Novel approaches for the characterisation of *Plasmodium* variant surface antigens

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Thesis submitted for the degree of Doctor of Philosophy to The University of Edinburgh

23rd November 2004

Abstract

The aim of this research was to identify the erythrocyte surface antigens of *P. falciparum*-infected erythrocytes (IEs) that are expressed following selection of the parasite for adhesion to the placental receptor chondroitin sulphate A (CSA). These surface antigens are considered potential vaccine targets against pregnancy-associated malaria (PAM).

Typically *P. falciparum* surface antigens are highly sensitive to treatment of the IE with the protease trypsin; however, variant surface antigen (VSA) mediated adhesion to CSA has often been found to be relatively trypsin-resistant. In this study the protease sensitivity of the VSA of CSA binding IEs was determined with regard to serum IgG recognition and placental receptor binding. Discordance between the protease sensitivity for these phenomena was identified; this may have implications for PAM vaccine design.

The completion of several *Plasmodium* genome sequences and advances in mass spectrometry have opened up the field of proteomics to the study of the malaria parasite. Proteomic methods have been applied to the study of *Plasmodium* VSA. However, the initial step of these methods, surface antigen labelling, proved problematic. Therefore, this thesis concentrates on the optimisation of these techniques. An antibody to the *Plasmodium falciparum* erythrocyte protein-1 (PfEMP1) family was raised and characterised. This reagent in combination with the advances made for IE surface labelling will be a valuable tool for future studies of VSA expression. Western blotting was also applied to investigate the developmental regulation, post-translational processing and surface trypsinisation sensitivity of two candidate IE surface antigens, *Plasmodium falciparum* Cysteine Repeat Modular proteins 1 and 2.

During the optimisation of biochemical techniques, a study to investigate the effect of mosquito passage on *Plasmodium chabaudi* variant surface antigen expression was initiated. Potential surface antigens whose expression associates with mosquito transmission were identified.

Declaration

The Ghanaian serum pools used for characterisation of *Pf*VSA protease sensitivity in Chapter 2 were collected and kindly donated by Mike Ofori, Maja Lundquist, Edmund Nii-Laryea and Victoria Ban.

Joanne Thompson had previously characterised the structural features of the PCRMP family and raised the antisera to PfCRMP1 and PfCRMP2 that were used in Chapter 4.

In Chapter 5, the *Plasmodium chabaudi* parasite material used for optimising surface labelling of AS infected erythrocytes was provided by Jaap de Roode. The experiment to label the IE surface of isolates pre- and post-mosquito transmission was carried out in collaboration with Dr. Mackinnon. Dr. Mackinnon passaged the *P. chabaudi* clones through mice and mosquitoes to derive the clones used in the experiment and performed mouse infections and rosetting assays.

Subject to the exceptions detailed above, I declare that the material presented in this thesis is my own work and has not been submitted to any other university or for any other degree.

Lisa Sharling

Edinburgh, 23rd November 2003

Acknowledgements

I thank the Wellcome Trust for funding and those people involved in the implementing a challenging and rewarding structured PhD programme. Special thanks go to Margaret Heck for organising symposia and her encouragement.

I am grateful to David Arnot for the opportunity to work in his lab. I appreciate his support for my attendance of conferences and meetings: excellent opportunities for keeping up with a fast moving field. Many thanks to the stars that make up the Arnot lab, particularly Mercy for providing melodies, muffins and for being the nearest thing to an intravenous coffee drip. Thanks Alison for sharing the drudgery of long-term *P. falciparum* culture. Thanks Clare and Saad for your understanding and encouragement.

I am much indebted to Joanne Thompson, Alex Rowe and Jana McBride for never failing to express their belief in me and for their continual support, which for each has been in numerous and different ways.

I thank those people that have been willing to collaborate with me on some really exciting projects, namely Margaret Mackinnon and Joanne Thompson. Jerry Thomas at York University, and Jim Creanor in Edinburgh University's Chemistry department introduced me to and provided training in the basics of MALDI-TOF mass spec.

Many thanks to David Walliker for willingly critiquing a number of chapters and ensuring a long-term collaboration with the Edinburgh transfusion unit. Thanks Sally for sharing IFA expertise, and along with Ahmed and Les, for maintaining a steady flow of blood and serum. David Cavanagh captained a tight ship in the culture room, in which I spent many hours, so for that I am very grateful. Rick Maizels, Sandie Cheesman and Richard Carter kindly made available invaluable pieces of equipment.

Thanks to Katelyn and Paulette for being a trusty pair of golden nuts.

Thanks to my parents for soothing periods of anxiety.

Last, but not least, thanks Jaap, your introduction to punctuation, punctuality and statistics were crucial for the completion of this thesis. Thanks for the sunshine despite Edinburgh's weather.

Abbreviations

2DGE	two-dimensional polyacrylamide gel electrophoresis
ATS	amino terminal sequence
BSA	bovine serum albumin
CIDR	cysteine-rich interdomain 1
CSA	chondroitin sulphate A
DAPI	4',6-diamidino-2-phenylindole
DBL	Duffy-like binding domain
DTE	dithioerythritol
DTT	dithiothreitol
ECL	enhanced chemiluminscence
EDTA	ethylenediamine-tetraacetic acid
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionisation
HA	hyaluronic acid
HEPES	4-(2-hydroxyethyl)-piperazine-ethanesulfonic acid
FACS	fluorescence activated cell sorting
FITC	fluorescein isothiocyanate
IE	infected erythrocyte
IgG	immunoglobulin G
IgM	immunoglobulin M
KLH	keyhole limpet hemocyanin
MADLI	matrix assisted laser desorption/ionisation
MS	mass spectrometry
OD	optical density
PAGE	polyacrylamide gel electrophoresis

PAM	pregnancy-associated malaria
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte protein 1
pI	isoelectric point
PVDF	polyvinylidene difluoride
RPMI	Roswell Park Memorial Institute
RT	room temperature
SDS	sodium dodecyl sulphate
s.e.m.	standard error of mean
SIRCAMS	Scottish Instrumentation & Resource Centre for Advanced Mass Spectrometry
TI	triton-X100 insoluble
TM	transmembrane
TNF	tumour necrosis factor
TOF	time-of-flight
TPCK	L-(tosylamido-2-phenyl) ethyl chloromethyl ketone
TS	triton-X100 soluble
VSA	variant surface antigen
VSA_{PAM}	variant surface antigens expressed by placental or CSA binding parasites

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Chapter 1. Introduction

Here I introduce the parasite *Plasmodium*, the causal agent of malaria. I describe the parasite's interaction with its mammalian host's immune system through antigenic variation. The burden of malaria is discussed with special consideration of the syndrome pregnancy-associated malaria. Recent advances in our understanding of the molecular mechanisms underlying this syndrome are reviewed and finally the aims of this thesis are outlined.

1.1 The malaria parasite

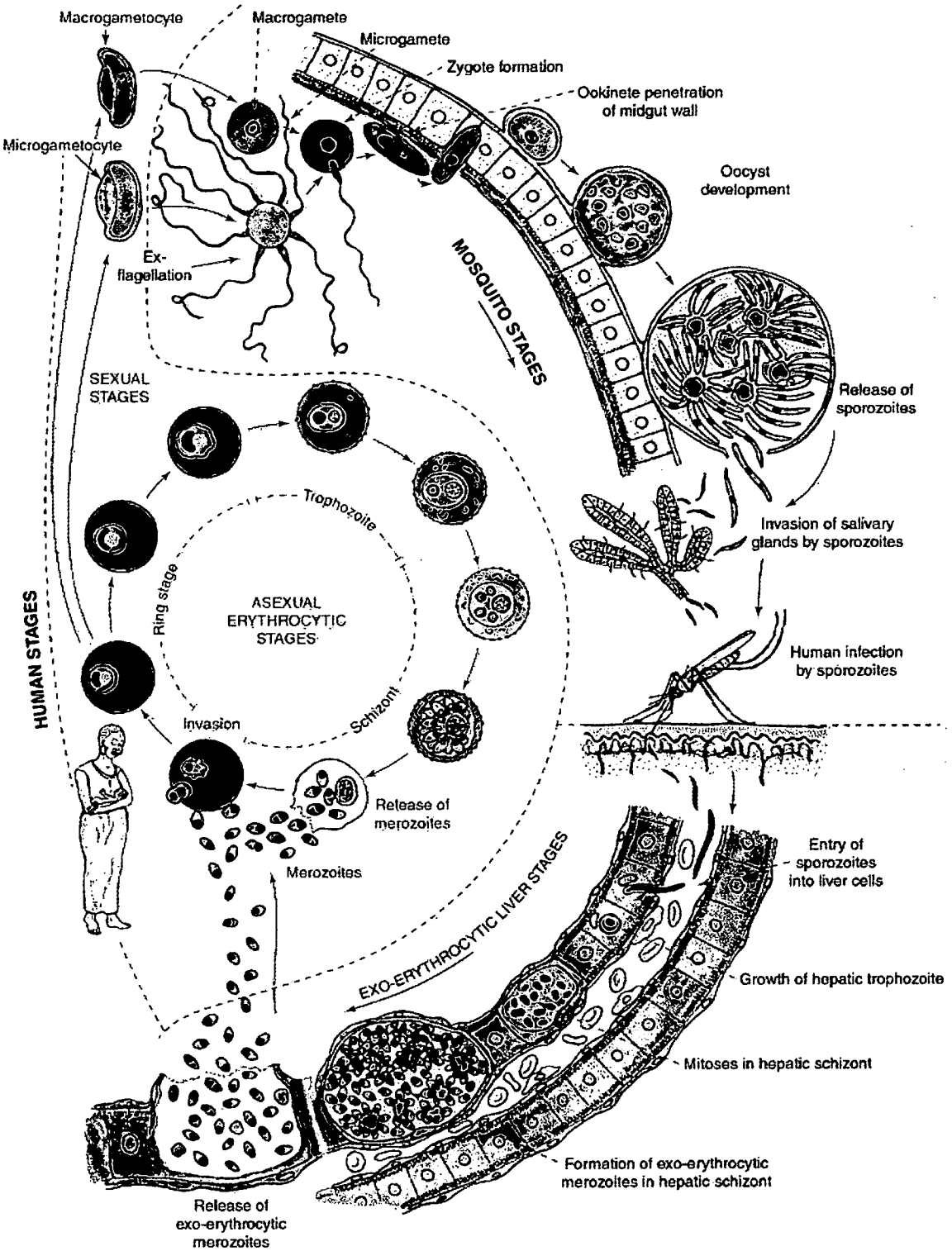
Plasmodium is a member of a monophyletic group, the Apicomplexa, which forms one of the three major super-groups of highly diverse eukaryotic protozoa comprising the Alveolata (Aravind *et al.*, 2003). All ~5000 known species of *Apicomplexan* are parasites of animals (Levine, 1988); they share a specialised cell invasion apparatus called the "apical complex", from which their name is derived.

Well over 200 *Plasmodium* species have been described, which parasitise many species of animal, including reptiles, birds and mammals (Rich & Ayala, 2003). *Plasmodium*, like the related apicomplexans *Babesia* and *Theileria* rely on a blood sucking dipteran for transmission between vertebrate hosts. Female mosquitoes of the genus *Anopheles* transmit the human malarias, *P. vivax*, *P. ovale*, *P. malariae*, and *P. falciparum*. *P. falciparum* is the species that is primarily studied in this thesis, although experiments have also been carried out using the rodent malaria *P. chabaudi*.

1.1.1 The life cycle of *Plasmodium falciparum*

With each probe into human skin, a *P. falciparum* infected mosquito injects tens to hundreds of sporozoite stages of the parasite (Rosenberg *et al.*, 1990); these are delivered to the liver via the circulation. Following sporozoite invasion of an hepatocyte, differentiation and intensive mitotic replication occur, processes taking approximately 6 days. The asexual intra-erythrocytic cycle is initiated when a mature hepatocyte ruptures, releasing an estimated 30 000 merozoites which upon encountering an erythrocyte, utilise their 'apical complex' for invasion. The erythrocytic cycle (Figure 1.2) involves erythrocyte invasion, intracellular growth, asexual reproduction by schizogony, erythrocyte rupture and reinvasion of new erythrocytes (described in detail in Section 1.1.2).

Following erythrocyte invasion a small proportion of merozoites differentiate into either male or female gametocytes, which can be taken up in the blood meal of a mosquito. In the mosquito's midgut male gametocytes undergo three rounds of DNA replication and mitosis and produces eight microgametes, each of which contains a haploid copy of its genome. Exflagellated male gametes fertilise a female gamete that has become primed to develop into an ookinete. Over a period of 5-18 h the fertilised zygote differentiates into a single motile ookinete. The first two meiotic divisions of the genome take place within a single nucleus that does not undergo karyokinesis. The motile ookinete then migrates through the mosquito midgut wall, but arrival at the basal lamina inhibits migration, and differentiation into the oocyst, a trophic and replicative form, occurs. The original polyploid nucleus divides approximately once each day such that after 12-18 days there are 2000-8000 haploid nuclei. When the oocyst bursts, the daughter cells, the sporozoites, are released into the haemocoel. Sporozoites migrate into the mosquito's salivary glands, to await the next blood-meal taken by the dipteran host (Sinden, 2002).



[1]Figure 1.1: The life cycle of *Plasmodium falciparum*

[1] Reprinted from Trends in Parasitology, Vol. 19, Bannister & Mitchell, The ins, outs and roundabouts of malaria, 209-213, Copyright (2003), with permission from Elsevier

1.1.2 A closer look at *P. falciparum's* erythrocytic cycle

The only extracellular stage of the asexual cycle is the small (~1.2 μm) invasive merozoite, but its exposure to the extracellular milieu of the blood stream and circulating host immune factors is brief. This compact stage of the parasite contains all the cellular machinery for establishing itself within the intraerythrocytic environment (figure 1.2A). A number of specialised apicomplexan and *Plasmodium* membranous and organellar cellular structures have evolved to accommodate the parasite's intra-erythrocytic niche. The apical complex consists of specialised secretory vesicles called the rhoptries, micronemes and dense granules; this complex mediates merozoite attachment to a fresh erythrocyte and invasion. The outer membrane of the merozoite is coated with short filaments that are thought to aid in attachment and invasion. During invasion a deep membrane pit is formed which eventually contains the parasite within the erythrocyte. In the parasitophorous vacuole (PV) the membrane lining the PV is called the parasitophorous vacuole membrane (PVM). Another feature of *Apicomplexa* is the presence of a plastid, thought to have arisen through the secondary endosymbiosis of an algal cell (Kohler *et al.*, 1997). The plastid contains a 35 kb circular genome such that the parasite bears two extrachromosomal DNA elements, the plastid genome like the mitochondrial genome is inherited maternally.

Following invasion of the erythrocyte the parasite flattens into a thin disc form that typically has a thick rim of cytoplasm containing the major organelles. In Giemsa stained blood smears viewed under light microscopy this stage appears as a 'ring' (figure 1.2B). The parasite begins to feed by engulfing in small aliquots of the erythrocyte cytoplasm through a small dense region on the surface of the parasite, the cytosome. Digestion of the erythrocyte's haemoglobin releases free haem as a toxic waste product which the parasite converts into a non-toxic crystalline material called haemozoin (Egan, 2002). Later in the erythrocytic cycle haemozoin is stored within another specialised organelle, the pigment vacuole (Bannister & Mitchell, 2003). Haemozoin appears as dark pigment on blood smears and histological sections, and can also be easily detected by polarized-light microscopy. The trophozoite and schizont stages of the parasite digest considerable quantities of haemoglobin, not only as a source of amino acids, but also to maintain a fine homeostatic balance of solutes across the erythrocyte membrane.

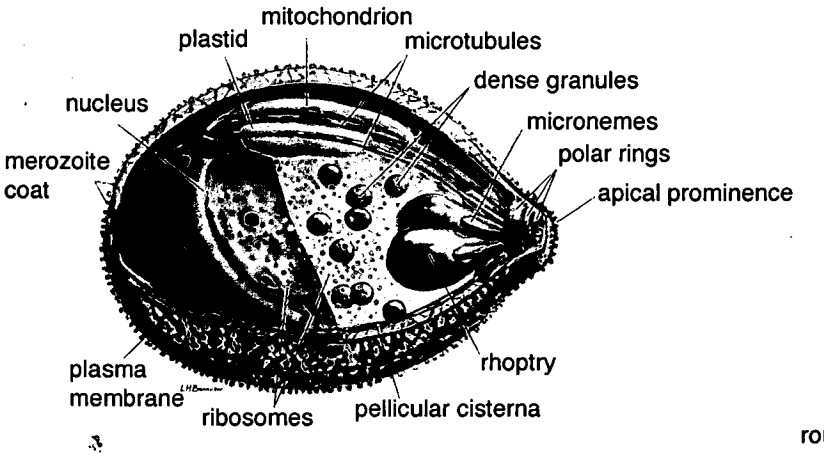
The trophozoite stage (figure 1.2C) is distinguished from the earlier ring stage by changes in cell size and shape. At this stage there is also an increase in protein synthesis and export of parasite proteins to the erythrocyte cytoplasm and

erythrocyte surface (Bannister *et al.*, 2000). Parasite proteins associate with the erythrocyte cytoskeleton and membrane to form protrusions at the erythrocyte surface that appear as electron dense knob-like structures by transmission electron microscopy. Since the erythrocyte lacks organellar and membrane trafficking pathways, in order to transport proteins to the erythrocyte membrane the parasite is required to synthesis trafficking pathways *de novo*. Proteins required at the erythrocyte membrane for the purpose of solute uptake, secretion or host interaction are thought to be trafficked via Maurer's clefts: structures proposed to arise through 'blebbing' and/or extension and elaboration of the PVM. During trophozoite development the number of free ribosomes increases, and the endoplasmic reticulum (ER) and putative Golgi complex increase in size and complexity.

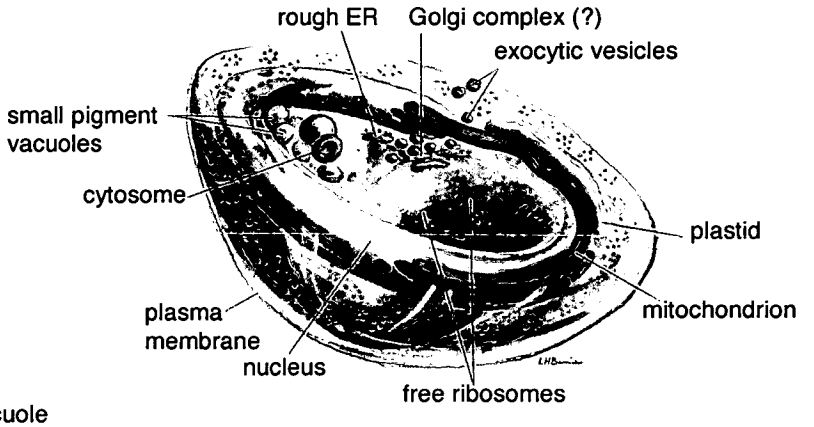
At the schizont stage the parasite undergoes repetitive nuclear division (figure 1.2D). The parasite nucleus divides around 4 times to produce about 16 nuclei and concurrently remodels its cytoskeleton and initiates organelle multiplication to lay down the elements of the merozoites. During the last nuclear division, a series of merozoite forming loci arrange around the circumference of the infected erythrocyte.

In *P. falciparum* infections of humans the asexual cycle takes 48 hours to complete and replication occurs synchronously. Rupturing *P. falciparum* schizonts release between approximately 8 and 20 merozoites, so that parasitaemias climb rapidly and exponentially in the absence of an effective immune response or drug treatment.

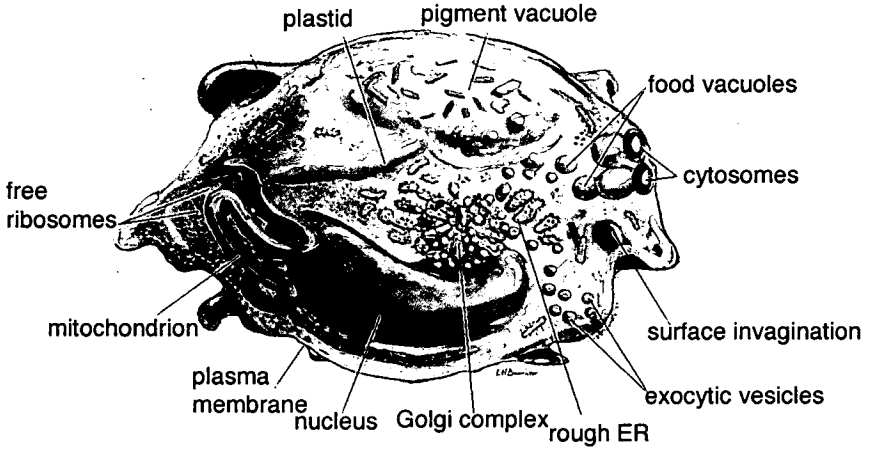
A. Merozoite



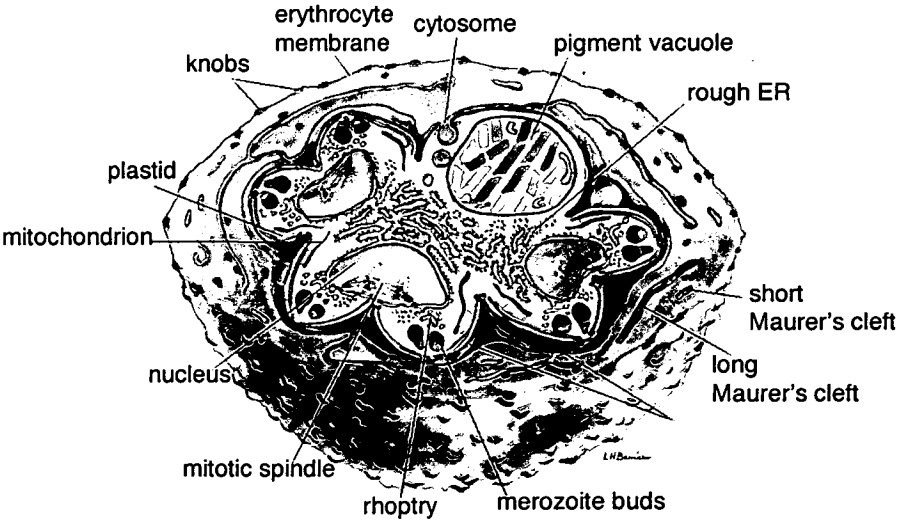
B. Ring stage



C. Mid-trophozoite stage stage



D. Maturing schizont



[2]Figure 1.2: Illustratrative depiction of the ultrastructure of *P. falciparum's* intraerythrocytic stages

Part A shows the internal organisation of the merozoite stage of the parasite. Several secretory vesicles, the rhoptries, the micronemes and the dense granules group at the apical end of the merozoite and release factors to permit erythrocyte invasion. Part B depicts the early ring stage; for clarity the red blood cell is not shown. Part C shows the organisation of the mid-trophozoite stage. An increase in protein synthesis and feeding is evident through the presence of multiple ribosomes, cytosomes and a pigment vacuole. Structures associated with the export of proteins (Golgi body and exocytic vesicales) are also apparent. Again for clarity the rbc is not shown. Part D shows a maturing schizont. Structures involved in protein trafficking (Maurer's clefts) are apparent and parasite-derived modifications to the erythrocyte membrane, knobs, are depicted. Developing merozoites are shown budding from the surface of the schizont. The relative sizes of the different stages have not been preserved.

1.1.3 An adaptable beast

The completion of *Plasmodium's* life cycle requires many stages of differentiation to enable it to replicate in three distinctly different cells in two radically different hosts. The intra-erythrocytic stages face continual assault from the vertebrate host's immune system; the parasite's response to this is described in the next section (1.2). The high level of digestive enzymes in the mosquito midgut also constitutes a hostile environment (Billingsley & Hecker, 1991).

Despite these adaptional capabilities, *Plasmodium* species are strict in their host species range and tissue specificity. A given species is transmitted by a restricted number of mosquito species to a limited number or a single vertebrate host species. Although some non-human primate malarias can infect humans and *vice-versa,* in generally the zoonotic capabilities of *Plasmodium* are considered to be limited. Recently, however, the macaque monkey parasite *P. knowlesi* was found to be responsible for over half of human malaria cases in a study in the Kapit division of Sarawak, Borneo (Singh *et al.,* 2004). Whether *P. knowlesi* in this region has undergone a host switch has yet to be established.

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[2] Reprinted from Parasitology Today, Vol. 16, Bannister *et al.,* A Brief Illustrated Guide to the Ultrastructure of *Plasmodium falciparum* Asexual Blood stages, 427-433, Copyright (2000), with permission from Elsevier.

Plasmodium is also restrictive in terms of cell selectivity; for example, *P. falciparum* sporozoites and merozoites invade only hepatocytes and mature erythrocytes respectively, whereas *P. vivax* merozoites selectively invade reticulocytes. By contrast, a single species of the apicomplexan *Toxoplasma* can infect virtually any nucleated cell of virtually any vertebrate (Roos *et al.*, 2002).

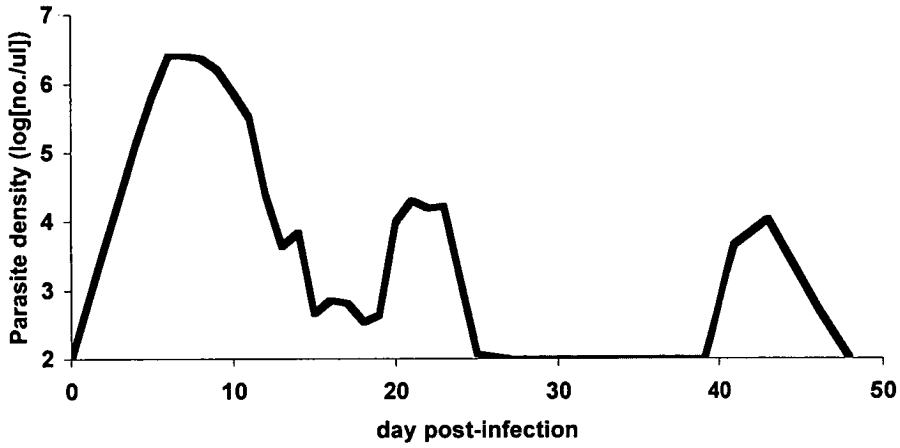
1.2 Antigenic variation

Maintaining chronic infections in the vertebrate host is considered desirable as it is thought to increase transmissibility. Chronic infection is a characteristic of many vector-borne parasitic protozoa, such *Trypanosoma, Babesia,* and *Plasmodium.* These organisms have evolved at least one common mechanism for evading their host's immune system; antigenic variation of molecules that are in direct contact with the host immune system.

During *Plasmodium's* erythrocytic cycle (figure 1.1) the parasite remains intracellular, except for a brief period between the release of merozoites from a rupturing schizont and invasion of a new cell. As human erythrocytes lack surface major histocompatibility complex molecules and a mechanism for antigen presentation, these particular cells of the host constitute a relatively safe haven. However, both *Plasmodium* and the related haemoparasite *Babesia bovis* (Allred *et al.*, 2000) disclose themselves to the mammalian immune system by exporting their proteins to the erythrocyte surface. Indications of this potentially counterintuitive phenomenon were first detected in the 1930s when serum from a monkey infected with *P. knowlesi* was found to agglutinate infected erythrocytes but not uninfected cells (Eaton, 1938). Species- and variant-specific antibodies to red cell surface antigens were subsequently shown for primate (Brown & Brown, 1965), rodent and bird malarias (reviewed in Kyes *et al.*, 2001). In 1983, direct biochemical evidence was presented to show that a primate *P. knowlesi* clone expressed surface antigens on the infected erythrocyte (IE) (Howard *et al.*, 1983).

It is thought that once an immune response has been mounted to one VSA phenotype there is selection for parasite clones expressing VSAs that are novel to the host immune system. The mechanism behind this 'switching' phenomenon is not understood. Recker et al. (2004) have constructed mathematical models to suggest that a new sub-population of antigenically dissimilar parasites, selected by the immune system, becomes the new dominant population. However, experiments with primate models suggest that an induced population-wide 'switch' in phenotype occurs (Galinski & Corredor, 2004).

Regardless of the underlying molecular mechanisms antigenic switches are thought to produce the recrudescing waves of parasitaemia that are characteristic of infection with many *Plasmodium* species (figure 1.3). Recrudescing waves of parasitaemia are especially apparent, or at least well characterised, in naïve hosts (Mackinnon & Read, 2004b).



[3]Figure 1.3: Recrudescing peaks of parasitaemia

Graph illustrates the dynamics of a clonal *P. chabaudi* infection in a malaria naïve laboratory mouse. Note that the y-axis is a log scale and therefore the recrudescent peaks of parasitaemia are considerably lower than the initial acute peak.

1.2.1 Parasite subversion and suppression of the host immune system

In addition to antigenic variation at the IE surface *P. falciparum* evades and subverts the host immune response via other mechanisms. Urban and colleagues (1999) have shown that intact IEs can bind to myeloid dendritic cells and furthermore modulate their maturation and function *in vitro*. Exposure to IEs inhibits the upregulation of major histocompatibility class II molecules, adhesion molecules and co-stimulatory molecules on stimulated dendritic cells. Furthermore, the ability of these cells to stimulate T-cell responses was profoundly reduced (Urban *et al.*, 2001). Studies in a rodent malaria model suggest that this phenomenon is not restricted to *P. falciparum*. Ocana-Morgnar and colleagues have shown in laboratory mice that blood stage infection with *Plasmodium yoelii* inhibits dendritic cell maturation and their capacity to initiate immune responses to the liver stages of the parasite (Ocana-Morgner *et al.*, 2003). That blood stages of *Plasmodium* can impair the host's immunity to liver stages of the parasite, thus aiding the establishment of re-infection, illustrates the extent of *Plasmodium's* adaptations to its mammalian host.

The ability of *Plasmodium* infection to suppress the host immune response in general has long been suspected. Williamson and Greenwood (1978) found immune

responses to measles vaccination to be impaired in children with acute malaria. In some malarious regions there is a significantly higher incidence of Burkitt's lymphoma, an otherwise rare tumour of children in which B cells are transformed by the Epstein-Barr virus (Whittle *et al.*, 1984), and also *Salmonella* infections (Mabey *et al.*, 1987). The increase in incidence of Burkitt's lymphoma and *Salmonella* infections in children living in malarious regions suggests that the immune surveillance mechanisms responsible for either detecting or eliminating Epstein-Barr virus transformed B cells and *Salmonella* infected cells may be dampened by malaria infections. Acute blood stage infection has been found to impair cell-mediated immunity (Brasseur *et al.*, 1983; Ho *et al.*, 1986; Hviid *et al.*, 1991) and lymphocyte proliferative responses are inhibited by serum from *P. falciparum* infected patients (Theander *et al.*, 1987). Recently, *P. falciparum* schizont lysate was shown to modulate plasmacytoid dendritic cells, one of the two dendritic cell precursor populations in humans, which in addition to antigen presentation are though to play an important role in innate immunity. PCDs serve as antigen presenting cells for initiating adaptive immune response, but also produce high levels of the proinflammatory cytokine interferon-gamma (IFN-δ) in response to a number of pathogen products, for example viral and microbial DNA, and CpG DNA stimulation. Plasmacytoid dendritic cells (PDCs) exposed to schizont lysate or intact schizont infected cells have been shown to produce interferon-gamma, but not produce tumour necrosis factor-gamma and fail to exhibit a cell morphology concomitant with DC maturation (Pichyangkul *et al.*, 2004). Schizont exposed PDCs were shown to upregulate the surface expression of the co-stimulatory molecule CD86, but did express the costimulatory molecule CD40, a marker of DC maturation. Furthermore, schizont exposed PDCs elicited poor T cell responses, but promoted γδT cell proliferation. The work of Pichyangkul *et al.* suggests that schizont exposed PDCs play an important role in mediating an innate immune response to *P. falciparum* through the production of IFN-δ. However, due to the failure of the PCDs to complete maturation, may not perform the role of antigen presentation efficiently during malaria infections.

Plasmodium clearly subverts and evades the host immune response at multiple levels, probably in order to maintain chronic infections. This may be particularly important in areas of seasonal transmission. Unlike *P. falciparum*, *P. vivax* sporozoites can differentiate into dormant liver stages, called hypnozoites, such that the parasite can re-establish a blood stage infection again after up to four years. The

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[3] *P. chabaudi* infection data kindly shared by J.C. de Roode.

importance of chronicity for *P. falciparum* blood stage infection is illustrated by debates over the source of infection following the dry season in regions where malaria transmission is highly seasonal. It is known that some individuals maintain a very low-grade parasitaemia over the dry season in areas of seasonal and unstable transmission, such as parts of Sudan (Babiker *et al.*, 2000; Hamad *et al.*, 2000). These individuals can provide the pool of transmissible *P. falciparum* gametocytes when rains and mosquitoes return.

1.2.2 Variant surface antigens as virulence factors: cytoadhesion

Of the four species of *Plasmodium* infecting humans, *P. falciparum* causes the most severe morbidity and the great majority of malaria-related mortality. *P. falciparum* is the most prominent species in tropical Africa; however, this alone does not account for its larger clinical impact. *P. vivax* is widely distributed and causes relatively 'mild' although distressing and debilitating clinical symptoms such as paroxysm, an acute fever typically preceded by chills and rigors (Karunaweera *et al.*, 2003). Nonetheless *P. vivax* infections, like *P. ovale* and *P. malariae* infections, rarely cause fulminating disease that kills the patient.

In malaria endemic areas most episodes of *P. falciparum* infection in adults result in 'mild' clinical symptoms such as fever, malaise, lethargy and headaches. Severe clinical symptoms are most common in young children, first-time pregnant mothers and non-immune adults. Cerebral malaria accounts for a significant proportion of the severe clinical manifestations and deaths caused by *P. falciparum* infection and is characterised by an unarousable coma, often with convulsions, although syndromes with varying degrees of impaired consciousness also occur. Severe anaemia also contributes significantly to morbidity and mortality, and probably results from the destruction of normocytes (older red blood cells) and reduced levels of erythropoiesis. Additional severe symptoms include respiratory distress, renal dysfunction or failure and severe haemoglobinuria. However, defining clinical syndromes can be difficult due to overlapping symptoms and varying degrees of severity. The burden of malaria and the epidemiology of *P. falciparum* malaria are discussed further in Section 1.3.

P. falciparum differs markedly from the other human malarias in that the VSA expressed by mature IEs adhere to the endothelium of the host micro-vasculature (Udeinya *et al.*, 1981), such that trophozoites and schizonts are rarely seen in peripheral blood smears. The selective driving-force for the cytoadherent capabilities of *P. falciparum* IEs is thought to be avoidance of splenic clearance. The spleen-dependent expression of *P. knowlesi* surface antigens in laboratory primate models supports this view (Barnwell *et al.*, 1983). All *P. knowlesi* blood stage forms circulate, but this parasite does exhibit some sequestration in primate models (Galinski & Corredor, 2004; Miller *et al.*, 1971). In primate models with *P. falciparum*, *PfVSA* expression at the IE surface is also modulated by the presence or absence of a spleen and by immune pressure (Hommel *et al.*, 1983). The spleen has also been shown to play a role in modulating cytoadherence properties in *Plasmodium fragile*

infections of its natural primate host (David *et al.*, 1988; Handunnetti *et al.*, 1987) and in *Plasmodium chabaudi* infections of laboratory mice (Gilks *et al.*, 1990).

The degree to which the rigidity of the infected cell is altered also differs between *Plasmodium* species. *P. vivax* infection increases erythrocyte deformability whereas *P. falciparum* decreases deformability (Suwanarusk et al., 2004). Suwanarusk and colleagues propose *P. vivax* IEs escape splenic entrapment by increasing the deformability of the infected cell. This would support the need for *P. falciparum* IEs, but not *P. vivax* IEs, to evolve a mechanism to avoid splenic clearance.

Cytoadherence of *P. falciparum* presumably promotes the elevated parasitaemias seen in these infections although the importance of alternative mechanisms for *P. falciparum* pathogenesis have been contentious (e.g. Berendt *et al.*, 1994; Clark & Rockett, 1994) and remain under review (e.g. Heddini, 2002). It is generally excepted that cytoadherence to the microvasulature endothelium and to other structures such as the placental syncytiotrophoblast will result in the release of cytokines and thereby trigger the pathogenic inflammatory responses associated with cerebral malaria (reviewed in Mackintosh *et al.*, 2004) and pregnancy-associated malaria (Fievet *et al.*, 2001; Fried *et al.*, 1998a; Okoko *et al.*, 2003). As *Pf*VSAs mediate cytoadhesion they are thus instrumental in bringing about *P. falciparum* malaria pathogenesis (reviewed in Beeson & Brown, 2002; Craig & Scherf, 2001), and are thereby considered virulence factors. In support of this view is the finding that *P. knowlesi* lines not expressing VSAs are less virulent in a primate malaria model (Galinski & Corredor, 2004). Cytoadherence is also a characteristic of *P. chabaudi* infection in mice: in this model *Pc*VSA-ve lines show the same characteristics as *Pc*VSA+ve lines over the acute stage of infection where parasitaemias and morbity are highest; *Pc*VSA-ve *P. chabaudi* does not, however, produce recrudescing waves of parasitaemia and chronic infection (Gilks *et al.*, 1990).

*Pf*VSAs also mediate rosetting, a cytoadherence phenomenon where infected erythrocytes bind two or more uninfected erythrocytes (Handunnetti *et al.*, 1989; Udomsangpetch *et al.*, 1989). Studies have indicated that rosetting in *P. falciparum* associates with an increased risk of severe disease (Carlson *et al.*, 1990; Ringwald *et al.*, 1993; Rowe *et al.*, 1995; Rowe *et al.*, 2002b); however, some studies have failed to find this association (al-Yaman *et al.*, 1995; Rogerson *et al.*, 1999). The rosetting cytoadherence phenotype was first described in *P. fragile* infections of toque monkeys (David *et al.*, 1988) and has since been shown to occur in all four human

malaria species (Angus *et al.*, 1996; Lowe *et al.*, 1998; Udomsanpetch *et al.*, 1995) and, primate and rodent malarias (Mackinnon *et al.*, 2002; Udomsangpetch *et al.*, 1991).

As discussed in section 1.2.1 recent studies show that *Pf*VSAs can interact and modulate immune cells and also suggests that *Pf*VSA might moderate virulence (Urban *et al.*, 1999; Urban *et al.*, 2001). Urban and Roberts (2002) propose that parasites adhering to immune cells might inhibit immune and inflammatory responses which are potentially harmful to both host and parasite.

Additional differences between *P. falciparum* and other human malarias such as cell selectivity, may influence parasite replication rate and thereby contribute to parasite virulence. Invasion of human erythrocytes by *P. vivax* seems to be dependent upon a single receptor, but *P. falciparum*, and some other species, can utilise multiple alternative invasion pathways (Michon *et al.*, 2002). Decreased cell selectivity has been shown to be associated with disease severity in *P. falciparum* infections (e.g. Chotivanich *et al.*, 2000; Simpson *et al.*, 1999). It is likely that parasite virulence is a multi-locus trait and plastic depending on factors such as immune status and age of the host. Plasticity is certainly reflected in the genes responsible for antigenic variation at the infected erythrocyte surface.

1.2.3 Molecular mechanisms of antigenic variation

By far the most extensively characterised *Pf*VSA, and thus far the only one shown to mediate adhesion to endothelial receptors, is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP-1 is a large protein encoded by members of the *var* multigene family (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995), which consists of around 50-60 copies per *P. falciparum* genome. *Var* genes are highly divergent in sequence. The variability for certain regions of the gene is found on average to be as great within an isolate as between isolates (Kyes *et al.*, 1997). In addition, very little cross-hybridisation has been found when a complex probe derived from the *var* genes of a *P. falciparum* clone was used to probe the genomic DNA of a different clone (Taylor *et al.*, 2000).

Var genes are found at central and subtelomeric loci within the parasite's genome (Gardner *et al.*, 2002; Rubio *et al.*, 1996). Telomeric *var* genes lie within ordered repeat sequences, and are flanked by members of other multigene families. In the protozoan parasite *Giardia duodenalis*, genes that mediate antigenic variation are also found at the polymorphic ends of chromosomes (Upcroft *et al.*, 1997). It has been suggested that the flexibility of chromosome ends during meiosis is exploited as a

common tool for generating diversity by several pathogenic agents (Lanzer *et al.*, 1995). Frequent meiotic recombination within sub-telomeric regions (Taylor *et al.*, 2000), the large number of *var* genes per genome and the multidomain structure of PfEMP-1 (figure 1.4) provide great potential for generating variation, although some PfEMP1 alleles have been found to be relatively conserved in field isolates (Fried & Duffy, 2002; Rowe *et al.*, 2002a; Salanti *et al.*, 2003; Winter *et al.*, 2003). Heterologous recombination may also occur during mitotic division of *P. falciparum* blood stages (Freitas-Junior *et al.*, 2000).

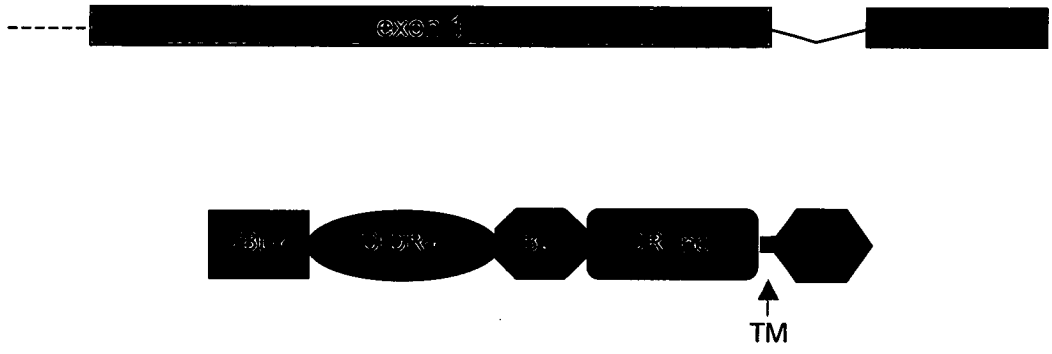


Figure 1.4: Schematic representation of a generic *var* gene and PfEMP1

Schematic based on the most common PfEMP1 structure in the genome of *P. falciparum* clone 3D7 (Gardner *et al.*, 2002); 38 out of 59 *var* genes encode this basic structure. The nomenclature follows that used by Gardner et al (2002). DBL-α: 'Duffy binding like domain of the α sub-class; only the α and γ sub-classes are present in the PfEMP1 depicted (3 further distinct DBL sub-classes are seen: δ, ε, and X); CIDR-α: 'cysteine rich interdomain region' type α (the remaining CIDR sequences do not form distinct tree branches and are therefore labelled not-α type); ATS: amino terminal sequence; TM: transmembrane sequence. Note this PfEMP1 does not incorporate a 'constant 2' region.

PfEMP1's cysteine-rich nature is shared by the surface antigens of *P. knowlesi* (Galinski & Corredor, 2004), a related apicomplexan haemoparasite *Babesia bovis* (Allred *et al.*, 2000), and the unrelated ancient eukaryotic parasite *Giardia duodenalis* (Upcroft *et al.*, 1997). The invasion receptors of *P. falciparum* and *P. vivax* from which PfEMP1 has arisen, due to gene duplication and diversification, are also cysteine-rich (Michon *et al.*, 2002). The significance of this is unknown, although a recent study by Hensmann *et al.* (2004) suggests the cysteine-rich nature of the 19 kDa C-

terminal fragment of the merozoite surface protein 1 (MSP1(19)), which confers a tightly folded structure and aspargine endopeptidase resistance, may impede the processing of this fragment by antigen presenting cells. Experimentally high levels of antibodies to MSP1(19) confer protective immunity against malaria, but natural antibody responses to this protein in human malaria infections are short-lived and relatively low, despite repeated exposure to infection.

PfEMP1 forms part of the Duffy binding-like (DBL) superfamily of proteins. Members of this family contain one or more domains with homology to a *P. vivax* merozoite surface protein that binds the Duffy blood group antigen, a host-cell invasion receptor (Adams *et al.*, 1992). The complete genome sequence of the *P. falciparum* clone 3D7 has shed more light on this interesting gene family (Gardner *et al.*, 2002). The *var* genes encoded by the 3D7 genome, and all other *vars* identified, thus far, share a similar gene structure (figure 1.4). A 5′ exon, between 3.5 and 9.0 kb in length, encodes the multidomain, extracellular portion of the protein which consists of both DBL domains and one or two cysteine rich interdomain regions (CIDR). A transmembrane region is also encoded at the 3′ end of the 5′ exon. A second smaller exon of between 1.0 and 1.5 kb encodes the intracellular amino terminal sequence (ATS) which is relatively well conserved (Gardner *et al.*, 2002). The *var* intron varies in length between 170 bp and 1.2 kb. Although the PfEMP1 structure shown in figure 1.4 is the most common in the 3D7 genome, it is the least well characterised. Selection for certain established cytoadherence phenotypes could explain the bias in the *var* genes studied thus far.

1.2.4 More multigene families and putative IE surface exposed proteins

In addition to the *var* multigene family, *P. falciparum's* genome contains two further highly variable multigene families: *rif* and *stevor* (Cheng *et al.*, 1998). The 3D7 genome encodes 59 *var*, 149 *rif* and 28 *stevor* genes, and additional pseudogenes and truncations are present for each family. The *rif* and *stevor* gene families encode proteins called repetitive interspersed family (rifin), and sub-telomeric variable open reading frame (stevor), respectively. Rifin proteins, like PfEMP1, are thought to be exported to the erythrocyte surface (Kyes *et al.*, 1999) and although stevor proteins have been found in Maurer's clefts (Kaviratne *et al.*, 2002), the IE surface has yet to be confirmed as their final destination.

Unlike the *var* gene family, homologues of *rif* genes are present in other *Plasmodium* species and together form the *Plasmodium* interspersed repeat (*pir*) gene superfamily

(Janssen *et al.*, 2004). The *pir* superfamily includes *cir*, *bir* and *yir* genes from the rodent malarias *P. chabaudi*, *P. bergei* and *P. yoelii* respectively (Carlton *et al.*, 2002; Janssen *et al.*, 2002), *kir* genes in *P. knowlesi* and *vir* genes in *P. vivax* (del Portillo *et al.*, 2001).

Finally, the *P. knowlesi* multi-gene family *sicavar* (al-Khedery *et al.*, 1999) and the *P. falciparum var* family are analogous in they encode variant surface antigens for which homologues have yet to be found in other *Plasmodium* species. Whether all or most *Plasmodia* possess species-specific VSA genes will soon come to light considering the current rate of genome sequencing for this genus.

In addition to variant multigene families a number of proteins, apparently encoded by single copy genes, have been proposed to be exposed at the surface of *P. falciparum* IEs, these proteins are summarised in Table 1.1. A mechanism by which proteins encoded by single copy genes would avoid recognition by the mammalian immune system and the subsequent destruction of the infected cell has yet to be proposed.

Table 1.1: Summary of *P. falciparum* IE surface exposed proteins

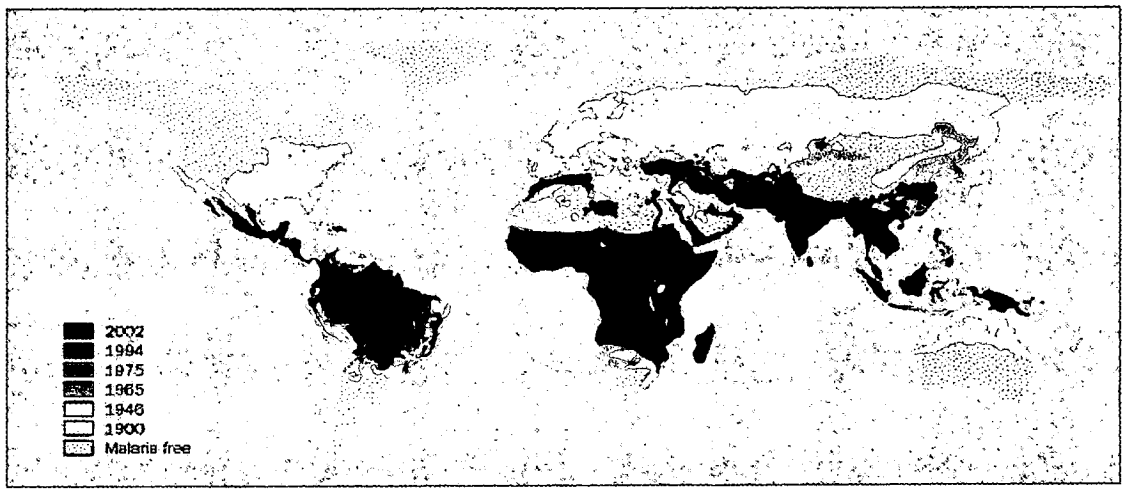
Abbreviations: Clag 9; cytoadherence associated gene 9, PIESP; parasite-infected erythrocyte surface protein, RSP; ring surface protein, RAP2; rhoptry associated protein-2

Protein	Gene	Function	References
PfEMP1	*var* family	Antigenic variation and cytoadherence to a number of endothelial cell and placental receptors.	(Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995)
Rifin	rifin family	Not known, but most likely antigenic variation due to the nature of the multicopy gene family.	(Fernandez *et al.*, 1999; Kyes *et al.*, 1999)
sequestrin	not known	Sequestrin is a ~270 kda protein and is thought to be encoded by a single copy gene, the protein has been proposed to mediate parasite adhesion to CD36.	(Ockenhouse *et al.*, 1991)
Clag 9	clag 9	Naturally occurring clag 9 knock-out parasites lose the ability to cytoadhere, therefore, a role for this gene in cytoadherence has been proposed. However, recent studies suggest that Clag9 is not on the IE surface, but may be necessary for the trafficking of VSAs or is required for the correct conformation of VSAs at the IE plasma membrane.	(Trenholme *et al.*, 2000) (Trenholme *et al.*, 2000) (Gardiner *et al.*, 2000) (Craig, 2000; Gardiner *et al.*, 2004)
PIESP1	PIESP1	A single copy gene recently identified through proteomic analysis, the function of this gene is currently unknown.	(Florens *et al.*, 2004)
PIESP2	PIESP2	A single copy gene identified along with PIESP1, like PIESP2 its function is currently unknown.	(Florens *et al.*, 2004)

RSP2 aka RAP2	RAP2	RAP2 is released from the micronemes of the merozoite during invasion and is transferred to the surface of the IE. In CSA binding parasite RSP2 is thought to mediate ring stage adhesion to the placental syncytiotrophoblast. How RSP2 would mediate ring stage adhesion for CSA binding parasites only, when it is present on ring stage parasites exhibiting an alternative adhesion phenotype is presently unclear.	(Douki *et al.*, 2003; Pouvelle *et al.*, 2000)
RSP1	Not known	Not known	(Pouvelle *et al.*, 2000)
Modified band III	anion exchange transporter-1	Band III is an abundant host anion-exchange transporter that is modified by the parasite *in situ*. Parasite mediated modifications to band III are thought to expose regions of the protein, such that cryptic adhesive domains are revealed. The cryptic binding domain has been named pfalhesin. Pfalhesin has been shown to bind thrombospondin and CD36.	(Crandall *et al.*, 1994; Crandall *et al.*, 1996; Crandall & Sherman, 1996)

1.3 The burden of malaria

While a single infection with some pathogens, principally viruses, is sufficient to elicit long-term 'sterile' immunity, sterile immunity to many parasites, including *P. falciparum*, is probably never achieved. The dynamics of acquired immunity to the *P. falciparum* malaria and the consequent severity of clinical symptoms depend on the intensity of transmission (e.g. Marsh & Snow, 1997). In regions of the world where malaria transmission is high, new-born infants are protected from severe malaria symptoms for their first 6 months of life, most likely due to passive transfer of immunity from their mothers (reviewed in Kyes *et al.*, 2001). As this immunity wanes children become susceptible to severe clinical symptoms. Severe malarial anaemia in areas of high malaria transmission is most frequently seen in young children (1-3 years old). Cerebral malaria, which may result in coma and death, is predominant in regions of moderate transmission and is often seen in somewhat older children (>3-4 years old) (Greenwood, 1997).



[4]Figure 1.5: Malaria remains a burden for many parts of the world

Distribution of malaria from pre-intervention to 2002. Data is presented for circa 1900 and for the years 1946, 1965, 1975, 1992, 1994, and 2002. Areas of high and low risk were merged to establish all-cause malaria transmission limits (Hay *et al.*, 2004).

[4] Reprinted from The Lancet, Vol. 4, Hay *et al*, The global distribution and population at risk of malaria: past, present, and future, pages 327-336, Copyright (2004), with permission from Elsevier

Despite severe malaria and malaria related mortality being relatively rare, due to the ubiquity of the parasite (figure 1.5), malaria kills up to 3000 children in Africa each day (0.5%-1% of all malaria cases) (Snow *et al.*, 1999; UNICEF, 2003). In malaria-endemic regions children under the age of five bear the brunt of malaria mortality. Immunity to non-cerebral, severe clinical manifestations is postulated by some to require few clinical episodes (Gupta *et al.*, 1999). Adults in areas of high transmission rarely suffer severe clinical symptoms. There is one exception: pregnant women (Menendez, 1995). This special case is discussed in more detail in the next section.

Falciparum malaria has a direct impact on the lives of the patient and their families, and its ramifications at the level of community and state, in terms of restraining economic growth and development, are also becoming realised (Breman, 2001; Russell, 2004). Although malaria has been eradicated from Europe and North America, long-term control has thus far proved impossible for many parts of the world. Malaria remains endemic in parts of Asia, Africa, Central and South America, Oceania, and certain Caribbean islands (figure 1.5, Hay *et al.*, 2004). Political and socio-economic factors, healthcare infrastructure, poor education and housing, high transmission intensities, drug resistance of the parasite and vector, and probably many more factors have played roles in ensuring only short-term success, or complete failure, of malaria control initiatives in those countries bearing the burden of malaria today.

1.4 Pregnancy-associated malaria

1.4.1 Pregnancy-associated malaria is an important public health problem

The remainder of this introduction is focused on one possible approach to alleviate one particular syndrome: pregnancy-associated malaria (PAM). PAM is estimated to result in 75 000 – 200 000 infant deaths per year (Steketee *et al.*, 2001). In areas of stable transmission, PAM increases the risk of low birth-weight babies, probably by intrauterine growth retardation, but possibly also by premature delivery. Although premature delivery is more difficult to establish without early prenatal screening and healthcare unit attendance by the mother (Menendez *et al.*, 2000; Meuris *et al.*, 1993; Shulman *et al.*, 1999). In sub-Saharan Africa, low birth-weight is a strong predictor of a child's fitness (Guyatt & Snow, 2001a) and PAM is thought to be one of the most preventable non-genetic causes of low birth weight in such endemic

areas (Brabin & Rogerson, 2001). For the mother there is a higher likelihood of severe malaria syndromes such as severe anaemia (Guyatt & Snow, 2001b). In areas of seasonal transmission, stillbirth rates are higher than in areas of high transmission and the symptoms suffered by mothers more severe (Duffy, 2003). At least 50 million pregnancies each year are at risk of malaria infection (Steketee *et al.*, 2001).

1.4.2 Pathogenesis of pregnancy-associated malaria

During pregnancy the immunological status of the mother is modified to prevent immune rejection of her allograft: her foetus. Several immunological factors are observed to change during gestation, notably a swing towards type 2 responses (non-inflammatory). However, epidemiological data indicate that most microbes do not pose an increased threat for pregnant women, the exceptions including cytomegalovirus reactivation, possibly leprosy relapses and higher parasite loads of the intracellular parasites *Trypanosoma* and *Plasmodium* (reviewed in Guilbert *et al.*, 2001). *P. falciparum* infection during pregnancy is characterised by the sequestration if IEs in the placenta such that parasite densities can become extreme. 90% parasitaemias have been reported (Brabin *et al.*, 2004). Non-falciparum PAM also occurs, but is not well documented, thus PAM shall refer to *P. falciparum* infections unless otherwise stated. *P. vivax* infection during pregnancy has been shown to cause lower birth weights and mild maternal anaemia in areas of low transmission (Nosten *et al.*, 1999), but the frequency of placental infection and the placental parasite densities tend to be much lower when compared to *P. falciparum* (McGready *et al.*, 2002; McGready *et al.*, 2004).

The hallmark of PAM is the sequestration of IEs on the maternal side of the placenta. IEs being detected in rare cases in Giemsa stained smears of chord blood, but not in foetal erythrocytes or foetal structures (Ismail *et al.*, 2000; Redd *et al.*, 1996). Haemozoin has also been found in the foetal trophoblastic cells (McGready *et al.*, 2002; McGready *et al.*, 2004), which cover the foetal villi of the placenta and act as a barrier between maternal and foetal blood (figure 1.6).

IEs are usually found in the intervillous spaces of the placenta closely associated with the syncytiotrophoblast cell layer (figure 1.6). Mature parasite stages have only been detected in the peripheral circulation in severe clinical cases of PAM (Beeson *et al.*, 2002a) and it is the accumulation of parasites in the placenta that is thought to lead to poor clinical outcomes for mother and child. As placental

histological examinations and placental blood smears can only be carried out at birth. Since pregnant women in endemic regions often have low peripherial parasitaemias, despite harbouring high-density placental infections (Brabin & Rogerson, 2001), infections in earlier stages of gestation are usually inferred by the presence of malaria pigment in monocytes and/or other immune cells in the placenta at birth.

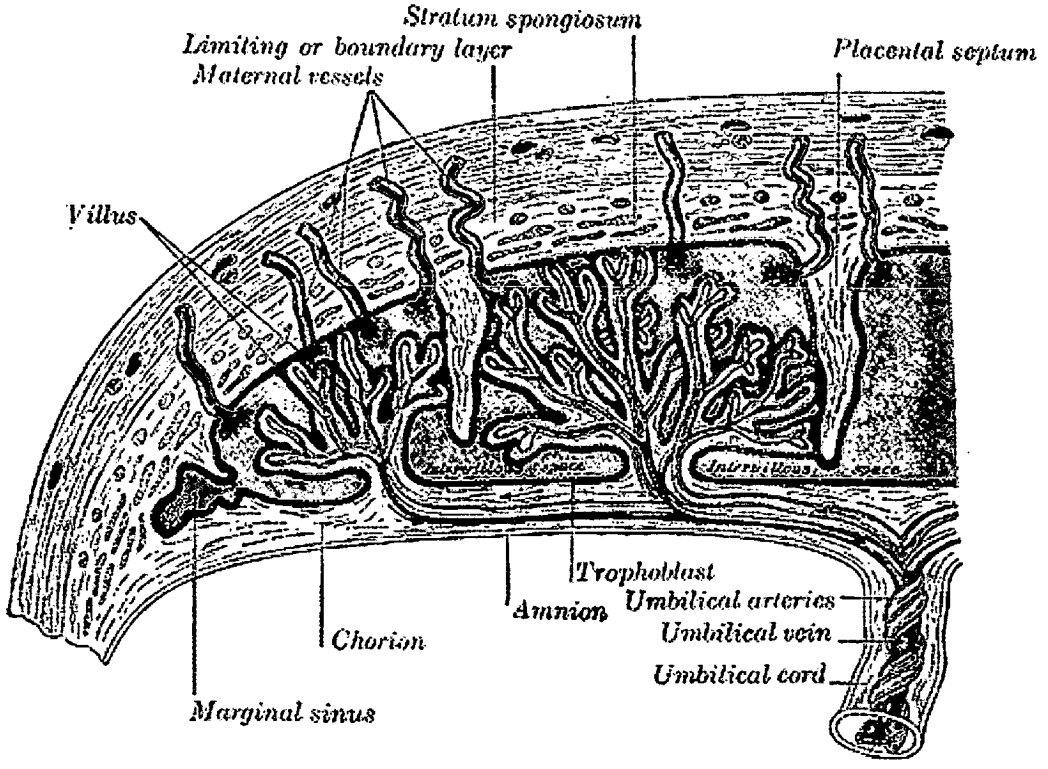


Figure 1.6: Schematic of the placental circulation

The placenta consists of a foetal and a maternal portion, the former consists of repeatedly branching villi suspended in an intervillous space. Maternal blood is carried into the intervillous space by uterine arteries where it bathes the foetal the villi. Uterine veins return maternal blood away from the placenta. A branch of an umbilical artery enters each villus and terminates in a capillary plexus from which blood is drained by a tributary of the umbilical vein. The foetal vessels within the villus are wrapped in a thin layer of mesoderm and two layers of cells derived from the trophoblast. The deeper layer of cells adjacent to the mesoderm is the cytotrophoblast layer and the superficial layer in contact with the maternal blood the syncytiotrophoblast layer. After the fifth month of gestation a single layer of flattened syncytiotrophoblast replaces these two cell layers. (Reproduced from: Gray, Henry. *Anatomy of the Human Body.* Philadelphia: Lea & Febiger, 1918; Bartleby.com, 2000. www.bartleby.com/107/.)

The infiltration of inflammatory cells into the intervillous space of the placenta (intervillousitis) has been closely associated with placental parasitaemia (Ismail *et al.*, 2000). This inflammatory infiltrate consists mainly of monocytes and macrophages (Bulmer *et al.*, 1993; Ordi *et al.*, 2001), and was found by Ordi *et al.* (1998) to be more prevalent in primigravidae and its occurrence to associate with low birth weight. Consistent with the observation of inflammatory cells in infected placentae is the imbalance in inflammatory (type 1) and non-inflammatory cytokines (type 2). Fried *et al.* (1998a) found in cases of PAM a decrease in the type 2 cytokine interleukin-10 and higher levels of the type 1 cytokines interferon-gamma, interleukin-12 and tumour necrosis factor-alpha in infected placentae. PAM-induced type 1 cellular responses are thought to be related to reduced foetal fitness (Okoko *et al.*, 2003).

1.4.3 Placental isolates exhibit a distinct adhesive phenotype

Sequestration and placental adhesion in the placenta is considered instrumental in triggering PAM pathogenesis. The characterisation of the host receptors mediating this parasite interaction has progressed significantly over recent years. A number of studies have shown that the glycosaminoglycan chondroitin-sulphate A (CSA) can support IE adhesion (Chaiyaroj *et al.*, 1996; Robert *et al.*, 1995; Rogerson *et al.*, 1995). CSA is a polymer comprising alternating glucuronic acid and 4-sulphated N-acetylglucosamine residues. In terms of polymer length and the pattern and degree of sulphation biological sources of CSA are highly heterogeneous (Karamanos *et al.*, 1994). Several studies have shown that placental IEs can bind many sources of CSA (Beeson *et al.*, 1999; Fried & Duffy, 1996), but binding is dependent on the degree of sulphation (Fried *et al.*, 2000) and molecular size, a tetradecasaccharide being the minimum size able to significantly inhibit IE binding. Achur and colleagues (2000) isolated placental CSA and identified several distinct types of chondroitin sulphates, but found the uniquely low sulphated extracellular CSA that localises to intervillous spaces (figure 1.6) bound IEs most efficiently. The validity of earlier studies suggesting the glycosaminoglycan hyaluronic acid (HA) is also a placental receptor (Beeson *et al.*, 2000; Beeson *et al.*, 2002b) have been questioned due to concerns with the purity of HA preparations and contamination with CSA (Duffy & Fried, 2003a). Recently, however, Beeson and colleagues (2004) have confirmed that HA can also support IE adhesion. HA like CSA can be extracted from placental tissue and is composed of repeating disaccharide units. *N*-acetylglucosamine and glucuronic

acid constitute HA, but it is not sulphated and its chemical composition is thus less variable than CSA (Smith & Miller, 2004). Interestingly, Beeson and colleagues (2004) have recently shown some parasites to express VSA with dual specificity, such that parasites can adhere to both CSA and HA.

Placental isolates also exhibit dichotomy in terms of their adhesive phenotype, in that they do not generally bind the endothelial receptors CD36 and ICAM1 (Beeson *et al.*, 1999; Beeson *et al.*, 2000; Fried & Duffy, 1996; Maubert *et al.*, 1997), which are commonly exploited by IEs isolated from the periphery of non-pregnant persons. *In vitro* binding studies by Maubert *et al.* (1997) using syncytiotrophoblasts found parasites to bind surface expressed ICAM1, but the parasites studied were not isolated from pregnant women.

It had become dogma that only the mature asexual stages of *P. falciparum* adhere and sequester, however, the work of Pouvelle and colleagues (2000) showing ring stage adhesion to brain and lung endothelial cells, and placental syncytiotrophoblasts challenges this. Interestingly, although ring stage adhesion was not inhibited by CSA, ring adherent parasites developed into trophozoites expressing exclusively CSA adhesion phenotypes. This was also the first demonstration that IEs can sequentially exhibit two distinct adhesion phenotypes during a single intraerythrocytic cycle. Ring stage adhesion may explain parasites being scarce or at undetectable levels in the peripheral circulation of women with PAM despite substantial placental parasitaemias (Ismail *et al.*, 2000).

1.4.4 Parity dependent acquisition of immunity

Although the severity of PAM symptoms has been shown to be dependent on transmission intensity, duration of infection and timing of infection (Brabin *et al.*, 2004), the most striking feature of PAM epidemiology is the parity dependence of susceptibility (McGregor *et al.*, 1983). Women in their first and second pregnancy are more susceptibility to infection and clinical consequences such as maternal anaemia (Matteelli *et al.*, 1994), intervilliousitis (Ismail *et al.*, 2000) and low birth weight (Watkinson & Rushton, 1983) are more severe than in women in later pregnancies. This parity dependent nature suggests immunity to PAM can be acquired as a function of parity in an analogous fashion to acquired immunity to non-PAM can be acquired with age and exposure. There is also a parity dependence for susceptibility to *P. vivax* infection during pregnancy, but in contrast

to *P. falciparum*, the negative impact of *P. vivax* infection on birth weight is more pronounced in multigravidae (Nosten *et al.*, 1999).

The findings of a large body of work suggest PAM's parity dependent nature lies in the ability of the parasites infecting the placenta to switch VSA expression to antigenically distinct variants. The development of the placenta during pregnancy is thought to provide novel adhesion receptors such that the VSA of placental binding parasites are structurally different to the VSA structure necessary to bind vascular endothelial receptors. The apparent mutually exclusive expression of VSA that bind either CSA or CD36 supports this theory. Immuno-epidemiological studies suggest the primigravidae lack substantial levels of antibodies to the VSA of placental isolates (VSA_{PAM}) and anti-VSA_{PAM} antibodies are acquired following *P. falciparum* infections during pregnancy (Fried *et al.*, 1998b; Maubert *et al.*, 1999; Ricke *et al.*, 2000). That men living in malaria endemic lack antibodies reactive to the surface of CSA-binding parasites further supports that the VSA of placental isolates are antigenically distinct (e.g. Ricke *et al.*, 2000). The primary role of VSA_{PAM} specific antibodies in protecting multigravidae is thought to be their ability to prevent IEs adhering to placental receptors (Fried *et al.*, 1998b; Maubert *et al.*, 1999; O'Neil-Dunne *et al.*, 2001; Ricke *et al.*, 2000).

The potential of anti-VSA antibodies in naturally acquired immunity to malaria in general was first investigated in longitudinal studies carried out by Marsh and colleagues (1989). Over the duration of a malaria transmission season in The Gambia *in vitro* assays to measure several types of immune response to asexual stage IEs were carried out using a large cohort of children. These studies found pre-existing levels of antibody to the IE surface to be the best predictor of protection against infection and clinical symptoms. Further studies in geographically diverse malarious areas found that during acute attacks of *P. falciparum* malaria in children, isolate-specific antibodies to the IE surface are developed, further supporting VSAs as targets for naturally acquired immunity (Bull *et al.*, 1998; Iqbal *et al.*, 1993). The acquisition of anti-VSA_{PAM} antibodies through exposure to infection following one or more pregnancies is analogous to the acquisition of immunity to VSA through childhood in malaria endemic regions.

Alternative and or additional immunological aspects of primigravidae such as generally dampened humoral and cellular mechanisms may contribute to the parity dependent nature of PAM (Fievet *et al.*, 2002). Rasheed *et al.* (1993) suggest primigravidae may be more susceptible to malaria because of physiologic factors, such as higher levels of circulating immunosuppressive corticosteroids.

1.4.5 A vaccine against PAM?

PAM is the clearest example in malaria pathology research of a strong association between infected erythrocyte sequestration and a particular disease syndrome (reviewed in Reeder, 1999). Antibodies against VSA_{PAM} that prevent adhesion to placental receptors (Duffy & Fried, 2003b) or at least antibodies that recognise the antigenically distinct surface of placental binding isolates (Staalsoe *et al.*, 2004) are associated with protection from the clinical symptoms of PAM for both mother and child. The VSA epitopes recognised by female-specific, parity-dependent antibodies are, therefore, considered rational candidates for inclusion in an experimental vaccine to protect women against PAM (e.g. Duffy & Fried, 1999; Duffy & Fried, 2003a; Fried *et al.*, 1998b; Staalsoe *et al.*, 2002). Since *P. falciparum* erythrocyte surface antigens are of a variant nature, their suitability as vaccines has been questioned. However, one of the initial serological studies of placental parasites from diverse geographical regions suggests the epitopes recognised by antibodies acquired during PAM are relatively conserved (Fried *et al.*, 1998b). Sera from Malawian, and Thai secundigravid and multigravid women were equally effective at blocking CSA adhesion of Kenyan parasites as Kenyan sera despite the parasite being geographically and presumably genetically distinct. These studies suggest that a limited number of VSA_{PAM} epitopes may be effective worldwide. As discussed earlier the host receptors mediating the interaction of parasite and placentae are relatively well defined. However, the identity of the parasite VSA interacting with placental CSA and HA *in vivo*, despite the question attracting the attention of many international groups, has remained contentious.

1.4.6 PfEMP1 and placental adhesion

The obvious suspect for mediating placental adhesion is PfEMP1 and two independent studies of laboratory isolates found two distinctly structured variants to bind CSA: FCR3*var1*CSA (Buffet *et al.*, 1999) and *var*CS2 (Reeder *et al.*, 1999). Binding assays using heterologous expressed domains from both FCR3*var*1CSA and *var*CS2 showed the DBL-γ sequence of both of these genes to encode CSA binding domains *in vitro* (Buffet *et al.*, 1999; Reeder *et al.*, 2000). *var* DBL-γ domains have thus received much attention as candidates for an adhesion blocking PAM vaccine. However, Reeder *et al.* (2000) also showed recombinant CIDR1-α of *var*CS2 to bind CSA and their findings were corroborated by Degen *et al.* (2000), who showed

recombinant CIDR1-α domains from two further PfEMP1 variants could also bind to cell surface CSA on Chinese hamster ovary (CHO) cells. Only antibodies raised to the recombinant *var*CS2 DBLγ domain however, inhibited CSA binding (Reeder *et al.*, 2000).

A number of studies have also described raising antisera to the recombinant DBL-γ FCR3*var*1CSA. Although anti-DBL-g sera were generally cross-reactive towards CSA-binding isolates and placental isolates, the CSA adhesion blocking capacity of different sera was variable. Lekana Douki *et al.* (2002) tolerised mice B cells to the surface of human erythrocytes or Chinese hamster ovary (CHO) cells prior to immunisation with CSA binding IEs or CHO cells expressing the DBL3γ domain of FCR*var*1CSA at their surface. 43 IE-surface-reactive monoclonal antibodies (MAbs) were derived from these mice, 70.8% of which were of the IgM subclass. Some of the MAbs immunoprecipitated a surface-exposed protein of ~400 kDa and several recognised the surface of CSA-binding parasites and placental isolates. About a third of the MAbs reacted to isolates with CD36 and ICAM1 adhesion phenotypes in immunofluorescence assays using fixed cells. The MAb's adhesion blocking capabilities were not determined in this study.

Costa and colleagues (2003) raised mouse MAbs against recombinant DBL3γ (FCR3*var*1CSA) produced in insect cells and two were characterised. They recognised the surface of a number of peripheral and placental isolates from West Africa, Brazil and Cameroon and the FCR3 parasite clone after selection for CSA adhesion but not after selection for CD36 adhesion. One of the mouse monoclonal antibodies of the IgG1 subclass was found to inhibit parasite binding to CSA.

Despite this apparently strong evidence supporting the expression of FCR3*var*1CSA and *var*CS2 at the erythrocyte surface of CSA-binding IEs, transcriptional studies have led some members of the field to question the involvement of these gene products in mediating adhesion of placental and CSA binding parasite. A more thorough analysis by RT-PCR and northern blotting of the *var* genes transcribed by the CSA-binding CS2 parasite showed neither FCR3*var*1CSA nor *var*CS2 to be the dominant transcripts in this parasite clone (Duffy *et al.*, 2002). Neither FCR3*var*1CSA nor *var*CS2 were up-regulated following CSA selection although a 13.2-14.0 kb *var* transcript whose identity was not revealed was significantly up-regulated. Further transcriptional analysis by Kyes and colleagues (2003) has cast more doubt on the role of FCR3*var*1CSA in placental adhesion as its 15 kb transcript was found to be constitutively expressed by several laboratory isolates, including FCR3, throughout the erythrocytic cycle and its expression was unrelated to the

parasite adhesion phenotype. Like Duffy *et al.* (2002), Kyes and colleagues (2003) also found a 14 kb *var* to be the only *var* transcript to be up-regulated following CSA selection of the FCR3 parasite clone. The 14 kb *var* transcript was also expressed abundantly in ring stages. Rings are considered the appropriate developmental stage in the erythrocytic cycle for *var* transcripts to be expressed if they are destined to be transcribed and expressed at the erythrocyte surface (Kyes *et al.*, 2003).

Transcriptional studies using field isolates also cast confusion over the role for the *var*CS2 and FCR3*var*1CSA genes in PAM. A number of studies have found DBLγ domains to be expressed by placental isolates from geographically diverse regions (e.g. Fried & Duffy, 2002; Khattab *et al.*, 2001; Khattab *et al.*, 2003). However, Rowe *et al.* (2002a) used degenerate primers to the *var* DBL-α domain and found conserved homologues of FCR3*var*1CSA to occur in the genomes of the majority of field isolates examined from around the world. Transcripts of the FCR3*var*1CSA gene were not expressed by placental isolates from Malawian primigravid women. Rowe *et al.* (2002a) did find the 3D7chr5*var* gene, which within its 3′ end (encoding a DBL5γ and DBL7ε) has high homology to the FCR3*var*1CSA gene, to be expressed by 4 out of 5 placental isolates. However, a subsequent study by Winter *et al.* (2003) suggested that 3D7chr5*var* related genes are also transcribed in non-placental isolates. Field studies have, thus far, not identified genes with homology to *var*CS2 to be the major transcripts in placental isolates. Transcriptional analysis of field samples appears to depend heavily on the design of the *var* DBL domain primer sequences. Combining this with a limited and potentially biased choice of isolates thus allows misleading associations to emerge.

An extensive real-time RT PCR analysis of the NF54 parasite, the parent of the completely sequenced 3D7 clone, pre- and post-CSA selection found a distinctly structured *var* transcript of 9.2 kb to be up-regulated following CSA selection (Salanti *et al.*, 2003). This gene was named *var*2csa and is highly conserved in field isolates. The CSA binding capacity of the domains encoded by this gene was not determined in this study.

It remains contentious as to which PfEMP1 or *Pf*VSA is expressed at the surface of the mature stages of CSA-binding parasite clones, and a HA binding *Pf*VSA has yet to be described. Recently, one of the molecules expressed at the erythrocyte surface of ring stage parasites that acquire a CSA-binding phenotype later in the erythrocytic cycle, however, has been identified. A ~40 kDa protein called ring surface protein-2 (RSP-2) was found to share antigenic similarity to the rhoptry associated protein (RAP-2) (Douki *et al.*, 2003). Interestingly, RSP-2 is also present

on the surface of uninfected erythrocytes in *P. falciparum* cultures. Douki *et al.*, (2003) suggest that RSP-2 is a remnant of abortive merozoite invasion on uninfected cells cultured with *P. falciparum*. Another surface-exposed protein of ~180 kDa was also described by Pouvelle *et al.* (2000) in their first study that reported ring stage adhesion; this protein was named ring stage protein–1 (RSP-1) but the gene encoding this protein remains unidentified.

1.5 Thesis aims

The erythrocyte surface antigens of *P. falciparum* IEs that mediate adhesion to the placenta are considered instrumental in bringing about PAM pathology. Immuno-epidemiological data supports the candidacy of these antigens for inclusion in a vaccine against PAM. The molecular identity of these surface antigens, however, remained contentious on initiation of this thesis. Therefore, my central aim was to further characterise the molecular nature of the surface antigens expressed by 'placental-like' *P. falciparum* laboratory clones and specifically those epitopes to which an antibody response is raised during PAM.

I use *in vitro* cultures of *P. falciparum* IEs that have been selected to bind the placental receptor CSA as a model for parasites that sequester in the placenta. Since CSA and placental binding parasites have previously been shown to express *Pf*VSA exhibiting a marked trypsin-resistant binding phenotype, my first set of experiments, described in Chapter 2, are aimed at defining the protease sensitivity of those surface epitopes recognised by antibodies from malaria-exposed pregnant women. The ultimate aim of these experiments is to identify VSA exhibiting differential protease sensitivities that might be exploited in subsequent proteomic analysis of CSA-binding *P. falciparum* clones.

The molecular mechanisms behind the regulation of *Pf*VSA expression are complex and not completely understood. The aim of a large part of this thesis, therefore, is to develop an alternative method to complement existing molecular techniques, ultimately for the identification of VSA_{PAM}. These experiments are described in Chapter 3. A non-radioactive cell surface protein labelling technique is sought that allows electrophoretic separation and/or the affinity purification of *Pf*VSA prior to identification using mass spectrometric techniques. Such a venture was considered timely due to the recent advances in mass spectrometry that have made protein identification possible when the genome sequence of an organism is available. The genome sequence of a *P. falciparum* clone was in its final stages of completion on initiation of this thesis. In Chapter 4 I apply a Western blotting technique developed in Chapter 3 to the further characterisation of two novel *Pf*VSA candidates, *Plasmodium falciparum* Cysteine Repeat Modular proteins 1 and 2.

I also apply an erythrocyte surface labelling technique to an *in vivo* model of malaria, using the rodent malaria *Plasmodium chabaudi*. This work was originally initiated to address problems faced with the surface labelling of *P. falciparum* IEs and is described in Chapter 5.

My main findings and their context within the field as it currently stands are discussed in Chapter 6. Chapter 7 contains a description of the materials and methods used during this body of work.

Chapter 2. CSA binding *Plasmodium falciparum* infected erythrocytes express trypsin resistant surface epitopes

2.1 Abstract

The ability of *Plasmodium falciparum*-infected erythrocytes to adhere to the microvasculature endothelium is thought to play a causal role in malaria pathogenesis. Cytoadhesion to endothelial receptors is generally found to be highly sensitive to trypsinisation of the infected erythrocyte surface. However several studies have found that parasite adhesion to placental receptors can be markedly trypsin-resistant. I have investigated this phenomenon in experiments testing whether chondroitin sulphate A (CSA)-binding parasites express trypsin-resistant variant surface antigens (VSA) and whether these trypsin-resistant VSA bind female-specific antibodies that are induced by pregnancy-associated malaria (PAM).

Fluorescence activated cell sorting (FACS) was used to measure the levels of adult, Scottish, Ghanaian male and Ghanaian pregnant female, plasma immunoglobulin G (IgG) that bind to the surface of infected erythrocytes. *P. falciparum* clone FCR3 cultures were used to assay surface IgG binding before and after selection of the parasite for adhesion to CSA. The effect of proteolytic digestion of parasite erythrocyte surface antigens on adhesion to CSA and hyaluronic acid (HA) was also determined.

I have shown that *P. falciparum*-infected erythrocytes selected for adhesion to CSA express trypsin-resistant VSA that are the target of naturally acquired antibodies from pregnant women living in a malaria endemic region of Ghana. However, *in vitro* adhesion to CSA and HA was relatively trypsin-sensitive. This study highlights discordance between the trypsin sensitivity of placental receptor binding and surface recognition of CSA selected parasites by serum IgG from malaria-exposed pregnant women. I conclude that the complete molecular definition of a *P. falciparum* erythrocyte surface protein that could be used as a malaria-in-pregnancy vaccine has yet to be achieved.

2.2 Introduction

The rapid clearance of parasites when IgG from malaria immune adults is transfused into clinically ill recipients illustrates that naturally acquired antibodies have a parasite clearing role in human malaria infection (Bouharoun-Tayoun *et al.*, 1990; Cohen *et al.*, 1961; Sabchareon *et al.*, 1991). Neither the nature of the protective immune response nor the target antigens and epitopes recognised by infection clearing antibodies are fully understood. Evidence is accumulating to suggest that the acquisition of antibodies binding the VSA on infected erythrocytes (IEs) plays a major role in the development of age- and exposure-dependent immunity (Bull *et al.*, 1998; Dodoo *et al.*, 2001; Marsh *et al.*, 1989; Nielsen *et al.*, 2002; Ofori *et al.*, 2002). The evidence for a protective anti-VSA response is particularly strong for the PAM syndrome (Duffy & Fried, 2003b; Staalsoe *et al.*, 2004).

In vitro selection of infected erythrocytes for adhesion to CSA concomitantly selects for expression of VSA that share characteristics with post-natal placental isolates. Thus plasma antibodies from malaria-exposed pregnant women, or multi-gravid women, recognise the VSA of CSA-binding parasites (here referred to as VSA_{PAM}). These sera can also block adhesion of CSA-selected IEs to CSA *in vitro* (Ricke *et al.*, 2000). Interestingly, as malaria-exposed males generally do not acquire antibodies that bind CSA-selected parasites such that there is a striking female-specific antibody response recognising both *in vitro* CSA-selected parasites (Ricke *et al.*, 2000; Staalsoe *et al.*, 2001) and *P. falciparum* isolates taken from infected placentae at delivery (Khattab *et al.*, 2004; Salanti *et al.*, 2003; Staalsoe *et al.*, 2004). Furthermore, the levels of CSA-adhesion blocking plasma IgG have been shown to increase with adult female parity. Recent immuno-epidemiological studies show a strong positive correlation between the levels of antibodies that recognise the IE surface (Staalsoe *et al.*, 2004), the level of CSA-adhesion blocking antibody (Duffy & Fried, 2003b) and positive birth outcomes as measured by birth weight.

Var genes encode large extracellular domains rich in lysine and arginine residues; it is therefore not surprising that PfEMP-1 molecules and adhesion to endothelial receptors have been reported to be highly sensitive to trypsin treatment (Baruch *et al.*, 1995; Biggs *et al.*, 1990; Howard *et al.*, 1988; Leech *et al.*, 1984; Magowan *et al.*, 1988). Less expected were studies showing parasite adhesion to the placental receptor CSA, when immobilized (Beeson *et al.*, 2000; Beeson & Brown, 2004; Chaiyaroj *et al.*, 1996) or when cell surface associated (Buffet *et al.*, 1999; Rogerson *et al.*, 1995), can be relatively trypsin-resistant. This chapter investigates the protease-

sensitivity profile of the VSA_{PAM} expressed by CSA-selected parasite clone FCR3 with regard to recognition by antibodies acquired during PAM and adhesion to placental receptors.

2.3 Results

2.3.1 Concomitant selection of a trypsin-resistant VSA following parasite selection for CSA adhesion

Erythrocytes infected with parasite clone FCR3 were repeatedly selected for adhesion to CSA by panning. Late stage parasites were incubated with CSA (Sigma) immobilised on polystyrene Petri dishes for 30 minutes, the non-CSA adherent IEs were removed by washing and the adherent population incubated in the Petri dish with fresh uninfected erythrocytes for 20 hours overnight to allow the parasites to develop and the merozoites from the CSA-adherent parasite population to reinvade. Ring stage parasites were then removed from the Petri dish and maintained under standard parasite culture conditions (see Chapter 7; Materials and Methods). IEs were selected for CSA adhesion on at least a fortnightly basis. It was then established that selection of clone FCR3 for adhesion to CSA resulted in the concomitant selection of VSA specifically recognised by plasma IgG from malaria exposed Ghanaian pregnant women (IgG*preg*) (figure 2.1). An increase in the binding of IgG from a pool of plasma from malaria-exposed Ghanaian men (IgG*male*) was not observed. The unselected FCR3 clone expressed VSA that were equally well recognised by antibodies in the IgG*male* and IgG*preg* serum pools (figure 2.1). These interactions between serum antibody binding and selection for CSA adhesion were highly significant ($F_{2,24}$ =9.5, p = 0.001).

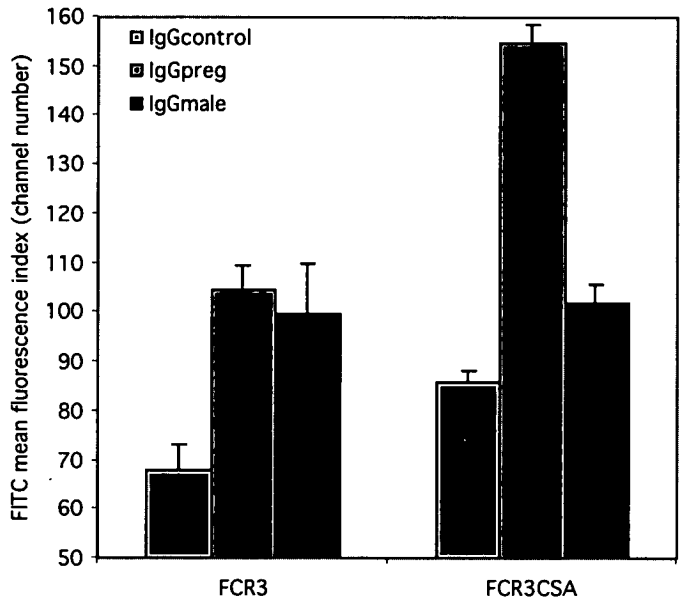


Figure 2.1: IgG recognition profiles of parasite clone FCR3 before and after selection for adhesion to CSA.

Totals of 5000-10000 late-stage parasites were gated using ethidium bromide, and FITC fluorescence due to serum IgG binging was measured. Serum samples from 6 Scottish malaria naïve individuals were pooled and used as a control (IgG*control*). Sera from 20 Ghanaian men were pooled for the malaria exposed male serum pool (IgG*male*). Sera collected at the time of birth from the placentae of 15 Ghanaian women were pooled for the malaria-exposed pregnant female pool (IgG*preg*). Bar chart shows means (+1 s.e.m.) for 5 independent experiments.

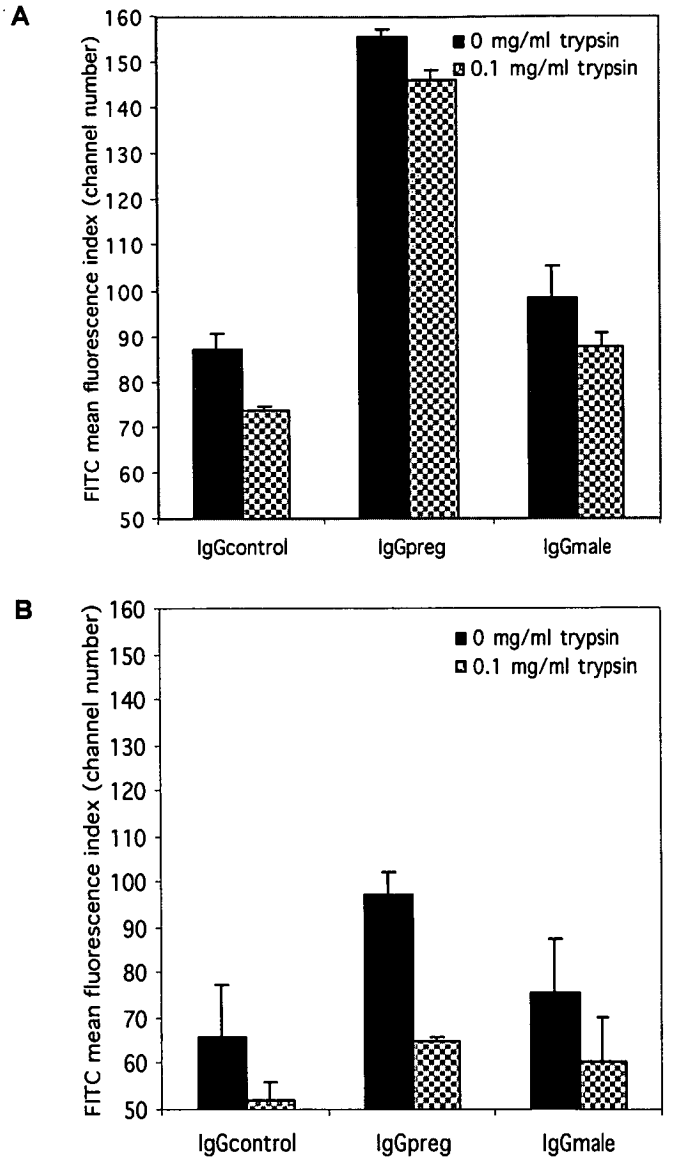


Figure 2.2: Serum IgG from malaria-exposed pregnant women recognise trypsin-resistant surface epitopes

Intact infected erythrocytes were treated with 0.1 mg/ml trypsin prior to FACS analysis. Panels A and B show serum IgG binding to the surface of FCR3CSA and FCR3 infected erythrocytes respectively. Serum pools are the same as those described in figure 2.1. Bar charts show means (+1 s.e.m.) for 2 independent experiments.

The trypsin sensitivity of this VSA/IgG binding interaction and of parasite adhesion to CSA was then measured. Parasitised erythrocyte surface trypsinisation at a concentration of 0.1 mg/ml showed that the IgG*preg* binding of FCR3CSA was significantly more trypsin-resistant than was binding of the same serum to the unselected clone (figures 2.2A&B; $F_{1,4}$=16.4, p=0.015). Although the mean surface fluorescence due to the IgG*preg* binding of FCR3CSA was slightly reduced by 0.1 mg/ml trypsin this reduction was not significant (figure 2.2A; $F_{1,2}$=11.3, p>0.05). The effect of 0.1 mg/ml trypsin on VSA recognition by IgG*male* and IgG*control* was comparable before and after CSA selection of the parasite (figure 2).

The effect of a 10-fold higher trypsin concentration and the effect of the non-specific protease, pronase, on IgG recognition of FCR3CSA were also determined. Trypsinisation with 1 mg/ml did not significantly reduce the mean surface fluorescence due to IgG*preg* binding to FCR3CSA; however, treatment of the intact infected erythrocyte with 0.1 mg/ml pronase did (figure 2.3; $F_{1,4}$=0.35, p=0.6). Pronase treatment also significantly reduced binding of the IgG*male* and IgG*control* serum pools (figure 2.3; $F_{4,18}$ =3.1, p= 0.04).

Surprisingly, IgG*control* binding to the infected erythrocyte surface increased following CSA selection of the parasite (figure 2.1); however, this non-immune recognition was found to be significantly more trypsin-sensitive than IgGpreg recognition (figure 2.3; $F_{4,18}$=3.11, p=0.041). This indicates that the epitopes recognised by the IgG*control* serum pool and the epitopes recognised by the IgG*preg* serum pool are distinct entities. An increase in apparent non-immune immunoglobulin binding to the infected erythrocyte surface has been observed for a number of parasite clones after selection for adhesion to CSA (data not shown). The source of this background labelling of FCR3CSA by naïve sera was found to be due to non-specific binding by the FITC-labelled tertiary rabbit anti-goat antibody. By using the modified antibody labelling procedure, which employs a biotin-labelled secondary antibody and FITC-labelled streptavidin, binding of malaria naïve IgG to FCR3CSA has been found to be comparable to the unselected parasite. Therefore the recognition of VSA_{PAM} by malaria naïve IgG is abolished (Sharling *et al.*, 2004).

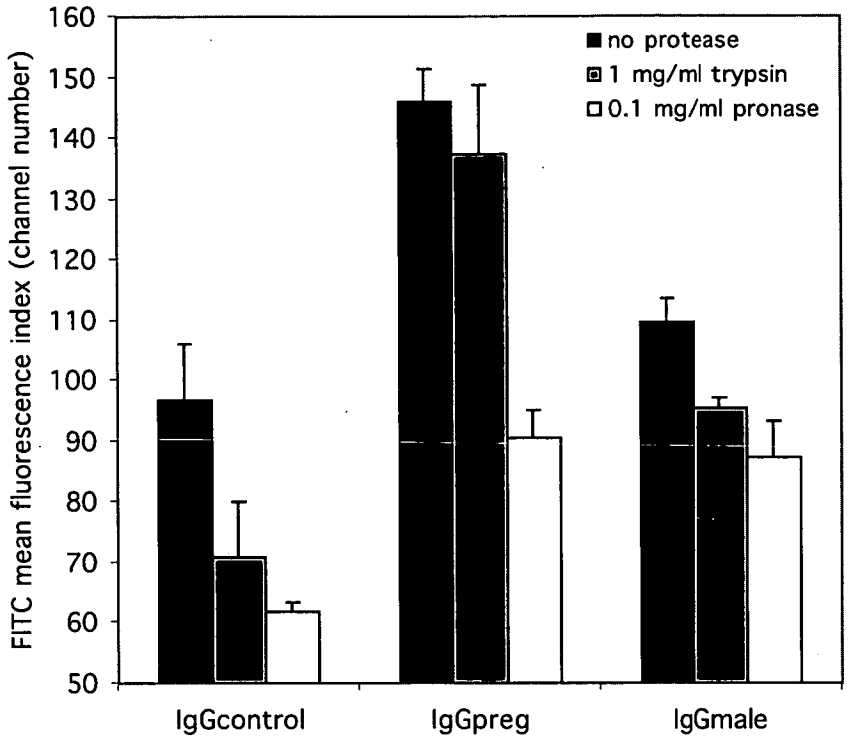


Figure 2.3: FCR3CSA expresses surface antigens exhibiting differential protease sensitivity

Intact infected erythrocytes were treated with 1.0 mg/ml trypsin or 0.1 mg/ml pronase prior to FACS analysis. Serum pools are the same as those described in figure 1. Bar chart shows means (+1 s.e.m.) for 3 independent experiments.

2.3.2 Discordance between the protease sensitivity of the CSA adhesion interaction and IgG binding

Following the identification of trypsin-resistant epitopes that appear to be concomitantly selected with CSA adhesion, the trypsin sensitivity of CSA adhesion itself was determined. FCR3CSA binding to immobilised CSA was markedly more sensitive to trypsin than IgG*preg* recognition of the infected erythrocyte surface (figure 2.4). Parasite adhesion was reduced by 81% and 91% following treatment with 0.1 mg/ml trypsin and 1 mg/ml trypsin respectively (figure 2.4). A trypsin concentration of 1 mg/ml reduced binding as efficiently as 0.1 mg/ml pronase, and although 0.1 mg/ml pronase significantly reduced cell surface fluorescence due to IgG*preg* antibody binding, 1 mg/ml trypsin had no significant effect on IgG*preg* antibody binding. There is, thus, significant discordance between the high trypsin sensitivity of CSA adhesion and the relatively trypsin-insensitive binding of IgG*preg* serum antibodies to the infected erythrocyte surface ($F_{1,8}$ =14.4, p= 0.005).

Human umbilical cord hyaluronic acid (HA) was also included in these assays to investigate the binding capacity of the CSA-selected clone with respect to this receptor. FCR3CSA was found to bind both HA and CSA, although binding to HA was significantly lower (figure 2.4B; $F_{3,19}$= 20.44, p<0.001), at 71% that observed for CSA. Interestingly, as has previously been shown for other *P. falciparum* isolates (Beeson & Brown, 2004), the trypsin-sensitivity of parasite adhesion to HA and CSA differed at low trypsin concentrations. Parasite adhesion to hyaluronic acid was found to be more sensitive to trypsinisation than adhesion to CSA (0.01 mg/ml; figure 2.4B; $F_{1,8}$= 7.7, p=0.02).

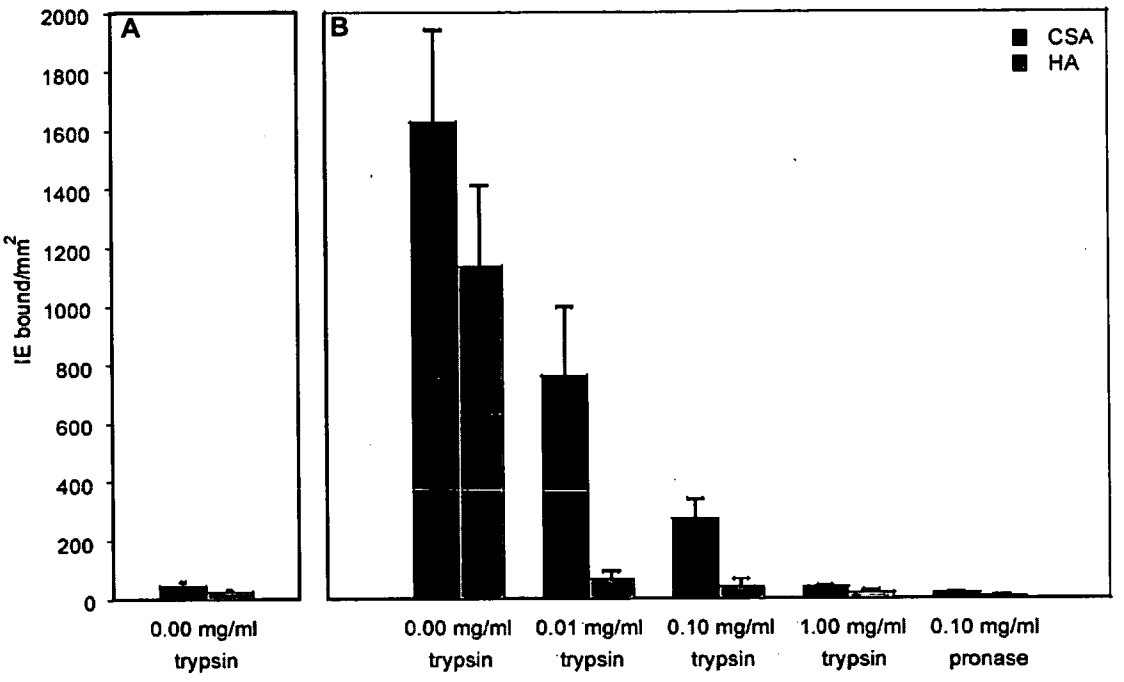


Figure 2.4: The effect of increasing concentrations of trypsin on parasite adhesion to immobilised CSA and HA.

Parasite adhesion to 10 μg/ml human umbilical cord HA and bovine trachea CSA, adsorbed onto plastic Petri dishes, was determined following protease treatment of the intact infected erythrocyte. Bound cells were Giemsa stained and counted by light microscopy. Panels A and B show receptor binding for FCR3 and FCR3CSA infected erythrocytes respectively. Bar chart shows means (+1 s.e.m.) for 3 independent experiments.

2.4 Discussion

The acquisition of antibodies to the surface of placental isolates correlates with protection from malaria in pregnancy and the targets of these antibodies are potential vaccine candidates (e.g. Staalsoe *et al.*, 2001; 2004). Two variants of the well characterized VSA PfEMP1 have been shown to have distinct CSA-binding domains (Buffet *et al.*, 1999; Reeder *et al.*, 1999). Antibodies raised against these domains have been reported to recognise the infected erythrocyte surface (Lekana Douki *et al.*, 2002) and in some cases block parasite adhesion (Costa *et al.*, 2003; Reeder *et al.*, 2000). However, a recent study by Salanti and colleagues (2003) of *var* gene transcription in CSA-selected clones identified a third potential CSA-binding PfEMP1 (*var*2csa). *Var*2csa is predicted to encode a number of atypical DBL-domains and appears to be the major *var* expressed by CSA-selected parasites that are recognised by parity-dependent antibodies (Salanti *et al.*, 2003). Proteomic analysis of CSA-selected parasites has also identified four additional potential CSA-binding PfEMP1 molecules (Fried *et al.*, 2004). The molecular identity of the surface antigens expressed at the infected erythrocyte surface remains unclear (Gamain *et al.*, 2004).

Although PfEMP1-mediated CSA adhesion appears to play a role in placental malaria, the molecular interactions triggering this syndrome are more complex than initially thought. Several studies implicate distinct binding phenotypes, and additional host receptors such as non-immune IgM (Creasey *et al.*, 2003), hyaluronic acid (Beeson *et al.*, 2000; Beeson & Brown, 2004; Chai *et al.*, 2001) and non-immune IgG (Flick *et al.*, 2001). CSA-binding laboratory clones and placental CSA-binding isolates also appear to express parasite-encoded surface antigens other than PfEMP1, such as ring surface proteins 1 and 2 (RSP 1 and 2) (Pouvelle *et al.*, 2000). Interestingly, a gene 'knock-out' of the CSA binding *var* (FCR3*var*CSA) in parasite clone FCR3 abolishes CSA binding, but the 'knock–out' parasites still bind the syncytio-trophoblast of *ex vivo* placental cryosections (Andrews & Lanzer, 2002). Monoclonal antibodies raised against the CSA-binding DBLγ domain also show this domain to be sensitive to surface proteolysis using relatively low trypsin concentrations (100 μg/ml) (Lekana Douki *et al.*, 2002). Is it possible that the trypsin-resistant VSA described here are not of the PfEMP1/CSA-binding type?

My work supports the view that major differences exist between VSA_{PAM} and previously characterised *Pf*VSA. Apart from being recognised only by female sera in a parity-dependent manner, VSA_{PAM} show other distinct characteristics such as:

(i) VSA_{PAM} rarely form infected-erythrocyte rosettes when compared to CD36-binding VSA (Beeson & Brown, 2004; Maubert *et al.*, 1998; Rogerson *et al.*, 2000); (ii) with the exception of rosetting isolates, non-immune IgM binding is a phenomenon only seen with CSA-binding clones (Creasey *et al.*, 2003); (iii) VSA_{PAM} do not generally mediate adhesion to CD36 (Beeson & Brown, 2004; Fried & Duffy, 1996); and (iv) VSA_{PAM}-mediated adhesion to the placenta and CSA can be resistant to concentrations of trypsin known to remove most PfEMP1 molecules from the infected cell surface. In combination with the findings of this study, these distinct properties of VSA_{PAM} suggest the involvement of either an unusually protease-resistant structure, such has been shown for the A4tres PfEMP1 molecule (Smith *et al.*, 2000), a non-CSA-binding PfEMP1 (Gamain *et al.*, 2002), or an alternative class of VSA in placental adhesion. The differential protease sensitivity exhibited by VSA_{PAM} could potentially be exploited in comparative proteomic analysis to aid in the identification of these molecules.

Surface epitopes of the FCR3CSA parasite are both highly resistant to trypsin and are recognised by antibodies from malaria-exposed pregnant women. However, binding assays with the parasite clone used in this study showed CSA and HA adhesion to be relatively trypsin-sensitive. This is compatible with a study by Beeson and colleagues (2004) demonstrating trypsin-resistant CSA adhesion to be a clone-dependent phenomenon. Another recent study by the same group showed naturally acquired human antitsera that are strongly reactive to the surface of CSA-selected parasites are not always capable of inhibiting CSA adhesion (Beeson *et al.*, 2004). Therefore, the findings of this Chapter supports the view that erythrocyte surface epitopes distinct from those involved in CSA adhesion may also be targets of antibodies acquired during PAM. One further implication is for vaccine development; a candidate vaccine raising only CSA adhesion-blocking antibodies may not mimic protective IE surface-reactive, gender-specific immune responses.

2.5 Future work

Phagocytic cell types are recruited in large numbers to malaria-infected placentae (e.g. Walter *et al.*, 1982). It is possible that the antibodies described here are cytophilic and aid in monocyte clearance of IE from the placenta. I have carried out steps to investigate the role of antibodies to trypsin-resistant surface epitopes in phagocytosis assays. Although cultures of monocytes were derived from whole blood time did not allow for a reproducible protocol to be established.

It would also be of interest to determine which IgG subclasses recognise the trypsin-resistant VSA_{PAM} epitopes, as previous work suggests specific IgG subclasses are important for phagocytosis of non-placental IEs. IgG1 has been shown to be the predominant subclass to react with the trophozoite surface (Piper *et al.*, 1999); however, *in vitro* antibody-dependent cellular inhibition (ADCI) assays using cultures of IEs and monocytes have shown that IgG3 cooperates with monocytes to inhibit parasite growth and promote phagocytosis. IgG2 has been found to antagonise the cooperation between IgG3 and monocytes (Bouharoun-Tayoun & Druilhe, 1992). Groux and Gysin (1990) have carried out phagocytosis assays with human monocytes to determine the relationship between the presence of opsonising antibodies, their IgG subclass and protective immunity. They found all IgG subclasses bound the surface of IEs, as determined by FACS analysis, and that only IgG1 and IgG3 could mediate opsonisation. Competition experiments also found IgG2 and IgG4 to inhibit the opsonising capabilities IgG1 and IgG3. Opsonising activity was only evident in individuals classified as immune, which were those with asymptomatic parasitaemias and subjects residing in endemic regions who presented neither symptoms nor parasites. The non-immune cohort included individuals who did not present with symptoms or had experienced only one malarial infection.

Although not concerned with antibody responses to the mature IE surface, studies by Beck *et al.* (1995) have monitored IgG subtypes responses to the ring-infected erythrocyte surface antigen (RESA) in a cohort of adults from a malaria endemic region. Although IgG responses to RESA were frequent for all subclasses, total IgG levels did not correlate with protection against re-infection or reduced health centre attendance. Only IgG3 responses were acquired in an age-dependent manner and only anti-RESA IgG1 and IgG3 responses were associated with reduced *P. falciparum* prevalence and reduced health centre attendance.

Kinyanjui *et al.* (2003) have shown the kinetics of antibody response to *Pf*VSA in children to be variable and children with short-term weak responses to be less efficient at acquiring resistance to re-infection. Isotype profiling suggested that short-lived antibody responses are associated with poor antibody class switching from IgM in these children. Since CSA-selected parasite lines bind non-immune IgM at the erythrocyte surface (Creasey *et al.*, 2003). it would be interesting to determine whether non-immune or parasite-specific IgM behaves antagonistically in phagocytosis assays with CSA-selected parasites. Currently, the significance of the co-selection of non-immune IgM binding when parasites are selected for CSA adhesion is not known. Determining which IgG subclasses are important for parasite clearance from the placenta and the role of surface bound IgM will be important for subsequent vaccine design.

Chapter 3. Towards a proteomics approach for *Pf*VSA identification

3.1 Abstract

Advances in proteomics and the completion of *Plasmodium falciparum's* genome sequence means parasite proteins, in complex mixtures or resolved electrophoretically, can be identified using mass spectrometry. These advances provide an additional tool with which to mine the static information held in *P. falciparum's* genome in order to understand dynamic events such as antigenic variation. A complementary approach to the existing molecular tools would be valuable as the variable and multi-copy nature of the genes mediating antigenic variation ensures that the design and production of specific molecular reagents is not trivial.

Recent studies suggest that placental *P. falciparum* isolates express antigenically distinct variant surface antigens (VSA_{PAM}). Results from traditional molecular based experiments carried out to ascertain the molecular identity of these antigens remain contentious. The aim of this chapter, therefore, was to develop a proteomics based technique for the identification of VSA_{PAM}. Here I describe a set of experiments to optimise the surface labelling of IEs with a membrane impermeable analogue of biotin. Unfortunately, the subsequent identification of biotinylated parasite-specific surface proteins by mass spectrometry was not achieved. However, the cellular fraction containing candidate biotinylated VSA_{PAM} represents an enriched and simple mixture of proteins suitable for future analysis. Specific antisera to the variant surface antigen *Plasmodium falciparum* erythrocyte surface 1 (PfEMP1) was also raised and used in the optimisation process. This serum will be a useful tool for future studies.

3.2 Introduction

3.2.1 *P. falciparum*: a molecular biologist's nightmare

P. falciparum's genome is extremely biased in terms of base composition: 80% of bases are either adensosine (A) or thymidine (T). Intergenic regions average more than 85% A's and T's. A similar degree of AT richness is also seen for *P. yoelii* (Carlton *et al.*, 2002), but is not the case for all *Plasmodium* genomes. *P. vivax's* genome, for intance, is composed of approximately 55% A's and T's (http://www.tigr.org/tdb/e2k1/pva1/intro.shtml). *P. falciparum's* AT bias makes primer design and stringent PCR amplification problematic and unpredictable recombination events often occur during the cloning of parasite DNA due its instability in bacterial plasmids. Obtaining the complete genome sequence for *P. falciparum* was therefore a major accomplishment (Doolittle, 2002).

P. falciparum proteins are on average 50% longer than their yeast orthologs, as they tend to be rich in stretches of low-complexity sequence when compared to other eukaryotic proteomes (Aravind *et al.*, 2003). Low-complexity sequence is characterised by homopolymeric runs of amino acids, runs of asparagine being common in *P. falciparum* protein; such sequence tends not to form globular structures. The abundance of non-globular structures in combination with an AT rich genome (which biases codon usage and is thought to affect translational rate) hampers the heterologous expression of *P. falciparum* proteins having a native conformation.

3.2.2 The *var* multigene family

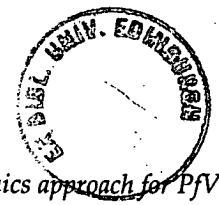
The molecular challenges posed by the extreme base composition are exacerbated by the *var* multigene family, the candidate receptors for placental adhesion (reviewed in Andrews & Lanzer, 2002; Beeson *et al.*, 2001; Craig, 2004; Duffy & Fried, 2003a). As discussed in chapter 2 the role of different variants of PfEMP1 in CSA remains unclear. The cause of the uncertainty regarding PfEMP1 and CSA adhesion lies partly in our poor understanding of the relationship between *var* gene transcription, translation and protein trafficking to the IE surface. In addition interpreting experiments on *var* gene transcription and the expression of their protein products is complicated by probe and primer bias (Duffy *et al.*, 2003; Gardner *et al.*, 2002) and antibody cross reactivity (Gamain *et al.*, 2004).

The expression of recombinant protein domains from PfEMP1 that conserve their native structural conformation in heterologous expression systems has proved difficult. This is probably due in part to PfEMP1's multi-domain structure and cysteine-rich nature. Therefore, anti-PfEMP1 antibodies raised to recombinant protein rarely react to the native molecule on the IE surface (critically reviewed in Sherman *et al.*, 2003). Furthermore, it is difficult, or at least not always attempted, to ascertain antiserum specificity and rule out cross reactivity. The VSA of CSA-selected clones also bind non-immune immunoglobulins (Creasey *et al.*, 2003; Flick *et al.*, 2001). Thus proving a specific antigen-antibody interaction at the IE surface for these clones is not a straightforward matter. Studying these molecules with existing molecular techniques has not proven to be easy.

The heterologous expression of PfEMP1 domains at the surface of mammalian cells has been used to determine the adhesive phenotype encoded by particular PfEMP1s and confirm associations of a transcript with an adhesive phenotype of the IE. However, due to the large multi-domain structure of PfEMP1 delineating a functional domain, extrapolating the *in vivo* interactions of that domain with neighbouring domains and the role of the overall structure of the intact protein is problematic and it has been shown that the adhesive phenotypes of PfEMP1 domains or fragments are very dependent on their context within the protein. The DBL-γ domain from the A4tres PfEMP1 surface expressed on a Chinese ovary cell line (CHO-745) binds CSA although the IE erythrocytes expressing this PfEMP1 do not (Gamain *et al.*, 2002). The CSA binding capacity of a 67 amino acid binding motif encoded by the FCR3*var*1CSA gene, when expressed at the surface of mammalian cell lines, is dependent on the extent of neighbouring sequence present in the expression constructs (Gamain *et al.*, 2004). Including more of the neighbouring sequence did in some instances ablate the CSA binding capacity of the binding motif.

3.2.3 Regulation of PfEMP1 'expression'

A number of studies have linked the level of a particular *var* gene transcript directly to the adhesive phenotype at the IE surface, however in order to relate a transcript to an adhesive phenotype the transcriptional regulation of *var* genes has first to be understood. Studies over the past 6 years on *var* gene transcriptional regulation through progression of the asexual cycle have failed to form a consensus as to when in the cell cycle the *var* transcript that is subsequently translated is transcribed.



Initial studies using reverse transcriptase (RT)-PCR suggested numerous *var* genes are transcribed in ring stages, but only the *var* destined to be translated and exported to the IE is transcribed in pigmented trophozoites (Chen *et al.*, 1998; Scherf *et al.*, 1998). This work was supported by later studies using single cell RT-PCR (Fernandez 2002). A model for the exclusive transcription of a single *var* was proposed. This model suggests that relaxed transcription of multiple *vars* in ring stages is followed by the silencing, in mature trophozoites, of all *var* transcripts except the one encoding the adhesive phenotype.

However, subsequent studies using RT-PCR on both cell populations (Noviyanti *et al.*, 2001) and single cells (Duffy *et al.*, 2002), detected numerous *var* transcripts in pigmented trophozoites. Although these studies identified a dominant *var* transcript, it was proposed that many full-length *var* genes are transcribed but not translated, thus suggesting a defined adhesive phenotype can be selected, but with the transcription of multiple *vars*, and little, or no, gene silencing.

Biologically, the relevance of *var* transcripts in mature trophozoite stages is unclear, since PfEMP1 protein expression peaks in late ring stages (Waterkeyn *et al.*, 2000) and exposure at the IE surface starts from 16 hours post-invasion (Gardner *et al.*, 1996). This biochemical evidence accords with the electron and atomic force microscopy data showing the accumulation of parasite induced knobs at the IE surface over the course of intraerythrocytic parasite development (Nagao *et al.*, 2000). In culture, translocation to the IE surface occurs over a narrow time window (16-20 hours post-invasion) and plateaus after 24 hours post-invasion, and there appears to be no mechanism for recycling PfEMP1 between intracellular compartments and the IE surface (Kriek *et al.*, 2003).

In closer agreement with patterns of PfEMP1 protein expression, recent findings from Northern blot analyses show abundant full-length *var* transcripts to be restricted to ring stages (3-18 hours post-invasion) (Kyes *et al.*, 2000; 2003). These findings are now considered to represent the true pattern of *var* transcription through the intraerythrocytic cycle. The alternative patterns reported may be explained by the presence of truncated *var* transcripts (Su *et al.*, 1995), the high sensitivity of RT-PCR, slightly asynchronous cultures, and amplification bias.

Although Northern blotting appears to be the preferred method for analysis, particularly for detecting full-length *var* transcripts, the choice of probe is crucial. A commonly used probe to the conserved exon 2 sequence of *vars* was recently shown to have limited cross-reactivity: for example FCR3*var*CSA and R29*var*1 both require specific probes for their exon 2 sequences in order to be detected by northern

blotting (Kyes *et al.*, 2003). Also, due to the genome containing numerous *var* pseudo genes, transcriptional analysis can prove biologically misleading (Winter *et al.*, 2003). In addition, early studies of CSA-selected parasites identified a full-length *var* transcript by Northern blotting (Buffet *et al.*, 1999), but it appears that this transcript may not be translated and/or transported to the IE surface (Gamain *et al.*, 2004; Kyes *et al.*, 2003). Full-length, developmentally regulated transcripts that are not translated have been described for other *P. falciparum* genes (Taylor *et al.*, 2001).

Until the relationship between *var* gene transcription, translation, and translocation of PfEMP1 to the erythrocyte surface is understood, studies linking *var* gene 'expression' at the RNA level to adhesion phenotypes and the clinical outcome of infection (Ariey *et al.*, 2001; Fried & Duffy, 2002; Jensen *et al.*, 2004; Kirchgatter & Portillo del, 2002; Rowe *et al.*, 2002a; Vazquez-Macias *et al.*, 2002) remain difficult to interpret.

3.2.5 Is VSA_{PAM} a PfEMP1?

Several groups internationally are working towards the characterisation of the surface antigens expressed by CSA-binding parasites and these studies have focused predominantly on PfEMP1. As discussed in Chapter 2, the VSA of CSA-selected and placental isolates in some respects do not exhibit PfEMP1-like characteristics. Therefore, the aim of this chapter was to develop an alternative approach for the study of VSA_{CSA}. A proteomics approach was considered attractive as it could potentially detect members of the relatively poorly characterised VSA family, rifin, whose potential in terms of CSA-binding and PAM has received little attention, and in addition any as yet uncharacterised VSA. If indeed PfEMP1 is the only VSA that is expressed by late stage placental isolates, then proteomics data should complement the existing data on PfEMP1 and CSA-adhesion.

3.2.6 MALDI-TOF mass spectrometry

The completion of a *P. falciparum* genome sequence along with advances in mass spectrometric technologies (Brancia *et al.*, 2000; Chalmers & Gaskell, 2000) has opened a proteomics toolbox for the study of *Pf*VSA expression. The potential to identify parasite proteins, separated by SDS-PAGE or in complex mixtures, means the static information held in the parasites' genome could in theory be mined to help understand dynamic events such as antigenic variation.

When this work was initiated the opportunity to gain training in protein identification using matrix assisted laser desorption/ionisation (MALDI) time-of-flight TOF) mass spectrometry (MS) was established with York University. To identify a protein resolved electrophoretically on SDS-PAGE gels using MALDI-TOF the protein spot is first excised from the gel and digested to completion with a sequence specific protease, for example trypsin. Tryptic peptides derived from a protein are then mixed with an excess of a proton rich matrix to prevent clumping, and the mixture is spotted onto a sample plate.

Within the spectrophotometer a vacuum is pulled such that the solvent of the matrix is drawn off. The tryptic peptides co-crystalise with the matrix molecules to create a homogeneously dispersed layer of peptide and matrix molecules. Short pulses of UV laser are then focused through a prism onto the sample spot to volatise the peptides and matrix. Since the matrices used contain a chromophore and are present in excess they absorb essentially all of the laser radiation. The matrix effectively isolates the tryptic peptides in a microenvironment that enhances the probability of peptide ionization (by the matrix donating a proton to the peptide), but not fragmentation. Ionised peptides are accelerated by a high voltage supply and are then allowed to drift down a flight tube where they separate according to their mass and charge (figure 3.1). The time taken (time-of-flight) for ions to be detected by a high-speed recording device at the end of the flight tube is recorded. In some circumstances the protonated peptides are reflected by an electrostatic mirror and subsequently detected with a reflector detector. When MALDI-TOF MS is performed in the positive mode (which is generally the case for peptides), with a suitable matrix, protonated molecular ions are the dominant species and all peptides are presumed to have a charge of +1.

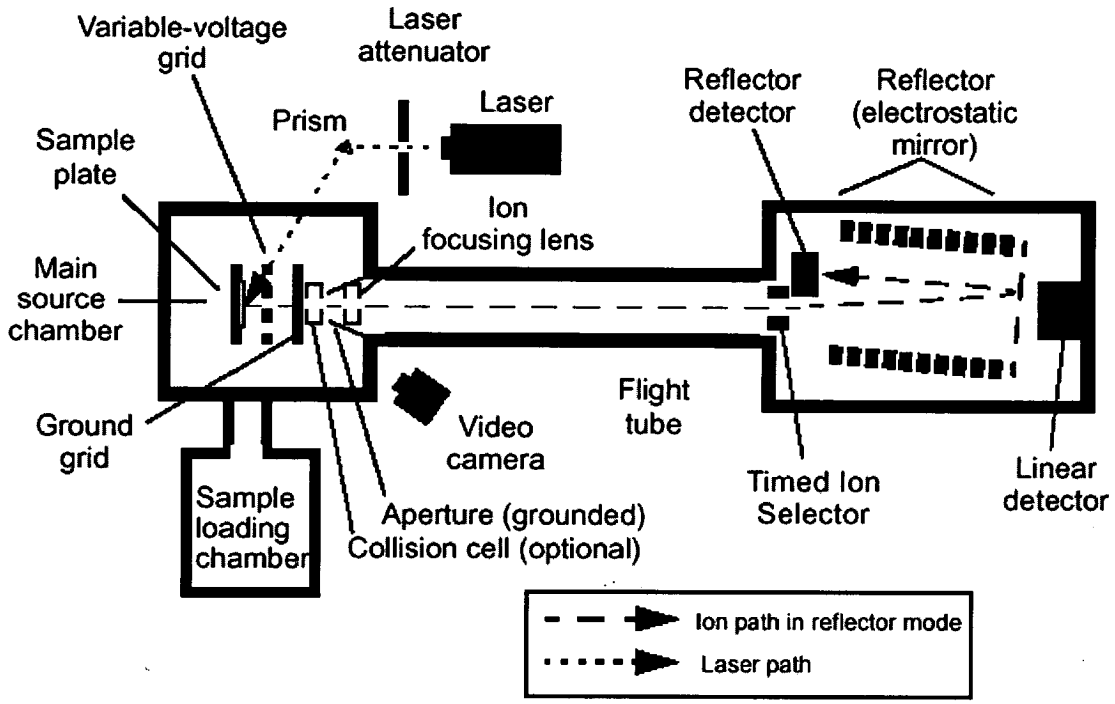


Figure 3.1: Simplified schematic of a MALDI-TOF mass spectrometer

By measuring the time-of-flight and using a set of peptide standards to obtain a number of constants for the spectrophotometer the mass of unknown ions can be determined using the following equation:

$$m/Z = B(t-A)2$$

t = time

m = mass

A & B = calibration constants derived from a number of peptides with known m/Z values

Z = charge of ion (all ions are presumed to have the same charge, +1)

Since, trypsin cleaves at the carboxyl side of lysine and arginine residues the tryptic peptides produced from a protein can be predicted from its sequence alone. Therefore an experimentally determined set of tryptic peptide masses from an unknown protein can be used as a fingerprint and searched against *in silico*

fingerprint predictions for proteins and potential open reading frames in genome sequence databases.

MALDI-TOF MS can also be applied in tandem to gain more information about a mixture of peptides. A small selection of tryptic peptides, usually the most abundant, are selected in real time and diverted into a collision cell where they are bombarded with a gas mixture to induce peptide fragmentation. The mass of fragmented peptides can then be determined in a similar fashion as described above for MALDI-TOF MS.

3.2.7 Outline of proteomics approach

A gel-based comparative proteomics approach was initiated using the parasite clone FCR3. This clone has been well relatively characterised with respect to CSA binding and associated surface antigen expression (Andrews *et al.*, 2003; Buffet *et al.*, 1999; Gamain *et al.*, 2004; Kyes *et al.*, 2003) and demonstrates a serological phenotype similar to placental isolates following CSA-selection (Chapter 2; figure 2.1). The 3D7 parasite clone used for the genome sequencing project would have been the ideal choice for proteomic analysis, however, this clone had proven difficult or impossible to select for CSA adhesion; the serological phenotype of CSA-selected 3D7 is also not particularly marked and is unstable (Salanti *et al.*, 2003).

The experimental plan was to label erythrocyte surface antigens with a commercially available analogue of the vitamin biotin (figure 3.2) that is predicted to be impermeable across cell membranes. Sulpho-NHS-LC-biotin contains a group that is reactive to primary amino groups and is predicted to label the lysine residues of surface proteins. PfEMP1 and rifins are lysine rich (Appendix 2), and lysine residues are relatively uniformly distributed throughout the proteins. Therefore, it was predicted that surface-expressed PfEMP1 and rifin molecules would have exposed lysine residues available for labelling. Biotin is a relatively small molecule (244 daltons) and can be conjugated to many proteins without altering their biological characteristics.

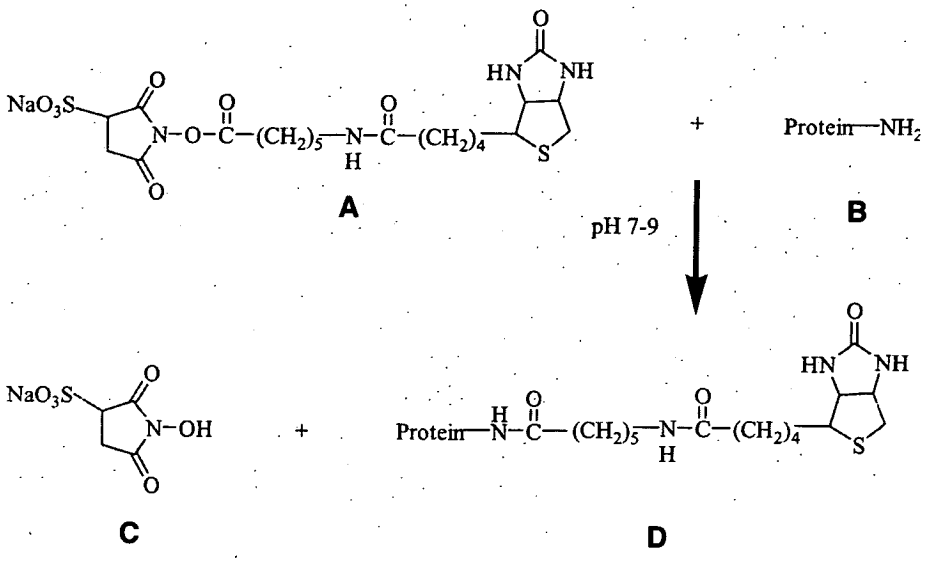


Figure 3.2: The reaction of sulpho-succinimidyl-6-(biotinamido) hexanoate with proteins

An amine group (B) of the protein to be labelled reacts with the ester group of sulpho-succinimidyl-6-(biotinamido) hexanoate (A) by nucleophilic attack to add a biotin label to the target protein (C). This reaction releases sulpho *N*-hydroxysulphosuccinimide (C) as a by-product. Sulpho-succinimidyl-6-(biotinamido) hexanoate (sulpho-NHS-LC-biotin) includes a spacer arm to minimise steric hindrance in antibody recognition of the biotinylated proteins.

Surface biointylation was chosen over surface iodination using I^{125} partly because it avoids the safety concerns associated with using high-energy radioactive isotopes. Combining I^{125} with the biohazard precautions necessary to work with live *P. falciparum* IEs was not feasible in the facilities available. Furthermore, biotinylated proteins can be detected by western blotting using a short chemiluminescence technique (Meier *et al.*, 1992) as opposed to the potentially long exposure times required for I^{125} labelled proteins. Detection methods for biotinylated proteins can also be very sensitive (Neumaier *et al.*, 1986). Biotin is a naturally occurring vitamin that binds the hen egg protein avidin with high avidity. This was considered one of the primary advantages of the sulpho-LC-NHS reagent, as biotin can be used as a tag for purification or enrichment using avidin (or streptavidin, a biotin-binding protein isolated from *Streptomycetes*) conjugated to a solid support.

As PfEMP1, is not present in abundance at the IE surface, a step for the enrichment of membrane-associated proteins by detergent extraction or membrane fractionation was included. Protein fractions were separated by 2D gel electrophoresis and

differences in the biotin-labelled proteins detected by western blotting, using horseradish peroxidase conjugated streptavidin. Those proteins showing differences in labelling patterns between the CSA-selected and unselected clone would be identified using tryptic peptide mass finger printing and database mining.

3.3 Results

3.3.1 IE erythrocyte biotinylation and MALDI-TOF mass spectrometry

Following steps to optimise the separation of biotin labelled proteins using 2DGE, silver stained protein spots were extracted from 2D gels for identification using MALDI-TOF mass spectrometry. Loosely synchronised trophozoite cultures of FCR3 and FCR3CSA at 10 % parasitaemia were cell surface labelled with sulpho-NHS-LC-biotin as described in section 7.3.1. Specifically, the biotinylation reaction was carried out using the sulpho-NHS-LC-biotin label at 0.25 mg/ml, with an incubation time of 30 minutes at 4 °C. The cells from one third of a 25 ml culture at 5% haematocrit were used for each labelling reaction. Cells were lysed and proteins extracted with triton-X100 as described in section 7.3.2 and both the soluble (figure 3.3) and insoluble fractions (figure 3.4) were separated by 2DGE. Protein extracts separated on duplicate gels were either stained with silver nitrate or transferred to PVDF for western blotting with horse radish peroxidase (HRP)-conjugated streptavidin. Figures 3.3 & 3.4 are representative of the labelling patterns seen for FCR3 and FCR3CSA.

York University's mass spectrometry facilities were used initially, as an automated tandem MALDI-TOF/TOF (Proteome Analyzer ABI 4700) instrument was available to trained users. Tandem MALDI-TOF MSMS identified proteins with a 30 % success rate; unfortunately, these did not include any clearly resolved labelled proteins. Furthermore, 92% of these proteins were identified using only MALDI-TOF (MS1) data. In only one case was MALDI-TOF MSMS data necessary. Therefore, training on the MALDI-TOF mass spectrometer (Voyager Applied Biosystems) available at Edinburgh University through the Scottish Instrumentation & Resource Centre for Advanced Mass Spectrometry (SIRCAMS) was sought. Although this instrument requires more user time than the automated MALDI-TOF/TOF proteome analyser at York University, due to manual acquisition, spectra processing, and database mining, since relatively small numbers of proteins were processed and a success rate of 50% was achieved, the user time required at Edinburgh was not considered a limitation. Table 3.1 shows a summary of the proteins identified using MALDI-TOF both at York and Edinburgh University.

MALDI-TOF analysis identified a number of host intracellular proteins, none of which were labelled, and biotin labelled host erythrocyte surface proteins such as band 3 and band 4.1 (Table 3.1). However, the parasite-specific biotin-labelled proteins identified were not surface antigens. Two labelled proteins in the

FCR3CSA triton-X100 soluble extract were identified as *P. falciparum* glycophorin binding protein 130 (*P.f*GBP130) and *P. falciparum* heat shock protein 70 (figure 3.3 ii). GBP130 (aka Ag78/96tR) resides within the erythrocyte cytosol, the vacuole and vacuolar space of the parasite (Ansorge *et al.*, 1996; Bianco *et al.*, 1987; Bonnefoy *et al.*, 1988).

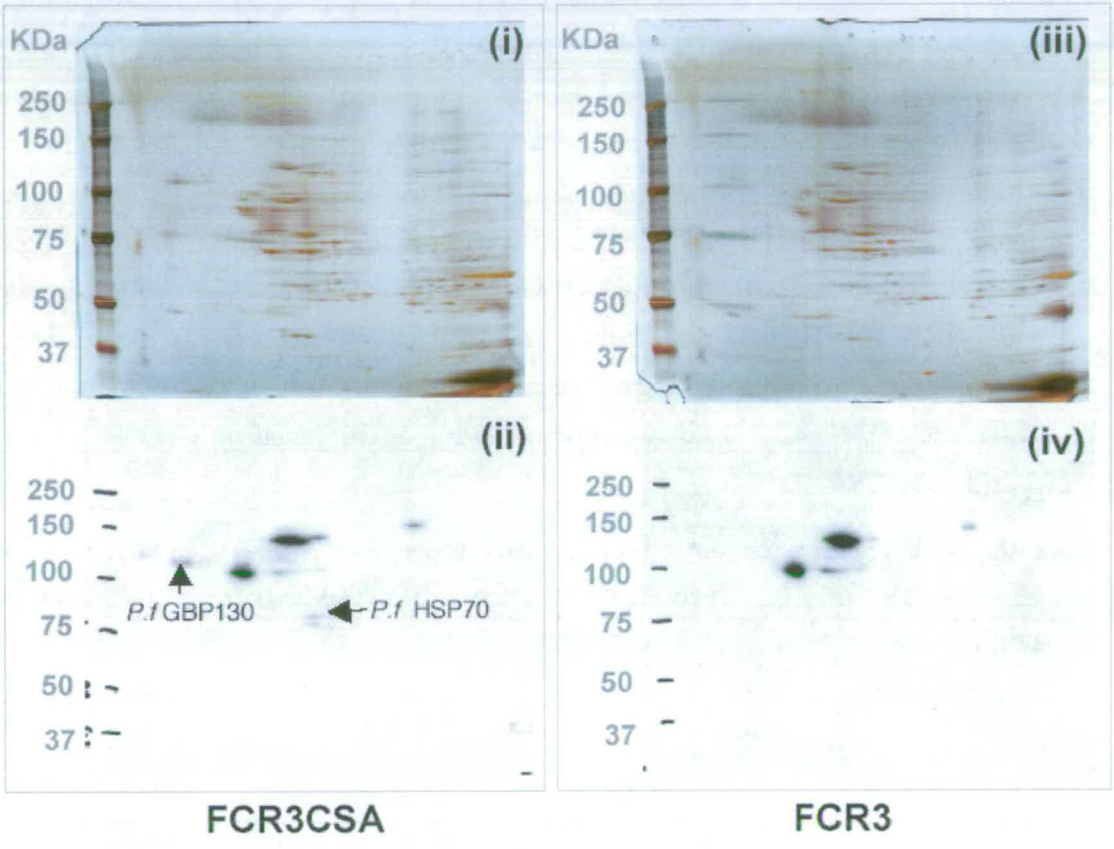


Figure 3.3: Triton-X100 soluble protein extracts resolved by 2DGE

Proteins were separated in the first dimension using an IPG strip with a pH range of 4-7; here the acidic end of the strip was placed adjacent to the molecular weight marker lane. Panels i & iii show silver stained gels and panels ii & iv duplicate gels of i and ii respectively transferred to PVDF and probed with HRP conjugated streptavidin to detect biotinylated proteins. Proteins were resolved in the second dimension using 4-12% bis-tris SDS-PAGE gels (Invitrogen).

Since a number of abundant intracellular host proteins such as spectrin and globin (figure 3.4) were not labelled, it seemed that the sulpho-NHS-LC-biotin reagent was not freely diffusing into intracellular milieu of the infected erythrocyte but that a

restricted number of parasite-derived proteins were labelled. To confirm that the biotin labelled parasite-specific proteins were not surface exposed the trypsin sensitivity of these proteins to trypsin treatment of the intact IE was investigated. However, before doing so, an alternative to detergent extraction was considered. An alternative extraction method was sought due to inconsistencies observed in the proteins represented in different batches of detergent extracted proteins (data not shown) and the overnight dialysis step required to remove SDS from detergent extracts prior to 2DGE prolonged the time required for sample preparation considerably.

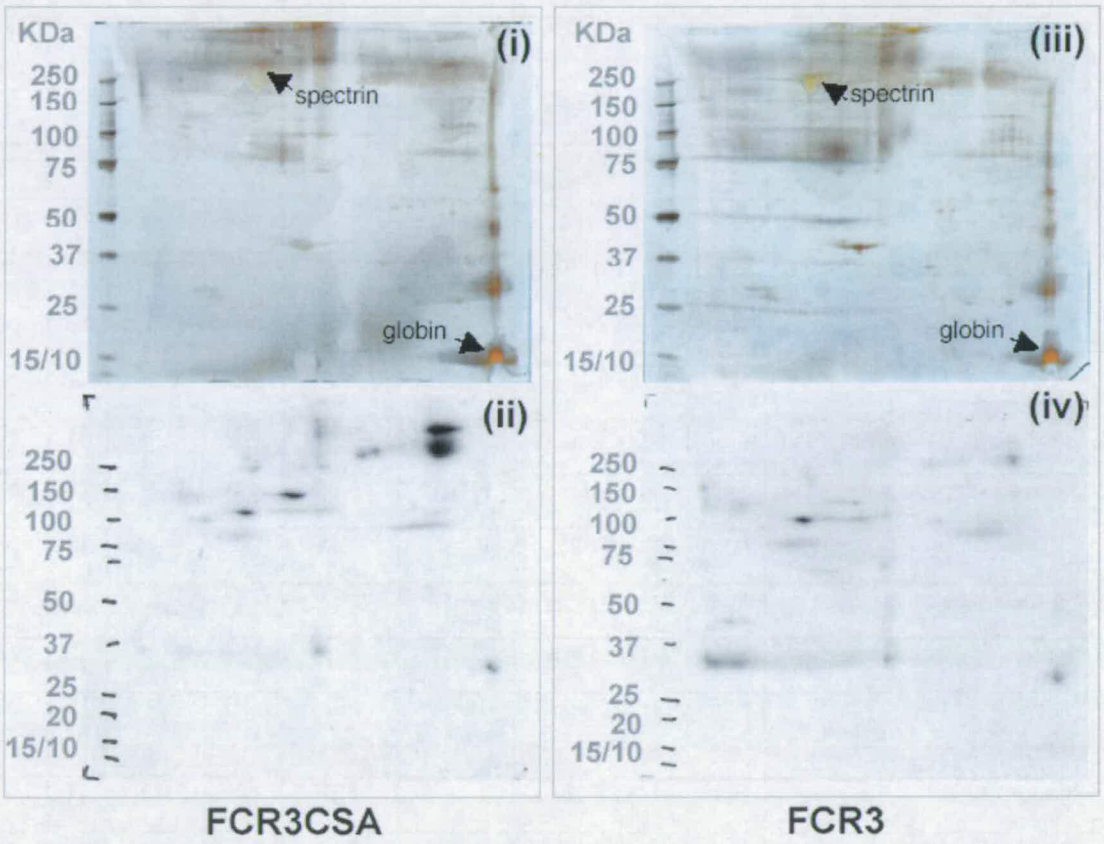


Figure 3.4: Triton-X100 insoluble protein extracts resolved by 2DGE

Proteins were separated using an IPG strip from pH 4-7, the acidic end of the strip was placed adjacent to the molecular weight marker lane. i & iii silver stained 4-12 % bis-tris SDS-PAGE gels (Invitrogen), ii & iv show western blots of duplicate gels of i and iii respectively. Blots were probed with HR- conjugated streptavidin to detect biotinylated proteins.

Table 3.1: Summary of mass spectrometric identification from 2DGEs

[1]University of York Technology facility, [2] Scottish Instrumentation & Resource Centre for Advanced Mass Spectrometry (SIRCAMS). TS: triton-X100 soluble fraction, TI; triton-X100 insoluble fraction, ND: not determined, *P.f*: *P. falciparum*, *H.s*: *Homo sapien*, Y: yes, N: no, *low mouse score due to keratin contamination. A MSFIT score of over 10^5 or a MASCOT score of over 50 was considered significant.

Protein	Species	Accession number/ locus tag	MSFIT MOWSE score	MASCOT MOWSE score	% coverage	Size (kDa)	Fraction	Biotin label?
[2]glycophorin binding protein-130	P.f	gi|1221507	$2.7e^{+07}$	ND	71	90.0	TS	Y
[2]heat shock protein 70	*P.f*	PF08_0054	$4.3e^{-10}$	ND	36	73.9	TS	Y
[1]acidic basic repeat antigen	*P.f*	gi|113007	ND	52	ND	69.3	TS	N
[1]serine rich protein	*P.f*	gi|160687	ND	142	ND	110.0	TS	N
[1]elongation factor 1α	*P.f*	gi|119153	ND	108	ND	49.0	TI	N
[1]ornithine aminotransferase	*P.f*	gi|585604	ND	107	ND	46.06	TS	N
[1]G3PDH	*P.f*	gi|19401842	ND	68	ND	36.64	TI	N
[1]phosphoglycerate kinase	*P.f*	gi|129926	ND	57	ND	45.4	TI	N
[2]heat shock protein 86	*P.f*	PF07_0029	$1.4\ e^{+09}$	ND	71	86.1	TS	N
[2]Ig binding protein (hsp 70)	*P.f*	PFI0875w	$8.7e^{+09}$	ND	31	72.4	TS	N
[2]N-methyltransferase	*P.f*	gi|21542912	$1.1e^{+07}$	ND	ND	31.0	TS	N
[2]actin	*P.f*	gi|27159630	$2.2e^{+06}$	ND	ND	42.5	TS	N
[2]enolase	*P.f*	gi|22794081	$1.8e^{+08}$	ND	ND	48.7	TS	N
[2]band 3	*H.s*	gi|4507020	$1.1e^{+07}$	ND	ND	101.8	TI	Y
[2]band 4.1	*H.s*	gi|42716288	*6702	ND	ND	86.6	TI	Y
[2]spectrin α chain (K12)	*H.s*	gi|1174412	$2.1e^{+32}$	ND	ND	280.0	TI	N
[1]spectrin β chain	*H.s*	gi|134798	ND	143	ND	242.5	TI	N
[2]ankyrin 1, isoform 2	*H.s*	gi:10947041	$2.5e^{+22}$	ND	ND	189.0	TI	N
[1]β-globin	*H.s*	gi|1066765	ND	261	ND	16.0	TS	N
actin, γ1 propeptide (E7)	*H.s*	gi|4501887	ND	75	ND	41.8	TS	N
[2]actin binding protein	*H.s*	gi|6273777	$4.3e^{+08}$	ND	ND	61.4	TS	N
[1]catalase	*H.s*	gi|7245758	ND	202	ND	56.6	TS	N
[2]heat shock protein 70	*H.s*	NP_006588	$4.6e^{+11}$	ND	43	70.9	TS	N

An erythrocyte membrane preparation method involving a simple lysis step in a hypo-osmotic buffer was compared directly to detergent extraction with triton-X100. The two methods were used to extract proteins from the same labelling reaction. The lysis step for each method was performed in equal volumes and an equivalent fraction of each extract was loaded.

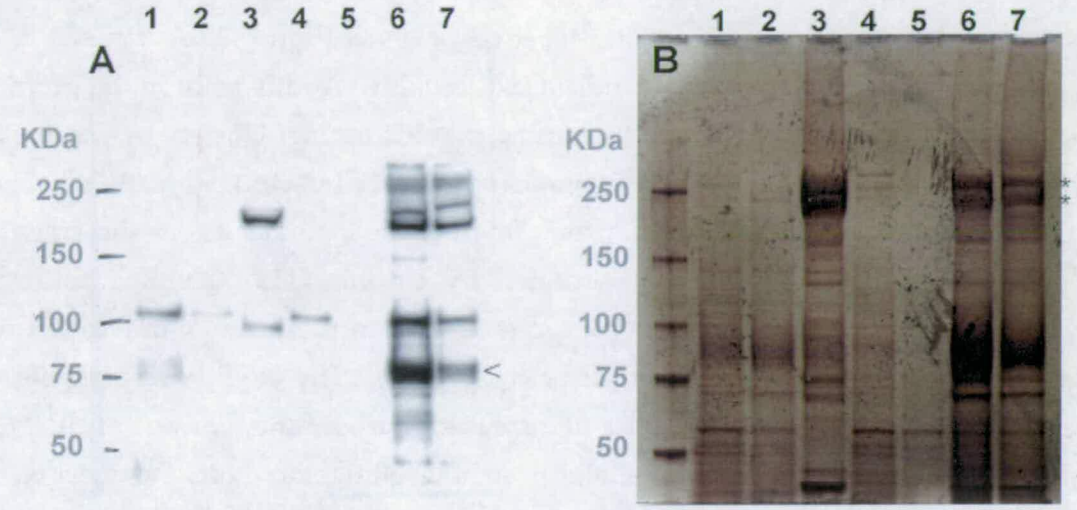


Figure 3.5: A comparison of detergent extraction and membrane fractionation

Panel A shows a western blot probed with streptavidin- HRP. Lanes 1 –3 are fractions from detergent extraction, lane 1: TS fraction, lane 2: wash from TI fraction and lane 3: TI fraction. Lanes 4-7 are fractions from membrane preparations, lane 4: lysate, lane 5: wash from membrane fraction, lane 6: membrane fraction, lane 7: ammonium acetate precipitated membrane fraction. Panel B shows a duplicate gel stained with silver nitrate. The arrowhead highlights biotin labelled band 3 and a pair of asterisks a α/β spectrin doublet. Proteins were separated on a 3-8 % Tris-acetate gel.

The complexity of proteins in the detergent extracts and membrane preparation was revealed by silver staining (figure 3.5B), and the biotin-labelling patterns detected by western blotting (figure 3.5A). This experiment showed that membrane fractions prepared by centrifugation following osmotic lysis enriched for a number of additional labelled proteins as detected by western blotting (figure 3.5, lanes 3 and 6). Although, the triton-X100 insoluble fraction enriched more for spectrin whose

location on the silver stained gel is highlighted by asterisks (figure 3.5B), biotin labelled erythrocyte surface exposed band 3 (figure 3.5A, lane 3) was more efficiently extracted in the membrane preparation procedure (figure 3.5A, lane 6).

After establishing that membrane preparation was a more reproducible method for enriching for host membrane proteins and biotin-labelled proteins, the protease sensitivity of the biotin-labelled proteins was investigated. Prior to biotinylation intact IE and non-IE were exposed to 1 mg/ml trypsin for 10 minute. This had no effect on the parasite specific biotin-labelled proteins (figure 3.6A, lanes 3 & 4), confirming that these proteins are indeed intracellular. At this point in the project a paper was published that also demonstrated that IEs but not UEs are permeable for the membrane 'impermeant' biotin label sulpho-NHS-LC-biotin (Nyalwidhe *et al.*, 2002). Fortunately the same group promptly published another paper showing that the labelling of IE intracellular proteins by sulpho-NHS-LC-biotin could be eliminated using a number of compounds that inhibit the parasite's novel permeation pathway (NNP) (Baumeister *et al.*, 2003). The NNP is responsible for the IE's increased permeability for small solutes. Furosamide was one of the NNP inhibitors found to prevent intracellular protein labelling, therefore, the effect of 100 μM furosamide on the biotinylation of FCR3CSA IEs was determined.

Figure 3.6 shows that in the presence of furosamide, no parasite specific biotinylated proteins are labelled (lanes 7&8). Unfortunately, it was impossible to determine whether the biotin labelled membrane proteins, detected by Baumeister and colleagues (2003), that remain labelled in the presence of NPP inhibitors are parasite derived, as a comparison of UEs and IEs was not presented. Although, the labelling conditions used by Baumeister and colleagues (2003) were followed in the experiment shown in figure 3.7, differences were found with regard to the labelling of host intracellular proteins. Baumeister and colleagues (2003) found spectrin and haemoglobin to be labelled in the absence of NNP inhibitors where as this was not the case in these experiments (figure 3.6A&B, lane 3 and figures 3.3 & 3.4). The restricted pattern of intracellular protein labelling seen in figure 3.6 may indicate that these protein are directly associated with the pore and/or channels that make up the NPP.

Although furosamide eliminated the problem of intracellular protein labelling, it became apparent that although furosamide did not inhibit host erythrocyte surface labelling parasite specific trypsin sensitive proteins were not present (figure 3.6A, lane 7). At this point it was considered that perhaps the western blotting technique was of insufficient sensitivity to detect low abundant *Pf*VSA, therefore, the surface

labelling of IEs was repeated in the presence of furosamide using a considerably higher parasitaemia.

Lane	1	2	3	4	5	6	7	8
U/I	U	U	I	I	U	U	I	I
Trypsin	-	+	-	+	-	+	-	+
Furosamide	-	-	-	-	+	+	+	+

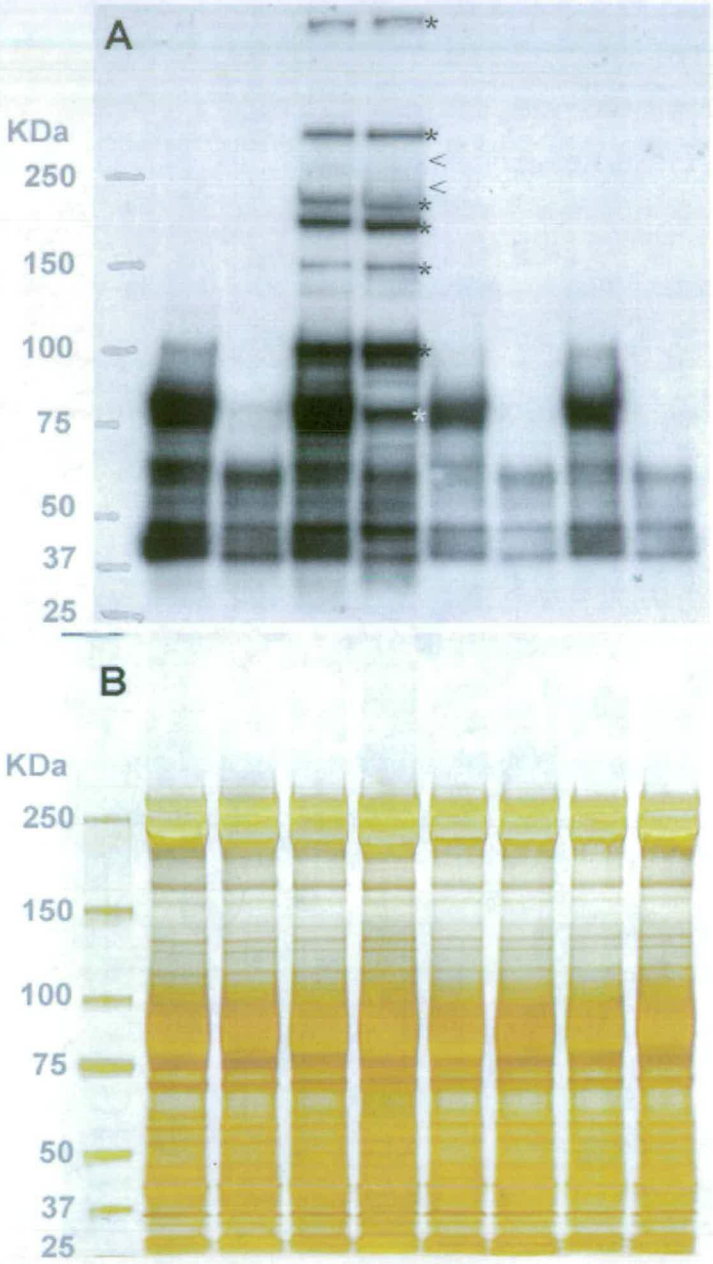


Figure 3.6: The effect of furosamide on biotin labelling of intact IE

Intact FCR3CSA IEs and UEs were labelled with 0.25 mg/ml sulpho-NHS-LC-biotin for 30 minutes at 4°C, in the presence or absence of 100 μM furosamide. Cells were fractionated following hypo-osmotic lysis. Membrane fractions were separated on 3-8% tris-acetate SDS-PAGE gels. (A) A western blot probed with HRP-streptavidin. (B) A duplicate gel where proteins were stained with silver nitrate. Parasite specific biotin-labelled proteins are highlighted with an asterisk. Sample loading is indicated in the table above.

Intact UEs and IEs were treated with either trypsin or pronase before labelling to test whether, if lower molecular weight PfVSA were being labelled, they could be revealed using an alternative protease, pronase. The supernatant formed following osmotic lysis and centrifugation of the membrane fraction was also analysed to determine whether labelled *Pf*VSA had become disassociated from the erythrocyte plasma membrane. Neither by increasing the parasitaemia by plasmagel flotation of late stage cultures (figure 3.7A), nor by pronase treatment (figure 3.7A), nor analysing the IE lysate fraction (figure 3.7B) revealed biotinylated candidate *Pf*VSA. To confirm the presence and abundance of parasite material in the membrane fractions a duplicate western blot was probed with a pool of purified plasma IgG from malaria exposed individuals (figure 3.7C). This confirmed that high molecular weight parasite proteins characteristic of PfEMP1 indeed could enter the gels used. Malaria immune IgG was also used in an attempt to reveal low molecular weight protease sensitive proteins that may have been masked by the heavily biotinylated host erythrocyte surface proteins. This however was unsuccessful.

It was considered at this point that it was necessary to have specific reagents to characterise the protein fractions. Therefore, an anti-peptide antibody to the conserved amino terminus sequence (ATS) of the PfEMP1 family of surface antigens was raised.

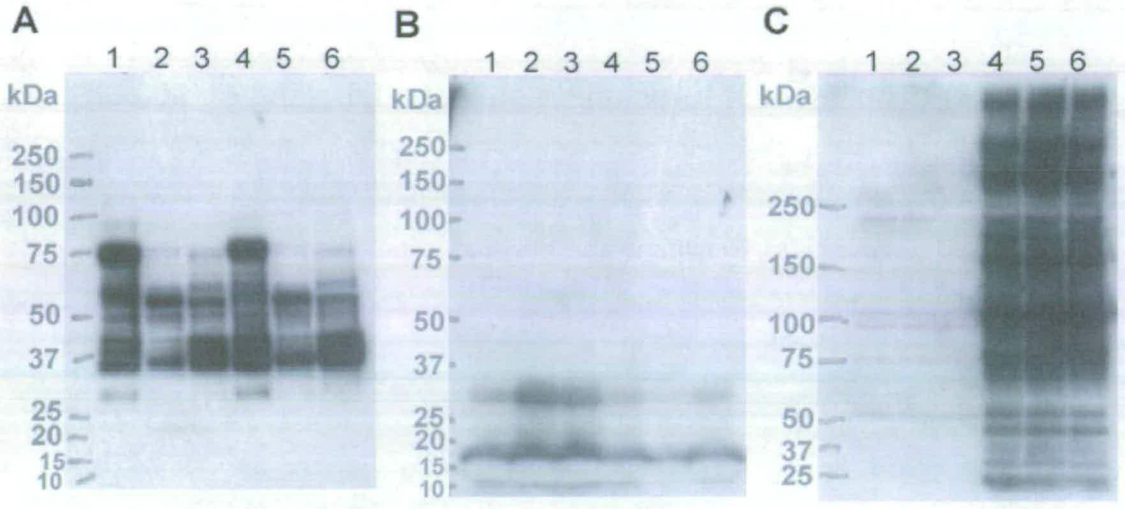
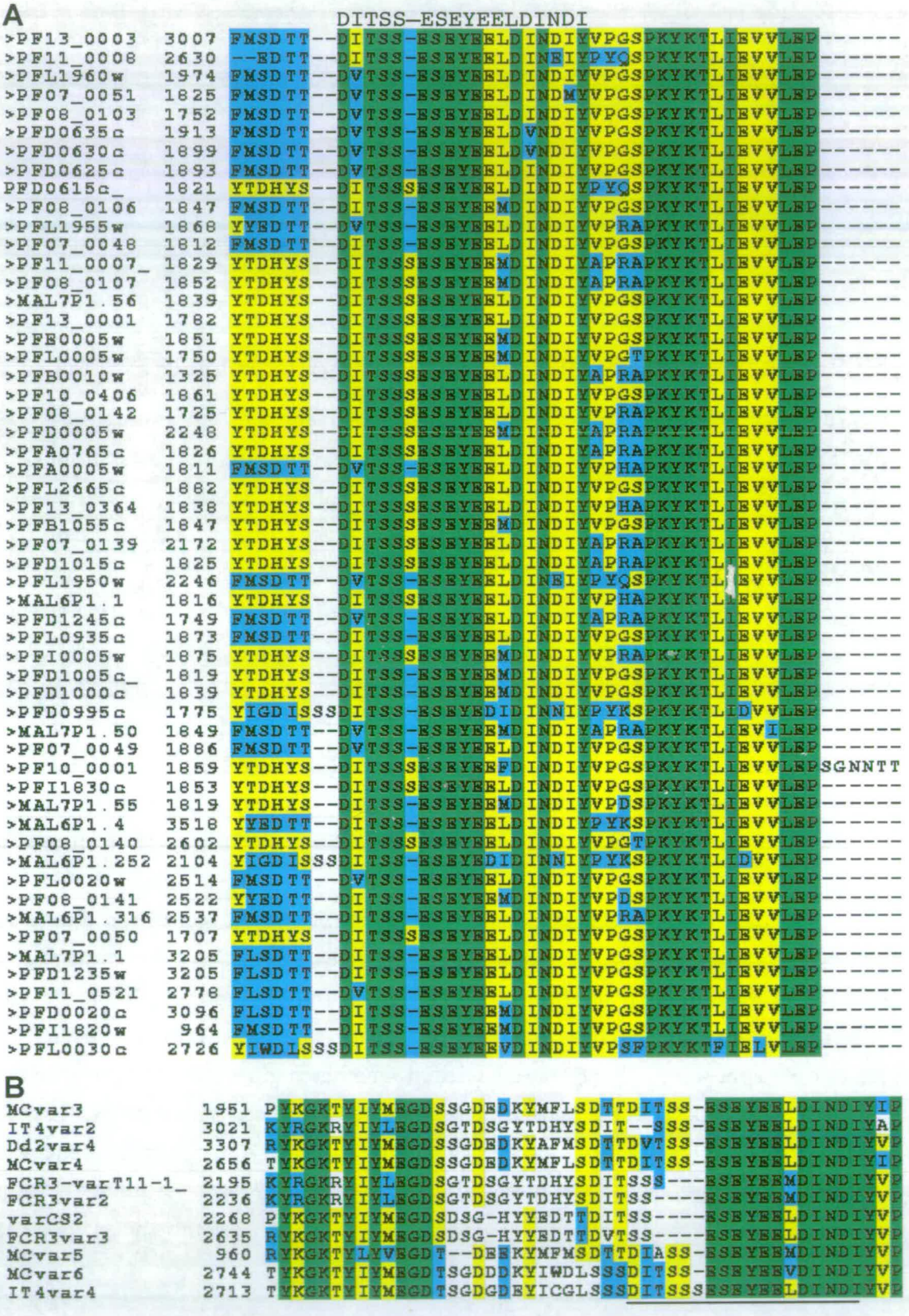


Figure 3.7: Biotin labelling of enriched IEs in the presence of 100 μM furosamide

Intact UEs (lanes 1-3) and plasmagel enriched FCR3CSA IEs (lanes 4-6) were labelled with 0.25 mg/ml sulpho-NHS-LC-biotin for 30 minutes at 37°C, and were then fractionated following hypo-osmotic lysis. Membrane fractions were separated on 3-8% tris-acetate SDS-PAGE gels (Bots A &C) and the lysate fraction was separated on a 4-12% tris-bis SDS-PAGE gel (Blot B). The blots shown in panels A and B were probed with HRP-streptavidin. The blot shown in panel C was probed with pooled IgG from malaria immune sera. Lane 1: UEs, lane 2: UEs treated with 1 mg/ml trypsin, lane 3: UEs treated with 0.1 mg/ml pronase, Lane 4: FCR3CSA IEs, lane 5: IEs treated with 1 mg/ml trypsin, lane 6: IEs treated with 0.1 mg/ml pronase.

3.3.2 Raising an anti-peptide antibody to the ATS region of PfEMP1

For the purpose of raising a universal anti-PfEMP1 antibody an anti-peptide was considered preferable since the heterologous expression of recombinant *P. falciparum* proteins can be problematic. A number of conserved regions within the C-terminal ATS of PfEMP1 were identified by constructing an alignment of 3D7 PfEMP1 sequences (figure 3.8A). Consideration of a hydrophobicity screen based on a Hopps-Woods plot of the 3D7 PfEMP1 PF13_0003 helped to identify a stretch within the ATS that was predicted to be both antigenic and conserved (figure 3.9A). The Hopps-Woods scale assigns values greater than zero to hydrophilic residues and positive peaks that are considered likely to be surface exposed segments of the folded protein. The highly conserved 14 mer, DITSSESEYEELDI, was predicted to be very hydrophilic (figure 3.9A) and although the residues NDI, that follow this sequence are not included in the predicted hydrophilic peak, they were included in the synthesised immunising peptide. Not only did this triplet complete an extremely well conserved sequence, it was encompassed in one of ten antigenic determinants predicted by a second antigenicity plot (figure 3.9B). The Antigenicity software programme used in figure 3.9A uses the method of Kolaskar and Tongaonkar to predict stretches in a protein sequence that are most likely to elicit an antibody response. Predictions are based on whether segments of a sequence have similarities to experimentally confirmed segmental epitopes. An alignment of the region containing the chosen immunising peptide for 3D7 PfEMP1 sequences is shown in figure 3.8A. A further alignment of PfEMP1 sequences from geographically diverse isolates was performed to ensure that the chosen peptide was not peculiar to the 3D7 clone (figure 3.8B).


A
DITSS—ESEYEELDINDI
>PF13_0003 3007 FMSDTT--DITSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF11_0008 2630 --EDTT--DITSS-ESEYEELDINEIYPYQSPKYKTLIEVVLEP------
>PFL1960w 1974 FMSDTT--DVTSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF07_0051 1825 FMSDTT--DVTSS-ESEYEELDINDMYVPGSPKYKTLIEVVLEP------
>PF08_0103 1752 FMSDTT--DVTSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PFD0635c 1913 FMSDTT--DVTSS-ESEYEELDVNDIYVPGSPKYKTLIEVVLEP------
>PFD0630c 1899 FMSDTT--DVTSS-ESEYEELDVNDIYVPGSPKYKTLIEVVLEP------
>PFD0625c 1893 FMSDTT--DVTSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
PFD0615c_ 1821 YTDHYS--DITSSSESEYEELDINDIYPYQSPKYKTLIEVVLEP------
>PF08_0106 1847 FMSDTT--DITSS-ESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
>PFL1955w 1868 YYEDTT--DVTSS-ESEYEELDINDIYVPRAPKYKTLIEVVLEP------
>PF07_0048 1812 FMSDTT--DITSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF11_0007_ 1829 YTDHYS--DITSSSESEYEEMDINDIYAPRAPKYKTLIEVVLEP------
>PF08_0107 1852 YTDHYS--DITSSSESEYEEMDINDIYAPRAPKYKTLIEVVLEP------
>MAL7P1.56 1839 YTDHYS--DITSSSESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF13_0001 1782 YTDHYS--DITSSSESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PFB0005w 1851 YTDHYS--DITSSSESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
>PFL0005w 1750 YTDHYS--DITSSSESEYEEMDINDIYVPGTPKYKTLIEVVLEP------
>PFB0010w 1325 YTDHYS--DITSSSESEYEELDINDIYAPRAPKYKTLIEVVLEP------
>PF10_0406 1861 YTDHYS--DITSSSESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF08_0142 1725 YTDHYS--DITSSSESEYEELDINDIYVPRAPKYKTLIEVVLEP------
>PFD0005w 2248 YTDHYS--DITSSSESEYEEMDINDIYAPRAPKYKTLIEVVLEP------
>PFA0765c 1826 YTDHYS--DITSSSESEYEELDINDIYAPRAPKYKTLIEVVLEP------
>PFA0005w 1811 FMSDTT--DVTSS-ESEYEELDINDIYVPHAPKYKTLIEVVLEP------
>PFL2665c 1882 YTDHYS--DITSSSESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF13_0364 1838 YTDHYS--DITSSSESEYEELDINDIYVPHAPKYKTLIEVVLEP------
>PFB1055c 1847 YTDHYS--DITSSSESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
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>PFL1950w 2246 FMSDTT--DVTSS-ESEYEELDINEIYPYQSPKYKTLIEVVLEP------
>MAL6P1.1 1816 YTDHYS--DITSSSESEYEELDINDIYVPHAPKYKTLIEVVLEP------
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>PFL0935c 1873 FMSDTT--DITSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
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>PFD1000c 1839 YTDHYS--DITSS-ESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
>PFD0995c 1775 YIGDISSSDITSS-ESEYEDIDINNIYPYKSPKYKTLIDVVLEP------
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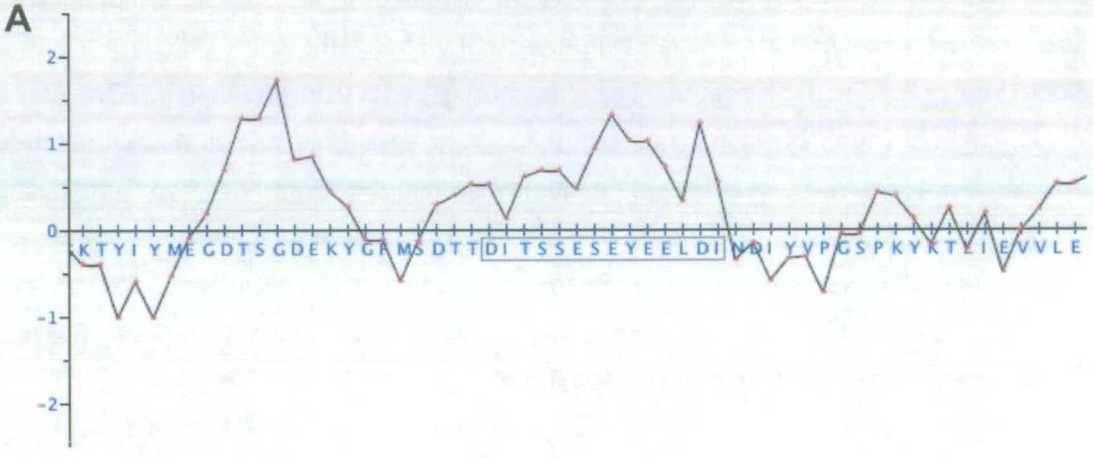
>MAL7P1.55 1819 YTDHYS--DITSS-ESEYEEMDINDIYVPDSPKYKTLIEVVLEP------
>MAL6P1.4 3518 YYEDTT--DITSS-ESEYEELDINDIYPYKSPKYKTLIEVVLEP------
>PF08_0140 2602 YTDHYS--DITSSSESEYEELDINDIYVPGTPKYKTLIEVVLEP------
>MAL6P1.252 2104 YIGDISSSDITSS-ESEYEDIDINNIYPYKSPKYKTLIDVVLEP------
>PFL0020w 2514 FMSDTT--DVTSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF08_0141 2522 YYEDTT--DITSS-ESEYEEMDINDIYVPDSPKYKTLIEVVLEP------
>MAL6P1.316 2537 FMSDTT--DITSS-ESEYEELDINDIYVPRAPKYKTLIEVVLEP------
>PF07_0050 1707 YTDHYS--DITSSSESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>MAL7P1.1 3205 FLSDTT--DITSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PFD1235w 3205 FLSDTT--DITSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PF11_0521 2778 FLSDTT--DVTSS-ESEYEELDINDIYVPGSPKYKTLIEVVLEP------
>PFD0020c 3096 FLSDTT--DITSS-ESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
>PFI1820w 964 FMSDTT--DITSS-ESEYEEMDINDIYVPGSPKYKTLIEVVLEP------
>PFL0030c 2726 YIWDLSSSDITSS-ESEYEEVDINDIYVPSFPKYKTFIELVLEP------
B
MCvar3 1951 PYKGKTYIYMEGDSSGDEDKYMFLSDTTDITSS-ESEYEELDINDIYIP
IT4var2 3021 KYRGKRYIYLEGDSGTDSGYTDHYSDIT--SSS-ESEYEELDINDIYAP
Dd2var4 3307 RYKGKTYIYMEGDSSGDEDKYAFMSDTTDVTSS-ESEYEELDINDIYVP
MCvar4 2656 TYKGKTYIYMEGDSSGDEDKYMFLSDTTDITSS-ESEYEELDINDIYIP
FCR3-varT11-1_ 2195 KYRGKRYIYLEGDSGTDSGYTDHYSDITSSS---ESEYEEMDINDIYVP
FCR3var2 2236 KYRGKRYIYLEGDSGTDSGYTDHYSDITSS----ESEYEEMDINDIYVP
varCS2 2268 PYKGKTYIYMEGDSDSG-HYYEDTTDITSS----ESEYEELDINDIYVP
FCR3var3 2635 RYKGKTYIYMEGDSDSG-HYYEDTTDVTSS----ESEYEELDINDIYVP
MCvar5 960 RYKGKTYLYVEGDT--DEEKYMFMSDTTDIASS-ESEYEEMDINDIYVP
MCvar6 2744 TYKGKTYIYMEGDTSGDDDKYIWDLSSSDITSS-ESEYEEVDINDIYVP
IT4var4 2713 TYKGKTYIYMEGDTSGDGDEYICGLSSSDITSSSESEYEELDINDIYVP

Figure 3.8: Multiple sequence alignment of a conserved region of the ATS region of PfEMP1

Panel A shows an alignment of 55 PfEMP1 sequences from the genome of the *P. falciparum* clone 3D7 (PlasmoDB.org). Panel B shows an alignment of 11 PfEMP1 sequences from three geographically diverse *P. falciparum* isolates, Malayan Camp, Dd2 and a parasite clone from the IT/FCR3 lineage. The immunising peptide is underlined. The Biology Workbench 3.2 (http://workbench.sdsc.edu) was used as an interface to access databases and sequence analysis tools. Multiple sequence alignments were compiled with ClustalW (Thompson *et al.*, 1994) using the Gonnet series weight matrix. Alignments were coloured using BOXSHADE version 3.1.1, where background shading in yellow highlights conserved residues; green identical residues, cyan similar residues, white different residues. Boxshade default settings were used for similarity definitions.

The peptide DITSSESEYEELDINDIC conjugated to KHL was used to immunise 2 white New Zealand rabbits as described in section 7.6.1. Initial screening of pre-immune sera highlighted cross reactivity to both uninfected and *P. falciparum* infected human erythrocytes (figure 3.10A&B). The cross reactivity to IEs was apparent as a punctate staining throughout the erythrocyte cytosol, a pattern similar to the anticipated pattern of reactivity following immunisation. Naturally occurring rabbit antibodies that recognise uninfected and *P. falciparum* human IEs have previously been reported (Crandall *et al.*, 1996). The immune sera was, therefore, affinity purify on the immunising peptide. For affinity purification, serum from rabbit SG-1780 was selected due to its lower cross-reactivity to uninfected cells, as detected by immunofluorescence assay (figure 3.10B). Although the intensity of staining in IFA increased following immunisation an ELISA was carried out to confirm that an antibody response specific to the immunising peptide had been raised and the efficiency of affinity purification. The ELISA confirmed that the pre-immune sera did not cross-react to the immunising peptide, that an antibody response specific to the peptide was raised and that peptide specific antibodies had been isolated by affinity purification (figure 3.11A). No reactivity was found to remain in the flow through from the affinity column used for purification (figure 3.11A). The optical density (OD) values for this ELISA were extremely low and serum dilution had no effect on reactivity. However, due to the small values of deviation between duplicate wells and consistent negative controls the small OD readings above the control values were considered to represent true reactivity. The low OD readings and lack of effect of serum dilution presumably resulted from the inefficient adsorption of the peptide to ELISA plate, such that the

small quantity of peptide adsorbed was completely bound by the highest dilutions of sera.



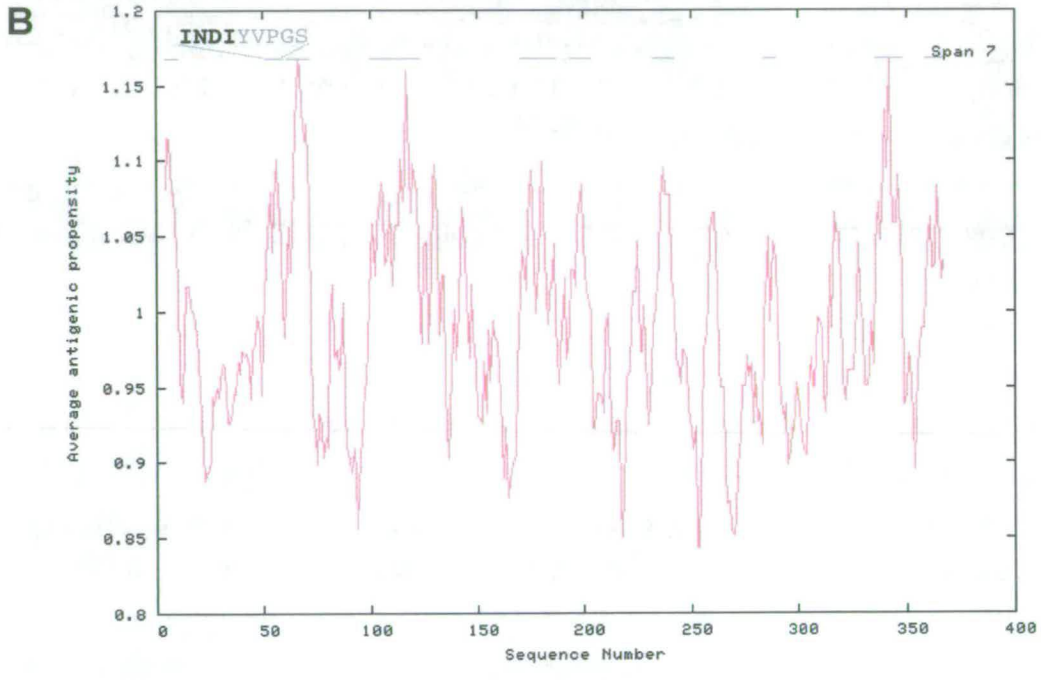


Figure 3.9: Antigenicity screening of the ATS sequence from 3D7 PfEMP1 PF13_0003

Panel A shows a Hopp-Woods plot carried out using HYDROPHOBICITY 1.0 software (http://www.bmm.icnet.uk) with a window size of seven. Boxed residues highlight the hydrophilic 14 mer included in the immunising peptide. For panel B an antigenicity plot was constructed using Antigenicity software (Kolaskar & Tongaonkar, 1990) (http://mif.dfci.harvard.edu). The sequence of the second antigenic determinant is shown above the corresponding peak. The first four residues highlighted in bold type were included in the immunising peptide.

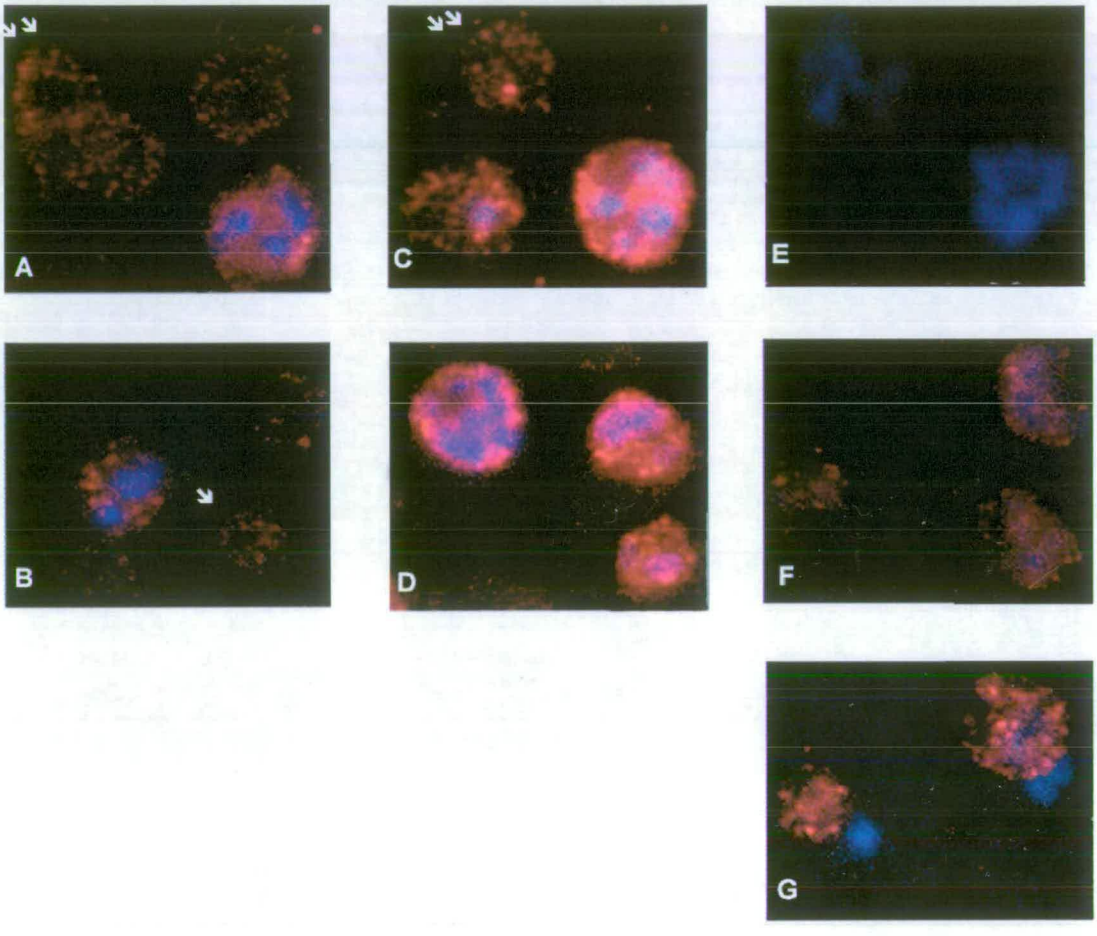


Figure 3.10: Immunofluorescence assay with anti-ATS rabbit antisera

Panels A-F show FCR3CSA-IEs and panel G A4-IEs. Nuclei are stained with DAPI (blue), red fluorescence shows rhodamine red-X-conjugated donkey (Jackson Laboratories) reactivity to rabbit immunoglobulins. A: pre-immune sera from rabbit 1779, B: pre-immune sera from rabbit 1780, C: Immune sera from rabbit 1779, D Immune sera from rabbit 1780, E secondary conjugate alone control, F&G: affinity purified sera from rabbit 1780. White arrows highlight uninfected cells.

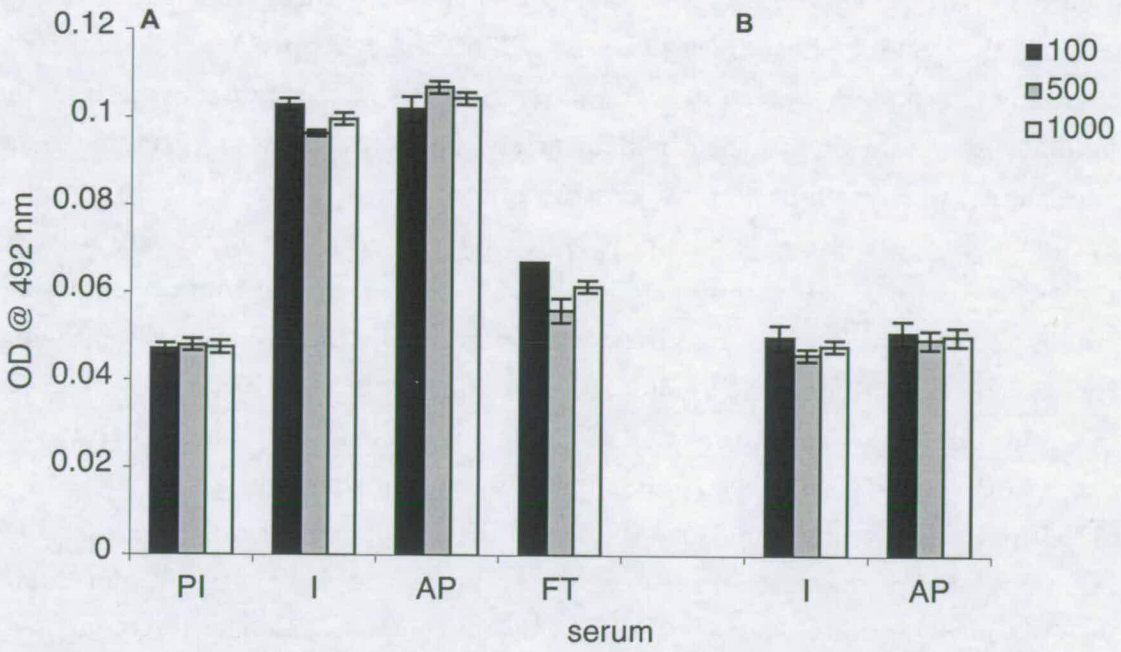


Figure 3.11: Peptide ELISA to establish specificity of rabbit sera

Rabbit immunoglobulins bound to the immunising ATS peptide immobilised to a microtitre plate were detected using HRP conjugated goat anti-rabbit Ig immunoglobulins. Panel A shows sera reactivity to 200 μg of peptide and panel B sera reactivity to coating buffer alone. The key denotes the serum dilution factors used. PI: pre-immune serum, I: immune serum, AP: affinity purified immunoglobulins, FT: flow through from affinity column.

In IFA affinity purified sera recognised erythrocytes infected with both FCR3CSA and A4 *P. falciparum* clones (figure 3.10 F&G), reactivity towards the later clone being of greater intensity (figure 3.10 G). A punctate pattern of staining was evident throughout the erythrocyte cytosol in a pattern previously reported for PfEMP1 and other proteins that are transported to erythrocyte plasma membrane. This staining pattern appeared in a developmental manner, whereby staining became more intense and filled more of the erythrocyte cytosol with increasing maturity of the parasite (data not shown).

The affinity purified anti-ATS antibody was then used in western blotting of membrane fractions from IEs prepared by osmotic lysis. The antibody consistently failed to recognise parasite specific proteins (data not shown) and blotting was complicated by consistent cross-reactivity to erythrocytic α and β spectrin, and an

additional host protein of 50 kDa, by the HRP-conjugated secondary antibody (figure 3.12A; I & ii). These spectrin isoforms span the predicted molecular weight of more than 50% of the PfEMP1s in the 3D7 genome (Appendix 3). Therefore alternative secondary detection methods were investigated along with steps to solubilise the pigmented nuclear pellet fraction that forms following osmotic lysis and membrane fractionation by centrifugation. An A4 infected culture was fractionated for this purpose due to its higher reactivity to the peptide antibody in IFA (figure 3.10G). HRP-conjugated to protein A, a bacterial immunoglobulin-binding protein, along with biotinylated affinity-purified anti-ATS antibody with HRP-conjugated streptavidin, as alternative secondary detection reagents were used in western blotting. To confirm that biotinylation of the affinity-purified antibody had been a success, the labelled and unlabelled immunoglobulins were analysed by SDS-PAGE and western blotting (figure 3.12B). This also confirmed that the immunoglobulins had not been excessively biotin labelled as their molecular weight, as determined by SDS-PAGE, was unchanged. Both HRP-conjugated protein A and streptavidin proved to be more suitable secondary reagents. Neither reagent was reactive towards high molecular weight host proteins, although slight reactivity to a host protein of approximately 75 kDa in the pigmented fraction was observed. HRP conjugated protein A was used for all subsequent western blotting, although the affinity purified biotin labelled antibody could potentially be a valuable reagent for future double labelling immunofluorescence assays.

In these experiments, the small, pigmented pellet than forms beneath the layer of red cell ghosts when IE cultures are fractionated was also analysed. This parasite pigment rich pellet also contains parasite nuclei and could not be loaded onto SDS-PAGE gels without nuclease treatment. The nuclease benzonase was used as it degrades genomic DNA rapidly at room temperature, therefore, avoiding the increased incubation times or temperatures required for alternative nucleases at which protease activity might increase. Erythrocytes infected with A4 schizonts expressed three protein species that are detected by the anti-ATS antibody, and these localise exclusively to the pigmented pellet fraction following osmotic lysis. Although membrane fractionation effectively enriches for band 3 and other host erythrocyte surface proteins (figure 3.5), PfEMP1 could not be detected in this fraction. The anti-ATS reactive proteins migrate on a SDS-PAGE gel above the 100 kDa, 150 kDa and 250 kDa molecular weight markers (figure 3.12A; iii & vi). The highest molecular weight band of over 250 kDa is the approximate size reported for PfEMP1, the two smaller bands however are not, although the genome of parasite

clone 3D7 does encode for one PfEMP1 with a predicted molecular weight of 150 kDa.

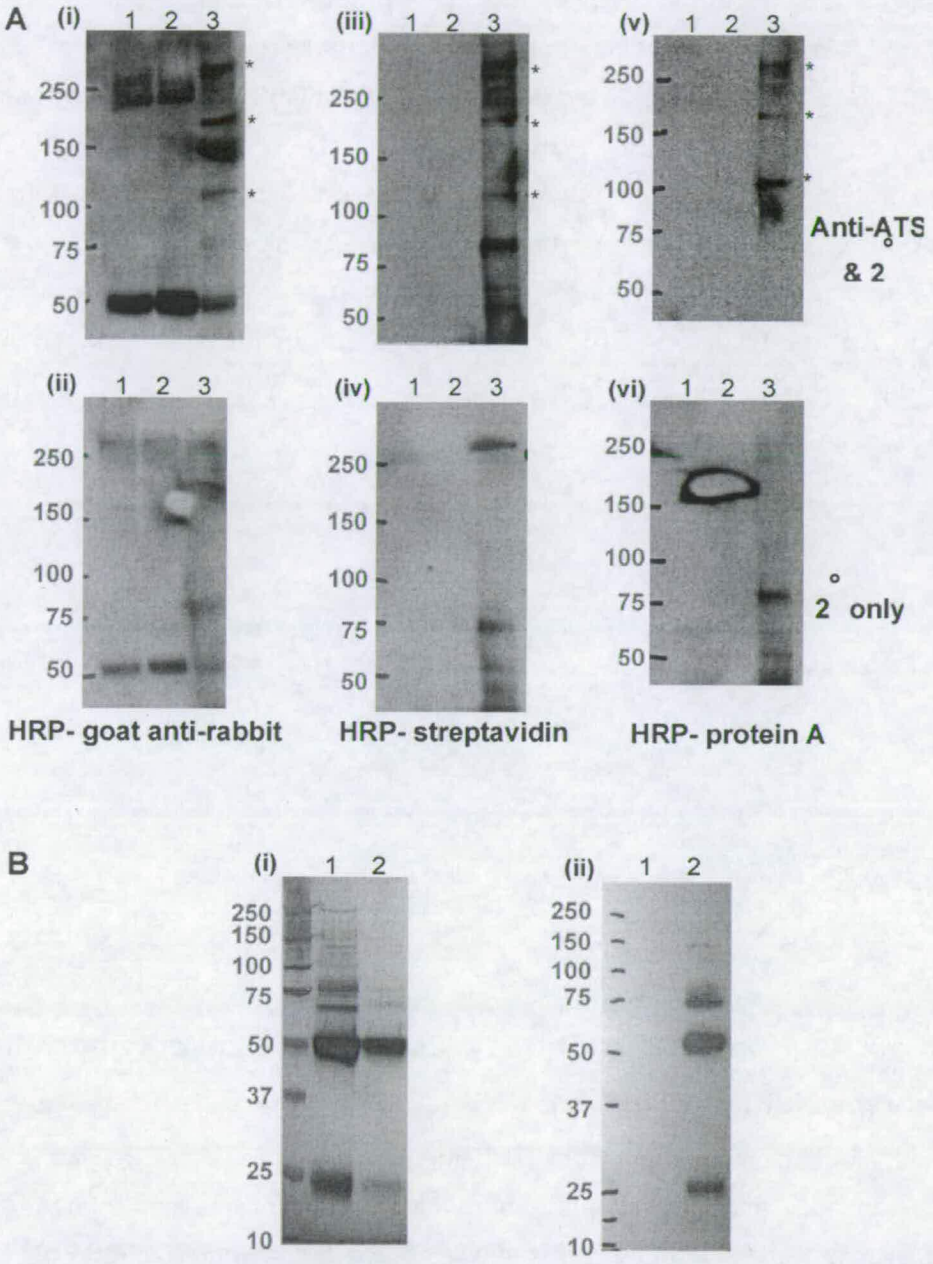


Figure 3.12: Secondary detection reagents for the detection of PfEMP1

Panel A, for blots i, iii, and v affinity purified anti-ATS immunoglobulins were used at 1:500 dilution and for blots ii, iv and vi the secondary reagent indicated was used alone. Lane 1: UE membrane fraction, lane 2: A4 IE membrane fraction, and lane 3: A4 IE pigmented fraction. Panel B, SDS-PAGE using 4-12 % bis-tris gels. Lane 1: unlabelled affinity purified immunoglobulins, lane 2: biotinylated affinity purified immunoglobulins. For gel i proteins are stained with silver nitrate and ii is a western blot using HRP-streptavidin. For blots i-vi SDS-PAGE was performed using 3-8% tris-acetate gels.

Following confirmation that the anti-ATS antibody reacted cleanly in western blot it was used to follow changes in PfEMP1 expression in the FCR3 parasite following selection for CSA binding (figure 3.13). The antibody failed to react to protein of over 250 kDa in FCR3, however, the lower molecular weight proteins of ~ 150 kDa and ~100 kDa were consistently detected in A4, FCR3 and FCR3CSA schizonts (highlighted with asterisk). A protein of > 250 kDa was detected in FCR3CSA, which showed size heterogeneity when compared to the largest protein species detected in A4 schizonts (figure 3.13; lanes 1 and 6). These higher molecular weight species detected by the anti-ATS peptide antibody will now be referred to as PfEMP1. An uninfected erythrocyte sample was not included as a negative control as the pigmented pellet does not form when mock cultures are fractionated by osmotic lysis.

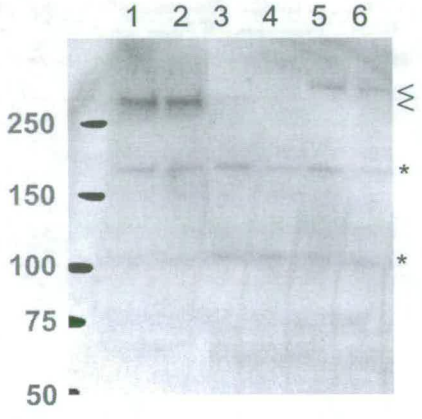


Figure 3.13: Anti-ATS antibody detects a size polymorphic high molecular weight protein

A western blot of pigmented pellet fractions from schizont-infected erythrocytes probed with anti-ATS antibody and HRP-streptavidin. Pigmented pellet fractions from A4 IEs were loaded in lanes 1 and 2, from FCR3 IEs in lanes 3 and 4, and from FCR3CSA IEs in lanes 5 and 6. For lanes 2, 4 and 6 intact IEs were treated with 1 mg/ml trypsin prior to osmotic lysis.

The surface expression of PfEMP1 species was investigated, by treating cells with 1 mg/ml trypsin prior to protein fractionation. Surface trypsinisation did not affect the band intensity of the PfEMP1 expressed by either A4 or FCR3CSA schizonts (figure 3.13; lanes 2 and 6). This is likely due to the fact that a large proportion of

PfEMP1 has been shown to remain within the infected erythrocyte such that only a small proportion of the total cell PfEMP1 is actually exposed to surface trypsinisation (Haeggstrom *et al.*, 2004; Kriek *et al.*, 2003). The SDS-PAGE gel used in figure 3.13 was silver stained post electrophoretic transfer. From the residual protein remaining in the gel it was possible to confirm the activity of trypsin, as proteolysis of host erythrocyte surface proteins was clearly apparent. This silver stained gel also confirmed that protein loading per lane was equivalent and efficient transfer across the gel had occurred. In addition, the lower molecular bands detected by the anti-ATS antibody also indicate that protein loading was equivalent (data not shown).

3.3.2 Identification of biotin labelled high-molecular weight candidate *Pf*VSA

Having localised PfEMP1 to the pigmented pellet fraction, FCR3CSA late trophozoite stages were labelled with biotin and the pigmented pellet fraction was included in western blot analysis. Figure 3.15 shows membrane fractions from an uninfected mock culture, and two FCR3 cultures that had been independently selected for CSA binding, and the pigmented pellet fractions for the two FCR3CSA cultures. Biotin-labelled, high molecular weight proteins that were also sensitive to cell surface trypsinisation were indeed also localised to the pigmented fractions (figure 3.15; lanes 7–10). Unfortunately the labelling appeared quite diffuse but asterisks highlight the clearest labelled proteins and shows variation in the labelling pattern between the two FCR3CSA cultures. Although these cultures were selected and synchronised in parallel there may have been slight variation in the parasite stages, or this variation may indicate that alternative proteins can be upregulated following selection for CSA adhesion. As previously mentioned the pigmented fraction does not form following membrane fractionation of uninfected erythrocyte cultures, but to address more closely the parasite origin of these proteins and their stage specific expression surface biotinylation was carried out over a time course of intraerythrocytic development.

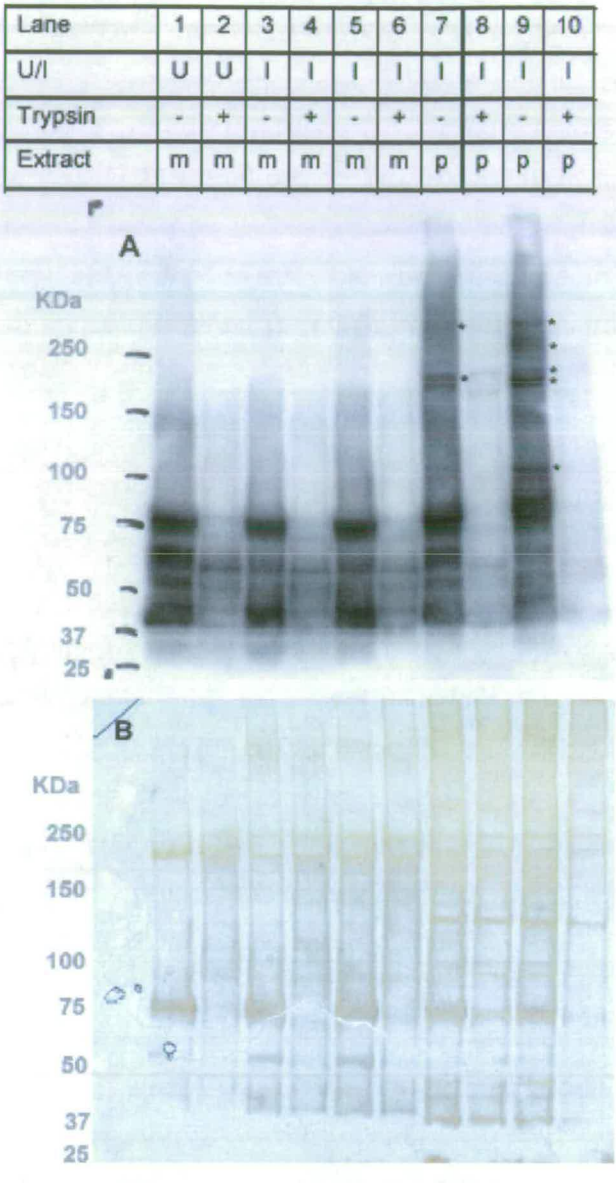


Figure 3.14: Parasite-specific biotinylated proteins

Panel A shows proteins extracts from surface-biotinylated UEs and late stage trophozoite and schizont IEs (FCR3CSA) separated by SDS-PAGE, transfered to PVDF membrane and probed with HRP-conjugated streptavidin. Panel B shows the SDS-PAGE gel post-electrophoretic transfer stained with silver nitrate. Cells were labelled with 0.5 mg/ml sulpho-NHS-LC-biotin in the presence of 100 μM furosamide.

A synchronised trophozoite culture of parasite clone FCR3CSA was used to seed three daughter flasks with equivalent numbers of parasites such that the parasitaemia in the next asexual cycle reached 10%. At the appropriate time points, flasks containing either ring, trophozoite or schizont stages were harvested for surface biotinylation and membrane fractionation. (The culture was synchronised and flasks harvested in parallel to the A4 cultures of which Giemsa stained slides are shown in figure 4.3A.) High molecular weight biotinylated proteins were not present in ring stage parasites (figure 3.15; lane 1). Although it was not possible to load an uninfected control for the pigment pellet fraction, the labelling pattern for ring stages is comparable to that seen for the membrane fraction from uninfected erythrocytes in this experiment (data not shown) and in an independent labelling experiment (see figure 3.14; lanes 1 and 2). A number of high molecular weight biotin labelled proteins that were sensitive to surface trypsinisation were apparent in the young trophozoite culture (figure 3.15; lanes 3 and 4) and the complexity of the labelled proteins increased in the schizont culture (figure 3.15; lanes 5 and 6).

This time course experiment showed the anticipated stage specific expression of surface exposed biotinylated proteins, if indeed these proteins are *Pf*VSAs. The pattern of biotinylated proteins that migrate around the 250 kDa marker in the schizont extract (figure 3.15; lane 5) is also comparable to the pattern seen in an independent labelling experiment of trophozoites and schizont stages (figure 3.14; lane 9), thus reflecting the reproducibility of this technique.

The SDS-PAGE gel used for preparation of the western blot shown in figure 3.15 was again silver stained post electrophoretic transfer to confirm that the integrity of the extracts prepared from trypsin treated samples was comparable to the non-trypsinised samples. The blot shown in figure 3.15 was also re-probed with the anti-ATS antibody to confirm the integrity of high molecular weight parasite proteins in trypsinised samples (data not shown). This showed that the highest molecular weight biotinylated protein in the schizont extract (figure 3.15; lanes 5, white asterisk) co-localises with the PfEMP1 expressed by this clone.

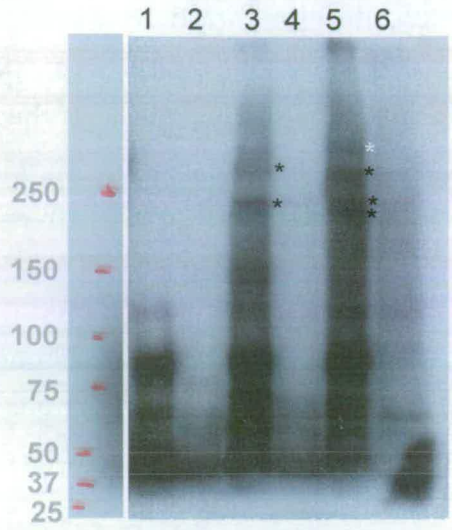


Figure 3.15: Intraerythrocytic developmental regulation of biotin labelled proteins

A western blot of pigmented pellet fractions prepared from synchronous cultures of FCR3CSA: ring IEs lanes 1 and 2, trophozoite IEs lanes 3 and 4 and schizont IEs lanes 5 and 6. In lanes 2, 4 and 6 intact IEs were treated with 1 mg/ml trypsin prior to osmotic lysis and protein extraction. Biotin-labelled proteins were detected using HRP-conjugated streptavidin and ECL detection. Proteins were separated by SDS-PAGE using a 3-8% tris-acetate gel. . Cells were labelled with 0.5 mg/ml sulpho-NHS-LC-biotin in the presence of 100 μM furosamide.

Finally, parasite specific, developmentally regulated, surface trypsinisation sensitive proteins were identified in a CSA selected parasite clone. Unfortunately time did not allow for further proteomic analysis of these proteins. Attempts were made to affinity purify biotin labelled proteins on immobilised streptavidin, for subsequent identification using mass spectrometry, however, despite numerous experiments to optimise this technique (data not shown) bands corresponding to the appropriate molecular weight could not be isolated. Upon further consideration of these experiments, blocking of non-specific protein and biotin binding sites may have been the main problem, in combination with the small quantities of extract available and the low abundance of the labelled proteins. Considering the undetectable effect of trypsin on the cell's pool of PfEMP1 suggests that for FCR3CSA at least, this VSA is exposed at the erythrocyte surface in relatively small amounts. Alternative approaches for the identification of the biotin labelled proteins identified here are

discussed in the discussion section of this chapter. That the pattern of high molecular weight biotinylated proteins appears more complex than the PfEMP1 species detected in FCR3CSA is also discussed.

3.4 Discussion and future work

Initial analysis of 'surface' biotinylated cell extracts using 2DGE and MALDI-TOF mass spectrometry unfortunately did not identify any *Pf*VSA (Table 3.1). The major problem was considered at the time to be the biotin labelling of internal proteins. Therefore a labelling protocol was optimised that detected biotinylated parasite specific, surface trypsinisation sensitive, developmentally regulated proteins by western blotting, however, time did not allow for the separation of these proteins by 2DGE and their subsequent identification. However, considering the average percentage identification rate for protein spots extracted from 2D gels only reached 50% using MALDI-TOF mass spectrometry, together with the recent exciting advances in non-gel based proteomic alternatives, a non-gel based technique is likely to be the way forward in this analysis. Such a technique is especially attractive in the case of PfEMP1 and the biotin-labelled proteins identified as they are both large molecular weight membrane-proteins (figure 3.14) and these are notoriously difficult to resolve by 2DGE (Santoni *et al.*, 2000).

Developments in the methods and instrumentation for automated, data-dependent tandem electrospray ionisation (ESI) mass spectrometry in combination with in-line microcapillary liquid chromatography (μLC) means large-scale identification of proteins in complex mixtures without prior gel electrophoretic separation has become more routine. ESI mass spectrometry can measure the mass of both positively and negatively charged ions, when used in tandem it can provide more information than tandem MALDI-TOF MS that only determines the mass of positively charged ions. Therefore, tandem ESI MS is considered the more powerful tool for identifying peptide sequence tags.

When analysing complex mixtures of proteins by μLC and tandem ESI MS a fractionation or partial purification step to enrich for candidate proteins of interest is considered advantageous to simplify the automated data analysis. The proteins that make up the pigmented pellet fraction represent a small fraction of the proteins present in the crude cell extract. This pellet fraction is quite a simple mixture and most of the highly abundant host cell proteins, that would create a significant amount of 'noise' in μLC and tandem ESI MS analysis, have been removed. The proteins of interest are also biotin labelled. Although affinity purification on immobilised streptavidin or monomeric avidin was not accomplished during this project, such a chromatographic purification step has been applied for isotope-coded affinity tag (ICAT) technology (Gygi *et al.*, 1999) to enrich for biotinylated

peptides following tryptic digestion of biotin labelled protein extracts (Ranish *et al.*, 2003). ICAT is a quantitative proteomics approach that uses μLC-MSMS in combination with an isotope-coded biotin tag. Instead of separating by 2DGE the biotin labelled proteins I have identified by 1D SDS-PAGE it might be feasible and preferable to digest the entire biotin labelled pigmented pellet extract and use avidin chromatography steps similar to those used in ICAT technology, in combination with μLC and tandem ESI mass spectrometry, as is applied in the ICAT approach. Although, FCR3 infected erythrocytes that had been 'mock selected' for CSA binding were used as a control for early surface biotinylation experiments, during the steps to optimise the labelling technique this control was not always included. This was due to the time necessary to culture FCR3 and FCR3CSA IEs in parallel while maintaining equivalent parasitaemias and synchronicity. Therefore, for initial analysis using μLC-MSMS a comparison of pigmented pellet extracts from the surface trypsinised FCR3CSA IEs and untreated IEs (figure 3.14) might be informative, at least for confirming that these biotin labelled proteins are indeed surface expressed, if not upregulated after CSA selection. FCR3 and FCR3CSA along with additional pairs of clones pre- and post CSA selection could be labelled to look for the up-regulation of specific biotinylated proteins following CSA selection. Ideally, a clone would be selected for an alternative adhesive phenotype, such as CD36 binding, to confirm any up-regulated proteins are not ubiquitously expressed by IEs following selection for any adhesive phenotype. Alternatively, analysis might be simplified by cutting slices from 1D SDS-PAGE gels at the molecular weights that correspond to the sizes of biotin labelled bands detected by western blotting and then attempting to analyse those quite simple mixtures of proteins by μLC-MS/MS.

During this project considerable time was invested in developing the surface biotinylation technique and detection of biotinylated proteins by western blotting. One could ask whether, in light of recent advances in high-throughput multidimensional protein identification technologies (MuDPIT) such μLC in combination with tandem electrospray ionisation mass spectrometry whether this optimisation will prove to be applicable. However, review of the studies that have applied MuDPIT-like proteomics approaches to *P. falciparum* during the course of this work suggests there are limitations, a major one being reproducibility. Screening labelled pigmented fractions prior to MuDPIT by western blotting would allow the reproducibility of independently labelled and extracted fractions to be determined. I am of the opinion that by enriching for specifically labelled surface

exposed proteins and having an estimation of their molecular weight will aid in refining the starting mixture of proteins to be analysed and thus reduce the complexity of data produced by MuDPIT. Perhaps a more stringent and thorough analysis of a smaller data set would then be feasible.

Over the course of this project four large-scale proteomic analyses of *P. falciparum* material have been carried out. The first two studies concentrated on defining the proteomes of different developmental stages for the parasite clone 3D7/NF54 (Florens *et al.*, 2002; Lasonder *et al.*, 2002). Lasonder and colleagues (2002) analysed trophozoites/schizont preparations, gametocyte and gamete preparations using a combination of 1D electrophoretic separation and in-line, high resolution μLC with tandem mass spectrometry. Their analysis identified 1289 parasite proteins, which comprised approximately 23% of the predicted *P. falciparum* proteome at that time. Also, 1000 human proteins and, importantly, a set of *P. falciprum* derived peptides that are not predicted by computational gene finding software were identified. A second study by Florens and colleagues (2002) used multidimensional protein identification technology (MuDPIT) using a method based on Washburn *et al.* (2001) which also involved in-line, high resolution μLC and tandem mass spectrometry. Relatively homogeneous preparations of merozoites, trophozoites, gametocytes and sporozoites were analysed which lead to the identification of 2425 parasite proteins (43% of predicted *P. falciparum* proteome).

Florens *et al.* and Lasonder *et al.* successfully described sets of stage specific proteins, however, a comparison of their findings highlights discrepancies with regard to the number of proteins and protein families found at different life cycle stages. Of interest to this work, there were discrepancies with regard to PfEMP1 and rifin proteins. Florens *et al.* (2002) reported that a repertoire of PfEMP1s and rifins are expressed by cultured gametocytes and that a preparation of sporozoites contained 21 different rifins and 25 PfEMP1 variants. They also detected PfEMP1s but not rifins in trophozoite extracts. Likewise Lasonder *et al.* (2002) did not detect rifins in their trophozoite/schizont preparation, however, unlike Florens *et al.* they did not detect PfEMP1 either.

The differences regarding stage specificity for PfEMP1 and rifin expression in Lasonder's and Florens' analyses may partly be explained by different stringency levels used for data filtering. For example the sequence coverage reported for PfEMP1s detected in any developmental stage by Florens *et al.* (2002) only reached 0.1%, although three of the rifins identified in the sporozoite extracts reached 80% sequence coverage. Because PfEMP1 is such a large protein and *var* genes are

present in multiple copies per genome the chance of hitting a peptide sequence within a PfEMP1 with similarity to a query peptide might be considered more likely, than for example an average sized single copy gene. Florens *et al.* (2002) also include half-tryptic peptides in their analysis, this may also increase the likelihood of mis-assigning peptides to proteins. These analyses, however, highlight the importance of considering the stringency levels applied to the analysis of MuDPIT data. Lasonder *et al.* (2002) also suggest that extraction methods may be responsible for their analysis not detecting PfEMP1 in preparations from asexual blood stage parasites.

PfEMP1 has primarily been studied in the asexual blood stages of the parasite, however, young gametocytes sequester so it will be interesting to discover whether a specific subset of PfEMPs are expressed at this stage or whether the expression pattern is analogous to that seen in asexual stages. The expression of PfEMP1 in sporozoites is supported by transcriptional microarray analysis of this developmental stage, Le Roch *et al.* (2003) identified transcripts for 22 of the 23 PfEMP1 variants identified by Florens *et al.* (2002).

More recently Florens *et al.* (2004) have used MuDPIT to analyse extracts from 'surface' biotinylated IEs. In this study IEs were bitoinylated with sulpho-NHS-LC-biotin and a biotin analogue that labels free sulfhydryl groups; however, an inhibitor of the parasite's novel permeation pathway, such as furosamide, was not included in the labelling step. Considering the findings of this chapter (figure 3.7), and those of Nyalwidhe *et al.* (2002) and Baumeister *et al.* (2003), the labelling conditions used by Florens *et al.* would have allowed for significant labelling of internal proteins. Especially considering their long incubation times for labelling (6 hours). Despite the internal labelling that occurred, following avidin affinity purification of biotin labelled proteins, two novel *P. falciparum* erythrocyte surface antigens were identified by MuDPIT analysis and have been designated parasite-infected erythrocyte surface protein 1 and 2 (PIESP1 and 2). Interestingly, PIESP1 and 2 are encoded by single-copy genes, which are conserved across *Plasmodium* species, and show little sequence diversity between different *P. falciparum* strains. Florens *et al.* (2004) confirmed erythrocyte surface expression by raising antisera to PIESP1 and 2 and performing IFA on live IEs. The work of Florens *et al.* (2004) suggests that ensuring surface specific labelling with biotin may not be crucial for proteomic analysis of surface proteins. Interestingly, despite Florens *et al.* confirming by western blotting that their 'surface preparations' contained PfEMP1 and rifin proteins they do not report detecting these proteins in their MuDPIT

analysis, although they did identify the secreted proteins (Exp1 and 2) and the rhoptry proteins (RAP-1 & 2 and RhopH-2 & –3).

Finally, the last proteomic study that has been published focused on the analysis of PfEMP1 variants expressed by both CSA selected clones and placental isolates. Fried *et al.* (2004) carried out μLC-MS/MS on tryptic peptides from PfEMP1 enriched detergent extracts that had been further fractionated on a strong cation exchange column. μLC-MS/MS analysis was also performed on tryptic peptides extracted from gel slices cut out from 1D SDS-PAGE gels loaded with the PfEMP1 enriched detergent extracts. The PfEMP1 variants detected in extracts from CSA selected and unselected IEs were compared to extracts from a number of placental isolates. A transcript encoding the PfEMP1 var2csa (PFL0030c) has recently been shown to be up-regulated following CSA-selection using real-time RT-PCR analysis (Salanti *et al.*, 2003), however, the MS/MS analysis of Fried *et al.* (2004) suggests var2csa is not associated with CSA-binding parasites. Peptides from FCR3*var*CSA-like PfEMP1 variants were detected in one parasite clone; however, this clone had not been selected to bind CSA. This analysis failed to support the role of any current candidate CSA binding PfEMP1s, but highlighted an additional four PfEMP1 molecules MAL6P1.4, PF07_0051, PFL1960w, and PF10005w as being associated with CSA-binding.

However, Fried *et al.* (2004) point out that the reproducibility between independent extracts from the same parasite clone or isolate was poor. For example, for an unselected and CSA-selected parasite clone, only 1 of 9 and 2 of 13 PfEMP1 variants respectively were detected in repeated assays. A comparable level of irreproducibility was also seen in Florens *et al.* (2002) MuDPIT analysis of the 3D7 parasite. None of the seven PfEMP1 variants identified were detected in more than one of four repeat assays. Also, as was the case with the analysis by Florens *et al.* (2002), Fried *et al.*, (2004) only achieved low sequence coverage (ranging from 0.5%-3.8%) for PfEMP1 variants. The PfEMP1 sequence coverage was particularly low compared to other membrane proteins, where coverage reached 26.7% for example.

The proteins I found to be selectively labelled during biotinylation of intact IEs in the absence of furosamide may be those proteins that are directly involved or associated with the NNP and transport of solutes in and out of the IE. The molecular components of the NNP pathway are currently not characterised, therefore, identification of these biotinylated proteins may aid in the delineation of this pathway. The apparent slight upregulation of some of these intracellular proteins, such as GBP-130, in the triton-soluble, FCR3CSA extract (figure 3.3) may

reflect a response to the selection for an adhesive phenotype and the upregulation of VSA transport to the IE surface.

The anti-ATS raised in this study will be a useful reagent for future studies, especially since the antibody appears to be relatively specific and versatile (working in IFA and western blotting). If conditions can be optimised to use this antibody for immunoprecipitation purposes, uLC-MSMS analysis could be performed on the precipitated PfEMP1 species to obtain peptide sequence tags for identification. By immunoprecipitating from extracts prepared from surface biotinylated IEs selected for CSA adhesion and western blotting the precipitated PfEMP1s with HRP-streptavidin this would confirm the surface expression of the precipitated PfEMP1 species. An effect of treating intact IEs with trypsin or pronase would also be anticipated to be evident for precipitated biotinylated PfEMP1 species.

Although the anti-ATS antibody was affinity purified on the immunising peptide two lower molecular weight proteins, one running just over 100 kDa and the other just over 150 kDa were consistently detected in trophozoite/schizont extracts from A4, FCR3 and FCR3CSA. A more tightly synchronised culture of A4 IEs revealed that only the schizont preparations contained these 2 proteins (See Chapter 4). Interestingly, no size polymorphism was apparent for these lower molecular weight species between A4 and FCR3, unlike the larger PfEMP1 species (>250 kDa) expressed by these two clones (figure 3.13). The genome of the 3D7 parasite clone contains 4 *var* genes that are predicted to encode proteins of less than 200 kDa; PFI1820, PFA0015c, and MAL6P1.314 are predicted to encode PfEMP1s of approximately 150 kDa and PRB0010w is predicted to encode a 200 kDa protein (Appendix 3). However, to see the pattern of recognition seen in the in figure 3.13 similar genes would have to be conserved in the genomes of the A4 and FCR3. It would be interesting to determine whether these two lower molecular weight proteins are expressed in other parasite clones.

An alignment of putative truncated *var* genes, from the 3D7 genome was also constructed to determine whether any of these genes might explain the lower molecular weight species that were recognised by the anti-ATS antibody. A number of putative truncated *var* genes and *var* pseudo genes were found to encode the sequence of the immunising peptide for the anti-ATS antibody (An alignment of truncated and pseudo *var* genes is included in Appendix 4). The lower molecular weight protein species detected by the anti-ATS antibody, however, did not correspond to the predicted molecular weights of the truncated *var* genes. Neither was a protein corresponding to the well-conserved single copy Pf60.1 protein (that

runs at about 74 kDa Bischoff *et al.*, 2000) detected by western blotting despite the immunising peptide being present in this protein (the final 3′ exon of the transcript encoding Pf60.1 shows considerable similarity with the exon 2 of *var* genes). As the Pf60.1 gene is well conserved between *P. falciparum* isolates the anti-ATS antibody was expected to be cross-reactive. However, Pf60.1 is a nuclear protein expressed in schizont stages and reactivity to the nuclei of schizont stages with the anti-ATS antibody was not apparent in IFA either (figure 3.10; F&G).

The literature was reviewed to determine whether on published western blots of PfEMP1 using an anti-ATS antibody lower molecular weight species of ~150 kDa and ~ 200 kDa are typically seen. In most publications a small molecular weight window is shown around the PfEMP1, presumably because the antibodies used do not cross-react to additional proteins (e.g. Florens *et al.*, 2004). Although in an immunoprecipitation by Kriek *et al.* (2003) of metabolically labelled PfEMP1 using an anti-ATS monoclonal antibody, a smaller molecular weight species (<220 kDa) is co-precipitated along with the larger molecular weight PfEMP1 protein (>220 kDa).

A discernable effect of surface trypsinisation could not be detected on PfEMP1 levels by western blotting (figures 3.13). This might be explained by the fact that only a small portion of the cells PfEMP1 is exposed at the erythrocyte surface. However, an intracellular fragment of PfEMP1 that is not accessible to surface trypsinistion that remains following trypsin treatment should be detected with an antibody to the intracellular ATS region of the protein. Such a tryptic fragment was not detected by western blotting however. This maybe due to the low avidity of the antibody and therefore overall low sensitivity of the western blotting method or due to the intracellular portion exhibiting different solubility characteristics to the intact PfEMP1 molecule and is, therefore, diluted to undetectable levels in the cell lysate fraction.

It is possible trypsin sensitive bands would have been apparent sooner had I continued to analyse detergent extracts following the revelation that furosamide could inhibit the parasite's novel permeation pathway and, therefore, the labelling of intracellular proteins of IEs. It was assumed that, since osmotic lysis produces red cell ghosts, PfEMP1 and other cell surface proteins would remain imbedded in the red blood cell membrane and remain in the membranous fraction. It became apparent, however, that PfEMP1 and potentially additional biotin labelled *Pf*VSA dissociated from the erythrocyte membrane and are found in the pigmented pellet fraction following centrifugation. This is most probably due to PfEMP1's poor solubility.

Chapter 4. *Plasmodium falciparum* Cysteine Modular Repeat Protein 2: a candidate erythrocyte surface antigen

4.1 Abstract

In Chapter 3 considerable effort was invested into the introduction of a reproducible method for western blotting high-molecular weight parasite proteins. These techniques were applied to a collaborative project with Dr. Thompson to further characterise two large molecular weight membrane proteins encoded by a novel gene family encoding the *Plasmodium* Cysteine Repeat Modular Proteins (PCRMP). Several interesting features of this gene family suggests the proteins they encode may be expressed at, or secreted from, the IE surface.

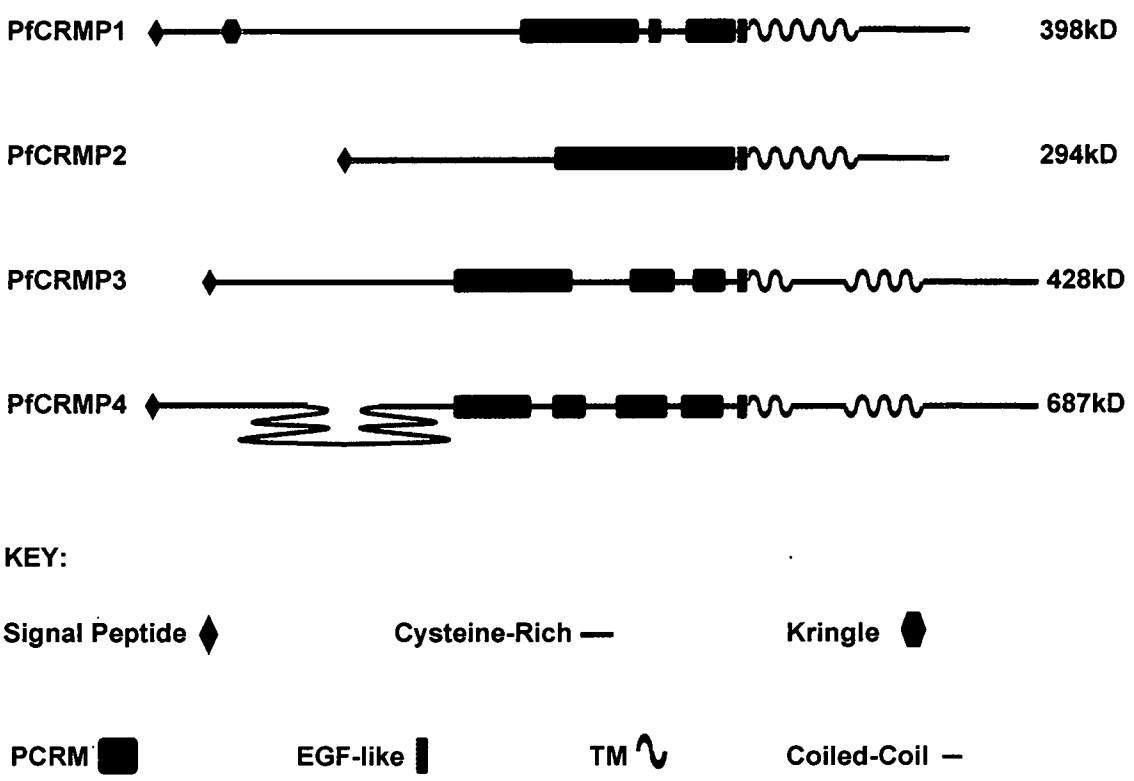
Rabbit serum raised against peptides from PfCRMP1 and PfCRMP2 was used for Western blotting to determine the intraerythrocytic developmental regulation of these proteins. Since they are predicted to possess several transmembrane regions and several distinct structural domains, evidence of post-translational processing of the nascent peptides was also anticipated. The sensitivity of PfCRMP1 and PfCRMP2 to cell surface trypsinisation was also determined.

This analysis showed both PfCRMP1 and PfCRMP2 expression is regulated in a similar fashion through intraerythrocytic development of the parasite and supports immunofluoresence data to suggest PfCRMP2 is translocated to the IE surface. The nascent PfCRMP2 polypeptide also appears to be cleaved during post-translational processing.

4.2 Introduction

Western blotting procedures optimised for the detection of candidate *Pf*VSA were applied to an exciting gene family encoding the *Plasmodium falciparum* cysteine-repeat modular proteins 1-4 (PfCRMP 1-4). A number of conserved structural features suggest these proteins are either present on the surface of the IE and/or parasite, or secreted. *P. falciparum* contains four genes encoding *Plasmodium* Cysteine Repeat Modular (CRM) regions, a schematic of the predicted features of these proteins is shown in figure 4.1. Sequence analysis of PfCRMP1 identified modularity with many reiterations of distinct types of cysteine-rich repeat giving rise to a characteristic recurrent C-x-C and C-x-x-C motif (Thompson *et al.*, In Prep.). The modular arrangement of PfCRMP1 is largely conserved in PfCRMP2-4 and the four genes encoding these proteins are highly conserved in all *Plasmodium* species examined so far. Furthermore, analyses of sequences released from the genome sequencing projects of the Apicomplexan parasites, *Theileria annulata, Theileria parva, Toxoplasma gondii* and *Eimeria tenella* suggests homologues of some PCRMPs are present throughout the phylum. In addition a low degree of polymorphism is found within the sequence for the CRM domain when seqiences from *P. falciparum* isolates from geographically diverse regions are analysed. The predicted secondary structure of the CRM region of PfCRMP1 suggests that it comprises an alpha-beta fold and disulphide-bonding to form a complex three dimensional structure. Interestingly, the tandem repeats of two or four cysteine modules are reminiscent of cysteine-repeat motifs that are a common feature of the TNF receptor family, members of this family generally form tight disulphide-bonded 3-dimensional structures and form either dimmers or trimers. The possibility that PCRMPs may have a role in molecular mimicry is interesting.

In addition to the CRM region, PfCRMP1-4 share; (i) an N-terminal cysteine-rich region, that in PfCRMP3 and 4 is interrupted by stretches of low complexity sequence, (ii) a hydrophobic leader sequence, which is encoded on a discrete exon in PfCRMP2, 3, and 4, (iii) multiple stretches of hydrophobic residues that are presented as putative multipass-transmembrane (m-TM) domains in figure 4.1, and (iv) a predicted epidermal growth factor (EGF)-like domain which lies adjacent to the most N-terminal putative transmembrane domain. Another unifying feature of the PfCRMPs is their large size; the predicted molecular weights of PfCRMP1-4 respectively are 389, 294, 428 and 687 kDa. PfCRMP1 is distinct from PfCRMP2-4 due to a putative Kringle domain in the N-terminal, cysteine-rich region.



[5]Figure 4.1: *Plasmodium falciparum* Cysteine Modular Repeat Proteins 1-4

Schematic showing the predicted features of PfCRMP1 (PFI0550w; chromosome 9), PfCRMP2 (MAL7P1.92; chromosome 7, PfCRMP3 (PFL0410w; chromosome 12), and PfCRMP4 (PF14_0722; chromosome 14). The proteins' predicted molecular weights are indicated and a key defines the symbols used; TM - transmembrane region; EGF - epidermal growth factor like domain. Due to the large size of PfCRMP4 the N-terminal region of the protein is drawn looped, this does not reflect a predicted structure, but merely allows the C-terminal portion of the protein to be shown while maintaining resolution of the PCRM containing C-terminal region.

[5] Figure 4.1 was a kind gift from J. Thompson

4.3 Results

4.3.1 Western blot analysis supports PfCRMP2 being an erythrocyte surface exposed protein

Several structural features of the recently described *Plasmodium* gene family PCRMP that encodes the *P. falciparum* proteins PfCRMP1-4 suggests that these proteins may be transported to the erythrocyte or parasite surface and/or secreted. IFA using affinity purified anti-peptide antibodies raised in rabbits against two of the these proteins, PfCRMP1 and PfCRMP2 reveals that both PfCRMP1 and 2 colocalise with PfEMP1 in structures that appear punctate and dispersed throughout the erythrocyte cytoplasm of the infected cell (data not shown). This pattern of staining in IFA is characteristic of proteins exported to the erythrocyte surface. This intriguing result supports the hypothesis that these proteins are trafficked to the erythrocyte surface. To confirm the specificity of these antisera and investigate the developmental regulation of these proteins through the erythrocytic cycle and the possibility of post-translational processing Western blot analysis was performed with extracts from synchronous asexual cultures. The effect of trypsinisation of the surface of intact infected erythrocytes was also determined.

Rabbits were immunised with 2 peptides for both PfCRMP1 and 2, the peptide sequences and their positions within the protein is shown in figure 4.2. Synchronous cultures of A4 and FCR3CSA infected erythrocytes were used to seed three daughter flasks, with equivalent numbers of parasites, such that the parasitaemia in the next asexual cycle would reach 10%. Flasks containing ring, trophozoite or schizont stages were harvested, half of each flask was exposed to surface trypsinisation and both halves were then processed for membrane fractionation. Giemsa stained smears of the A4 cultures prior to harvesting are shown in figure 4.3A to illustrate the stage and synchronicity of these cultures. A mock culture of uninfected cells was established using the same aliquot of uninfected erythrocytes used for the parent and daughter parasite cultures. The lysate, membrane and pigmented pellet fractions from the time course were analysed by western blotting.

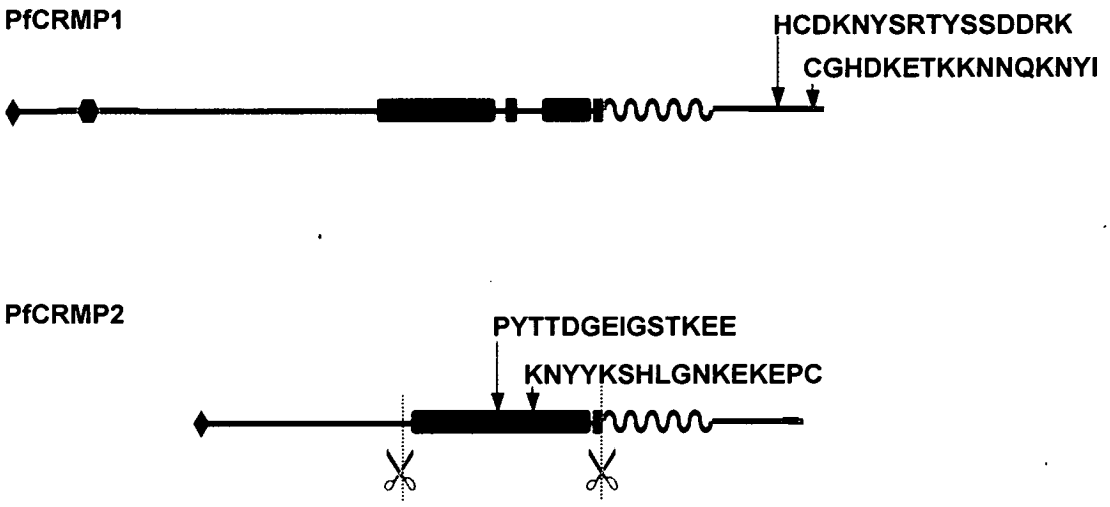


Figure 4.2: Schematic showing peptides used to raise anti-PfCRMP antisera

Sera against two KHL-conjugated peptides from PfCRMP1 and two from PfCRMP2 were raised in rabbits by Eurogentec S. A. The sequence of the peptides and their position within the proteins is shown in the schematic above. Peptides were chosen considering possible cross reactivity to other members in the family. Antisera were affinity purified against neighbouring pairs of peptides. Scissors highlight predicted proteolytic cleavage sites in PfCRMP2.

PfCRMP1 and PfCRMP2 anti-sera detected proteins in both the membrane fraction (figure 4.3C; i) and pigmented pellet fractions (figure 4.3B; i & ii) although reactivity to the pigmented pellet was more intense. The predicted molecular weight of PfCRMP1 is 389 kDa and although a large molecular weight protein of over 250 kDa was detected with the anti-PfCRMP1 antisera the exact molecular weight was not determined. Anti-PCRMP2 antisera detect a protein of approximately 200 kDa, this is roughly 100 kDa smaller than the predicted size of 294 kDa. This smaller molecular protein could be explained by the presence of a number of predicted proteolytic cleavage sites that lie adjacent to the CRM domain in PfCRMP2. These sites could be involved in post-translational processing of the protein.

Neither PfCRMP1 nor PfCRMP2 were detected in A4 ring stages (figure 4.3B; & C i), although a small amount of PfCRMP2 was detected in the FCR3CSA ring stage

cultures (figure 4.3C; ii, lane 1). PfCRMP1 was expressed in comparable amounts in both trophizoite and schizont stages. In neither the membrane fraction pool (figure 4.3C; i) nor the pigmented pellet fraction pool (figure 4.3B; i) could an effect of surface trypsinisation be detected. However, PfCRMP2 protein expression appeared to be upregulated in schizont stages when compared to the trophozoite stages (figure 4.3B, ii) and following surface trypsinisation an additional lower molecular weight band of 150 kDa was apparent for the schizont stage of the A4 culture (figure 4.3B; i, lane 6). The effect of trypsin was more marked in the pigmented pellet fractions analysed from FCR3CSA trophozites and schizonts (figure 4.3C; ii, lanes 3 and 5). In both the A4 and FCR3CSA schizont extracts a lower molecular weight band of approximately 40 kDa was also detected (e.g. figure 4.3C; ii, lanes 4 and 5).

Western blots of A4 pigmented pellet fractions were also probed with the anti-ATS PfEMP1 antibody, this showed the developmental regulation of PfCRMP1 and 2 to be similar to PfEMP1 (figure 4.3B; iii). It was also apparent that the protein of approximately 150 kDa detected by the anti-ATS antibody is limited to later stage parasites and is not present in early trophozoites (figure 4.3B; iii, lanes 5 and 6). Silver staining of the gel used for blotting in figure 4.3B confirmed the positive effects of trypsin (data not shown). That a trypsin sensitive pool of PfCRMP2 and PfEMP1 localise to the pigmented pellet fraction may suggest that the additional biotinylated proteins (identified in Chapter 3) that localise to this cell fraction are parasite derived and associated with the IE surface.

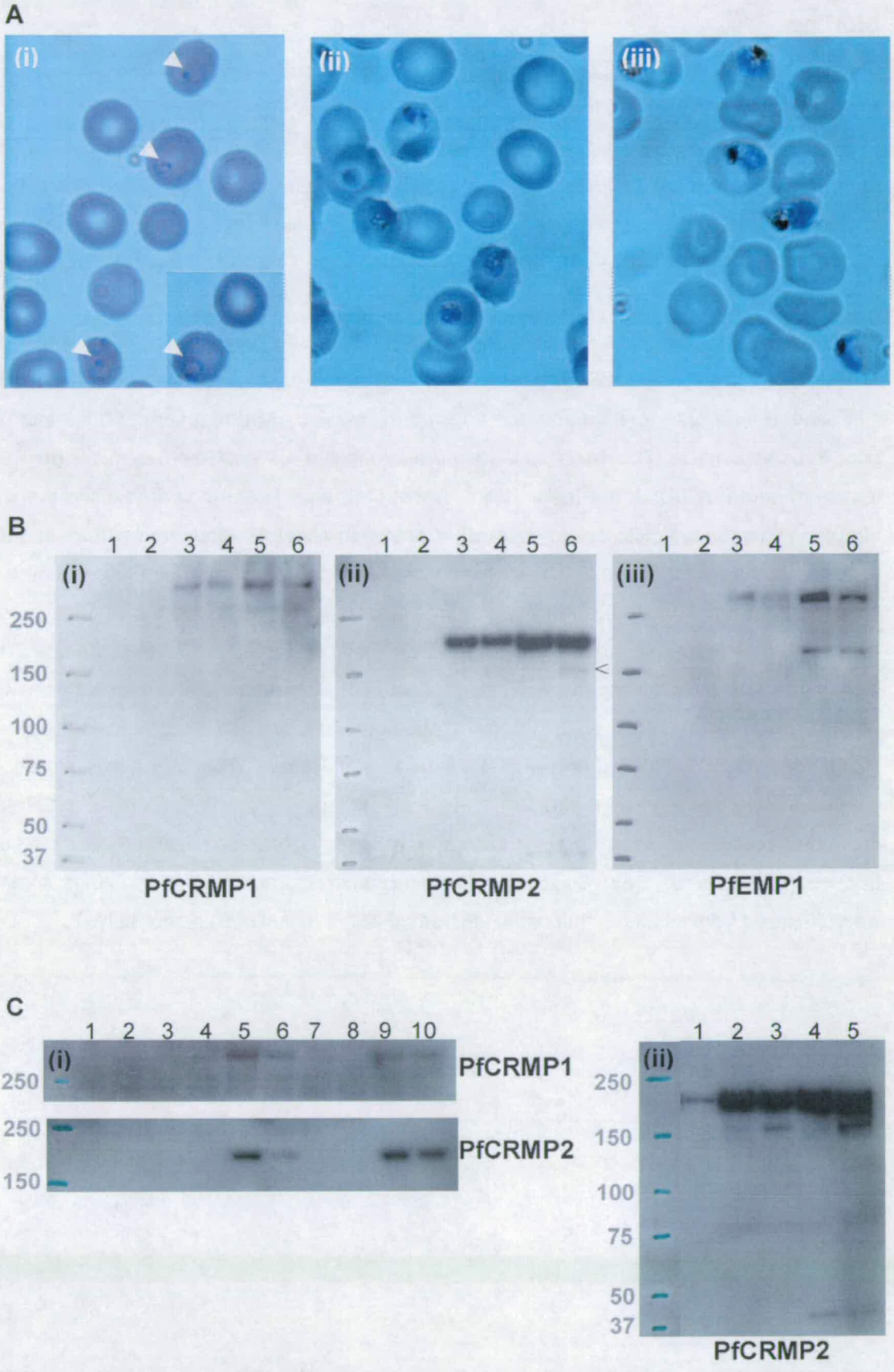

A
(i)
(ii)
(iii)
B
1 2 3 4 5 6
(i)
(ii)
(iii)
250
150
100
75
50
37
PfCRMP1
PfCRMP2
PfEMP1
C
1 2 3 4 5 6 7 8 9 10
(i)
250
PfCRMP1
250
150
PfCRMP2
1 2 3 4 5
(ii)
250
150
100
75
50
37
PfCRMP2

Figure 4.3: Characterisation of PfCRMP1 and PfCRMP2 by western blotting

Panel A shows Giemsa stained blood smears of sychronised A4 cultures immediately prior to harvesting: (i) ring IEs, (ii) trophozoite IEs and (iii) schizont IEs. White arrowheads in panel (i) highlight ring infected erythrocytes. Panel B shows a western blot of pigmented pellet fractions prepared from the synchronous cultures of A4 shown in panel A. Ring IEs lanes 1 and 2, trophozoite IEs lanes 3 and 4 and schizont IEs lanes 5 and 6. In lanes 2, 4 and 6 intact IEs were treated with 1 mg/ml trypsin prior to osmotic lysis and protein extraction. The blot was probed initially with an affinity purified anti-CRMP1 antibody and subsequently probed with both an affinity purified anti-CRMP2 antibody and the anti-ATS antibody. Antibodies were stripped from the blot between antibody incubations. The arrowhead in blot ii in panel B highlights a faint band that appears following trypsin treatment of the intact IEs prior to osmotic lysis. This band appears more strongly in the equivalent extract from from trypsin treated FCR3CSA IEs (panel C ii). Panel C (i) shows membrane fractions prepare from the synchronous cultures of A4 parasites shown in panel A. Membranes from; ring IEs were loaded in lanes 1 and 2, UE extracted at the trophozoite time point were loaded in lanes 3 and 4, trophozoite IEs were loaded into lanes 5 and 6, UE extracted at the schizont time point were loaded in lanes 7 and 8, schizont IEs were loaded into lanes 9 and 10. The blot was probed initially with an affinity purified anti-CRMP1 antibody and then with an anti-CRMP2 antibody as in panel B the blot antibodies were stripped from the blot between incubations. All SDS-PAGE gels were 3-8% tris-acetate. Panel C (ii) shows a western blot of pigmented fractions from a FCR3CSA time course, the blot was probed with the anti-CRMP2 antibody. Loaded into lane 1 was an extract from ring stages, lanes 2 and 3 extracts from trophozoites and lanes 5 and 6 exrtracts from schizionts. Extracts loaded into lanes 3 and 5 were prepared from intact IEs that had been treated with 1 mg/ml trypsin prior to lysis.

Figure 4.3: Characterisation of PfCRMP1 and PfCRMP2 by western blotting

Panel A shows Giemsa stained blood smears of sychronised A4 cultures immediately prior to harvesting: (i) ring IEs, (ii) trophozoite IEs and (iii) schizont IEs. White arrowheads in panel (i) highlight ring infected erythrocytes. Panel B shows a western blot of pigmented pellet fractions prepared from the synchronous cultures of A4 shown in panel A. Ring IEs lanes 1 and 2, trophozoite IEs lanes 3 and 4 and schizont IEs lanes 5 and 6. In lanes 2, 4 and 6 intact IEs were treated with 1 mg/ml trypsin prior to osmotic lysis and protein extraction. The blot was probed initially with an affinity purified anti-CRMP1 antibody and subsequently probed with both an affinity purified anti-CRMP2 antibody and the anti-ATS antibody. Antibodies were stripped from the blot between antibody incubations. The arrowhead in blot ii in panel B highlights a faint band that appears following trypsin treatment of the intact IEs prior to osmotic lysis. This band appears more strongly in the equivalent extract from from trypsin treated FCR3CSA IEs (panel C ii). Panel C (i) shows membrane fractions prepare from the synchronous cultures of A4 parasites shown in panel A. Membranes from; ring IEs were loaded in lanes 1 and 2, UE extracted at the trophozoite time point were loaded in lanes 3 and 4, trophozoite IEs were loaded into lanes 5 and 6, UE extracted at the schizont time point were loaded in lanes 7 and 8, schizont IEs were loaded into lanes 9 and 10. The blot was probed initially with an affinity purified anti-CRMP1 antibody and then with an anti-CRMP2 antibody as in panel B the blot antibodies were stripped from the blot between incubations. All SDS-PAGE gels were 3-8% tris-acetate. Panel C (ii) shows a western blot of pigmented fractions from a FCR3CSA time course, the blot was probed with the anti-CRMP2 antibody. Loaded into lane 1 was an extract from ring stages, lanes 2 and 3 extracts from trophozoites and lanes 5 and 6 exrtracts from schizionts. Extracts loaded into lanes 3 and 5 were prepared from intact IEs that had been treated with 1 mg/ml trypsin prior to lysis.

4.4 Discussion and future work

Western blotting with anti-PfCRMP2 antibodies suggests the protein is processed, as the major band detected is about 100 kDa smaller than the predicted molecular weight. A protein of the predicted full-length nascent polypeptide was not detected. The anti-peptide antibody was raised against peptides in the CRM domain of PfCRMP2, to investigate how the protein is processed. Antibodies against the carboxyl region and the amino terminal region of the PfCRMP2 protein would provide valuable tools for investigating the fate of the full-length protein. Western blotting with the anti-PfCRMP1 antibody suggests that the antibody used in IFA, which reveal a similar punctate staining in the erythrocyte cytosol, is specific to a large molecular weight protein. Although, anti-peptide antibodies avoid the potentially time consuming step of optimising recombinant protein expression and peptides being quite easily used for the affinity purification of sera, short stretches of peptide are unlikely to raise antibodies that are reactive to the native conformation of the protein. A panel of antibodies recognising several regions of native conformation along these large proteins would be ideal for live cell immunofluorescence assays to confirm that these proteins are transported to the IE surface. Such antisera would also be useful for revealing the membrane topology of these interesting proteins. Of course because the PfCRMP gene family show reasonable sequence identity selecting suitable regions of the protein that would not illicit cross-reactive antibodies might be difficult. In addition several regions of PfCRMP1 and 2 are predicted to be highly cysteine rich, it therefore may prove difficult to get portions of the proteins containing cysteines to fold correctly.

A complementary approach to raising a panel of antisera to these proteins might be μLC-MSMS. My work on these proteins has determined the mobility of the protein species detected by the anti-PfCRMP1 and 2 antisera, and therefore, it would now be possible to cut slices from the appropriate molecular weight sizes on 1D SDS-PAGE gels firstly to confirm that peptides derived from these proteins are present at the molecular weights indicated by the antisera. As was the case for taking such an approach for investigating the protein species that are biotinylated, by virtue of the cellular fractionation steps and 1D gel electrophoresis the complexity of peptides present in a gel slice incorporating the molecular weight of interest would be limited. Depending on the success of this approach it might be possible to explore the processing of PfCRMP2, especially as it appears that a substantial portion of this large protein (100 kDa) is cleaved from the predicted full-length polypeptide.

Although, it might be difficult to interpret the absence of tryptic peptides, positive peptide identifications by μLC-MSMS would be informative. Although, it appears that only a small portion of PfCRMP2 is affected by surface trypsinisation it may be possible to look at the lower molecular weight species, that appear following surface trypsinisation, by μLC-MSMS also.

The developmental regulation of PfCRMP1 and 2 through the intraerythrocytic cycle would suggest that these proteins are not transporters for essential metabolic intermediates, since the proteins are not expressed in ring stage IEs. PfCRMP1 and 2 protein expression commences at the trophozoite stage, therefore it may be possible that the predicted seven transmembrane regions produce some kind of cell surface receptor, on either the parasite or the IE that is important for development through the schizont stage.

Chapter 5. Biotin tagging of *P. chabaudi* erythrocyte surface antigens

5.1 Abstract

Experiments described in Chapter 3 showed biotin-labeled *Plasmodium falciparum* VSA expressed by cultured IEs were not detectable in membrane fractions. At that time, the possibility that IEs maintained under *in vitro* culture conditions may not express VSA at detectable levels was considered. Another concern was that the large molecular weight of PfEMP1 might perturb the entry of the protein into SDS-PAGE gels. An *in vivo* model of malaria infection was therefore considered, as host factors, such as the absence of a spleen, have been shown in a number of malaria to reduce VSA expression. The surface biotinylation procedure described in chapter 3, has thus been applied to *Plasmodium chabaudi*-infected mouse erythrocytes, as not only does *P. chabaudi* infection of laboratory mice replicate many aspects of *P. falciparum* infection of humans, but all current candidate *P. chabaudi* VSA genes (as predicted from genome sequencing) are small compared to PfEMP1.

Initial *P. chabaudi* labeling experiments identified two erythrocyte surface exposed parasite specific proteins in membrane preparations. One is a 120 kDa protein. Another candidate, of approximately 30 kDa, approximates to the molecular weight of the candidate *Pc*VSA multigene family *cir*. Neither cir proteins nor any other candidate *Pc*VSA have definitively been proven to be exposed at the IE surface. Tryptic peptides were prepared from candidate *Pc*VSA for future identification using recently developed mass spectrometric techniques.

An experiment to investigate changes in the expression of biotin labeled parasite specific proteins following mosquito passage was also initiated. Mosquito passage of three *P. chabaudi* lines had been shown to result in a marked increase in rosetting rate. Surface labeling of these lines prior to and post-mosquito passage highlighted the differential expression of a biotin-labeled protein of ~120 kDa. This protein also displayed a pattern of expression in erythrocytic stages that is characteristic of *Pc*VSA-mediated traits, such as sequestration and rosetting.

These experiments using *P. chabaudi* IEs have confirmed that a biotin label can be used to tag *Plasmodium*-specific cell surface exposed proteins. This work spurred

further critical assessment of the failure of this approach in *P. falciparum.* In addition, this work has identified two candidate *P. chabaudi* VSA.

5.2 Introduction

During the optimisation work on the detection of biotin-tagged candidate *Pf*VSAs, a transient mystery was the absence of biotinylated candidate *Pf*VSA in membrane enriched fractions from enriched *P. falciparum* cultures (figures 3.6 & 3.7). Prior to the realisation that PfEMP1 and biotinylated parasite specific proteins were sedimenting with a small, insoluble, pigmented pellet, consideration was given to applying the technique to an alternative model of *Plasmodium* infection. This addressed two concerns. Firstly, whether failing to detect biotinylated candidate *Plasmodium* VSA in membrane fractions was specific to *P. falciparum*. My primary concern was that, despite parasites being selected for an adhesive phenotype, *in vitro* culture did not mimic the necessary conditions for the full expression of higher, more detectable levels of *Pf*VSA. For example, the spleen has been demonstrated to play a role in the modulation of *Plasmodium* VSA expression in a number of systems (David *et al.*, 1983; Gilks *et al.*, 1990; Handunnetti *et al.*, 1987; 1983) and is perhaps a more significant factor in *P. falciparum* PfEMP1 expression than has been appreciated. Another concern was that due to the large molecular weight of PfEMP1 the protein may have failed to enter SDS-PAGE gels efficiently. This, in combination with only a small portion of cellular PfEMP1 being transported to the erythrocyte surface (Haeggstrom *et al.*, 2004; Kriek *et al.*, 2003), may have led to the levels of biotinylated PfEMP1 being below the detection limits of the Western blotting methods. To investigate these concerns I applied the surface biotinylation procedure and membrane fractionation method described in Chapter 3 to an *in vivo* mouse malaria model; *P. chabaudi*. This model system was especially attractive because not only do *P. chabaudi* infections of mice reflect many aspects of *P. falciparum* infections of humans, but also *P. chabaudi* material was readily available through collaboration within the department.

5.2.1 *P. chabaudi* infections of the laboratory mouse

The thicket rat *Thamnomys rutilans* is the natural host of *P. chabaudi*; however, this rodent malaria has been used extensively as a model for *in vivo* malaria infection using a number of strains of laboratory mouse. *P. chabaudi* infections in mice show numerous similarities to *P. falciparum* infections of humans: (i) *P. chabaudi* undergoes rapid asexual growth in naïve hosts; (ii) mature erythrocytic stages sequester from the circulation and can form rosettes; (iii) *P. chabaudi* exhibits antigenic variation in the laboratory mouse (Phillips *et al.*, 1997); and (iv) immunity

to infection is incomplete and, partly, strain- and variant-specific (Cox, 1988; Mota *et al.*, 2001). There are differences in the sites of sequestration however; for the *P. chabaudi* clone AS, schizont stages have been shown to sequester in the liver (Gilks *et al.*, 1990), an organ not reported as a site for sequestration for *P. falciparum* infections of humans. Despite differences in organ specificity, *P. chabaudi* IEs bind purified CD36 (Mota *et al.*, 2000), which is a major endothelial receptor for *P. falciparum* (e.g. Baruch *et al.*, 1995; Baruch *et al.*, 1996). Like *P. falciparum*, *P. chabaudi* also preferentially invades older red blood cells; however, the acute stage parasitaemias often achieved in *P. chabaudi* infections of laboratory mice are considerably higher (>30%) than those generally reached in human *P. falciparum* infections (Taylor-Robinson, 1995). *P. chabaudi* infections of its natural host are generally less acute during the early stages of infection and chronic infections are more persistent. *P. chabaudi* infection may persist for the entire life of the natural host (Phillips *et al.*, 1997).

The course of *P. chabaudi* infection in malaria naïve laboratory mice is typified by an acute peak of parasitaemia, lasting approximately 10-15 days, followed by recrudescing peaks of parasitaemia, characteristic of waves of antigenic variants (McLean *et al.*, 1982). Using immune sera against parasites isolated from the acute peak and sera raised to parasites isolated from subsequent recrudescent peaks, Phillips and colleagues (1997) established that parasites in recrudescing peaks express antigenically distinct VSA when compared to those expressed by the same clone during the acute stage of infection. The importance of the spleen for surface antigen expression and sequestration by *P. chabaudi* in mouse infections was demonstrated by the work of Gilks and colleagues (1990). The kinetics of the acute stage of infection in splenectomised mice was indistinguishable from infections of intact animals. However, in splenectomised mice, chronic recrudescing peaks were not apparent. Sequestration-associated variant antigen expression in *P. chabaudi* seems to enable the establishment of chronic infections in the presence of a spleen.

Mouse models of rodent malaria have increased our understanding of many aspects of *Plasmodium*, including the pathology induced by the erythrocytic stages of the parasite (Li *et al.*, 2001). Interestingly, a large molecular weight protein comparable in size to PfEMP1 (~ 250 kDa) has been identified either at the surface of *P. chabaudi* and *P. bergei* IEs or at the merozoite surface (Newbold *et al.*, 1982). This antigen was also proposed to be important in the induction of antibody-mediated immunity to *P. chabaudi* infection (Boyle *et al.*, 1982). However, the *P. chabaudi* proteins expressed at the erythrocyte surface of mature asexual stages that interact with the mammalian

host's immune system and microvasculature endothelium have yet to be identified. Due to the similarities *P. chabaudi* infections share with Falciparum malaria, *P. chabaudi* infections of mice thus provide an accessible *in vivo* malaria model for dissecting the molecular mechanisms underlying VSA switching at the cellular and population level, and the role of VSA in pathology and parasite virulence.

5.2.2 *P. chabaudi* multigene familes: candidate VSA genes

Although the genes encoding the VSA of *P. chabaudi* IEs have not been definitively characterised, a number of gene families identified through analysis of *P. chabaudi's* genome are strong candidates. Through sequencing 80 kb from the end of a *P. chabaudi* chromosome Fischer and colleagues (2003) identified ten variant multigene families. These families are exclusively located at the subtelomeric regions of chromosomes (a characteristic of genes mediating antigenic variation) and their copy numbers range from four to several hundred per genome. One of these gene families the *cir* family, which is present in ~130 copies per genome, was initially described by Janssen and colleagues (2002). The *cir* coding sequences are quite variable with only short stretches exhibiting high sequence conservation although the non-coding sequences surrounding *cir* genes are relatively well conserved. Long stretches of intergenic regions that neighbour *cir* genes reach 75% identity. As discussed earlier (section 1.2.4) *cir* genes belong to the *Plasmodium* interspersed repeat (*pir*) superfamily, members of which have been found in several rodent, human and non-human primate malarias (Janssen *et al.*, 2004). *Cirs* encode relatively small proteins of ~30 – 40 kDa and their transcripts are regulated through the asexual blood stages.

Fischer *et al.* (2003) proposed that two further *P. chabaudi* multigene families encode variant surface proteins. The first of these families includes three genes labelled 5c, 7c and 10c and there are an estimated 75 copies of related genes per genome. Fischer *et al.* (2003) have not named this gene family, and for simplicity I shall refer to the protein products of this family as *P. chabaudi* cir-like proteins (PcCLP). Although the 5c, 7c, and 10c genes vary considerably in size, they share a similar gene structure, not only with each other, but also to *cir* genes. They possess three exons, the small 5′ exon and the 3′ exon are conserved in size, but the second is variable. The second exon of the 7c gene is considerably larger than that observed in *cir* genes. The first 200 amino acids and last 140 amino acids of 5c, 7c and 10c show high sequence conservation and the central part of the protein, encoded by the 3′ end of exon 2, contains varying lengths of low complexity amino acid

composition and internal repeats. The 3′ end of exon 2 also encodes a conserved putative transmembrane domain. It is the relatively conserved 5′ end of exon two which shows similarity to cir proteins, this cir-like region contains four conserved cysteine residues and a number of conserved rare amino acids such as tryptophan and tyrosine. Considering their subtelomeric location, a single putative transmembrane sequence and regions of similarity to cir proteins Fischer *et al.* (2003) propose the PcCLP family is derived from the same common ancestral genes that gave rise to all members of the *pir* superfamily. Like cirs and other VSA, such as PfEMP1, a signal sequence is not apparent at the amino terminus of the PcCLPs.

Two genes, given the identities 1w and 14w,define the third putative *Pc*VSA multigene family proposed by Fischer *et al.* (2003). There are an estimated 13 copies of related genes in the *P. chabaudi* genome. These genes again vary considerably in size (~2 fold), but are clearly related and show similarities to the *P. chabaudi* erythrocyte membrane antigen 1 (EMA1) protein (Deleersnijder *et al.*, 1992). EMA1 itself is not thought to belong to a multigene family although a related gene called EMA1-R has been identified (Favaloro & Kemp, 1994). The EMA1 protein contains a major tandem repeat array of 16 octapeptides and has a predicted molecular weight of 50 kDa. EMA1 is associated with the erythrocyte membrane of the IE throughout the intraerythrocytic cycle, but is thought to reside on the cytoplasmic face (Deleersnijder *et al.*, 1992). Members from the three families described above were found to be transcribed in blood stages (Fischer *et al.*, 2003).

Initially, I have applied the biotin surface labelling technique to erythrocytes infected with the AS clones of *P. chabaudi* as parasite material was available following a large-scale transmission study in the department. As the AS clone was used for the *P. chabaudi* genome sequencing project, this clone was ideal for any subsequent mass spectrometry analysis of candidate *Pc*VSA. AS infected and uninfected control mice were bled when parasites were ring stages, serum was removed and the cells kept in short term culture until they had developed into trophozoite stages. Cultures were then surface labelled with biotin and the membrane fractions analysed by western blotting. This approach successfully identified two candidate *Pc*VSA in the equivalent membrane fraction to that in which *Pf*VSA could not be detected in analogous labeling experiments of *P. falciparum*. Success at the initial stage of the originally planned multi-step proteomics approach (detailed in section 3.2.7) with the *P. chabaudi* model led to experiments to investigate the expression of candidate *Pc*VSA in a model of rosetting in *P. chabaudi*.

established. Tryptic peptides were extracted from fine gel slices and prepared for analysis by μLC and tandem ESI MS. Figure 5.2 shows the primary MALDI-TOF quality control screen of a selected number of extracts to confirm the presence and quality of tryptic peptides. Although this quality control step suggests these samples are suitable for further analyses, the collaboration to analyse the tryptic peptides by μLC and tandem ESI MS unfortunately did not come to fruition. Attempts were made to find alternative facilities for the analysis of these peptides. However, since such multidimensional mass spectrometric technologies are costly to establish, and have only recently been applied to complex protein mixtures, suitable facilities are scarce and an alternative could not be found.

Lane	1	2	3	4	5	6	7	8
U/I	U_a	U_a	U_b	U_b	I_a	I_a	I_b	I_b
Trypsin	+	-	+	-	+	-	+	-

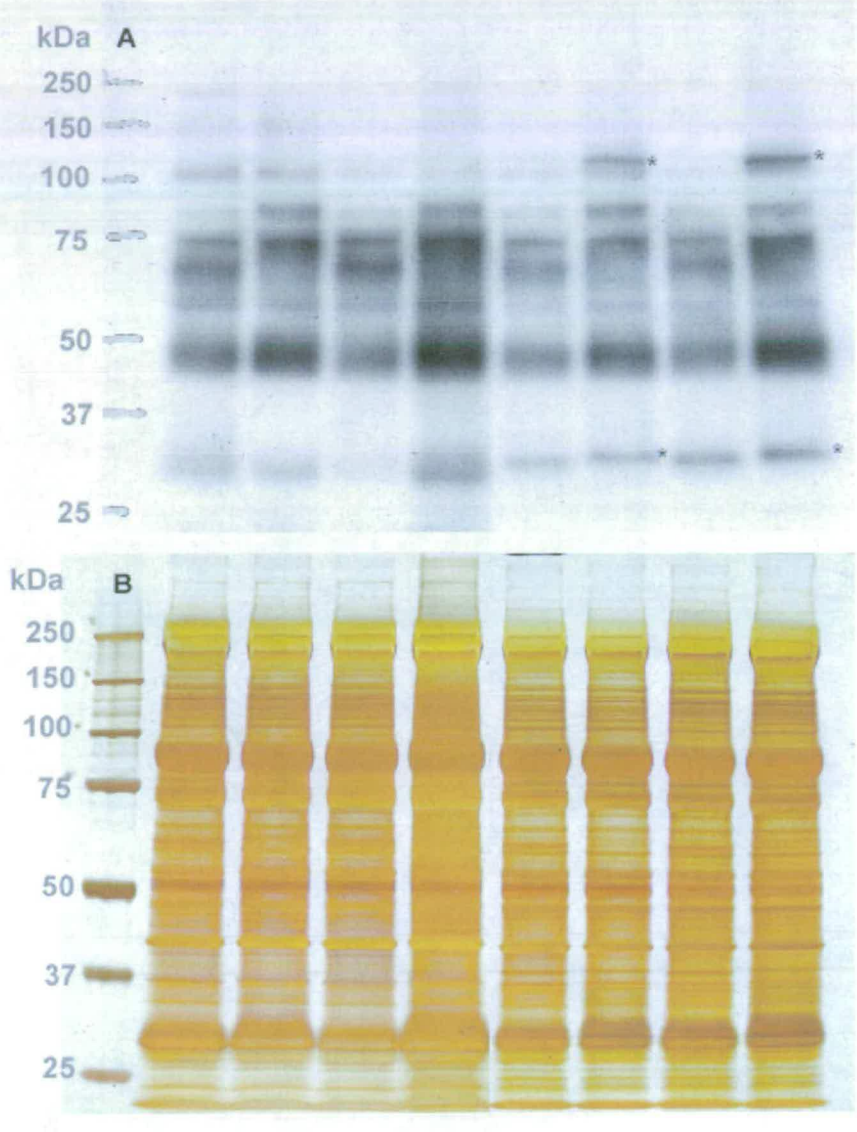


Figure 5.1: Surface labelling of *P. chabaudi* (clone AS) infected erythrocytes using sulpho-NHS-LC-biotin

Panel A shows a western blot of membrane extracts separated on a 4-12% gradient bis-tris SDS-PAGE gel and probed with HRP-conjugated streptavidin. Panel B shows a duplicate gel of that shown in A stained with silver. Lanes containing extracts from uninfected erythrocytes are indicated in the table by U, and extracts from C57 mouse erythrocytes infected with the AS clone of *P. chabaudi* are indicated by I. Surface exposed potential *P. chabaudi* derived proteins are highlighted with an asterisk.

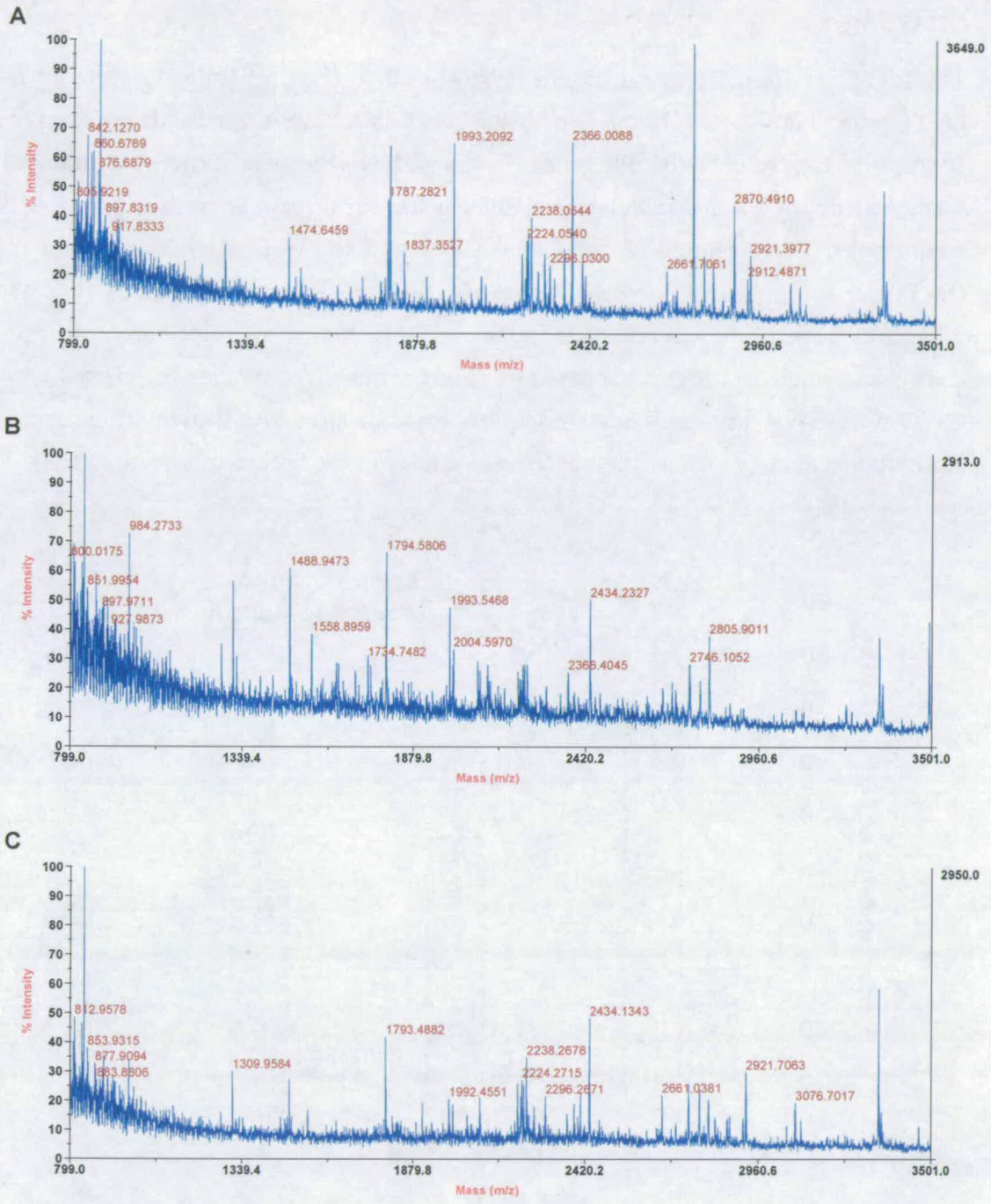


Figure 5.2: MALDI-TOF spectra of tryptic peptides form *P. chabaudi* IE membrane fractions

Tryptic peptides were prepared from gel slices from lane 8 of the silver stained gel shown in figure 5.1B, the exact locations are shown in Appendix 5. Spectra A, B and C were obtained for tryptic peptides extracted from 1.5 mm gel slices cut from ~120 kDa, ~100 kDa and ~37 kDa respectively.

5.4.2 Candidate *Pc*VSA expression, mosquito transmission and rosetting

The identification of two candidate *Pc*VSAs led to the application of the surface labeling technique to study the expression candidate *Pc*VSA in rosetting clones of *P. chabaudi*. Mackinnon and colleagues (In Press) have recently shown that mosquito transmission of *P. chabaudi* isolates selects for significant increases in rosetting frequencies. Three related clones CWA, CWO and CWV used by Mackinnon *et al.* (In Press) which were shown to increase their rosetting frequencies following mosquito transmission were surface labeled with biotin to follow any associated changes in surface antigen expression. The rosetting frequencies for clones CWA, CWO and CWV pre and post mosquito transmission are shown in figure 5.3. Rosetting frequency was defined as the percentage of IEs bound to 2 or more IEs.

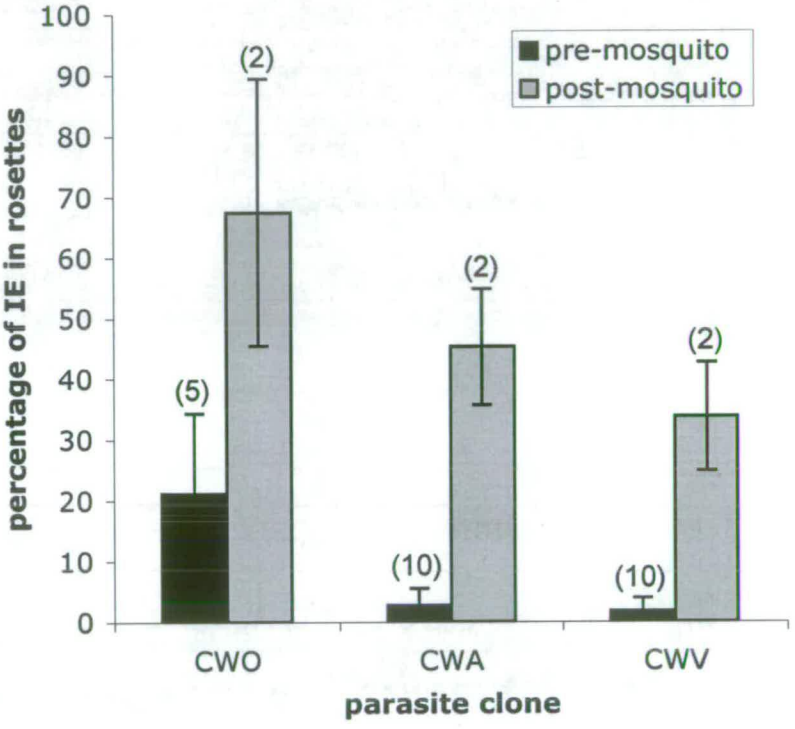


Figure 5.3: Rosetting frequencies of CW isolates pre- and post-mosquito transmission

The rosetting frequencies of *P. chabaudi* isolates on days 5-7 post-infection were assayed as described in Mackinnon *et al.* (2002). The mean rosetting rate is shown and the number of observations indicated in brackets above the error bars. Error bars indicate standard deviation.

Clones CWA and CWV had very low rosetting rates prior to mosquito transmission (~2%), but post-mosquito transmission rosetting frequencies were ~ 40% and 30% respectively. Clone CWO exhibited a moderate rosetting frequency (~20%) prior to mosquito challenge (CWOpre). This may reflect CWO being recently derived from the natural host and consequently having been recently transmitted through mosquitoes. Both CWApre and CWVpre were derived from CWOpre by extensive serially blood passage through mice (the passage history of CWA and CWV is described in more detail in section 7.7.3). Despite CWOpre exhibiting a moderate rosetting frequency, mosquito transmission increased this further and by a comparative degree to that seen as a result of mosquito transmission of the CWApre and CWVpre clones. The rosetting frequency of CWO post-mosquito challenge (CWOpo) was ~ 60 %.

The experimental design for the surface labeling of CW derived isolates pre- and post-mosquito transmission is outlined in figure 5.4. The experiment was carried out in duplicate and prior to surface biotinylation half of the erythrocytes from each infected and control uninfected mouse were treated with 1 mg/ml trypsin. Both ring infected and schizont IEs were labeled to determine the stage specificity of candidate *Pc*VSA. Figure 5.5, panels A-C show western blots of membrane extracts from surface biotinylated ring IEs. Parasite specific biotinylated proteins similar to those identified in the membrane extracts of AS trophozoite IEs were not detected in extracts of ring stage parasites for any of the six CW isolates. Western blots of membrane extracts from surface biotinylated schizont IEs are shown in figure 5.6. A biotinylated parasite-specific trypsin-sensitive protein having a similar molecular weight to the 110 kDa protein seen in AS membrane extracts (figure 5.1) was identified in schizont extracts (figure 5.6B; lanes 3,7 & 9 and 5.6C; lanes 3,5,9 & 11). However, this band was intense in only the three CW isolates that had recently been transmitted through mosquitoes and was less intense in the CWOpre schizont extract. The presence and intensity of the biotinylated 110 kDa protein correlated with the rosetting rates of the CW clones. The stage specific expression of the biotinylated 110 kDa protein is also in agreement with *Pc*VSA mediated cytoadherence phenotypes, such as sequestration and rosetting, being restricted to the mature erythrocytic stages of the parasite (Gilks *et al.*, 1990). Membrane extracts from duplicate mice were also consistent with regard to the pattern of biotinylated proteins and the presence or absence of the 110 kDa protein.

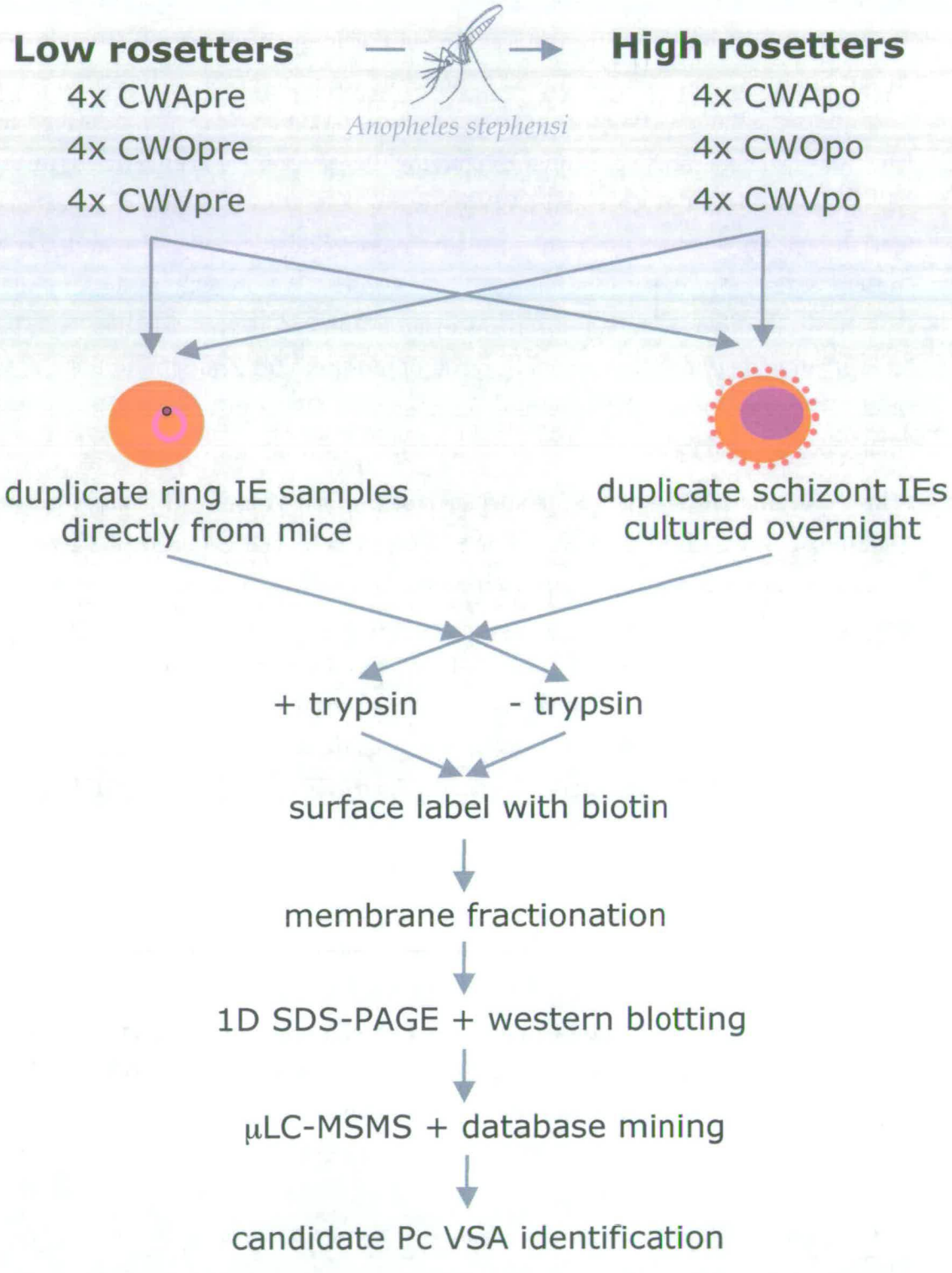
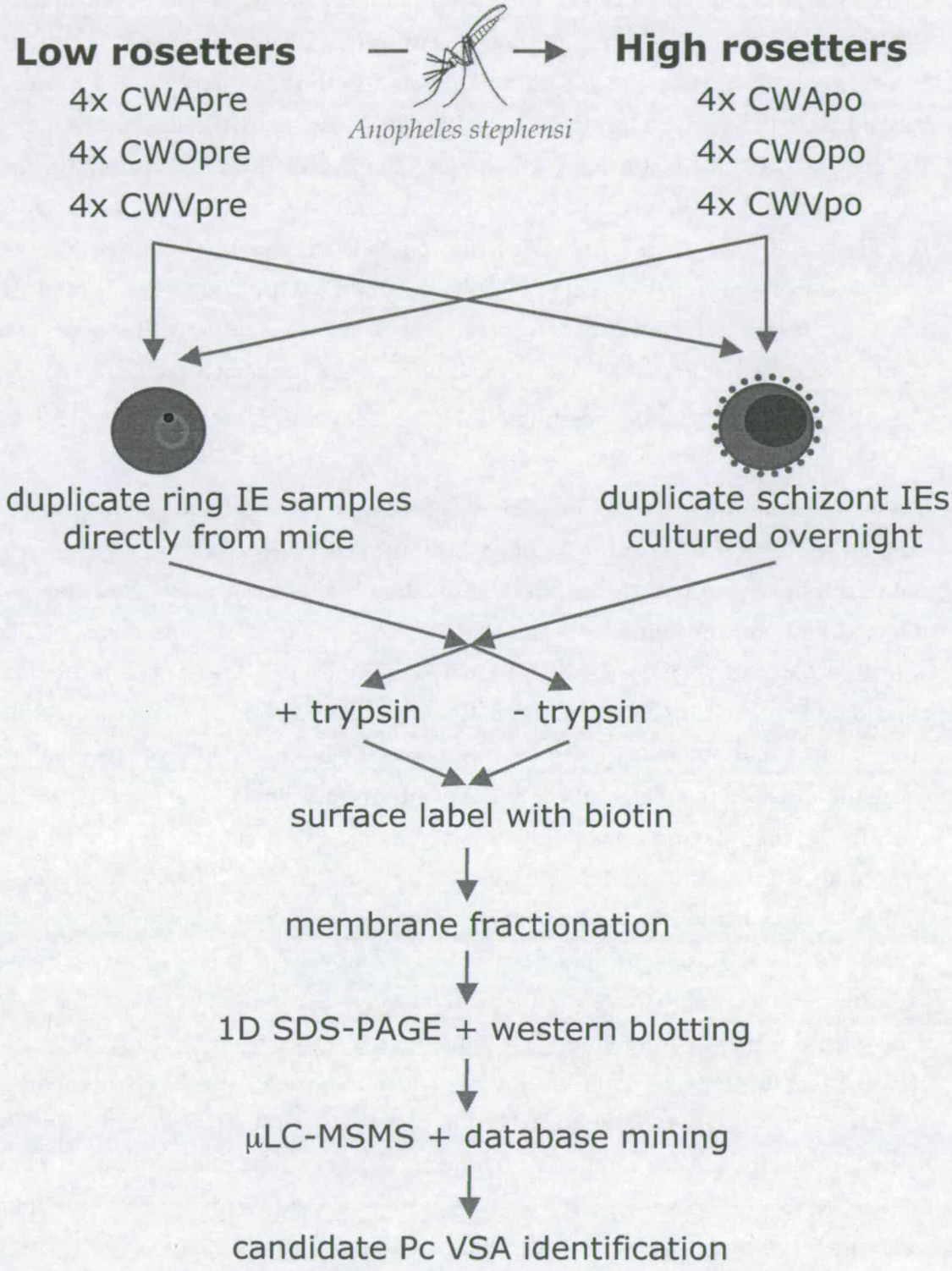


Figure 5.4: Experimental outline of surface biotinylation experiment using *P. chabaudi* CW isolates pre- and post-mosquito passage



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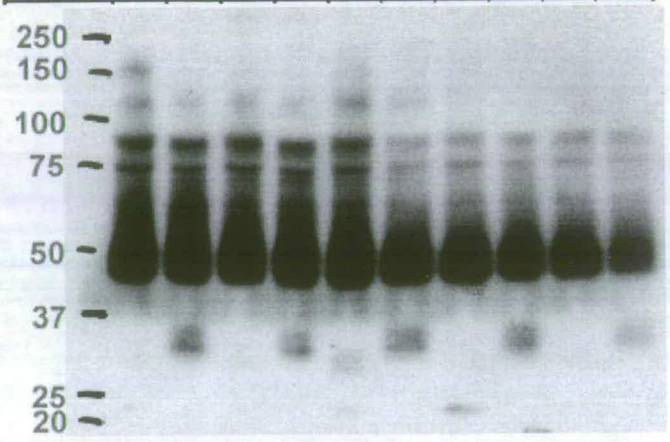
Using immune sera in live cell immunofluorescence assays it has been shown that *Pc*VSA are sensitive to trypsin (Gilks *et al.*, 1990). This is in agreement with the trypsin sensitivity exhibited by the parasite specific biotin labelled proteins detected in membrane extracts from both AS and CW clones (figures 5.1 and 5.6). A lower molecular weight parasite specific protein equivalent to the ~30 kDa protein identified in AS IE extracts was not detected in any of the membrane extracts from CW isolates. This may reflect the different developmental stage at which membranes were extracted. AS infected erythrocytes were cultured for 6 hours after bleeding infected mice and the CW isolates were cultured overnight. Biotinylated proteins characteristic of *Pc*VSA were not detected in extracts from CWA and CWV isolates pre-mosquito passage.

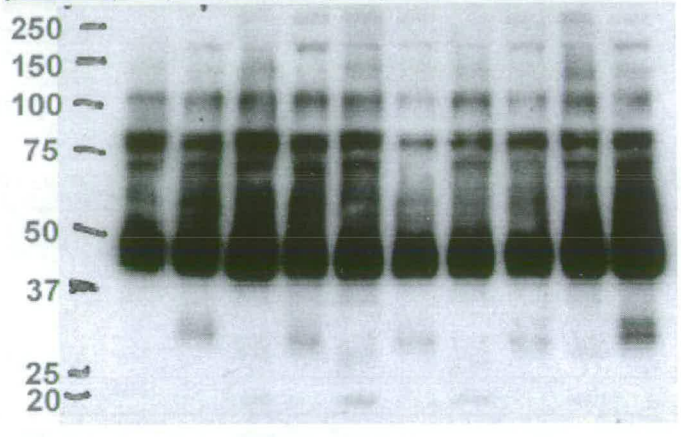
A

Lane	1	2	3	4	5	6	7	8	9	10
Cells	U	U	A	A	A	A	V	V	V	V
Trypsin	-	+	-	+	-	+	-	+	-	+



B

Lane	1	2	3	4	5	6	7	8	9	10
Cells	U	U	O	O	O	O	O_p	O_p	O_p	O_p
Trypsin	-	+	-	+	-	+	-	+	-	+



C

Lane	1	2	3		4	5	6	7	8
Cells	U	V_p	V_p		U	A_p	A_p	A_p	A_p
Trypsin	-	-	+		-	-	+	-	+

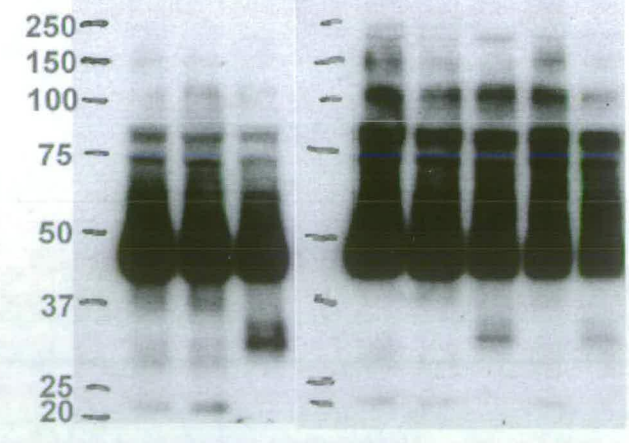
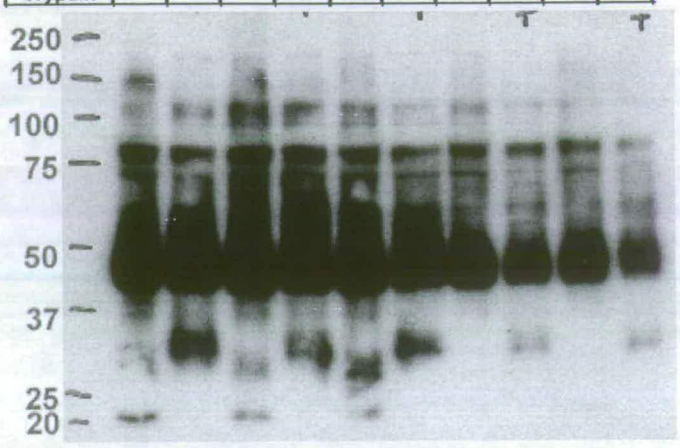


Figure 5.5: Membrane extracts from *P. chabaudi* ring IEs labelled with sulpho-NHS-LC-biotin

Ring stage parasites isolated directly from C57Bl/6J mice were washed and labelled with sulpho-NHS-LC-biotin. Membrane extracts were extracted and separated on 4-12% gradient bis-tris SDS-PAGE gels. Western blots of these gels probed with HRP-conjugated streptavidin are shown. Lanes labelled A, O, and V contain extracts from clones CWA, CWO and CWV respectively pre-mosquito transmission. Lanes Ap, Op, and Vp contain extracts from clones CWA, CWO and CWV respectively post-mosquito transmission. Lanes loaded with extracts from uninfected erythrocytes are labelled U. Extracts from intact IEs treated with 1 mg/ml trypsin or not were loaded into lanes labelled with + or - respectively. Panel A shows duplicate extracts from CWA and CWV IEs pre-mosquito transmission, panel B shows duplicate extracts from CWO IEs pre and post-mosquito transmission and panel C shows duplicate extracts from CWA, and a CWV extract, post mosquito transmission.

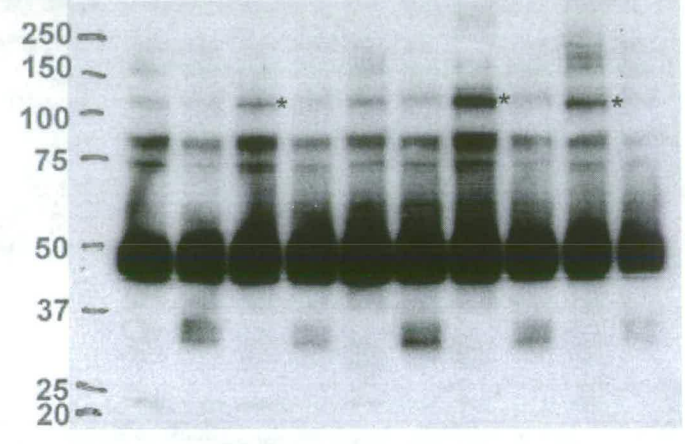
A

Lane	1	2	3	4	5	6	7	8	9	10
Cells	U	U	A	A	A	A	V	V	V	V
Trypsin	-	+	-	+	-	+	-	+	-	+



B

Lane	1	2	3	4	5	6	7	8	9	10
Cells	U	U	O	O	O	O	O_p	O_p	O_p	O_p
Trypsin	-	+	-	+	-	+	-	+	-	+



C

Lane	1	2	3	4	5	6	7	8	9	10	11	12
Cells	U	U	V_p	V_p	V_p	V_p	U	U	A_p	A_p	A_p	A_p
Trypsin	-	+	-	+	-	+	-	+	-	+	-	+

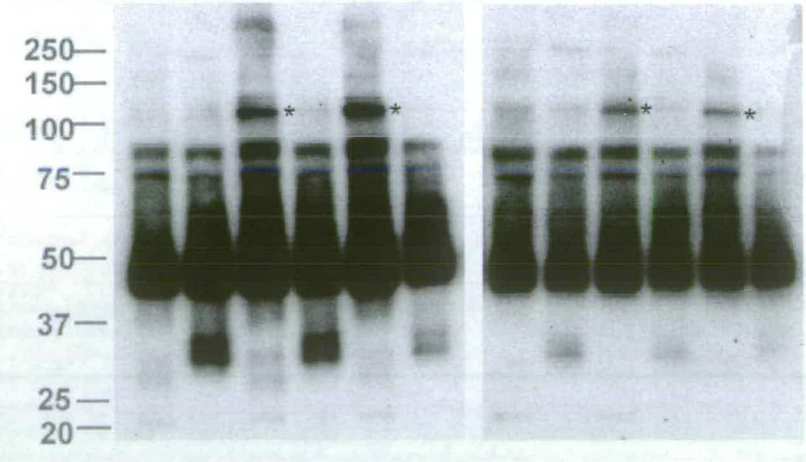


Figure 5.6: Membrane extracts from *P. chabaudi* schizont IE labelling with sulpho-NHS-LC-biotin

Late ring stage infected erythrocytes were isolated from C57Bl/6J mice and matured in short-term culture overnight. Cultured cells were washed and labelled with sulpho-NHS-LC-biotin. Membrane extracts were prepared and separated on 4-12% gradient bis-tris SDS-PAGE gels (Invitrogen). Western blots of these gels probed with HRP-conjugated streptavidin are shown. Lanes labelled A, O, and V contain extracts from clones CWA, CWO and CWV respectively pre-mosquito transmission. Lanes Ap, Op, and Vp contain extracts from clones CWA, CWO and CWV respectively post-mosquito transmission. Lanes loaded with extracts from uninfected erythrocytes are labelled U. Extracts from intact IEs treated with 1 mg/ml trypsin or not were loaded into lanes labelled with + or - respectively. Candidate *Pc*VSA bands are highlighted with an asterisk. Panel A shows duplicate extracts from CWA and CWV IEs pre-mosquito transmission, panel B shows duplicate extracts from CWO IEs pre and post-mosquito transmission and panel C shows duplicate extracts from CWA and CWV post-mosquito transmission.

5.5 Discussion

Surface labelling experiments using *P. chabaudi* IEs were initiated to address concerns regarding the detection of biotinylated *P. falciparum* erythrocyte surface antigens following the fractionation of red blood cell ghosts. Trials of the biotinylation protocol with *P. chabaudi* IEs from mice led to the identification of two parasite specific biotinylated proteins. These experiments confirmed that sulpho-NHS-LC-biotin could successfully label *Plasmodium*-specific, surface-trypsinisation-sensitive proteins. These candidate *Pc*VSAs were present in the equivalent membrane fraction for which biotin tagged *Pf*VSA could not be detected. Following further experiments with *P. falciparum* IEs it was apparent, however, that the original concerns regarding the abundance of *Pf*VSA at the IE surface and the large molecular weight of PfEMP1 were not warranted. As discussed in chapter 3, the dissociation of PfEMP1 from the red blood cell membrane and PfEMP1's insoluble nature is most likely responsible for the absence of biotin tagged *Pf*VSA in membranous fractions. However, it was partly the successes with the labelling of *P. chabaudi* IEs that fuelled the continuation of experiments to investigate the small, pigmented pellet fraction that forms following osmotic lysis and centrifugation. The pigmented pellet was the fraction in which biotin labelled *Pf*VSA were subsequently found.

Parasite specific biotinylated proteins of ~110 kDa and ~30 kDa were detected in extracts from *P. chabaudi* clone AS clone (figure 5.1). A protein with similar characteristics to the AS 110 kDa protein was also identified in extracts from CW isolates (figure 5.6), however, this protein is larger than the predicted molecular weights of any members of the multigene families described by Fischer *et al.* (2003). The largest PcCLP identified is predicted to encode a 74 kDa protein, although, considering the extent of size polymorphism for the three members of the PcCLP family described by Fischer *et al.* (2003), it is possible that one or more of the 75 predicted copies of PcCLP genes encodes a larger protein. The lower molecular weight protein of ~30 kDa detected only in AS extracts has characteristics of a cir protein. Slight size polymorphism in the 100 kDa and 30 kDa proteins is congruent with these proteins being encoded by variant multigene families. Using CW infected mice the ~110 kDa protein was also expressed in an appropriate stage specific manner for a *Pc*VSA.

In 1982 Newbold and colleagues (1982) identified a candidate VSA of approximately 250 kDa in *P. chabaudi* and another rodent malaria *P. bergei*. I did not identify a

protein of equivalent size, although it is possible that the antigen described by Newbold *et al.* (1982) lacks surface exposed lysine residues to which the sulpho-NHS-LC-biotin reagent would have access. That the gene encoding the 250 kDa candidate *Pc*VSA has yet to be identified further illustrates the challenges faced when studying *Plasmodium* VSAs. However, considering the speed at which *Plasmodium* genome sequences are becoming available, it is anticipated that the pace at which the molecular mechanisms underlying the biology of this parasite are revealed will continue to accelerate. Indeed, the *P. chabaudi* genome sequence aided the description of the prime *Pc*VSA candidates, *cirs*. However, further characterisation of the cir proteins will face some of the difficulties evidenced in recent studies of *var* gene transcription and expression (reviewed in sections 3.2.2 and 3.2.3). The design of specific molecular probes for *cir* genes and an appreciation of reagent cross-reactivity will be important issues. The surface biotinylation technique described labelled proteins with cir characteristics (figure 5.3.1) and may offer a complementary approach to current molecular biology techniques.

Although *P. chabaudi's* genome does not encode PfEMP, this parasite still exhibits cytoadherence phenotypes, such as rosetting, sequestration, CD36 binding and antigenic variation. I have followed the expression of candidate *Pc*VSA for three related clones that had been selected for high rosetting frequencies via passage through *Anopheles stephensi* mosquitoes (Mackinnon *et al.*, In Press). This analysis suggests a surface exposed protein of ~110 kDa is associated with rosetting and/or mosquito challenge. Although the contribution of rosetting to parasite virulence remains unclear, anti-rosette vaccines have been considered for *P. falciparum* malaria to protect against severe disease (e.g. Chen *et al.*, 2004). Naturally acquired antibody responses to the IE surface seem to be important for the acquisition of protective immunity to *P. falciparum*, but the relative importance of surface reactive antibodies, versus invasion blocking antibodies for example, is not known.

Mota and colleagues (1998; 2000; 2001) have carried out a number of studies to characterise the acquisition of antibodies to the surface of *P. chabaudi* erythrocytes. Their work suggests *P. chabaudi* infections of mice are analogous to *P. falciparum* in respect to acquired immunity to VSAs. The *P. chabaudi* model could circumvent many of the experimental limitations of human malaria in order to further characterize the mechanisms of naturally acquired immunity to the IE surface and their relative importance for protection. The whole life cycle of *P. chabaudi* can be studied with relative ease in the laboratory and the genetic background, age, sex, immune status of the host controlled and contrasted. The diversity of mice with

genetically manipulated immune systems also allows for the contribution and importance of different immune factors to be addressed.

When the host and parasite molecular 'players' behind rosetting in *P. chabaudi* are revealed, this *in vivo* mouse model might provide a suitable system for investigating the effect of rosette disruption on the course and severity of infection. Anti-sera against recombinant rosette mediating domains of PfEMP1 have been shown to disrupt rosettes (Chen *et al.*, 2004). Mice could be immunised with *P. chabaudi* rosetting ligands to raise antibodies that inhibit rosetting and the effects of an anti-rosetting vaccine on the selection of alternative *Pc*VSA variants determined. Such experiments would be a criterion for assessing the effects of such a vaccine for *P. falciparum*. Assaying the protective effects of the passive transfer of purified immunoglobulins from mice, immunised with a *P. chabaudi* anti-rosetting vaccine, to naïve mice prior or during *P. chabaudi* infection could help determine the protective properties of anti-VSA antibodies alone. Mota *et al.* (1998) have demonstrated that naturally acquired anti-*Pc*VSA antibodies are important for phagocytosis *in vitro*. It would be interesting to carry out similar experiments to address the mechanism of anti-IE surface antibody mediated parasite clearance of rosetting versus non-rosetting parasite clones to address whether rosette formation effects parasite clearance. As *P. chabaudi* has a relatively less A/T biased genome in terms of base composition, the heterologous expression of *P. chabaudi* recombinant proteins, for the purpose of binding studies and raising antibodies, should be more straightforward than with *P. falciparum*. Also the candidate *Pc*VSA proteins are significantly smaller than PfEMP1 thus potentially easier to characterise.

5.6 Future work

A collaboration to analyse tryptic peptides, prepared for *P. chabaudi* surface extracts, by μLC-MSMS was established during this project, but unfortunately it did not come to fruition. Therefore, the first experiments to carry out in the future would be the analysis of tryptic peptides to determine the identity of the proteins present in gel slices containing biotin labelled candidate *Pc*VSA. There is currently no evidence that the parasite-specific biotin-labelled proteins are parasite derived and it is possible that host serum proteins bound to *Pc*VSA have been labelled. Whether host or parasite derived these biotinylated surface exposed proteins warrant further investigation.

Chapter 6: Main Discussion

The central aim of this thesis was to apply proteomics to investigate *Pf*VSA$_{PAM}$ expression. In Chapter 2 I have shown for parasite clone FCR3 that CSA selection results in the concomitant expression of trypsin resistant VSA epitopes that are recognised by IgG in a pool of sera from malaria exposed pregnant women. While adhesion of this parasite to the placental receptors CSA and HA was comparatively trypsin sensitive. My findings suggest that antibodies acquired during PAM are not simply recognising the IE surface epitopes involved in CSA adhesion. Whether distinct genes in the FCR3 genome encode for the CSA binding epitopes and the trypsin resistant epitopes has yet to be determined. It was my intention that the differential protease sensitivity exhibited by VSA$_{PAM}$ epitopes would be exploited in comparative proteomic analysis. Although this was not achieved in the course of this project, it is possible that the trypsin resistant phenotype may still prove valuable in the future.

The surface labelling and gel-based proteomic approach that was developed has proven unsuccessful in terms of VSA$_{PAM}$ identification. However, the initial step of cell surface specific labelling has been optimised. Several, large molecular weight, surface trypsinisation sensitive, biotinylated proteins were detected in extracts of FCR3CSA from surface labelled IEs. The largest of these proteins co-localised with a variant of PfEMP1 in Western blotting. That the other high molecular weight species are PfEMP1 variants that are not reactive to the anti-ATS antibody is a possibility.

Increasingly sophisticated mass spectrometric techniques are being developed for the identification of proteins in complex mixtures, circumventing the need for absolute electrophoretic separation and isolation from gel matrices. I had a relatively poor success rate (~50%) when identifying proteins by MALDI-TOF mass spectrometry of tryptic peptides extracted from proteins resolved by 2D SDS-PAGE, and considering the large molecular weight of the biotin labelled candidate *Pf*VSA, a non-gel based technique combining micro-liquid chromatography (μLC) and tandem ESI MS might prove a more appropriate alternative. The cell fraction (pigmented pellet) to which parasite-specific labelled proteins sediment represents a rather small portion of the IE total protein, but is enriched for PfEMP1, surface trypsinisation sensitive PfCRMP2, and additional biotin labelled candidate *Pf*VSA. 1D SDS-PAGE then provides yet another round of separation, such that Western blotting of the pigmented pellet fraction with streptavidin can identify fine gel slices containing the biotinylated surface trypsinisation sensitive candidate *Pf*VSA.

Therefore these gel slices should prove relatively simple to analyse by μLC and tandem ESI MS. The differential protease sensitivity of the VSA_{PAM} epitopes would also allow for the treatment of the infected erythrocyte surface with relatively low concentrations of trypsin to remove trypsin sensitive CSA-binding eptiopes, but leave the more trypsin resistant epitopes intact. This protease treatment would simplify the surface complexity, and potentially provide a marker for the identification of the trypsin resistant VSA_{PAM} epitopes described in Chapter 2. It would therefore be interesting to look at the effect of titrating the concentration of trypsin on the pattern of IE surface labelling. During my experiments to optimise IE surface biotinylation only high concentrations of trypsin (1 mg/ml) were employed solely in order to ascertain whether labelling was cell surface specific.

6.1 PfEMP1 and PAM

The decision to develop a proteomics method for the study of VSA_{PAM} expression was made in part due to conflicting data regarding the identity of the *var* gene transcribed and expressed at the erythrocyte surface of CSA selected parasite clones and placental isolates. However, over the last few months, two particularly relevant papers have been published that have cleared the fog of confusion surrounding this matter slightly. The group that initially identified the FCR3*var*1CSA gene published the first of these. Gamain *et al.* (2004) have further defined a 67 amino acid (aa) CSA binding motif that is relatively well-conserved between *var*1csa homologs from different parasite isolates. However their paper also states that a monoclonal antisera against the recombinant FCR3*var*1CSA DBL-δ domain, that inhibits CSA binding (Costa *et al.*, 2003), and antisera raised against the 67 aa binding motif, may not specifically recognise their immunising antigens. Cross-reactivity is evidently possible as a monoclonal anti-sera raised against the DBL-δ domain of FCR3*var*1CSA recognises the surface of FCR3*var*1CSA-knockout parasites (after reselection on CSA) (Andrews *et al.*, 2003). Interestingly, antisera raised against the 67 aa CSA binding domain recognises CSA selected clones specifically but does not inhibit binding (Gamain *et al.*, 2004). Since the role of anti-VSA_{PAM} antibodies in opsonisation dependent phagocytosis and/or alternate parasite clearing pathways in the placenta is unknown, it is therefore difficult to predict the efficacy of a vaccine raising surface reactive antibodies that do not block CSA adhesion. The findings of chapter 2 and work by Beeson *et al.* (2004) certainly suggest that non-adhesion blocking antibodies are raised naturally to VSA_{PAM}. The paper by Gamain *et al.* (2004) further supports the view that FCR3*var*1CSA may not be expressed by CSA

binding parasites and that additional or alternative genes in FCR3's genome can support CSA adhesion.

The second papers provides evidence for the involvement of the *var*2csa gene in PAM (Salanti *et al.*, 2004). This unusually structured *var* gene lacks a conventional CIDR1 domain and is also relatively well conserved. It was originally shown to be transcriptionally up-regulated in the parasite clones NF54 and FCR3CSA, following selection for adhesion to CSA (Salanti *et al.*, 2003). Salanti and colleagues (2004) demonstrate that antibodies raised in rabbits to two of the six DBL domains (DBL1-X and DBL5-ε) encoded by *var*2csa recognise both of the CSA selected clones in which the gene was originally found to be transcriptionally upregulated. Furthermore, their ELISA data suggests recombinant DBL1-X and DBL5-ε domains are well recognised by IgG from serum of a group of term pregnant women residing in an area of hyper-endemic stable malaria transmission, but not by sera from sympatric males. They also found high levels of *var*2csa-specific IgG to associate with favourable birth outcomes. Salanti *et al.* (2004), however, did not report on the CSA binding capacity of the recombinant DBL1-X and DBL5-ε domains nor on the anti-adhesion properties of the antisera they raised against these domains. Interestingly, Gamain *et al.* (2004) report that the minimal 67 aa CSA binding motif from the DBL-δ of FCR3*var*1CSA is related to the DBL1-X of *var*2csa. Since genes closely related to *var*2CSA are commonly found, and a homolog occurs in the genome of FCR3 (Salanti *et al.*, 2003), it is possible that this may explain the antisera cross-reactivity seen by Gamain *et al.* (2004).

Interestingly the 13-14 kb *var* transcripts shown to be up-regulated in the CSA selected FCR3/IT lineage and the FCR3 clone, by Duffy *et al.* (2002) and Kyes *et al.* (2003) respectively are too large to be a close homolog of *var*2csa. The identity of these transcripts is still unknown. Thus, development of an alternative complementary method, and perhaps further development of the approach I have worked towards here, for the study of VSA_{PAM} remains justified, especially considering a concluding statement of Gamain *et al.* (2004); 'Given the uncertainties about FCR3-CSA protein translation and the complete lack of knowledge about what is expressed at the surface of placental isolates, the precise targets of these antibodies are still not known'.

Recent, quantitative proteomics studies in *P. falciparum* again highlight that mRNA levels do not generally correlate with protein abundance in this parasite. Nirmalan and colleagues (2004b) found patterns of mRNA expression and protein levels for different genes differed significantly, which indicates differences in mRNA stability,

and/or different efficiencies and timing of translation. Although these studies were not concerned with PfEMP1 they further support the need to look at the level of protein expression. Nirmalan *et al.* (2004b) designed a rather elegant quantitative proteomics method specifically for *P. falciparum* that relies on the metabolic labelling of parasite proteins with alternatively labelled isoleucine which contains different numbers of stable isotopes of carbon and hydrogen (Nirmalan *et al.*, 2004b). Their method allows for experimental cultures of the parasite, for example a drug treated culture, to be compared directly to a control untreated culture (Nirmalan *et al.*, 2004a). Although this approach was considered for studying VSA_{PAM} expression, the method of Nirmalan and colleagues requires that you know the position of your protein(s) of interest on a 2D SDS-PAGE gel.

6.2 Is PfEMP1 everything in the case of VSA_{PAM}?

Since the identification of the *var* multigene family, studies of *P. falciparum* VSA involving assaying naturally acquired antibody responses and/or binding phenotypes have generally presumed that PfEMP1 is solely responsible for mediating these traits. However, considering: (1) members of the rifin family are now known to be expressed on the IE surface (Kyes *et al.*, 1999); (2) the recent discovery of *Plasmodium* infected erythrocyte surface proteins 1 an 2 (Florens *et al.*); and (3) the possibility that members of the PfCRMP family are surface exposed (Thompson *et al.* In Prep), it seems PfEMP1 is not alone on the IE surface. The VSA_{PAM} of mature stage IEs exhibits several qualities uncharacteristic of PfEMP1; however, studies to define the molecular identity of VSA_{PAM} have focused solely on this protein (discussed in section 1.4.6).

It seems that many species of malaria can exhibit cytoadhesion phenotypes, such as sequestration (e.g. *P. knowlesi* and *P. chabaudi*) and rosetting (all *Plasmodium spp* studied so far) without PfEMP1. *Var* gene homologs have yet to be found outside of *P. falciparum*. With respect to malaria in pregnancy, *P. vivax* infection can bring about negative birth outcomes, albeit to a lesser extent than *P. falciparum* (McGready *et al.*, 2004). In the small number of studies looking at malaria animal models during pregnancy, higher parasitaemias, accentuated pathology, and immuno-pathological changes comparable to that of *P. falciparum* infected human placentae, have been recorded (reviewed in Desowitz, 2001). Furthermore, a presentation at the Molecular Approaches to Malaria 2004 conference reported 30 out of 30 *P. vivax* isolates examined bound specifically to immobilised CSA and HA (Abstract

published as Chotivanich *et al.*, 2003). *P. vivax* adhesion to these placental receptors was reported to begin ~16 hours post invasion and peak 32 hours post invasion. *P. vivax* IEs were not found to adhere to CD36, ICAM1 or thromospondin, which are major endothelial receptors for *P. falciparum*. It will be interesting to investigate whether members of the *P. vivax* interspersed multigene family, *vir* (del Portillo *et al.*, 2001), mediate adhesion to CSA and HA.

6.3 A small animal model of antigenic variation in a post-genomics era

In Chapter 4 I have applied a surface biotinylation technique to *P. chabaudi* IEs. This project was initiated partly to test the ability of the technique to label *Plasmodium* VSA, but also to detect surface exposed proteins having similar characteristics to the *P. chabaudi* interspersed repeat family, *cirs* (Fischer *et al.*, 2003; Janssen *et al.*, 2002). Surface labelling of the *P. chabaudi* AS clone highlighted a surface exposed protein of the anticipated molecular weight (~30 kDa) for members of this family (Janssen *et al.*, 2004). A similar protein was not seen in subsequent studies using isolates derived from the clone CW. However, when three isolates derived from CW were surface labelled pre- and post transmission through *Anopheles stephensi* mosquitoes, a 120 kDa surface exposed protein was upregulated in the clones that had been mosquito transmitted when compared to the isolates that had previously undergone several rounds of serial blood passage. The hypothesis that mosquito transmission selects for a general up-regulation of surface antigen expression has not been directly tested. However, that rosetting rates also increase following mosquito transmission of these *P. chabaudi* clones (Mackinnon *et al.*, In Press) supports this hypothesis.

As discussed in chapter 5, *P. chabaudi* infections of laboratory mice reproduce many of the features of *P. falciparum* infections of humans. In *in vivo* experiments in mice lend themselves to manipulation and controlled experimentation far more easily then their equivalent in humans. Although, *P. chabaudi's* genome does not encode the infamous *var* gene family, this parasite exhibits several VSA mediated phenotypes, such as, antigenic variation, sequestration and rosetting. The *P. chabaudi* sequencing project is well underway (Janssen *et al.*, 2001) and nearing completion, a combination of increasingly sophisticated molecular and proteomic approaches is now possible for this *in vivo* malaria model. *P. chabaudi* now offers a powerful accessible model with which to further our understanding of the molecular mechanisms of VSA 'switching', VSA associated pathogenesis, and the impact of adhesion blocking vaccines.

Chapter 7: Materials and Methods

7.1 *P. falciparum* culture and manipulation

7.1.1 *P. falciparum* parasite isolates and culture

Parasites were maintained in group O erythrocytes under standard conditions (Trager & Jensen, 1976), using RPMI 1640 medium containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 μg/ml gentamycin and 10% pooled normal human serum. The pH was adjusted to between 7.2 and 7.4 with 1 M NaOH. Culture flasks at 5% haematocrit were gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. The laboratory clone FCR3 originates from peripheral blood collected in the Gambia. FCR3CSA was obtained from the Malaria Research and Reference Reagent Resource Centre (ATCC) (Scherf *et al.*, 1998), and was confirmed using genetic markers to be identical to the laboratory clone FCR3 kept in the original W.H.O. strain registry collection in Edinburgh (pers. Comm. D. Walliker).

7.1.2 Giemsa staining of thin blood smears

Thin blood smears were prepared using a pipette to transfer approximately 10 μl of the cell layer that settles at the bottom of the culture flask to a microscope slide and then spreading the cells thinly with a clean microscope slide. Blood smears were air-dried and fixed with 100% methanol for about 30 seconds and then air-dried again. Slides were stained by immersion for 20 minutes in a 10% Giemsa stain solution (BDH) diluted in Giemsa buffer (prepared using Giemsa buffer tablets; BDH). Slide were washed thoroughly under tap water and then air-dried. Slides were observed by light microscopy using a 100x objective under immersion oil (BDH).

7.1.3 Sorbitol synchronization of *P. falciparum* infected erythrocytes

Sorbitol synchronisation was to obtain only ring stage parasites was performed following the method of Lambros and Vanderberg (1979). Parasite cultures containing ring stage parasites were transferred to centrifuge tubes and centrifuged for 5 minutes at 500 x*g*. Culture medium was removed and the cell pellet resuspended in 5 ml of 5% sorbitol (w/v; Sigma) and incubated at RT for 5 minutes.

Cells were then centrifuged again for 5 minutes at 500 x*g*, washed with 10 ml of RMPI-1640 and returned to standard culture conditions.

7.1.4 Enrichment of trophozoite and schizont stages

Trophozoite and schizont stages were enriched by sedimentation using a commercially available high-molecular-weight polymer Plasmagel (Cellular Products, Inc., Buffalo, N.Y.). 5 ml 5% haematocrit IE cultures were washed once with RPMI-1640 in a 15 ml centrifuge tube. The washed pellet of cells were resuspended in a 5 ml of Plasmagel pre-warmed to 37°C and the tube incubated upright at 37°C for 15-20 minutes or until a clear layer of late stage IE erythrocytes was evident above the sediment of uninfected and ring stage parasites. The upper layer of IEs was carefully transferred to a fresh tube and washed twice with 10 ml of RPMI-1640.

7.1.5 Selection of *P. falciparum* infected erythrocytes for adhesion to CSA

CSA binding was maintained by panning late stage infected erythrocytes on bovine tracheal CSA (Sigma) immobilized on polystyrene Petri dishes (Falcon) essentially as described by Chaiyaroj *et al.* (1996). 2 ml of CSA (10 μg/ml) prepared in PBS (pH 7.2) was adsorbed onto 2.5 cm diameter petri dishes (Falcon) overnight at 4°C. Immediately prior to panning CSA was removed from the dishes and overlaid with 2 ml of 2% BSA (essentially immunoglobulin free; Sigma) prepared in PBS to block non-specific protein binding site. Dishes were blocked for 30 minutes at RT. Following the removal of this blocking solution, late-stage parasites, suspended in 2ml of complete RPMI-HEPES medium (generally 8-10% parasitaemia, 5% haematocrit), were added to the Petri dish. Parasites were incubated with the immobilised receptor for 1 hr at 37 °C with occasional agitation. Then unbound cells were removed by four gentle washes with incomplete RPMI-HEPES medium. Bound IEs were cultured with 2ml of complete RPMI-HEPES medium and 50 μl of packed erythrocytes for 20- 24 hours at 37 °C in an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen. Ring stage infected erythrocytes were removed form the dishes the following day and returned to standard culturing conditions. The maximum time interval between panning was two weeks but on averaged was performed weekly. Prior to protease treatment and analysis by flow cytometry, cultures were synchronised at least twice by sorbitol treatment.

7.1.6 Binding assays

Human umbilical cord hyaluronic acid (Sigma) and bovine trachea CSA (Sigma) were used at a concentration of 10 μg/ml in PBS (pH 7.2). 20 μl of each receptor was spotted in triplicate onto 5 cm diameter petri dishes (Falcon). Receptors were adsorbed onto the plastic petri dishes overnight at 4°C. 10 μg/ml BSA in PBS was similarly adsorbed as a negative control. Plates were then blocked by removing the receptor solution and adding 20 μl of 2% BSA in PBS. Following the removal of this blocking solution, late-stage parasites, suspended in 2ml of complete RPMI-HEPES medium (8-10% parasitaemia, 5% haematocrit), were added to the petri dish. Parasites were incubated with the immobilised receptor for 60 minutes at 37 °C with occasional agitation. Unbound cells were removed by four gentle washes with incomplete RPMI-HEPES medium; bound cells were fixed with 0.5% (v/v) glutaraldehyde in PBS for 10 minutes and Giemsa stained. Bound cells were assayed by light microscopy. Protease treatment of intact cells was carried out as described in section 7.1.7.

7.1.7 Cell surface protease treatment

Protease treatment of infected erythrocytes was carried out as previously described (Chaiyaroj *et al.*, 1996). Samples, containing 3 x 10^6 cells from sorbitol-treated late-stage cultures of 8-10 % parasitaemia were washed twice with phosphate-buffered saline (PBS; pH7.2) and then incubated with the appropriate concentration of trypsin-TPCK (Worthington Biochemicals) or pronase (Boehringer-Mannheim) in a final volume of 1.0 ml in PBS, for 10 minutes at 37 °C. The reaction was terminated either by adding soybean trypsin inhibitor (Worthington Biochemicals) to a final concentration of 1 mg/ml or by adding 10% human serum. Cells were washed twice with PBS before further use.

7.2 Fluorescence activiated cell sorting

7.2.1 Plasma donors

Serum samples from 20 men living in a malaria endemic region of Ghana were pooled to produce the male serum pool. Serum samples collected at the time of birth from the placentae of 15 women living in a malaria endemic region of Ghana were pooled to produce the pregnant female serum pool. This pool included five

primigravidae, nine secundigravidae and one multigravid woman. Serum samples from six Scottish malaria naïve individuals were pooled and used as a control.

7.2.2 Analysis of VSA specific antibodies by flow cytometry

Flow cytometry was used to measure the levels of plasma IgG binding to the VSA of late stage parasites essentially following the method previously described by Staalsoe *et al* (1999; 2001). 3 x 10^6 cells from late stage *P. falciparum* cultures of 8-10 % parasitaemia were washed twice with PBS. Cells were incubated sequentially with plasma antibodies diluted 1:20 in PBS, goat anti-human IgG diluted 1:200 in PBS (Dako Cytomation) and fluorescein isothiocyante (FITC)-conjugated rabbit anti-goat (Dako) diluted 1:25 in PBS. All incubations were in a total volume of 100 µl for 30 minutes at room temperature and were followed by two washes with 1 ml of PBS. Samples were analysed immediately on a FACSCAN apparatus (Becton-Dickinson). FITC fluorescence due to cell surface antibody recognition was determined for 5000-10000 ethidium bromide gated infected erythrocytes.

7.2.3 Statistical analysis

Statistical analyses were performed using Analyses of Variance in Minitab 13.30 (Minitab Inc.), using protease, protease concentration and serum pool as explanatory variables. Statistical models were tested for homogeneity of variance and normality of error distributions. Where possible, maximal models with interactions between these variables were fitted first, after which models were minimised by removing non-significant ($p>0.05$) terms.

7.3 Biotinylation and cell fractionation

7.3.1 Labelling of surface proteins using sulpho-NHS-LC-biotin

The lyophilised sulpho-NHS-LC-biotin label was stored in sealed aliquots to minimise exposure to air and hydrolysis. Labelling of proteins with sulpho-NHS-LC-biotin is efficient at a broad range of label concentrations and temperatures within a pH range of 7-9. Parasite cultures were washed with PBS (pH7.2) and transferred to eppendorf tubes. Cells were either treated or mock treated with proteases as described in section 7.1.7. Cell surface labelling was carried out simply by estimating the pack cell volume and resuspending the cells in 10 volumes of PBS

(pH7.2) containing 0.5 mg/ml sulpho-NHS-LC-biotin. Intact cells were incubated with the biotin label at RT with gentle agitation for 30 minutes and then washed twice with 1 ml of PBS (pH7.2).

For surface biotinylation in the presence of furosamide the pack cell volume of washed cells was estimated and the cell pellet resuspended in 10 volumes of 100 mM furosamide in PBS supplemented with 0.6 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-2). Cells were incubated in furosamide for 1 minute before adding sulpho-NHS-LC-bioin to a final concentration of 0.5 mg/ml. Cells were incubated at RT with gentle agitation for 30 minutes in the presence of biotin and furosamide. Cells were pelleted and resuspended in PBS-2 100 mM glycine. Cells were then washed with PBS-2 alone.

7.3.2 Detergent extraction of proteins

Cells were washed twice with PBS and transferred to 1.5 ml eppendorf tubes prior to lysis on ice with 5 volumes of a solution 150 mM NaCl, 5 mM EDTA, 50 mM tris pH8.0, 1% triton-X100 (w/v) and a commercially available protease inhibitor cocktail (Complete mini, Roche). The cell lysates were centrifuged at 13 000 rpm in a microcentrifuge at 4°C for 10 minutes, the supernatants containing triton-X100 soluble (TS) fractions were removed to a fresh tube and the triton-X100 insoluble pellet resuspended in a solution containing 20 mM tris-HCl pH 8.0, 150 mM HCl, 2% (w/v) SDS and a protease inhibitor cocktail (Complete mini; Roche). The solubilised TI fraction was pipetted up and down several times to disrupt the parasite DNA and the samples centrifuged at 13 000 rpm in a microcentrifuge at 4°C for 10 minutes. The SDS soluble TI fraction was transferred to a fresh tube and stored at –30 °C.

7.3.3 Membrane fractionation by osmotic lysis

Cells were washed twice with PBS and transferred to 1.5 ml eppendorf tubes prior to lysis with 10 volumes of a ice-cold solution of 5 mM Na_2HPO_4 (pH 8.0) containing protease inhibitor cocktail (Roche) (Olivieri *et al.*, 2001). Cell lysates were centrifuged in a benchtop microfuge at 13 000 rpm for 10 minutes at 4°C. The supernatant was removed and the fluffy layer of erythrocyte membranes removed to a fresh tube. The remaining pigmented pellet was either discarded or stored directly at - 30°C. The membrane fraction was washed twice with 1 ml of ice-cold

5P8 containing a protease inhibitor cocktail (Complete mini Roche) prior to storage at –30°C.

7.3.4 Nuclease treatment of pigment containing fraction

The pigmented pellet was cleared by resuspending it in 60 µl of bugbuster protein extraction reagent (Novagen), a recommended buffer for the genetically engineered endonulease from *Serratia marcescens*, benzonase (Novagen). 30 units of the endonuclease were added to each reaction (Novagen). Benzonase is highly active at RT such that nuclease treatment could be carried out swiftly (15-30 minutes) at RT. Thus avoiding higher temperatures and longer incubation periods at which proteolysis would be favoured. The tubes were briefly vortexed 3 times during the reaction.

7.4 SDS-PAGE and Western blotting

7.4.1 Protein assays

Protein concentrations were determined using a commercially available detergent compatible protein assay kit (Biorad, Hercules, CA, Catalogue number 500-0114) in a microtitre plate following the manufacturers protocol.

7.4.2 2D SDS-PAGE

Prior to isoelectric focusing, 25 µl aliquots of triton-X-100 insoluble extracts that had been solubilised with 2% SDS (w/v) were dialysed against 50 ml of 7M urea (Sigma), 2 M thiourea (Sigma) overnight at 4°C. Considering the initial protein concentration for each extract an appropriate volume to load 100 µg of protein per immobilised pH gradient strip was combined with strip rehydration buffer such that the final constituents were 7 M urea, 2 M thiourea, 2% 3-[3-Cholamidopropyl) dimethylammonio]-1-propanesulphonate (Amersham), 65 mM. DTE (Sigma), 0.8% resolytes pH 4-7 (Amersham), 1% triton-X100 (Amersham) and a trace of bromophenol blue (BDH), in a final volume of 125 µl. Likewise, 125 µg of triton-X100 soluble extract was combined directly with strip rehydration buffer to a final volume of 125 µl. Extracts were incubated with rehydration buffer for 1 hour at RT. Protein samples were then loaded onto immobilised immobilised pH gradient (IPG)

strips (7 cm; pH 4-7; Amersham) via the cup loading method. Proteins were separated by virtue of their pI using the conditions described in Table 7.1.

IPG strips were equilibrated directly for SDS-PAGE or stored at -70°C until directly prior to SDS-PAGE. A primary equilibration step with 2 ml of 1.5 M tris, pH 8.8, 6 M urea, 30% glycerol (v/v) (Sigma), 2% (w/v) SDS (Sigma), a trace of bromophenol blue and 1% DTE, was carried out for 15 minutes at RT. A second step followed as the first except for 1% DTE was replaced with 4% iodoacetamide (Sigma). Equilibrated IPG strips were then loaded onto pre-cast 4-12% bis-tris gels with the appropriate 2D well format (Invitrogen). Loaded IPG strips were overlaid with 0.8 ml of molten 1% agarose prepared in SDS-PAGE running buffer containing 50 mM MOPS, 50 mM tris-base, 0.1% SDS (w/v) and 1 mM EDTA (pH 7.7). After the agarose had hardened pre-stained precision protein molecular weight markers (Biorad) were loaded into the designated marker lane and gels were ran at 100 V until the dye front arrived at the foot of the gel.

Table 7.1: Optimised conditions for IPG strips

Tables shows the conditions used for isoelectric focusing on an Ettan IPGphor isoelectric focusing apparatus (Amersham).

Step	Parameter	Voltage (V)	Time (hours)
1	Gradient	500V	0.1
2	Gradient	4000V	2.5
3	Step and hold	5000V	1.5

7.4.3 1D SDS-PAGE

SDS-PAGE was performed using the NuPAGE precast gel system initially to allow greater reproducibility for 2D SDS-PAGE, however due the reliability of the gradient gels this system was subsequently used routinely. Bis-tris gradient gels were run using a buffer containing 50 mM 3-Morpholinopropanesulphonic acid, 50 mM tris-base, 0.1% SDS (w/v) and 1 mM EDTA (pH 7.7). A buffer containing 2.5 mM tricine, 2.5 mM Tris base, and 0.005 % SDS (pH8.24) was used for tris-acetate gradient gels. Samples were prepared using a 4X SDS sample buffer containing 106

mM tris-HCl, 141 mM tris-base, 2% SDS (w/v), 10% glycerol, 0.51 mM EDTA, 0.22 mM SERVA® Blue G250, 0.175 mM phenol red (pH 8.5) and 2.8% β-mercaptoethanol. Samples were incubated at 90 °C for 10 minute and then centrifuged briefly prior to loading. Proteins were separated at 150V for ~1 hour. Pre-stained precision protein molecular weight markers (10-250 kDa; Bio-rad) were used throughout.

7.4.4 Western Blotting

Electrophoretic transfer of proteins from SDS-PAGE gels to Westran® polyvinylidene difluoride (PVDF; Schleicher & Schuell BioScience) membrane was performed at 30 V for one hour at RT using a transfer buffer containing 1.25 mM bicine, 1.25 mM bis-trs, 0.05 mM EDTA (pH 7.2) and 10 % methanol (BDH). Prior to transferring the PVDF membrane onto the gel the membrane was soaked in 100% methanol for 5 seconds. Since biotin is present in commonly used biological blocking reagents such as dried milk and bovine serum albumin, a number of alternative blocking reagents were tried for western blotting with HRP-conjugated streptavidin. A commercially available blocking reagent from QIAGEN was found to be the most suitable. Unless otherwise stated, this blocking reagent was used for all western blots presented in this thesis. Blocking was performed for between 1 hour and overnight at RT, using a solution of the QIAGEN blocking reagent pre-heated to 70°C and cooled to RT. HRP-conjugated streptavidin was added directly to the blocking buffer to a final concentration of 0.02 μg/ml and incubated for 1 hour at RT. Blots were washed twice with PBS/0.1% tween-20 and once with PBS alone prior to processing the blot with an enhanced chemiluminescence (ECL) detection kit (Amersham). Chemiluminescence was detected using Hyperfilm™ ECL (Amersham).

The affinity purified anti-ATS (1:000), anti-PfCRMP1 and anti-PfCRMP2 rabbit sera were detected using peroxidase conjugated ImmunoPure® Recomb® protein A (Pierce Biotechnology) diluted in QIAGEN blocking reagent to a final concentration of 2.5 μg/ml.

7.4.5 Stripping antibodies from PVDF membrane

Western blots were incubated for 30 minutes at 50°C with agitation in a solution of 62.5 mM tris-HCl (pH6.7) containing 2 % SDS (w/v) and 100 mM β-mercaptoethanol. Blots were then washed twice with copious amounts of PBS/0.1%

tween-20 for 20 minutes and either immediately blocked prior to re-probing or stored at 4°C in PBS/0.1% tween-20 in a sealed polythene bag.

7.5 MALDI-TOF

7.5.1 Mass spectrometry compatible silver-staining

Proteins resolved using SDS-PAGE were stained with silver by sequential incubations in the following solutions at RT for the times stated: (1) 40% ethanol (BDH)/10% acetic acid (BDH) for 30 minutes; (2) 30% ethanol/160 mM sodium thiosulphate (Fluka)/490 mM sodium acetate (Fluka) for 30 minutes; (3) three times 5 minute incubations in distilled water; (4) 15 mM silver nitrate (Fluka) for 20 minutes; (5) two times 1 minute incubations in distilled water; (6) 236 mM sodium carbonate (Sigma)/0.75% (w/v) formaldehyde (BDH) for 2-5 minutes or until protein spots visible; (7) 10 minutes in 40mM ethylenediaminetetraacetic acid ($N_2.2H_20$) (Sigma). Following staining gels were stored at 4 °C in distilled water.

7.5.2 In-gel trypsin digest protocol (York)

Protein spots excised from silver-stained gels were transferred to 1.5 ml eppendorf tubes and destained using 100 µl of Farmer's reducing agent (1:1 (v:v) 20% sodium thiosulphate and 1% potassium ferricyanide). Gel pieces were washed thrice for 20 minutes in 100 µl of 20m mM ammonium bicarbonate/50% acetonitrile (BDH) at RT. A Speed Vac was used to dry the gel pieces for 15 minutes using a medium drying rate. To dried gel pieces, 10 µl of 0.02 µg/µl sequencing grade modified porcine trypsin (Promega) in 20 mM ammonium bicarbonate/ 0.1% octyl-β-D-glucopyranoside was added and gel pieces allowed to swell for 10 minutes. 20 µl of 20 mM ammonium bicarbonate/0.1% octyl-β-D-glucopyranoside was then overlaid over the gel pieces and incubated at 37 °C overnight. The supernatants were removed and a fraction stored at -70°C. The gel pieces were washed twice with 50% acetonitrile and the washes combined with the remainder of the supernatants and dried in a speed vac and stored at –70°C. A fraction of the tryptic digests were used directly for MALDI-TOF analysis, whereas the remainder, that had been dried, was resuspended prior to analysis and desalted following the procedure described in section 7.5.4.

7.5.3 SIRCAMS in-gel trypsin digest protocol

Silver stained proteins resolved by SDS-PAGE were excised from the gel and destained. Gel pieces were then washed twice with 100 µl 50% acetonitrile (BDH) for 5 minutes at RT and dried in a Speed Vac using a medium drying-rate for 15 minutes.

For proteins resolved by standard one-dimensional (1D) SDS-PAGE, reduction and alkylation steps were included prior to trypsin digestion. Dried gel slices were covered with 15 µl of 10 mM dithiothreitol (DTT; Sigma)/0.2% EDTA (w/v) in 100 mM ammonium bicarbonate (Sigma) and incubated at 56 °C for 30 minutes. This solution was then removed and 50 µl of 50 mM iodoacetamide (Sigma) in 100 mM ammonium biocarbonate was added. The gel pieces were incubated at RT in the dark for 30 minutes. This solution was then removed from the gel pieces. Gel slices were washed with 200 µl 100 mM ammonium bicarbonate for 10 minutes and then dehydrated by incubating the gel slice twice in 200 µl neat acetonitrile for 10 minutes. Gel slices were dried as described earlier. Trypsin digestion of washed and dried protein spots from 2D SDS-PAGE or reduced and alkylated protein spots from 1D SDS-PAGE were carried out as described in section 7.5.2 with the omission of octyl-β-D-glucopyranoside.

7.5.4 Peptide desalting and application to MALDI plate

Tryptic peptides were desalted using a 10 µl C18 zip-tip (Millipore) and a P10 pipette (Gilson). Briefly, a C18 zip-tip was washed twice with 10 µl elution buffer (1:1 water: 0.1% formic acid in acetonitrile) and then twice with 10 µl 0.1% formic acid before sample loading. Tryptic digest reactions were loaded onto the zip-tip by aspirating the sample up and down 10 times and then washing twice with 10 µl of 0.1% formic acid. Finally, desalted peptides were eluted from the zip-tip with 10 µl elution buffer. Samples were either analysed by MALDI-TOF directly or stored at –30 °C. For each sample, 0.7 µl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.3% trifluroacetic acid was placed onto a spot of a 100 well stainless steel MALDI plate (Applied Biosystems) and 1.5 µl of desalted tryptic peptide solution overlaid.

7.5.5 SIRCAMS MALDI-TOF data acquisition

Mass spectra were acquired using a Voyager DEtmSTR MALDI-TOF instrument (Applied Biosystems) with an accelerating voltage of 20000V. For each acquisition 200 shots were fired using a laser intensity of between 2069 - 2200V. The acquisition mass range was set to 800-3000 Da. Spectra were processed manually using Data Explorer software. Isotopic peaks were converted into their monoisotopic peak. Initially peak detection was set to 0% maximum peak area and then each spectrum was split into segments to set an appropriate baseline to allow detection of the 100-200 most intense peaks per spectrum. Trypsin autolytic peptides were used to calibrate each spectrum independently. The resulting mass lists were searched against the National Centre for Biotechnology Information non-redundant (NCBInr) database via two proteomics web interfaces, MASCOT and MSFIT.

7.5.6 York MALDI-TOF data acquisition

An Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF instrument was used to acquire tandem MS data. Calibration was set, prior to acquiring data for a plate, using a standard mixture of peptides. For each acquisition 500 shots were fired with a laser intensity of between 2000 - 3000V for MS1. The 10 most abundant peptides for each tryptic digest from the first round of MALDI-TOF were selected for MS2. Spectrum processing was automated and involved steps to deisotope and detect peaks over a 2% maximum peak area cut off. Batch MSMS data was searched against the NCBInr database using the web interface MASCOT (www.matrixscience.com) or MSFIT (http://prospector.ucsf.edu).

7.5.6 Database mining

The MSFIT and MASCOT database searching interfaces both score the likelihood of a list of tryptic peptides being derived from predicted protein sequences in sequence databases using a MOWSE score (Pappin *et al.*, 1993). A MOWSE score is a scoring method analogous to e-value assigned to a hit derived through BLAST searching sequence databases with a nucleic acid or protein sequence. Although, MSFIT and MASCOT use the same scoring systems the numerical value assigned to a score is on a different scale. A MSFIT score of over 10^5 a MASCOT score of over 50 is considered a significant hit although the significance may be interpreted differently depending on the size of the database. For database searching using

spectra from tryptic digests the mass list was searched against all species in the NCBInr database. Allowed modifications were the oxidation of methione and acrylamide modifications of cysteines. The maximum number of miscleavages was set at 1 and miscleaved peptides were given a pFactor of 0.4.

7.6 Anti-ATS antibody purification and characterisation

7.6.1 Immunisation protocol for anti-peptide antibody

Two New Zealand white rabbits were immunised with the PfEMP1 ATS peptide (DITSSESEYEELDINDIC), conjugated to the carrier protein keyhole limpet hemocyanin (KLH), using the following schedule. Pre-immune serum was collected on day 1 of the immunisation schedule when the rabbits were immunised with 200 μg of KLH- peptide conjugate in Complete Freund's Adjuvant. Five further immunisations with 100 μg of KLH-peptide conjugate in Incomplete Freund's Adjuvant were carried out with 7-day intervals, starting on day 14. 5 ml test bleeds were taken on days 35, 49, 70 and 77. Final bleeds of approximately 85 ml were taken on day 79. Sera were divided into aliquots and stored at –30°C. The peptide was synthesised and immunisations carried out by Sigma Genosys.

7.6.2 Affinity purification and biotinylation of anti-ATS antibody

To affinity-purify immune sera against the immunising peptide, the peptide was covalently immobilised to a SulphoLink® column (Pierce Biotechnology) via the sulfhydryl group of the carboxyl terminal cysteine residue. This column contains 6% cross-linked beaded agarose. 5 mg of peptide was conjugated to a 1.5 ml SulphoLink® column following the manufacturer's instructions. Briefly, the peptide was resuspended in 1 ml of the sample preparation buffer provided, to which 2-mercaptoethylamine•HCl was added to a final concentration of 50 mM. This solution was incubated at 37°C for 1.5 hours and then allowed to cool to RT. The reduced peptide was applied to a P10 desalting column (Amersham) that had been equilibrated with 25 ml SulfoLink® coupling buffer (5 mM EDTA disodium salt/tris (hydroxymethyl aminomethane). 3.5 ml of SulfoLink® coupling buffer was then added to the column and the peptide collected in a 3 ml fraction after discarding the first 0.5 ml of flow through.

The reduced and desalted peptide was then applied to a SulphoLink® column that had also been pre-equilibrated with SulfoLink® coupling buffer. The column was rocked for 15 minutes at RT and allowed to stand for 30 minutes. 6 ml of coupling buffer was used to wash the column and free binding sites blocked by applying 2 ml of 0.05 M L-cysteine. The column was rocked for 15 minutes at RT, allowed to stand for 30 minutes and then washed with 12 ml PBS (pH 7.2).

Immune sera in 1.5 ml aliquots were affinity-purified on the peptide column by incubating the sera on the pre-equilibrated column for 1 hour at RT. The flow-through was collected and the column washed with 12 ml of PBS (pH7.2). Immunoglobulins were eluted by applying 4 ml ImmunoPure® IgG elution buffer (Pierce) and were collected in a 3 ml fraction after discarding the first 1 ml. To each 1 ml of eluent, 50 µl of 1 M Tris, pH 9.5, was added to neutralise the acidic elution buffer. The affinity-purified immunoglobulins were buffer-exchanged into PBS (pH 7.2)/0.05% (w/v) sodium azide, and volume reduced to ~ 0.3 ml, using a 6 ml Vivaspin column with a 10 kDa cut off. The average immunoglobulin yield was 0.3 mg/ml.

7.6.3 Immunofluorescence assay

Small aliquots (~10 µl) of parasite culture at 50 % haematocrit and 10% parasitaemia were smeared directly onto glass microscope slides (60 mm x 20 mm). Air dried smears were immediately fixed by immersion in using ice cold 2% paraformaldehyde (Sigma)/PBS (pH7.2) for 5 minutes and then immersion in 90% methanol/10% acetone (BDH) for 2 minutes at RT. Using a glass pen a 1 cm^2 square was scored within the smear and the surrounding cells removed gently with a tissue. Slides were air dried once more and pre-equilibrated by overlaying 100 µl of (Sigma) PBS/0.1% tween-20 and incubating the slides in a humidity chamber for 15 minutes at RT. All antibody dilutions were made with 5% donkey serum/PBS/0.1% tween-20 in a final volume of 100 µl and incubations were for one hour in a humidity chamber at RT. Following antibody incubations slides were washed by immersion in a staining trough containing 100 ml of PBS/0.1% tween-20, for 10 minutes at RT, with gentle agitation, this was repeated twice more with buffer changes. Primary whole rabbit serum was diluted 200 fold and affinity purified rabbit sera 50 fold. The secondary rhodamine red™-x-conjugated affinity purified donkey anti-rabbit IgG (Jackson Laboratories) was diluted 200 fold. Following the final antibody incubation and washes slides were overlaid with 100 µl of 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI)/ PBS/0.1% tween-20 for 5 minutes and then

were washed once with PBS prior to mounting. Slides were mounted by overlaying one drop of Vectashield mounting medium (Vector Laboratories) and a 22 mm x 22 mm coverslip. Coverslips were sealed with nail varnish. Immunofluorescence was observed using an Olympus BX-50 microscope. Images were captured with a digital camera and OpenLab software.

7.6.4 Peptide enzyme linked immunosorbent assay

Wells of a 96-well plate (Immunolon4, Dynatech) were coated with either 200 µg of ATS peptide in 100 µl coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.3) or coating buffer alone and incubated overnight at RT while shaking. Wells were washed three times with 200 µl washing buffer (PBS/0.05% tween-20) using a plate washer and all subsequent washing steps were carried out in this manner. Unoccupied protein binding sites were blocked by applying 200 µl of blocking buffer (1% Immunoglobulin free BSA (Sigma)/PBS/0.1% tween-20) per well and incubating the plate at RT for 2 hours. The plate was then washed. Serum and affinity purified immunoglobulin dilutions were prepared with blocking buffer, 100 µl of which were applied to the appropriate wells, and incubated at RT overnight. The plates were washed prior to applying 200 µl of a 2000 fold dilution of HRP-conjugated goat anti-rabbit Ig antibody (DAKO Cytomation). The secondary conjugate was incubated on the plate for 2 hours at RT and washed once more before developing the plate with O-phenylenediamine dihydrochloride (OPD) (Sigma). A solution containing 10 mg of OPD dissolved in 35 ml of 17 mM citric acid/37 mM Na_2HPO_4/29% H_2O_2 was prepared and 100 µl applied and incubated for 30 minutes at RT before stopping the reaction with 25 µl of 2.0 M H_2SO_4. Absorbance was measured at 492 nm.

7.6.5 Biotinylation of anti-ATS immunoglobulins

2 mg of affinity purified anti-ATS immunoglobulins were labelled in 1 ml of PBS (pH 7.2) containing 0.3 mM sulpho-NHS-LC-biotin. Using this molar ratio of IgG to label was anticipated to result in 8 or more biotin molecules pre IgG molecule. The reaction was carried out at RT for 30 minutes. The immunoglobulins were then buffer exchanged into PBS alone and concentrated to 0.04 mg/ml using a 6 ml vivaspin column with a 10 kDa cut-off.

7.7 *P. chabaudi*, infections, isolates and culture

7.7.1 *P. chabaudi* isolates

The AS *P. chabaudi* clone used in this study was cloned by serial dilution of isolates from wild-caught thicket rats of the species *Thamonmys rutilans* (Beale *et al.*, 1978). The CWOpre clone had been recently derived from *Thamnomys rutilans* and, therefore, had recently undergone mosquito transmission (Mackinnon & Read, 1999). CWApre and CWVpre were derived from the CWOpre clone, but had undergone many serial blood passages through C57Bl/6J mice. Blood was passaged on day 12 post-infection for 12 passages. During these passages parasite clone CWVpre was selected for high virulence and CWApre for low virulence. Virulence was defined by weight loss in the mouse (Mackinnon & Read, 1999). CWApre and CWVpre also underwent a further 11-13 passages on days 5-8 post infection for routine purposes prior to this experiment (Mackinnon & Read, 2004a). CWApre, CWOpre and CWVpre were transmitted through *Anopheles stephensi* mosquitoes and the parasites isolated from C57Bl/6J mice infected by these mosquitoes were designated CWApo, CWOpo and CWVpo.

7.7.2 AS infections and collection of parasites

P. chabaudi infected erythrocytes were obtained from infected CBA mice which had been used for mosquito feeds for the purpose of an independent long term transmission experiment. Two uninfected mice and two mice inoculated with AS parasites were sacrificed 7 days post infection. The two AS infected mice and one control mouse had been anaesthetised and exposed to bites from *Anopheles stephensi* mosquitoes prior to sacrifice. For the purpose of the transmission experiment for which these mice were originally infected one control-uninfected mouse was not exposed to mosquito bites. The asexual parasitaemia of the AS infected mice was 40-45 %. Mice were bled at 4 pm and the blood from each mouse cultured for 6 hours to allow late-ring stage parasites to mature. The four cultures were then processed for surface biotinylation and membrane extraction as described below. The integrity of the IEs was confirmed following surface biotinylation by microscopy of Giemsa stained blood smears.

7.7.3 CW infections and collection of parasites

Six groups of four female C57Bl/6J mice aged between 4 and 8 weeks were inoculated with 10^5 parasites per mouse. All mice in each of the 6 groups were infected with one of the following isolates, CWApre, CWOpre, CWVpre, CWApo CWOpo, and CWVpo. At various time points on days 6-7 post-infection, experimental mice and 6 uninfected control mice were sacrificed. Parasitaemias ranged from 15-40%. 2 mice from each group plus a control mouse were sacrificed in the morning and bled immediately to obtain ring stage IEs. The serum was removed following centrifugation of the blood and the ring stage IEs and control samples were processed directly for surface biotinylation and membrane extraction. The remaining 2 mice in each group were bled in the afternoon immediately after sacrifice and the blood from each mouse, following the removal of serum, was cultured overnight to allow late-ring stage parasites to mature into schizonts. Overnight cultures were processed between 8-9 am for surface biotinylation and membrane extraction as described below.

7.7.4 Short-term *in vitro* culture of *P. chabaudi*

Mice were sacrificed using a CO_2 chamber and cardiac blood was then collected using a x gauge needle and 1 ml syringe containing 0.1 ml of citrate saline. Blood was transferred into 1.5 ml eppendorf tubes and cells sedimented by centrifugation at 2 500 rpm in a microcentrifuge. Serum was removed and cells from one mouse cultured with RPMI 1640 medium containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 μg/ml gentamycin and 10% normal mouse serum (heat inactivated at 56 °C for 30 minutes; Sigma). The pH of the medium was adjusted to between 7.2 and 7.4 with 1 M NaOH. Cultures were set up at 5% haematocrit and gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. The length of culture was dependent on the experiment.

7.7.5 Biotinylation of *P. chabaudi* IEs

Cell surface biotinylation of *P. chabaudi* IEs was carried out as described in section 6.3.1. Prior to labelling with biotin, cells were washed with once with RPMI 1640 medium and then PBS.

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Appendix Contents

Publications resulting from this PhD

Sharling, L., Enevold, A., Sowa, K.M., Staalsoe, T., and Arnot, D.E. (2004) Antibodies from malaria-exposed pregnant women recognise trypsin resistant epitopes on the surface of *Plasmodium falciparum*-infected erythrocytes selected for adhesion to chondroitin sulphate A. *Malar J* **3**: 31

(based on the findings of Chapter 2)

Publications resulting from collaborative projects carried out during this PhD

Sowa, M.P.K., Sharling, L., Humphreys, G., Cavanagh, D.R., Gregory, W.F., Fenn, K., Creasey, A.M., and Arnot, D.E. (2004) High throughput imuno-screening of cDNA expression libraries produced by in vitro recombination; exploring the *Plasmodium falciparum* proteome. *Mol Biochem Parasitol* **133**: 267

Appendix 1: Source of enzymes, immunoglobulins and non-standard reagents

Reagent	Supplier	Catalogue Number
FACS		
Goat anti-human IgG	DAKO cytomation	A0473
Rabbit anti- goat FITC	DAKO cytomation	F0250
IMMUNOFLUORESCENCE ASSAY/ELISA		
Rhodamine Red™-X-conjugated AffiPure donkey anti-rabbit IgG (H+L)	Jackson ImmunoResearch	711-295-152
Donkey Serum	Sigma	D 9663
Vectashield Mounting Medium	Vector Labs	H1000
Peroxidase conjugated goat anti-rabbit immunoglobulins	DAKO cytomation	P0448
2DGE		
Immobine dry strips pH 4-7, 7 cm	Amersham	17-6001-10
IPG resolytes, pH 4-7	Amersham	17-600-86
IPG sample cups	Amersham	80-6459-81
PlusOne mini dialysis kit, 8 kDa cut off, 250 µl volume	Amersham	80-6484-13
Ettan IPGphor cup loading strip holder	Amersham	80-6459-62
Immobline DryStrip reswelling tray, 7-18 cm	Amersham	80-6371-84
Ettan IPGphor isoelectric focusing apparatus	Amersham	80-6414-02
WESTERN BLOTTING		
ImmunoPure® Recomb® Protein A-peroxidase conjugated	Pierce Biochemicals	32400
Detergent compatible protein assay kit	Bio-rad Laboratories	500-0114
ImmunoPure Streptavidin-HRP	Pierce/Perbio	21126
QIAGEN-Blocking Reagent	Qiagen	34460
4-12 % NuPAGE Bis-Tris SDS-PAGE gels	Invitrogen	NP0322
3-8 %Tris-Acetate SDS-PAGE gels	Invitrogen	EA03752
Westran® PVDF	Schleicher & Schuell	10413096
Hyperfilm ECL 18 x 24 cm	Amersham	RPN3103K
ECL Plus Western Detection Reagent Kit	Amersam	RPN-2132
Prestained Precision Plus Protein (10-250 kDa)	Bio-rad Laboratories	161-0372

Bugbuster protein extraction reagent	Novagen	70584-4
Benzonase endonuclease	Novagen	70664-3
MALDI-TOF		
P10 C18 Zip Tips	Millipore	ZTC18 S096
Sequencing grade modified porcine trypsin	Promega	V5111
Voyager stainless steel sample plate, 100 positions	Applied Biosystems	V700666
MISCELLANEOUS		
Mouse serum	Sigma	S7273
Trypin-TPCK	Worthington Biochemicals	LS003740
Soya bean trysin inhibitor	Worthington Biochemicals	LS003570
EZ-Link Sulpho-NHS-LC- biotin	Pierce/Perbio	21335
Pronase	Roche	0165921
PBS tablets, pH 7.2	Sigma	P-4417
Complete mini protease inhibitor cocktail tablets	Roche	1836153

Appendix 2: Lysine content of PfEMP1s and rifins

Table 1: Lysine content of 20 PfEMP1 molecules

SAP Number	Protein ID	Lysine number (%)
1	PFA0005w	239 (11.0%)
2	PFA0765c	257(11.7%)
3	PRB0010w	178(10.4%)
4	PFB1055c	259(11.8%)
5	PFD0625c	239(10.5%)
6	PFD1235w	411(11.6%)
7	PFD1245c	247(11.5%)
8	PFD0615c	260(11.8%)
9	PFD0995c	252(11.7%)
10	PFD1000c	267(12.1%)
11	PFD1005c	262(12.0%)
12	PFD1015c	252(11.5%)
13	PFD0630c	235(10.2%)
14	PFD0635c	237(10.2%)
15	PFD0005w	307(11.6%)
16	PFD0020c	394(11.4%)
17	PFE0005w	243(11.0%)
18	MAL6P1.316	323(11.2%)
19	MAL6P1.252	295(12.3%)
20	MAL6P1.1	252(11.3%)

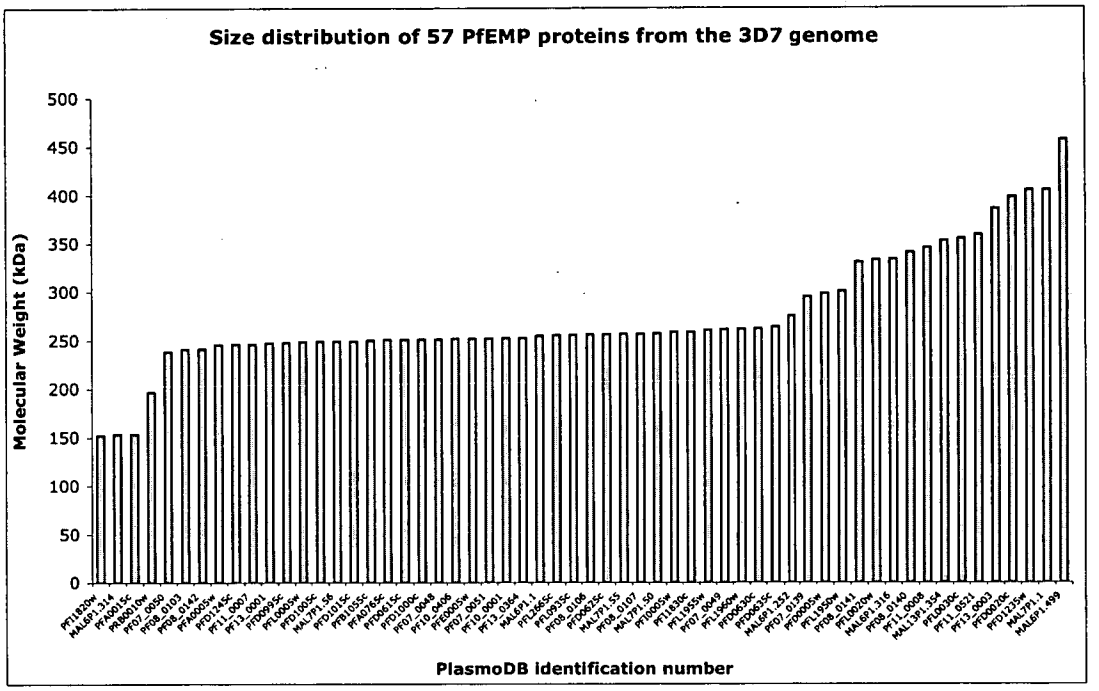
Average K content of 20 PfEMP1s = 10.84

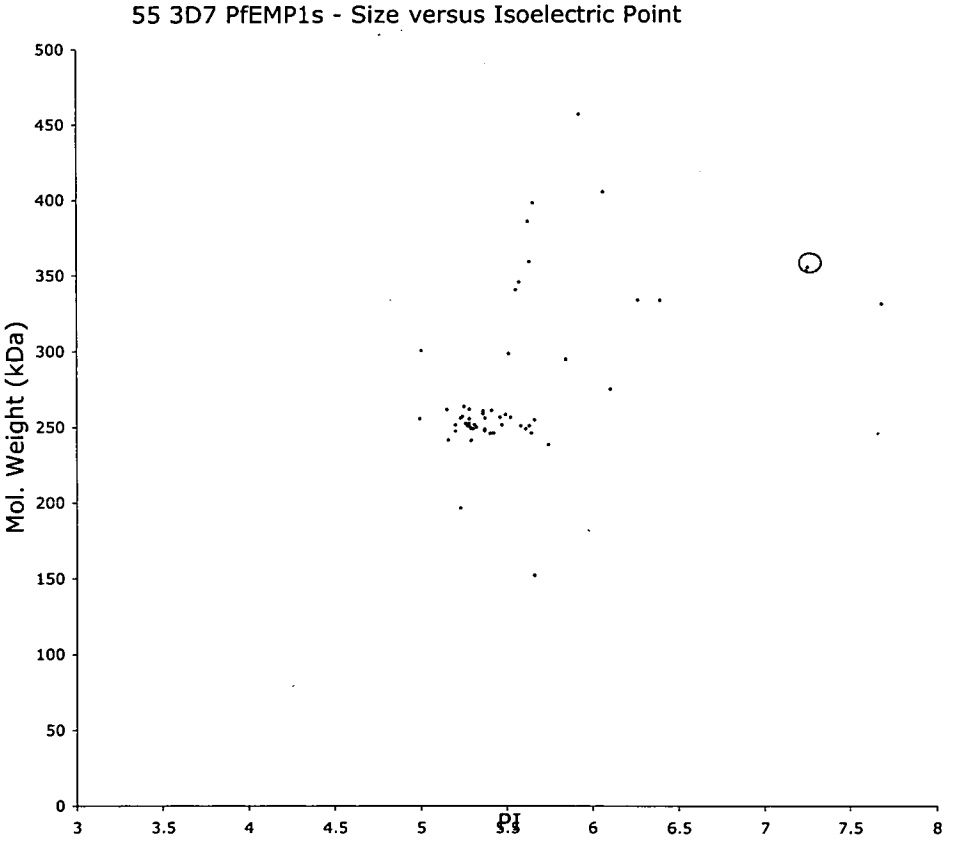
Table 2: Lysine content of 20 rifin molecules

SAP Number	Protein ID	Lysine number (%)
1	PFA0710c	37(11.5%)
2	PFA0740w	37(10.1%)
3	PFA0745w	31(9.2%)
4	PFA0760w	36(9.5%)
5	PFA0010c	31(9.4%)
6	PFA0020w	35(9.4%)
7	PFA0030c	35(9.7%)
8	PFA0040w	38(11.0%)
9	PFA0045c	39(11.1%)
10	PFA0050c	39(10.9%)
11	PFA0080c	36(9.8%)
12	PFA0095c	35(10.2%)
13	PFB0015c	28(8.4%)
14	PFB1005w	33(10.4%)
15	PFB1000w	27(9.6%)
16	PFB0060w	42(11.6%)
17	PFB0055c	32(10.3%)
18	PFB1050w	31(9.5%)
19	PFB1040w	36(10.4%)
20	PFB1035w	32(10.0%)

Average K content of 20 rifins = 10.1%

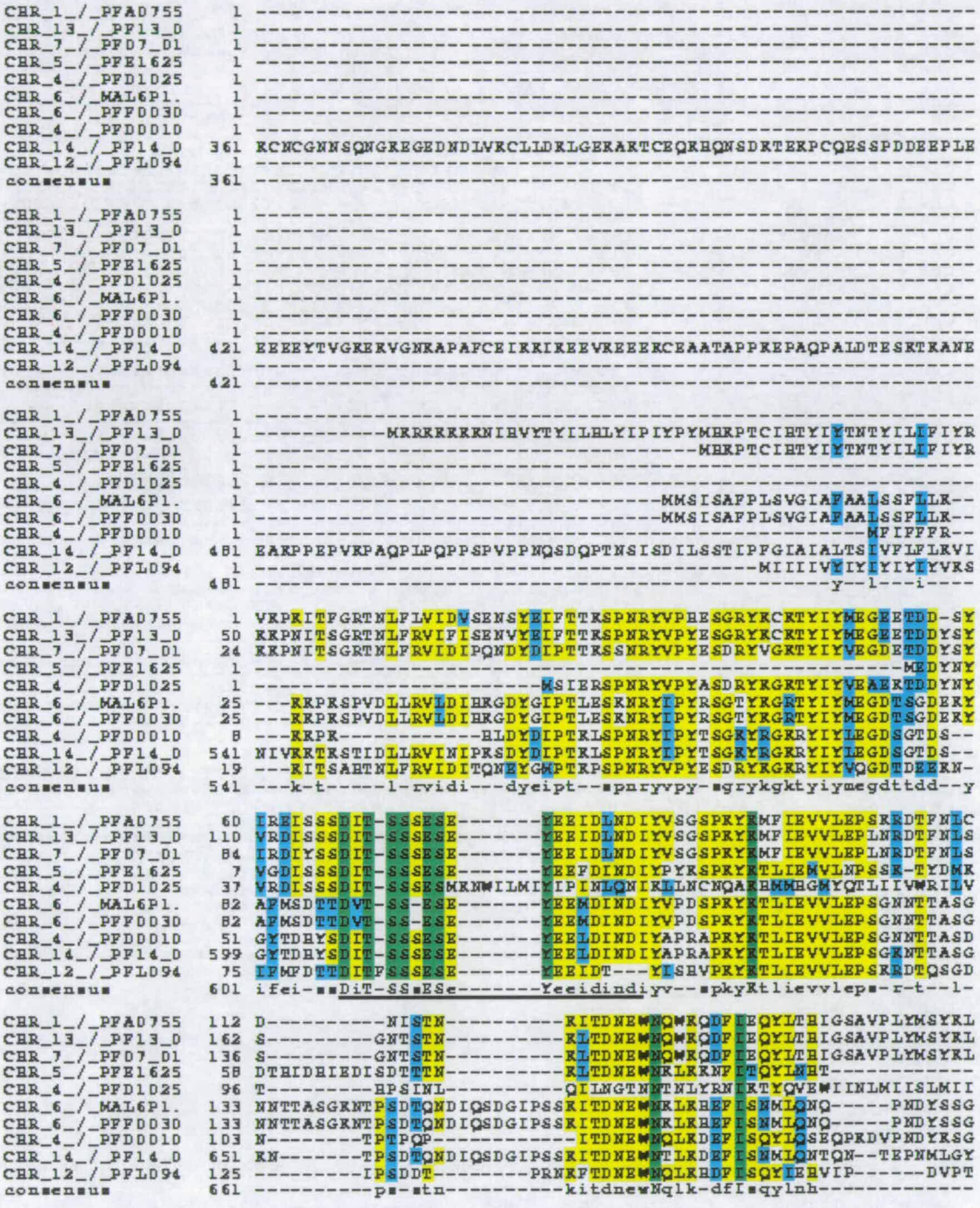
Appendix 3: Size distribution of 3D7 PfEMP1s



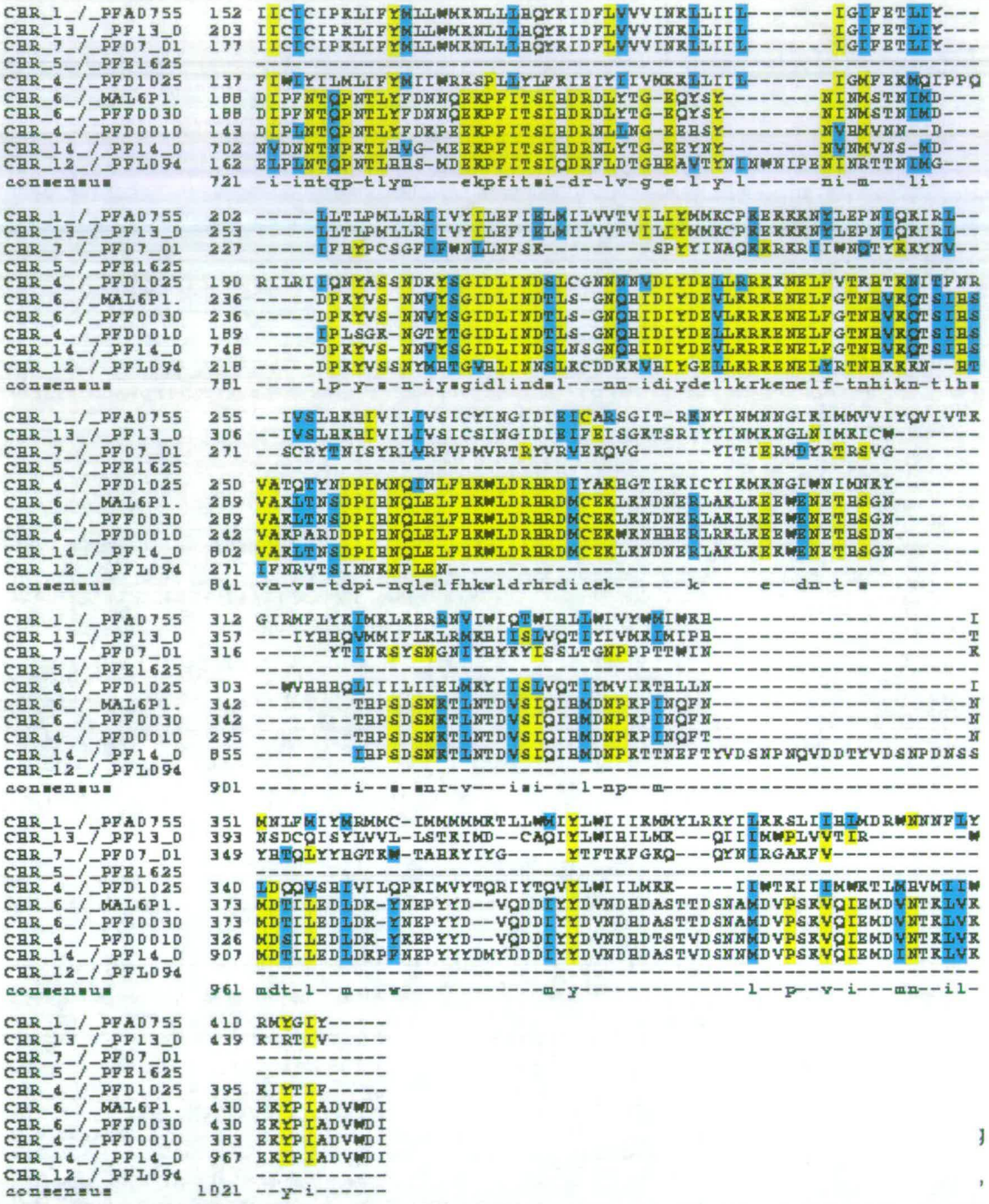


Appendix 4: Alignment of 3D7 truncated and/or pseudo *var* genes encoding the ATS of PfEMP1

Part 1

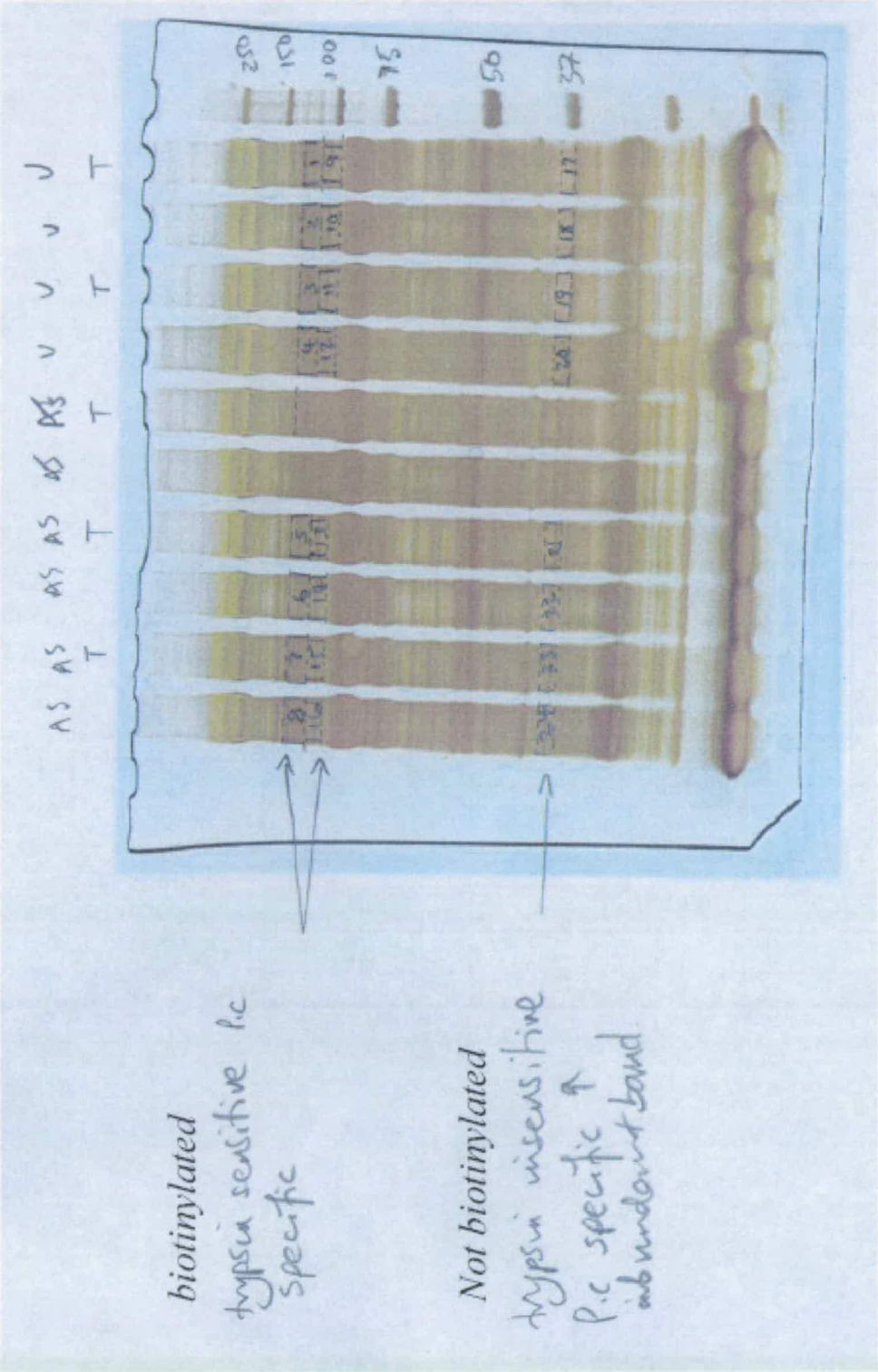


Part 2



where background shading in green highlights completely conserved residues; yellow identical residues, cyan similar residues, white different residues. Boxshade default settings were used for similarity definitions. The immunising peptide sequence for the anti-ATS antibody is the consensus sequence formed and is underlined in the alignment in Part A.

Appendix 5: Gel showing location of gel slices from *P. chabaudi* membrane extracts for MALDI-TOF analysis



Spectra label	Gel slice	Source	Size (kDa)	
ztMS1	3	Uninfected	~120	
ztMS2	4	Uninfected +	~120	
ztMS3	7	*P. chabaudi*	~120	Contains Pc specific, trypsin sensitive surface biotinylated protein
ztMS4	8	*P. Chabaudi* +	~120	
ztMS5	11	Uninfected	~110	
ztMS6	12	Uninfected +	~110	
ztMS7	15	*P. chabaudi*	~110	Contains Pc specific, trypsin sensitive surface biotinylated protein
ztMS8	16	*P. Chabaudi* +	~110	
ztMS9	19	Uninfected	~37	
ztMS10	20	Uninfected +	~37	
ztMS11	23	*P. chabaudi*	~37	Contains abundant Pc specific protein
ztMS12	24	*P. Chabaudi* +	~37	

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Antibodies from malaria-exposed pregnant women recognize trypsin resistant epitopes on the surface of *Plasmodium falciparum*-infected erythrocytes selected for adhesion to chondroitin sulphate A

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Published: 06 September 2004

Malaria Journal 2004, **3**:31 doi:10.1186/1475-2875-3-31

Received: 01 June 2004

Accepted: 06 September 2004

This article is available from: http://www.malariajournal.com/content/3/1/31

Abstract

Background: The ability of *Plasmodium falciparum*-infected erythrocytes to adhere to the microvasculature endothelium is thought to play a causal role in malaria pathogenesis. Cytoadhesion to endothelial receptors is generally found to be highly sensitive to trypsinization of the infected erythrocyte surface. However, several studies have found that parasite adhesion to placental receptors can be markedly less sensitive to trypsin. This study investigates whether chondroitin sulphate A (CSA) binding parasites express trypsin-resistant variant surface antigens (VSA) that bind female-specific antibodies induced as a result of pregnancy associated malaria (PAM).

Methods: Fluorescence activated cell sorting (FACS) was used to measure the levels of adult Scottish and Ghanaian male, and Ghanaian pregnant female plasma immunoglobulin G (IgG) that bind to the surface of infected erythrocytes. *P. falciparum* clone FCR3 cultures were used to assay surface IgG binding before and after selection of the parasite for adhesion to CSA. The effect of proteolytic digestion of parasite erythrocyte surface antigens on surface IgG binding and adhesion to CSA and hyaluronic acid (HA) was also studied.

Results: *P. falciparum* infected erythrocytes selected for adhesion to CSA were found to express trypsin-resistant VSA that are the target of naturally acquired antibodies from pregnant women living in a malaria endemic region of Ghana. However *in vitro* adhesion to CSA and HA was relatively trypsin sensitive. An improved labelling technique for the detection of VSA expressed by CSA binding isolates has also been described.

Conclusion: The VSA expressed by CSA binding *P. falciparum* isolates are currently considered potential targets for a vaccine against PAM. This study identifies discordance between the trypsin sensitivity of CSA binding and surface recognition of CSA selected parasites by serum IgG from malaria exposed pregnant women. Thus, the complete molecular definition of an antigenic *P. falciparum* erythrocyte surface protein that can be used as a malaria in pregnancy vaccine has not yet been achieved.

Background

Rapid clearance of parasitaemia following transfusion of IgG from malaria immune adults to clinically ill recipients illustrates that naturally acquired antibodies have a parasite clearing role in human malaria infection [1-3]. Neither the nature of the protective immune response nor the target antigens and epitopes recognized by infection clearing antibodies are fully understood. Evidence is accumulating to suggest that the acquisition of antibodies binding the VSA on infected erythrocytes plays a major role in the development of age and exposure dependent immunity [4-8]. The evidence for protective anti-VSA responses is particularly strong for the PAM syndrome [9,10].

PAM is characterized by the sequestration of *Plasmodium falciparum* infected erythrocytes in the intervillous spaces of the placenta. Infected erythrocytes adhere to low-sulphated forms of CSA present on the extracellular proteoglycan matrix of syncytiotrophoblasts [11]. *In vitro* selection of infected erythrocytes for adhesion to CSA concomitantly selects for expression of VSA that share characteristics with postnatal placental isolates. Thus plasma antibodies from malaria exposed pregnant, or multigravid women, recognize the VSA of CSA binding parasites (here referred to as VSA_{PAM}). These sera can also block adhesion of CSA-selected infected erythrocytes to CSA *in vitro* [12]. Interestingly, antibodies that bind CSA-selected parasites and block adhesion are not acquired by malaria-exposed males. There is a striking female-specific antibody response recognizing both *in vitro* CSA-selected parasites [12,13] and *P. falciparum* isolates taken from infected placentae at delivery [14-16]. Furthermore, the levels of CSA-adhesion blocking plasma IgG have been shown to increase with adult female parity. Recent immuno-epidemiological studies also show a strong positive correlation between the levels of antibodies that recognize the infected erythrocyte surface[15], the level of CSA-adhesion blocking antibody [17] and positive birth outcomes as measured by birth weight.

PAM is, thus, the clearest example in malaria pathology research of a strong association between infected erythrocyte sequestration and a particular disease syndrome. The VSA recognized by female-specific, parity-dependent antibodies are, therefore, rational and exceptionally interesting candidates for inclusion in an experimental vaccine to protect women against PAM, a major cause of stillbirth, maternal anaemia and low birthweight.

To date, the best characterized VSA is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a polymorphic, high molecular weight membrane protein (200–450 kDa) encoded by the *var* multi-gene family [18-20]. Members of the PfEMP-1 family function as adhesion molecules binding to various host endothelial receptors. They are situated in the knob-like protrusions associated with the parasitized erythrocyte surface.

Since *var* genes encode large extracellular domains rich in lysine and arginine residues, it is not surprising that PfEMP-1 molecules and adhesion to endothelial receptors have been reported to be highly sensitive to trypsin treatment [18,21-24]. Less expected was the finding that parasite adhesion to the placental receptor CSA, when immobilized [25-27] or when cell surface associated [28,29], can be relatively trypsin resistant. This study investigates the protease-sensitivity profile of the VSA_{PAM} expressed by CSA-selected parasite clone FCR3 with regard to recognition by antibodies acquired during PAM and adhesion to placental receptors.

Methods

Parasite isolates

Parasites were maintained in group O erythrocytes under standard conditions [30], using RPMI 1640 medium containing 25 mM HEPES, supplemented with 20 mM glucose, 2 mM glutamine, 25 μg/ml gentamycin and 10% pooled normal human serum. The pH was adjusted to between 7.2 and 7.4 with 1 M NaOH. Culture flasks at 5% haematocrit were gassed with 96% nitrogen, 3% carbon dioxide and 1% oxygen. The laboratory clone FCR3 originates from peripheral blood collected in the Gambia. FCR3CSA was obtained from the Malaria Research and Reference Reagent Resource Centre (ATCC) [31], and was confirmed, using genetic markers to be identical to the laboratory clone FCR3 kept in the original W.H.O. strain registry collection in Edinburgh (D. Walliker, pers. comm.). CSA binding was maintained by panning late stage infected erythrocytes fortnightly on bovine tracheal CSA (10 μg/ml) (Sigma) immobilized on polystyrene Petri dishes (Falcon), as previously described [26]. Prior to protease treatment and analysis by flow cytometry, cultures were synchronized by sorbitol treatment to obtain cultures enriched for late stage parasites.

Plasma donors

Serum samples from 20 men living in a malaria endemic region of Ghana were pooled to produce the male serum pool. Serum samples collected at the time of birth from the placentas of 15 women living in a malaria endemic region of Ghana were pooled to produce the pregnant female serum pool. This pool included five primigravidae, nine secundigravidae and one multigravid woman. Serum samples from six Scottish malaria naïve individuals were pooled and used as a control.

Protease treatment

Protease treatment of infected erythrocytes was carried out as previously described [26]. Briefly, samples containing 3 × 10^6 cells from sorbitol treated late stage cultures of 8–10

% parasitaemia were washed twice with phosphate-buffered saline (PBS) and then incubated with the appropriate concentration of trypsin-TPCK (Worthington Biochemicals) or pronase (Boehringer-Mannheim) in a final volume of 1.0 ml in PBS, for 10 minutes at 37°C. The reaction was terminated either by adding soybean trypsin inhibitor (Worthington Biochemicals) to a final concentration of 1 mg/ml or by adding 10% human serum. Cells were washed twice with PBS before further use.

Analysis of VSA specific antibodies by flow cytometry

Flow cytometry was used to measure the levels of plasma IgG binding to the VSA of late stage parasites essentially following the method previously described by Staalsoe *et al* [13,32]. 3 × 10^6 cells form late stage *P. falciparum* cultures of 8–10 % parasitaemia were washed twice with PBS. Cells were incubated sequentially with plasma antibodies diluted 1:20 in PBS, goat anti-human IgG diluted 1:200 in PBS (Dako) and fluorescein isothiocyante (FITC)-conjugated rabbit anti-goat (Dako) diluted 1:25 in PBS. All incubations were in a total volume of 100 µl for 30 minutes at room temperature and were followed by two washes with 1 ml of PBS. Samples were analysed immediately on a FACSCAN apparatus (Becton-Dickinson). FITC fluorescence due to cell surface antibody recognition was determined for 5000–10000 ethidium bromide gated infected erythrocytes.

Modified labeling procedure for FACS analysis

In order to circumvent the non-specific labeling of the VSA by the tertiary antibody, new reagents have been introduced. The procedure follows the method detailed above with the following modifications. A biotinylated rabbit anti-human IgG antibody (DAKO) was used diluted 1:25 to replace the secondary antibody. In the place of a tertiary antibody, FITC-conjugated streptavidin (DAKO) was used at a 1:2000 dilution. In these experiments the control sera was a pool of malaria naïve Danish volunteer serum.

Binding assays

Human umbilical cord hyaluronic acid (Sigma) and bovine trachea CSA (Sigma) were used at a concentration of 10 µg/ml in PBS (pH 7.2). 20 µl of each receptor was spotted in triplicate onto 5 cm diameter petri dishes (Falcon). Receptors were adsorbed onto the plastic petri dishes overnight at 4°C. 10 µg/ml BSA in PBS was similarly adsorbed as a negative control. Plates were then blocked by removing the receptor solution and adding 20 µl of 2% BSA in PBS. Following the removal of this blocking solution late stage parasites, suspended in 2 ml of complete RPMI-HEPES medium (8–10% parasitaemia, 5% haematocrit), were added to the petri dish. Parasites were incubated with the immobilized receptor for 60 minutes at 37°C with occasional agitation. Unbound cells were removed by four gentle washes with incomplete RPMI-HEPES medium; bound cells were fixed with 0.5% (v/v) glutaraldehyde in PBS for 10 minutes and Giesma Stained. Bound cells were counted by light microscopy. Protease treatment of intact cells was carried out as described above.

Statistical analysis

Statistical analyses were performed using Analyses of Variance in Minitab 13.30 (Minitab Inc.), using protease, protease concentration and serum pool as explanatory variables. Statistical models were tested for homogeneity of variance and normality of error distributions. Where possible, maximal models with interactions between these variables were fitted first, after which models were minimized by removing nonsignificant ($p > 0.05$) terms.

Results

Concomitant selection of a trypsin-resistant VSA following parasite selection for CSA adhesion

It was first established that selection of clone FCR3 for adhesion to CSA resulted in the concomitant selection for VSA specifically recognized by plasma IgG from malaria exposed Ghanaian pregnant women (IgG*preg*) (figure 1). However there was no increase in the binding of IgG from a pool of plasma from malaria exposed Ghanaian men (IgG*male*). The unselected FCR3 clone expressed VSA that were equally well recognized by antibodies in the IgG*male* and IgG*preg* serum pools (figure 1). These interactions between serum antibody binding and selection for CSA adhesion were highly significant ($F_{2,24}$ 9.5, P = 0.001).

The trypsin sensitivity of this VSA/IgG binding interaction and of parasite adhesion to CSA was then measured. Parasitized erythrocyte surface trypsinization at a concentration of 0.1 mg/ml showed that the IgG*preg* binding of FCR3CSA was significantly more trypsin-resistant than was binding of the same serum to the unselected clone (figure 2A &2B; $F_{1,4}$16.4, p = 0.015). Although the mean surface fluorescence due to the IgG*preg* binding of FCR3CSA was slightly reduced by 0.1 mg/ml trypsin this reduction was not significant (figure 2A; $F_{1,2}$ 11.3, p = 0.078). The effect of 0.1 mg/ml trypsin on VSA recognition by IgG*male* and IgG*control* was comparable before and after CSA selection of the parasite (figure 2).

The effect of a 10-fold higher trypsin concentration and the effect of the non-specific protease, pronase, on IgG recognition of FCR3CSA was also determined. Trypsinization with 1 mg/ml did not significantly reduce the mean surface fluorescence due to IgG*preg* binding to FCR3CSA (figure 3; $F_{1,4}$ 0.35, p = 0.587). However, treatment of the intact infected erythrocyte with 0.1 mg/ml pronase did significantly reduce IgG*preg* recognition of FCR3CSA (figure 3). Pronase treatment also significantly reduced

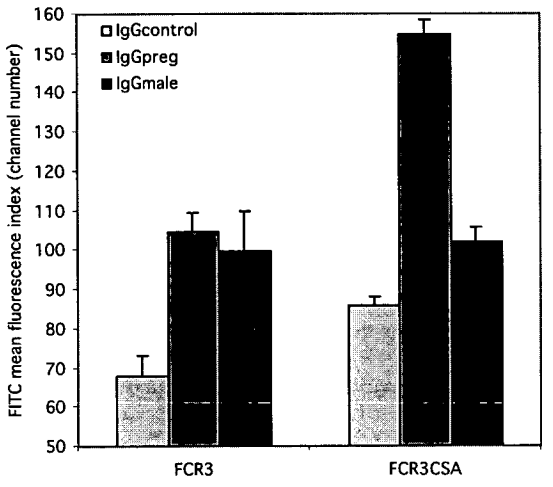

Figure 1
IgG recognition profiles of parasite clone FCR3 before and after selection for adhesion to CSA. Following selection of parasite clone FCR3 for adhesion to CSA the expression of variant surface antigens was investigated using FACS. 5000–10000 late stage parasites were gated using ethidium bromide and FITC fluorescence due to serum IgG binding was measured. Serum samples from six Scottish malaria naïve individuals were pooled and used as a control (IgG*control*). Sera from 20 Ghanaian men were pooled to produce the malaria exposed male serum pool (IgG*male*). Sera collected at the time of birth from the placentas of 15 Ghanaian women were pooled to produce the malaria exposed pregnant female serum pool (IgG*preg*). The bar chart shows mean and standard error of the means for five independent experiments.

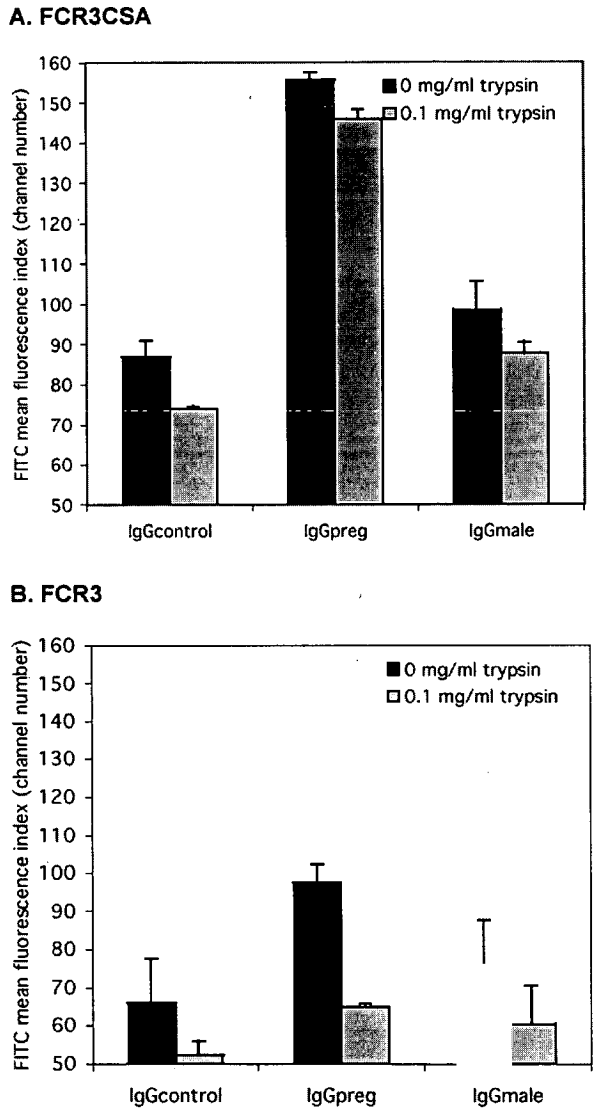

Figure 2
Serum IgG from malaria exposed pregnant women recognises trypsin-resistant surface epitopes. Intact infected erythrocytes were treated with 0.1 mg/ml trypsin prior to FACS analysis. Panels A and B show serum IgG binding to the surface of FCR3CSA and FCR3 infected erythrocytes respectively. Serum pools are the same as those described in Figure 1. The bar chart shows mean and standard error of the means for two independent experiments.

binding of the IgG*male* and IgG*control* serum pools (figure 3; $F_{4,18}3.1$, $p = 0.041$).

Surprisingly, IgG*control* binding to the infected erythrocyte surface increased following CSA selection of the parasite (figure 1); however, this non-immune recognition was found to be significantly more trypsin sensitive than IgGpreg recognition (figure 3; $F_{4,18}3.11$, $p = 0.041$). This indicates that the epitopes recognized by the IgG*control* serum pool and the epitopes recognized by the IgG*preg* serum pool are distinct entities. An increase in apparent non-immune immunoglobulin binding to the infected erythrocyte surface has been observed for a number of parasite clones after selection for adhesion to CSA (data not shown). The source of this background labelling of FCR3CSA by naïve sera was found to be due to non-specific binding by the FITC-labelled tertiary rabbit anti-goat antibody. By using the modified antibody labelling procedure, which employs a biotin-labelled secondary antibody and FITC-labelled streptavidin, binding of malaria

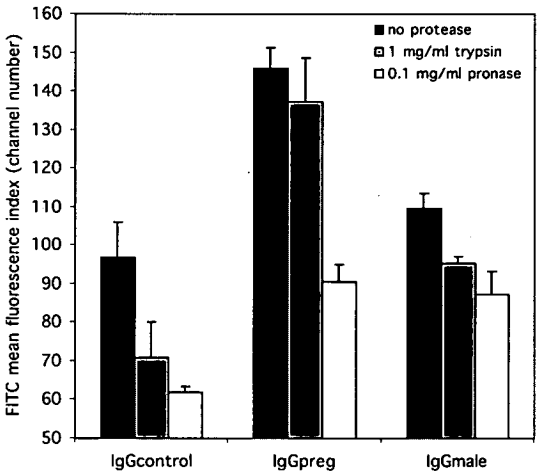

Figure 3
FCR3CSA expresses surface antigens exhibiting differential protease sensitivity. Intact infected erythrocytes were treated with 1.0 mg/ml trypsin or 0.1 mg/ml pronase prior to FACS analysis. Serum pools are the same as those described in Figure 1. The bar chart shows mean and standard error of the means for three independent experiments.

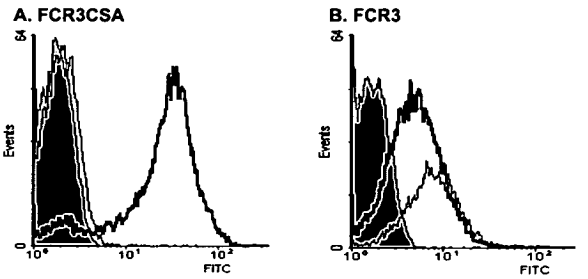

Figure 4
A modified antibody labelling procedure for FACS analysis of CSA selected parasites. In order to circumvent the non-specific labelling of FCR3CSA VSA seen when using the FITC rabbit anti-goat tertiary antibody, a biotinylated rabbit anti-human antibody in combination with FITC-conjugated streptavidin was used. Panels A and B show FCR3CSA and FCR3 infected erythrocytes respectively. In these experiments the control serum was a pool of sera from malaria naïve Danish volunteers, here shown as a solid grey histogram. The IgG*male* serum pool is shown as a lightweight line and the IgG*preg* serum pool as a heavyweight line.

naive IgG to FCR3CSA (mean fluorescence index = 16) was comparable to the unselected parasite (mean fluorescence index = 17). Thus the recognition of VSA_{PAM} by malaria naive IgG was abolished (figure 4).

Discordance between the protease sensitivity of the CSA adhesion interaction and IgG binding

Following the identification of trypsin-resistant epitopes that appear to be concomitantly selected with CSA adhesion, the trypsin sensitivity of CSA adhesion itself was determined. FCR3CSA binding to immobilised CSA was markedly more sensitive to trypsin than IgG*preg* recognition of the infected erythrocyte surface (figure 5). Parasite adhesion was reduced by 81% and 91% following treatment with 0.1 mg/ml trypsin and 1 mg/ml trypsin respectively (figure 5). A trypsin concentration of 1 mg/ml reduced binding as efficiently as 0.1 mg/ml pronase, and although 0.1 mg/ml pronase significantly reduced cell surface fluorescence due to IgG*preg* antibody binding, 1 mg/ml trypsin had no significant effect on IgG*preg* antibody binding. There is, thus, significant discordance between the high trypsin sensitivity of CSA adhesion and the relatively trypsin-insensitive binding of IgG*preg* serum antibodies to the infected erythrocyte surface ($F_{1,8}$ 14.4, p = 0.005).

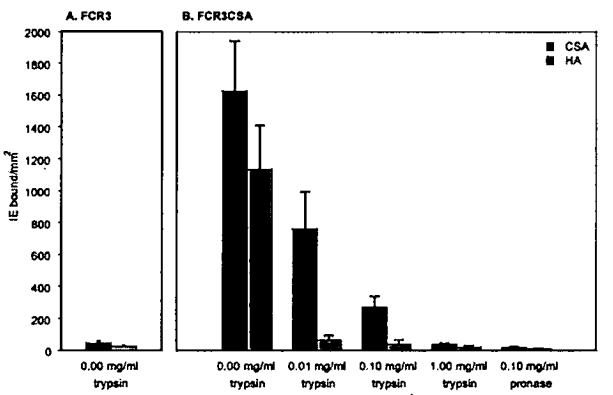

Figure 5
The effect of increasing concentrations of trypsin on parasite adhesion to immobilised CSA and HA. Parasite adhesion to 10 μg/ml human umbilical cord HA and bovine trachea CSA, adsorbed onto the plastic petri dishes, was determined following protease treatment of the intact infected erythrocyte. Bound cells were Giemsa stained and counted by light microscopy. Panels A and B show receptor binding for FCR3 and FCR3CSA infected erythrocytes respectively. The bar chart shows mean and standard error of the means for three independent experiments.

Human umbilical cord hyaluronic acid (HA) was also included in these assays to investigate the binding capacity of the CSA selected clone with respect to this receptor. FCR3CSA was found to bind both HA and CSA, although binding to HA was significantly lower (figure 5B; $F_{3,19}$ 20.44, $p < 0.001$), at 71% that observed for CSA. Interestingly, as has previously been shown for other *P. falciparum* isolates [27], the trypsin-sensitivity of parasite adhesion to HA and CSA differed at low trypsin concentrations (0.01 mg/ml) (figure 5B; $F_{1,8}$ 7.7, $p = 0.024$). Parasite adhesion to hyaluronic acid was found to be more sensitive to trypsinization than adhesion to CSA.

Discussion

The acquisition of antibodies to the surface of placental isolates correlates with protection from malaria in pregnancy and the targets of these antibodies are potential vaccine candidates [13,15]. Two variants of the well characterized VSA, PfEMP1, have been shown to have distinct CSA-binding domains [29,33] and antibodies raised against these domains have been reported to recognize the infected erythrocyte surface [34] and in some cases block parasite adhesion [35,36]. However, in a recent study of *var* gene transcription in CSA-selected clones, a third potential CSA-binding PfEMP1 (var2csa) was identified. Var2csa is predicted to possess distinctly different DBL domains and appears to be the major *var* expressed by CSA-selected parasites that are recognized by parity-dependent antibodies [14]. Proteomic analysis of CSA-selected parasites has also identified four additional potential CSA binding PfEMP1 molecules [37]. The molecular identity of the surface antigens expressed at the infected erythrocyte surface remains unclear [38]. However, the differential protease sensitivity of the epitopes described here would allow treatment of the infected erythrocyte surface with trypsin thereby simplifying the surface complexity, thus, potentially making proteomic approaches more straightforward.

Although PfEMP1-mediated CSA adhesion appears to play a role in placental malaria the molecular interactions triggering this syndrome are more complex than initially thought. Several studies implicate additional receptors and binding phenotypes of placental parasites, such as non-immune IgM [39], hyaluronic acid [25,27,40] and non-immune IgG [41]. CSA-binding laboratory clones and placental CSA binding isolates also appear to express some parasite encoded surface antigens other than PfEMP1, such as ring surface proteins 1 and 2 (RSP 1 and 2) [42]. Interestingly, a gene 'knock-out' of the CSA binding *var* (FCR3*var*CSA) in parasite clone FCR3 abolishes CSA binding, but the 'knock-out' parasites still bind the syncytio-trophoblast of *ex vivo* placental cryosections [43]. Monoclonal antibodies raised against the CSA binding DBLγ domain also show this domain to be sensitive to surface proteolysis using relatively low trypsin concentrations (100 μg/ml) [34]. It is certainly possible that the trypsin-resistant VSA described here are not of the PfEMP1/CSA binding type.

Surface epitopes of the FCR3CSA parasite are both highly resistant to trypsin and are recognized by antibodies from malaria-exposed pregnant women. This agrees with a number of studies that have found parasite adhesion to placental receptors to be resistant to surprisingly high trypsin concentrations. However, binding assays with the parasite clone used in this study showed CSA and HA adhesion to be relatively trypsin-sensitive. This is also compatible with the results of Beeson and his colleagues who demonstrated trypsin-resistant CSA adhesion to be a clone dependent phenomenon [27]. Another recent study by the same group showed sera that is strongly reactive to the surface of CSA selected parasites is not always capable of inhibiting CSA adhesion [44]. Thus this study supports the view that erythrocyte surface epitopes distinct from those involved in CSA adhesion may be targets of the antibodies acquired during PAM and suggests that these two epitopes could be on different molecules. One further implication for vaccine development is that a candidate vaccine raising only CSA adhesion blocking antibodies may not mimic protective surface reactive gender-specific immune responses.

Conclusion

This study supports the view that major differences exist between VSA_{PAM} and previously characterized VSA. Apart from being recognized only by female sera in a parity-dependent manner, VSA_{PAM} show other distinct characteristics such as; i) VSA_{PAM} rarely form infected erythrocyte rosettes when compared to CD36 binding VSA [27,45], ii) with the exception of rosetting isolates, non-immune IgM binding is a phenomenon only seen with CSA-binding clones [39], iii) VSA_{PAM} do not generally mediate adhesion to CD36 [27,46], and iv) VSA_{PAM} mediated adhesion to the placenta and CSA can be resistant to concentrations of trypsin known to remove most PfEMP1 molecules from the infected cell surface. In combination with the findings of this study, these distinct properties of VSA_{PAM} suggest the involvement of either an unusually protease-resistant PfEMP1 structure, such has been shown to exist in the A4tres PfEMP1 molecule [47] or an alternative class of VSA in placental adhesion. The differential protease sensitivity exhibited by VSA_{PAM} can be exploited in comparative proteomic analysis to aid in the identification of the molecules whose phenotype is described here.

List of abbreviations

TPCK – L-(tosylamido-2-phenyl) ethyl chloromethyl ketone, CSA – chondroitin sulphate A, PfEMP1 – *P. falciparum* erythrocyte protein 1, PAM – Pregnancy associated

malaria, VSA – variant surface antigens, VSA_{PAM} – variant surface antigens expressed by placental or CSA binding parasites, IgG – immunoglobulin G, DBL-γ-Duffy like binding domain-gamma, FITC – fluorescein isothiocyanate.

Authors' contributions

LS conceived of the study, maintained *P. falciparum* culture, performed FACS analysis and binding assays, AE performed the modified labelling FACS experiments and participated in manuscript preparation, MS participated in the design of the study, TS helped develop some methodologies used in this study. DA helped conceive and fund the study and write the manuscript. All authors read and approved the final manuscript.

Declaration

None declared.

Acknowledgements

LS is sponsored by a Wellcome Trust Studentship. This work was also supported by EU PAMVAC contract QLK2-CT-2001-01302. We thank Andrew Sanderson for his help with performing the FACS analysis, David Walliker for his help in strain genotyping, Mike Ofori, Maja Lundquist, Edmund Nii-Laryea Browne and Victoria Bam for serum collection. Jaap de Roode helped with statistical analysis and Alex Rowe, Lars Hviid and anonymous reviewers gave helpful comments and suggestions on an earlier version of this manuscript.

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Molecular & Biochemical Parasitology 133 (2004) 267–274
MOLECULAR & BIOCHEMICAL PARASITOLOGY

High throughput immuno-screening of cDNA expression libraries produced by in vitro recombination; exploring the *Plasmodium falciparum* proteome

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Received 15 June 2003; received in revised form 29 October 2003; accepted 29 October 2003

Abstract

Improved *Plasmodium falciparum* cDNA expression libraries were constructed by combining mRNA oligo-capping with in vitro recombination and directional cloning of cDNA inserts into a plasmid vector that expresses sequences as thioredoxin fusion proteins. A novel procedure has also been developed for the rapid identification of seropositive clones on high-density filters, using direct labelling of *P. falciparum* immune immunoglobulin with fluorescein isothiocynate (FITC). This approach combines the advantages of recombination-assisted cDNA cloning with high throughput, non-radioactive serological screening of expression libraries. Production of replicate colony matrices allows the identification of antigens recognised by different pools with different specificities from residents of a malaria endemic region. Analyses of DNA sequences derived from sero-reactive colonies indicate that this is an effective method for producing recombinant proteins that react with antibodies from malaria-exposed individuals. This approach permits the systematic construction of a database of antigenic proteins recognised by sera from malaria-exposed individuals.

Keywords: Plasmodium falciparum; cDNA; In vitro recombination; Protein macro-array

1. Introduction

Serological screening of expression libraries from microbial pathogens has a revived role in the exploitation of genomic data for development of novel drugs and recombinant vaccines. Defining the portion of the pathogen proteome recognised by the host immune system—its 'immunome'—is a necessary step in identifying pathogen epitopes that stimulate protective immunity. However, systematic mapping of the immunome of complex parasites requires faster and more efficient methods than those originally developed for serological screening of expression libraries [1–3].

This approach simplifies the construction of *P. falciparum* cDNA expression libraries by using recombination cloning (RC) [4], resulting in libraries with better representation of mRNA species than conventional restriction-ligation dependent cloning [5]. We have combined production of cDNA from "oligo-capped" RNA [6] with PCR, to amplify cDNA and introduce the 5′ *att*B1 and 3′ *att*B2 sites necessary for site-specific recombination of the cDNA into a Gateway™ donor vector. This oligo-capping/PCR RC-method is an alternative to the adaptor ligation method for recombination cloning developed by Ohara and Temple [5].

We have combined these library construction methods with a novel antigen detection process. Purified immunoglobulins from malaria-exposed donors were directly labelled with fluorescein isothiocyanate (FITC) and used to screen the expression library derived from *P. falciparum* cDNA. Like radio-iodination, direct FITC labelling of antibodies and their subsequent detection is more sensitive than secondary-antibody based detection, but FITC labelled antibodies have the advantage of being cheaper to produce, do not require radio-chemical handling and disposal and have a

Abbreviations: FITC, fluorescein isothiocyanate; CSA, chondroitin sulphate A; Ig, immunogobulins; TBS, Tris buffered saline; TBS-TT, Tris buffered saline with 0.2% Tween 20 and 0.05% Triton-X 100; PMSF, Phenylmethylsulphonylfluoride

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doi:10.1016/j.molbiopara.2003.10.013

longer useful 'shelf life'. By pre-incubating this library with unlabelled antibodies from a control pool with different or shared specificities, the library can then be differentially screened with FITC-labelled antibodies for specificities unique to the labelled antibody or serum pool [7,8].

2. Materials and methods

2.1. Parasites

P. falciparum SD2H3/5-CSA was cloned by limiting dilution from SD2H3, a parasite isolate from a Sudanese female and cultured using standard methods [9]. The parasites were kept under chondroitin sulphate A (CSA) selection in order to maintain the CSA adhesion phenotype [10]. A subset of antibodies on infected red blood cells containing these parasites are recognised by adult female sera from malaria endemic areas, but not by endemic adult male sera. This sex-dependent serum recognition profile has been described in *P. falciparum* isolates implicated in pregnancy-associated malaria [11].

2.2. RNA isolation, oligo-capping and reverse transcription

Total RNA was isolated from 100 μl of the SD2H3/5-CSA parasite pellet obtained after centrifugation of saponin lysed asynchronous parasites (5 ml culture) using the Trizol extraction method (Gibco). Extracted total RNA was resuspended in RNA Storage Solution (Ambion) before DNAse 1 treatment (Ambion). Aliquots of the isolated RNA were checked for genomic DNA contamination using PCR [12]. cDNA was synthesised immediately after RNA isolation using a tagged oligo $dT_{(18)}$. Oligo-capping was carried out as described [13,14] using a synthetic RNA primer with the sequence: 5′CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA3′. Total RNA was dephosphorylated and the methyl cap removed using tobacco acid pyrophosphatase. The synthetic RNA primer was ligated onto the de-capped mRNA using RNA ligase. Reverse transcription was carried out using a tagged oligo dT primer (GeneRacer, Invitrogen) and SuperScript II (Gibco).

2.3. Second strand synthesis and attachment of recombination sites

Introduction of the *att*B1 and *att*B2 recombination sites was carried out during second strand synthesis by PCR, using a specific forward primer, *att*B1/RNA oligo with the sequence: 5′*GGGGACAAGTTTGTACAAAAAAGCAGGC*TTGACATGGACTGAAGGAGTA3′ and a specific reverse primer, the *att*B2/tagged oligo dT with the sequence: 5′*GGGGACCACTTTGTACAAGAAAGCTGGGT*CGGCATGACAGTGTTTTTTTTTT3′. The *att* sites are italicized. 50 μl PCR reactions were prepared and the custom synthesised primers added to a final concentration of 1 μM. The reactions were cycled as follows: 1 cycle at 94 °C for 2 min, followed by 15 cycles at 94 °C for 30 s, 50 °C for 30 s and 68 °C for 10 min. A final extension step was carried out at 68 °C for 12 min. PCR products of less than 300 base pairs were removed by PEG 8000 (Gibco) precipitation of the reaction mix. Double stranded cDNA was fractionated on agarose gels and the purified individual fractions used to make size-fractionated "entry" libraries.

2.4. Generation of the P. falciparum cDNA entry library

A tenth of the volume of the PCR amplification reaction was incubated with 250 ng of the 'donor' vector, pDONR-222 (a gift of Dr. Mark Smith, Invitrogen), BP Clonase enzyme and reaction buffer (Gateway Cloning Technology, Invitrogen). The reaction mix was incubated at 25 °C for 24 h before precipitation with 0.1 volume of 3 M sodium acetate pH 5.2, 0.1 volume of glycogen and 2.5 volumes of ethanol. The pellet was re-suspended in 1× Tris–EDTA and electro-competent Top10 *Escherichia coli* cells (Invitrogen) were transformed with 1 μl of the mixture. The transformed cells were rescued in SOC medium at 30 °C for 1 h and immediately spread on LB agar plates containing 50 μg ml^{-1} kanamycin. Transformants were incubated overnight at 30 °C. Colonies were picked, used in the inoculation of LB containing 50 μg ml^{-1} kanamycin, grown overnight at 30 °C and insert sizes checked by PCR. The entry library with an insert range of 0.3–3 kb was used in the production of the expression library.

2.5. Generation of the cDNA expression library

The library was constructed in the plasmid pBAD-DEST49 (Gateway Destination Vector, Invitrogen) a 6.2 kb arabinose-inducible expression vector that fuses an N-terminal thioredoxin domain onto the recombinant protein. The vector also fuses a six-histidine polypeptide as a 'tag' to the C-terminal of the fusion protein to facilitate detection and purification. 10% of the DNA from the entry library was incubated with 300 ng of pBAD-DEST49 and LR Clonase was added following the manufacturers protocol (Invitrogen). The reaction mix was incubated at 25 °C for 24 h, precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, 0.1 volume of glycogen and 2.5 volumes of ethanol. After centrifugation, the pellet was re-suspended in 12 μl of 1 × Tris-EDTA and 1.5 μl of the mix used to transform 100 μl of electro-competent Top10 *E. coli* (Invitrogen). Transformed cells were rescued in 1 ml of SOC medium and grown with aeration at 30 °C for 1 h before plating aliquots on LB agar plates supplemented with 1% glucose and 300 μg ml^{-1} ampicillin overnight at 30 °C. Transformants were subsequently grown in either liquid or semi-solid medium with reduced ampicillin (100 μg ml^{-1}).

2.6. Labelling of antibodies

Two different pools of sera were obtained, with informed consent, from 25 males and 25 females between the ages of

20–39 residents in Ejisu, a malaria hyper-endemic region in Ghana. Pooled malaria naïve serum (used as a negative control) was obtained from The Scottish Blood Transfusion Centre, Edinburgh, Scotland. Serum immunogobulins (Ig) were affinity purified on Protein L columns (Pierce), to select IgA, D, E and M, as well as the four subclasses of IgG. Eluted antibodies were dialysed against 0.15 M NaCl overnight at 4 °C, prior to FITC labelling. FITC in 0.5 M sodium carbonate-bicarbonate buffer pH 9.0 was added to the purified Ig to a final concentration of 0.05 mg of FITC/mg of protein and incubated at room temperature for 2 h with gentle agitation. Unbound FITC was removed from the Ig solution by overnight dialysis against several changes of PBS at 4 °C.

2.7. Bacterial colony growth and induction of protein expression on high-density filters

Six thousand, nine hundred and twelve colonies were picked into seventy-two 96-well plates containing LB medium supplemented with 1% glucose, 8% glycerol and 100 μg ml^{-1} ampicillin and grown overnight at 30 °C prior to arraying on filters. Replicate bacterial colony arrays were prepared by cutting High Bond-C Extra filters (Amersham) to size (23 cm × 23 cm). The replicate filters were inked using filter sterilised black ink, prior to robotically spotting bacterial cultures onto the filters using a PBA Flexys Gridder and Replicator. The order of colony spotting from the 96-well plates was based on a programmed pattern, with each 25 spot square containing 12 duplicated colonies arranged in such a way that no duplicates border each other (a 'non-contiguous array'). One blank spot was left in each square to facilitate orientation. This arraying pattern produced six 'patches', each patch containing duplicated colonies from twelve 96-well plates. The colonies were grown by placing the filters on LB agar supplemented with 1% glucose and 100 μg ml^{-1} ampicillin, in 24.5 cm × 24.5 cm bioassay dishes (Nunc) overnight at 30 °C. The arrayed filters were transferred from the 1% glucose and 100 μg ml^{-1} ampicillin supplemented LB agar after overnight growth, onto LB agar plates supplemented with 0.2% arabinose and 100 μg ml^{-1} ampicillin, for induction of fusion protein expression at 37 °C for 4 h.

2.8. Preparation of induced colony arrays for screening with antibodies

After 4 h of induction, the arrayed colonies were lysed in situ by placing the filters onto Whatman 3 M filter paper pre-soaked with BugBuster HT lysis mix (Novagen), supplemented with 0.1 mM PMSF for twenty min at room temperature. Lysis was completed by incubating each filter in 50 ml of Lysis Buffer (50 mM Tris–HCl pH 7.5, 3% BSA, 0.15 M NaCl, 5 mM $MgCl_2$, 0.1 mM PMSF, 40 μg ml^{-1} lysozyme and 300 units L^{-1} benzonase) in different bioassay dishes for 3 h at room temperature with gentle agitation. Filters were subsequently blocked overnight at 4 °C in 3% BSA and 0.1 mM PMSF in TBS-TT with gentle agitation in individual bioassay dishes.

2.9. Detection of antibody positive clones

After blocking, filters were washed four times for 15 min per wash in approximately 100 ml per filter of Tris buffered saline (TBS) with 0.2% Tween 20 and 0.05% Triton-X 100 (TBS-TT), followed by two washes in 100 ml/filter of TBS to remove residual detergent. To detect colonies producing recombinant protein, one of the replicate filters was probed with HRP-conjugated anti-His tag antibody (Penta His, Qiagen, at a 1:2000 dilution of the supplied antibody in the blocking buffer provided by the manufacturer for 1 h at room temperature). Positive colonies were detected using the ECL Plus kit (Amersham).

To detect malaria seropositive clones, a second replicate filter was incubated with FITC-labelled total immunoglobulin from a pool of highly malaria antigen-reactive sera from Ghanaian adult female donors (2 μg/ml in 3% BSA, 0.1 mM PMSF in TBS-TT) for 3 h at room temperature. Positive colonies from this screen were picked and re-arrayed on replicate High Bond C-Extra membranes. The re-arrayed filters containing a range of malaria Ig-reactive colonies from the initial screen, were subjected to a more rigorous re-screening after overnight blocking of the filter in 3% BSA, 0.1 mM PMSF in TBS-TT in order to screen for female specific parasite antigens.

To screen for female specific parasite antigens, unlabelled protein L-purified Ig from the male serum pool (0.5 μg/ml in 30 ml of 3% BSA, 0.1 mM PMSF in TBS-TT), was added to the filters, in sealed bags, to allow binding of unlabelled antibody to any specifically male-recognised antigens, or those antigenic specificities that are well-recognised by both male and female adults. Filters were washed in TBS-TT and TBS as previously described and antigens specifically recognised only by female antibodies were detected as follows: the washed filters were incubated in 30 ml per filter of a solution containing 2 μg ml^{-1} of FITC-labelled female Ig in 3% BSA, 0.1 mM PMSF in TBS-TT for 3 h in the dark at 4 °C. After washing the filters, colonies producing recombinant proteins recognised by FITC labelled purified Ig from the female pool were detected with the STORM 860 fluorescent imager (Amersham). As a control, the replicate filter was screened with FITC-labelled purified Ig from Scottish malaria naïve donors in the same way as described above. Filters were washed in TBS-TT and TBS as described and FITC-labelled colonies were detected using the STORM 860 fluorescent imager (Amersham).

3. Results and discussion

P. falciparum cDNA libraries were constructed using the method illustrated in Fig. 1. This consisted of oligo-capping

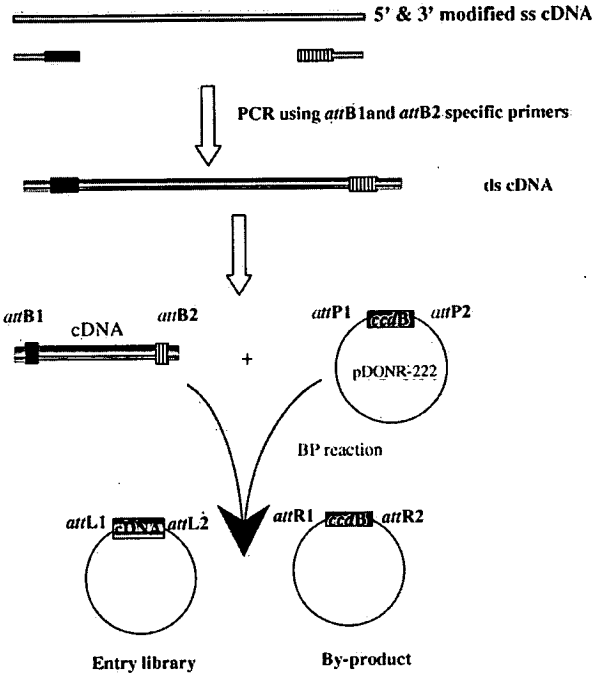

Fig. 1. Flow chart outlining the incorporation of the *att*B1 and *att*B2 recombination site sequences with second strand cDNA synthesis and integration of double stranded cDNA into the donor vector pDONR-222. The *att*B1 and *att*B2 recombination site-specific sequences are depicted by the black and hatched boxes respectively. The *ccd*B gene, the grey box in pDONR-222 and by-product of the reaction, is a negative selection marker removed by successful reciprocal recombination. The protein it encodes interferes with DNA gyrase and is lethal to non-recombinant bacteria; ds: double stranded; ss: single stranded.

total parasite RNA, followed by cDNA synthesis with concurrent introduction of recombination sites by PCR amplification and in vitro recombination into a donor vector (pDNR-222) to produce an entry library. The entry library was then shuttled into an arabinose-inducible plasmid expression vector, pBAD DEST-49 to produce the expression library in *E. coli*. 6912 cDNA clones were cultured in seventy-two 96-well microtitre plates and robotically arrayed onto replica high-density High Bond C-Extra filters, using a PBA Flexis robot. The *P. falciparum* expression library had an initial titre of around 100,000 cfu ml^{-1} with inserts ranging in size from 0.3 to 3.0 kb (mean of 0.8 kb).

The grid plan for arraying the cDNA library is shown in Fig. 2A. Clones were arrayed in duplicate on replica filters prior to in situ induction of expression of the recombinant proteins, lysis and immuno-screening. One of the replicates was probed with HRP-conjugated anti-His antibody. This was to detect the presence of the C-terminal His tag, attached to recombinant proteins. The second replicate, after blocking with 3% BSA and 0.1 mM PMSF in TBS-TT, was probed with labelled total immunoglobulin from a pool of highly malaria antigen-reactive sera from Ghanaian adult female donors. The initial screening process detected many antigens common to both adult male and female pooled sera and was used to produce a library of clones that react with sera from malaria-exposed individuals. These 'enriched' replicate arrays were prepared using the antibody-reactive colonies from the initial screen.

One of the enriched replicate filters was then pre-incubated with pooled unlabelled male immune Ig prior to screening with FITC-labelled pooled female immune Ig, in order to detect strongly female pool-specific parasite antigens. A control replicate filter was probed with FITC labelled malaria naive Ig. Fig. 2B shows the result of one of these screens, a typical fluorescence image of FITC-labelled antibodies bound to duplicate reactive clones. A replica filter screened with FITC-labelled malaria naive Igs is shown in the lower panel of Fig. 2B.

A number of duplicate clones appeared to react consistently with both FITC-labelled Ghanaian female 'immune' sera and Scottish malaria naïve sera. These clones were re-screened and the plasmid inserts sequenced, to determine if this was a screening artifact caused by free FITC binding or binding of anti-*E. coli* antibodies present in both screening sera. Cross-reacting clones were detected consistently and sequencing showed that they all contained *P. falciparum* sequences rather than *E. coli* or human sequences. These clones recognised by Scottish malaria naive Ig were therefore screened with a commercial preparation of naïve human IgM (Rockland, USA) and all colonies tested were shown to bind this IgM preparation (data not shown). A modified protocol including a pre-incubation step with unlabelled malaria naïve antibodies therefore seems more selective than pre-binding antibodies to *E. coli* lysates, as recommended in some earlier protocols.

Initial screening with Ghanaian 'malaria immune' Ig resulted in 27% of the library clones reacting positively. This was a low stringency screen performed without pre-binding of either naïve or male malaria specific-Ig and therefore was most likely to contain a number of reactivities with non-coding "nonsense" clones and some with non-specific cross-reactivities. These clones, together with clones of low reactivity were removed in an additively stepwise manner (see below). The initial apparent sero-reactivity of 27% may be overestimated due to these non-specific reactivities, but is still a significant increase on the 1–5% sero-reactivity detected in earlier expression library immuno-screening experiments [2,3]. This increase in efficiency of detecting antigenic epitopes from *P. falciparum* proteins can be ascribed to two features of this system: (1) the directional cloning scheme that doubles the chances of a cDNA fragment being inserted 'in-frame', thus producing a functional fusion protein and (2) the improved efficiency of translation and increased protein solubility resulting from fusion to the 11 kDa thioredoxin molecule rather than the 112 kDa β-galactosidase carrier used in λgt11 expression vectors [1–3,7,8]. The reducing environment induced by attachment to thioredoxin also aids folding and solubility of the fusion

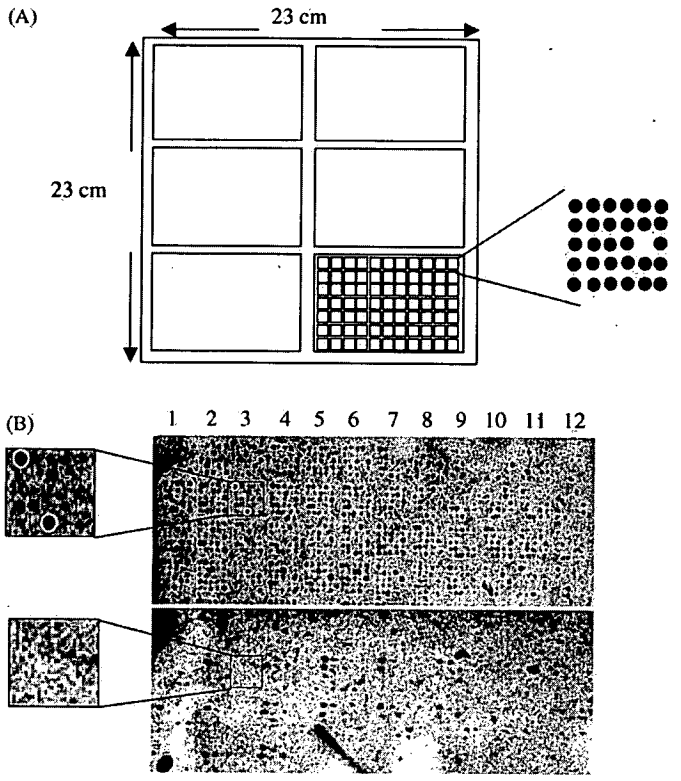

Fig. 2. (A) Schematic diagram of the layout of bacterial colonies on a 23 cm × 23 cm high-density filter. The six patches result from duplicate spotting of twelve 96-well plates in a non-contiguous pattern. The lower right box illustrates the 8 × 12 lay out of the squares. Twelve duplicated colonies are spotted in a 5 × 5 sub-grid (one blank spot for orientation). (B) Scanned image of a differential screening assay in which previously identified female sero-reactive expressed recombinant protein from colonies on high density filters blocked with purified unlabelled Ig from pooled adult Ghanaian male sera were treated as follows: (i) incubated with FITC-labelled Ig from Ghanaian female serum pools (ii) probed with FITC-labelled Ig from pooled malaria naive sera to detect and eliminate cross reactive antigens that may also be recognised by malaria immune Ig. The inset shows a sub-grid of 12 duplicated colonies, with one blank spot. The white circles illustrate 'true' duplicate positive colonies on the filter screened with malaria immune labelled female Igs; the same subgrid from the filter screened concurrently with malaria naive pooled Scottish serum is also shown, highlighting the specificity of the malaria immune Igs with the immobilized protein.

proteins as the intracellular environment of *P. falciparum* is known to be thioredoxin-rich [15].

One of our aims was to identify clones that react strongly with the female malaria immune Ig. Due to the marked differences in the intensities of fluorescent antibody binding between clones, two further rounds of immuno-screening were performed using decreasing concentrations of malaria-exposed female purified Ig (1 and 0.4 $\mu g\,ml^{-1}$) with more stringent filter washes between screens. Sero-positive clones could thus be divided into 'weak' and 'strong' binders. Table 1 summarises the results from three rounds of immuno-screening with antibodies from Ghanaian malaria endemic sera. Seventy percent of the strongly positive sero-reactive clones have been sequenced to date. The sequences obtained were compared to those in the National Centre for Biotechnology Information (NCBI), PlasmoDB and the FULL-malaria databases [16]. Eighty-nine percent of 200 strongly antibody positive colonies sequenced have cDNA inserts that are identical or almost identical to sequences in the recently published genome sequence of *P. falciparum* clone 3D7A [17]. Table 2 shows the functional categories into which the sequenced immuno-reactive clones fall. Eleven percentage of these clones encode antigens previously considered as malaria vaccine candidates and a further 5% encode antigens whose homologues have been considered as vaccine candidates in other parasites. It should be noted that the antigens we

Table 1
The proportion of library expressing sero-reactive proteins after three rounds of immunoscreening with increasing stringency

	Number of colonies (%)
Total array	6912 (100)
Low stringency primary malaria endemic Ig screen	1866 (27)
Immunoscreen with 1.0 $\mu g\,ml^{-1}$ female malaria immune Ig (weak binders)	1120 (16)
Immunoscreen with 0.4 $\mu g\,ml^{-1}$ female malaria immune Ig (strong binders)	260 (3.7)

Table 2
The range of sequenced *P. falciparum* clones expressing sero-reactive recombinant protein

Clone ID	Accession number match	Expect (E) value/3D7 chromosomal location (c)	Description of clone based on match (functional category)
1C11	AF061080	2.8E−22/c11	Circumsporozoite related antigen (V/MTP)[a]
1D5	AF348158	2.4E−45/c14	Glyceraldehyde-3 phosphate-dehydrogenase (E/MP)[b]
1G6	X77854	5.1E−84/c2	Serine repeat antigen (V/MTP)
3A8	PFU21939	1.8E−82/c5	28S rRNA asexual stage type
3B5	PFMAL4P3	5.7E−55/c4	Ribosomal protein
3E2	AF170073	9.9E−29/c12	Sexual stage specific protein kinase (S)[c*]
6D4	AF034389	4.9E−59/c4	Pfs 16 (gMP)[d]
7F10	AE014851	6.4E−71/c12	DEAD/DEAH box (NAP)[e]
11A9	AF142344	1.0E−152/(NCBI)	Histidine rich protein II (SP)[f]
16F1	PFU27338	1.2E−36/c14	*P. falciparum* erythrocyte membrane protein1 (eMP)[d]
18B7	PFE1590w	2.5E−106/c5	Early transcribed ring stage membrane protein (rMP)[d]
21B2	PF10_0121	3.3E−47/c10	Hypoxanthine-guanine-phosphoribosyl transferase (E/MP)
25H1	AF420310	3.5E−108/c14	Calmodulin gene (S)
26A3	AF302208	1.6E−97/c12	Translation initiation factor (TAP)[g]
29C4	AF056936	7.3E−87/c5	*P. falciparum* erythrocyte membrane protein2 (eMP)[d]
29F10	NP701198.1	3.8E−171/c11	Aquaglyceroporin (V/MTP)
44E3	M86865	2.9E−46/c6	Histone H2A (NAP)
44F10	M19753	1.5E−07/c8	Heat shock protein 70 (CA)[h]
64H11	AE0148481	2.0E−54/c12	Putative macrophage inhibition factor (SP)
67F2	PFB0120w	5.3E−129/c2	Hypothetical proteins with transmembrane domains (pMP)[d]

Expect values are from the PlasmoDB database unless otherwise stated.
[a] V/MTP: vessicle/membrane trafficking proteins.
[b] E/MP: enzyme/metabolic protein.
[c*] S: signalling.
[d] e/r/g/pMP: erythrocyte/ring/gametocyte/probable membrane protein.
[e] NAP: nuclear associated proteins.
[f] SP: secreted protein.
[g] TAP: translation associated proteins.
[h] CA: cytoplasmic antigen.

have identified by our differential screening method are a female biased sample, as antigen specificities that were strongly recognised by the pool of Ghanaian male sera had been excluded by this stage of the screening process. This may explain the non-appearance of the prominent class of antigens found on the merozoite surface, such as AMA-1 and MSP-1 and 2.

Approximately 25% of all sequenced inserts have been matched to locations in the genome sequence to which no function has yet been assigned. These sequences fit criteria established by the Malaria Genome Project for 'hypothetical proteins' (referred to as 'orphan genes' in the text).

A proportion of the sero-positive clones detected by both male and female Ig pools, were found to encode rRNA, particularly 28S rRNA. This was not unexpected as total RNA, rather than purified mRNA, was used to construct the libraries to avoid loss of any *P. falciparum* mRNA species that bind poorly to oligo dT columns [18]. Serum reactivity with colonies containing plasmids with rRNA inserts has been previously observed by other malaria researchers, but rarely commented on, although the phenomenon has an interesting basis in some other systems. Tenson et al. [19] demonstrated the presence of a functional peptide encoded by an open reading frame (ORF) within *E. coli* 23S rRNA in vitro and Jansson et al. [20] have reported the presence of a small ORF that encodes a hemolysin protein in the large subunit rRNA of *Entamoeba histolytica*. The persistent isolation of rRNA-containing clones, particularly 28S rRNA, when screening *P. falciparum* expression libraries may be explained by some specific anti-parasite response. However, natural malaria infections are known to induce anti-host nuclear antibodies [21]. An alternative explanation for the detection of colonies containing plasmids with *P. falciparum* rRNA inserts is that they are recognised by infection-induced anti-nucleic acid antibodies. Therefore, at present, the reasons for the strong reactivity of *P. falciparum* rRNA in immuno-screened expression libraries remain uncertain.

Eight percent of the clones sequenced bore no homology to any sequence in any database. Since *E. coli* or other microbial contaminating sequences would be detected as 'hits' in such searches, a non-*P. falciparum* origin for these sequences can probably be excluded. Furthermore, since most of them contain A + T rich sequences with open reading frames and some contain repetitive sequences, a common feature of malaria parasite proteins, it is likely that these are *P. falciparum* sequences. Some may originate from the residual portion of the *P. falciparum* 3D7A genome that has not yet been added to the database, or may be on the small, existing portion of incompletely sequenced chromosomes. For example, one of the sero-reactive clones that we identified has 100% homology to the Histidine Rich Protein II (HRPII, accession number—AF142344) gene sequence of *P. falci-*

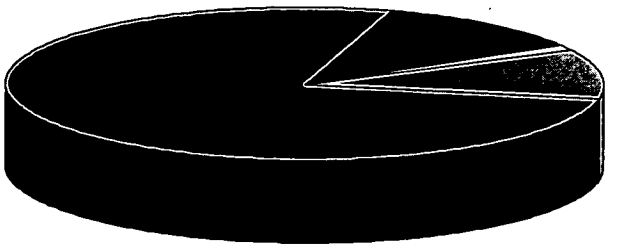

Fig. 3. (■) Identity with sequence from known *P. falciparum* proteins with assigned function and known chromosomal location; (▣) identity with sequence from known chromosomal location of *P. falciparum* genes but of unknown function; (▥) putative open reading frames; (□) identity with sequence from known *P. falciparum* proteins with assigned function but unknown chromosomal location.

parum, which can be found in the NCBI database, but not in PlasmoDB. This protein may be localised on one of the chromosomes that has not yet attained gap closure, i.e chromosomes 6–8, 10, 11, 13 and 14 [17] and therefore has not yet been assigned a locus. It is also likely that some of these unidentified sequences are polymorphic in *P. falciparum* and are allelic variants or multigene family members present in SD 2H3/5-CSA but not in 3D7A. Fig. 3 shows the chromosomal distribution of the sequenced sero-reactive clones and highlights the percentage of sequenced clones with chromosomal location but without functional assignation.

As yet there are no completely predictive correlates of immunity to malaria with specific immunological responses to particular antigens. The novel combined cDNA library construction and antibody screening methods outlined here will facilitate efforts to systematically organise data on the antibody responses to immunogenic antigens of *P. falciparum* and help identify minimal epitopes for incorporation into novel malaria vaccine candidates [22]. The data presented further confirm the view that B cell responses against malaria are directed against a large number of antigens [2,3,23]. It has also recently been demonstrated that T cell responses in humans immunised with irradiated sporozoites are not concentrated on a few immuno-dominant epitopes but that T cells also respond to a relatively large number of antigens [24].

Nonetheless there are recent indications that antibody responses to variant antigens on the surface of *P. falciparum* infected erythrocytes are strongly correlated with protection against certain severe malaria syndromes such as pregnancy-associated malaria [25]. The extent to which this serological screening method can detect such syndrome-specific immunogenic antigens is under investigation.

Acknowledgements

We thank Dr. Cecilia Fernandez, University of Montevideo, for critical comments and Drs. Linda Brooks and Mark Smith of Invitrogen for advice. We would also like to thank our collaborators at the Panum Institute, University of Copenhagen and Rigshospitaleit, Copenhagen, for 2H3/5-CSA and the malaria immune serum. This work was funded by the Wellcome Trust through a University Fellowship to DEA and the Commission of the European Union, through grants QLK2-CT-1999-01293 (EUROMALVAC-1) and QLK2-CT-2001-01302 (PAMVAC)

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