across all strains, in contrast to a strain-specific response indicated by antibodies against the intragenic repeats of the 3D7 allele. Immunogenicity of the 3D7 family-specific region could protect against all infecting parasites with an MSP2 of this family. We also investigated whether responses against different regions of the family-specific domain of the alternative FC27 allelic family were elicited. To quantify

* Corresponding author. Mailing address: Swiss Tropical Institute, Socinstrasse 57, P.O. Box CH-4002, Basel, Switzerland. Phone: 41-61-284 81 17. Fax: 41-61-271 86 54. E-mail: ingrid.felger@unibas.ch.

TABLE 1. Sequences and accession numbers of MSP2 constructs

Antigen	Primer sequence (restriction site)[a]	Accession no. (allele)	Recombinant protein sequence
3D7 family-specific domain	5′-cgggatcccgTGGTAATGGTGCT-3′ (BamHI) 5′-ggggtaccccAGATTGTAATTCG-3′ (KpnI)	M60188 (FCR3)	MRGS-HHHHHH-GSR-(FCR3 aa[b] 109–214)-GYPGSTCSQA
FC27 family-specific domain			
5′ part	5′-cgggatcccgTAAGAGTGTAGGTG-CAAATGCTCCAAAAggaattcc-3′ (BamHI/EcoRI) 5′-ggaattcc (EcoRI)	M59766 (K1)	MRGS-HHHHHH-GSRKSVGANAPK-GIP-(FC27 fsp)-APQEP
Fused to 3′ part	5′-gggattccAGAAAGTTCAAGTT-3′ (EcoRI) 5′-ggggtaccccAGCAGGATTTTCA-3′ (KpnI)	MS9766 (K1)	QTAENENPA-GYPGSTCSQA
FC27-type repeat $(32aa)_4$	5′-cgggatcccgTGCTCCAAAAGCT-3′ (BamHI) 5′-ggggtaccccAGGGGTATCAGCA-3′ (KpnI)	AY532388 (lfa 45)	MRGS-HHHHHH-GSR-APK-(32-aa repeat)$_4$-ADTP-GYPGSTCSQA

[a] Lowercase nucleotides indicate mismatches with respect to the *msp2* nucleotide sequence; underlined nucleotides indicate restriction sites used for cloning.
[b] aa, amino acid.

immune responses against the conserved, variable, or repetitive domains of MSP2, the corresponding fragments of the gene were cloned and expressed in *Escherichia coli*.

In the PNG trial, antibody responses were elicited against all three antigens included in the vaccine (11). A significant increase in the anti-MSP2 antibody level was observed in response to vaccination. The serological analysis was performed using the full-length 3D7 type of MSP2 antigen, which was included in the Combination B vaccine. These tests did not permit delineation of the response with respect to the different domains of MSP2.

In anticipation of further vaccine trials of MSP2, there is a need to analyze what effects could have been responsible for the protection observed in the recent trial and why these effects did not protect completely. We were particularly interested in exploring whether vaccination induced an immunological response against the conserved parts of the molecules or specifically against the 3D7 family-specific domain. In light of the limited natural recognition of the conserved domains of MSP2 (14), performing such analyses with samples from immunized children becomes crucial for deciding the composition of the next generation of an MSP2 vaccine. We also investigated whether antimalarial pretreatment led to increased immunogenicity of the vaccine, as has been proposed previously (3, 10).

MATERIALS AND METHODS

Study area and study design. The study was conducted in four villages of the Wosera District of the East Sepik Province of PNG. The study area shows intense and perennial malaria transmission. Entomological inoculation rates were estimated to be 35 infectious bites per year for *P. falciparum*. One hundred twenty children from ages 5 to 9 years were enrolled in the double-blind, block-randomized (within age groups), four-arm, placebo-controlled trial to assess safety, immunogenicity, and pilot efficacy of the Combination B vaccine. Since it has been debated whether preexisting infections should be cleared before immunization, half the children were pretreated with sulfadoxine pyrimethamine (SP) (Fansidar; Hoffmann LaRoche, Basel, Switzerland) at baseline (week −1). Injections were given twice, at weeks 0 and 4.

Blood samples were collected at baseline and every 2 weeks from weeks 4 to 18. The primary outcomes of the trial were the rate of adverse events and the geometric mean *P. falciparum* parasite density (assessed for all positive samples from weeks 8 to 18).

Ethical clearance was obtained from the PNG Medical Research Advisory Committee. Detailed study procedures were described previously (11).

Cloning of recombinant *msp2* constructs. The different MSP2 domains were PCR amplified and cloned in the pQE30 expression vector (QIAGEN, Valencia, Calif.), providing an N-terminal His_6 tag. The primers and *msp2* alleles used as a template for amplification are given in Table 1. A recombinant FC27 family-specific domain was generated by ligating two PCR products representing the region upstream of the 32-mer repeats and the region downstream of the 12-mer, respectively. The restriction sites necessary for ligating the two PCR fragments gave rise to three additional residues, arginine, asparagine, and serine, which are not found at this position in wild-type MSP2 variants. Cloning of N- and C-terminal constant domains was described previously (8). All constructs cloned in *E. coli* were confirmed by DNA sequencing on an ABI Prism 310 genetic analyzer (Perkin-Elmer). Accession numbers are given in Table 1.

Antigen preparation. Five recombinant antigens corresponding to different MSP2 domains were expressed as His_6-tagged proteins in *E. coli* strain M15 (QIAGEN) and purified under denaturing conditions (8 M urea) by use of a Ni-nitrilotriacetic acid column in accordance with the manufacturer's protocol (QIAGEN). The reactivities of the antigens were assessed by immunoblot analysis using a serum pool of 20 semi-immune adults from PNG. The concentrations of purified antigens were determined with a bicinchoninic acid protein assay (Pierce). The recombinant proteins correspond to the conserved N-terminal part, the conserved C-terminal part, the 3D7 family-specific part, the FC27 family-specific part, and an FC27-type 32-amino-acid sequence repeated four times. We failed to recombinantly express the 4-mer repeat glycine-glycine-serine-alanine (GGSA) of the 3D7 strain. Therefore, a synthetic peptide (molecular size, 1,379 Da) corresponding to $(GGSA)_5$ was used. This peptide was kindly provided by Giampietro Corradin, Lausanne, Switzerland.

Determination of antibody titers. Titers of antibody against the above-mentioned antigens were determined by a standard enzyme-linked immunosorbent assay (ELISA). Immulon 2HB plates (Thermo Labsystems, Franklin, Mass.) were coated overnight with 50 μl of antigen at a concentration of 2 μg/ml (recombinant proteins) or 10 μg/ml (synthetic peptide). Plates were blocked for 1 h at room temperature in phosphate-buffered saline containing 5% nonfat milk powder. Antibody reactions were carried out in phosphate-buffered saline containing 0.5% milk powder and 0.05% Tween 20. Serum samples were serially diluted threefold starting from a 1:50 dilution. A pool of serum from 20 adults from PNG was used as an internal standard at a dilution specific for each antigen to give an optical density of about 1. Dilutions of the serum pool were 1:10,000 for the 3D7 family-specific domain and for the FC27-type repeat, 1:1,200 for the FC27 family-specific domain, and 1:100 for the two conserved domains and for the 3D7-type repeat.

The plates were incubated for 2 h at room temperature. Plate washing was

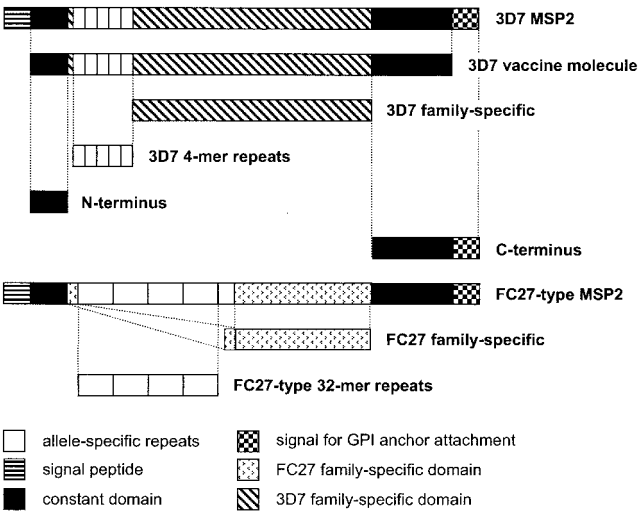


FIG. 1. Schematic diagram of recombinant and synthetic MSP2 antigens used for ELISA. The constructs are aligned with full-length MSP2 alleles representing the two allelic families of MSP2. The 3D7 vaccine molecule included in Combination B is also shown.

performed with an ELISA washer with water containing 0.05% Tween 20. Horseradish peroxidase-labeled goat anti-human immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used as a secondary antibody at a 1:4,000 dilution and incubated for 2 h at room temperature. After extensive washing, ABTS [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] peroxidase substrate (Kirkegaard & Perry) was added. The reaction was stopped after 30 min with 1% sodium dodecyl sulfate, and the optical densities of the plates were read at 405 nm. Antibody titers were determined from the last dilution giving an optical density above 0.1 after standardization and background subtraction (same serum dilution on the uncoated plate).

PCR-restriction fragment length polymorphism genotyping. IsoCode STIX PCR template preparation dipsticks (Schleicher & Schuell, Inc., Keene, N.H.) were used for transport and storage of blood pellets after removal of serum from all blood samples collected during the study. Isolation of *P. falciparum* DNA and *msp2* genotyping were performed as previously described (7). Genotyping of FC27-type alleles was done by a HinfI restriction digestion. To identify each 3D7-type allele unequivocally, additional DdeI and ScrFI digestions were done.

Statistical analysis. For each child, the ratio of the titer at week 12 to that at baseline (week −1) was computed. Two sample *t* tests were used to test the statistical significance of the difference in this ratio between the placebo and vaccine groups. To evaluate whether the diversity of infecting parasites modified the level of antibody, Spearman correlations were computed between the mean multiplicity of infection (MOI) from weeks 4 to 12 and the specific antibody titers at week 12. To ascertain whether any specific antibody appeared to be protective, the correlations were computed between the mean MOI from weeks 14 to 18 and the antibody titers at week 12.

RESULTS

The different MSP2 domains used for ELISA are depicted schematically in Fig. 1. We failed to express the 3D7 repeats in *E. coli*; therefore, a synthetic peptide was used.

Effect of vaccination. The responses against the MSP2 component of the Combination B vaccine were compared among the four study groups, i.e., those receiving either (i) vaccine with no SP, (ii) placebo with no SP, (iii) vaccine with SP, or (iv) placebo with SP. Serum samples analyzed were derived from the baseline survey of the vaccine trial at week −1 and from the parasitological survey at week 12 after vaccination. We chose sera collected at week 12 because titers of IgG against the full-length MSP2 vaccine molecule were found to peak in vaccinated children at this time (11). Figure 2 gives the average titers to all six antigens for the four treatment groups. To test for vaccine effects, the ratio of the antibody titers of the two time points was determined for each child. Table 2 shows the effect of vaccination on this ratio. A significant increase in anti-MSP2 antibody titers in vaccinated children was found for the 3D7-specific antigens. The increase was 4.9-fold (95% confidence interval, 2.72 to 8.83) for the 3D7 family-specific domain and 3.1-fold (95% confidence interval, 1.97 to 4.86) for the 3D7 repeats ($P < 0.0001$). The Combination B vaccine did not significantly increase the titers of antibody against the FC27 32-mer repeats or the conserved N- or C-terminal domains. In contrast, titers of antibody against the FC27 family-specific domain decreased in the placebo group but remained almost constant in the vaccine group, leading to an overall positive effect of vaccination (Fig. 2). Thus, this latter result must be viewed with care.

Effect of SP treatment prior to vaccination. Half of the children (30 vaccine treated and 30 placebo treated) were pretreated with SP in the week prior to the first immunization. To identify whether this pretreatment modified the antibody response, we carried out a comparison of the responses in the SP-treated and untreated groups. We found no significant effect of SP treatment on the ratio of week 12 titer to baseline titer for any of the antigens corresponding to the vaccine molecule, nor were the responses against the N- and C-terminal constant domains or to the FC27 32-mer repeat affected by prevaccine treatment. The only effect of SP treatment was observed for the FC27 family-specific domain. Children who did receive SP showed reduced titers of antibody against this FC27 domain (Table 2).

We also tested for a possible interaction between antimalarial treatment and vaccine effect. SP treatment did not significantly modify the effect of vaccination on any of the titers.

Effect of present or new infections. In order to assess whether *P. falciparum* infections present during the trial period influenced the serological outcomes independently of the effect of vaccination, we calculated Spearman correlations between antibody titers and the mean MOIs determined by PCR-restriction fragment length polymorphism. This was done separately for the MOIs of FC27- and 3D7-type parasites. The MOI was chosen as the end point because it reflects the exposure during that period. The initial analyses considered only children who were not treated with SP (59 children in the placebo and vaccine groups), since SP-treated children remained largely uninfected. Among the untreated children, there were no significant relationships between the mean MOI from weeks 4 to 12 of either the 3D7 or the FC27 type of parasites and any antibody at week 12, suggesting that stimulation by recent infections had little effect on the antibody levels. In particular, titers of antibody against the 3D7 family-specific domain of MSP2, which were significantly increased by vaccination, showed no significant correlation to the MOI of 3D7-type parasites nor to the MOI of FC27-type parasites (correlation coefficients were −0.15 [$P = 0.3$] and 0.09 [$P = 0.5$], respectively).

When the SP-treated group was analyzed, no relationship was detected between the MOI and the levels of antibody against any of the antigens tested. However, when we determined the prospective effect of antibody levels measured at

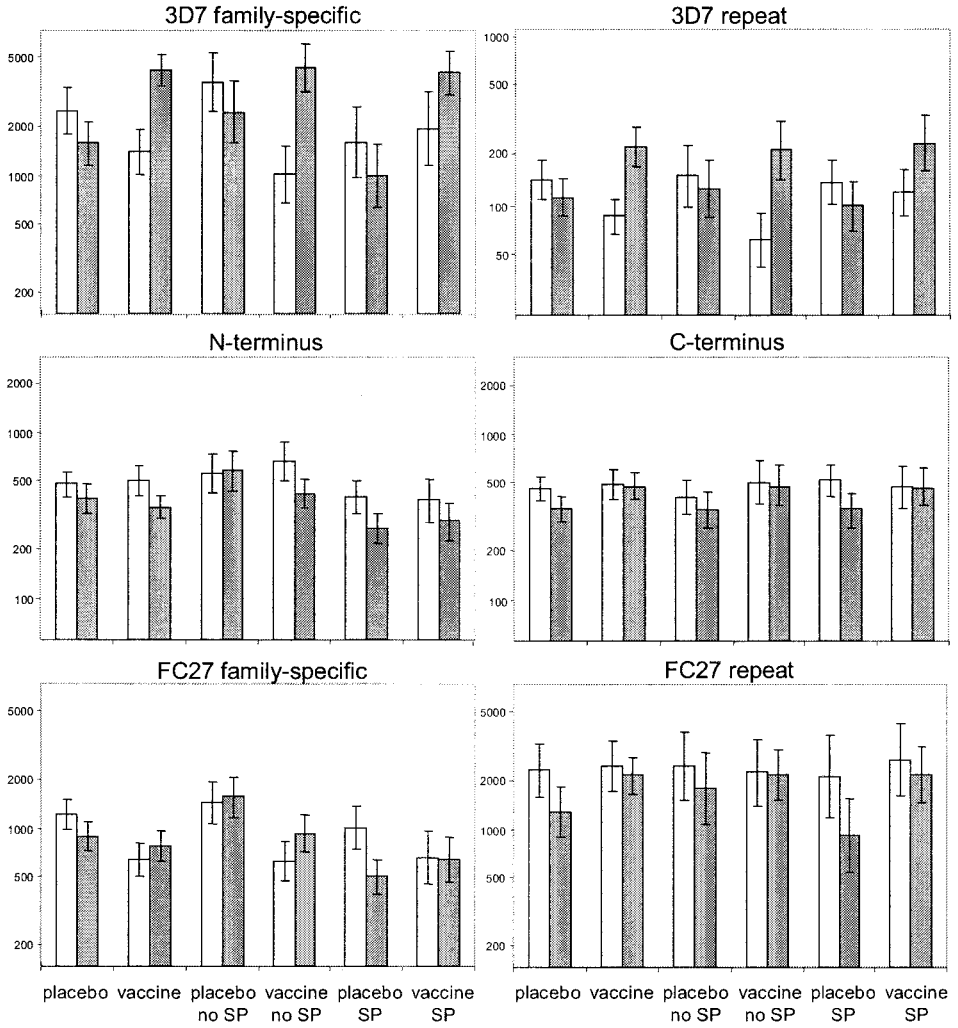


FIG. 2. IgG responses of different treatment groups to recombinant and synthetic MSP2 constructs. Geometric means of titers are shown for each treatment group to all tested antigens: conserved N terminus, conserved C terminus, 3D7 family-specific domain, 3D7 4-mer repeat, FC27 family-specific domain, and FC27 32-mer repeat. White columns represent titers at baseline; gray columns represent titers at week 12 postvaccination with combination B. Standard errors are indicated. The numbers of tested sera at baseline and week 12, respectively, for the different groups were as follows: placebo, 56 and 57; vaccine, 56 and 58; placebo with no SP, 29 and 29; vaccine with no SP, 29 and 30; placebo with SP, 27 and 28; and vaccine with SP, 27 and 28.

week 12 in all non-SP-treated children, irrespective of whether they had received placebo or vaccine, we found positive associations between antibodies against nonvaccine epitopes (FC27) and the mean MOI from weeks 14 to 18 (Table 3). This finding suggests that these antibodies reflect the long-term history of exposure of the host. There was little or no relationship between titers of antibody against vaccine epitopes (3D7) and post-week 12 MOIs, probably because the levels of these antibodies were modified by vaccination so that they no longer represented long-term exposure. This analysis included non-SP-treated children only in order to exclude the effects of this long-acting antimalarial drug on MOI.

TABLE 2. Effect of vaccine and of SP pretreatment[a]

Antigen	Vaccine				SP pretreatment			
	ψ_v	95% CI	t	P	ψ_t	95% CI[b]	t	P
Conserved N terminus	0.84	0.561.27	−1.24	0.2		NS		
Conserved C terminus	1.23	0.871.76	1.17	0.2		NS		
3D7 family specific	4.90	2.728.83	5.12	<0.0001		NS		
3D7 repeat	3.10	1.974.86	4.88	<0.0001		NS		
FC27 family specific	1.57	1.042.37	2.11	0.037	0.59	0.39–0.89	−2.49	0.01
FC27 repeat	1.42	0.902.23	1.5	0.14		NS		

[a] $\psi_v = x_{v,12}x_{p,-1}/x_{v,-1}x_{p,12}$, where $x_{v,-1}$ is the geometric mean titer in the vaccine group at baseline, $x_{p,-1}$ is the geometric mean titer in the placebo group at baseline, $x_{v,12}$ is the geometric mean week 12 titer in the vaccine group, $x_{p,12}$ is the geometric mean week 12 titer in the placebo group, and ψ, is the corresponding ratio testing the effect of SP treatment rather than of vaccination. A value of 1 for ψ_v or ψ_t corresponds to no effect.

[b] CI, confidence interval; NS, not significant.

DISCUSSION

The aim of including the conserved N- and C-terminal domains of MSP2 in a multicomponent vaccine was to elicit a

TABLE 3. Correlations between MOI (weeks 14 to 18) and antibody titer at week 12 irrespective of vaccination[a]

Antigen	3D7 family		FC27 family	
	Spearman's ρ	*P* value	Spearman's ρ	*P* value
Conserved N terminus	0.41	0.001	0.27	0.04
Conserved C terminus	0.19	0.14	0.27	0.04
3D7 family specific	0.11	0.4	0.11	0.4
3D7 repeat	0.14	0.3	0.09	0.5
FC27 family specific	0.38	0.003	0.35	0.007
FC27 repeat	0.26	0.05	0.49	<0.001

[a] Results shown are for only the 59 non-SP-treated children with complete data. Calculation of MOI included samples with no infections. Mean MOI of 3D7-type infections, 0.24 (standard deviation, 0.40); mean MOI of FC27-type infections, 0.24 (standard deviation, 0.46).

strain-transcending immune response. We found that vaccination with Combination B did not induce an antibody response to either of the conserved termini of MSP2. This finding is consistent with generally low titers of naturally occurring antibodies to the conserved parts of MSP2 (14, 15, 20, 22, 24). Also, our recombinant constructs of the N- and C-terminal constant domains had been tested previously in immunoblots with a panel of sera of malaria-exposed adults and had shown only limited reactivities (8).

This finding contrasts with previous results from immunizations of mice suggesting that the conserved parts are immunogenic. Both a recombinant fusion of the conserved N and C termini (14) and short synthetic peptides representing parts of the conserved MSP2 regions (13) were immunogenic in mice. Humoral responses were elicited against the immunogens, but both studies reported very little reactivity with the full-length MSP2 protein.

Despite not being immunogenic in response to vaccination with Combination B, the conserved domains of MSP2 were recognized to be highly prevalent but generally to exist at low titers in all study groups. Our findings suggest that the conserved domains are antigenic to some extent in naïve individuals. However, vaccination or continuous natural exposure does not boost the response, indicating some tolerogenic properties of these parts of the molecule. This finding is also supported by our observation (unpublished) that titers of antibodies to conserved regions are generally found to be higher in young children than in semi-immune adults.

The repetitive domain of MSP2 is allele specific, and repeat sequences differ considerably between individual 3D7-type alleles. At week 12 postvaccination, we found significantly increased titers of IgG against the central repeat region in vaccinees. But it remains unclear how much the observed antirepeat response has contributed to protection against other 3D7-type parasite infections via cross-protective epitopes. The repeat unit GGSA of the 3D7 vaccine molecule occurs only rarely in the study area (unpublished observation). This finding is reflected by low baseline titers of anti-GGSA antibodies as opposed to high titers of antibodies to the FC27-type repeat (mean titers of 107 and 2,479, respectively). It might be possible that we failed to measure the entire anti-GGSA response in our ELISA because we used a short synthetic peptide [$(GGSA)_5$] which may present the repeat in a conformation different from that in the full-length vaccine molecule. However, using this synthetic peptide for measuring the anti-GGSA response seems adequate in view of the high titers (>12,000) found in some sera after vaccination.

The anti-3D7 responses were impressive, with a 4.9-fold increase in titers of antibody against the family-specific domain in vaccinated children irrespective of SP treatment. This result is consistent with the previously described 2.5-fold increase in antibody titers against the entire 3D7 vaccine molecule (11). The result is also in line with a significant reduction in the prevalence of 3D7-type infections in the non-SP-treated vaccinated children (12).

The immunogenicity result supports the inclusion of the 3D7 family-specific domain in further vaccine formulations. Our finding suggests, but does not prove, that vaccine-induced anti-3D7 antibodies specifically protect against infections with parasites of the same allelic family, consistent with findings of cross-reactivity within the allelic family (8, 9). Whether the specific anti-3D7 response accounted for the vaccine-induced reduction in parasite densities remains open.

In trials of malaria vaccines (1, 2), insecticide-treated mosquito nets (for examples, see references 16 and 19), and drugs (21), participants are often pretreated with antimalarials in order to clear parasitemia and to allow determination of time to infection. Such treatment also has been advocated because of the immunosuppression during immunization caused by acute malaria infections and asymptomatic malaria parasitemia (3, 23). But pretreatment does reduce the statistical power to determine the primary end point for efficacy (e.g., parasite density as in the Combination B trial) and molecular measurements by preventing infections for much of the follow-up period. The benefits of pretreating vaccine trial participants have also been questioned on the grounds of effects on immune responses (4), adverse events, or resistance of parasites (5, 17). Our results from the PNG trial of Combination B showed no interaction between SP treatment and vaccine effect on anti-MSP2 antibody titers but dramatically reduced the statistical power of the trial to detect the parasitological effects of the vaccine.

SP treatment had a small but significant effect in reducing titers of antibody against the FC27 family-specific domain, the antigen not represented by the MSP2 variant included in the Combination B vaccine, presumably due to the reduction in exposure caused by the clearance of FC27-type parasites at baseline.

Genotyping all blood samples gave important information on whether the immune response to the MSP2 vaccine was modified by the presence of parasites, which is a topic of considerable interest in malaria vaccine trial design. There was no indication that exposure of the children studied to naturally presented malaria antigen primed them for additional boosting by the 3D7 vaccine component because antibody titers were unaffected by SP treatment. Also, multiplicity of 3D7-type infections did not affect titers of anti-3D7 antibody titers. Our finding is in agreement with a previous indication that a particular infecting MSP2 variant was not associated with the boosting of a strain-specific antibody response in semi-immune adults from Vietnam (24).

During the 1-year follow-up period after the trial of Combination B, an increase in morbidity associated with FC27-type alleles was observed, suggesting that the vaccine caused a se-

lective effect favoring the allelic family not represented in the vaccine (12). Here we showed that the vaccine had only a small effect on specific anti-FC27 antibody titers, consistent with evidence that there is a limited degree of cross-reactivity between the two allelic families (8). However, by reducing the stimulus caused by 3D7-type infections, the vaccine may have reduced immune stimulation against other malaria epitopes and hence made the hosts more vulnerable to FC27 infections. Our results strongly encourage inclusion of both variants of the central dimorphic region in a future MSP2 vaccine.

ACKNOWLEDGMENTS

We thank the study subjects and their families, the staff of the PNG Institute of Medical Research, and S. Märki and S. Steiger of the Swiss Tropical Institute for technical assistance.

This study was funded by the Swiss National Science Foundation (grant 31-062951).

REFERENCES

1. **Alonso, P. L., T. Smith, J. R. Schellenberg, H. Masanja, S. Mwankusye, H. Urassa, I. Bastos de Azevedo, J. Chongela, S. Kobero, C. Menendez, N. Hurt, M. C. Thomas, E. Lyimo, N. A. Weiss, R. Hayes, A. Y. Kitua, M. C., Lopez, W. L., Kilama, T. Teuscher, and M. Tanner.** 1994. Randomised trial of efficacy of SPf66 vaccine against *Plasmodium falciparum* malaria in children in southern Tanzania. Lancet **344:**1175–1181.
2. **Bojang, K. A., P. J. Milligan, M. Pinder, L. Vigneron, A. Alloueche, K. E. Kester, W. R. Ballou, D. J., Conway, W. H., Reece, P. Gothard, L. Yamuah, M. Delchambre, G. Voss, B. M. Greenwood, A., Hill, K. P. McAdam, N. Tornieporth, J. D. Cohen, T. Doherty, and RTS,S Malaria Vaccine Trial Team.** 2001. Efficacy of RTS,S/AS02 malaria vaccine against *Plasmodium falciparum* infection in semi-immune adult men in The Gambia: a randomised trial. Lancet **358:**1927–1934.
3. **Bradley-Moore, A. M., B. M. Greenwood, A. K. Bradley, A. Bartlett, D. E. Bidwell, A. Voller, J. Craske, B. R. Kirkwood, and H. M. Gilles.** 1985. Malaria chemoprophylaxis with chloroquine in young Nigerian children. II. Effect on the immune response to vaccination. Ann. Trop. Med. Parasitol. **79:**563–573.
4. **Bygbjerg, I. C., N. Odum, and T. G. Theander.** 1986. Effect of pyrimethamine and sulphadoxine on human lymphocyte proliferation. Trans. R. Soc. Trop. Med. Hyg. **80:**295–300.
5. **Coulibaly, D., D. A. Diallo, M. A. Thera, A. Dicko, A. B. Guindo, A. K. Kone, Y. Cissoko, S. Coulibaly, A. Djimde, K. Lyke, O. K. Doumbo, and C. V. Plowe.** 2002. Impact of preseason treatment on incidence of falciparum malaria and parasite density at a site for testing malaria vaccines in Bandiagara, Mali. Am. J. Trop. Med. Hyg. **67:**604–610.
6. **Felger, I., B. Genton, T. Smith, M. Tanner, and H.-P. Beck.** 2003. Molecular monitoring in malaria vaccine trials. Trends Parasitol. **19:**60–63.
7. **Felger, I., A. Irion, S. Steiger, and H.-P. Beck.** 1999. Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. Trans. R. Soc. Trop. Med. Hyg. **93**(Suppl. 1)**:**3–9.
8. **Felger, I., S. Steiger, C. Hatz, T. Smith, and H.-P. Beck.** 2003. Antigenic cross-reactivity between different alleles of the *Plasmodium falciparum* merozoite surface protein 2. Parasite Immunol. **25:**531–543.
9. **Franks, S., L. Baton, K. Tetteh, E. Tongren, D. Dewin, B. D. Akanmori, K. A. Koram, L. Ranford-Cartwright, and E. M. Riley.** 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.
10. **Fryauff, D. J., S. J. Cryz, H. Widjaja, E. Mouzin, L. W. Church, M. A. Sutamihardja, A. L. Richards, B. Subianto, and S. L. Hoffman.** 1998. Humoral immune response to tetanus-diphtheria vaccine given during extended use of chloroquine or primaquine malaria chemoprophylaxis. J. Infect. Dis. **177:**1762–1765.
11. **Genton, B., F. Al-Yaman, I. Betuela, R. F. Anders, A. Saul, K. Baea, M. Mellombo, J. Taraika, G. V. Brown, D. Pye, D. O. Irving, I. Felger, H.-P. Beck, T. A., Smith, and M. P. Alpers.** 2003. Safety and immunogenicity of a three-component blood-stage malaria vaccine (MSP1, MSP2, RESA) against *Plasmodium falciparum* in Papua New Guinean children. Vaccine **22:**30–41.
12. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, D. Pye, D. O. Irving, T. A. Smith, H.-P., Beck, and M. P. Alpers.** 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.
13. **Jones, G. L., H. M. Edmundson, R. Lord, L. Spencer, R. Mollard, and A. J. Saul.** 1991. Immunological fine structure of the variable and constant regions of a polymorphic malarial surface antigen from *Plasmodium falciparum*. Mol. Biochem. Parasitol. **48:**1–9.
14. **Lawrence, G., Q. Q. Cheng, C. Reed, D. Taylor, A. Stowers, N. Cloonan, C. Rzepczyk, A. Smillie, K. Anderson, D. Pombo, A. Allworth, D. Eisen, R. Anders, and A. Saul.** 2000. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. Vaccine **18:**1925–1931.
15. **Metzger, W. G., D. M. Okenu, D. R. Cavanagh, J. V., Robinson, K. A. Bojang, H. A. Weiss, J. S. McBride, B. M., Greenwood, and D. J. Conway.** 2003. Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite Immunol. **25:**307–312.
16. **Msuya, F. H., and C. F. Curtis.** 1991. Trial of pyrethroid impregnated bednets in an area of Tanzania holoendemic for malaria. Part 4. Effects on incidence of malaria infection. Acta Trop. **49:**165–171.
17. **Owusu-Agye, S., F. Binka, K. Koram, F. Anto, M. Adjuik, F. Nkrumah, and T. Smith.** 2002. Does radical cure of asymptomatic *Plasmodium falciparum* place adults in endemic areas at increased risk of recurrent symptomatic malaria? Trop. Med. Int. Health **7:**599–603.
18. **Smythe, J. A., M. G. Peterson, R. L. Coppel, A. J., Saul, D. J., Kemp, and R. F. Anders.** 1990. Structural diversity in the 45-kilodalton merozoite surface antigen of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **39:**227–234.
19. **Stich, A. H., C. A. Maxwell, A. A. Haji, D. M. Haji, A. Y. Machano, J. K. Mussa, A. Matteelli, H. Haji, and C. F. Curtis.** 1994. Insecticide-impregnated bed nets reduce malaria transmission in rural Zanzibar. Trans. R. Soc. Trop. Med. Hyg. **88:**150–154.
20. **Taylor, R. R., D. B. Smith, V. J., Robinson, J. S. McBride, and E. M. Riley.** 1995. Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is serogroup specific and predominantly of the immunoglobulin G3 subclass. Infect. Immun. **63:**4382–4388.
21. **Taylor, W. R., T. L. Richie, D. J., Fryauff, H. Picarima, C. Ohrt, D. Tang, D. Braitman, G. S. Murphy, H. Widjaja, E. Tjitra, A. Ganjar, T. R. Jones, H. Basri, and J. Berman.** 1999. Malaria prophylaxis using azithromycin: a double-blind, placebo-controlled trial in Irian Jaya, Indonesia. Clin. Infect. Dis. **28:**74–81.
22. **Thomas, A. W., D. A. Carr, J. M., Carter, and J. A. Lyon.** 1990. Sequence comparison of allelic forms of the *Plasmodium falciparum* merozoite surface antigen MSA2. Mol. Biochem. Parasitol. **43:**211–220.
23. **Usen, S., P. Milligan, C. Ethevenaux, B. Greenwood, and K. Mulholland.** 2000. Effect of fever on the serum antibody response of Gambian children to *Haemophilus influenzae* type b conjugate vaccine. Pediatr. Infect. Dis. J. **19:**444–449.
24. **Weisman, S., L. Wang, H. Billman-Jacobe, D. H. Nhan, T. L., Richie, and R. L. Coppel.** 2001. Antibody responses to infections with strains of *Plasmodium falciparum* expressing diverse forms of merozoite surface protein 2. Infect. Immun. **69:**959–967.

Editor: W. A. Petri, Jr.

3. Effect of the malaria vaccine Combination B on subsequent merozoite surface protein 2 diversity

Draft for Infection, Genetics and Evolution

Effect of the malaria vaccine Combination B on merozoite surface antigen 2 diversity

Christian Flück, Sonja Schöpflin, Tom Smith, Blaise Genton, Michael P. Alpers, Hans-Peter Beck, Ingrid Felger

Swiss Tropical Institute, Socinstrasse 57, Postfach, CH 4002 Basel, Switzerland

Address for Correspondence:

Dr I. Felger,

Swiss Tropical Institute,

Socinstrasse 57,

PO Box,

CH-4002, Basel, Switzerland.

Email: ingrid.felger@unibas.ch

Tel. +41-61-2848117

Facsimile: +41-61-2718654

ABSTRACT

Extensive genetic polymorphism is generally found in *Plasmodium falciparum* surface antigens. This poses a considerable obstacle to the development of a malaria vaccine. In order to assess possible effects of a polymorphic vaccine, we have analyzed the genetic diversity of parasites collected in the course of a phase 2b field trial of the blood stage vaccine Combination B in Papua New Guinea. The full length 3D7 allele of the merozoite surface protein 2 (MSP2) was included in Combination B as one of three subunits. Vaccinees had a lower prevalence of parasites carrying a 3D7-type allele (corresponding to that in the vaccine) and selection appeared to favour the alternative FC27-type alleles resulting in a higher incidence of morbid episodes associated with FC27-type parasites. We sequenced MSP2 alleles detected in study participants after vaccination to identify breakthrough genotypes. Extensive genetic diversity of MSP2 was observed in both the repetitive and family-specific domains, but alleles occurring in vaccine recipients were no different from those found in placebo recipients. A phylogenetic analysis showed no clustering of 3D7-type breakthrough infections from vaccine recipients. The repeat unit present in the vaccine molecule occurred in a number of alleles from the trial area and was also observed in vaccinated individuals. Thus the anti-repeat immune response did not lead to elimination of parasites carrying the same repeat unit. We conclude that the conserved epitopes in the family-specific domain were the most important determinants of the vaccine effect against new 3D7-type infections and that the hypervariable domains were not subject to selective effects of the vaccine.

Keywords: Plasmodium falciparum, malaria vaccine, breakthrough infection, merozoite surface protein 2, genetic diversity, phylogenetic analysis

INTRODUCTION

Plasmodium falciparum surface proteins, and in particular those coating the invasive merozoite stage, are considered prime candidates for vaccine development. Merozoite surface proteins are accessible to the immune system between the rupture of a schizont-infected erythrocyte and reinvasion. Antigens located on the merozoite surface are generally well recognized by the immune system, but unfortunately these

antigens also exhibit extensive polymorphism. Antigenic diversity is thought to help the parasite in escaping human immune defences (Anders, 1986).

Extensive genetic polymorphism poses considerable obstacles to vaccine design. If only a single allele of a polymorphic antigen is used as a malaria vaccine, the protection achieved might be directed only against the same or similar variants. The merozoite surface protein 2 (MSP2) of *P. falciparum*, which constitutes a major component of the surface coat of the merozoite, is an example of a highly polymorphic antigen that has been used for vaccination. The recombinant, full length MSP2 molecule was one component of the Combination B subunit vaccine along with MSP1 (190LCS.T3), and the Ring-infected Erythrocyte Surface Antigen (RESA). In 1998 Combination B was tested in a randomised, 4-armed placebo-controlled, double-blind Phase I/IIb trial (natural challenge) in 120 Papua New Guinean children aged 5-9 years. Vaccinated children were found to have reduced parasite densities, with an efficacy of 62% (Genton et al., 2002).

A single allele of the highly polymorphic MSP2, deriving from the 3D7 strain, was included in the Combination B vaccine. The major portion of MSP2 is polymorphic, only the N- and C-terminal domains are conserved. A dimorphic region flanking a repetitive domain identifies the two allelic families of MSP2, the 3D7-type and FC27-type alleles. The units of tandem repeats vary considerably in length and sequence between different *msp2* alleles. Difference in repeat copy number causes extensive length polymorphism which forms the basis of various *msp2* genotyping schemes.

Genotyping all blood samples collected during the trial at fortnightly intervals over 18 weeks revealed that the vaccine efficacy exhibited specificity for infections belonging to the 3D7 allelic family. Genotyping of blood samples from morbid episodes, collected from these 120 children over a period of one year following the trial, revealed that vaccination favoured FC27-type alleles, thus increasing morbidity in vaccinated children. These were the first reports of a selective effect exerted by vaccination with a polymorphic malaria vaccine (Genton et al., 2002; Felger et al., 2003).

Combination B, as most other malaria vaccines currently in development, was not expected to provide sterilizing immunity. Such imperfect vaccines may select for specific breakthrough parasites, comprising variants not cleared by vaccine-induced immunity. While genotyping of all samples from the vaccine trial had shown that the 3D7 vaccine differentially affected the alleles of the FC27 and 3D7 family (Genton et al., 2002), the vaccine effect on individual 3D7-type MSP2 alleles from subsequent infections has not yet been examined. To monitor the impact of the imperfect MSP2 vaccine subunit of Combination B on the subsequent genetic diversity of MSP2, we have now sequenced *msp2* alleles from both breakthrough and placebo group infections. Phylogenetic sequence analysis was applied to identify characteristics of genotypes resistant to vaccine effects. Such analysis has been postulated to be of great relevance, since partially effective vaccines could potentially select for more virulent pathogens (Gandon et al., 2001).

MATERIALS AND METHODS

Study population. 120 children aged 5 – 9 years from the Wosera area of Papua New Guinea were recruited for a phase I/IIb field trial of Combination B, starting in February 1998. The study was designed as a 4-armed placebo controlled trial with one half of the children being pretreated with SP at baseline. Venous or finger prick blood samples were collected at baseline and during 8 consecutive cross-sectional surveys as described by Genton et al. (2002). During a one-year morbidity follow-up finger prick blood samples were taken from all children reporting with a history of fever in the last three days.

Sample collection. From the 120 children enrolled in the trial, 1079 blood samples were collected at baseline and during the 18 weeks follow-up period. Isocode stix dip sticks (Schleicher and Schuell) were used for transport and storage of blood pellets after removal of serum. All samples were analyzed by PCR, and 257 of these were found to be positive for *P. falciparum*. In addition, 449 blood samples were collected from those 120 children during the one-year morbidity follow-up (296 samples from self-reported case detection at the health center and 153 samples from community-

based case detection through weekly visits by village reporters). From these 449 morbidity follow-up samples, 202 were positive for *P. falciparum* by PCR.

Genotyping. Isolation of *P. falciparum* DNA and *msp2* genotyping was performed as previously described (Felger et al., 1994; Felger and Beck, 2002). The variable central part of the *P. falciparum msp2* gene was amplified by PCR. Subsequent restriction digests produced a genotype-specific RFLP pattern for each different parasite clone in a blood sample. PCR-RFLP genotyping distinguished 39 *msp2* alleles in 257 parasite-positive blood samples of the trial. During the following year of morbidity follow-up, 3 additional *msp2* alleles were detected in morbid episodes. This amounts to 42 *msp2* alleles (8 of the FC27 family, 34 of the 3D7 family) present in the study area.

Sequencing. During the entire study we sequenced 32/34 different 3D7-type *msp2* alleles identified by PCR-RFLP. Alleles from single infections were chosen for analysis. The nested *msp2* PCR product was directly sequenced using both PCR primers and internal sequencing primers. The sequencing reaction was performed with Big Dye sequencing reagents (Applied Biosystems) according to the supplier's instructions and loaded to an ABI PRISM 310 genetic analyzer. The ABI Sequence Navigator program was used for sequence analysis. Sequences were submitted to Genbank under the accession numbers: U07001, AY534507, U07009, U16840, U16842, DQ162622, DQ168572, DQ166534, DQ185319, AJ318755, AJ318753, AJ318752, AJ318754, DQ166535, DQ174442, DQ166536, DQ158904, DQ185320, DQ168571, DQ166545, DQ166537, DQ166538, DQ166546, DQ166539, DQ166540, DQ166541, DQ166542, DQ166543, DQ166544, DQ171731, DQ171732, M73810.

Phylogenetic analysis. For sequence alignment the ClustalX program (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) was used. Phylogenetic analysis was performed with PHYLIP (Phylogeny Inference Package) version 3.6.a3 (Felsenstein, 2002). Distance and parsimony methods were chosen to calculate the fractions of sites that differ between MSP2 alleles. Trees were constructed by the Neighbor Joining as well as by the parsimony algorithm using 1000 bootstrap replicates. Phylogenetic trees were viewed by the TreeView program version 1.5.2. (Page, 1996).

Definition. A breakthrough infection was defined as a new infection which occurred from week 8 onwards in children immunized with Combination B, and which has not been present in the blood sample of the same individual at baseline and at weeks 4 and 6. Immunizations were carried out at baseline and at week 4.

RESULTS

Genetic diversity of 3D7-type msp2 alleles

DNA sequence data was generated for 32 out of a total of 34 3D7-type alleles identified by PCR-RFLP genotyping in the course of the entire study. Seven alleles were detected in baseline samples only, or persisted from baseline onwards but did not occur in new infections in either of the trial groups. 25 alleles were detected in the 80 3D7-type new infections detected in all trial groups after week 8. Among these 80 new infections, 37 occurred in children immunized with Combination B and thus were considered breakthrough infections. These 37 breakthrough infections corresponded to 19 different alleles; only 6 alleles were absent from breakthrough infections. This finding suggests that most alleles were able to resist the effects of vaccination. The fact that 6 alleles of a total of 34 different 3D7-type MSP2 alleles detected in this study were absent from breakthrough infections might reflect chance effects in transmission. In the small number of 37 breakthrough infections, it would be unlikely that all 34 3D7-type MSP2 alleles would occur.

An answer to the question whether this involves any selective effect as a consequence of vaccination, can best be given by measuring the sequence similarity between breakthrough alleles and the 3D7 vaccine molecule.

MSP2 Gene trees

Phylogenetic analysis of genes with intragenic repeats is difficult. Sequence alignment of 3D7-type *msp2* alleles was problematic because repeat units of the various alleles differed in sequence and lengths and were often scrambled. The repeat region includes tandem and scrambled repeats and is defined on nucleotide sequence level by regular spacing of thymidines, giving rise to solely (XXT) codons

(Felger et al., 1997). This previous observation was confirmed by all new *msp2* alleles sequenced in this study. **Figure 1** shows an alignment of the polymorphic domains of 32 3D7-type MSP2 alleles detected in the trial area. For comparison, the 3D7 vaccine molecule is added to the alignment, despite the fact that this variant was not present in the study area. The repeats are followed by a non-repetitive but polymorphic region of variable length, which reveals several clusters of similar sequences. Downstream of this variable region all 3D7-type *msp2* alleles contain a poly-threonine (T) stretch, also varying in lengths, which represents on the nucleotide sequence level in fact another tandem repeat. Further downstream of the poly-threonine stretch follows the so-called family-specific domain of 90 residues that in parts is well conserved with the exception of a region of microheterogeneity (starting at position 14 after poly-T). This region of microheterogeneity revealed 10 SNPs within a stretch of 25 residues, and synthetic peptides representing a part of this region bound to human red blood cells and inhibited parasite invasion of erythrocytes (Ocampo et al., 2000). Just upstream of the repeats, a second small region of microheterogeneity within the family-specific domain is located, spanning six residues of which only the serine at position 3 is conserved.

In order to quantify the sequence similarity between each allele and the 3D7 vaccine molecule, we performed a distance analysis and applied the Neighbour Joining algorithm. First the analysis was performed with the entire polymorphic region between the N- and C-terminal constant domains. The resulting MSP2 gene tree is shown in **Figure 2a**. The vaccine molecule 3D7 (accession number M28891) was chosen as outgroup when drawing the tree. We found that breakthrough infections were not clustered in any branch of the tree, and that alleles not found in breakthrough infections were distributed over all clusters. The same analysis was performed with alignments from which increasing portions of the variable regions had been removed. **Figure 2b** shows the MSP2 gene tree obtained after the (XXT)n repeat region had been removed. Both trees were consistent in their key finding, that breakthrough infections found in vaccinated individuals showed no greater distance to the vaccine molecule than alleles from the placebo group. In addition to the distance method for constructing a gene tree, we also applied the maximum parsimony algorithm. Trees obtained were similar with the major clusters maintained

(data not shown). Breakthrough alleles were again evenly spread over all clusters of the tree.

Because the region of microheterogeneity located downstream of the poly-T stretch and spanning 25 residues was implied in competitive invasion inhibition (Ocampo et al., 2000), we also analyzed this region in greater detail. For **Figure 2c** this region of 25 residues was aligned together with a stretch of 6 residues preceding the tandem repeats and also displaying sequence heterogeneity. The gene tree showed that breakthrough infections were not clustered and did not reveal less similarity to the 3D7 vaccine molecule than sequences found in the placebo group. Thus, vaccination obviously did not select for alternative variants. However, the considerable number of variants detected in our samples suggests that this region is nevertheless under selection.

Repeats of breakthrough infections

The intragenic repeats of 3D7-type alleles are mainly responsible for length polymorphism. Some alleles share the same repeat units, but vary in copy number of repeats (Felger et al., 1997). We were interested in how far the 3D7 repeats, which had been found to be immunogenic in the Combination B vaccine trial (Flück et al., 2004), might have cross-protected vaccinated children against becoming infected with an allele carrying the same repeat unit. We found that, while the 3D7 allele representing the vaccine molecule 3D7, was not present in the study area, its 4-mer repeat Glycine-Glycine-Serine-Alanine (GGSA) was present in three alleles from the trial area. One allele harbouring a GGSA repeat (accession number DQ166535) was detected in two vaccinated children. The same 4-mer motif was also represented in another, larger repeat unit present in four additional alleles (DQ174442, DQ171732, DQ166539, DQ166543), three of which were found in vaccinees.

Sequence fidelity and persistence in time of msp2 alleles in the study area

At the site of the vaccine trial, in the Wosera area in PNG, *msp2* diversity has been studied six years prior to the trial by using the same genotyping technique. In this previous cross-sectional survey in 1992, 38 different *msp2* alleles of both families were detected in two villages (Felger et al., 1994). When we compared the nucleotide sequence and frequencies of RFLP-genotypes, we found that the most frequent

genotypes in 1992 were still frequent six years later, and that some alleles had been maintained without a single point mutation. Alleles of low allelic frequency seem to fluctuate and were mostly new.

Because PCR-RFLP detects length polymorphism and mutations at restriction sites, it does not reveal all sequence diversity present. In order to establish sequence fidelity within a RFLP genotype, we chose the most frequent alleles, KF1916, for a detailed sequencing analysis. Seven nested *msp2* PCR products, all classified as KF1916 genotype by PCR-RFLP, were directly sequenced. These KF1916 sequences were aligned together with the original KF1916 sequence from Genbank (accession number M73810) that derived from a PNG isolate adapted to culture in the 1980s (Marshall et al., 1992). Three KF1916 sequences from the 1992 survey were also added to the alignment. **Figure 3** shows that KF1916 is well conserved, mutations did not accumulate over time, and SNPs were found at an average frequency of one per clone sequenced. The repetitive domain and most part of the family-specific domain were totally conserved whereas SNPs were only detected in the two regions of microheterogeneity. From these data we concluded that PCR-RFLP genotypes are stable over time.

In summary, our results showed a high amount of sequence heterogeneity at the trial site. Allelic diversity in *msp2* was not restricted to the actual repetitive domain alone. High diversity was also found in a considerable part of the dimorphic or family-specific domain. Strictly conserved within all 3D7-type MSP2 alleles was a stretch of 53 residues upstream of the C-terminal constant domain. Specifying the boundaries of strictly conserved dimorphic domains has implications for design of other MSP2 vaccine molecules. We showed that in the Combination B vaccine trial, the diverse domains seem to have not contributed to vaccine efficacy, because breakthrough infections in vaccinated individuals were independent of similarity with the 3D7 vaccine molecule. This suggests that the well conserved epitopes in the family-specific domain must have been the important determinants of the vaccine effect against 3D7-type infections.

DISCUSSION

Antigenic diversity in *P. falciparum* represents a significant challenge for the development of a malaria vaccine. As polymorphism is prevalent in most *P. falciparum* antigens, it is unrealistic to expect complete parasite clearance in vaccinated individuals. Not even natural immunity prevents infection entirely, and sterilizing immunity is never achieved in individuals from endemic areas. Most current efforts in malaria vaccine development consider partially effective vaccines and combination of multiple subunits consisting of several candidate antigens or of several variants of a polymorphic vaccine molecule is the currently favoured strategy (Mahanty et al., 2003).

Little evidence from field data is available on selective effects in malaria vaccine trials. Despite the extensive polymorphism of MSP2, the MSP2 allele of the 3D7 strain was included as a subunit in the Combination B malaria vaccine. Thus, it is likely that escape mutants emerge in the population by filling ecological niches emptied by variants eliminated by vaccine-induced immunity. Our previously published genotyping results from the Combination B trial had shown selection acting on the level of allelic families (Genton et al., 2002). Now we have investigated whether a vaccine effect is evident also on the level of individual alleles. The impact of the imperfect 3D7-MSP2 vaccine on breakthrough infections occurring in vaccinated children was assessed by sequence analysis of all *msp2* alleles detected during the trial.

Msp2 repeat units as smoke screen epitopes

The function of intragenic tandem repeats in plasmodial surface antigens remains obscure. It has been speculated that the arrays of repeats represent “smoke screen” epitopes, which divert the immune system from protective responses by directing the response to irrelevant repetitive epitopes (Anders, 1986, Kemp et al., 1987). It has been proposed that repeats are immunodominant, but even so induce only non-neutralizing antibodies by crosslinking hapten-specific surface immunoglobulin on B cells thus providing a thymus-independent activation with no memory elicited (Schofield, 1991). It is generally assumed that levels of anti-repeat antibodies are not correlated with protection, but this has not yet been shown conclusively for MSP2.

Our data cannot provide much evidence to either support or reject this hypothesis, mainly because the $(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ repeat of the vaccine molecule was found only rarely in alleles in the study area. Nevertheless we inspected the anti-$(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ response at week 12 post vaccination in the few vaccinated individuals infected with a parasite displaying the (Gly-Gly-Ser-Ala) motif, either as tandem repeat or as part of a larger repeat unit. From those individuals antibody titres were available in the serological data base of Flück et al. (2004). Antibody titres were either not raised at week 12 compared to baseline values, or only a minor increase was observed (data not shown). Thus, it remains unclear whether infection by these genotypes could have been prevented if an anti-$(Gly\text{-}Gly\text{-}Ser\text{-}Ala)_5$ response had been induced in these vaccinated children.

Variable non-repetitive domain

After deleting the repeat regions from all sequences of the alignment of 3D7-type MSP2 alleles, a region of about 50 residues of the dimorphic domain proved totally conserved. This region is located adjacent to the C-terminal conserved region. Further upstream, flanking the repeats, highly polymorphic non-repetitive blocks are found characterized by an accumulation of point mutations. We have tested particularly whether these regions of microheterogeneity were subject to selection in vaccinated individuals. We found that sequence similarity of this SNP-rich region to the vaccine molecule did not affect a genotype's presence or absence in the immunized group. Therefore it has to be assumed that responses elicited against these regions were not protective. It follows that the 50-residues-long totally conserved dimorphic region might have been responsible for the selective effect acting on the level of the allelic family.

We assume that the regions of microheterogeneity were immunogenic, because several studies have mapped immunogenicity to these regions. Immunization with a short peptide including a part of the C-terminal region of microheterogeneity (peptide 40 in Jones et al., 1992) elicited IFA-positive antibodies. Lawrence and co-workers (2000) have mapped linear antibody epitopes within MSP2 after vaccinating a human volunteer with the 3D7 variant of MSP2, corresponding to the 3D7 component of Combination B. The same analysis was performed after immunizing mice with the same molecule. Both experiments showed in parallel that the regions of local

microheterogeneity were found to be the major targets of antibody response in the family-specific domain of MSP2. This hypervariable region downstream of the poly-threonine stretch revealed 10 sites of non-synonymous mutations clustered in a stretch of 25 amino acids (underlined in Figure 3). It is exactly this block that seems to play an important role in merozoite invasion of human red blood cells. Ocampo et al. (2000) have identified an MSP2 peptide with high specific binding to human erythrocytes, which is identical with this block. The peptide also inhibited *in vitro* parasite invasion by up to 95%. It remains unclear how a possible function in invasion can be reconciled with the hypervariability we have documented in our small sample size.

Conserved regions within the family-specific domain

The Combination B vaccine was effective in reducing parasite densities, yet the effect was incomplete. Despite vaccination, some 3D7-type infections could establish themselves in immunized children but the vaccine effect may have lead to faster elimination and thus to the reduced prevalence of 3D7-type parasites observed in the trial (Genton et al., 2002). In search of new improved vaccine formulations, the question arises which domain of MSP2 could have caused the selective effect? The 3D7 family-specific domain contains 50 residues of invariant sequence. This is a likely candidate to account for selection observed on the level of the allelic family. We have previously shown that antibody levels against the recombinant 3D7 family-specific domain and against the 3D7 repeats were significantly higher in vaccinated children (Flück et al., 2004). However, our phylogenetic analysis does not allow us to pinpoint the active component of the 3D7 subunit vaccine; it can only indicate whether a polymorphic region is selected and thus subject to protective antibodies.

As with naturally induced immune responses, responses elicited by vaccination are directed against many different epitopes, only a fraction of which might lead to protection. We assume that an antibody response against both the repeats and regions of microheterogeneity was elicited, but did not protect the vaccinated children against new infections. This does, however, not exclude activity of these antibodies against high parasite densities.

This analysis does not take into account the pre-existing acquired immunity to 3D7 genotypes nor the possibility that an ongoing 3D7-type infection inhibits a newly occurring infection of the same allelic family via within-host competition (e.g. for resources). The extent of competitive interactions in multiple clone infections is only recently being studied (de Roode et al., 2004). It is unclear whether such conditions can be ignored in the analysis of selectivity.

Conclusion

The vaccine trial has shown that the 3D7-MSP2 component had some efficacy, though this was imperfect and numerous breakthrough infections occurred. When analyzing the breakthrough genotypes, we found no evidence that responses against the Gly-Gly-Ser-Ala repeats and hypervariable stretches might have protected against new infection by similar variants. We conclude that a response against the conserved stretches within the family-specific dimorphic domain is more likely to account for the MSP2 family-specific selective effect seen in the Combination B trial.

The occurrence of replacement by FC27-type infections in vaccinees in the Combination B trial, leading to increased morbidity, confirms that selective effects of imperfect polymorphic malaria vaccines are of real concern. As more results from other and bigger trials of polymorphic vaccines become available, it will become clear whether such selective effects are a general side effect of vaccine interventions. There is a clear need to include several variants in a single vaccine formulation.

ACKNOWLEDGEMENTS

Financial support was obtained from the Swiss National Science Foundation (grant 3100-062951).

References

Anders RF. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. Parasite Immunol. 1986 Nov; 8(6):529-39.

de Roode JC, Culleton R, Bell AS, Read AF. Competitive release of drug resistance following drug treatment of mixed Plasmodium chabaudi infections. Malar J. 2004 Sep 14; 3:33

Felger I, Tavul L, Kabintik S, Marshall V, Genton B, Alpers M, Beck HP. *Plasmodium falciparum*: extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. Exp Parasitol. 1994 Sep;79(2):106-16.

Felger I, Marshal VM, Reeder JC, Hunt JA, Mgone CS, Beck HP. Sequence diversity and molecular evolution of the merozoite surface antigen 2 of *Plasmodium falciparum*. J Mol Evol. 1997 Aug; 45(2):154-60

Felger I, Beck HP. Genotyping of *Plasmodium falciparum*. PCR_RFLP analysis. Methods Mol Med. 2002; 72:117-29.

Felger I, Genton B, Smith T, Tanner M, Beck HP. 2003. Molecular monitoring in malaria vaccine trials. Trends Parasitol. 19(2):60-3. Review.

Felsenstein, J. 2002. PHYLIP (Phylogeny Inference Package) version 3.6.a3. distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Flück C, Smith T, Beck HP, Irin A, Betuela I, Alpers MP, Anders R, Saul A, Genton B, Felger I. Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun. 2004 Nov;72(11):6300-5

Gandon S, Mackinnon MJ, Nee S, Read AF. 2001. Imperfect vaccines and the evolution of pathogen virulence. Nature. 414(6865):751-6.

Genton B, Betuela I, Felger I, Al-Yaman F, Anders RF, Saul A, Rare L, Baisor M, Lorry K, Brown GV, Pye D, Irving DO, Smith TA, Beck HP, Alpers MP. 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(6):820-7.

Jones GL, Edmundson HM, Lord R, Spencer L, Mollard R, Saul AJ. 1992. Immunological fine structure of the variable and constant regions of a polymorphic malarial surface antigen from *Plasmodium falciparum*. Mol Biochem Parasitol 48:1-10.

Kemp DJ, Coppel RL, Anders RF. Repetitive proteins and genes of malaria. Annu Rev Microbiol. 1987;41:181-208. Review.

Lawrence N, Stowers A, Mann V, Taylor D, Saul A. Recombinant chimeric proteins generated from conserved regions of *Plasmodium falciparum* merozoite surface protein 2 generate antiparasite humoral responses in mice. Parasite Immunol 2000;22(5):211-21

Mahanty S, Saul A, Miller LH. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. J Exp Biol. 2003 Nov;206(Pt21):3781-8. Review

Marshall VM, Coppel RL, Anders RF, Kemp DJ. Two novel alleles within subfamilies of the merozoite surface antigen 2 (MSA-2) of *Plasmodium falciparum*. Mol Biochem Parasitol. 1992;50(1):181-4.

Ocampo M, Urquiza M, Guzman F, Rodriguez LE, Suarez J, Curtidor H, Rosas J, Diaz M, Patarroyo ME. Two MSA 2 peptides that bind to human red blood cells are relevant to *Plasmodium falciparum* merozoite invasion.
J Pept Res 2000 Mar;55(3):216-23

Page RD. TreeView: an application to display phylogenetic trees on personal computers. Comput Appl Biosci. 1996 Aug;12(4):357-8

Schofield L. On the function of repetitive domains in protein antigens of Plasmodium and other eukaryotic parasites. Parasitol Today. 1991 7(5):99-105.

Figure legends

Figure 1: Sequence alignment of the 3D7-MSP2 vaccine molecule and 32 3D7-type MSP2 alleles that were detected in children participating in the Combination B vaccine trial in PNG. Names of sequences correspond to Genbank accession numbers. Stretches of sequence microheterogeneity are bold.

Figure 2: Phylogenetic analysis of 3D7-type *msp2* alleles detected in children from the Combination B trial. Sequences occurring in breakthrough infections (new infections after week 8 post vaccination) of vaccinated children are underlined. The phylogenetic tree was built using the Neighbor Joining method with 1000 bootstrap replicates. The tree was drawn with the vaccine molecule 3D7 as outgroup. The scale bar indicates sequence distance. a) *msp2* gene tree obtained from an alignment of the entire variable region including repeats. *b) msp2* gene tree based on 3D7-type *msp2* sequences from which the repeats had been deleted. c) *msp2* gene tree based on regions of microheterogeneity flanking the repetitive domain.

Figure 3: Sequence fidelity of the PCR-RFLP genotype KF1916 of MSP2 over a period of 20 years. Isolates were collected in Papua New Guinea in 1992 and 1998 and compared to the KF1916 sequence submitted to Genbank that derived from an isolate collected in the 1980s. The polymorphic central part of *msp2* corresponding to the nested PCR product is shown. Underlined residues correspond to the two regions of microheterogeneity where SNPs are frequent.

```
DQ166534  SIRRSMAESNPSTGAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------------------------------
DQ166540  SIRRSMAESNPSTGGSGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------
DQ166535  SIRRSMAESNPSTGAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------------------------
DQ166539  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSAGGSGSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSAGS----------------------------------------------------------
DQ166543  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSAGGSAGS----------------------------------------------------
DQ174442  SIRRSMAESNPSTGAGGSGSAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSAGGSGSAGGSGSAGGSAGGSAGS----------------------------------------------------
DQ171732  SIRRSMAESNPSTGAGASGSAGGSGSAGGSGSAGGSGSAGGSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSAGS----------------------------------------------------------
3D7       SIRRSMAESKPSTGAGGTAGGSAGGSAGGSAGGSAGGSAGS----------------------------------------------------------------------------------------
DQ162622  SIRRSMEESKPPTGASGSAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGNGAVASA----------------------------------------------------------------
DQ168572  SIRRSMEESKPPTGASGSAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGSGAVASAGNGAVASA----------------------------------------------------------
M73810    SIRRSMAESKPPTGTGASGSAGSGAGASGSAGSGDGAVASA----------------------------------------------------------------------------------------
U16840    SIRRSMAESKSPTGTGASGSAGSGDGASGSAGSGDGASGSAGSGDGAVASA------------------------------------------------------------------------------
U07001    SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASA----------------------------------------------------------
DQ158904  SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASA----------------------------------------------
DQ166536  SIRRSMAESKPPTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASARN----------------------------------
DQ166544  SIRRSMEESNPSTGAGGSGSAGSRDGAVASAGSRDGAVASAGSRDGAVASAGSRDGAVASARN----------------------------------------------------------------
DQ166545  SIRRSMAVSNPPTGAGASGRAGAGAGAGASGRAGAGGASGRAGSGDGAVASAGSGDGAVASAGSGDGAVASARN----------------------------------------------------
U07009    SIRRSMTESNPPTPTGAGAGAGDGAVASAGSGDGAVASA------------------------------------------------------------------------------------------
DQ166537  SIRRSMAESKPPTGAGAGAGASGSAGS----------------------------------------------------------------------------------------------------
U16842    SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGASA----------------------------------------------------------
DQ185319  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAVAGSGAGAVAGSGAGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGASA----------------------------------------------
DQ185320  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAVAGSGAGAGAGAVAGSGAGAGAVAGSGAGAGAGSGAGASA----------------------------------------------------
DQ171731  SIRRSMEESNPPTGAGAVAGSGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGAGAVAGSGAGASA----------------------------------------------------
AY534507  SIRRSMTESNPPTGASGSAGAGAGASGSAGAGASGSAGAGASGSAGAGASGSASGSAGASGSAGAGASGSAGS----------------------------------------------------
DQ166541  SIRRSMAESNPPTGASGSAGAGAGASGSAGAGASGSAGAGASGSAGAGAGASGSASGSAGASGSAGAGASGSAGS----------------------------------------------------
DQ166538  SIRRSMAESKPPTGASGSAGAGAGASGSAGASGSAGAGASGSAGAGASGSASGSAGASGSAGAGASGSAGS----------------------------------------------------
DQ168571  SIRRSMAESKPPTPTGAGAGAGASGSAGSGDGASGSASGSAGASGSASGSAGASGSASGSA----------------------------------------------------------------
AJ318755  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAAGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA
AJ318752  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA----------------
AJ318754  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA----------
AJ318753  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA----------------------
DQ166542  SIRRSMAVSKAPTSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSAGASGSA------------------------------------------------------------
DQ166546  SIRRSMAESNPPAGAGASGNPPAGAGASGNPPAGAGASGNPPAGAGASGNPPAGASGSA----------------------------------------------------------------
```

```
DQ166534  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ166540  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ166535  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ166539  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ166543  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ174442  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ171732  GDGNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAKTNPKGNGGVQKPNQANKETQNNSNVQQDSQTKSNVPP
3D7       GDGN----GADAEGSSSTPATTTTKTTTTTTTTNDAEASTSTSSENPNHKNAETNPKGKGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ162622  --GNGANPGADAAGSSSTRATTTTTTTTTTT---NDAEASTSTSSENPNHKNAETNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ168572  --GNGANPGADAAGSSSTRATTTTTTTTTTT---NDAEASTSTSSENPNHKNAETNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ158904  --RNGANPGADAEGSSSTRATTTTTTTT------NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPP
M73810    --RNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAETNPKGKGEVQKSNQANKETQNNSNVQQDSQTKSNVPP
U16840    --RNGANPGADAEGSSSTPATTTTTTTTTTTTTTNDAEASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
U07001    --RNGANPGADAEGSSSTRATTTTTTTT------NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPP
DQ166536  ----GANPGADAEGSSSTRATTTTTTTT------NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPP
DQ166544  ----GANPGADAEGSSSTRATTTTTTTT------NDAEASTSTSSENPNHKNAETNPKGKGEVQKTNQANKETQNNSNVQQDSQTKSNVPP
DQ166545  ----GANPGADAKRSPSTPATTTTTTTT------NDAEASTSTSSENSNHNNAETNPKGKGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
U07009    --GNGANPGADAEGSSSTPATTTTTTTT------NDAEASTSTSSENTNHNNAETNPKGNGKVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ166537  GDGNGANPGADAERSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
U16842    --GN----GADAKRSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ185319  --GN----GADAKRSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ185320  --GN----GADAKRSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ171731  --GN----GADAKRSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAETNPKGKGQVQEPNQANKETQNNSNVQQDSQTKSNVPP
AY534507  --------GADAERSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ166541  --------GADAERSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ166538  --------GADAERSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAKTNPKGKGEVQKPNQANKETQNNSNVQQDSQTKSNVPP
DQ168571  --------GA--ERSPSTPATTTTTTTT------NDAEASTSTSSENPNHNNAKTNPKGK-EVQEPNKANTETQNNSNVQQDSQTKSNVPP
AJ318755  --------GA--EGSSSTPATTTTTTTT------NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
AJ318752  --------GA--EGSSSTPATTTTTTTT------NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
AJ318754  --------GA--EGSSSTPATTTTTTTT------NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
AJ318753  --------GA--EGSSSTPATTTTTTTT------NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ166542  --------GA--EGSSSTPATTTTTTTT------NDAKASTSTSSENPNHNNAETNPKGNGEVQEPNQANKETQNNSNVQQDSQTKSNVPP
DQ166546  --------GA--EGSSSTPATTTTT---------NDAEASTSTSSENPNHNNAKTNPKGNGGVQEPNQANKETQNNSNVQQDSQTKSNVPP
```

```
DQ166534  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166540  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166535  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166539  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166543  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ174442  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ171732  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
3D7       TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ162622  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ168572  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
M73810    TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U16840    TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U07001    TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ158904  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166536  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166544  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166545  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U07009    TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166537  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
U16842    TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ185319  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ185320  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ171731  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AY534507  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166541  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166538  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ168571  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318755  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318752  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318754  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
AJ318753  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166542  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
DQ166546  TQDADTKSPTAQPEQAENSAPTAEQTESPELQSAPEN
```


Figure 1

Figure 2 a

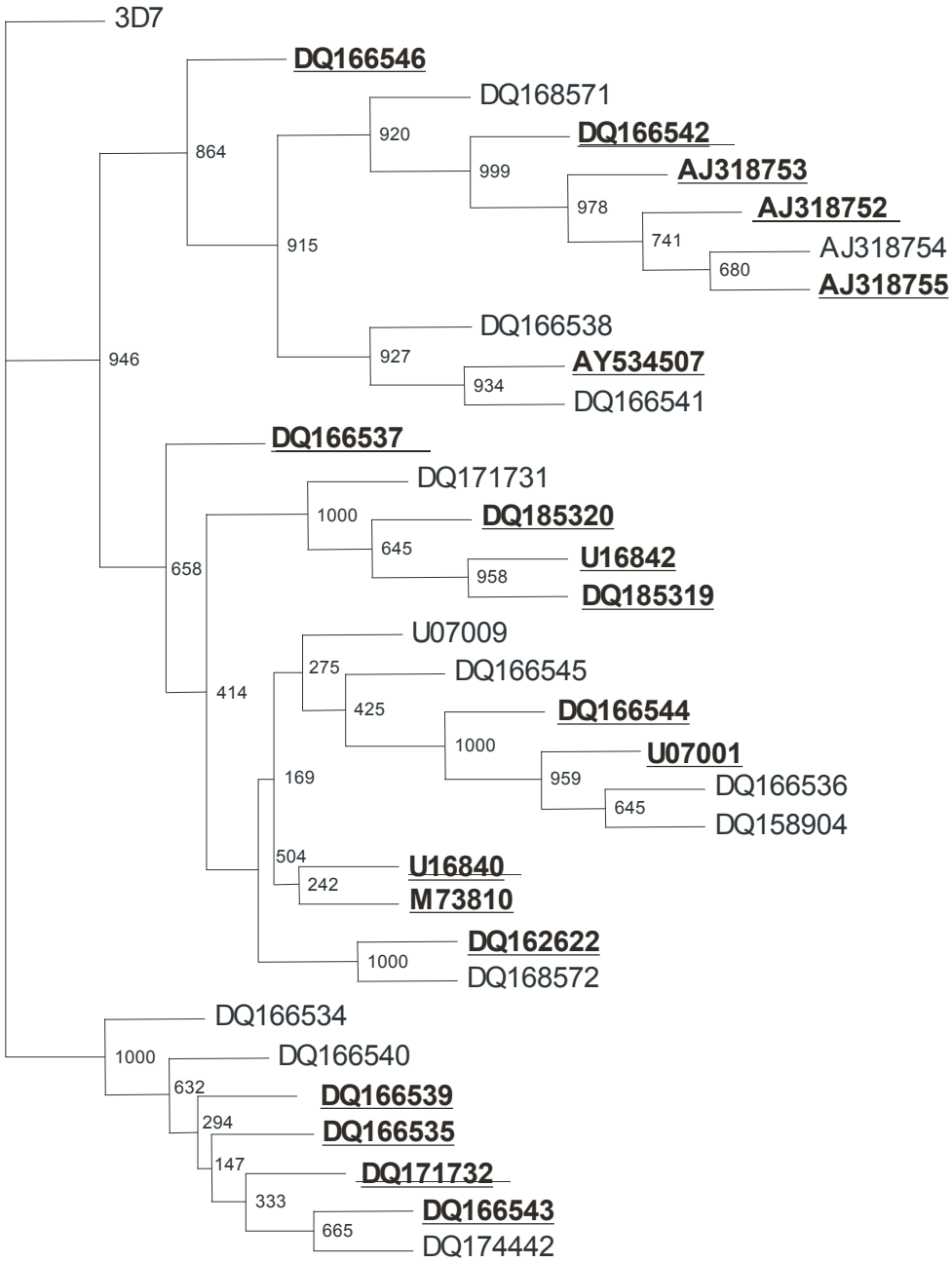
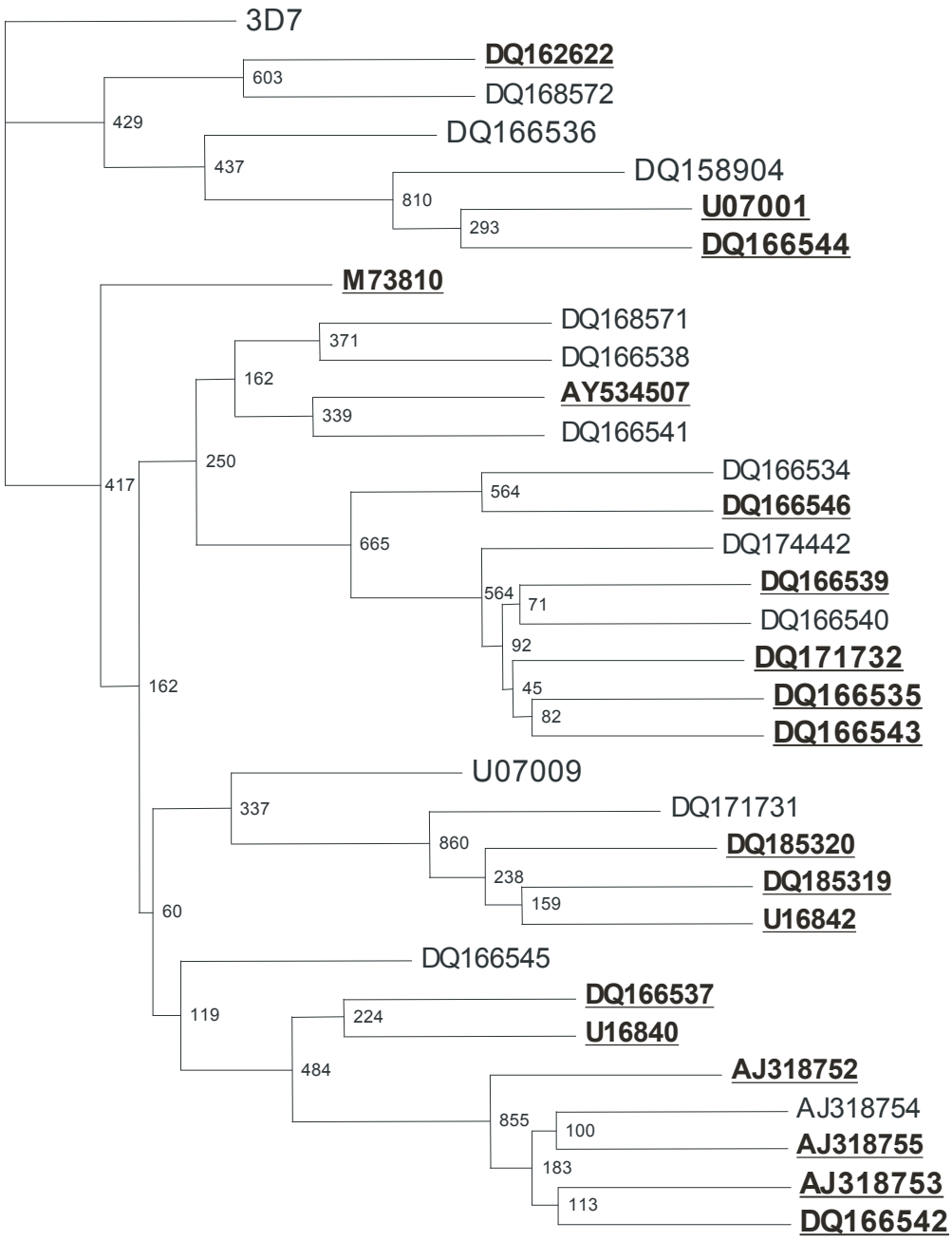

3D7
DQ166546
DQ168571
DQ166542
AJ318753
AJ318752
AJ318754
AJ318755
DQ166538
AY534507
DQ166541
DQ166537
DQ171731
DQ185320
U16842
DQ185319
U07009
DQ166545
DQ166544
U07001
DQ166536
DQ158904
U16840
M73810
DQ162622
DQ168572
DQ166534
DQ166540
DQ166539
DQ166535
DQ171732
DQ166543
DQ174442
864
920
999
978
741
680
915
946
927
934
658
1000
645
958
414
275
425
1000
959
645
169
504
242
1000
1000
632
294
147
333
665

Figure 2 b


3D7
M73810
DQ162622
DQ168572
DQ166536
U07001
DQ158904
DQ166544
DQ166537
U16840
U07009
DQ166534
DQ166540
DQ174442
DQ166535
DQ166539
DQ166543
DQ171732
DQ166545
DQ171731
DQ185320
U16842
DQ185319
DQ166538
AY534507
DQ166541
DQ166546
DQ168571
AJ318754
AJ318752
AJ318755
AJ318753
DQ166542
332
970
659
392
986
345
286
178
374
837
647
186
45
47
57
143
998
274
188
176
394
349
760
359
187
468
992
211
110
113

Figure 2 c


3D7
DQ162622
603
DQ168572
429
DQ166536
437
DQ158904
810
U07001
293
DQ166544
M73810
DQ168571
371
DQ166538
162
AY534507
339
DQ166541
250
DQ166534
417
564
DQ166546
665
DQ174442
DQ166539
564
71
DQ166540
92
DQ171732
162
45
DQ166535
82
DQ166543
U07009
337
DQ171731
860
DQ185320
238
DQ185319
159
60
U16842
DQ166545
DQ166537
119
224
U16840
484
AJ318752
855
AJ318754
100
AJ318755
183
AJ318753
113
DQ166542

```
KF1916 M73810 S I R R S M A E S K P P T G T G A S G S A G S G A G A S G
1992 (n=3)    - - - - - - S(2/3)- - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - E(1/6)- S(3/6)- - - - - - - - - - - - - - - - -

KF1916_M73810 S A G S G D G A V A S A R N G A N P G A D A E G S S S T P
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

KF1916_M73810 A T T T T T T T T T T T T T N D A E A S T S T S S E N P
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

KF1916_M73810 N H N N A E T N P K G K G E V Q K S N Q A N K E T Q N N S
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - N(1/6)- - - P(1/6)- - - - - - - - -

KF1916_M73810 N V Q Q D S Q T K S N V P P T Q D A D T K S P T A Q P E Q
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

KF1916_M73810 A E N S A P T A E Q T E S P E L Q S A P E N
1992 (n=3)    - - - - - - - - - - - - - - - - - - - - - - -
1998 (n=7)    - - - - - - - - - - - - - - - - - - - - - - -
```

Figure 3

4. Evaluation of two long synthetic merozoite surface protein 2 peptides as malaria vaccine candidate

Christian Flück[1)], Géraldine Frank[2)], Ahmed Bolad[3)], Julia Birk[1)], Klavs Berzins[3)], Ingrid Felger[1)], Giampietro Corradin[2)]

(Manuscript)

[1)] Swiss Tropical Institute, Basel, Switzerland
[2)] Institut de Biochimie, Université de Lausanne, Epalinges, Switzerland
[3)] Stockholm University, Department of Immunology, Stockholm, Sweden

Abstract

Merozoite surface protein 2 (MSP2) is a promising candidate for a vaccine against *Plasmodium falciparum* bloodstages. A recombinant 3D7 form of MSP2 was a subunit of Combination B, a bloodstage vaccine tested in the field in Papua New Guinea. A selective effect in favour of the allelic family not represented by the vaccine argued for a MSP2 vaccine consisting of both dimorphic variants. An alternative approach to recombinant manufacture of vaccines is the production of long synthetic peptides. Long peptides exceeding a length of well over 100 amino acids can now be routinely synthesized. Synthetic production of vaccine antigens cuts the often time-consuming steps of protein expression and purification short. This considerably reduces the time for a candidate to reach the phase of clinical trials. Here we present the evaluation of two long synthetic peptides representing both allelic families of MSP2 as potential vaccine candidates. The peptides were well recognized by human immune sera from different locations and different age groups. The peptides share major antigenic properties with native MSP2. Immunization of mice with these peptides yielded high titre antibody responses and monoclonal antibodies recognized parasite-derived MSP2. Antibodies affinity-purified from human immune sera on our peptides inhibited parasite growth *in vitro* in cooperation with human monocytes. Our results justify to take these candidate peptides on into further vaccine development. The results obtained encourage the development of synthetic peptides as malaria vaccine candidates.

Introduction

Plasmodium falciparum, the causative agent of the most severe form of malaria infects 500 million people per year and kills at least one million. One of the most cost-effective intervention would be a vaccine, which to date is not available (Mahanty et al., 2003). The reasons for this are mainly the complex life cycle of the parasite, its antigenic variation and diversity, the wide variety of immune responses it induces, and the incomplete knowledge of protective immunity. Individuals living in malaria endemic regions develop a clinical immunity associated with high antibody titers against major surface molecules of the merozoite stage, however, immunity is never sterile. Passive transfer studies have shown that immunoglobulins from semi-immune individuals can confer clinical immunity to individuals exposed to geographically diverse parasite strains (McGregor et al., 1963; Sabchareon et al., 1991). Vaccines against the blood stages of the parasite could accelerate the acquisition of natural immunity. They do not aim at preventing infection, but at protecting from morbidity and mortality. An advantage of this type of vaccine is constant boosting of the immune response by naturally occuring infections.

The polymorphic merozoite surface portein 2 (MSP2) is considered a vaccine candidate because several studies have shown that high antibody titres to MSP2 are associated with protection against *P. falciparum* malaria (Al-Yaman et al., 1994, Al-Yaman et al., 1995; Aucan et al., 2000; Metzger et al., 2003). MSP2-specific antibodies in immune individuals are found to be predominantly of the cytophilic IgG3 subclass (Taylor et al., 1995; Taylor et al., 1998; Metzger et al., 2003). The major part of MSP2 is polymorphic. A central amino acid repeat region is flanked by a dimorphic region that defines the two allelic families 3D7 and FC27. The function of MSP2 remains unclear but appears to be essential, because targeted gene disruption failed (Cowman et al., 2000). The location of MSP2 on the merozoite surface suggests a role in invasion, and monoclonal antibodies to MSP2 inhibited the invasion of merozoites into erythrocytes (Epping et al. 1988; Ramasamy, 1990). Natural antibody reponses have been shown to be directed mainly against the dimorphic and polymorphic regions but not against the highly conserved termini (Thomas et al., 1990; Lawrence et al., 2000a; Metzger et al., 2003; Weisman et al., 2001). Peptides corresponding to polymorphic and conserved parts of the molecule were immunogenic in mice (Jones et al., 1991; Saul et al., 1992; Lougovskoi et al., 2000).

Combination B was the only subunit vaccine against *P. falciparum* blood stages so far that showed a promising efficacy against parasite density (62%) when tested in a phase I-IIb field trial in Papua New Guinean children (Genton et al., 2002; Genton et al., 2003). The MSP2 component consisted of a recombinant version of the full-length protein of strain 3D7. The activity of this subunit against parasite density was suggested by a selective effect of the vaccine in favour of parasite strains carrying an MSP2 allele belonging to the allelic family not represented by the vaccine (Genton et al., 2002). The finding of a selective effect strongly indicated inclusion of both allelic families in an improved MSP2 vaccine.

The 3D7 MSP2 component of Combination B had been produced as recombinant protein in *Escherichia coli* (Genton et al., 2000). The limitations of recombinant expression of vaccine molecules are folding and purification of the antigens. Since the function of MSP2 is not known, it is difficult to prove that the protein expressed in *E. coli* or other expression systems is correctly folded. Demonstration of cross-antigenicity to native protein is a means to evaluate correct conformation of a recombinant antigen.

Production of correctly folded recombinant antigen is a major problem in vaccine development. It could be tackled using a eukaryotic secretory expression system. However, this would lead to N- and O-linked glycosylation of the recombinant antigen, a feature that is very limited in *P. falciparum.* (Kimura et al., 1996; Gowda et al, 1997; Gowda and Davidson, 1999). In particular, no N- or O-linked glycosylation has been found for MSP2 (Berhe et al., 2000) despite the presence of potential glycosylation sites. When producing MSP2 for a malaria vaccine, it is not desirable to introduce inappropriate modifications by the expression system, which could compromise the efficacy of the humoral response. Problems of purification and undesired glycosylation can be overcome by producing the antigens synthetically. Also, recombinantly expressed antigens often contain unrelated amino acids resulting from affinity tags, linkers, or protease cleavage sites that can alter the antigenic properties of the molecule. Thus, the use of synthetic peptides as vaccines has some advantages over recombinant antigen production.

The absence of a suitable animal model and lacking knowledge of surrogate markers for protective immune reponses in humans constitute major limitations to vaccine development. Due to this lack, other approaches to assess the potential of vaccine candidates need to be taken to carry malaria vaccines from the bench to the clinic. Before a vaccine is tested in a

human trial, the candidate antigen is evaluated by immunogenicity studies in rodents, immuno-epidemiological studies, and *in vitro* assays. These tests provide justification for further development of candidate vaccines. While there is no agreed developmental pathway for vaccine candidates, several empirically found assessment tools are available. (1) The vaccine potential of antigens can be assessed by immuno-epidemiological data, e.g. by a prospective study design, where protection is defined by absence of a malaria episode during the time following blood sampling. (2) Immunoassays with monoclonal antibodies are used to establish the antigenicity of the candidate molecule. In particular, it is important to show that antibodies raised against the vaccine candidate react with parasite-derived protein, i.e. in an indirect immunofluorescence assay. (3) Two *in vitro* assays are available. First, a direct growth inhibition assay can be used to assess the potential of specific antibodies to inhibit parasite growth (Epping et al., 1988; Egan et al., 1999; O'Donnell et al., 2001; Mueller et al., 2003). Such inhibitory antibodies are thought to interfere with the invasion process. Antibodies of any isotype purified from human sera or mouse monoclonal antibodies can be used likewise. Second, the inhibitory potential of an antibody in cooperation with human monocytes can be determined. Human monocytes have been shown to act with merozoite-specific cytophilic antibodies in a cooperative manner against intraerythrocytic *P. falciparum* stages via a mechanism termed antibody-dependent cellular inhibition (ADCI) that involves secretion of TNF-α and other factors. (Bouharoun-Tayoun et al, 1990; Oeuvray et al., 1994a, Oeuvray et al., 1994b; Bouharoun-Tayoun et al., 1995; Theisen et al., 2000; Singh et al., 2004).

All these approaches to measuring protection fall short of a definite proof of concept, since they are no absolute correlates of protection. This remains to be shown in efficacy field trials. We have applied the experimental tests 1-3 for the evaluation of a new MSP2 vaccine candidate consisting of two long synthetic peptides representing both allelic families.

Material and Methods

Synthetic peptides

All of the peptides used in this study were synthesized at the Institute of Biochemistry, Lausanne, using solid-phase Fmoc chemistry (Applied Biosystem 431A, Foster City, California). Briefly, peptides were prepared on a *p*-alkoxybenzylalcohol resin (Wang resin).

After cleavage from the resin, the crude peptide was purified by RP-HPLC (C18 preparative column; H_2O 0.1% TFA/ acetonitrile 0.1% TFA from 90/10 to 20/80 in 70 minutes with a flow rate of 10 mL/min). The purity (>80%) was determined by analytical C18 HPLC and mass spectroscopy (MALDI-TOF, Applied Biosystem, Foster City, California). Lyophilised peptides were dissolved in phosphate buffered saline (PBS) at a concentration of 1 mg/ml. All peptide sequences are shown in figure 1.

Human Sera

Sample set I: Sera had been collected in the framework of the The Malaria Vaccine Epidemiology and Evaluation Project of Papua New Guinea (Alpers et al., 1992) in the Wosera area of the Maprik District in the East Sepik Province, Papua New Guinea (PNG), an area which is highly endemic for malaria. 214 adults from 2 villages (Kunjingini 1 and 2) had been recruited, consisting of 125 females and 89 males, aged 12 to 72 years (mean age 35.5 years). Sera of twenty of these subjects were pooled and used as positive control in enzyme linked immunosorbent assays (ELISAs).

Sample set II: Sera had been collected during an antigenicity study conducted in Burkina Faso (Meraldi et al., 2004). 37 adult volunteers from three different villages situated 50 km from Ouagadougou were bled in March and/or December 2000.

Sample set III: Sera had been collected for a study conducted to determine the effect of insecticide-treated bednets on prevalence and multiplicity of *P. falciparum* infections in infants in the village of Kiberege, Kilombero District, southern Tanzania (Fraser-Hurt et al., 1999). The area is holoendemic for malaria with approximately 300 infectious bites per year with perennial transmission and little seasonal changes of parasite prevalence and density (Smith et al. 1993). Two finger-prick blood samples were collected seven months apart from 30 children initially aged 5 to 15 months who used insecticide-treated nets (ITNs), and from 30 children of the same age group not using ITNs.

Immunization of mice with long synthetic MSP2 peptides MR141 (3D7 MSP2) and MR144 (FC27 MSP2)

Groups of five mice each were immunized three times subcutaneously with 20 µg of peptide (MR141 (3D7 MSP2) or MR144 (FC27 MSP2), respectively in 50 µl adjunvant. Group 1: BALB/c mice, 20 µg peptide MR141 + montanide ISA 720; Group 2: CB6F1 mice, 20 µg

peptide MR141 + montanide ISA 720; Group 3: BALB/c mice, 20 µg peptide MR141 + incomplete Freund's adjuvant (IFA); Group 4: CB6F1 mice, 20 µg peptide MR144A + montanide ISA 720.

Production of monoclonal antibodies (mAbs)

Mice giving the highest titres against the immunogen were chosen for production of monoclonal antibodies (mAbs). Mice were injected a fourth time intraperitoneally with 1 µg of the peptide in 100 µl PBS. Three days after the fourth immunization, spleens were sterilely removed and fused with the mouse myeloma cell line X63.Ag8.653. Culture supernatants of growing hybrids were screened for antibodies by ELISA and indirect immunofluorescence assays (IFA). Positive hybrids, based on ELISA and IFA were cloned by limiting dilution. Hybridoma clones secreting the monoclonal antibodies of interest were grown in serum-free OPTIMEM medium.

Enzyme linked immunosorbent assays (ELISAs)

Recognition of the synthetic peptides by human sera and monoclonal antibodies was assessed by ELISA. The optimal coating concentration of the peptides was determined by a checkerboard titration with positive and negative control sera. The optimal concentration was 1 µg/ml for MR141 and MR144A, and 5µg/ml for MR140.

Immulon® 2HB plates (Thermo Labsystems) were coated overnight with 50 µl of peptide at the appropriate concentration. Plates were blocked for one hour at room temperature in phosphate buffered saline (PBS) containing 5% non fat milk powder. Antibody reactions were carried out in PBS containing 0.5% milk powder and 0.05 % Tween 20. Human sera were diluted 1:400 for assays on MR140 and 1:1000 for assays on MR141, and MR144A (sample sets I and III) or serially diluted 1:3 (sample set II). Supernatants from hybridoma cultures were also serially diluted 1:3. A serum pool of 20 semi-immune adults from PNG was used as internal standard. Pooled sera from 40 non-exposed European children aged 5-15 months were used to determine the cutoff value of our ELISAs. The plates were incubated for two hours at room temperature. Plate washing was performed in an ELISA washer with water containing 0.05% Tween 20. Secondary antibodies were incubated for two hours at room temperature. Goat anti-human IgG-γ specific HRP-conjugated antibody from Kirkegaard and Perry Laboratories (KPL) was used at a dilution of 1:2000 and Goat anti-mouse IgG (heavy+light chain) HRP-conjugated antibody from Biorad was used at a 1:5000 dilution. After extensive washing, ABTS peroxidase substrate (KPL) was added. The reaction was

stopped after 30 minutes with 1% sodium dodecyl sulfate and the plates were read at 405 nm. For IgG subclass-specific ELISAs alkaline phosphatase (AP)-labelled anti-human IgG1, IgG3, and IgG4 antibodies (Southern Biotech) were used at a dilution of 1:1000 and an AP-labelled anti-human IgG2 antibody (Zymed) was used at a dilution of 1:500.

Affinity purification of antibodies from human sera and hybridoma supernatants

5 mg of each peptide (MR141, MR144) were coupled to CNBr-activated sepharose with a final column volume of 1ml. The column was equilibrated with 50 ml of phosphate buffered saline (PBS) pH 7.3. Approximately 80ml of human sera were pooled, centrifuged for 10 minutes at 6000 g, decanted, and diluted 1:5 in PBS and filtered through a 0.22 μm bottle top filter before loading to the sepharose column. Binding was performed at 0.5 ml/min at 4°C. The column was washed with 100 ml PBS and antibodies eluted with 0.1M glycine, 0.15M NaCl, pH 3.4 to 2.9 at 0.8 ml/min at room temperature. 2 ml fractions were collected and neutralised immediately after elution with 100 μl of 1 M Tris-HCl, pH 8.5. Fractions containing antibody were pooled and dialysed twice against 2 litres of PBS. Purified antibodies were concentrated using Centricon YM-10 centrifugal filter units (Millipore, Billerica, MA, USA). Antibodies were sterile filtered through a 0.22 μm syringe filter, aliquoted and stored at -80°C. Concentration of purified antibodies was determined with a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using a bovine serum albumin (BSA) standard.

Hybridoma supernatants were sterile filtered through a 0.22 μm bottle top filter and purified on a HiTrap Protein G column (Amersham Biosciences) according to manufacturer's protocol. Eluted fractions were processed as described above. The titre of purified antibodies against the corresponding peptide was determined by a standard ELISA as described above.

***Plasmodium falciparum in vitro* cultures**

Plasmodium falciparum strains 3D7, K1, and FC27 were grown in human O+ red blood cells (Blutspendezentrum SRK beider Basel, Basel, Switzerland) at 5% hematocrit in RPMI 1640 supplemented with Albumax (GibcoBRL) to a final concentration of 0.5% and gassed with 5% CO_2 and 0.5% O_2 in N_2 at 37 °C as described previously (Trager and Jensen, 1976).

Parasite cultures were synchronised by two sorbitol treatments 10 hours apart (Lambros and Vanderberg, 1979).

Immunofluorescence assays (IFAs)

IFAs were performed on *Plasmodium falciparum* cultures containing at least 5% schizonts. Blood smears from strains 3D7 or FC27 were fixed with acetone/methanol (1:1). Slides were blocked for 30 minutes with 3% BSA in PBS. Primary antibody reactions were carried out for 1 hour at room temperature in 3% BSA/PBS. Secondary antibodies were applied after 5 washes with PBS and incubated for 1 hour at room temperature in 3% BSA/PBS. Cy3-conjugated goat anti-human IgG (H+L) (Jackson Immunoresearch) and Cy3-conjugated goat anti-mouse IgG(γ) (Jackson Immunoresearch) were used at a dilution of 1:500. Slides were washed 5 times with PBS and mounted with Vectashield mounting medium H-100 (Vector Laboratories, Burlingame, CA, USA) containing DAPI at a concentration of 1μg/ml. Fluorescense images were captured using a Leitz Dialux 20 fluorescence microscope and a Leica DC 200 digital camera (Leica Microsystems AG, Germany). Images were processed using Adobe PhotoshopCS.

***In vitro* growth inhibition assays**

Assays were done as described by Mueller et al. (2003). Parasite cultures were synchronized with 5% sorbitol 3 days and 1 day before the assay. The cultures were diluted with fresh erythrocytes to a parasitaemia of 0.5%. The final hematocrit in the test was 0.5%. Antibody solutions or control PBS (up to 50% of total culture volume) were pipetted into 96-well flat bottom microtitre plates and parasite cultures were added (final volume 100 μl). Each test was set up in sextuplicate. The assay plates were put in air tight boxes which were gassed with 4% CO_2, 3% O_2 and 93% N_2 and incubated at 37°C. 96 hours later, plates were centrifuged at 180 g for 5 minutes, the supernatants pipetted off and parasite cultures incubated with 15 μg/ml hydroethidine (Polysciences Inc., Warrington, PA.) in PBS for 30 minutes at room temperature. The red blood cells were washed twice with PBS, resuspended in a final volume of 200 μl PBS and analysed in a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) with CellQuest 3.2.1fl software. The hydroethidine emission was detected in the FL2 channel by logarithmic amplification, and the erythrocytes were gated on the basis of their forward and side scatters. 30,000 cells were analysed per sample. Percent inhibition was calculated from the geometric mean parasitemias of sextuplicate test and control wells as 100 x [(control - test)/control]. Statistical significance was calculated by a two-sided *t* test.

Antibody-dependent cellular inhibition (ADCI) assays

White blood cells were separated from erythrocytes on a Ficoll gradient. 10^7 white blood cells were incubated in a petri dish for 1 – 2 hours at 37°C. Non-adherent cells were washed away. The adherent monocytes were harvested using a cell-lifter and washed before use. 0+ erythrocytes were used for all assays at 2 % hematocrit and 1% parasitemia. The assays were set up in 96-well tissue culture plates. Assays were performed with monocytes alone, the test antibody alone, and the antibody together with monocytes. Antibodies were serially diluted in RPMI. Approximately 10^5 monocytes were used per well. The cultures were incubated at 37°C with CO_2 and harvested 22 hours and 42 hours later. The cultures were washed and fixed on 8-well slides with glutaraldehyde. They were stained with acridin orange and fluorescence microscopy was performed. 25 random fields were counted per well, each field representing 200 cells (total 40 000 cells). The growth inhibition was calculated with the formula: % inhibition = (% parasitemia in parasite control - % parasitemia test)/ % parasitemia in parasite control.

Results

Design of long synthetic MSP2 peptides. Two long synthetic MSP2 peptides were synthesized and evaluated. Due to the selective effect seen in the Combination B trial (Genton et al., 2002), where only one allelic MSP2 family had been represented by the vaccine, we designed two synthetic peptides representing both allelic families. Despite the presence of immunodominant epitopes within the polymorphic repeat regions, we excluded repeats from our candidate peptides because antibodies against repetitive sequences are likely to be non-protective. It was proposed that such sequences present in malarial antigens, similarly to bacterial carbohydrates, induce T-cell-independent B-cell activation by crosslinking of surface immunoglobulin (Schofield, 1991). A T-cell-independent response is considered to be inferior to a T-cell-dependent response, because it lacks affinity maturation and formation of T- and B-cell memory. Furthermore, if repeats are immunodominant, they could suppress induction of antibody responses to neighbouring regions on the same antigen. After excluding the repetitive region, the dimorphic non-repetitive domain and the cnserved domains were considered.

The Combination B vaccine elicited considerable levels of antibodies specific to the dimorphic part of the 3D7 vaccine molecule (Flück et al., 2004). These dimorphic domains are likely to contain protective epitopes and therefore constituted the major part of our synthetic peptides. The C-terminal conserved domain has been found to be only weakly antigenic in naturally exposed humans and Combination B did not induce a significant antibody response to it (Flück et al., 2004). This suggested that this conserved domain has anergic properties. However, the C-terminal conserved domain was still included in both peptides because it contains the only two cysteines of MSP2. Cysteines might be important determinants of protein structure. The peptide sequences are shown in figure 1. Peptide MR141 (3D7-MSP2) includes 88 amino acids of the non-repetitive semi-conserved part of the 3D7 molecule and 40 amino acids of the C-terminal conserved part. Peptide MR144 (FC27-MSP2) is its counterpart representing the other allelic family (67 amino acids of the non-repetitive dimorphic part) plus the 40 amino acids of the C-terminal conserved part. Two versions of the FC27-MSP2 were synthesized, MR144 and MR144A. MR144 includes one copy of the 12 amino acid repeat preceding the non-repetitive part and being present in 1 to 7 copies in the majority of alleles sequenced to date. A shorter version of this peptide, MR144A, was made for antigenicity studies. The latter peptide does not include the 12 amino acid repeat. An additional peptide covering the 40 amino acids of the C-terminal conserved part (MR140) was also synthesized. The two C-terminal cysteine residues were reduced in all peptides.

Antigenicity of long synthetic MSP2 peptides. The antigenicity of the synthetic peptides was evaluated by IgG ELISA using three sets of human sera from different age groups and different malaria endemic areas. The peptides MR141 and MR144A included the family-specific part of 3D7 or FC27 MSP2. The prevalence of antibody against both peptides was high. 96% of the tested adult sera from Papua New Guinea (n=80) recognized the 3D7 peptide MR141. 93% recognized the FC27 peptide MR144A, and 43% recognized peptide MR140 that represents the conserved C-terminus. The median OD value was 0.84 for the peptide representing the 3D7 family (quartiles: 0.31; 1.83). The median OD value for the FC27 peptide was 0.42 (quartiles: 0.09; 0.49) and 0.16 (0.11; 0.27) for MR140. Dilution of Sera was 1:1000 for assays on peptides MR140 and MR144A and 1:400 for assays on MR140.

High antibody titres to peptides MR141 (3D7-MSP2) and MR144A (FC27-MSP2) were also found in adults from Burkina Faso (n=47). The geometric mean antibody titre was 17788 to peptide MR141 (quartiles: 5400; 437400) and 1345 to MR144A (quartiles: 600; 48600).

Recognition of the peptides by sera of 6 to 14 months old Tanzanian children was also assessed. The ELISA results were compared to those previously obtained from the same sera but using recombinant proteins (Irion et al., 2002). These antigens were expressed in *E. coli* and corresponded to the two MSP2 family-specific domains (3D7 and FC27) or to a fusion of the conserved N- and C-terminus. The prevalence of sera with IgG reactivity to the synthetic peptides agreed well with the positivity obtained for the recombinant proteins (Table 1). This indicates similar antigenic properties of the synthetic peptides and the recombinant proteins. Compared to the results obtained in adults, positivity against the dimorphic parts was higher in adults than in young children, but positivity against the conserved C-terminal part was higher in children, which is a surprising finding.

Immunogenicity of peptides representing the two allelic MSP2 families. We immunized mice with MR141 (3D7-MSP2) and MR144 (FC27-MSP2) to determine immunogenicity of the peptides. CB6F1 mice injected 3 times with 20 µg of peptide with Montanide consistently gave antibody titres of $4x10^5$. The C-terminal conserved part of MSP2 contained in peptides MR141 and MR144 also proved to be immunogenic giving titres ranging from $5x10^4$ to $4x10^5$. This is consistent with the findings of previous studies also showing that peptides and recombinant antigens corresponding to the C-terminal conserved part of MSP2 were immunogenic in mice (Saul et al., 1992; Lougovskoi et al., 2000; Lawrence et al., 2000b).

Monoclonal antibodies raised against synthetic peptide MR141 recognize parasite-derived MSP2. Monoclonal antibodies were raised against peptide MR141 (3D7-MSP2). 24 hybridoma cultures were positive against the peptide used for immunization (MR141). 5 of the 24 positive culture supernatants were also positive against peptide MR140, suggesting that they recognize an epitope in the conserved C-terminal part of MSP2. 11 of 24 of the ELISA-positive cultures produced antibody that reacted with the merozoite surface of 3D7 parasites in indirect immunofluorescence assays (IFA). Four surface-reactive cultures were chosen for cloning. IgG subclasses IgG1, IgG2a, and IgG2b were found among the hybridoma clones. Figure 3C shows an immunofluorescence image with monoclonal antibody from a clone recognizing an epitope in the 3D7 family-specific domain. Figure 3D shows

immunofluorescence reactivity of antibody from another clone, recognizing an epitope in the C-terminal conserved domain. Both monoclonal antibodies gave a pattern characteristic for surface staining in mature schizonts (Smythe et al., 1988). The production of hybridomas from mice immunized with MR144 failed twice for unknown reasons.

Synthetic peptide MR140 was recognized by a monoclonal antibody that had been raised against a recombinant fusion of the two conserved terminal regions (Irion, 2000). This corroborates the statement that structural differences between the recombinant protein and the synthetic peptide are limited and do not lead to differential recognition by antibodies.

Naturally occuring human antibodies purified on synthetic MSP2 peptides recognize parasite-derived MSP2 at the merozoite surface and are mainly of the IgG3 subclass.
Sera from Papua New Guinean adults that gave high OD values (>1) in ELISA to MR141 (3D7-MSP2) or MR144A (FC27-MSP2) were pooled for affinity purification of antibodies on the corresponding peptides. The affinity purification yielded 2 mg of anti-MR141 and 0.8 mg of anti-MR144 antibody. The antibodies purified on peptide MR141 represented ~1/700 of total IgG and corresponded to a serum concentration of 21 µg/ml. The antibody purified on peptide MR144 represented ~1/800 of total IgG and a serum concentration of 18 µg/ml. The reactivity and specificity of purified antibodies was confirmed by ELISA. A weak cross-reactivity to the peptides representing the alternative allelic forms of MSP2 was found. This reactivity can be attributed to the conserved C-terminal part common to both peptides (Fig. 1), as it has been shown before that antibodies to the dimorphic part of one MSP2 family do not cross-react to a greater extent with the other MSP2 family (Lawrence et al., 2000a; Felger et al., 2003).

Affinity-purified antibodies were used in immunofluorescence assays. The antibody staining obtained was typical for a merozoite surface protein (Fig. 3A, B). Thus, naturally occuring antibodies reactive to our synthetic peptides also recognize native parasite-derived MSP2. This indicates that the antigenic properties of our peptides are comparable to those of native MSP2.

IgG subclasses of the purified human MSP2 antibodies were determined and cytophilic IgG3 was found to be the dominant subclass in antibody preparations (Fig. 4). Such a high proportion of IgG3 antibodies is unusual for protein antigens but has been reported for MSP2-

specific antibodies in immune sera in several studies (Taylor et al., 1995; Rzepczyk et al., 1997; Ferrante and Rzepczyk, 1997). Furthermore, *in vitro* IgG responses to MSP2 were also studied and found to be entirely restricted to IgG3 (Garraud et al., 2002).

***In vitro* assays for the assessment of the inhibitory potential of peptide-purified MSP2 antibodies.** Antibodies to *falciparum* antigens are predominantly of the cytophilic and complement-fixing subclasses IgG1 and IgG3 in immune adults and of non-cytophilic subclasses (IgM, IgG2) in children (Bouharoun-Tayoun and Druilhe, 1992). We tested the inhibitory potential of the affinity-purified IgG antibodies in a direct growth inhibition assay and in cooperation with human monocytes in an antibody-dependent cellular inhibition (ADCI) assay. In the direct growth inhibition assay the antibodies did not inhibit parasite growth on their own at concentrations of 160 µg/ml (data not shown). This was not surprising since only a few MSP2-specific monoclonal antibodies to epitopes in the repeat region were shown to inhibit parasite growth at concentrations similar to those we used (Epping et al., 1988; Ramasamy et al., 1990) while other monoclonals (Ramasamy et al., 1987) and rabbit sera against recombinant full-length MSP2 and homopolymeric peptides (Ramasamy et al., 1999a; Ramasamy et al., 1999b) did not show an inhibitory effect.

Since our affinity-purified human antibodies were predominantly of the cytophilic subclass IgG3, we tested their inhibitory potential in an ADCI assay. In cooperation with monocytes, the antibody purified on peptide MR141 (3D7-MSP2) gave 72% inhibition of parasite growth at a concentration of 32 µg/ml and 61% inhibition at a concentration of 16 µg/ml in a 22 hour assay. The control (no antibody) with only monocytes gave an inhibition of 20% at 22 hours. Microscopic examination of cultures showed that inhibition by monocytes alone was mainly caused by phagocytosis of erythrocytes infected with late stage parasites. Inhibition mediated by antibodies plus monocytes was mainly due to an attack of the intraerythrocytic stages, as plenty of parasites with a condensed morphology were seen. These data were obtained from a single experiment and are thus to be regarded as preliminary. To confirm the inhibitory activity of those antibodies, the assays have to be repeated. Also, antibodies purified on peptide MR144 will be assessed for ADCI activity.

Discussion

The results from the Combination B trial in Papua New Guinea justified further development of MSP2 as a component to be included in a subunit blood stage malaria vaccine. Selection induced by the Combination B was acting in favour of MSP2 genotypes not represented by the 3D7-MSP2 vaccine molecule. This identified MSP2 as a major active component in this vaccine (Genton et al., 2002) and further suggested to include the family-specific domain of both allelic families in an improved MSP2 vaccine. In search of an optimized MSP2 vaccine we produced and evaluated two long synthetic peptides representing both allelic families of MSP2.

The MSP2 peptides presented here comprise the non-repetitive semi-conserved family-specific domain plus the C-terminal domain that is highly conserved in all MSP2 alleles. Our antigenicity studies on immune sera from different malaria endemic areas and different age groups showed a high prevalence of antibody recognizing both long synthetic peptides. Furthermore, prevalences of antibodies to recombinant proteins corresponding to the family-specific parts and the conserved parts were in accordance with results obtained with our peptides. This finding indicates similar structural and immunological properties of recombinantly produced and synthetic MSP2 antigens. In addition, we showed that antibodies from human immune sera purified on our candidate peptides react with native MSP2 on the surface of merozoites, showing cross-antigenicity with native MSP2. Taken together, these data provide evidence for our synthetic peptides to share major epitopes with parasite-derived MSP2. Presence of high titre antibodies to MSP2 was shown previously to be associated with protection against *P. falciparum* malaria (Al-Yaman et al., 1994; Al-Yaman et al., 1995; Aucan et al., 2000). Association with protection remains to be shown for our peptides.

Circular dichroism spectra of 3D7 MSP2 showed that MSP2 is an intrinsically unstructured protein, random coil being the dominant conformation (Adda et al., 2004). The lack of a folded structure implies that it barely contains conformational epitopes. Thus, a peptide approach to an MSP2-based vaccine is promising.

Both the family-specific and the C-terminal conserved regions present on the peptides MR141 and MR144A elicited high titre IgG responses in CB6F1 mice when administered with montanide ISA 720. It was reported previously that the C-terminal conserved part of MSP2 is

immunogenic in mice (Saul et al., 1992; Lougovskoi et al., 2000; Lawrence et al., 2000). This contrasts with the findings in naturally exposed humans where no high titre responses to this part are found, suggesting anergic properties of this part of the molecule (Thomas et al., 1990; Stower et al., 1997; Lawrence et al., 2000a). Also, vaccination with Combination B did not induce a response to the conserved regions of MSP2 (Flück et al., 2004). In general, conserved domains from *Plasmodium falciparum* antigens are thought to be poorly antigenic (Früh et al., 1991; Dodoo et al., 1999). Replacing some amino acids was shown to improve immunogenicity of conserved domains without affecting cross-reactivity with the parasite-derived molecule (Torres et al., 2003; Espejo et al., 2004).

We were able to generate monoclonal antibodies directed to both the 3D7 family-specific and the C-terminal conserved part in peptide MR141. These monoclonal antibodies were also reactive to the merozoite surface, confirming that antibodies were elicited that were cross-reactive to the peptide and to native MSP2.

An important criterion for the decision to carry on further development of a molecule as a vaccine is proof of efficacy *in vitro*. It needs to be shown that antibodies to the candidate can inhibit parasite growth in an *in vitro* assay. We tested both, affinity-purified antibodies from human immune sera and mouse monoclonal antibodies in direct growth inhibition assays. We did not get significant growth inhibition with either antibody preparation. Antibodies to merozoite surface antigens can act against parasite growth in different ways. In addition to directly blocking invasion into erythrocytes they can agglutinate merozoites (Lyon et al., 1989; Lyon et al., 1997; Thomas et al., 1990) or induce monocytes to release soluble immune mediators killing parasites (Bouharoun-Tayoun et al., 1995). It has been reported that the inhibitory activity of immune sera was 22-fold increased in the presence of normal human monocytes (Khusmith and Druilhe, 1983; Bouharoun-Tayoun et al., 1990). This suggested that direct inhibition of merozoite invasion by binding to essential invasion molecules is not a major function of parasite-specific antibodies *in vivo*. Cytophilic subclasses, especially IgG3 have been implicated in an antibody-dependent cellular inhibition (ADCI) involving merozoite-specific antibodies and the FC-gamma II receptor on blood monocytes (Bouharoun-Tayoun et al., 1990; Bouharoun-Tayoun et al., 1992; Bouharoun-Tayoun et al., 1995). Subclass typing of antibodies purified on peptides MR141 and MR144 showed that IgG3 was the predominant subclass. This unusual subclass distribution in MSP2-specific antibodies is in line with previous reports (Taylor et al., 1995; Taylor et al., 1998; Rzepczyk

et al., 1997; Ferrante and Rzepczyk, 1997). In a preliminary experiment antibodies purified on peptide MR141 showed ADCI activity reducing parasite growth by 41% at a concentration of 16 μg/ml. This concentration compares well to the concentration of MR141-specific antibody in the serum pool we used for affinity purification.

In summary, our results encourage the use of long synthetic peptides in subunit malaria vaccines. synthetic production of a vaccine seems to be justified, in particular, for MSP2, due to its lack of cysteines in all but the C-terminal domain and its predicted highly flexible random-coil conformation,. The peptides presented here have proven to be valuable candidates to be taken one step further in the pathway of vaccine development.

The strategy of using long synthetic peptides as subunits of malaria vaccines as described here for MSP2, can be taken further. Availability of the *P. falciparum* genome allows the identification of novel vaccine molecules through bioinformatic analysis. In particular, unstructured domains could exhibit the same antigenic properties as in the native protein. By chemical peptide synthesis it is now possible to produce a large number of such unstructured domains within a very short time. They can then be screened for their recognition by human immune sera and promising candidates can be further tested.

Acknowledgments

We thank Annelies Oettli for help with establishing ELISAs, Diana Diaz and Jean-Pierre Dangy for assistance with antibody purification, Shinji Okitsu for help with parasite growth inhibition assays, and Julia Birk for help with subclass ELISAs. This work was supported by the Swiss National Science Foundation (Grant # 3100-062951).

References

1. **Adda, C., V. Murphy, K. Vingas, S. Abdulnour, L. Waddington, M. Sunde, A. Hodder, R. Masciantonio, and R. Anders.** 2004. *Plasmodium falciparum* merozoite surface protein 2 forms beta-sheet-containing fibrils in vitro. molecular approaches to malaria (MAM) conference, Lorne, Vic. 26 January - 1 February 2004.

2. **al-Yaman, F., B. Genton, R. Anders, J. Taraika, M. Ginny, S. Mellor, and M. P. Alpers.** 1995. Assessment of the role of the humoral response to *Plasmodium falciparum* MSP2 compared to RESA and SPf66 in protecting Papua New Guinean children from clinical malaria. Parasite Immunol **17:**493-501.

3. **al-Yaman, F., B. Genton, R. F. Anders, M. Falk, T. Triglia, D. Lewis, J. Hii, H. P. Beck, and M. P. Alpers.** 1994. Relationship between humoral response to *Plasmodium falciparum* merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. Am J Trop Med Hyg **51:**593-602.

4. **Alpers, M. P., F. al-Yaman, H. P. Beck, K. K. Bhatia, J. Hii, D. J. Lewis, R. Paru, and T. A. Smith.** 1992. The Malaria Vaccine Epidemiology and Evaluation Project of Papua New Guinea: rationale and baseline studies. P N G Med J **35:**285-97.

5. **Aucan, C., Y. Traore, F. Tall, B. Nacro, T. Traore-Leroux, F. Fumoux, and P. Rihet.** 2000. High immunoglobulin G2 (IgG2) and low IgG4 levels are associated with human resistance to *Plasmodium falciparum* malaria. Infect Immun **68:**1252-8.

6. **Berhe, S., P. Gerold, M. H. Kedees, A. A. Holder, and R. T. Schwarz.** 2000. *Plasmodium falciparum*: merozoite surface proteins 1 and 2 are not posttranslationally modified by classical N- or O-glycans. Exp Parasitol **94:**194-7.

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:**1633-41.

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:**1473-81.

9. **Bouharoun-Tayoun, H., C. Oeuvray, F. Lunel, and P. Druilhe.** 1995. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. J Exp Med **182:**409-18.

10. **Cavanagh, D. R., and J. S. McBride.** 1997. Antigenicity of recombinant proteins derived from *Plasmodium falciparum* merozoite surface protein 1. Mol Biochem Parasitol **85:**197-211.

11. **Cowman, A. F., D. L. Baldi, J. Healer, K. E. Mills, R. A. O'Donnell, M. B. Reed, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2000. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Lett **476:**84-8.

12. **Dodoo, D., T. G. Theander, J. A. Kurtzhals, K. Koram, E. Riley, B. D. Akanmori, F. K. Nkrumah, and L. Hviid.** 1999. Levels of antibody to conserved parts of *Plasmodium falciparum* merozoite surface protein 1 in Ghanaian children are not associated with protection from clinical malaria. Infect Immun **67:**2131-7.

13. **Egan, A. F., P. Burghaus, P. Druilhe, A. A. Holder, and E. M. Riley.** 1999. Human antibodies to the 19kDa C-terminal fragment of *Plasmodium falciparum* merozoite surface protein 1 inhibit parasite growth in vitro. Parasite Immunol **21:**133-9.

14. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. Mol Biochem Parasitol **28:**1-10.

15. **Espejo, F., A. Bermudez, E. Torres, M. Urquiza, R. Rodriguez, Y. Lopez, and M. E. Patarroyo.** 2004. Shortening and modifying the 1513 MSP-1 peptide's alpha-helical region induces protection against malaria. Biochem Biophys Res Commun **315:**418-27.

16. **Felger, I., S. Steiger, C. Hatz, T. Smith, and H. P. Beck.** 2003. Antigenic cross-reactivity between different alleles of the *Plasmodium falciparum* merozoite surface protein 2. Parasite Immunol **25:**531-43.

17. **Ferrante, A., and C. M. Rzepczyk.** 1997. Atypical IgG subclass antibody responses to *Plasmodium falciparum* asexual stage antigens. Parasitol Today **13:**145-8.

18. **Fluck, C., T. Smith, H. P. Beck, A. Irion, I. Betuela, M. P. Alpers, R. Anders, A. Saul, B. Genton, and I. Felger.** 2004. Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun **72:**6300-5.

19. **Fraser-Hurt, N., I. Felger, D. Edoh, S. Steiger, M. Mashaka, H. Masanja, T. Smith, F. Mbena, and H. P. Beck.** 1999. Effect of insecticide-treated bed nets on haemoglobin values, prevalence and multiplicity of infection with *Plasmodium falciparum* in a randomized controlled trial in Tanzania. Trans R Soc Trop Med Hyg **93 Suppl 1:**47-51.

20. **Fruh, K., O. Doumbo, H. M. Muller, O. Koita, J. McBride, A. Crisanti, Y. Toure, and H. Bujard.** 1991. Human antibody response to the major merozoite surface antigen of *Plasmodium falciparum* is strain specific and short-lived. Infect Immun **59:**1319-24.

21. **Garraud, O., R. Perraut, A. Diouf, W. S. Nambei, A. Tall, A. Spiegel, S. Longacre, D. C. Kaslow, H. Jouin, D. Mattei, G. M. Engler, T. B. Nutman, E. M. Riley, and O. Mercereau-Puijalon.** 2002. Regulation of antigen-specific immunoglobulin G subclasses in response to conserved and polymorphic *Plasmodium falciparum* antigens in an in vitro model. Infect Immun **70:**2820-7.

22. **Genton, B., F. Al-Yaman, R. Anders, A. Saul, G. Brown, D. Pye, D. O. Irving, W. R. Briggs, A. Mai, M. Ginny, T. Adiguma, L. Rare, A. Giddy, R. Reber-Liske, D. Stuerchler, and M. P. Alpers.** 2000. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua

New Guinea. Vaccine **18:**2504-11.

23. **Genton, B., F. Al-Yaman, I. Betuela, R. F. Anders, A. Saul, K. Baea, M. Mellombo, J. Taraika, G. V. Brown, D. Pye, D. O. Irving, I. Felger, H. P. Beck, T. A. Smith, and M. P. Alpers.** 2003. Safety and immunogenicity of a three-component blood-stage malaria vaccine (MSP1, MSP2, RESA) against *Plasmodium falciparum* in Papua New Guinean children. Vaccine **22:**30-41.

24. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, D. Pye, D. O. Irving, T. A. Smith, H. P. Beck, and M. P. Alpers.** 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:**820-7.

25. **Gowda, D. C., and E. A. Davidson.** 1999. Protein glycosylation in the malaria parasite. Parasitol Today **15:**147-52.

26. **Gowda, D. C., P. Gupta, and E. A. Davidson.** 1997. Glycosylphosphatidylinositol anchors represent the major carbohydrate modification in proteins of intraerythrocytic stage *Plasmodium falciparum*. J Biol Chem **272:**6428-39.

27. **Irion, A.** 2000. Molecular diversity and immunological properties of the *Plasmodium falciparum* merozoite surface protein 2 (MSP2). PhD Thesis.

28. **Irion, A., H. P. Beck, and T. Smith.** 2002. Assessment of positivity in immuno-assays with variability in background measurements: a new approach applied to the antibody response to *Plasmodium falciparum* MSP2. J Immunol Methods **259:**111-8.

29. **Jones, G. L., H. M. Edmundson, R. Lord, L. Spencer, R. Mollard, and A. J. Saul.** 1991. Immunological fine structure of the variable and constant regions of a polymorphic malarial surface antigen from *Plasmodium falciparum*. Mol Biochem Parasitol **48:**1-9.

30. **Khusmith, S., and P. Druilhe.** 1983. Cooperation between antibodies and monocytes that inhibit in vitro proliferation of *Plasmodium falciparum*. Infect Immun **41:**219-23.

31. **Kimura, E. A., A. S. Couto, V. J. Peres, O. L. Casal, and A. M. Katzin.** 1996. N-linked glycoproteins are related to schizogony of the intraerythrocytic stage in *Plasmodium falciparum*. J Biol Chem **271:**14452-61.

32. **Lambros, C., and J. P. Vanderberg.** 1979. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J Parasitol **65:**418-20.

33. **Lawrence, G., Q. Q. Cheng, C. Reed, D. Taylor, A. Stowers, N. Cloonan, C. Rzepczyk, A. Smillie, K. Anderson, D. Pombo, A. Allworth, D. Eisen, R. Anders, and A. Saul.** 2000a. Effect of vaccination with 3 recombinant asexual-stage malaria antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. Vaccine **18:**1925-31.

34. **Lawrence, N., A. Stowers, V. Mann, D. Taylor, and A. Saul.** 2000b. Recombinant chimeric proteins generated from conserved regions of *Plasmodium falciparum*

merozoite surface protein 2 generate antiparasite humoral responses in mice. Parasite Immunol **22:**211-21.

35. **Lougovskoi, A. A., N. J. Okoyeh, and V. S. Chauhan.** 1999. Mice immunised with synthetic peptide from N-terminal conserved region of merozoite surface antigen-2 of human malaria parasite *Plasmodium falciparum* can control infection induced by Plasmodium yoelii yoelii 265BY strain. Vaccine **18:**920-30.

36. **Lyon, J. A., J. M. Carter, A. W. Thomas, and J. D. Chulay.** 1997. Merozoite surface protein-1 epitopes recognized by antibodies that inhibit *Plasmodium falciparum* merozoite dispersal. Mol Biochem Parasitol **90:**223-34.

37. **Lyon, J. A., A. W. Thomas, T. Hall, and J. D. Chulay.** 1989. Specificities of antibodies that inhibit merozoite dispersal from malaria-infected erythrocytes. Mol Biochem Parasitol **36:**77-85.

38. **Mahanty, S., A. Saul, and L. H. Miller.** 2003. Progress in the development of recombinant and synthetic blood-stage malaria vaccines. J Exp Biol **206:**3781-8.

39. **McGregor I, C. S., Cohen S.** 1963. Treatment of East African *Plasmodium falciparum* malaria with West African gammaglobulin. Trans R Soc Trop Med Hyg.

40. **Meraldi, V., I. Nebie, A. B. Tiono, D. Diallo, E. Sanogo, M. Theisen, P. Druilhe, G. Corradin, R. Moret, and B. S. Sirima.** 2004. Natural antibody response to *Plasmodium falciparum* Exp-1, MSP-3 and GLURP long synthetic peptides and association with protection. Parasite Immunol **26:**265-72.

41. **Metzger, W. G., D. M. Okenu, D. R. Cavanagh, J. V. Robinson, K. A. Bojang, H. A. Weiss, J. S. McBride, B. M. Greenwood, and D. J. Conway.** 2003. Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite Immunol **25:**307-12.

42. **Mueller, M. S., A. Renard, F. Boato, D. Vogel, M. Naegeli, R. Zurbriggen, J. A. Robinson, and G. Pluschke.** 2003. Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of *Plasmodium falciparum* apical membrane antigen 1. Infect Immun **71:**4749-58.

43. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med **193:**1403-12.

44. **Oeuvray, C., H. Bouharoun-Tayoun, H. Gras-Masse, E. Bottius, T. Kaidoh, M. Aikawa, M. C. Filgueira, A. Tartar, and P. Druilhe.** 1994b. Merozoite surface protein-3: a malaria protein inducing antibodies that promote *Plasmodium falciparum* killing by cooperation with blood monocytes. Blood **84:**1594-602.

45. **Oeuvray, C., H. Bouharoun-Tayoun, H. Grass-Masse, J. P. Lepers, L. Ralamboranto, A. Tartar, and P. Druilhe.** 1994a. A novel merozoite surface antigen of *Plasmodium falciparum* (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. Mem Inst Oswaldo Cruz

89 Suppl 2:77-80.

46. **Ramasamy, R.** 1987. Studies on glycoproteins in the human malaria parasite *Plasmodium falciparum*. Identification of a myristilated 45kDa merozoite membrane glycoprotein. Immunol Cell Biol **65 Pt 5:**419-24.

47. **Ramasamy, R., G. Jones, and R. Lord.** 1990. Characterisation of an inhibitory monoclonal antibody-defined epitope on a malaria vaccine candidate antigen. Immunol Lett **23:**305-9.

48. **Ramasamy, R., R. Kanagaratnam, P. D. Chandanie, K. Kulachelvy, M. S. Ramasamy, and P. M. Dharmasena.** 1999b. Model multiple antigenic and homopolymeric peptides from non-repetitive sequences of malaria merozoite proteins elicit biologically irrelevant antibodies. Biochim Biophys Acta **1453:**115-25.

49. **Ramasamy, R., S. Yasawardena, R. Kanagaratnam, E. Buratti, F. E. Baralle, and M. S. Ramasamy.** 1999a. Antibodies to a merozoite surface protein promote multiple invasion of red blood cells by malaria parasites. Parasite Immunol **21:**397-407.

50. **Rzepczyk, C. M., K. Hale, N. Woodroffe, A. Bobogare, P. Csurhes, A. Ishii, and A. Ferrante.** 1997. Humoral immune responses of Solomon Islanders to the merozoite surface antigen 2 of *Plasmodium falciparum* show pronounced skewing towards antibodies of the immunoglobulin G3 subclass. Infect Immun **65:**1098-100.

51. **Sabchareon, A., T. Burnouf, D. Ouattara, P. Attanath, H. Bouharoun-Tayoun, P. Chantavanich, C. Foucault, T. Chongsuphajaisiddhi, and P. Druilhe.** 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. Am J Trop Med Hyg **45:**297-308.

52. **Saul, A., R. Lord, G. L. Jones, and L. Spencer.** 1992. Protective immunization with invariant peptides of the *Plasmodium falciparum* antigen MSA2. J Immunol **148:**208-11.

53. **Schofield, L.** 1991. On the function of repetitive domains in protein antigens of Plasmodium and other eukaryotic parasites. Parasitol Today **7:**99-105.

54. **Singh, S., S. Soe, J. P. Mejia, C. Roussilhon, M. Theisen, G. Corradin, and P. Druilhe.** 2004. Identification of a conserved region of *Plasmodium falciparum* MSP3 targeted by biologically active antibodies to improve vaccine design. J Infect Dis **190:**1010-8.

55. **Smith, T., J. D. Charlwood, J. Kihonda, S. Mwankusye, P. Billingsley, J. Meuwissen, E. Lyimo, W. Takken, T. Teuscher, and M. Tanner.** 1993. Absence of seasonal variation in malaria parasitaemia in an area of intense seasonal transmission. Acta Trop **54:**55-72.

56. **Smythe, J. A., R. L. Coppel, G. V. Brown, R. Ramasamy, D. J. Kemp, and R. F. Anders.** 1988. Identification of two integral membrane proteins of *Plasmodium falciparum*. Proc Natl Acad Sci U S A **85:**5195-9.

57. **Stowers, A., D. Taylor, N. Prescott, Q. Cheng, J. Cooper, and A. Saul.** 1997.

Assessment of the humoral immune response against *Plasmodium falciparum* rhoptry-associated proteins 1 and 2. Infect Immun **65:**2329-38.

58. **Taylor, R. R., S. J. Allen, B. M. Greenwood, and E. M. Riley.** 1998. IgG3 antibodies to *Plasmodium falciparum* merozoite surface protein 2 (MSP2): increasing prevalence with age and association with clinical immunity to malaria. Am J Trop Med Hyg **58:**406-13.

59. **Taylor, R. R., D. B. Smith, V. J. Robinson, J. S. McBride, and E. M. Riley.** 1995. Human antibody response to *Plasmodium falciparum* merozoite surface protein 2 is serogroup specific and predominantly of the immunoglobulin G3 subclass. Infect Immun **63:**4382-8.

60. **Theisen, M., S. Soe, S. G. Jessing, L. M. Okkels, S. Danielsen, C. Oeuvray, P. Druilhe, and S. Jepsen.** 2000. Identification of a major B-cell epitope of the *Plasmodium falciparum* glutamate-rich protein (GLURP), targeted by human antibodies mediating parasite killing. Vaccine **19:**204-12.

61. **Thomas, A. W., D. A. Carr, J. M. Carter, and J. A. Lyon.** 1990. Sequence comparison of allelic forms of the *Plasmodium falciparum* merozoite surface antigen MSA2. Mol Biochem Parasitol **43:**211-20.

62. **Torres, E., L. M. Salazar, M. Vanegas, F. Guzman, R. Rodriguez, Y. Silva, J. Rosas, and M. E. Patarroyo.** 2003. Modified merozoite surface protein-1 peptides with short alpha helical regions are associated with inducing protection against malaria. Eur J Biochem **270:**3946–52.

63. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673-5.

64. **Weisman, S., L. Wang, H. Billman-Jacobe, D. H. Nhan, T. L. Richie, and R. L. Coppel.** 2001. Antibody responses to infections with strains of *Plasmodium falciparum* expressing diverse forms of merozoite surface protein 2. Infect Immun **69:**959-67.

Table 1

Comparison of prevalence of antibodies to synthetic MSP2 peptides and recombinant MSP2 domains in Tanzanian infant sera.

Antigen	Data description		Classical approach used to define positivity [1]		Latent class model (% positive, 95% confidence levels) [2]
	Mean OD in negative controls (S.D.)	Mean OD in test sera (S.D.)	Cutoffs (units) mean OD + 2 S.D.)	Proportion above cutoff (positivity)	
MR141 (3D7-cons)	0.002 (0.004)	0.221 (0.293)	0.011	0.728	0.73 (0.62, 0.83)
MR144A (FC27-cons)	0.003 (0.005)	0.214 (0.353)	0.012	0.772	0.82 (0.72, 0.90)
MR140 (cons)	0.009 (0.009)	0.566 (0.707)	0.028	0.781	0.82 (0.72, 0.91)
recombinant 3D7 fsp	0.005 (0.005)	0.05 (0.08)	0.015	0.525	0.75 (0.65, 0.84) [3]
recombinant FC27 fsp	0.090 (0.095)	0.30 (0.24)	0.280	0.443	0.78 (0.64, 0.89) [3]
rec. N-C-conserved	0.281 (0.284)	2.07 (3.07)	0.848	0.540	0.84 (0.78, 0.91) [3]

[1] Determination of proportion of positive sera by classical approach (cutoff = OD of control sera + 2* standard deviation (S.D.))
[2] Proportion of positive sera according to latent class model (Irion et al., 2002)
[3] Data taken from Irion et al. (2002)

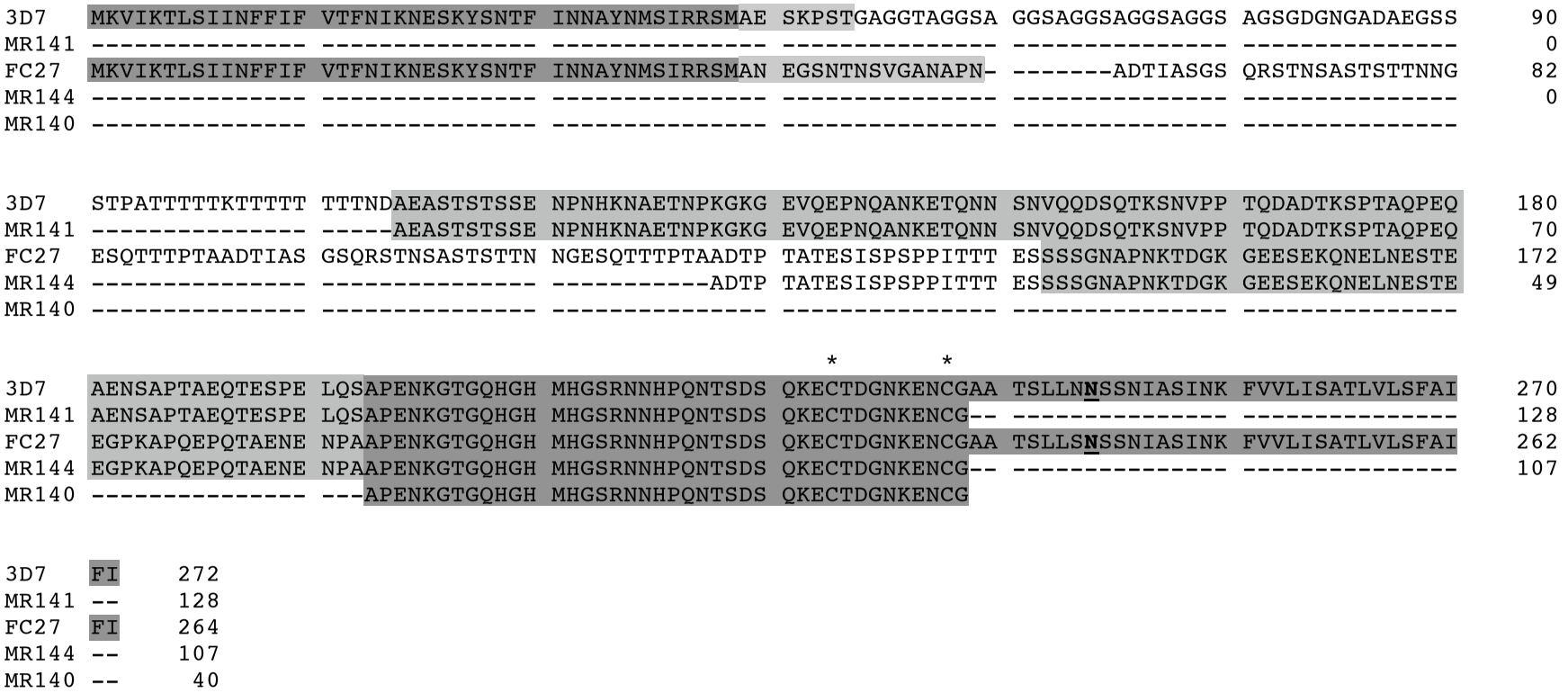


Fig. 1 Amino acid sequence alignment of full-length MSP2 alleles and long synthetic peptides. Peptides MR141 (3D7 family-specific domain + conserved C-terminal part) and MR144 (FC27 12mer repeat-family-specific domain + conserved C-terminal part) were used for affinity-purification of antibody from human immune sera. Peptide MR141 was also used to immunise mice for production of monoclonal antibodies. Peptide MR144A is a shorter version of MR144 starting from (SSSGN...). Non-repetitive family-specific parts are shaded in light gray, conserved sequences are shaded in dark gray. The bold and underlined asparagine residue (N) represents the putative cleavage site for the addition of the glycosylphosphatidylinositol (GPI) anchor. The two cysteine residues in the C-terminus (marked with asterisks) were reduced in the peptides used for affinity purification.

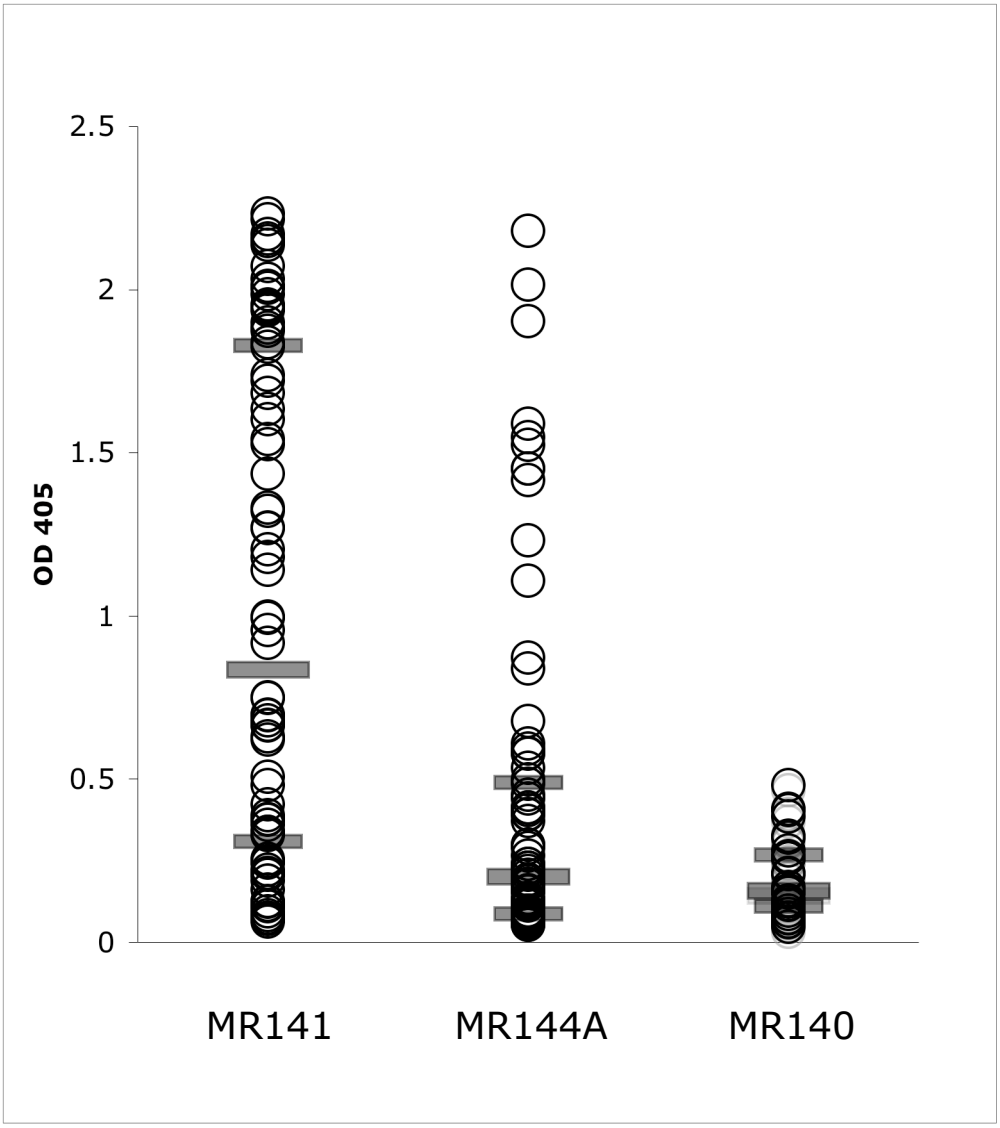


Fig. 2 Recognition of long synthetic MSP2 peptides by PNG human immune sera. Enzyme-linked immuno sorbent assays (ELISA) with adult sera from a region in Papua New Guinea where malaria is highly endemic on synthetic MSP2 peptides. 96% of the tested sera recognized peptide MR141 (3D7-cons), 93% recognized peptide MR144A (FC27-cons), and 43% recognized peptide MR140 (cons). The median OD value was 0.84 for the 3D7-cons peptide with 50% of the values lying between OD 0.31 and 1.83. The median OD value for FC27-cons was 0.42 (50% percentile from 0.09 to 0.49) and 0.16 (0.11 to 0.27) for the C-terminal conserved peptide (cons).

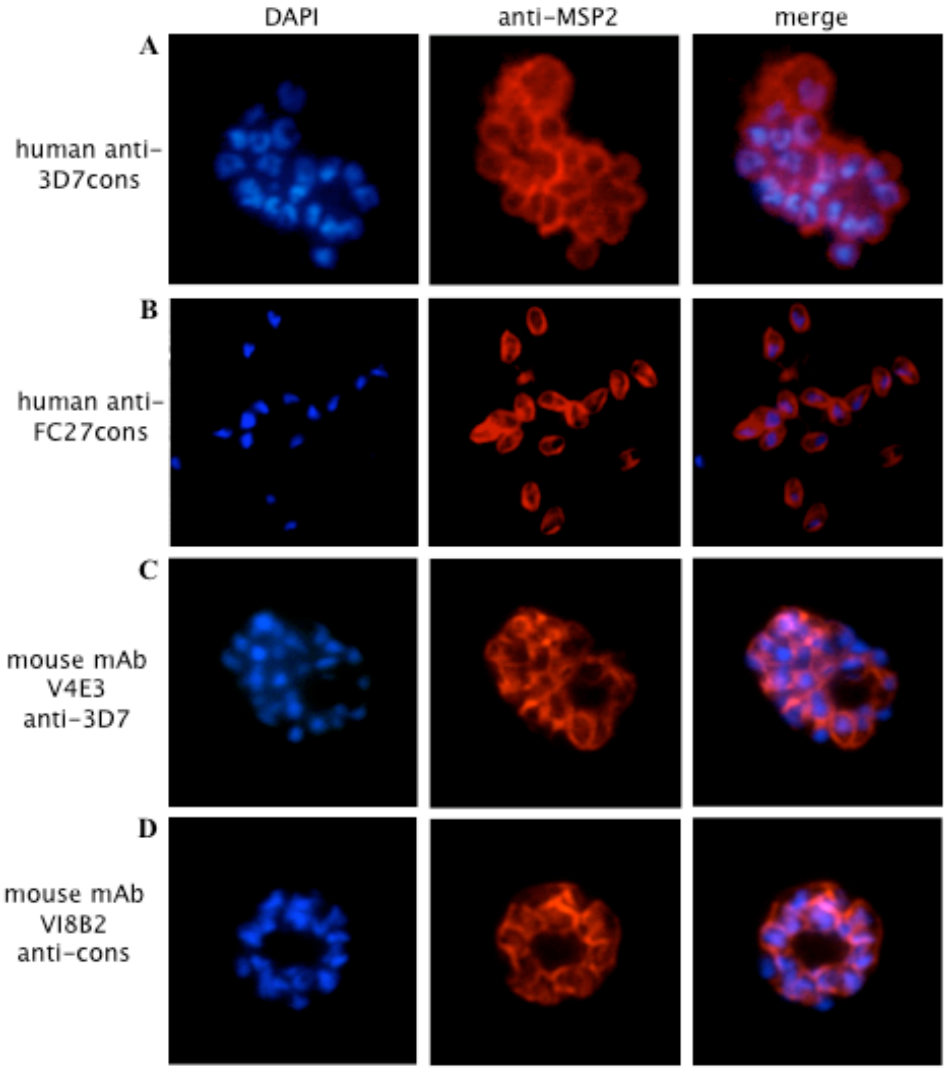


Fig. 3. Immunofluorescence microscopy analysis of *P. falciparum* parasites with MSP2-peptide specific antibodies. Acetone / methanol-fixed *P. falciparum* schizonts and merozoites were reacted with MSP2-specific antibodies. (A) human antibody affinity-purified on peptide MR141 (3D7cons) on 3D7 parasites. (B) human antibody affinity-purified on peptide MR144 (FC27cons) on K1 parasites. (C) Mouse monoclonal antibody raised against peptide MR141 with an epitope mapped to the 3D7-specific region (C) and to the C-terminal conserved region (D) on 3D7 parasites. Left hand panels show parasite nuclei stained with DAPI, central panels show the MSP2 antibody labelling followed by a Cy3-conjugated anti-human IgG-specific (A, B) or anti-mouse IgG-specific antibody (C, D), respectively. The right hand panels are a merge of the blue and red fluorescence channels.

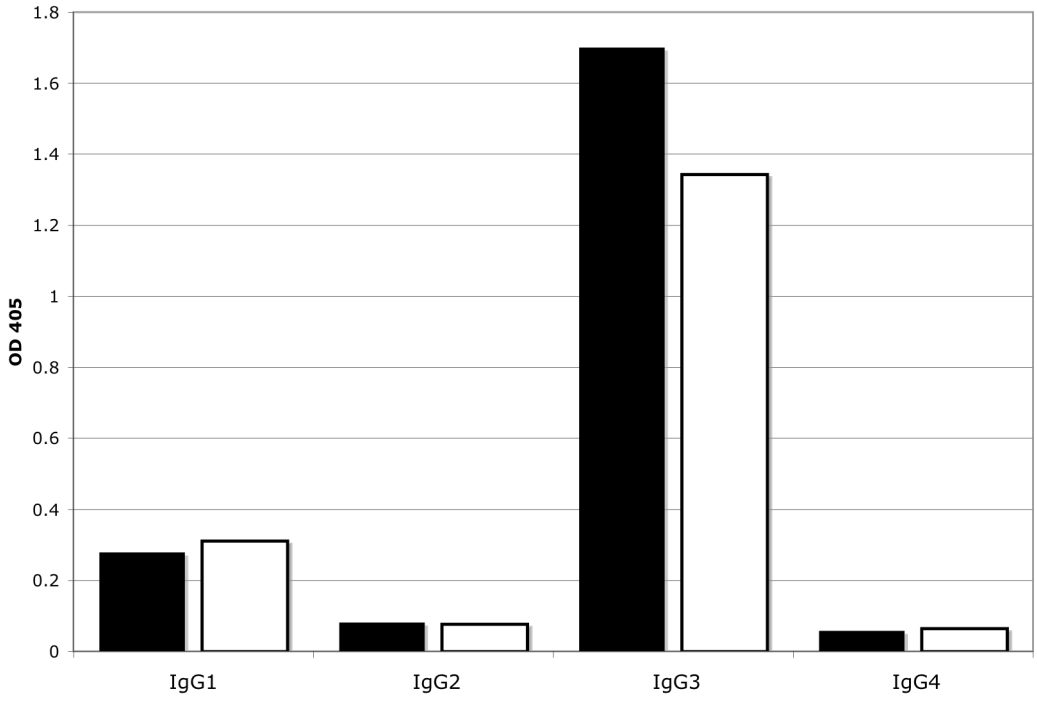


Fig. 4. IgG3 is the dominant subclass of MSP2-specific antibodies affinity-purified from human immune sera. Standard ELISA on human MSP2 antibody affinity-purified on synthetic peptides MR141 (black columns; antibody dilution 1:450) and MR144 (white columns; antibody dilution 1:150). Microtitre plates were coated with 1 µg/ml of peptide and reacted with affinity-purified human antibodies followed by subclass-specific anti-human antibodies. OD values are given for each IgG subclass. Values represent mean values of one experiment run in duplicates.

5. Replacement of the *Plasmodium falciparum* merozoite surface protein 2 gene by partially deleted coding regions

Christian Flück[1), 2)], Hans-Peter Beck[1)], Alan F. Cowman[2)], Ingrid Felger[1)]

(Manuscript)

[1)] Swiss Tropical Institute, Basel, Switzerland

[2)] Walter & Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia

Abstract

Merozoite surface protein 2 (MSP2) is a polymorphic component of the merozoite surface coat of *Plasmodium falciparum*. Its polymorphism is restricted to the central part of the molecule while the two terminal domains are highly conserved. The function of MSP2 is yet unknown. We hypothesized that the polymorphic repeat region is only of immunological importance and is dispensable for *in vitro* growth. To investigate the importance of the repeat domain and the central dimorphic domain *in vitro* we conducted gene replacements at the *msp2* locus. The endogenous 3D7 *msp2* gene was to be replaced by the FC27 allele lacking the polymorphic repeat region or a fusion of the two conserved termini. We were able to target the *msp2* locus with plasmid constructs containing the partially deleted *msp2* genes. Plasmids recombined via a single cross-over. However, double cross-over recombination that would have led to a replacement of the *msp2* gene was not obtained. This indicates that the polymorphic and dimorphic parts of the molecule are essential for *in vitro* growth.

Introduction

Merozoite surface protein 2 (MSP2) is the second most abundant antigen on the surface of *Plasmodium falciparum* merozoites. It is a 45 to 51 kDa protein anchored to the plasma membrane via a glycosylphospatidylinositol (GPI) anchor. Its surface localisation has been proven by immuno-electron microscopy (Epping et al., 1988). MSP2 is highly polymorphic.

The polymorphism is restricted to the central part of the molecule, with semi-conserved dimorphic domains that flank a highly polymorphic repeat region. According to their dimorphic domains, *msp2* alleles are grouped into the 3D7 or FC27 family. Common to all alleles from both families are the highly conserved N- and C-terminal regions. The conserved N-terminal domain spans 43 amino acids including a classical signal peptide at the N-terminus. The C-terminal domain includes 74 amino acids and shows a putative cleavage site for addition of the lipid anchor 34 residues upstream of the stop codon.

The protein's function is unknown. It is thought to be involved, like other MSPs, in the primary attachment of the merozoite to the erythrocyte. However, MSP2 has never been shown to bind to red blood cells although two MSP2 peptides showed high specific binding, which also inhibited parasite growth *in vitro* (Ocampo et al., 2000). Its sequence does not predict any stable secondary structures and is thought to be intrinsically unstructured (Adda et al., 2004). Another possibility is that MSP2 functions as a scaffold protein underlying other components of the merozoite coat. MSP2 has been shown to have the propensity to polymerise into amyloid-like fibrils *in vitro* (Adda et al., 2004).
The function of the protein appears to be essential since several attempts to disrupt the *msp2* gene have failed (Cowman et al., 2000; Paul Sanders, personal communication). Allelic exchange experiments showed that an FC27-type gene could replace an endogenous 3D7-type gene. This indicated that an allele from one family can fully complement the function of an allele belonging to the other family (Wickham 2003).

MSP2-specific antibodies are abundant in sera of protected individuals from malaria endemic areas (Al-Yaman et al., 1994). The reactivity of the MSP2-specific antibody response has been investigated and it was found that most of it is directed against the central dimorphic and repetitive parts and only to a minor extent to the conserved terminal regions (Al-Yaman et al., 1994; Lawrence et al., 2000). It was proposed that the conformation of the protein on the surface allows the exposure of the central variable part only. Furthermore it was suggested that the function of the polymorphic repeat region, which is highly antigenic, is merely to mislead the host's immune system to produce large amounts of non-protective antibodies (Anders, 1986). The high degree of length and sequence polymorphism within the repeat region indicates that it is unlikely to play an important functional role like in protein interaction.

Despite the fact that the functional and immunological importance of the different domains of MSP2 remain to be established, MSP2 is considered a vaccine candidate and underwent first testing in a field trial in PNG (Genton et al., 2002). We therefore undertook a study on the functional role of the central polymorphic domain of MSP2. We hypothesised that the repeat region is not required for *in vitro* growth of the parasite. To answer this question we constructed mutant parasites expressing MSP2 with the repeat region deleted. By successively extending this deletion we wanted to test whether the semi-conserved dimorphic domain or parts of it are also dispensable for parasite survival in culture. With this approach we aimed at elucidating the functional importance of the central domains of MSP2. The new *msp2* genes generated by the internal deletions are shown schematically in figure 1.

The functional complementation of a 3D7-type *msp2* gene by an FC27 allele has been successfully performed (Wickham, 2002). The approach for this replacement had been a single cross-over strategy involving a plasmid vector with a human dihydrofolate reductase (h*dhfr*) selection cassette, a transgene cassette designed for expression of the *msp2* gene from the FC27 strain, and a single target sequence consisting of a truncated 3D7 *msp2* gene with mutated start codon and lacking the signal for GPI-anchor addition. Upon integration via a single crossover between the endogenous *msp2* of the 3D7 line and the target sequence, one or more copies of the plasmid were integrated into the *msp2* locus, leading to gene disruption by insertion-duplication that yields two unfunctional copies of the 3D7 *msp2* gene while the FC27 *msp2* transgene is expressed. Initially, we had chosen a similar approach to produce a mutant expressing 3D7 MSP2 lacking the central repeat region on a 3D7 background but could not achieve integration into the *msp2* locus (data not shown).

Here we based our replacement constructs on plasmid pHTK (Duraisingh et al., 2002). This parental plasmid (Fig. 2A) also contains a human *dhfr* cassette to select for transfectants. In addition, it carries a *Herpes simplex* thymidine kinase (*tk*) gene cassette that can be used to select against the plasmid backbone. This allows for active selection of double cross-over recombination events.

Material and Methods

Construction of *Plasmodium falciparum* transfection vectors

We generated three different transfection plasmids designed for integration in the *msp2* locus of the 3D7 parasite line. All constructs were based on the transfection vector pHTK (Duraisingh et al., 2002).

Plasmid pHTK (Fig. 2A) was cut with restriction enzymes *Sac*II and *Spe*I (New England Biolabs) to be ligated to a PCR product amplified from pBS-M2 (a derivative of pM2F (Wickham et al., 2003)) consisting of the whole *msp5-msp2* intergenic region, a multiple cloning site and the *P. berghei dhfr-ts* 3'UTR with primers 5'flank-F AGTC**CCGCGG**GAAaTCTTATTCTT GCCATCC and 5'flank-R AGTC**ACTAGT**CTACCCTGAAGAAGAAAAGTC (sequences in bold mark restriction sites) to yield plasmid pTK-M2-5'flank. The *msp5-msp2* intergenic region served as 5'target sequence for integration of the constructs in the *P. falciparum* 3D7 *msp2* locus. The *Nhe*I site located in the introduced multiple cloning site was used for a further cloning step involving insertion of different *msp2* coding regions.

The 3' target sequence consisting of the conserved C-terminal part of *msp2* and the *msp2* 3'UTR from the 3D7 line was PCR amplified from 3D7 genomic DNA using primers 3'flank-F AGTC**ACGCGT**CACCAGAGAATAAAGGTACAG and 3'flank-R GACACTGCAAGTT GGGCAC with insertion of an *Afl*III site (ACATGT) at the 5' end. The PCR product was cut with *Afl*III (an endogenous *Afl*III site is located near the end of the 3'UTR) and ligated into pTK-M2-5' digested with *Nco*I making use of compatible overhangs to yield pTK-M2.

Msp2 coding regions with internal deletions were generated by separate amplification of the corresponding 5' and 3' parts of the gene from D10 genomic DNA. Primer M2F-Avr CAAA**CCTAGG**CAAAATGAAGGTAATTAAAACATTGTC and M2R-Avr ATCC **CCTAGG**AAGAGAATTATATGAATATGGC include the *msp2* start and stop codon, respectively (underlined) and were used for all amplifications of coding sequences. The 5' conserved region and the 5'-conserved plus 5'-FC27 (D10) family-specific part was amplified using primer M2F-Avr and Cons-N-term-R-Xba GATC**TCTAGA**TGCCATACTTCTCCTT ATACTCAT or FC27-5'fsp-R-Xba GATC**TCTAGA**ATTTGGAGCATTTGCACCTAC, respectively. The corresponding 3'-conserved and the 3'-FC27 family-specific parts were

amplified separately using primers Cons-C-term-F-Nhe GATC**GCTAGC**GCTGCACCAGAGAATAAAGGTAC or FC27-3'fsp-F-Nhe GATC**GCTAGC**GAAAGTTCAAGTTCTGGCAATG, respectively. The corresponding 5' and 3' PCR products were then digested with either *Xba*I or *Nhe*I, ligated, and redigested with *Xba*I and *Nhe*I, leaving only correctly ligated fragments. These ligated products were used as PCR templates for an amplification with the outer primers only (primer M2F-Avr and primer M2R-Avr) to yield FC27 (D10) *msp2* coding regions lacking the repeat or the complete variable domain. Similarly, the D10 full-length coding region was amplified from genomic DNA.

These PCR products, after digestion with *Avr*II, were finally ligated into the *Nhe*I site of pTK-M2 making use of the compatible overhangs. The ligations were transformed into *E.coli* PMC 103 (ATCC) by electroporation and plated on LB-agar containing ampicillin at 100 µg/ml. The integrity of the plasmids was checked by restriction digests, the correctness of coding regions and 500bp of the upstream region were checked by sequencing (micromon sequencing facility, Monash University, Clayton, Victoria, Australia). The newly constructed *Plasmodium falciparum* transfection vectors were named pTK-M2-FC27-full-length, pTK-M2-FC27-delta-rep, and pTK-M2-N-C-cons.

Parasite cultures and transfection

The 3D7 cloned line of *P. falciparum* was used for all transfections. Parasites were grown in human O+ red blood cells (erythrocyte concentrate from blood bank at Royal Melbourne Hospital) at 4% hematocrit in RPMI 1640 supplemented with Albumax (GibcoBRL) to a final concentration of 0.5% and gassed with 5% CO_2 and 0.5% O_2 in N_2 at 37 °C as described previously (Trager and Jensen, 1976). Cultures to be transfected were synchronised by treatment with 5% sorbitol 48 hours before transfections. 5ml of a ring-stage culture with at least 5% parasitaemia was used per transfection. 80 to 100 µg of maxi prep DNA (QIAGEN, Valencia, CA, USA) was transfected by electroporation with a single pulse of high capacitance (950 µF) and low voltage (310V) as described (Wu et al., 1995; Fidock and Wellems, 1997). The antifolate drug WR99210 (kindly provided by Jacobs Pharmaceuticals Co. Inc.) was added to the cultures 6 hours post transfection at a concentration of 5nM to select for parasites expressing human dihydrofolate reductase (*h*dhfr) from episomes. Culture medium was changed daily for the first week and every second day thereafter. Fresh red blood cells were added weekly to maintain 4% hematocrit until a population of WR99210-resistant

parasites was established. WR99210 was withdrawn and re-applied 3 weeks after (cycle 1). Without drug selection parasites lose episomal plasmids. Thus, after re-application of WR99210 transfectants having integrated the plasmids in the chromosome are enriched. This drug cycling was repeated (cycle 2) and negative selection applied after the second cycle. The pro-drug ganciclovir (Cytovene®, provided by Roche, Basel, Switzerland) selecting against the presence of the *Herpes simplex* thymidine kinase (*tk*) gene on episomes and integrated plasmid copies was added at a concentration of 20 µM after re-establishment of WR99210-resistant cultures and the parasites were cultivated under both selections to select for double cross-over integration events. After establisment of double drug resistant parasite populations ganciclovir selection was omitted.

Isolation of genomic DNA from *P. falciparum*

Genomic DNA was extracted from the 3D7 parental strain, WR99210-resistant transfectant cultures after drug cycling, and ganciclovir-selected parasite cultures. 10ml parasite cultures with at least 5% late stages (late trophozoites/schizonts) were subjected to saponin lysis (0.05% Saponin in PBS incubated on ice for 5 minutes) and washed with PBS. Parasite pellets were resuspended in 400 µl resuspension buffer A (500 mM Na Acetate pH 5.2, 100mM NaCl, 1mM EDTA), ½ volume 10% Sodium-dodecyl-sulfate (SDS) was added and mixed. The DNA was extracted twice with 1 volume of a phenol / chloroform mixture (1:1) followed by a chloroform extraction. The DNA was precipitated with 2 volumes of 100% ethanol, washed, dissolved in TE and stored at 4°C.

Southern blotting

Genomic DNA from different transfected parasite cultures and maxiprep plasmid DNA was completely digested with *Xmn*I (New England Biolabs) overnight and separated on a 0.6% agarose gel. The gel was soaked in 0.25M HCl for 10 minutes for depurination of the DNA and subsequently DNA was denatured by soaking the gel in 0.4M NaOH for 15 minutes. A capillary blot setup was used to transfer the DNA to a Hybond XL Nylon membrane (Amersham, Uppsala, Sweden). Subsequently the DNA was crosslinked to the membrane using an Amersham UV crosslinker (700 energy units). The membrane was prehybridized in hybridization solution (6x SSC, 5% Denhardt's, 0.5% SDS) for 1 hour at 62°C. Hybridization solution was discarded and replaced by 15 ml of hybridization solution containing denatured herring sperm DNA at a concentration of 200 µg/ml and prehybridized for at least 2 h at 62°C. Radioactive probes were made using the random prime labelling kit Decaprime II

(Ambion, Austin, TE, USA) according to the manufacturer's protocol. In brief, 2.5 µl decamers were mixed with 10 ng purified PCR product (*msp2* 5'UTR) or linearized plasmid (pGEM-T easy, Promega, Madison, WI, USA) in 11.5 µl H_20 and denatured for 5 minutes at 100°C, then put on ice. 5 µl 5x reaction buffer – dATP, 1 µl Klenow and 5 µl ^{32}P α-dATP were added and incubated for 30 to 60 minutes at 37°C. A prepacked sephadex G50 spin column was spun at 0.8 g for 1 minute. 50 µl TE (with 2x EDTA) was added to the labelling reaction and the reaction loaded on the spin column to remove unincorporated nucleotides. Labelling of the probe was checked with a Geiger counter, the probe denatured for 2 minutes at 100°C, and added to the hybridisation solution. Hybridisation was carried out overnight at 62°C. Washing was performed twice in 2x SSC for 10 minutes and once in 0.2 x SSC/0.05% SDS. The membrane was removed from the hybritube, wrapped in saran wrap and exposed to an X-ray film in a cassette with two enhancer screens at -70°C for 2 to 15 hours, depending on signal intensity.

Western blot analysis

Late stage *P. falciparum* cultures were lysed with saponin, parasite pellets washed twice with PBS before resuspension in 2x Laemmli buffer containing β-mercaptoethanol. Equivalents of 200 µl of a culture with 5% parasitaemia were boiled for 2 minutes and separated on a 12% SDS-polyacrylamide gel. The proteins were transferred to nitrocellulose membranes by tank blotting (Biorad) in Tris-Glycine buffer containing 20% methanol. The membranes were blocked in phosphate-buffered saline (PBS) containing 5% non-fat milk powder. Antibody reactions were carried out in PBS/0.1% Tween 20 (PBS-T) with 1% non-fat milk powder. Primary antibodies were used at the following dilutions: Rabbit anti-FVO-MSP2 affinity-purified on recombinant full-length FVO-MSP2 (3D7-type) at a dilution of 1:80 (Wickham, 2002); second bleed rabbit sera R633 and R634 raised against recombinant 3D7-family-specific domain of MSP2 (Irion, 2001) as well as R630 and R631 raised against recombinant FC27-family-specific domain D61/9 (Irion, 2001) were used at a 1:200 dilution. Primary antibodies were reacted for 2 hours at room temperature. Washing was performed in PBS-T with 0.5% milk 3 times for 10 minutes. The blots were then incubated with horse raddish peroxidase-labelled goat anti-rabbit IgG (Southern Biotech, Birmingham, AL, USA) for 1 hour and washed as above. Binding was visualized with enhanced chemiluminescence (ECL) (Amersham, Uppsala, Sweden).

Results

Our aim was the expression of internally deleted *msp2* variants from the *msp2* locus instead of the endogenous *msp2* gene. The resulting mutants can be expected to be potentially deleterious. Using WR99210 drug cycling to enrich for recombinants is a rather weak selection and may not yield the desired mutants. A recently established negative selectable marker makes it possible to actively select against the plasmid backbone and for double cross-over recombination (Duraisingh et al., 2002). This strategy was used for gene disruptions (Duraisingh et al., 2003; Wiek et al., 2004; Triglia et al., 2005) but never for gene replacements. The *msp2* replacement constructs presented here are based on the pHTK vector of Duraisingh et al. (2002).

Figure 2B shows the basic structure of the plasmids designed for *msp2* replacements. They contain a human dihydrofolate reductase gene (*hdhfr*) cassette for positive selection with WR99210 flanked by an *msp2* transgene expression cassette and the 3'conserved part of the *msp2* gene plus the *msp2* 3'untranslated region (UTR). The 1100 bp long *msp5-msp2* intergenic region contains the *msp2* promoter that drives expression of the *msp2* transgenes and also serves as 5'target sequence for homologous recombination. The 800 bp fragment encompassing the 3' conserved part of the *msp2* gene and its 3'UTR serves as 3' target sequence for recombination. The plasmid also contains a *Herpes simplex thymidine kinase* (*tk*) gene cassette that can be selected against with ganciclovir. This allows for selection of double cross-over recombination events in the *msp2* locus leading to replacement of the endogenous *msp2* gene by a full-length or partially deleted *msp2* transgene.

Figure 3 shows the three possible recombination events that can be in principle expected after targeting of the *msp2* locus with the replacement constructs. Figure 3A depicts the desired double cross-over event. The plasmid with its target sequences, the wild-type *msp2* locus, and the situation at the *msp2* locus after a double cross-over recombination event are shown. Such a recombination leads to disruption of the endogenous *msp2* gene. Single cross-over recombination within either the 3' or the 5' target sequence leads to insertion of the entire plasmid as shown in figure 3B and 3C. These integrations do not disrupt the endogenous *msp2* gene and are thus not desirable.

Southern blot Analysis of transfected *P. falciparum* cultures

The three plasmid constructs were transfected into *P. falciparum* strain 3D7 and the cultures selected for expression of human dihydrofolate reductase with WR99210 (WR). Drug resistant populations were subjected to drug cycling (off/on WR) and negative selection against the presence of thymidine kinase with ganciclovir. Genomic DNA from the three different WR-cycled and ganciclovir-treated cultures was subjected to Southern blot analysis to check for integration in the *msp2* locus.

For the control construct **pTK-M2-FC27-full-length** (Fig. 4A) Southern blot analysis showed no evidence for integration in the *msp2* locus after one WR drug cycle. Sequences of plasmid origin were still present. However, the plasmid was no more in its original form. Two major fragments of 9 kb and 6 kb were detected by Southern blot probably representing re-arranged plasmid integrated into a locus other than *msp2*. The faint band of around 8 kb could represent episomes of the same re-arranged plasmid. After the second WR cycle and negative selection with ganciclovir none of the three expected recombination events depicted in Figure 3 had taken place in this culture. Plasmid sequences were still present as detected with a pGEM-specific probe. A single band of around 5.5kb was detected with the *msp2* 5'UTR probe while the band corresponding to the wild-type *msp2* locus was absent, indicating that a re-arranged plasmid had integrated into the *msp2* locus. Parasites survived well the addition of ganciclovir. This points towards mutations in the thymidine kinase gene rendering it unfunctional or to a further recombination event that had led to loss of the TK gene.

For the culture transfected with the plasmid carrying the FC27-type *msp2* gene with the internal repeat deletion (**pTK-M2-FC27 delta-Rep**) no integration was detected when the parasites were hit with ganciclovir without previous WR cycling (Fig. 4B). The plasmid was present in its original form, which showed that direct selection with ganciclovir was not efficient. However, after two cycles off/on WR but without ganciclovir (Fig. 4B, lane 3), the whole population had integrated several copies of the plasmid in the *msp2* locus via a cross-over with the 3'target sequence. This result corresponds to the expected integration depicted in Fig. 3B. Integration of multiple plasmid copies has been reported previously and is due to episomal plasmids being present as concatamers (Crabb et al., 1997; O'Donnell et al., 2001). Therefore it is possible that all tandemly repeated plasmid integrations in this culture originated from a single recombination event. Our plasmids were designed in a way that a single cross-over integration does not lead to disruption of the endogenous *msp2* gene.

Negative selection with ganciclovir after two WR cycles (Fig. 4B, lane 4) led to excision and loss of all integrated plasmids resulting in parasites that had converted the *msp2* locus back to wild-type. In this case the negative selection had worked well. However, the parasites must have developed a resistance to WR in the course of double selection because they maintain resistance despite excision of integrated plasmids from the *msp2* locus. A WR resistance in *P. falciparum* has not been reported to date. Another more likely explanation is that the human *dhfr* gene has integrated at another locus, i.e. *calmodulin* or *hrp2*. This is possible because regulatory sequences in the selection cassette originated from these loci. Such recombination events were not detectable with our probes.

For the culture transfected with **pTK-M2-N-C-cons,** Southern blot also revealed other than the desired integration event. Direct treatment with ganciclovir selected for parasites carrying a mutated form of the plasmid (Fig. 4C). A single band was detected with the pGEM probe that is around 3 kb smaller than the originally transfected plasmid and does not correspond to one of the expected integration events. This recombined plasmid is likely to lack the thymidine kinase gene since parasites carrying it were not eliminated by ganciclovir treatment. The *msp2* 5'UTR probe only detected the wild-type *msp2* locus confirming that no integration had occurred. However, the majority of the parasites in this culture lost all episomes after two drug cycles and integrated several plasmid copies in the *msp2* locus via a cross-over with the 3' target sequence (Fig. 4C, lane cycle 2). Subjecting these cycled parasites to ganciclovir selected for parasites carrying the mutated, ganciclovir-resistant episome. But parasites with 3' cross-over integrations of the full plasmid were eliminated by ganciclovir.

The expected band for a double cross-over recombination was around 10 kb. A band of that size was detected in WR-cycled cultures transfected with pTK-M2-FC27-delta-Rep and pTK-M2-N-C-cons (Fig. 4B and 4C) before ganciclovir treatment. The Southern blot analysis however shows clearly that this band originates from several integrated plasmid copies and hence a single cross-over recombination, since the ratios of band intensities are similar for the *msp2* 5'UTR- and pGEM-specific probes.

Summarizing the results of both deletion constructs we have shown that only single cross-over recombination events were selected by WR. Selection resulted in the insertion of

multiple full length copies of the plasmid constructs. No double cross-over integrants were obtained and allelic replacement was thus not achieved.

Analysis of transgene expression

Since the transfected plasmids contain a complete transgene cassette with a full-length promoter that has been shown to drive expression of similar transgenes from episomes (Wickham et al., 2003) it was reasonable to assume that our FC27-type *msp2* transgenes would be co-expressed with the endogenous 3D7-type *msp2*. We conducted western blot analyses on total parasite protein extracts using antibodies specific for either of the two allelic MSP2 families. The rabbit sera used were raised against recombinant proteins corresponding to the non-repetitive family-specific parts of MSP2 (D46/1 and D61/9 for 3D7 or FC27 family, respectively) (Irion, 2001). The antibodies have proven to be highly specific for MSP2 of the corresponding family. While the chromosomal MSP2 was well detected, we could not detect any protein of episomal origin (Fig. 5) in the transfectant cultures 3D7-TK-M2-FC27-full-length and 3D7-TK-M2-FC27-delta-rep. The culture transfected with plasmid pTK-M2-N-C-cons does not contain any FC27-type-specific sequences and therefore constitutes a perfect negative control. The lack of episomal expression could be due to a deleterious promoter. The promoter of the transgene cassette on the plasmids could have contained mutations that completely abolish episomal expression. Such mutations or deletions could arise in the course of cloning and even after parasite transfection. This remains to be shown by sequencing. Despite of the lack of an intact promoter the transgene could still be expressed upon integration via a 5' single cross-over or a double cross-over. But neither of these events were selected in our transfectant cultures.

Discussion

An allelic replacement of the 3D7 *msp2* with a full-length *msp2* gene from the heterologous allelic family has been achieved previously (Wickham et al., 2003). This experiment showed the functional equivalence of members of the two families *in vitro*. This successful allelic replacement led us further to expect that also other, modified and deleted *msp2* alleles could replace a wild-type gene – provided the introduced construct does not compromise the essential function of MSP2. MSP2 appears to be essential since efforts to knock out *msp2* failed so far. We hypothezised that the central polymorphic regions may well be of great

immunological importance but have no essential function for parasite survival *in vitro*. This was to be tested by allelic replacement with an *msp2* construct carrying an internal repeat deletion. Furthermore we were interested whether the semi-conserved family-specific part is essential. We addressed this question of the functional importance of the dimorphic domains of the molecule by a replacement with a fusion of the conserved terminal regions completely devoid of any family-specific sequences. We transfected parasites with three plasmid constructs that would disrupt the endogenous *msp2* gene in strain 3D7 upon double cross-over recombination within the *msp2* locus and instead express a transgenic *msp2* gene of the FC27 family (control construct) or genes with internal deletions.

Southern blot analysis of our transfectants clearly showed that the plasmid episomes had re-arranged during culturing. This was most evident in the case of the positive control (the 3D7 – FC27 exchange) where parasites with plasmids survived ganciclovir selection well. It is probable that the re-arrangement of the plasmid had led to loss of the thymidine kinase gene. Plasmid sequences are still detected after ganciclovir selection, but do not seem to be associated with the *msp2* locus. The plasmid probably had integrated into the *calmodulin* or *hrp2* locus since regulatory sequences from these genes were also present in the human *dhfr* selection cassette. It is unlikely that the plasmid was still present episomally, since two WR cycles should have sufficiently selected against them (O'Donnell et al., 2002). Though, stably replicating forms consisting of large concatamers have been reported that are not lost when WR selection was omitted (O'Donnell et al., 2001). As an alternative explanation to insertion of plasmid in another locus, concatamerization could have led to persistence of the plasmid despite WR cycling.

Southern blot analysis of maxi-prep plasmid DNA revealed a band at 7.5 kb although *Xmn*I digestion should linearize the plasmids (~11 kb). Such a band was detected in all three plasmid maxi-preps but was by far most prominent in the control plasmid. It had not been seen on ethidium bormide-stained gels and was first thought to have resulted from star activity due to the high enzyme concentration in the overnight digestion. Alternatively, a subpopulation of re-arranged plasmid carrying one or more deletions could have been present before transfection. This is a likely explanation considering that the plasmid copy numbers for these constructs were very low, a sign for plasmid instability in bacteria. Smaller, re-arranged plasmids could have been taken up preferentially during parasite transfection or been selected for during the long culture period.

In summary, the results gained from Southern blot analysis of the full-length control transfectants can explain why no FC27-*msp2* transgene was expressed at any time. The failure to generate a positive control parasite line was due to recombination in the 3D7-FC27 allelic exchange construct and makes it difficult to identify reasons for the failure to achieve double cross-overs with the two internal deletion constructs.

Transfection with deletion constructs and subsequent WR drug cycling led to selection of 3' single cross-over events with insertion of multiple plasmid copies that do not disrupt the endogenous *msp2* gene. Multiple insertion of plasmids is a known phenomenon (Maier et al., 2003). Negative selection with ganciclovir against the plasmid backbone led to reversion to the wild-type *msp2* locus showing that the integrated plasmids contained a functional thymidine kinase gene. However, ganciclovir selection did not lead to the desired double cross-over recombinations. It remains open whether this is due to all domains of MSP2 being essential for survival or whether this is an artifact of unstable plasmid constructs. No plasmid sequences were detected after ganciclovir selection in the culture transfected with the construct with internal repeat deletion. It is likely that the parasites surviving ganciclovir treatment had the *dhfr* gene integrated in a locus other than *msp2* (i.e. *calmodulin* or *hrp2*). In the culture transfected with the construct with the deletion of the entire dimorphic part, ganciclovir treatment selected for parasites carrying the re-arranged plasmid of 7.5 kb that was present at time of transfection. This shows that it is crucial that the transfected plasmid population is uniform and does not contain traces of any recombined molecules.

In none of the three transfectant cultures we could detect the expected double cross-over integration leading to *msp2* gene disruption and expression of the introduced *msp2* transgene, although the negative selection with ganciclovir was principally effective in eliminating plasmid backbone sequences in one case (3D7-pTK-M2-FC27-delta-rep). All single cross-over recombinants selected for with drug cycling arose from recombinations with the 3'target sequence on the plasmids, consisting of the conserved 3'part of the *msp2* gene plus its 3'UTR. No cross-overs within the 5'target sequence were found. The 5'target sequence largely consisted of non-coding sequence with a high A/T content and only ~ 120 bp coding region. It was found previously that A/T-rich intergenic sequences do not promote plasmid integration (Alan Cowman, personal communication). However, although desirable, it was not possible to use large stretches of coding region for a target sequence because the entire N-terminal conserved region spans only 40 amino acids. The 5'target sequences also included the entire

promoter, which could therefore drive expression of the *msp2* transgene. But we did not find expression from episomes. It is likely that parts of the promoter were lost. This is also suggested by the findiing that the construct for FC27-*msp2*-full-length expression was clearly re-arranged before ganciclovir selection and no plasmid-associated *msp2* 5'UTR was detected (Fig. 4A). For the other two constructs, the plasmids appear to be slightly smaller in the transfectants than the corresponding maxi-prep plasmids indicating that short sequence stretches may have been deleted. Such a deletion occurring within the 5'target sequence could likewise affect expression from the episome and recombination efficiency. Still, integration involving a cross-over in the promoter or 5'UTR would inevitably provide the transgene with a full-length promoter and it would be expressed at the expense of the endogenous *msp2* gene. The fact that this event was never found in the cultures transfected with plasmids with the partially deleted *msp2* genes suggests that the repeat region is not dispensable for *in vitro* growth. However, the full-length control is crucial to corroborate this assumption. It is also possible that not the repeat sequence itself is essential but that a spacer is needed between the semi-conserved dimorphic parts. However, our attempts to replace the repeat region with multiple epitope tags (3x c-myc) was not successful.

We generated family-specific anti-MSP2 rabbit sera that could be used for detection of the full-length FC27-type MSP2 and the FC27-type MSP2 with deleted repeat region. In contrast to what was found by Wickham and co-workers, our *msp2* transgenes were not expressed from the episome (Fig. 5). Reasons for this could be mutations or deletions in the promoter region as discussed above.

Thymidine kinase as negative selectable marker has basically worked in selecting aginst the plasmid backbone but did not lead to selection of double cross-overs. It has become clear that this selection can only work if i) the transfected plasmid population is unifom and ii) the plasmids are stable in the transfected parasites. Both preconditions were not met in our experiments. A further technical weakness became apparent after Southern blot analysis of transfected cultures. Ganciclovir selection without WR drug cycling did not lead to plasmid loss, confirming that this negative selection is not 100 % effective as seen by others (Tony Triglia, personal communication) and that previous WR drug cycling is advisable. To date, no double cross-over gene replacement was achieved in *Plasmodium falciparum* by use of the TK vector. Several publications show the successful use of the system for the generation of gene knock-outs by double cross-over recombination since the proof of principle in 2002

(Duraisingh et al., 2003a; Duraisingh et al., 2003b; Gilberger et al., 2003; Wieks et al., 2004; Triglia et al., 2005). Yet the value of TK as a negative selectable marker for gene replacements remains to be established. Our results do not support its further use.

Acknowledgments

We thank Andrea Irion for antigens, Brendan Crabb for antibody production, Melanie Rug and Till Voss for assistance with microscopy and parasite cultures.

References

1. **Adda, C., V. Murphy, K. Vingas, S. Abdulnour, L. Waddington, M. Sunde, A. Hodder, R. Masciantonio, and R. Anders.** 2004. Plasmodium falciparum merozoite surface protein 2 forms beta-sheet-containing fibrils in vitro. molecular approaches to malaria (MAM) conference, Lorne, Vic. 26 January - 1 February 2004.

2. **al-Yaman, F., B. Genton, R. F. Anders, M. Falk, T. Triglia, D. Lewis, J. Hii, H. P. Beck, and M. P. Alpers.** 1994. Relationship between humoral response to Plasmodium falciparum merozoite surface antigen-2 and malaria morbidity in a highly endemic area of Papua New Guinea. Am J Trop Med Hyg **51:**593-602.

3. **Anders, R. F.** 1986. Multiple cross-reactivities amongst antigens of Plasmodium falciparum impair the development of protective immunity against malaria. Parasite Immunol **8:**529-39.

4. **Cowman, A. F., D. L. Baldi, J. Healer, K. E. Mills, R. A. O'Donnell, M. B. Reed, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2000. Functional analysis of proteins involved in Plasmodium falciparum merozoite invasion of red blood cells. FEBS Lett **476:**84-8.

5. **Crabb, B. S., T. Triglia, J. G. Waterkeyn, and A. F. Cowman.** 1997. Stable transgene expression in Plasmodium falciparum. Mol Biochem Parasitol **90:**131-44.

6. **Duraisingh, M. T., A. G. Maier, T. Triglia, and A. F. Cowman.** 2003a. Erythrocyte-binding antigen 175 mediates invasion in Plasmodium falciparum utilizing sialic acid-dependent and -independent pathways. Proc Natl Acad Sci U S A **100:**4796-801.

7. **Duraisingh, M. T., T. Triglia, and A. F. Cowman.** 2002. Negative selection of Plasmodium falciparum reveals targeted gene deletion by double crossover recombination. Int J Parasitol **32:**81-9.

8. **Duraisingh, M. T., T. Triglia, S. A. Ralph, J. C. Rayner, J. W. Barnwell, G. I. McFadden, and A. F. Cowman.** 2003b. Phenotypic variation of Plasmodium falciparum merozoite proteins directs receptor targeting for invasion of human erythrocytes. Embo J **22:**1047-57.

9. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in Plasmodium falciparum. Mol Biochem Parasitol **28:**1-10.

10. **Fidock, D. A., and T. E. Wellems.** 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci U S A **94:**10931-6.

11. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, D. Pye, D. O. Irving, T. A. Smith, H. P. Beck, and M. P. Alpers.** 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:**820-7.

12. **Gilberger, T. W., J. K. Thompson, T. Triglia, R. T. Good, M. T. Duraisingh, and A. F. Cowman.** 2003. A novel erythrocyte binding antigen-175 paralogue from Plasmodium falciparum defines a new trypsin-resistant receptor on human erythrocytes. J Biol Chem **278:**14480-6.

13. **Irion, A.** 2000. Molecular Diversity and Immunological properties of the Plasmodium falciparum Merozoite surface protein 2 (MSP2). PhD thesis University of Basel.

14. **Lawrence, N., A. Stowers, V. Mann, D. Taylor, and A. Saul.** 2000. Recombinant chimeric proteins generated from conserved regions of Plasmodium falciparum merozoite surface protein 2 generate antiparasite humoral responses in mice. Parasite Immunol **22:**211-21.

15. **Maier, A. G., M. T. Duraisingh, J. C. Reeder, S. S. Patel, J. W. Kazura, P. A. Zimmerman, and A. F. Cowman.** 2003. Plasmodium falciparum erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. Nat Med **9:**87-92.

16. **O'Donnell, R., P. R. Preiser, D. H. Williamson, P. W. Moore, A. F. Cowman, and B. S. Crabb.** 2001. An alteration in concatameric structure is associated with efficient segregation of plasmids in transfected Plasmodium falciparum parasites. Nucleic Acids Res **29:**716-24.

17. **O'Donnell, R. A., L. H. Freitas-Junior, P. R. Preiser, D. H. Williamson, M. Duraisingh, T. F. McElwain, A. Scherf, A. F. Cowman, and B. S. Crabb.** 2002. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of Plasmodium falciparum chromosomes. Embo J **21:**1231-9.

18. **Ocampo, M., M. Urquiza, F. Guzman, L. E. Rodriguez, J. Suarez, H. Curtidor, J. Rosas, M. Diaz, and M. E. Patarroyo.** 2000. Two MSA 2 peptides that bind to human red blood cells are relevant to Plasmodium falciparum merozoite invasion. J

Pept Res **55:**216-23.

19. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673-5.

20. **Triglia, T., M. T. Duraisingh, R. T. Good, and A. F. Cowman.** 2005. Reticulocyte-binding protein homologue 1 is required for sialic acid-dependent invasion into human erythrocytes by Plasmodium falciparum. Mol Microbiol **55:**162-74.

21. **Wickham, M.** 2002. Molecular analysis of Plasmodium falciparum pathogenesis. PhD thesis, Melbourne University.

22. **Wickham, M. E., J. K. Thompson, and A. F. Cowman.** 2003. Characterisation of the merozoite surface protein-2 promoter using stable and transient transfection in Plasmodium falciparum. Mol Biochem Parasitol **129:**147-56.

23. **Wiek, S., A. F. Cowman, and K. Lingelbach.** 2004. Double cross-over gene replacement within the sec 7 domain of a GDP-GTP exchange factor from Plasmodium falciparum allows the generation of a transgenic brefeldin A-resistant parasite line. Mol Biochem Parasitol **138:**51-5.

24. **Wu, Y., C. D. Sifri, H. H. Lei, X. Z. Su, and T. E. Wellems.** 1995. Transfection of Plasmodium falciparum within human red blood cells. Proc Natl Acad Sci U S A **92:**973-7.

Figures

Fig. 1. Schematic representation of MSP2 constructs. The basic structure of MSP2 is shown of representatives for the two allelic families (3D7 and FC27). FC27delta-Rep and N-C-cons display the internal deletion constructs to be expressed from the *msp2* locus. They lack the FC27 repeat or the complete central domains, respectively. For amino acid sequences of the constructs see supplementary figure S5 (appendix)

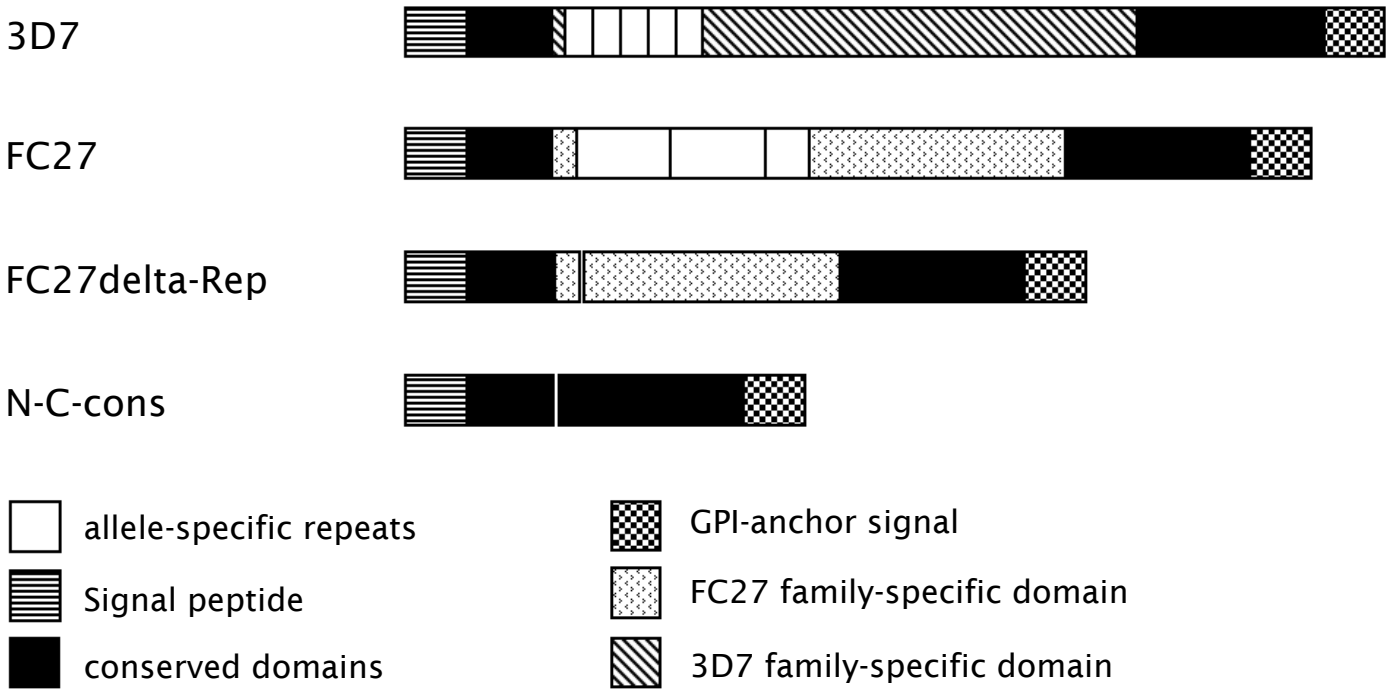

3D7
FC27
FC27delta-Rep
N-C-cons
allele-specific repeats
Signal peptide
conserved domains
GPI-anchor signal
FC27 family-specific domain
3D7 family-specific domain

Fig.1

Fig. 2. *Plasmodium falciparum msp2* gene replacement constructs.
(A) Parental plasmid pHTK is shown. It contains a human dihydrofolate reductase (*dhfr*) cassette for positive selection with WR99210 and a *Herpes simplex* thymidine kinase (*tk*) cassette for negative selection with ganciclovir. The *P. falciparum* calmodulin (*cam*) promoter and 5' untranslated region (UTR) drive expression of human *dhfr* while *P. falciparum* heat shock protein 86 (*hsp86*) promoter/5'UTR drives expression of the *tk* gene. The transcriptional terminators used in the selection cassettes are the 3'UTRs from *P. falciparum* histidine rich protein 2 (*hrp2*) gene (*dhfr* cassette) and *P. berghei* dihydrofolate reductase-thymidine synthase (*dhfr-ts*) gene (*tk* cassette), respectively. Two multiple cloning sites (MCS) flanking the *dhfr* cassette facilitate the insertion of target sequences for homologous recombination. Restriciton sites relevant for cloning are underlined.
(B) Schematic representation of the transfection constructs designed for double cross-over integration into the *msp2* locus on chromosome 2. The constructs derive from the plasmid described in (A). A complete *msp2* transgene expression cassette was inserted into MCS 1. It contains 1.1kb of target sequence for homologous recombination within the *msp2* upstream region followed by a full-length or partially deleted *msp2* gene. The transcriptional terminator used is the *P. berghei dhfr-ts* 3'UTR. A second target sequence for homologous recombination consisting of the 3'conserved part of the *msp2* gene and its 3'UTR was inserted into MCS 2.

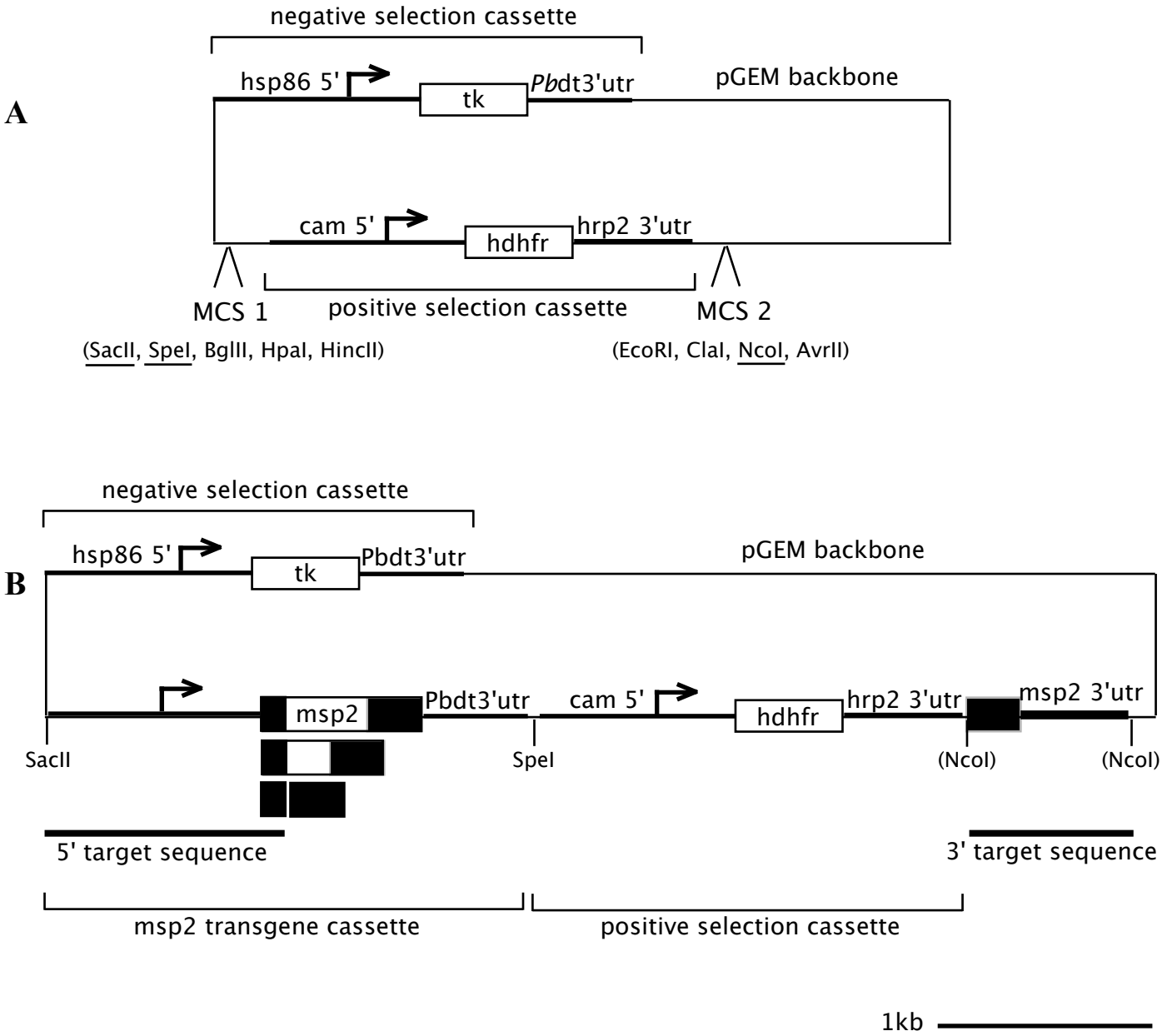

A
negative selection cassette
hsp86 5'
tk
Pbdt3'utr
pGEM backbone
cam 5'
hdhfr
hrp2 3'utr
MCS 1
positive selection cassette
MCS 2
(SacII, SpeI, BglII, HpaI, HincII)
(EcoRI, ClaI, NcoI, AvrII)
B
negative selection cassette
hsp86 5'
tk
Pbdt3'utr
pGEM backbone
msp2
Pbdt3'utr
cam 5'
hdhfr
hrp2 3'utr
msp2 3'utr
SacII
SpeI
(NcoI)
(NcoI)
5' target sequence
3' target sequence
msp2 transgene cassette
positive selection cassette
1kb

Fig.2

Fig. 3. Integration of replacement constructs into *msp2* locus.
Expected recombination events at the *msp2* locus are displayed schematically. The 5' and 3' target sequences for homologous recombination are represented by black or gray boxes, respectively. The selection cassettes and the *msp2* transgene are represented by open boxes. The endogenous *msp2* coding region is marked with asterisks. The *Xmn*I restriciton sites relevant for Southern blotting are indicated (X) and fragment sizes for the control construct given in kilobases. Sizes in brackets are for the cultures transfected with the two partial *msp2* deletion constructs. Regions used as hybridization probes for Southern blotting are indicated by black (*msp2* 5'UTR) and open (pGEM) bars, respectively. (**A)** double cross-over recombination and the resulting *msp2* locus with a disrupted *msp2* gene. (**B)** single cross-over recombination in the 5' target sequence. (**C)** single cross-over recombination in the 3' target sequence.

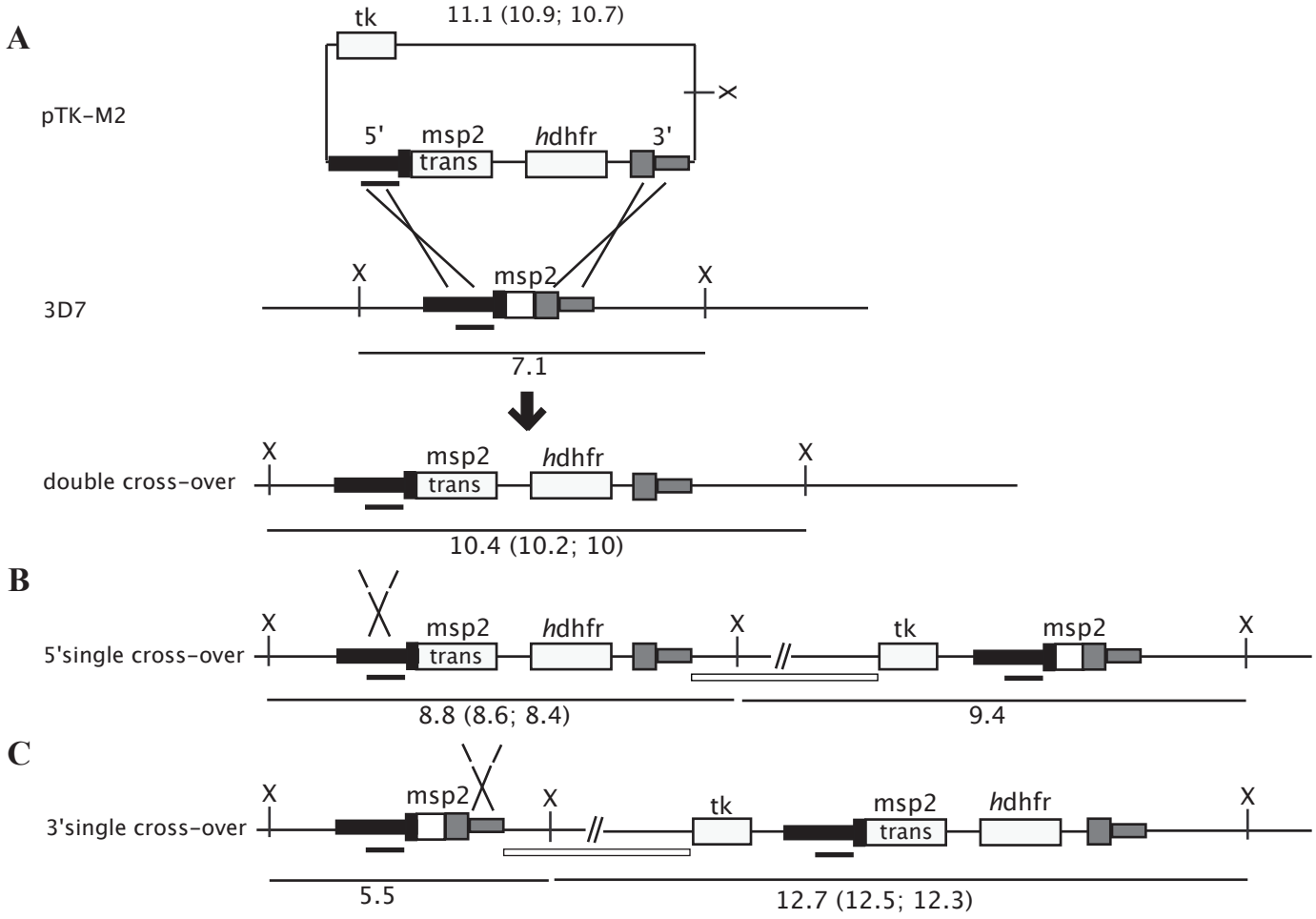

A
tk
11.1 (10.9; 10.7)
pTK-M2
5'
msp2
trans
hdhfr
3'
X
msp2
X
3D7
7.1
X
msp2
hdhfr
X
double cross-over
trans
10.4 (10.2; 10)
B
X
msp2
hdhfr
X
tk
msp2
X
5'single cross-over
trans
8.8 (8.6; 8.4)
9.4
C
X
msp2
X
tk
msp2
hdhfr
X
3'single cross-over
trans
5.5
12.7 (12.5; 12.3)

Fig.3

Fig. 4. Southern blot analysis of transfected *Plasmodium falciparum* cultures.
Genomic DNA of the parental 3D7 strain and different transfectant cultures as well as purified plasmid DNA digested with *Xmn*I was subjected to Southern blotting. The membrane was probed for the *msp2* 5'UTR, stripped, and re-hybridized to a pGEM plasmid probe. The size standard is in kilobases. **(A)** 3D7 parental line and 3D7-pTK-M2-FC27-full-length (control). **(B)** 3D7-pTK-M2-FC27-delta-rep transfectant cultures. **(C)** 3D7-pTK-M2-N-C-cons transfectant cultures.

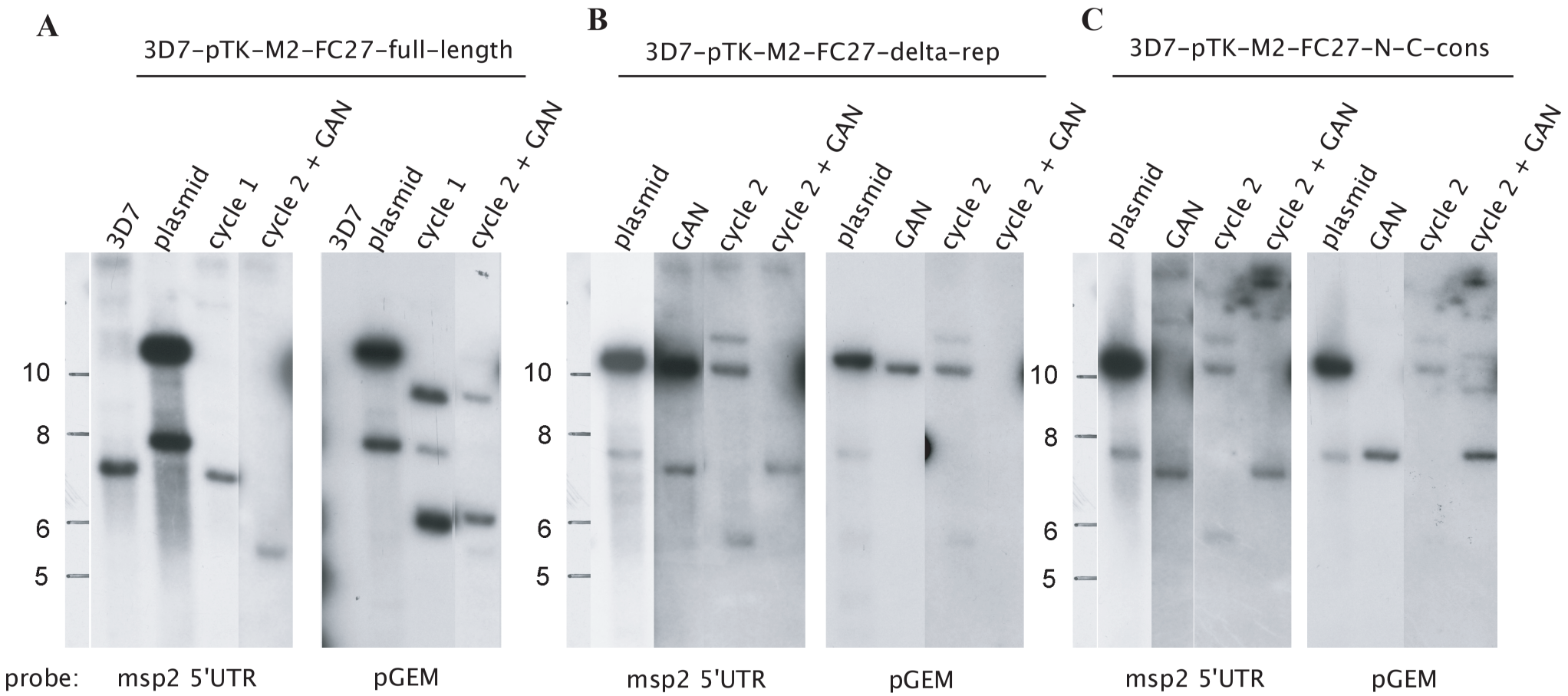

A
3D7-pTK-M2-FC27-full-length
3D7
plasmid
cycle 1
cycle 2 + GAN
3D7
plasmid
cycle 1
cycle 2 + GAN
B
3D7-pTK-M2-FC27-delta-rep
plasmid
GAN
cycle 2
cycle 2 + GAN
plasmid
GAN
cycle 2
cycle 2 + GAN
C
3D7-pTK-M2-FC27-N-C-cons
plasmid
GAN
cycle 2
cycle 2 + GAN
plasmid
GAN
cycle 2
cycle 2 + GAN
10
8
6
5
10
8
6
5
10
8
6
5
probe:
msp2 5'UTR
pGEM
msp2 5'UTR
pGEM
msp2 5'UTR
pGEM

Fig.4

Fig. 5. Analysis of MSP2 expression in cultures transfected with *msp2* replacement constructs.
Total lysates from late stage cultures of 3D7 and D10 wild-type parasites as well as non-cycled transfectants 3D7-pTK-M2 FC27-full-length, 3D7-pTK-M2 FC27-delta-rep, and 3D7-pTK-M2-N-C-cons were subjected to Western blotting and reacted with anti-MSP2 rabbit sera. (**A)** Reacted with rabbit sera specific for the 3D7 family-specific part. (**B)** Reacted with rabbit sera specific for FC27-type MSP2. Relevant size standards are indicated.

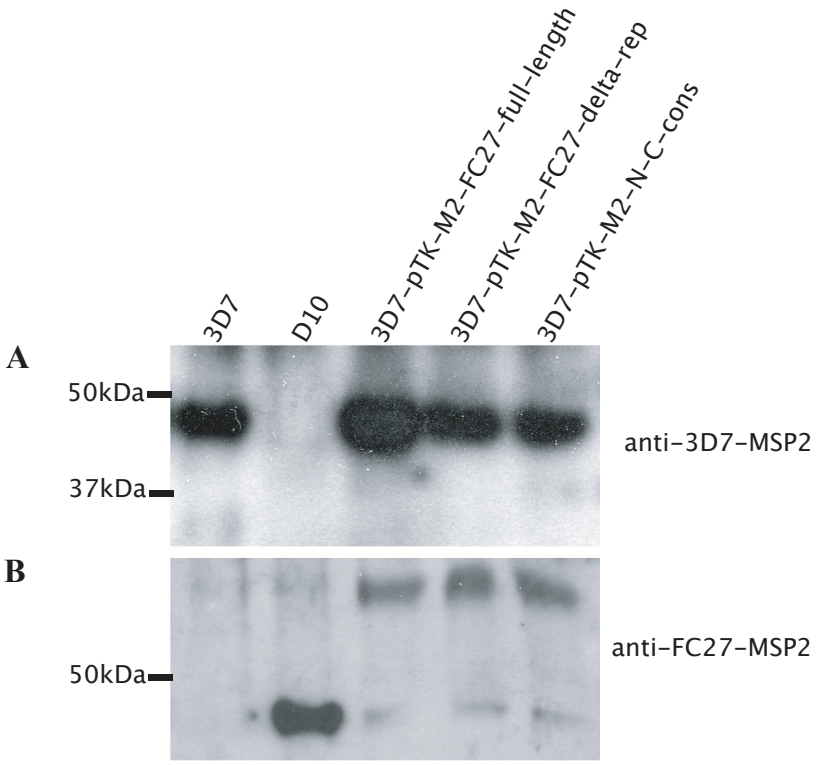

3D7
D10
3D7-pTk-M2-FC27-full-length
3D7-pTk-M2-FC27-delta-rep
3D7-pTk-M2-N-C-cons
A
50kDa
37kDa
anti-3D7-MSP2
B
50kDa
anti-FC27-MSP2

Fig.5

6. Sequence requirements for a GPI-anchored protein to be transported to the surface of *Plasmodium falciparum* merozoites

Christian Flück[1), 2)], Ingrid Felger[1)], Alan F. Cowman[2)]

(Manuscript)

[1)] Swiss Tropical Institute, Basel, Switzerland

[2)] Walter & Eliza Hall Institute, 3050, Victoria, Australia

Abstract

Attachment of a glycosylphosphatidylinositol (GPI) moiety is one of several mechanisms for membrane anchhoring of proteins. In protozoa, GPI-anchored proteins often constitute the most abundant cell-surface proteins and have important roles in defence against the host immune system. In *Plasmodium falciparum* all membrane-associated merozoite surface proteins (MSPs) are GPI-anchored. It is not known by which mechanisms and pathways GPI-anchored proteins are transported from the endoplasmatic reticulum (ER) to the cell surface. The GPI anchor is necessary for surface targeting. We investigated whether further essential transport signals are present on the protein moiety of a mature GPI-anchored merozoite surface protein. In order to identify such signals, we generated a series of transgenic *P. falciparum* parasites expressing Yellow Fluorescent Protein (YFP) flanked by different portions of the conserved terminal regions of the merozoite surface protein 2 (MSP2). A fluorescent reporter flanked by conserved N- and C-terminal MSP2 sequences was correctly trafficked to the plasma membrane in schizonts and merozoites. In contrast, a fluorescent reporter only flanked by the MSP2 signal peptide and the GPI anchor signal remained in the ER. This suggested the presence of crucial transport signals within the conserved regions of MSP2. We further investigated whether the transmembrane domain from apical membrane antigen (AMA-1) can mimick a GPI anchor signal when placed at the very C-terminus of a fluorescent reporter. Our results do not show whether this reporter was porvided with a GPI anchor, however, it was not targeted to the plasma membrane. A further YFP reporter fused N-terminally to the AMA-1 transmembrane domain and cytoplasmic tail was correctly trafficked to the micronemes, suggesting that the C-terminus of this protein contains important transport signals. However, trafficking of this reporter was not efficient.

Introduction

The merozoite is the invasive stage in the erythrocytic cycle of the malaria parasite *Plasmodium falciparum*. A number of merozoite surface proteins (MSPs) have been identified and all MSPs directly associated with the parasite plasma membrane are attached by a glycosyl-phosphatidyl-inositol (GPI)-anchor. These are MSP1, MSP2, MSP4, MSP5, and MSP-10. Functional redundancy among these proteins is unlikely since attempts to disrupt these genes were not successful (Cowman et al., 2000). Proteins provided with a GPI anchor display an N-terminal signal peptide for entering the secretory pathway and a predicted C-terminal signal for addition of the lipid anchor. The latter signal consists of a hydrophobic C-terminus preceded by a short hydrophilic spacer and the actual site of cleavage, the ω-site, which is the first of three amino acids with small side chains (Furukawa et al., 1997; Kodukula et al., 1993). Cleavage and lipid anchor addition is catalyzed by the GPI-transamidase complex and takes place at the lumenal side of the endoplasmatic reticulum (ER) membrane (Amthauer et al., 1993).

The structure of *P. falciparum* GPIs is known. The parasite GPIs differ from those of the host in that they contain palmitic and myristic acids at C-2 of inositol, and do not contain additional phosphoethanolamine substitution in their core glycan structures. The components of the GPI biosynthetic pathway have been identified and offer potential drug targets (Delorenzi et al., 2002). The purified parasite GPIs can induce tumor necrosis factor release from macrophages (Naik et al. 2000) and several studies have shown that GPI functions as the dominant parasite toxin in a malaria infection (Schofield et al., 1993; Schofield et al., 2002).

In yeast GPI-anchored proteins are transported from the ER to the Golgi apparatus in vesicles distinct from those containing non-GPI-anchored proteins (Muniz et al., 2001). This finding demonstrated that protein sorting can occur upon exit from the ER. However, it is not known how this sorting and the trafficking to the plasma membrane is achieved. Therefore, we aimed at identifying putative transport signals present on a mature GPI-anchored merozoite surface protein. For our analysis we chose the polymorphic merozoite surface protein 2 (MSP2) that is transcribed in late blood stages and localizes to the surface of schizonts and merozoites.
We hypothesized putative transport signals to be present in the conserved N- or C-terminal domains of MSP2. After cleavage of the N-terminal signal peptide and the C-terminal GPI anchor signal, 20 amino acids of the N-terminal and 47 amino acids of the C-terminal

conserved domain remain on the mature protein. Due to their sequence conservation it is more likely that putative transport signals are located within these terminal regions than in polymorphic central domains. To examine this, we made a panel of transgenic parasites expressing reporter constructs. These involve enhanced yellow fluorescent protein (eYFP) flanked by various portions of the MSP2 conserved terminal domains.

Transmembrane domains resemble GPI anchor signals in that they show a similar hydrophobicity. They are usually followed by a hydrophilic tail. We wanted to test whether a transmembrane domain of another merozoite-associated protein, apical membrane antigen 1 (AMA-1), can function as GPI-anchor addition signal when placed at the very C-terminus of a reporter construct. To localize our YFP reporter constructs we used live fluorescent microscopy and indirect immunofluorescence assays.

Material and Methods

Construction of *Plasmodium falciparum* transfection vectors using Multisite Gateway™ recombinational cloning technology.

Construction of *P. falciparum* transfection vectors has been difficult since plasmids containing large stretches of *P. falciparum* DNA appear to be unstable in *E.coli*, presumably due to the high A/T content of *P. falciparum* DNA. We have used Multisite Gateway™ cloning for the construction of transfection vectors carrying MSP2-reporter constructs to be expressed episomally in *Plasmodium falciparum* parasites. The MultiSite Gateway™ cloning system (Invitrogen, Carlsbad, CA, USA) is generally used when a larger number of similar reporter constructs are to be cloned. It allows three different DNA fragments to be combined in a single reaction to yield the final expression clone by using mixtures of the λ Integrase (Int), Excisionase (Xis), and *E.coli* Integration Host Factor (IHF) to insure efficient homologous recombinations between DNA molecules (PCR products and supercoiled plasmids). In addition, it utilizes a negative selection that greatly enhances cloning efficiency.

Construction of entry clones

A panel of entry clones, depicted in figure 1A, were constructed by recombination of PCR-products carrying specific *att* sites for recombination with the appropriate pDONR vectors in BP clonase reactions according to the manufacturer's protocol to generate 5' entry clones 4/1

(*att*L4/R1), central entry clones 1/2 (*att*L1/L2), and 3' entry clones 2/3 (*att*R2/L3). The primers used for PCR are listed in table 1. *Att* sites for site-specific recombination with DONR vectors are underlined. *Msp2*-specific sequences to generate 4/1, 1/2, and 2/3 entry clones were amplified from the allelic exchange vector pM2F (Wickham, 2003) or from 3D7 genomic DNA (for the 4/1 entry clone msp2 prom+N-cons*, which carries a full-length *msp5* to *msp2* intergenic region and the N-terminal conserved part of *msp2*). *Apical membrane antigen 1* (*ama1*)-specific sequences were amplified from 3D7 genomic DNA. The gene for enhanced yellow fluorescent protein (eYFP) was amplified from a plasmid carrying the gene (Marti et al, 2004). The triple hemagglutinin tag (3xHA) was amplified with primers containing *att*L1 and *att*L2 sequences from a 3' entry clone containing the same tag (kindly provided by Kylie Mullin, Melbourne University). The 1/2 entry clones FC27central and eYFPcentral were generated by directional TOPO cloning into pENTR™/D-TOPO that already contains *att*L1 and *att*L2 sites.

The generated entry clones are depicted in figure 1A. All 5' entry clones contain a *msp2* promoter and N-terminal parts of the *msp2* gene, consisting of either the *msp2* signal peptide or the complete conserved 5' region of the gene. Central entry clones carry the reporters (the family-specific part of FC27-type *msp2*, the enhanced yellow fluorescent protein (eYFP) gene without initiating methionine, or a triple hemagglutinin epitope tag). 3' entry clones again carry parts of the gene of interest, (the *msp2* 3' conserved region including the GPI-anchor addition signal, the *msp2* GPI-anchor addition signal only, the *ama-1* transmembrane domain plus cytoplasmic tail, the *ama-1* transmembrane domain only), or a reporter (triple hemagglutinin tag).

The BP recombination reactions were transformed into *E.coli* PMC 103 (ATCC) and plated on LB agar containing kanamycin at a concentration of 50 µg/ml. Positive clones were identified by restriction digests of mini prep plasmid DNA (QIAGEN, Valencia, CA, USA). The correctness of the inserted fragments was checked by sequencing (micromon sequencing facility, Monash University, Clayton, Australia). Compared to the sequence in the *P. falciparum* genome data base (www.plasmoDB.org) a 80 bp deletion was found in entry clones carrying the *msp2* promoter, msp2 prom+N-cons and msp2 prom+SP, around 700 bp upstream from the *msp2* start codon marked with a Δ in figure 1. According to Wickham and co-workers (2003) this region contains a positive regulatory element. A 5' truncation deleting this element led to a drop-off in promoter activity of 45% compared to a truncation retaining

this region. This deletion must have occured during propagation of plasmid pM2F in *E.coli*. Despite an expected drop in promoter activity we continued to work with constructs I to VII (including the deletion) since our reporter constructs could be toxic, making it desirable not to have full promoter activity. The same constructs carrying the 3D7 full-length *msp2* promoter and no deletion were also generated (constructs VIII to XIII) (Fig.1C).

Generation of *P. falciparum* transfection vectors by MultiSite Gateway LR reactions

The generated entry clones were used in MultiSite Gateway LR reactions together with the *P. falciparum* destination vector pHH-VP-*att*4/1 (Marti et al. 2004) displayed in figure 1B to create expression clones according to the manufacturer's protocol (Invitrogen). pHH-VP-*att*4/1 is a derivative of pHH, containing a human dihydrofolate reductase (*hdhfr*) selection cassette to confer resistance to the antifolate WR99210. The destination vector also contains a cassette with a chloramphenicol resistance gene (Cam^R) and a *ccdB* killer gene for negative selection against non-recombined plasmids in *E.coli*. The *ccdB* gene product is a potent poison of gyrase. The Cam^R/*ccdB* gene cassette is flanked by *att*R4 and *att*R1 sites that facilitate site-specific recombination with *att*L4 and *att*L1 sites present in entry clones. Adjacent to the Cam^R/*ccdB* cassette, the plasmid contains the *P.berghei dhfr-ts* 3'untranslated region (UTR) that is fused to the reporter construct upon recombination, completing the transgene expression cassette. The cassette is separated from the *hdhfr* selection cassette by 0.5 kb of telomeric Rep20 repeat for better plasmid segregation (O'Donnell et al., 2002). The destination vector carries an ampicillin resistence gene (Amp^R) while all entry clones carry a kanamycin resistence (Kan^R). Three entry clones (1x *att*L4/*att*R1, 1x *att*L1/*att*L2, and 1x *att*R2/*att*L3) and the destination vector (*att*R4/*att*R1) were combined in LR recombination reactions according to manufacturer's protocol. The reactions were extracted with a phenol/chloroform mixture, precipitated, and transformed into electrocompetent *E.coli* PMC103 and plated on LB agar plates containing ampicillin at a concentration of 100 µg/ml. Positive clones were identified by PCR screening. Plasmid mini preps (QIAGEN, Valencia, CA, USA) were analysed by restriciton digests. Plasmid maxi preps (QIAGEN) were made of correct clones. The *P. falciparum* expression plasmids obtained from LR recombination reactions are listed in figure 1C.

Parasite culture and transfection

The cloned line 3D7 of *P. falciparum* was used for all transfections. Parasites were grown in human O+ red blood cells (erythrocyte concentrate from blood bank at Royal Melbourne

Hospital) at 4% hematocrit in RPMI 1640 supplemented with Albumax (GibcoBRL) to a final concentration of 0.5% and gassed with 5% CO_2 and 0.5% O_2 in N_2 at 37 °C as described previously (Trager and Jensen, 1976). Cultures to be transfected were synchronised by treatment with 5% sorbitol 48 hours before transfection. 5ml of a ring-stage culture with at least 5% parasitaemia was used per transfection. 80 to 100 µg of maxi prep DNA (QIAGEN) was transfected by electroporation with a single pulse of high capacitance (950 µF) and low voltage (310V) as described (Wu et al., 1995; Fidock and Wellems, 1997). The antifolate drug WR99210 (kindly provided by Jacobs Pharmaceuticals Co. Inc.) was added to the cultures 6 hours post transfection at a concentration of 5nM to select for parasites expressing human dihydrofolate reductase (*h*dhfr) from episomes. Culture medium was changed daily for the first week and every second day thereafter. Fresh red blood cells were added weekly to maintain 4% hematocrit until a population of WR99210-resistant parasites was established.

Fluorescence microscopy

Transfected *P. falciparum* cultures containing at least 5% schizonts or magnet-purified late stages were mounted wet on microscope slides, covered with glass cover-slips and imaged at room temperature using a Zeiss Axioscop 2 microscoppe equipped with a PCO SensiCam (12-bit) camera and Axiovision 3 software. Captured images were processed using Adobe Photoshop (version 7). Prior to microscopy live cells were incubated for 20 minutes at 37°C with either DAPI (4',6-diamidino-2-phenylindole) DNA stain to a final concentration of 5 µg/ml to visualize nuclei, or with ER-Tracker™ Blue-white (molecular probes) to a final concentration of 0.25µM to stain the parasite endoplasmatic reticulum.

Indirect immunofluorescence assays were performed on *p*-formaldehyde/glutaraldehyde-fixed infected erythrocytes using a batch protocol as described previously (Tonkin et al., 2004) on Methanol/Acetone-fixed blood smears. Antibody reactions were carried out in PBS containing 3% BSA for 1 hour at room temperature. As primary antibodies an affinity-purified rabbit serum against FVO (3D7-type) MSP2 (Wickham et al, 2003) and mouse monoclonal antibody 8G10/48 (Epping et al., 1988) specific for FC27-type MSP2 were used at a dilution of 1:20 or 1:32, respectively. A mouse monoclonal antibody against GFP (Roche diagnostics) was used at a dilution of 1:50. Secondary antibodies used were Alexa Fluor 568-conjugated anti-rabbit IgG and Alexa Fluor 488-conjugated anti-mouse IgG (Molecular probes) diluted 1:500. The cells were mounted on glass slides with Vectashield mounting

medium H-1000 (Vector Laboratories, Burlingame, CA, USA) containing DAPI at a concentration of 1 μg/ml.

Purification of late stage parasites using MACS columns

50ml of *P. falciparum* cultures containing at least 5% late trophozoites and schizonts were centrifuged at 1200g for 5 minutes and the cell pellet was resuspended in 10ml wash buffer (Hepes-buffered RPMI 1640 with 0.2% $NaHCO_3$). The suspension was loaded on a MACS CS column equilibrated with 20ml wash buffer attached to a varioMACS magnet (Miltenyi Biotech, Germany). The column was washed with 20ml wash buffer followed by 10ml of culture medium. The column was then removed from the magnet and bound infected erythrocytes eluted with 12ml culture medium.

FACS sorting of YFP-expressing *Plasmodium falciparum* parasites

MACS-purified late stages were resuspended to $4x10^7$ cells per ml in culture medium and sorted according to presence or absence of green fluorescence (YFP) using a Moflo fluorescence-activated cell sorter (FACS) at a speed of 16'000 cells per second. Populations of fluorescence positive and negative cells were collected in culture medium. Fluorescence positive cells were put back to culture in a 2 ml dish containing fresh red blood cells (4% hematocrit) and sorting repeated upon establishment of the culture. Alternatively, sorted cells were spun down and resuspended in 2x Laemmli buffer for SDS-PAGE and western blotting.

Table 1. Primers used for amplification of DNA fragments for the production of Gateway™ entry clones.

Primer name	Sequence 5' to 3'	Used for entry clones
5' entry clones		
attB4 fwd m2-prom	GGGGACAACTTTGTATAGAAAAGTTGGAAATCTTATTCTTGCCATCC	msp2 prom+N-cons; msp2 prom+SP
attB4 fwd m2-prom*	GGGGACAACTTTGTATAGAAAAGTTGGAATTCTTATTCTTGCCATCCA	msp2 prom+N-cons* 2)
attB1 rev m2-SP	GGGGACTGCTTTTTTGTACAAACTTGCATTTTTAATATTAAAGGTAACAAAAA	msp2 prom+SP
attB1 rev m2-N-cons	GGGGACTGCTTTTTTGTACAAACTTGCCATACTTCTCCTTATACTCAT	msp2 prom+N-cons; msp2 prom+N-cons* 2)
central entry clones		
attB1 fwd HA	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCCTAGGTACCCGTACGAC	3xHA
attB2 rev HA	GGGGACCACTTTGTACAAGAAAGCTGGGTAGAGCTCGGCATAATCTGGA	3xHA
eYFP fwd	caccGTGAGCAAGGGCGAGGAGCTG	eYFPcentral 1)
eYFP rev	CTTGTACAGCTCGTCCATGC	eYFPcentral 1)
FC27-central-F	caccGCAAATGAAGGTTCTAATACTA	FC27central 1)
FC27-central-R	AGCAGGATTTTCATTTTCTGC	FC27central 1)
3' entry clones		
attB2 fwd m2-C-cons	GGGGACAGCTTTCTTGTACAAAGTGGCTGCACCAGAGAATAAAGGTAC	msp2 C-cons
attB2 fwd m2-GPI	GGGGACAGCTTTCTTGTACAAAGTGGCCCTCTTAAATAACTCTAGTAATA	msp2 GPI
attB2 fwd ama1-C	GGGGACAGCTTTCTTGTACAAAGTGGATAAACCAACTTATGATAAAATGAAA	ama1 C-term; ama1 TM
attB3 rev m2-STOP	GGGGACAACTTTGTATAATAAAGTTGaTTATATGAATATGGCAAAAGATAAAAC	msp2 C-cons; msp2 GPI
attB3 rev ama1-STOP	GGGGACAACTTTGTATAATAAAGTTGtTTAATAGTATGGTTTTTCCATCAG	ama1 C-term
attB3 rev ama1-TM	GGGGACAACTTTGTATAATAAAGTTGtTTAAGCATTTCCTTTTCTTTTATAAAGA	ama1 TM

1) entry clones obtained from directional topo cloning
2) primers used for amplification of the full-length *msp2* promoter from 3D7 genomic DNA

Results

We used Gateway™ recombinational cloning to generate the *P. falciparum* transfection constructs I to XIII shown in figure 1C (The amino acid sequences of the reporter proteins are shown in supplementary figure S6 A to D). These constructs are designed to be maintained episomally in the parasite nuclei and to express fluorescent or other reporter genes. All reporters are driven by the *msp2* promoter to insure correctly timed expression at the schizont stage. A schizont-specific promoter is important since inappropriate timing of expression can interfere with correct trafficking (Rug et al., 2004). Expression of reporter genes in the first set of plasmid constructs (I to VII) was driven by a *msp2* promoter with an 80 bp deletion ~700 bp upstream of the start codon. The deleted region was shown to contain a positive promoter element (Wickham et al., 2003) and transgene expression in these transfectants was generally infrequent. This prompted us to re-clone some of the constructs with a full-length *msp2* promoter.

All constructs (I to XIII) contain the hydrophobic N-terminal signal peptide of MSP2 for entry to the endoplasmatic reticulum (von Heijne, G. 1990). In *P. falciparum* bloodstages, a classical signal sequence in the absence of other transport signals directs transport of a fluorescent reporter to the parasitophorous vacuole (Waller et al., 2000; Wickham et al., 2001; Adisa et al., 2003). We aimed at identifying putative signals on GPI-linked merozoite surface protein 2 (MSP2) necessary for transport to the merozoite surface. For this we looked at the localisation of reporter constructs flanked by different proportions of the MSP2 conserved terminal domains.

Double immuno-fluorescence reveals transgenic FC27-type MSP2 expressed at the merozoite surface. As control for the design of our reporter constructs we transfected 3D7 parasites with plasmid construct (I) that expresses a reconstituted full-length FC27-type MSP2 (clone D10). Thus, transgenic parasites were expected to express both, a 3D7 endogenous and a FC27 transgenic MSP2 at the merozoite surface. This vector was constructed with the MultiSite Gateway recombination system, using the same 5' and 3' entry clones as for other reporter constructs. The resulting recombinant FC27-type protein differs from its parental, the MSP2 of clone D10, in some additional amino acids introduced by *att* sites used for homologous recombination. Fifteen foreign amino acids were introduced before and after the FC27 family-specific domain (supplementary figure S6A).

Indirect immuno-fluorescence microscopy on the 3D7 parental strain was carried out with an antibody against 3D7 MSP2 to show parasite surface localization in schizonts and free merozoites (Fig. 2A). Double immuno-fluorescence on transfectant parasites (construct I) with MSP2 family-specific antibodies revealed that the introduced FC27-type MSP2 co-localizes with the endogenous 3D7 MSP2 at the merozoite surface (Fig. 2B).

YFP fused to MSP2 conserved terminal domains localizes to the merozoite surface. We conducted live fluorescence microscopy of parasites transfected with YFP reporter constructs. Constructs II and IX were designed to yield a GPI-anchored YFP flanked on each side by the conserved terminal domains of MSP2 (Fig. 1C). The only difference between these two constructs lay in the *msp2* promoter driving expression of the transgene. Construct IX carried a full-length promoter while construct II had a deletion of 80 basepairs ~700 bases upstream from the start codon. The protein expressed by construct II and IX was expected to localize to the merozoite surface since it contains all the conserved MSP2 sequences that are most likely to contain transport signals. We did live fluorescence microscopy on transfectant cultures II and IX. Late trophozoites, schizonts, and merozoites from transfectant IX are shown in figure 3. YFP fluorescencee appeared in unsegmented parasites with multiple nuclei (Fig. 3A) and localized to the confines of the parasites, indicative of the parasite plasma membrane. In later stages, where the formation of merozoites has started or been completed (Fig. 3B to E), the fluorescence pattern matched that of a merozoite surface staining by MSP2-specific antibodies (see Fig. 2B). In ruptured schizonts the fluorescence remains associated with individual merozoites, ruling out the parasitophorous vaculole as a possible localization for the YFP reporter construct. We also conducted fluorescence microscopy on transfectant parasites labelled with ER-tracker to show that the reporter protein is not retained in the ER (Fig. 3 F and G). Construct (XI) that consists of a triple haemagglutinin (HA) tag flanked by both conserved terminal MSP2 regions was also expressed at the merozoite surface (data not shown).

'Naked' GPI-anchored YFP is not transported to the merozoite surface. Expression of the reporter constructs (III) and (IV) was too low and infrequent to allow a reliable localisation for the fluorescence signal. Construct (V) was expressed at slightly higher rates (>0.1%) and three consecutive FACS sortings for green fluorescence enriched for parasites expressing the YFP reporter by ~50 fold. YFP reporter in construct (V) is only flanked by MSP2 sequences that constitute known signals, but both signals are absent from the mature

protein. These are the N-terminal signal peptide for entry to the endoplasmatic reticulum (ER) and the C-terminal signal for addition of the GPI-anchor. Thus, no MSP2-specific sequences were present in the mature reporter protein. We found that in mature segmenters and free merozoites the fluorescence was not associated with the merozoite surface (Fig. 4). The location was perinuclear (Fig. 4A), indicative of ER localization (Leann Tilley, personal communication). Van Dooren and co-workers followed ER morphology over the erythrocytic cycle and described a pattern similar to the bottom pannel of figure 4B for segmenters (van Dooren et al., 2005). Labelling of parasites with ER tracker showed a partial colocalization of the YFP fluorescence with ER (Fig. 4C). Therefore we assume that the ER tracker we used stained other membranes in addition to the ER.

Enrichment of fluorescent parasites using fluorescence-activated cell sorting allowed colocalization studies with MSP2 as a merozoite surface marker. Figure 4D shows schizonts labelled with anti-GFP and anti-3D7-type MSP2 antibodies. We found that YFP fluorescence did not colocalize with MSP2. The schizonts depicted are not fully segmented and the YFP fluorescence is surrounded by the MSP2 fluorescence.

These data indicate that GPI-anchored YFP is not expressed at the merozoite surface nor is it secreted into the parasitophorous vacuole. It is probably retained at some point along the secretory pathway. This suggests that crucial transport signals are absent from the reporter and that GPI-addition alone is not sufficient for surface targeting.

The AMA-1 transmembrane domain and cytoplasmic tail are sufficient for reporter transport to the micronemes. Transmembrane domains and GPI-anchor addition signals are similar in that they basically consist of a hydrophobic stretch of amino acids. GPI-signals are located at the extreme C-terminus while transmembrane domains are usually followed by a hydrophilic cytoplasmic tail.

The apical membrane antigen 1 (AMA-1) is a micronemal protein (Bannister et al., 2003) that is secreted and distributed over the merozoite surface from the apical tip in mature merozoites and during invasion. It has been shown before for EBA-175, another micronemal transmembrane portein, that the cytoplasmic domain is not essential for correct trafficking (Gilberger et al., 2003). Our reporter construct (VI) has an N-terminal conserved part of MSP2 including the signal peptide followed by YFP and the AMA-1 C-terminal part

including the transmembrane domain (TM) and the cytoplasmic tail. Live fluorescence microscopy of schizonts showed a pattern of bright spots that appeared to be associated with the apical end of merozoites (Fig. 5A). This suggested that the AMA-1 C-terminus directed the YFP reporter to the micronemes. A weak fluorescence underlied the bright spots in figure 5A, This pattern was similar to that observed in parasites transfected with construct (V). The weak background fluorescence probably represents a pool of immature or not properly trafficked reporter in the ER.

We were interested, whether truncation of the C-terminus of a transmembrane protein downstream of the transmembrane domain would result in GPI-anchor addition. If this addition was achieved, the transmembrane domain would act as GPI anchor addition signal. Construct (VII) consists of the MSP2 signal peptide followed by YFP and the AMA-1 transmembrane domain. Fluorescence microscopy of schizonts (Fig. 5B) showed a picture similar to that seen for the YFP-GPI construct (V). Micronemal targeting was abolished and fluorescence was associated with the ER. Whether the reporter constructs with the TM were linked to a GPI-anchor remains to be examined. The Big-PI GPI anchor signal prediction software does not predict a potential ω-site for anchor addition in this reporter construct (Eisenhaber et al., 1999; http://mendel.imp.univie.ac.at/sat/gpi/gpi_server.html).

Low and infrequent transgene expression. We found that our reporter genes were generally expressed at very low frequencies (~0.1%). Our first set of transfection constructs (I to VII) had a deletion in the *msp2* promoter driving expression of the reporter constructs that may have been responsible for the infrequent expression. Alternatively, toxicity of the YFP reporter proteins may also have impaired growth. We also observed infrequent expression of construct I, which is a reconstitution of an FC27-type MSP2. This contrasted the findings of Wickham and co-workers who created a transgenic parasite line co-expressing MSP2 of both allelic families at comparable levels (Wickham et al., 2003). That led us to suppose that episomal expression of our transgenes might be abolished due to the 80 bp promoter deletion and that the occasional parasites expressing the reporter gene could have integrated the plasmid into the chromosomal *msp2* locus.

Aiming at enriching for parasites expressing the transgene and at analysing endogenous MSP2 expression in these parasites, we sorted cultures transfected with constructs (II) and (V) for YFP fluorescence in a fluorescence-activated cell-sorter. The first sort yielded 30’000

YFP-positive schizonts for culture (II) and 70'000 for culture (V). The frequency of fluorescent parasites were 0.1% and 0.17%, respectively. 30'000 parasites each were put back in a culture dish with fresh red blood cells. A culture with 1% parasitaemia was established after two weeks for the transfectants carrying the construct with the YFP-GPI reporter (V). These parasites showed normal growth rates thereafter and were subjected to a second round of sorting. The YFP-positive parasites represented 2.3% of the population at the time of the second sorting and 9% one week after the a third sorting. From the sorted parasites transfected with the YFP construct (II) no culture could be re-established after two months of culturing although the size of the inoculum was the same as for (V).

For transfectant (V) MSP2 expression was analysed by western blot directly after FACS sorting. No reduction in endogenous MSP2 expression was found (data not shown). This indicates that the chromosomal *msp2* locus was not targeted by the reporter plasmid.

To further investigate whether the 80 bp promoter deletion in our first set of constructs led to infrequent expression of the reporter genes, we re-cloned some of the constructs with a full-length MSP2 promoter (constructs VIII, IX, X) (Fig. 1C). In addition, we made new constructs with a triple heamagglutinin (3xHA) tag as a reporter instead of YFP (constructs XI and XII). A control construct with both reporters (YFP followed by 3xHA) without C-terminal signal was also constructed. But from these six new transfectants only transfectants (IX) and (XI) expressed the transgene at detectable levels.
We conclude that the promoter deletion in the first set of constructs was not the only reason for the infrequent transgene expression since the use of the full-length *msp2* promoter does not guarantee expression of similar reporter genes. The finding that drug resistant cultures were established but did barely or not express the transgenes can be explained by plasmid instability due to AT-rich *P. falciparum* DNA stretches. At the time of transfection, a small and undetectable proportion of the plasmid preparation may already have contained deletions due to instability of our large constructs in *E. coli*. If a parasite is transformed with such a plasmid that does not allow reporter gene expression, it has an overwhelming advantage over those forced to express an unfavourable gene.

The triple haemagglutinin (HA) tag flanked by both conserved terminal MSP2 regions (XI) was expressed at the merozoite surface (data not shown).

Discussion

Localization of YFP reporter constructs

We made a series of *P. falciparum* mutants designed to express different YFP-reporter constructs in order to identify putative transport signals on a mature GPI-anchored merozoite surface protein. The YFP reporter was flanked by different portions of the MSP2 N- and C-terminal regions including sequences that represent known signals for entry into the secretory pathway and for GPI-anchor attachment. In addition, we included sequences of the conserved parts of MSP2 that were most likely to contain trafficking signals because these domains are strictly conserved in all alleles of the polymorphic MSP2.

The YFP reporter flanked by the conserved N- and C-terminal regions of MSP2 (constructs II and IX) was expressed at the merozoite surface as judged by the fluorescence pattern in schizonts. To our knowledge this is the first report on a GPI-linked surface-expressed fluorescent reporter in apicomplexan parasites. Our data show that a GPI-linked fluorescent reporter can be correctly trafficked to the merozoite surface. YFP trafficking to the surface was uncertain since it was shown repeatedly that a fluorescent reporter can interfere with trafficking of the fusion protein in *P. falciparum* (Adisa et al., 2003; Knuepfer et al., 2005).

In contrast, a YFP reporter flanked only by the two MSP2 signal sequences that are cleaved in the ER (V), was clearly not trafficked to the merozoite surface. The perinuclear fluorescence pattern in merozoites indicated that the fluorescent protein remained in the ER. This suggested that transport signals essential for targeting to the surface were missing. Such signals might be provided by the MSP2 conserved regions in construct IX, that enabled transport of the fusion protein to the plasma membrane. The difference between the two constructs (V and IX) consists of 23 amino acids from the conserved N-terminal domain of MSP2 and 45 amino acids from the conserved C-terminal domain of MSP2. In order to search for a signal in these MSP2 regions, we performed sequence alignments with other GPI-anchored merozoite surface proteins (MSP1, MSP4, MSP5, MSP10) but they did not reveal a conserved motif. This is not surprising since the GPI-anchor addition signal among these proteins is also not conserved on amino acid sequence level. The lack of a common targeting signal in our alignment may also indicate that different merozoite surface proteins use different transport mechanisms. One possibility is the use of specific escorter proteins. Our data do not allow to assign the putative transport signal to either of the conserved MSP2

domains since localisation of reporter constructs (III) and (IV) was not achieved (Fig. 1 C) due to infrequent reporter expression. However, the need of a transport signal in addition to the GPI-anchor signal in MSP2 contradicts what is known from mammalian proteins. Experiments involving a C-terminal fusion of a GPI-anchor signal to an otherwise secreted protein led to its insertion into the plasma membrane (Caras et al., 1989). Furthermore, a null mutant for the major GPI-anchored surface glycoproteins of procyclic forms of African trypanosomes was found to express high levels of free GPI anchors at the surface (Vassella et al., 2003). This also suggests that the GPI anchor lead to its association with transport vesicles that are destined to the plasma membrane.

If it holds true as a general rule that the GPI anchor is sufficient as a signal for plasma membrane targeting, the question arises whether our reporter construct (V), consisting of YFP flanked by both signals but not targeted to the surface, was provided with a GPI anchor. The MSP2 GPI-anchor signal in construct (V) included three amino acids N-terminal to the only predicted GPI anchor addition site (Big-PI predictor, Eisenhaber et al., 1999). The proximity of the YFP to the cleavage site may influence the efficiency of anchor addition. Transport of proteins with an uncleaved GPI anchor signal was found to be blocked between the ER-Golgi intermediate compartment and the Golgi stacks (Field et al., 1994). However, it has not been reported that cleavage and GPI anchor addition were inhibited by the amino acid composition of sequences N-terminal to the cleavage site. Furthermore, in our reporter constructs the YFP is separated from the C-terminal portion of MSP2 by a spacer of 15 amino acids that was introduced by GATEWAY cloning. Therefore it is unlikely that construct (V) is not GPI-linked.

In agreement with our results, a green fluorescent protein (GFP) fusion with the corresponding MSP1 signals (signal peptide and GPI anchor signal) and a spacer of 10 alanine residues between GFP and the GPI anchor signal was also trapped in the ER (Meissner et al., 2005; Paul Gilson, personal communication). In conclusion, to prove that transport signals were missing from our construct (V) it remains to be shown that the reporter protein was provided with a GPI anchor and that this was not sufficient for plasma membrane targeting.

The finding that the AMA-1 C-terminal part (transmembrane domain plus cytoplasmic tail) appears to be sufficient to target a YFP reporter to the micronemes was surprising. Gilberger

and co-workers found that a truncation of the cytoplasmic tail of EBA-175, another micronemal type 1 transmembrane protein, was trafficked correctly. This suggested that sorting signals lie in the domain facing the microneme lumen or in the transmembrane domain (Gilberger et al., 2003). We didn't incorporate any sequences upstream of the transmembrane domain and still got correct trafficking (construct VI). This suggests that the signal for micronemal targeting lies within the transmembrane domain. However, our results obtained with construct (VII) did not support this suggestion. When we placed the AMA-1 transmembrane domain at the very C-terminus trafficking to the micronemes was abolished. The fluorescence pattern seen in parasites transfected with construct (VII) is typical for ER. This could mean that the YFP in this case interferes with correct trafficking or that the AMA1 cytoplasmic domain is not dispensable in contrast to the situation in EBA-175.

We also investigated whether the AMA-1 transmembrane domain can trigger GPI anchor addition. The big-PI predictor software (Eisenhaber et al., 1999) did not find an appropriate GPI-addition site in this construct. Our results indicated that the reporter remained in the ER. This would be expected if no anchor were added. Proteins with an uncleaved GPI anchor signal (i.e. a hydrophobic C-terminus) were shown not to be secreted (Field et al., 1994). To confirm that a C-terminal transmembrane domain does not act as a GPI anchor signal, it needs to be shown in our transfectant, that the reporter found in the ER found in the ER indeed has no GPI anchor attached.

Lack of efficient expression of reporter constructs

All reporters from the first set of transfection constructs were expressed at low frequencies (~0.1%). The question arose whether the low frequencies are due to the 80 bp deletion in the *msp2* promoter of this first set of constructs. This would be a likely explanation since a positive promoter element was mapped to the deleted region (Wickham et al., 2003). A 5' truncation to 770 base pairs upstream of the ATG led to 60% of full-length promoter activity. Truncation of another 200 base pairs led to a further drop of activity to 15% (Wickham et al., 2003). Our deletion may have removed a positive regulatory element, thus leading to down regulation. However, it was not expected that promoter activity was abolished completely. The promoter element deleted in our constructs represents a conserved tandem repeat with an underlying 11 nucleotide repeat unit $(TAAATATAATA)_7$.

The occasional parasites that were able to express the reporter gene probably differed genetically from the bulk of non-expressing ones. They could have integrated the plasmid into the *msp2* locus. Recombination within the *msp2* promoter, the only matching target sequence on our plasmid constructs, can lead to a promoter swap providing the YFP reporter with the endogenous promoter that allows expression. In exchange, the endogenous *msp2* gene will aquire the promoter with the deletion and might not be expressed to detectable levels. Analysis of FACS sorted transfectant (V) did not support this hypothesis since the levels of endogenous MSP2 was unchanged. It would have been surprising to find an MSP2 knock-down since MSP2 is an abundant component of the merozoite surface and is probably essential (Cowman et al., 2000).

Expression of the reporter constructs with the full-length *msp2* promoter showed that the deletion in the promoter was not responsible for infrequent expression. As a general rule expression frequencies were not higher for reporter genes driven by the full-length promoter. We also designed and transfected some new constructs using a heamagglutinin (HA) epitope tag instead of the fluorescent reporter (Figure 1C). Drug resistant populations were readily established for five of the six transfectants (VIII, X, XI, XII, and XIII). However, only transfectant (XI) (N-cons-3xHA-C-cons) expressed its transgene at detectable levels. This also indicated that the promoter deletion in the first set of constructs was not the sole reason for the infrequent expression.

High level expression of GPI-anchored YFP at the merozoite surface is toxic. At first, no drug resistant population was established for the culture transfected with construct (IX), which is a repetition of construct (II) with the full-length promoter. Occasionally, some parasites could be detected in Giemsa-stained blood smears but the parasitaemia did not increase over several weeks. When we omitted the drug pressure, the culture reached 1% parasitemia within two weeks, indicating that the parasites had been forced under selection to express a YFP-reporter that was toxic to them. Without drug pressure, the plasmid copy number may have decreased, thus also reducing the level of transgene expression. It is unlikely that the plasmid was lost completely during this short removal of drug since it contains a Rep20 telomeric repeat sequence that was shown to improve plasmid segregation (O'Donnell et al., 2002). When the drug was re-applied, no parasite dying was observed, supporting the idea that most parasites still contained plasmid. However, it was surprising to find that ~10% of the parasites expressed the fluorescent reporter to a level detectable with

fluorescence microscopy. The parasites now grew at normal growth rates at a drug concentration they had not grown at initially. It is therefore probable that short-term removal of the drug led to reduction of plasmid copy number and thus selected for parasites that express the reporter at lower levels, no more reaching toxic concentrations.

Our attempts to enrich transfectant culture (II) (expressing the same reporter gene as IX) for YFP fluorescence by FACS sorting had also indicated toxicity of the reporter. No culture was established from the sorted culture in contrast to transfectant (V). This supports the idea that expression of this particular reporter construct at high levels may be toxic.

In conclusion, we have shown that a YFP reporter flanked by the conserved terminal domains of MSP2 is expressed at the surface of merozoites. Its transport to the plasma membrane is mediated by MSP2 sequences present on the mature protein since a reporter lacking these sequences and only containing the N-terminal signal peptide and the GPI anchor signal does not show surface localisation. This implies that surface transport of endogenous MSP2 also depends on sequences present on the mature protein and that addition of the GPI anchor is not sufficient for correct targeting. The sequence motif necessary for surface targeting remains to be identified. Furthermore it remains to be shown if trafficking of other GPI-anchored merozoite surface proteins also relies on sequence motifs present on the processed molecule. This could lead to identification of a conserved motif.

In this work a we have established a transgenic parasite that expresses a GPI-anchored YFP reporter flanked by the conserved terminal domains of MSP2 at the merozoite surface. This parasite may be a valuable tool for analysing the function of MSP2.

Acknowledgments

We thank Cornelia Spycher, Melanie Rug, and Till Voss for assistance with parasite cultures and microscopy. Viki Milovac and Catherine Tarlington are thanked for technical assistance with FACS sorting. This work was supported by the Swiss National Science Foundation (Grant # 3100-062951) and C.F. by the Basler Stiftung für experimentelle Zoologie.

References

1. **Adda, C., V. Murphy, K. Vingas, S. Abdulnour, L. Waddington, M. Sunde, A. Hodder, R. Masciantonio, and R. Anders.** 2004. *Plasmodium falciparum* merozoite surface protein 2 forms beta-sheet-containing fibrils in vitro. molecular approaches to malaria (MAM) conference, Lorne, Vic. 26 January - 1 February 2004.

2. **Adisa, A., M. Rug, N. Klonis, M. Foley, A. F. Cowman, and L. Tilley.** 2003. The signal sequence of exported protein-1 directs the green fluorescent protein to the parasitophorous vacuole of transfected malaria parasites. J Biol Chem **278:**6532-42.

3. **Amthauer, R., K. Kodukula, L. Gerber, and S. Udenfriend.** 1993. Evidence that the putative COOH-terminal signal transamidase involved in glycosylphosphatidylinositol protein synthesis is present in the endoplasmic reticulum. Proc Natl Acad Sci U S A **90:**3973-7.

4. **Bannister, L. H., J. M. Hopkins, A. R. Dluzewski, G. Margos, I. T. Williams, M. J. Blackman, C. H. Kocken, A. W. Thomas, and G. H. Mitchell.** 2003. *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-1) is translocated within micronemes along subpellicular microtubules during merozoite development. J Cell Sci **116:**3825-34.

5. **Bohme, U., and G. A. Cross.** 2002. Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in Trypanosoma brucei. J Cell Sci **115:**805-16.

6. **Caras, I. W., G. N. Weddell, and S. R. Williams.** 1989. Analysis of the signal for attachment of a glycophospholipid membrane anchor. J Cell Biol **108:**1387-96.

7. **Cowman, A. F., D. L. Baldi, J. Healer, K. E. Mills, R. A. O'Donnell, M. B. Reed, T. Triglia, M. E. Wickham, and B. S. Crabb.** 2000. Functional analysis of proteins involved in *Plasmodium falciparum* merozoite invasion of red blood cells. FEBS Lett **476:**84-8.

8. **Delorenzi, M., A. Sexton, H. Shams-Eldin, R. T. Schwarz, T. Speed, and L. Schofield.** 2002. Genes for glycosylphosphatidylinositol toxin biosynthesis in *Plasmodium falciparum*. Infect Immun **70:**4510-22.

9. **Eisenhaber, B., P. Bork, and F. Eisenhaber.** 1999. Prediction of potential GPI-modification sites in proprotein sequences. J Mol Biol **292:**741-58.

10. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. Mol Biochem Parasitol **28:**1-10.

11. **Fidock, D. A., and T. E. Wellems.** 1997. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. Proc Natl Acad Sci U S A **94:**10931-6.

12. **Field, M. C., P. Moran, W. Li, G. A. Keller, and I. W. Caras.** 1994. Retention and degradation of proteins containing an uncleaved glycosylphosphatidylinositol signal. J Biol Chem **269:**10830-7.

13. **Furukawa, Y., K. Tsukamoto, and H. Ikezawa.** 1997. Mutational analysis of the C-terminal signal peptide of bovine liver 5'-nucleotidase for GPI anchoring: a study on the significance of the hydrophilic spacer region. Biochim Biophys Acta **1328:**185-96.

14. **Gilberger, T. W., J. K. Thompson, M. B. Reed, R. T. Good, and A. F. Cowman.** 2003. The cytoplasmic domain of the *Plasmodium falciparum* ligand EBA-175 is essential for invasion but not protein trafficking. J Cell Biol **162:**317-27.

15. **Knuepfer, E., M. Rug, and A. F. Cowman.** 2005. Function of the plasmodium export element can be blocked by green fluorescent protein. Mol Biochem Parasitol **142:**258-62.

16. **Kodukula, K., L. D. Gerber, R. Amthauer, L. Brink, and S. Udenfriend.** 1993. Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: specific amino acid requirements adjacent to the site of cleavage and GPI attachment. J Cell Biol **120:**657-64.

17. **Marti, M., R. T. Good, M. Rug, E. Knuepfer, and A. F. Cowman.** 2004. Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science **306:**1930-3.

18. **Meissner, M., E. Krejany, P. R. Gilson, T. F. de Koning-Ward, D. Soldati, and B. S. Crabb.** 2005. Tetracycline analogue-regulated transgene expression in *Plasmodium falciparum* blood stages using Toxoplasma gondii transactivators. Proc Natl Acad Sci U S A **102:**2980-5.

19. **Muniz, M., P. Morsomme, and H. Riezman.** 2001. Protein sorting upon exit from the endoplasmic reticulum. Cell **104:**313-20.

20. **Naik, R. S., O. H. Branch, A. S. Woods, M. Vijaykumar, D. J. Perkins, B. L. Nahlen, A. A. Lal, R. J. Cotter, C. E. Costello, C. F. Ockenhouse, E. A. Davidson, and D. C. Gowda.** 2000. Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. J Exp Med **192:**1563-76.

21. **O'Donnell, R. A., L. H. Freitas-Junior, P. R. Preiser, D. H. Williamson, M. Duraisingh, T. F. McElwain, A. Scherf, A. F. Cowman, and B. S. Crabb.** 2002. A genetic screen for improved plasmid segregation reveals a role for Rep20 in the interaction of *Plasmodium falciparum* chromosomes. Embo J **21:**1231-9.

22. **Rug, M., M. E. Wickham, M. Foley, A. F. Cowman, and L. Tilley.** 2004. Correct promoter control is needed for trafficking of the ring-infected erythrocyte surface antigen to the host cytosol in transfected malaria parasites. Infect Immun **72:**6095-105.

23. **Schofield, L., M. C. Hewitt, K. Evans, M. A. Siomos, and P. H. Seeberger.** 2002. Synthetic GPI as a candidate anti-toxic vaccine in a model of malaria. Nature **418:**785-9.

24. **Schofield, L., L. Vivas, F. Hackett, P. Gerold, R. T. Schwarz, and S. Tachado.** 1993. Neutralizing monoclonal antibodies to glycosylphosphatidylinositol, the dominant TNF-alpha-inducing toxin of *Plasmodium falciparum*: prospects for the immunotherapy of severe malaria. Ann Trop Med Parasitol **87:**617-26.

25. **Tonkin, C. J., G. G. van Dooren, T. P. Spurck, 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.

26. **Trager, W., and J. B. Jensen.** 1976. Human malaria parasites in continuous culture. Science **193:**673-5.

27. **van Dooren, G. G., M. Marti, C. J. Tonkin, L. M. Stimmler, A. F. Cowman, and G. I. McFadden.** 2005. Development of the endoplasmic reticulum, mitochondrion and apicoplast during the asexual life cycle of *Plasmodium falciparum*. Mol Microbiol **57:**405-19.

28. **Vassella, E., P. Butikofer, M. Engstler, J. Jelk, and I. Roditi.** 2003. Procyclin null mutants of Trypanosoma brucei express free glycosylphosphatidylinositols on their surface. Mol Biol Cell **14:**1308-18.

29. **von Heijne, G.** 1990. The signal peptide. J Membr Biol **115:**195-201.

30. **Waller, R. F., M. B. Reed, A. F. Cowman, and G. I. McFadden.** 2000. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. Embo J **19:**1794-802.

31. **Wickham, M. E., M. Rug, S. A. Ralph, N. Klonis, G. I. McFadden, L. Tilley, and A. F. Cowman.** 2001. Trafficking and assembly of the cytoadherence complex in *Plasmodium falciparum*-infected human erythrocytes. Embo J **20:**5636-49.

32. **Wickham, M. E., J. K. Thompson, and A. F. Cowman.** 2003. Characterisation of the merozoite surface protein-2 promoter using stable and transient transfection in *Plasmodium falciparum*. Mol Biochem Parasitol **129:**147-56.

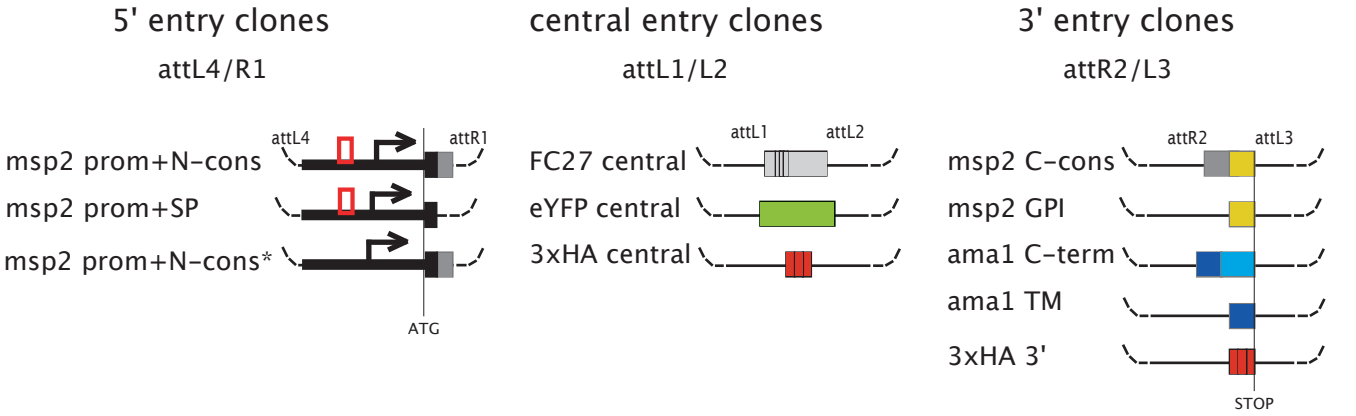
33. **Wu, Y., C. D. Sifri, H. H. Lei, X. Z. Su, and T. E. Wellems.** 1995. Transfection of *Plasmodium falciparum* within human red blood cells. Proc Natl Acad Sci U S A **92:**973-7.

Figures

Fig. 1. Schematic representation of *P. falciparum* Gateway™ transfection plasmids. Figure 1A shows all generated entry clones. The plasmids contain a kanamycin resistance gene and *att* sites for site-specific recombination. *Att* L4 from 5' entry clones recombines with *att* R4 on the destination vector shown in 1B, *att* R1 recombines with *att* L1 from a central entry clone, *att* L2 of central entry clones recombines with *att* R2 of a 3' entry clone and *att* L3 again with the destination vector in an LR reaction. Figure 1C shows the *P. falciparum* expression constructs gained from Gateway recombinational cloning. The red Δ marks the 80 base pair deletion in the *msp2* promoter.

Figure 1

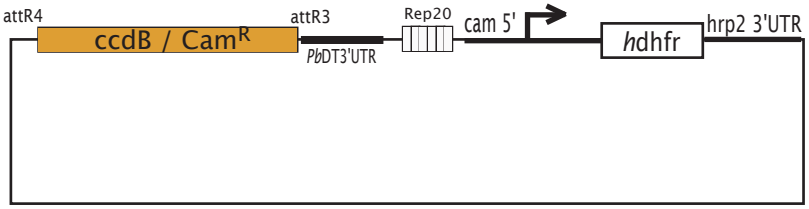
A



B

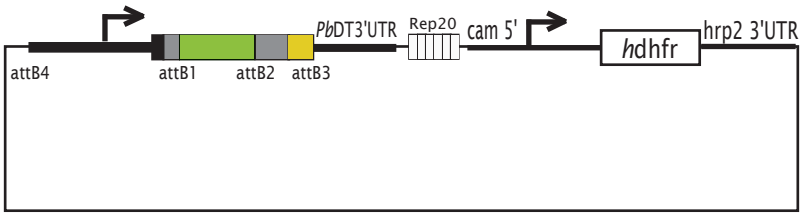
destination vector pHH-VP-att-R4/R1

attR4/R1



C

P.falciparum expression vectors



I	FC27 msp2 control	
II	N-cons-YFP-C-cons	
III	N-cons-YFP-GPI	
IV	SP-YFP-C-cons	
V	SP-YFP-GPI	
VI	N-cons-YFP-ama1-C-term	
VII	SP-YFP-ama1-TM	
VIII	FC27 msp2 control*	
IX	N-cons-YFP-C-cons*	
X	N-cons-YFP-GPI*	
XI	N-cons-3xHA-C-cons	
XII	N-cons-3xHA-GPI	
XIII	N-cons-YFP-3xHA	

Fig. 2. Immunofluorescence microscopy of MSP2 in 3D7 wild-type and transfected parasites. **(A)** 3D7 parasites were labelled with a 3D7-type-specific anti-MSP2 rabbit serum followed by Alexa 568-conjugated anti-rabbit IgG to show parasite surface staining in schizonts (upper panel) and free merozoites (lower panel). **(B)** Double staining of a schizont from a culture transfected with construct (I) with 3D7-type-specific anti-MSP2 rabbit serum and an FC27-type-specific anti-MSP2 mouse monoclonal antibody followed by Alexa 488-conjugated anti-rabbit IgG and Alexa 568-conjugated anti-mouse IgG. The left panels show nuclear staining with DAPI. Merge 1 in (A) is an overlay of blue and red fluorescence channels, merge 1 in (B) is an overlay of green and red fluorescence channels.

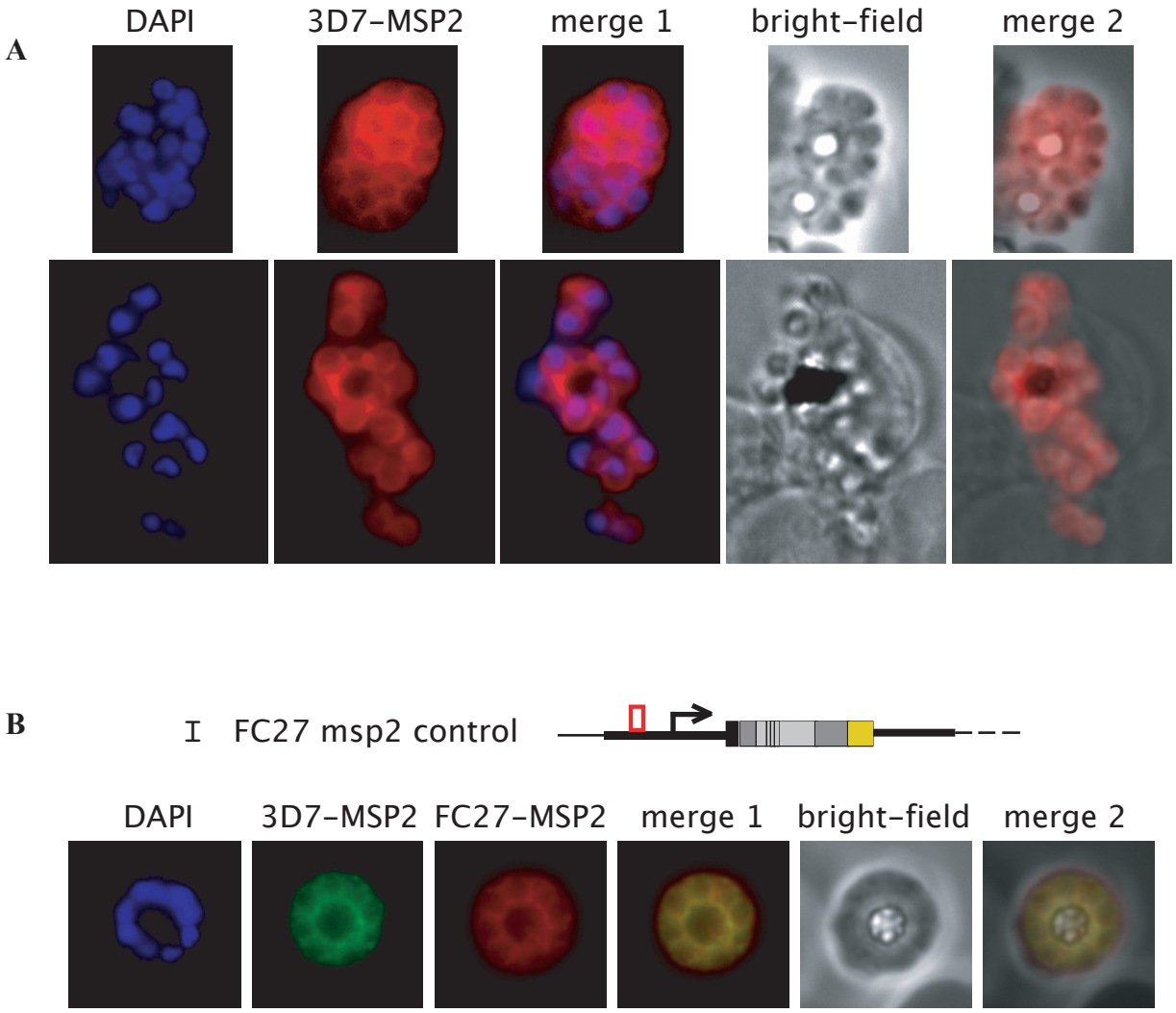

A
DAPI
3D7-MSP2
merge 1
bright-field
merge 2
B
I FC27 msp2 control
DAPI
3D7-MSP2
FC27-MSP2
merge 1
bright-field
merge 2

Figure 2

Fig. 3. Live fluorescence microscopy of transfectant IX. 3D7 parasites transfected with construct IX expressing YFP reporter flanked by the conserved terminal domains of MSP2 are shown. (A) late trophozoite. (B to D) Schizonts of increasing maturity. (E) ruptured schizont with free merozoites. (F) late trophozoite. (G) Schizont. YFP fluorescence is shown in green (first column). Live cultures were stained with DAPI to visualize nuclei (A to E, blue), or ER-tracker (F and G, red) (second column). Merge 1 is an overlay of the green and blue fluorescence channel (A to E) or the green and red fluorescence channel (F and G). Merge 2 schows an overlay of the bright field image and the green fluorescence channel.

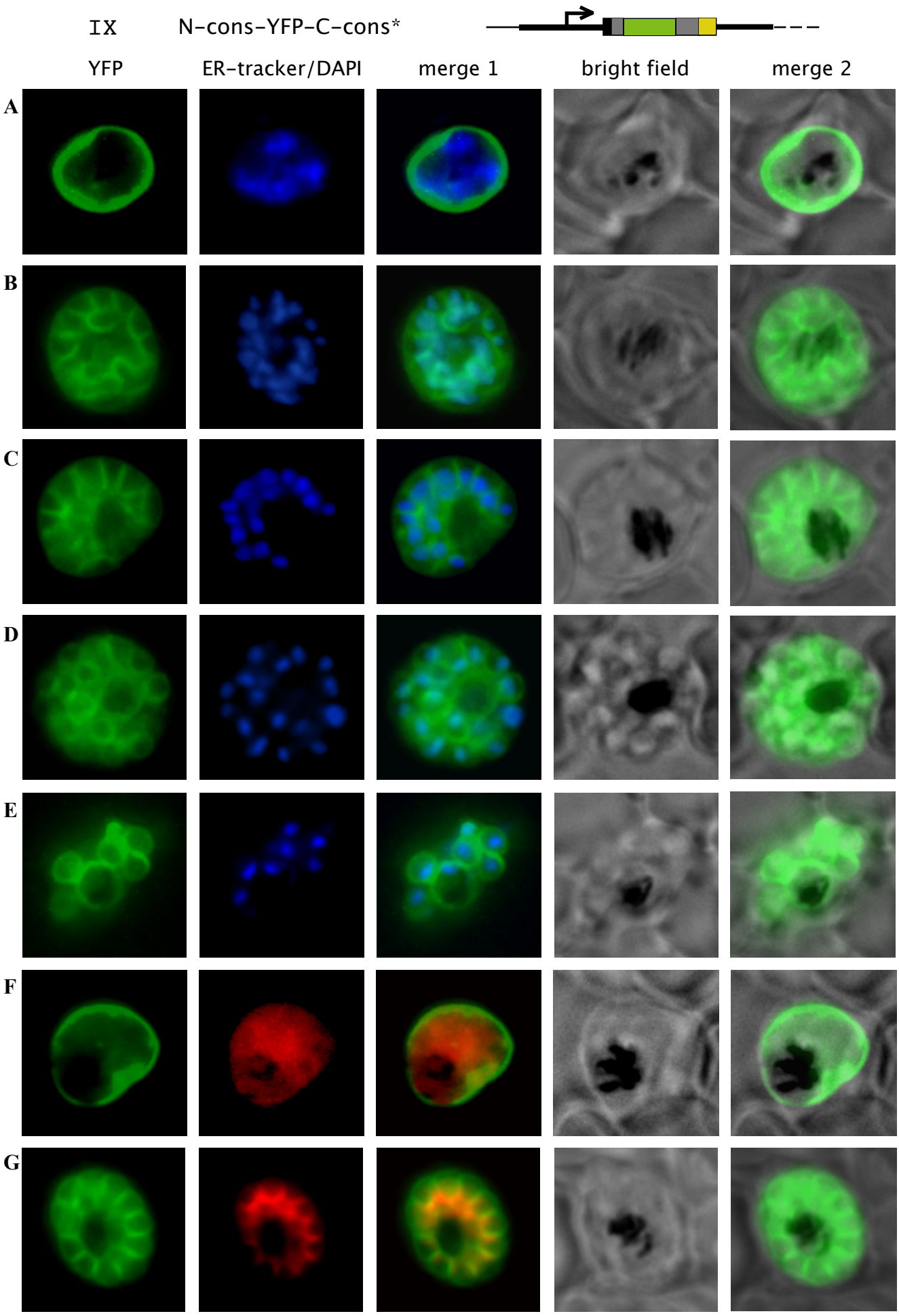

IX
N-cons-YFP-C-cons*
YFP
ER-tracker/DAPI
merge 1
bright field
merge 2
A
B
C
D
E
F
G

Figure 3

Fig. 4. Fluorescence microscopy of parasites transfected with construct V. (**A** to **C**) Live fluorescence microscopy of late stages. (**A**) cultures were treated with DAPI to visualize nuclei (blue): segmenter with ruptured parasitophorous vaculolar membrane, upper panel, and free merozoites (lower panel). (**B**) YFP fluorescence in two segmenters. (**C**) Cultures were treated with ER tracker. YFP fluorescence is shown in green and ER tracker staining in red. (**D**) Indirect immunofluorescence double labelling on methanol-fixed schizonts using a mouse monoclonal antibody against GFP followed by an Alexa 488-conjugated anti-mouse IgG antibody (green) and anti-MSP2 rabbit serum followed by a Alexa 568-conjugated anti-rabbit IgG antibody (red).

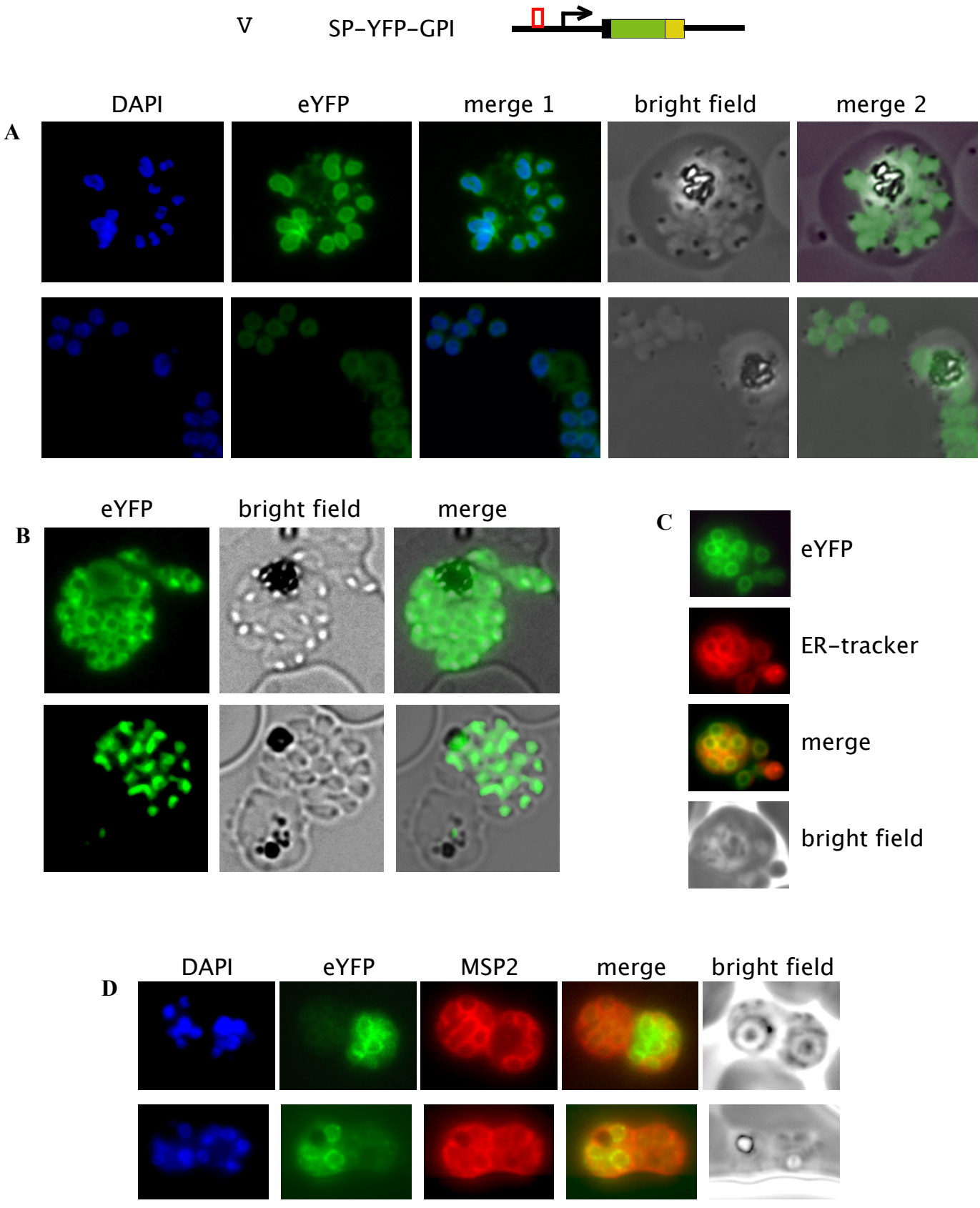

V
SP-YFP-GPI
A
DAPI
eYFP
merge 1
bright field
merge 2
B
eYFP
bright field
merge
C
eYFP
ER-tracker
merge
bright field
D
DAPI
eYFP
MSP2
merge
bright field

Figure 4

Fig. 5. Live fluorescence microscopy of parasites expressing YFP reporters with AMA-1 C-terminal domains. YFP fluorescence is shown in a segmenter of transfectant VI (**A**). White arrowheads indicate apical organelles that appear dark in the bright field image. (**B**) YFP fluorescence in a segmenter of transfectant VII. YFP autofluorescence is shown. Corresponding reporter constructs are represented schematically.

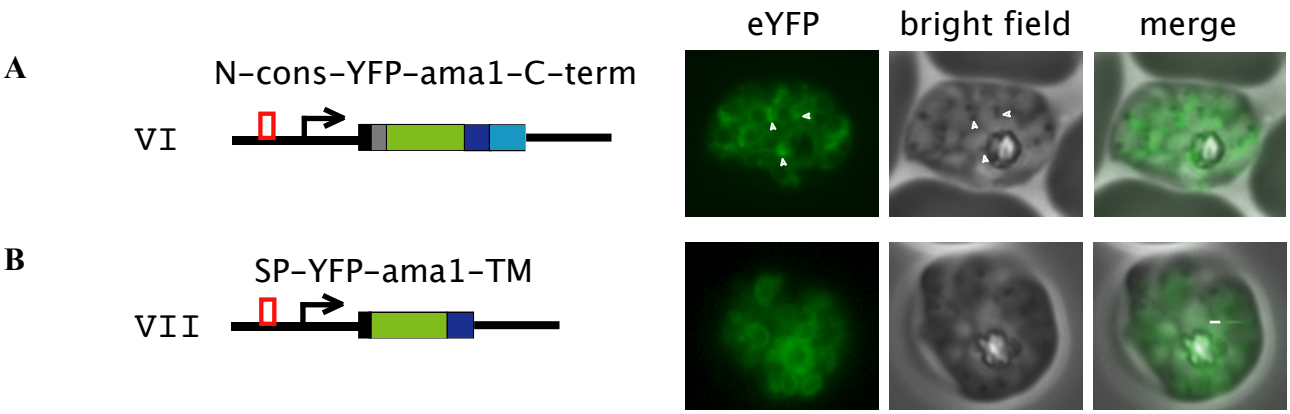

A
N-cons-YFP-ama1-C-term
VI
B
SP-YFP-ama1-TM
VII
eYFP
bright field
merge

Figure 5

7. General Discussion and Conclusions

Malaria is still one of the top ranking causes of morbidity and death in tropical countries. Per year, 500 million people become infected and 1 to 2 millions die, the majority of deaths occurring in children under the age of 5 and caused by *Plasmodium falciparum*. A vaccine would be one of the most cost-effective interventions, but is still not available. The reasons for this are mainly the complex life cycle of the parasite, its antigenic diversity and variation, the incomplete knowledge of the immune responses it induces, and of factors determining the outcome of an infection. However, there are reasons to believe that development of an effective vaccine is feasible. 1) Transient sterile immunity was demonstrated by repeated vaccination with attenuated sporozoites (Clyde et al., 1973). 2) Continuous natural exposure leads to development of a clinical immunity associated with high antibody titers against major surface molecules of the merozoite stage, however, immunity is never sterile. 3) It is well established that anti-parasite antibodies are important in controlling blood stage parasite growth. Passive transfer of immunoglobulin fractions from immune donors can protect naïve individuals from clinical malaria (Cohen et al., 1961; McGregor et al., 1963; Cohen and Butcher, 1970). Furthermore, antibodies specific to antigens of the merozoite stage can inhibit parasite growth *in vitro* by directly blocking important invasion molecules (Epping et al., 1988; Ahlborg et al., 1993; O'Donnell et al., 2001; Moreno et al., 2001; Müller et al., 2003) or by interaction with blood monocytes via FC-gammaII receptors inducing killing of intra-erythrocytic parasites (Khusmith and Druilhe, 1983; Bouharoun-Tayoun et al., 1990; Bouharoun-Tayoun et al., 1995; Oeuvray et al., 1994; Singh et al., 2004; Theisen et al., 2000) In addition to antibody responses and the required T-cell help, cell-mediated immunity is probably also important in controlling blood stage parasites, however, its regulation is poorly understood. It is known from animal models that CD4+ T-cells can limit parasite growth in the absence of B-cells (van der Heyde HC et al., 1994). Gamma delta T-cells were shown to target extracellular merozoites (Elloso et al., 1994). High parasite densities can lead to apoptosis of effector and helper CD4+ T cells as well as antibody-producing B cells. These regulatory mechanisms may limit harmful effects on host tissues but also result in ineffective antiparasite immunity (reviewed in Good et al., 2005)

Although irradiated sporozoites proved to be an effective vaccine, such a whole organism approach is not practical, hindered by technical difficulties to produce large amounts of

antigen. Thus, research has been focussed mainly on the development of recombinant subunit vaccines during the last three decades.

Three stages of the parasite, pre-erythrocytic, blood stages, and sexual stages of the parasite can be targeted by subunit vaccines. An anti-sporozoite and anti-hepatocytic stage vaccine would ideally lead to sterile immunity. The disadvantage of such a vaccine is that development of natural immunity would be prevented (Reeder, 2001). Sexual stage vaccines aim at blocking the transmission to the mosquito. The benefit of such a vaccine is for the community, not for the vaccinated individual. The aim of vaccines against the blood stages is primarily to reduce the parasite load and hence clinical symptoms and complications. It is hoped that such vaccines also accelerate the acquisition of natural immunity.

The only pure blood stage vaccine tested in the field to date was Combination B. Combination B is a three component vaccine consisting of an N-terminal fragment of merozoite surface protein 1 (MSP1), the full-length MSP2 from strain 3D7, and the ring-infected erythrocyte surface antigen (RESA). Combination B showed an efficacy of 62% in reduction of parasite density in Papua New Guinean children aged 5 to 9 years. Since a selecting effect on breakthrough parasites was observed, favouring genotypes carrying an MSP2 of the alternate allelic family, it was suggested that the MSP2 component of the vaccine had some protective effect against parasites carrying a 3D7-type MSP2 allele (Genton et al., 2002). Humoral and cellular responses to the individual vaccine components were analysed. Antibody titres were increased to all three antigens, while only the MSP1 component elicited a significant T-cell response (Genton et al., 2002; Genton et al., 2003).

We have addressed the question, which domains of the 3D7-MSP2 vaccine molecule in Combination B were effective in reducing parasite density. We therefore analysed the Combination B-induced antibody response to conserved, repetitive, and dimorphic domains of MSP2 (chapter 2). The antibody titres against individual MSP2 domains of the vaccine molecule were determined in vaccinees and placebo recipients, thus providing a measure of immunogenicity of each domain. The key finding was that the elicited response was exclusively directed against strain- and family-specific domains. The vaccine increased antibody titres to the Gly-Gly-Ser-Ala (GGSA) repeat by 3-fold and those against the non-repetitive family-specific part by almost 5-fold. The identified targets of the response to the 3D7-MSP2 vaccine molecule highlights the problems when using polymorphic antigens as

vaccines. The elicited response was strain-specific. However, an ideal malaria vaccine would confer strain-transcending protection. To reach this, the vaccine molecule must either consist of conserved epitopes or several variants of the antigen must be combined. The conserved parts of MSP2 were shown not to be immunogenic in the Combination B trial. This result is consistent with low antibody titres found in naturally exposed individuals (Lawrence et al., 2000; Metzger et al., 2003; Weisman et al., 2001). However, inclusion of these domains in future MSP2-based vaccines should be considered since they were shown to contain T-cell epitopes in mice (Rzepczyk et al., 1992). Also, minor changes in the amino acid sequence might improve immunogenicity without changing the antigenic properties. This has been demonstrated for peptides from conserved MSP1-19 sequences (Torres et al., 2003; Espejo et al., 2004).

The vaccine effect of Combination B was imperfect. Vaccination did not entirely protect against breakthrough infections with parasites carrying a 3D7-type MSP2. Genotyping of all blood samples of the Combination B trial had shown reduced prevalence of 3D7-type parasites in vaccinees. We have aimed at characterizing the selective effect on sequence level (chapter 3). We sequenced all 3D7-type *msp2* alleles detected in the trial. Alleles occurring in vaccine recipients were no different from those in placebo recipients. A phylogenetic analysis showed no clustering of alleles from breakthrough infections in vaccinees. This argues against a strain-specific protective response but in favour of family-specific protection. This indicates that conserved stretches within the family-specific part were most likely to have contributed to protection.

Repetitive domains in *Plasmodium* antigens are generally immunodominant and were proposed to function as smoke screens in that they elicit a massive, non-protective antibody response distracting the immune system from mounting a response to adjacent protective epitopes (Anders, 1986; Schofield, 1991). These anti-repeat responses may result from T-cell-independent B-cell activation by crosslinking of surface immunoglobulin and would therefore be short-lived and of low affinity. Parasites carrying MSP2 alleles containing the 3D7 repeat sequence GGSA were present in five children vaccinated with Combination B. These children had consistently low titre antibodies to the $(GGSA)_5$ repeat of the vaccine molecule and vaccination did not considerably increase these titres. It remains open whether they would have been protected against these particular genotypes if a high titre anti-repeat response had been induced. However, it became evident that the anti-repeat responses to Combination B

varied considerably in their magnitude between individual vaccinees. This indicates that anti-repeat responses may be restricted in some individuals.

Apart from choosing appropriate antigens to confer a strain-transcending protection, a very important feature of an effective malaria vaccine is the induction of a persistent protection. It is known that anti-parasite antibody levels in naturally exposed indiviuals drop rapidly in the absence of natural challenge (Cavanagh et al., 1998; Giha et al., 1999; Genton et al., 2000). The drop of antibody levels varies considerably depending on the antigen. MSP2 antibody titres were found to be high but also to drop rapidly without exposure. This can be due to the fact, that MSP2-specific antibodies are predominantly of the short-lived IgG3 subclass. This indicates a defect in IgG subclass switching to the more durable IgG1 but also a defect in generating specific memory B-cells as it has been proposed for some malarial antigens (Dorfman et al., 2005). It remains open whether the unusual subclass distribution of MSP2-specific antibodies is beneficial or detrimental to the host. IgG3 has been implicated in antibody-dependent cellular inhibition, a proposed effector mechanism of antibodies targeted to merozoites (Bouharoun-Tayoun et al., 1992; Tebo et al., 2001). However, since heavy-chain switching and the generation of memory cells are largely dependent on the antigen, it will be difficult to produce an MSP2-based malaria vaccine giving long term protection.
Nevertheless, in infants and young children, even a vaccine inducing short-term, partial protection may be a valuable measure to reduce parasite densities and concomitantly severe disease.

It was shown that MSP2 forms amyloid-like fibrils *in vitro* and it was proposed that these fibrils are also formed on the surface of merozoites (Adda et al., 2004). Through fibril formation, individual MSP2 molecules would probably be placed in very close proximity to eachother and display a parallel and thus repetitive structure. It is known that densely packed repetitive and highly ordered viral epitopes are potent inducers of T-cell-independent B-cell responses (Bachmann et al., 1995; Jegerlehner et al., 2002). Similarly, antigen organization on the surface of *Plasmodium* merozoites could influence induction of T-cell-independent B-cell responses. Assuming that MSP2 is organized as a polymer at the merozoite surface, that could mean that antibodies to non-repetitive epitopes of the MSP2 molecule are also produced in a T-cell-independent manner. T-cell-independent antibody responses may be potent in neutralizing viruses but are probably disadvantageous in the context of a malaria infection (Schofield, 1991). However, presenting MSP2 as a monomer in a vaccine would, with

appropriate T-cell help provided, give rise to high affinity antibodies that exceed the quality of naturally acquired antibodies. Circular dichroism spectra suggest that random coil is the dominant conformation in both, monomeric and polymeric 3D7-type MSP2 (Adda et al., 2004). This indicates that most antibodies raised against the monomer will also react with the polymer.

The result of the Combination B trial suggested to include both family-specific domains in an optimized MSP2-based vaccine. If the family-specific non-repetitive part of the alternative family (FC27) proves to be as effective in inducing a protective antibody response against most members of the own family, then a combination of the two molecules in one vaccine could result in a strain-transcending protection. Nevertheless, it is advisable to also include other antigens since it is unlikely that antibodies to a single target will give maximal protection.

We evaluated two long synthetic MSP2 peptides as vaccine candidates (chapter 4). They each represent either of the two allelic MSP2 families (3D7 and FC27). They contain the non-repetitive dimorphic domain starting downstream of the repeat region plus the C-terminal conserved domain. The peptides proved to be recognized by immune sera at high prevalences and were immunogenic in mice. Monoclonal antibodies raised from mice immunized with the 3D7-MSP2 peptide as well as antibodies purified from human immune sera on both peptides reacted with parasite-derived MSP2. The peptide-purified antibodies were mainly directed against the family-specific part. Preliminary experiments also showed that the antibody purified on the 3D7-MSP2 peptide inhibits parasite growth in cooperation with monocytes. Taken together, these data suggest that the peptides are recognized by antibodies relevant to an anti-parasite response and that they have the potential to elicit a protective response.

To date most malaria vaccine candidates have been produced as recombinant proteins. As an example, the 3D7 molecule that was part of Combination B and the FC27 variant are currently being developed for large scale manufacture in *Escherichia coli* for clinical trials in humans (Robin Anders, Latrobe University, Bundoora, Australia and GroPep Ltd, Adelaide, Australia). The large scale production of recombinant proteins is a time-consuming process. The advantage of producing an antigen synthetically is that any sequence can be synthesised within weeks and purification from *E. coli* protein contamination is obsolete. The peptide synthesis approach has great implication in view of a genome-wide approach to screening and

development of new vaccine candidates. With our work we could demonstrate that synthetic peptides can replace recombinantly produced antigens in some cases.

Another aim of this thesis was to address the question of the functional importance of the different domains of MSP2. We hypothesised that the polymorphic repeat region of MSP2 was dispensable for *in vitro* growth. This is based on the smoke screen hypothesis, only attributing an immunological relevance but no essential function to this domain. However, two monoclonal antibodies directed to epitopes in the repeat region were shown to inhibit parasite growth *in vitro* (Epping et al., 1988; Ramasamy, 1990). This would argue for an important functional role of the repetitive domain. This view contrasts with the immunological smoke screen hypothesis that suggests that these repeats are primarily a means to diverge the immune response from other important antigenic determinants. It is also conceivable that low-affinity anti-repeat antibodies elicited during natural infections actually help to protect the function of the repeats by impeding binding of rare high-affinity antibodies.

To investigate the importance of the repeat domain, we targeted the *msp2* locus of 3D7 parasites with a plasmid construct designed to replace the endogenous *msp2* gene with an FC27 *msp2* gene lacking the repeat region (chapter 5). The plasmid integrated into the *msp2* locus via a single cross-over. A double cross-over, which would have led to gene replacement, was not achieved. Replacement with the full-length control construct was also not achieved, because this plasmid had recombined, rendering it resistant to negative selection with ganciclovir. It remains open whether gene replacement with the internally deleted *msp2* gene was not achieved due to the importance of the missing repeat region or for technical reasons. The major problem of our approach consisted in recombination events either during propagation in *E. coli* or in the course of drug selection of transfected *P. falciparum* cultures. For designing alternative approaches to clarify the functional role of repeats, the choice of deletion constructs needs to be reconsidered.

An ancestral MSP2 molecule might have carried just one copy of the repeat unit. This is suggested by some wild-type FC27 alleles carrying only a single copy or a deletion of one repeat, while presence of two repeat units is the rule in FC27-type alleles. Therefore, the repetitive nature of the domain as such may not be essential. Our approach of deleting all repeats might have been too radical. In a more conservative approach, a gene replacement

could be attempted with a minimized *msp2* gene containing only one copy of a repeat unit. Problems could also arise here when spacing is important. However, this is unlikely considering the great length polymorphism observed among different *msp2* alleles, although, there may be a minimal allowed length.

We also investigated the putative function of the conserved terminal domains (chapter 6). MSP2 is a GPI-anchored protein and therefore the mechanism of its transport to the cell surface is uncertain. It could be that the addition of the anchor is sufficient as a signal for plasma membrane targeting. In addition, a specific sequence on the processed protein could be necessary. Our data obtained from localisation studies on YFP-fusion proteins suggest that the conserved terminal domains do contain sequences acting as transport signal. To conclusively prove the involvment of the conserved domains in transport processes, it needs to be determined whether the fusion construct lacking the two conserved MSP2 domains, that was not transported to the surface, was provided with a GPI-anchor. After confirming the role of conserved MSP2 domains, the putative transport signals need to be assigned to either the N- or C-terminal domain, followed by identification of the actual sequence involved.

In conclusion, we have addressed several aspects of the functional and immunological role of the different domains of MSP2, a major surface antigen of the invasive stage of *P. falciparum*. A detailed molecular and serological analysis of a vaccine field trial proved highly informative for our goal to identify the MSP2 domains most relevant for inclusion in a subunit vaccine. Both, *msp2* sequence analysis and anti-MSP2 antibody responses highlighted a non-repetitive region that is totally conserved within the allelic family and could be considered as a potential vaccine subunit. Following this line of evidence, we were able to evaluate two synthetic peptides in a formalized pathway, that showed promising results. Our parallel approach to assess the function of the repetitive and conserved domains was based on technically challenging transfection experiments and produced several successfully transfected parasite lines. These experiments suggested a new role for the conserved MSP2 domains in transport to the merozoite surface, yet this awaits definitive experimental verification.

References

1. **Adda, C., V. Murphy, K. Vingas, S. Abdulnour, L. Waddington, M. Sunde, A. Hodder, R. Masciantonio, and R. Anders.** 2004. *Plasmodium falciparum* merozoite surface protein 2 forms beta-sheet-containing fibrils in vitro. molecular approaches to malaria (MAM) conference, Lorne, Vic. 26 January - 1 February 2004.

2. **Ahlborg, N., B. W. Flyg, J. Iqbal, P. Perlmann, and K. Berzins.** 1993. Epitope specificity and capacity to inhibit parasite growth in vitro of human antibodies to repeat sequences of the *Plasmodium falciparum* antigen Ag332. Parasite Immunol **15:**391-400.

3. **Anders, R. F.** 1986. Multiple cross-reactivities amongst antigens of *Plasmodium falciparum* impair the development of protective immunity against malaria. Parasite Immunol **8:**529-39.

4. **Bachmann, M. F., H. Hengartner, and R. M. Zinkernagel.** 1995. T helper cell-independent neutralizing B cell response against vesicular stomatitis virus: role of antigen patterns in B cell induction? Eur J Immunol **25:**3445-51.

5. **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:**1633-41.

6. **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:**1473-81.

7. **Bouharoun-Tayoun, H., C. Oeuvray, F. Lunel, and P. Druilhe.** 1995. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. J Exp Med **182:**409-18.

8. **Cavanagh, D. R., I. M. Elhassan, C. Roper, V. J. Robinson, H. Giha, A. A. Holder, L. Hviid, T. G. Theander, D. E. Arnot, and J. S. McBride.** 1998. A longitudinal study of type-specific antibody responses to *Plasmodium falciparum* merozoite surface protein-1 in an area of unstable malaria in Sudan. J Immunol **161:**347-59.

9. **Clyde, D. F., H. Most, V. C. McCarthy, and J. P. Vanderberg.** 1973. Immunization of man against sporozite-induced falciparum malaria. Am J Med Sci **266:**169-77.

10. **Cohen, S., and G. A. Butcher.** 1970. Properties of protective malarial antibody. Nature **225:**732-4.

11. **Dorfman, J. R., P. Bejon, F. M. Ndungu, J. Langhorne, M. M. Kortok, B. S. Lowe, T. W. Mwangi, T. N. Williams, and K. Marsh.** 2005. B cell memory to 3 *Plasmodium falciparum* blood-stage antigens in a malaria-endemic area. J Infect Dis **191:**1623-30.

12. **Elloso, M. M., H. C. van der Heyde, J. A. vande Waa, D. D. Manning, and W. P. Weidanz.** 1994. Inhibition of *Plasmodium falciparum* in vitro by human gamma delta T cells. J Immunol **153:**1187-94.

13. **Epping, R. J., S. D. Goldstone, L. T. Ingram, J. A. Upcroft, R. Ramasamy, J. A. Cooper, G. R. Bushell, and H. M. Geysen.** 1988. An epitope recognised by inhibitory monoclonal antibodies that react with a 51 kilodalton merozoite surface antigen in *Plasmodium falciparum*. Mol Biochem Parasitol **28:**1-10.

14. **Espejo, F., A. Bermudez, E. Torres, M. Urquiza, R. Rodriguez, Y. Lopez, and M. E. Patarroyo.** 2004. Shortening and modifying the 1513 MSP-1 peptide's alpha-helical region induces protection against malaria. Biochem Biophys Res Commun **315:**418-27.

15. **Genton, B., F. Al-Yaman, R. Anders, A. Saul, G. Brown, D. Pye, D. O. Irving, W. R. Briggs, A. Mai, M. Ginny, T. Adiguma, L. Rare, A. Giddy, R. Reber-Liske, D. Stuerchler, and M. P. Alpers.** 2000. Safety and immunogenicity of a three-component blood-stage malaria vaccine in adults living in an endemic area of Papua New Guinea. Vaccine **18:**2504-11.

16. **Genton, B., F. Al-Yaman, I. Betuela, R. F. Anders, A. Saul, K. Baea, M. Mellombo, J. Taraika, G. V. Brown, D. Pye, D. O. Irving, I. Felger, H. P. Beck, T. A. Smith, and M. P. Alpers.** 2003. Safety and immunogenicity of a three-component blood-stage malaria vaccine (MSP1, MSP2, RESA) against *Plasmodium falciparum* in Papua New Guinean children. Vaccine **22:**30-41.

17. **Genton, B., I. Betuela, I. Felger, F. Al-Yaman, R. F. Anders, A. Saul, L. Rare, M. Baisor, K. Lorry, G. V. Brown, D. Pye, D. O. Irving, T. A. Smith, H. P. Beck, and M. P. Alpers.** 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:**820-7.

18. **Giha, H. A., T. Staalsoe, D. Dodoo, I. M. Elhassan, C. Roper, G. M. Satti, D. E. Arnot, T. G. Theander, and L. Hviid.** 1999. Nine-year longitudinal study of antibodies to variant antigens on the surface of *Plasmodium falciparum*-infected erythrocytes. Infect Immun **67:**4092-8.

19. **Good, M. F., H. Xu, M. Wykes, and C. R. Engwerda.** 2005. Development and regulation of cell-mediated immune responses to the blood stages of malaria: implications for vaccine research. Annu Rev Immunol **23:**69-99.

20. **Jegerlehner, A., T. Storni, G. Lipowsky, M. Schmid, P. Pumpens, and M. F. Bachmann.** 2002. Regulation of IgG antibody responses by epitope density and CD21-mediated costimulation. Eur J Immunol **32:**3305-14.

21. **Khusmith, S., and P. Druilhe.** 1983. Cooperation between antibodies and monocytes that inhibit in vitro proliferation of *Plasmodium falciparum*. Infect Immun **41:**219-23.

22. **Lawrence, G., Q. Q. Cheng, C. Reed, D. Taylor, A. Stowers, N. Cloonan, C. Rzepczyk, A. Smillie, K. Anderson, D. Pombo, A. Allworth, D. Eisen, R. Anders, and A. Saul.** 2000. Effect of vaccination with 3 recombinant asexual-stage malaria

antigens on initial growth rates of *Plasmodium falciparum* in non-immune volunteers. Vaccine **18:**1925-31.

23. **McGregor, C. S, and C. S.** 1963. Treatment of East African *Plasmodium falciparum* malaria with West African gammaglobulin. Trans R Soc Trop Med Hyg.

24. **Metzger, W. G., D. M. Okenu, D. R. Cavanagh, J. V. Robinson, K. A. Bojang, H. A. Weiss, J. S. McBride, B. M. Greenwood, and D. J. Conway.** 2003. Serum IgG3 to the *Plasmodium falciparum* merozoite surface protein 2 is strongly associated with a reduced prospective risk of malaria. Parasite Immunol **25:**307-12.

25. **Moreno, R., F. Poltl-Frank, D. Stuber, H. Matile, M. Mutz, N. A. Weiss, and G. Pluschke.** 2001. Rhoptry-associated protein 1-binding monoclonal antibody raised against a heterologous peptide sequence inhibits *Plasmodium falciparum* growth in vitro. Infect Immun **69:**2558-68.

26. **Mueller, M. S., A. Renard, F. Boato, D. Vogel, M. Naegeli, R. Zurbriggen, J. A. Robinson, and G. Pluschke.** 2003. Induction of parasite growth-inhibitory antibodies by a virosomal formulation of a peptidomimetic of loop I from domain III of *Plasmodium falciparum* apical membrane antigen 1. Infect Immun **71:**4749-58.

27. **O'Donnell, R. A., T. F. de Koning-Ward, R. A. Burt, M. Bockarie, J. C. Reeder, A. F. Cowman, and B. S. Crabb.** 2001. Antibodies against merozoite surface protein (MSP)-1(19) are a major component of the invasion-inhibitory response in individuals immune to malaria. J Exp Med **193:**1403-12.

28. **Oeuvray, C., H. Bouharoun-Tayoun, H. Grass-Masse, J. P. Lepers, L. Ralamboranto, A. Tartar, and P. Druilhe.** 1994. A novel merozoite surface antigen of *Plasmodium falciparum* (MSP-3) identified by cellular-antibody cooperative mechanism antigenicity and biological activity of antibodies. Mem Inst Oswaldo Cruz **89 Suppl 2:**77-80.

29. **Ramasamy, R., G. Jones, and R. Lord.** 1990. Characterisation of an inhibitory monoclonal antibody-defined epitope on a malaria vaccine candidate antigen. Immunol Lett **23:**305-9.

30. **Reeder, J. C.** 2001. Towards a malaria vaccine for Papua New Guinea. PNG Med J **44:**17-23.

31. **Rzepczyk, C. M., P. A. Csurhes, A. J. Saul, G. L. Jones, S. Dyer, D. Chee, N. Goss, and D. O. Irving.** 1992. Comparative study of the T cell response to two allelic forms of a malarial vaccine candidate protein. J Immunol **148:**1197-204.

32. **Schofield, L.** 1991. On the function of repetitive domains in protein antigens of Plasmodium and other eukaryotic parasites. Parasitol Today **7:**99-105.

33. **Singh, S., S. Soe, J. P. Mejia, C. Roussilhon, M. Theisen, G. Corradin, and P. Druilhe.** 2004. Identification of a conserved region of *Plasmodium falciparum* MSP3 targeted by biologically active antibodies to improve vaccine design. J Infect Dis **190:**1010-8.

34. **Tebo, A. E., P. G. Kremsner, and A. J. Luty.** 2001. *Plasmodium falciparum*: a major role for IgG3 in antibody-dependent monocyte-mediated cellular inhibition of parasite growth in vitro. Exp Parasitol **98:**20-8.

35. **Theisen, M., S. Soe, S. G. Jessing, L. M. Okkels, S. Danielsen, C. Oeuvray, P. Druilhe, and S. Jepsen.** 2000. Identification of a major B-cell epitope of the *Plasmodium falciparum* glutamate-rich protein (GLURP), targeted by human antibodies mediating parasite killing. Vaccine **19:**204-12.

36. **Torres, E., L. M. Salazar, M. Vanegas, F. Guzman, R. Rodriguez, Y. Silva, J. Rosas, and M. E. Patarroyo.** 2003. Modified merozoite surface protein-1 peptides with short alpha helical regions are associated with inducing protection against malaria. Eur J Biochem **270:**3946–52.

37. **van der Heyde, H. C., D. Huszar, C. Woodhouse, D. D. Manning, and W. P. Weidanz.** 1994. The resolution of acute malaria in a definitive model of B cell deficiency, the JHD mouse. J Immunol **152:**4557-62.

38. **Weisman, S., L. Wang, H. Billman-Jacobe, D. H. Nhan, T. L. Richie, and R. L. Coppel.** 2001. Antibody responses to infections with strains of *Plasmodium falciparum* expressing diverse forms of merozoite surface protein 2. Infect Immun **69:**959-67.

8. Appendix

Supplementary figures

Supplementary figure to chapter 5

Fig. S5 Deduced amino acid sequences of *msp2* transgenes to replace the endogenous *msp2* gene in strain 3D7. Note: A serine and an arginine residue were introduced by restriction sites (bold).

FC27 full-length (265 amino acids)

MKVIKTLSIINFFIFVTFNIKNESKYSNTFINNAYNMSIRRSMANEGSNTNSVGANAPN
ADTIASGSQRSTNSASTSTTNNGESQTTTPTAADTIASGSQRSTNSASTSTTNNGESQT
TTPTAADTPTATESISPSPPITTTESSSSGNAPNKTDGKGEESEKQNELNESTEEGPKAP
QEPQTAENENPAAPENKGTGQHGHMHGSRNNHPQNTSDSQKECTDGNKENCGAAT
SLLSNSSNIASINKFVVLISATLVLSFAIFI

FC27 delta rep (184 amino acids)

MKVIKTLSIINFFIFVTFNIKNESKYSNTFINNAYNMSIRRSMANEGSNTNSVGANAPN
SRESSSSGNAPNKTDGKGEESEKQNELNESTEEGPKAPQEPQTAENENPAAPENKGT
GQHGHMHGSRNNHPQNTSDSQKECTDGNKENCGAATSLLSNSSNIASINKFVVLISA
TLVLSFAIFI

N-C-cons (122 amino acids)

MKVIKTLSIINFFIFVTFNIKNESKYSNTFINNAYNMSIRRSMA**SR**AAPENKGTGQHGH
MHGSRNNHPQNTSDSQKECTDGNKENCGAATSLLSNSSNIASINKFVVLISATLVLSF
AIFI

Supplementary figures to chapter 6

```
     1             15 16            30 31            45 46            60 61            75 76            90
FC27 MKVIKTLSIINFFIF VTFNIKNESKYSNTF INNAYNMSIRRSMA- --------------N EGSNTNSVGANAPNA DTIASGSQRSTNSAS     75
I    MKVIKTLSIINFFIF VTFNIKNESKYSNTF INNAYNMSIRRSMAS LYKKAGSAAAPFTAN EGSNTNSVGANAPNA DTIASGSQRSTNSAS     90

     91          105 106          120 121          135 136          150 151          165 166          180
FC27 TSTTNNGESQTTTPT AADTIASGSQRSTNS ASTSTTNNGESQTTT PTAADTPTATESISP SPPITTTESSSSGNA PNKTDGKGEESEKQN    165
I    TSTTNNGESQTTTPT AADTIASGSQRSTNS ASTSTTNNGESQTTT PTAADTPTATESISP SPPITTTESSSSGNA PNKTDGKGEESEKQN    180

     181         195 196         210 211         225 226         240 241         255 256         270
FC27 ELNESTEEGPKAPQE PQTAENENP-------- ------AAPENKG TGQHGHMHGSRNNHP QNTSDSQKECTDGNK ENCGAATSLLSNSSN    241
I    ELNESTEEGPKAPQE PQTAENENPAKGGRA DPAFLYKVAAPENKG TGQHGHMHGSRNNHP QNTSDSQKECTDGNK ENCGAATSLLNNSSN    270

     271         285 286
FC27 IASINKFVVLISATL VLSFAIFI    264
I    IASINKFVVLISATL VLSFAIFI    293
```

Fig. S6A. Deduced amino acid sequences of wild-type FC27 MSP2 and Multisite Gateway-constructed reconstitution of the gene (construct I). Conserved MSP2 sequences are marked gray, amino acids introduced by Gateway cloning are in bold.

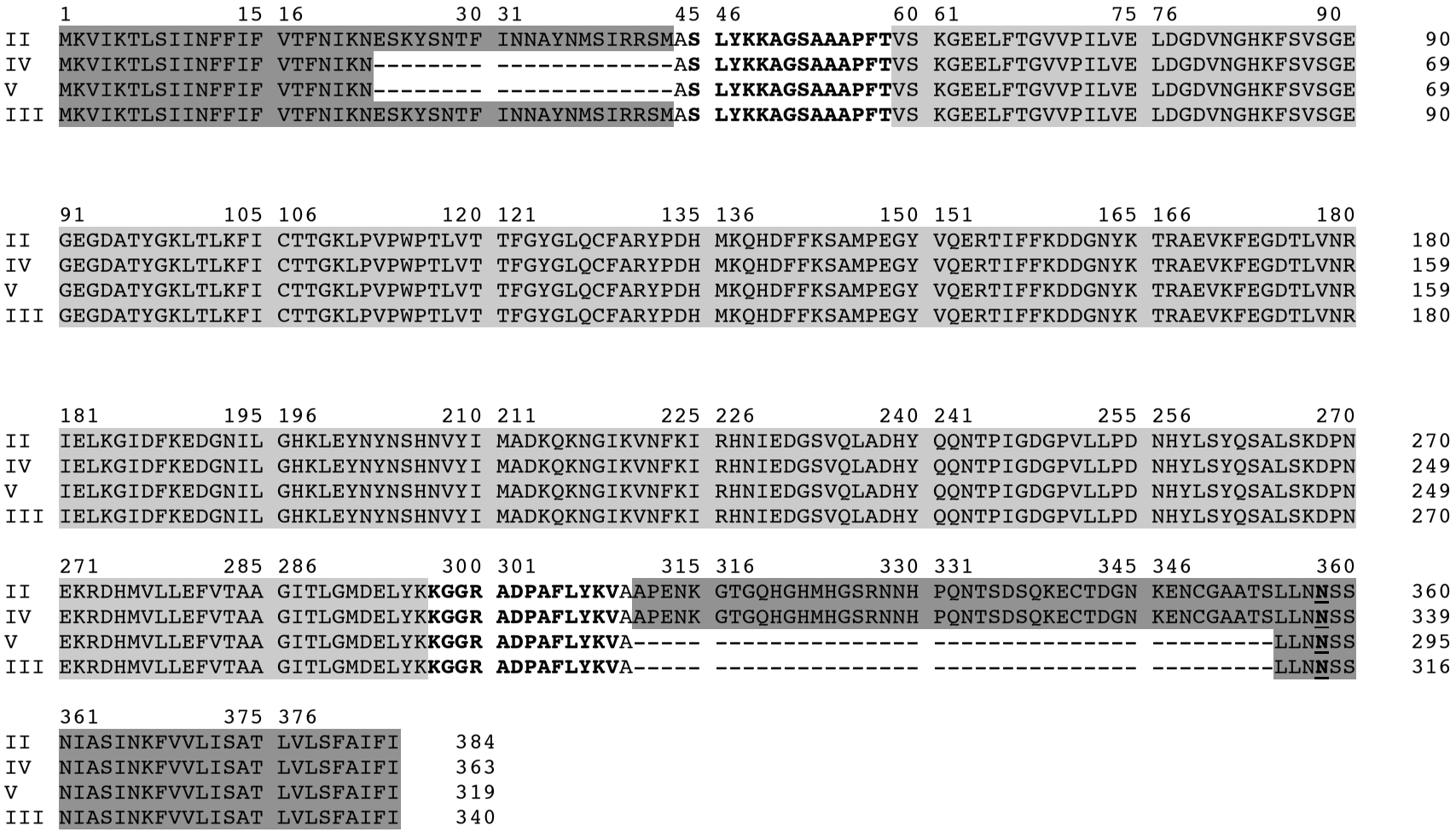


Fig. S6B. MSP2-YFP fusions. Conserved MSP2 parts are shaded dark gray, YFP sequences light gray. Residues introduced by Gateway cloning are in bold. Note: Construct II and construct IX have identical sequences.

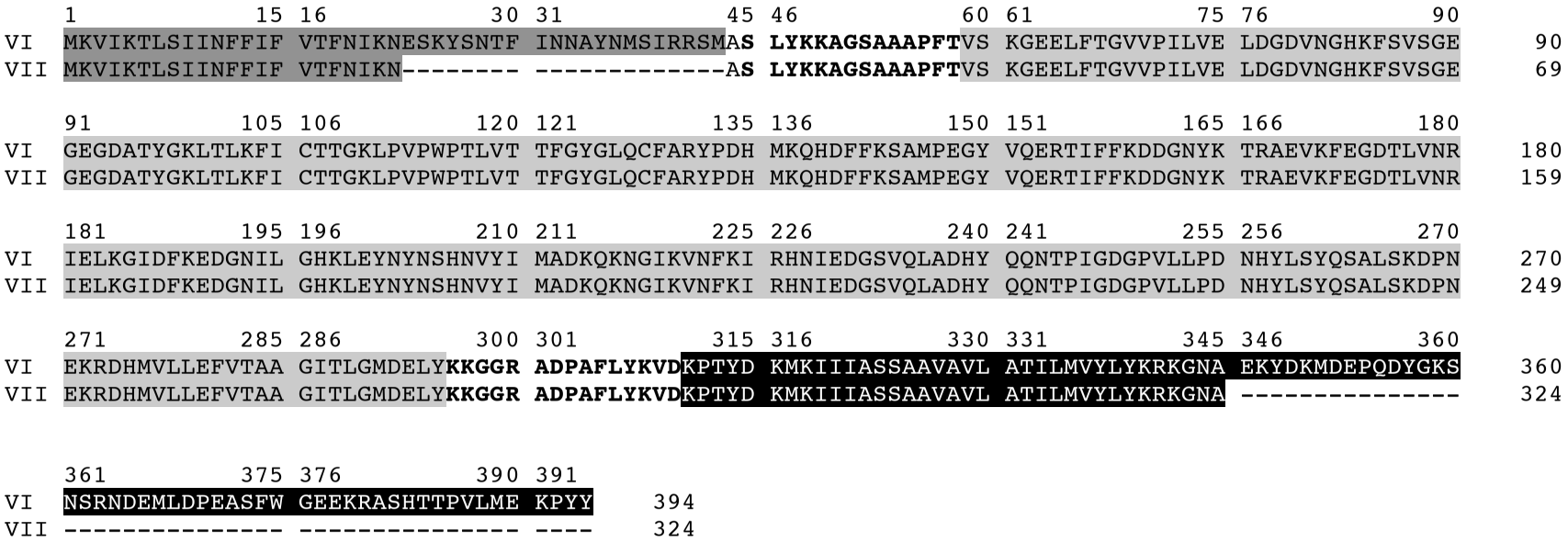


Fig. S6C. MSP2-YFP-AMA1 fusions. Conserved MSP2 sequences are shaded dark gray, YFP light gray, and AMA-1 C-terminal sequences black. Amino acids introduced by Gateway cloning are bold.

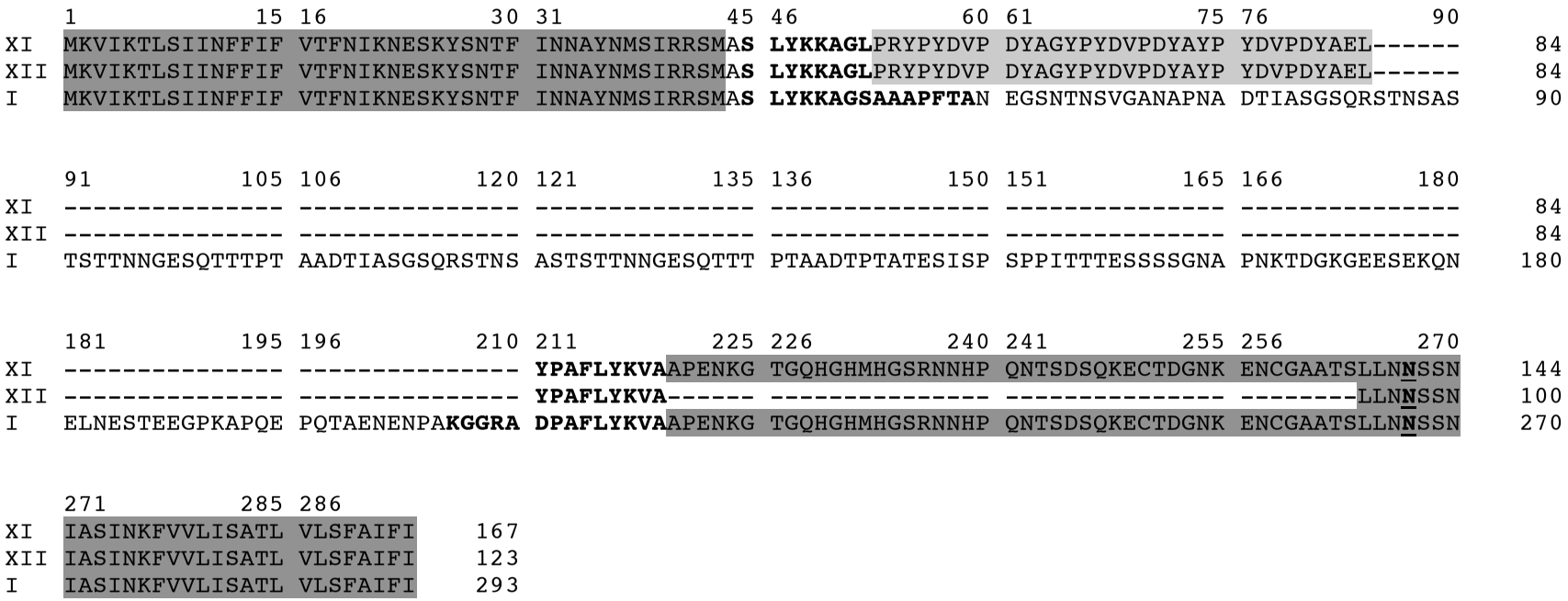


Fig. S6D. MSP2-3x-haemagglutinin tag fusions. Conserved MSP2 sequences are shaded dark gray, and the triple haemagglutinin tag light gray. Amino acids introduced by Gateway cloning are in bold.

Curriculum Vitae

Personal data

Name	Flück
First name	Christian
Citizenship	Swiss
Birth date	06/07/1975
Place of birth	Berne, Switzerland
Address	Wasgenring 121 4055 Basel Switzerland
Affiliation	Swiss Tropical Institute, Socinstr. 57, 4051 Basle, Switzerland Tel: +41 61 284 82 37; Fax: +41 61 271 86 54 e-mail: christian.flueck@unibas.ch

Professional training

Apr 1991 – May 1995	General qualification for university entrance Gymnasium Koeniz, Berne, Switzerland Type E (economy)
Oct 1995 – Sep 1997	Basic studies in biology, University of Berne
Oct 1997 – Oct 1998	Advanced studies in microbiology, molecular biology, genetics and parasitology, University of Berne
Nov 1998 – May 2000	Diploma thesis in molecular parasitology (*Trypanosoma brucei*) at the University of Berne: "The search for regulatory elements mediating the induction of procyclin mRNA levels in response to protein synthesis inhibitors" Supervisor: Isabel Roditi
Feb 2001 – Nov 2005	PhD thesis: Functional and immunological analysis of Merozoite Surface Protein 2 of *Plasmodium falciparum* Supervisor: PD Dr. Ingrid Felger Research advisors: PD Dr. Ingrid Felger, Prof Dr. H.-P. Beck, Prof. Dr. Isabel Roditi
Feb 2004 –Dec 2004	Research stay at The Walter and Eliza Hall Institute, Melbourne, Australia: "Determination of the essential parts of Merozoite Surface Protein 2 by stable transfection of *Plasmodium falciparum*" and "Sequence requirement of a GPI–anchored protein to be transported to the surface of *Plasmodium falciparum* merozoites" Supervisor: Prof. Dr. Alan Cowman

Related activities

Oct 2000– Jan 2001	University Hospital Berne, research assistant in the neurosurgery research laboratory. Supervisor: Dr. Hans-Rudolf Widmer

Meetings (oral presentations)

Jan 2000	Swiss trypanosomatid meeting, Les Diablerets, Switzerland: "Regulatory elements mediating the induction of procyclin mRNA levels in bloodstream form trypanosomes in response to cycloheximide.
Aug 2001	PhD student meeting, Swiss Society of Tropical Medicine and Parasitology, in Münchenwiler, Switzerland. "Strategies to analyse the function of Merosoite Surface Protein 2 of *Plasmodium falciparum*"

Meetings (poster presentations)

Sep 2004	The Australian Society for Parasitology, annual meeting 2004, Fremantle, Australia. "GPI-linked yellow fluorescent protein on the surface of *Plasmodium falciparum* merozoites"
May 2005	COST action 857: Apicomplexan biology in the post genomic era. "Sequence requirements for a GPI-anchored protein to be transported to the surface of *P. falciparum m*erozoites"

Invited seminars

Feb 2004	The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. "Dissection of the humoral immune response to Merozoite Surface Protein 2 in the Combination B malaria vaccine trial"
Apr 2004	Queensland Institute of Medical Research, Brisbane, Australia "Strain-specific humoral response to a polymorphic malaria vaccine"
Jun 2004	NHMRC seminar, Botany Department, University of Melbourne "Sequence requirements for a GPI-anchored protein to be transported to the merozoite surface"

Workshops

Apr 2002	Malaria Transfection Workshop, International Centre for Genetic Engineering and Biotechnology, New Delhi, India (WHO/TDR and MR4)

Publications

Flück C, Salomone JY, Kurath U, Roditi I. Cycloheximide-mediated accumulation of transcripts from a procyclin expression site depends on the intergenic region. Mol Biochem Parasitol. 2003 Mar;127(1):93-7.

Flück C, Smith T, Beck HP, Irion A, Betuela I, Alpers MP, Anders R, Saul A, Genton B, Felger I. Strain-specific humoral response to a polymorphic malaria vaccine. Infect Immun. 2004 Nov;72(11):6300-5.

During my studies I attended lectures by the following lecturers:	R. Braun, D. Schümperli, A. Scholl, C. Wedekind, W. Nentwig, M. Milinski, B. Lanzrein, D. Felix, H. Imboden, H. Riedwyl, H. Balsiger, T. Stocker, B. Erni, A. Boschetti, C. Rothen, G. Pfander, M. Page, A. Azzi, C. Kuhlemeier, K. Ammann, B. Ammann, C. Brunold, D. Newbery, J. Kohli, P. Munz, M. Schweingruber, I. Roditi, T. Seebeck, O. Fleck, B. Stadler, T. Jungi, B. Gottstein, N. Müller, A. Zurbriggen, T. Schirmer, N. Weiss, H.P. Beck, I. Felger, G. Pluschke, T. Bickle, P. Matthias, S. Arber