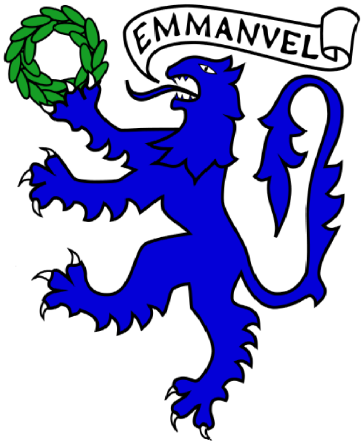
Identification and characterisation of host-pathogen protein-protein interactions in the blood stages of malaria



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For Julia, Nick and Tim

Declaration

I hereby declare that the contents of this thesis are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other, University. This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. This thesis does not exceed the word limit set by the Faculty of Biology.

Abigail Perrin
2014

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Abstract

Malaria is a widespread and serious disease which affects billions of people. Protein-protein interactions occurring between host and *Plasmodium* parasites are critical to the pathogenesis of malaria and thus represent prime targets for greatly-needed novel therapeutics. Identifying these host-pathogen interactions is challenging, but recent advances in our understanding of parasite biology and in high-throughput biomolecular interaction detection methods have paved the way to a number of successes.

In this work I produced a library of recombinant *Plasmodium falciparum* proteins to screen for interactions with human receptors in a number of high-throughput assays. Using an established ELISA-based protein-protein interaction detection method, I identified an interaction between *P. falciparum* merozoite protein 7 (PfMSP7) and human P-selectin (SELP). I used surface plasmon resonance and flow cytometry approaches to validate this interaction and, by screening more widely across the MSP7 protein family, identified that SELP-binding is a conserved property of multiple MSP7s in at least three *Plasmodium* species. The evolutionary conservation indicates that SELP-MSP7 interactions might have an important function in malaria. Isolating the interacting regions of SELP and PfMSP7 to the secreted, flexible N-terminus of PfMSP7 and the known ligand-binding domains of SELP led to a hypothesis that PfMSP7 could prevent the leukocyte recruitment and activation properties of SELP. I used PfMSP7 to block the interactions between SELP and leukocyte ligands *in vitro*, providing support to this hypothesis. Further evidence will be required to determine whether *Plasmodium* MSP7 proteins and their interactions with SELP mediate an immunomodulatory mechanism in malaria, and whether the MSP7 proteins represent useful therapeutic targets.

I also developed a biochemical co-purification assay aiming to detect additional interactions between recombinant *P. falciparum* merozoite extracellular proteins and those present in human serum. This assay was successful in detecting previously-identified interactions but did not identify novel binding partners for 56 *P. falciparum* ligands. By expanding the screen or by decreasing its stringency this method could facilitate the identification of further receptors for *Plasmodium* ligands which could in turn, like the interaction between SELP and MSP7s, aid our understanding of how host and pathogen interact to cause disease.

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General introduction

1.1 An introduction to malaria

1.1.1 Malaria as a major global health problem

Caused by protozoa of the *Plasmodium* genus, malaria is a disease that has plagued humanity since antiquity. Challenges in surveillance make it difficult to quantify the current global burden of malaria; the World Health Organisation (WHO) estimated the number of clinical cases in 2010 to be over 200 million, with over one million being fatal, but it is possible that this is a significant underestimate[136, 359]. Approximately half of the world's population live in areas where malaria is endemic and the disease persists particularly in some of the world's poorest countries. Sub-Saharan Africa bears the largest burden of this severe disease, which has its most devastating effects in young children and represents a major barrier to development[360].

One of the United Nations' Millennium Development Goals was to bring about a decrease in the global incidence of malaria by 2015. This has catalysed truly international research efforts, with an annual investment in excess of $2billion towards combating what was previously a neglected, understudied disease[266]. Progress has been abundant: half of all sub-Saharan African households are now thought to own a protective insecticide treated bednet[360]; annotated genome sequences are available for a growing number of *Plasmodium* isolates[48, 49, 107, 255, 260]; and the world's first malaria vaccine (albeit only partially effective) has been developed[244]. Such efforts are estimated to have prevented a quarter of a billion cases and saved over one million lives[360].

Despite ongoing successes in disease prevention and treatment, there is still a great need to better understand the pathology of malaria, the biology of *Plasmodium* parasites and the dy-

namics of transmission to confront challenges in the future. We still lack an effective vaccine and are encountering a growing number of drug-resistant parasites. Furthermore, it is possible that changes in global climate and human behaviours will bring the threat of malaria to immunologically naïve populations[88].

1.1.2 *Plasmodium* parasites cause malaria

Apicomplexan *Plasmodium* parasites are single-celled eukaryotes, over 200 species of which have been identified. *Plasmodium* species have parasitised birds, reptiles and mammals long before the evolution of humans, with the earliest evidence for their existence dating as far back as 30 million years[267]. Human infection is thought to have originated on multiple occasions from ape-infecting parasites and is thought to have become prevalent around the time of the Agricultural Revolution over 10,000 years ago[191, 194, 195], with *Plasmodium* DNA still detectable in human remains from Ancient Egypt[182]. These protozoan parasites were identified by microscopy as the causative agent of malaria in the late 19th century, and mosquitoes were soon after identified as being responsible for transmitting these parasites to humans[283].

All *Plasmodium* species complete a complex life cycle between a vertebrate and mosquito host, progressing through a series of morphologically-distinct forms, first described over 200 years ago[121](Figure 1.1). Human infection begins when a host is bitten by an infected *Anopheles* mosquito. When the mosquito takes a blood meal, *Plasmodium* sporozoites enter the human host via the skin. These sporozoites migrate to the liver where they replicate asymptomatically to create thousands of merozoites, which are released into the bloodstream. Here the parasites undergo further cycles of asexual reproduction. These cycles begin with the merozoites' invasion of a red blood cell (RBC), within which they replicate. These new merozoites cause lysis of the RBC, such that they are released back into the blood where they rapidly invade a new RBC. A subset of merozoites will develop into gametocytes, which go on to produce micro- and macro-gametes that fuse as part of sexual reproduction in the mosquito host. Fused gametes develop into diploid ookinetes, which are able to cross the mosquito midgut wall and form an oocyst, where sporozoites develop. Once these sporozoites are released they invade the mosquito salivary gland, allowing them to be transmitted to a new human host when the mosquito feeds.

At least five *Plasmodium* species naturally infect humans. *P. falciparum* is responsible for the most severe cases of malaria, and for the majority of fatalities. It is found throughout South East Asia and in parts of South America but is hyperendemic in most of Sub-Saharan Africa,

with as many as 50% of people infected[310]. *P. vivax* is thought to cause the largest number of infections globally, but has been less widely studied, perhaps owing to its lower mortality rate[219]. Whilst *P. vivax* infections do occur in Africa, the majority of clinical cases occur in Central Asia[114]. Infections with *P. malariae,* or one of the two sub-species of *P. ovale* are not known to be as prevalent, but are likely under-reported[44, 322]. These particular parasites have thus received comparatively little attention in the form of research effort. *P. knowlesi* was long-considered a macaque pathogen, but is now recognised to cause disease in people living in the regions of South East Asia where humans and macaques interact[162]. Mixed infections, whereby hosts are simultaneously infected with more than one *Plasmodium* species are also thought to be common[208].

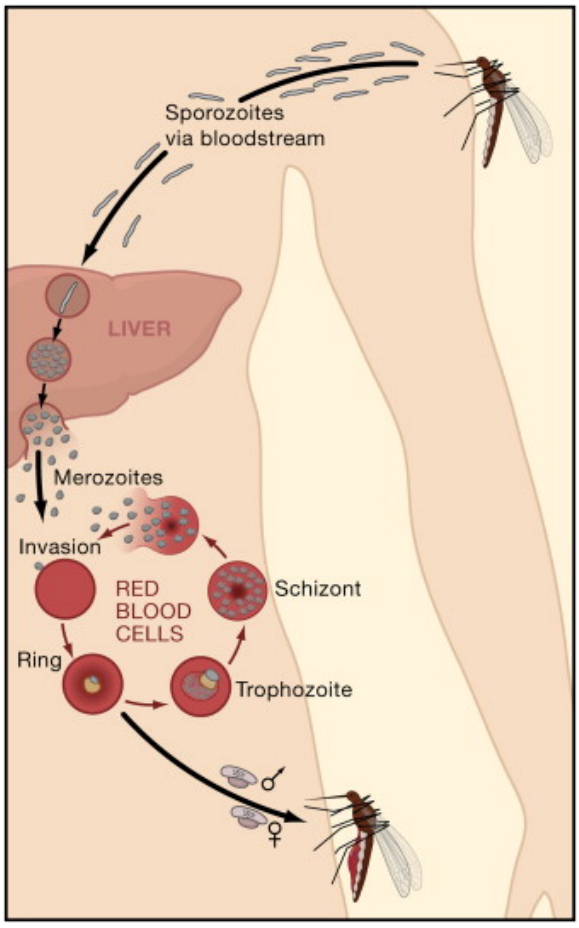


Figure 1.1: **Parasite life cycle in the human host**
Figure from *Invasion of red blood cells by malaria parasites* by Cowman and Crabb (2006)[61]. Reproduced with permission.

1.1.3 *Plasmodium* merozoites invade red blood cells

1.1.3.1 Merozoite structure

The blood-stage merozoite form of the parasite has been the most intensively studied, and is the primary focus of the work described in this thesis. At just over 1µm in length *P. falciparum* merozoites are amongst the smallest known eukaryotic cells. Free merozoites have an elliptical cross section with a projected apical end where secretory organelles - rhoptries, micronemes and dense granules - are located (Figure 1.2). Parasites also possess a highly specialised endomembrane system known as the inner membrane complex (IMC) located just below the plasma membrane. This structure and its connections to the cytoskeleton are thought to play critical roles in determining and maintaining the shape of the merozoite, in cell division, and in mediating its RBC-invasion mechanism[3, 22, 39]. The merozoite surface is covered in a 15-20nm thick filamentous coat[14] comprised primarily of a number of glycosylphosphatidylinositol (GPI)-anchored membrane proteins and peripherally associated proteins[115, 293]. Amongst these integral membrane proteins are those described as MSPs (merozoite surface proteins), including PfMSP1, 2, 4, 5 and 10 and a family of cysteine-rich proteins which are-associated with adhesive properties in *P. falciparum*[221]. These proteins interact with other

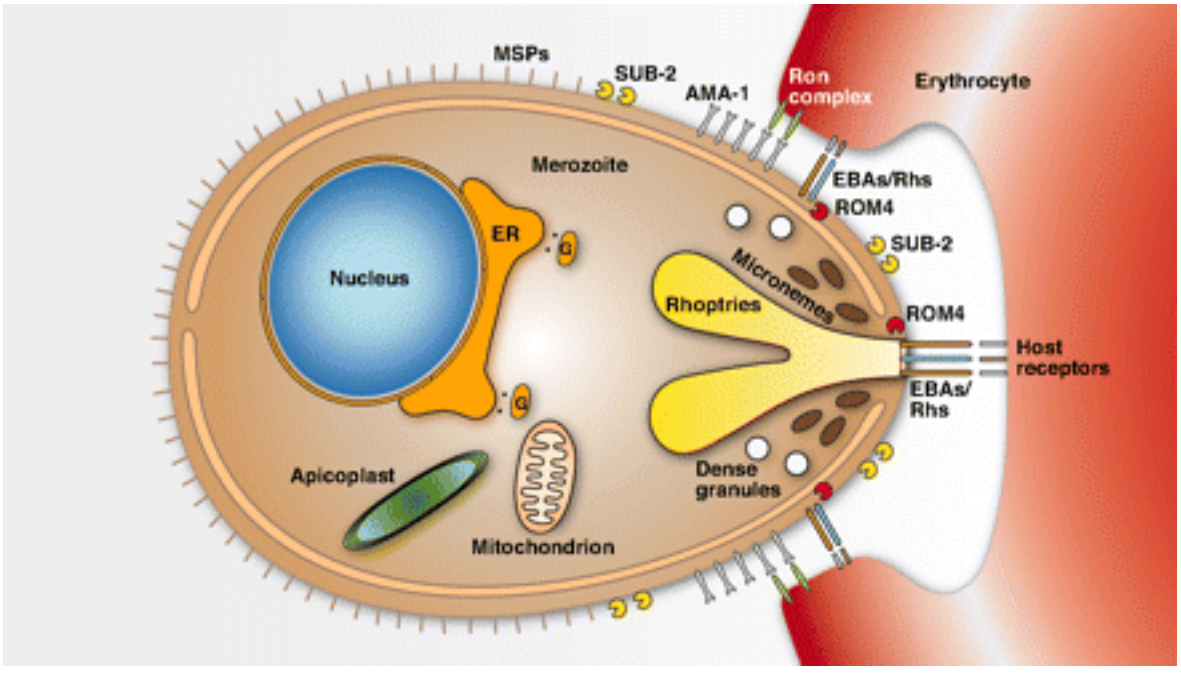


Figure 1.2: **Merozoite structure and RBC invasion ligands**
Figure from *That was then but this is now: malaria research in the time of an eradication agenda* by Kappe *et al.*, (2010)[163]. Reproduced with permission from AAAS.

parasite proteins, including those from the PfMSP3- and PfMSP7-families, which themselves do not possess a GPI anchor or transmembrane domain, to tether them to the merozoite surface. The PfMSP1 and PfMSP2 proteins are thought to comprise about two-thirds of the total protein content of the merozoite surface[115]. PfMSP1 is proteolytically processed such that four associated PfMSP1 peptides derived from a single precursor are present on the merozoite surface[28]. A fragment of each PfMSP6 and PfMSP7 are also peripherally associated with these PfMSP1 fragments to constitute an assembly known as the MSP1 complex[257, 336]. A number of other membrane proteins and peripherally-associated proteins have been identified as more minor components of the merozoite surface, many of which are delivered from *Plasmodium*'s secretory organelles prior to RBC invasion[61, 293].

1.1.3.2 RBC invasion and parasitism

In order to invade a host RBC, the merozoite carries out a number of steps in a defined sequence, which has been elucidated by microscopy[4, 81]. The invasion process involves molecular recognition events which cause the initial physical association between the two cell types. The merozoite then re-orientates such that its apical pole is in contact with the RBC surface, with which a tighter interaction is then made. Powered by its actin-myosin motor complex, the merozoite actively drives entry into the RBC, such that this tight junction moves from the apical to the posterior end of the merozoite[166]. During this invasion process, merozoite surface proteins and those involved in the tight junction are proteolytically cleaved and released into the bloodstream[135]. The final result of this process is that the merozoite ends up in the RBC cytoplasm surrounded in host-derived membrane in what is known as the parasitophorous vacuole. Whilst inside the RBC, the parasite digests a substantial proportion of the host cell's haemoglobin. Some of this haemoglobin consumption provides nutrition for parasite growth but it is thought that it plays a more pivotal role in regulating the osmotic stability of the parasitised RBC (pRBC), so as to prevent lysis before the parasite has undergone asexual reproduction[192]. After merozoites have divided several times, the pRBC, at this point termed a schizont, ruptures, releasing as many as 32 merozoites into the bloodstream. Within a matter of seconds, these merozoites invade new RBCs and the cycle repeats. Merozoites are known to progress through their erythrocytic cycle in synchrony, resulting in waves of RBC rupture, occurring approximately every 48 hours in *P. falciparum* malaria[1]. The mechanism and function of this synchrony is not fully understood; it could potentially be driven by the host responses to infection, or conversely be an adaptation of the parasite to overwhelm

[1] Synchronicity in merozoite invasion/lysis cycles is not observed in all infections, and is less common when the host is simultaneously infected by *Plasmodium* parasites with a diverse range of genotypes[334]

the immune system when merozoites are exposed[127, 176].

Whilst intracellular, *P. falciparum* is known to modify the surface of the pRBC by trafficking a subset of its own proteins to the membrane. Amongst these is PfEMP1[214], deriving from a family of around 60 highly variable proteins, encoded by *var* genes. PfEMP1 is a critical component of 'knob' structures on pRBCs which enable them to adhere to vascular endothelia[62], thus avoiding passage to, and destruction by, the spleen. This process is referred to as cytoadherence or sequestration and is thought to account for the much of increased morbidity associated with *P. falciparum* infections compared with other *Plasmodium* species. PfEMP1 is also thought to mediate platelet-mediated clumping of pRBCs[259] and 'rosetting' whereby pRBCs aggregate with uninfected RBCs[54].

1.1.4 Malaria can have life-threatening sequelae

The first symptoms of infection with *Plasmodium* parasites occur after an incubation period of at least one week. Although malaria is often described as being characterised by a periodic fever in the human host, it usually presents as a combination of symptoms including, but not limited to, fever, chills, headaches, nausea and malaise. In uncomplicated infections these symptoms can be temporarily debilitating but usually resolve within a few weeks. However, a proportion of infections do progress to severe, life-threatening syndromes. Severe Malarial Anaemia (SMA) is defined by the WHO as having detectable parasitaemia and a hematocrit lower than 15%, indicating that RBC counts are at least half healthy levels[248]. SMA is responsible for a large proportion of childhood deaths in malaria-endemic regions[84]. Anaemia results not only from the direct parasite-driven lysis of pRBCs but primarily from the destruction of uninfected RBCs[150], by macrophage-mediated phagocytosis or clearance by the spleen[179]. Respiratory distress, renal failure and septic shock-like symptoms can develop in a subset of cases and are responsible for a small proportion of fatalities.

Certain sequelae are particular to *P. falciparum* malaria. Cerebral malaria, thought to occur in about 1% of clinical *P. falciparum* cases[360], is characterised by neurological impairment and can lead to coma and death. Adherence of pRBCs in the brain is thought obstruct blood flow through cerebral microvasculature, which might deprive the brain of oxygen. At the same time, the pro-inflammatory cytokines produced as part of the anti-parasite immune response are thought to destabilise the blood-brain barrier, exacerbating the neurological impairment[142]. Pregnant women are at particular risk from *P. falciparum* malaria. Parasites express a specific PfEMP1 variant that is able to bind to placental CSA, which causes the adherence of pRBCs to blood vessels in the placenta[344]. This blocks the flow of nutrients

to the developing foetus and as such can negatively affect the outcome of the pregnancy.
Although re-infection is common in malaria-endemic regions, those who recover from *P. falciparum* malaria and remain unexposed do not develop any further symptoms. However, recurrence of disease can be observed many years after exposure to *P. vivax* or *P. ovale,* the parasite remaining dormant in the liver in a form known as the hypnozoite[57].

1.1.5 Natural immunity to malaria provides incomplete protection from *Plasmodium* infections

Immunity to malaria is enigmatic. Even people living in endemic regions are not thought to develop true sterile immunity to malaria. However, provided they are continually exposed, people gradually develop a level of protection against life-threatening disease, even when parasites are detectable in their blood (Figure 1.3). This slow development of immunity is widely considered to be the result of strain-specificity in immune responses[153], such that apparent immunity results from the generation of a repertoire of strain-specific responses to a diverse range of *Plasmodium* parasite infections[70]. This would explain why the majority of deaths occur in infants and why protection from severe disease appears to wane in the absence of continual exposure.

1.1.5.1 Innate and adaptive immune responses

The immunological basis of protection against malaria is still unclear and has been investigated at multiple stages of the parasite lifecycle. Whilst the induction of anti-sporozoite antibodies has been shown to provide protection against disease[165, 269, 301], the sporozoite stage has not been clearly demonstrated to be a target of protective antibody responses in natural infections. Since blood-stage infection is so commonly observed it is often assumed that naturally-induced immune responses against sporozoites are largely ineffective at preventing disease. This might result from the low number of sporozoites transmitted by the mosquito[2], which provides a scarcity of antigen against which the immune system can respond. There is also a body of evidence suggesting that sporozoites drive the suppression of anti-parasite immune response from the moment they are injected into the skin[130].
The mechanisms by which the host controls blood-stage *Plasmodium* parasites have been investigated extensively, however a unified picture of which responses are protective has not yet emerged. Antibody responses have long been recognised as important in controlling infection, since the observation that immunoglobulins prepared from adults living in endemic areas could

[2]Studies in rodent models suggest a median of about 18 sporozoites are injected per bite[212]

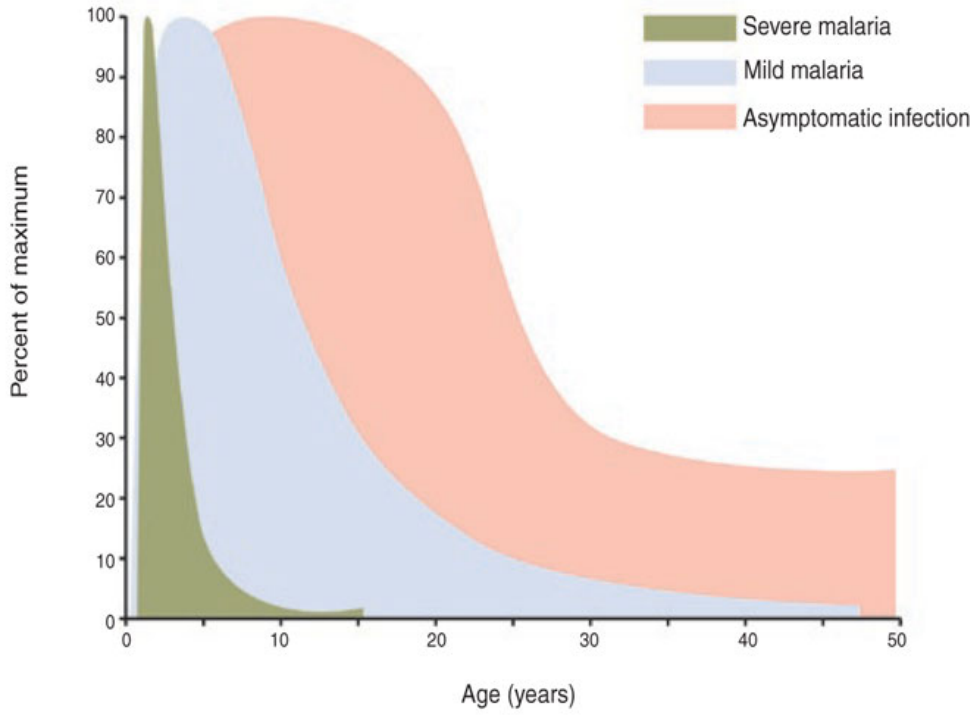


Figure 1.3: **Acquisition of immunity to malaria**
Data collected from a population living in an endemic region of *P. falciparum* transmission show that immunity to severe disease is acquired during infancy, but infections still persists into adulthood.
Figure from *Immunity to malaria: more questions than answers* by Langhorne *et al.*, (2008)[185]. Reproduced with permission.

have a therapeutic effect on children suffering from malaria[58]. A dependence on antibody to limit parasitaemia has been corroborated by studies in B-cell deficient mice, that are unable to clear *P. chabaudi* from the blood[184]. Antibody may function to control infection in several ways; by directly binding to merozoite antigens marking the parasite for antibody-dependent cell mediated killing and phagocytosis[149, 252], by binding to merozoite antigens to prevent them from invading RBCs[29], or by binding to PfEMP1 on pRBCs to induce their clearance from the bloodstream[37]. A broad range of parasite-specific antibodies are detectable in the serum of *P. falciparum*-exposed individuals, but it is still unclear as to which confer protection from severe disease[91]. PfMSP2, PfMSP3, PfMSP7, PfSEA1, PfAMA1, PfGLURP and PfSERA9 are amongst a growing number of candidate proteins against which antibody is thought to protect individuals in specific populations [124, 145, 243, 251, 273, 311]. More thorough analyses of a larger range of candidate antigens will be required to develop a complete picture

of naturally-protective antibody responses.

Antibody-independent mechanisms also play a role in the host immune response to infection by *Plasmodium* parasites. A number of PAMPs (pathogen-associated molecular patterns), including GPI (by which merozoite surface proteins are anchored to the membrane) and haemozoin (produced from the parasites' digestion of RBC haemoglobin), are thought to bind to Toll-like receptors (TLRs), and stimulate both $CD4^+$ and $CD8^+$ T-cells to produce pro-inflammatory cytokines[56, 93, 236]. These cytokines include Interferonγ (IFNγ) and Tumour Necrosis Factor α (TNFα), which are thought to contribute to parasite killing in the early stages of infection[92, 231, 232]. However, the activation of an inflammatory immune response in malaria has more often been associated with a negative outcome for the host, the over-production of cytokines being considered highly immunopathological[199].

1.1.5.2 Genetic resistance to malaria

The human race has evolved alongside *Plasmodium* parasites since antiquity, and its selective pressures have left their mark on the human genome, such that resistance to certain strains of malaria can be afforded by host genetic factors. Notably, a number of blood disorders are commonly associated with protection against malaria. The HbS variant of the HBB gene - which encodes RBC β-globin component of haemoglobin - has arisen on multiple independent occasions and been maintained in malaria-endemic populations, even though homozygotes suffer from life-threatening sickle-cell disease. This risk is balanced against the ten-fold reduced incidence of *P. falciparum* malaria in heterozygotes[1]. A number of other variants in the HBA and HBB globin genes are also found in malaria-endemic regions, again affording a degree of protection from malaria at the expense of sub-optimal haemoglobin functionality[55, 97, 226]. Similarly, a number of RBC enzyme deficiencies are thought to have been maintained in populations as they prevent *Plasmodium* parasites from surviving intracellularly. The most common example is glucose-6-phosphate dehydrogenase (G6PD) deficiency which is observed in African and Mediterranean populations that have evolved with *P. falciparum* infections[198]. Interestingly, it is thought that the parasite has adapted to produce its own G6PD, counteracting the host's once-innate defence[341]. Similarly *P. vivax* transmission in Africa was thought to be restricted by the lack the FY Duffy blood group antigen, which was thought to be required by the parasite for entry into the RBC[222][3]. However it is now recognised that *P. vivax* isolates from Madagascar are able to invade Duffy-negative RBCs indicating that parasites have once again evolved to overcome this restriction[217]. Recent large-scale genome-wide studies linking host genotypes with disease outcomes have helped to clarify the role of these host loci

[3] *P. knowlesi* is also thought to require Duffy antigen to enter RBCs

believed to affect malaria susceptibility and have identified many more host genes that might also contribute[151, 202].

1.1.6 Blood-stage *P. falciparum* parasites are highly adept at immune-evasion

Part of the reason why long-lasting, sterile immunity to malaria is has eluded mankind may lie in the ability of the parasite to overcome the defences of the host immune system. The largely intracellular habitat of the parasite within RBCs shields *Plasmodium* parasites from the onslaught of immune effector cells and molecules present in the blood. RBCs are one of the few cell types that do not present intracellular antigen on their surface via MHC Class I molecules, further hiding parasite-derived antigens from immune surveillance. The spleen mechanically filters damaged RBCs from the blood and thus might be expected to bring about the destruction of pRBCs. However, *P. falciparum*-infected RBCs adhere to endothelia (as described in 1.1.3.2) so as to avoid passage to the spleen. PfEMP1 protein, displayed on the surface of the pRBC, is critical for this adhesion process and is one of the few parasite proteins that is directly exposed to immune effectors in the blood for more than a matter of seconds. *P. falciparum* has evolved a sophisticated mechanism to prevent PfEMP1 from recognition by the immune system. It only expresses one of a diverse range of approximately 60 PfEMP1-encoding *var* genes at any one time, such that antibodies raised against one PfEMP1 variant will be ineffective against parasites that display another[295]. Though their role in disease is less well-characterised, antigenic switching of this kind has also been observed in a number of pRBC surface proteins belonging to other multi-gene families. These include the *rif* family[178] and *stevor* family which has been recently implicated in RBC invasion and rosetting[238].

Plasmodium parasites are also thought to actively subvert the functioning of the immune system; this may be particularly important in protecting free merozoites from destruction when they are exposed between cycles of RBC lysis and invasion. There have been many reports that *Plasmodium* parasites can impair the maturation of antigen-presenting dendritic cells (DCs), possibly preventing them from stimulating parasite-killing activity in T-cells[197, 315, 340]. It has been suggested that the haemozoin, produced by the parasites' digestion of haemoglobin, can mediate the observed suppression of DC function[223]. A parasite-encoded homologue of human macrophage migration inhibitory factor (MIF) might also affect the function of antigen-presenting cells (APCs), stimulating them to produce cytokines that drive the premature maturation of $CD4^+$ T-cells such that their anti-parasite specificity is rapidly lost and does

not contribute to immunological memory[320].

Whilst there is increasing evidence that certain naturally-induced antibodies do protect hosts from infection (Section 1.1.5.1), there are a number of mechanisms by which parasites are thought to suppress the generation of an effective, long-lasting antibody response. Memory B-cell populations studied from people living in areas of high malaria transmission are considered under-responsive and potentially impaired in their ability to combat infection with *P. falciparum*[143]. Many merozoite antigens display a great deal of sequence diversity between isolates[18], indicating that they are under strong balancing selection exerted by the human immune system. Alongside the antigen-switching behaviour exhibited by *P. falciparum* proteins on the pRBC surface, this variability impairs the effectiveness of antibody repertoires generated during previous infections to neutralise new infecting strains of the parasite. The inability of the immune system to respond effectively to a malarial challenge, and the lack of long lasting immunological memory present obstacles to making an effective vaccine.

1.1.7 Current anti-malarial measures

1.1.7.1 Chemotherapeutic agents

A wide range of anti-malarial drugs has been developed and used both as treatments and as prophylaxis in immunologically naïve travellers to malaria zones. 4-Aminooquinolines, such as chloroquine, are safe and affordable drugs that have been effective against all species of *Plasmodium* known to affect humans. They form a complex with a waste product of haemoglobin digestion that is toxic to the parasite, and prevent its degradation to haemozoin[245]. Arylaminoalcohols (structural relatives of aminoquinolones) and artemisinin derivatives have been used for hundreds of years. There has been much speculation over the mechanism of action of these drugs; arylaminoalcohols (such as quinine) have been suggested, like aminoquinolones, to prevent the detoxification of haem degradation products. Artemisinin is thought to cause oxidative damage to the parasite but the precise targets are unclear[220]. More recently, drugs that target the parasite respiratory chain or biosynthetic pathways have been developed[356]. A number of antibiotics also have an antimalarial activity, acting on the prokaryote-derived apicoplast organelle (see Figure 1.2) upon which the parasite relies for fatty acid and isoprenoid biosynthesis[353]. However these antibiotics are not used as standalone treatment as they have a delayed-killing effect, dependent on the parasites' completion of an erythrocytic cycle[66]. In an effort to prevent the spread of drug resistance, many of these classes of drugs are used in combination with one another, and new anti-malaria compounds are being sought actively. Of note, nearly two million chemicals were recently screened for

anti-parasite activity, which has led to the identification of novel drug targets and many chemical starting-points for potential new drugs[104, 129].

1.1.7.2 Environmental management

There is currently no widely-available vaccine, so prevention of malaria in people living in endemic regions has relied solely on environmental management aimed at preventing infectious bites from occurring. Interventions that target mosquito vectors have been a major contributor to the eradication of the disease from many areas of the world, notably the USA where a relatively short campaign saw the eradication of malaria by the 1950s. This elimination process has involved the use of insecticides and the destruction of the stagnant-water habitats where mosquitoes breed. For the areas of the world where infected *Anopheles* vectors still persist, the WHO advocate that at-risk people sleep under an insecticide-treated bed net (ITN) and that indoor surfaces are regularly sprayed with insecticides (indoor residual spraying, IRS). They estimate that almost 60% of sub-Saharan African households have now been supplied with ITNs and that 135 million people (about 4% of the at-risk population) are protected by IRS[360]. In the small number of areas, often urban areas, where mosquito breeding sites are easily-identifiable, accessible and few, biological or chemical agents are sometimes used to eliminate developing larvae[247]. Innovative vector control strategies have been emerging in recent years. These include the generation of genetically-modified mosquitoes that are resistant to infection[204] or that produce predominantly male offspring, so as to drive a population crash[102]. Interventions of this kind rely on the large-scale generation and release of genetically-modified insects, which is currently a logistical challenge.

1.1.8 Threats to the elimination of malaria

1.1.8.1 Antimalarial drug resistance

Whilst prophylactic drug treatment for malaria is effective for travellers who visit malaria zones, the lifelong treatment of the billions at risk from malaria would not be practicable. The spread of drug-resistant parasites threatens the utility of these preventative and curative treatments. Chloroquine, once the first-line treatment for *P. falciparum* malaria, was considered to be a highly effective treatment until the emergence of resistant strains in the 1960s. Resistance is thought to have evolved in at least four independent incidences and has now spread such that chloroquine treatment failure rates are high in almost every country studied

to date[4], approaching 100% in a number of areas[246]. Similar emergences of drug-resistant parasite strains have led to an over-reliance on artemisinin-based therapies, but in recent years resistance to this drug has also been identified[225]. The use of combination therapies has been an important step in preventing the spread of antimalarial resistance, however multi-drug resistant strains are emerging, which is a serious concern. Adaptations in transporter proteins which allow the parasite to expel these drugs from their sites of action include mutations in the *pfcrt* gene and amplification of the *pfmdr1* gene, and have become particularly widespread[170, 246]. This does not necessarily mean that our repertoire of antimalarial drugs is now useless; just ten years after the withdrawal of chloroquine treatment in Malawi, chloroquine-sensitive parasites dramatically re-emerged such that treatment failure rates returned to negligible levels[188]. However the potential for chloroquine-resistant parasites to re-surface under the selective pressure of drug treatment is all too clear.

Many existing treatments, such as quinine and artemisinin, are derived from naturally occurring remedies discovered hundreds of years ago. We can no longer rely on chance discoveries; an in-depth knowledge of the molecular biology of the parasite will better inform the rational design of new drugs and the combinations of treatments that will best guarantee long-term treatment success.

1.1.8.2 Insecticide-resistant mosquitoes

Whilst the use of insecticides has been arguably the biggest contributor to a recent reduction in the global incidence of severe malaria, and is still effective in most areas, it is widely believed that the spread of resistant mosquitoes will threaten the continuation of this decline. Four main classes of insecticides are used against malaria, and mosquitoes resistant to each of them have been identified[11, 271]. Furthermore, almost all ITNs are treated with pyrethroids, against which there are the largest number of reported cases of resistant mosquitoes. It is estimated that if pyrethroids lose their efficacy over half of the benefit of current vector control programmes would be lost, resulting in over 100,000 additional deaths every year[249]. These concerns make the establishment of new preventative measures an urgent priority.

1.1.8.3 Challenges of vaccine development

Although antimalarial drugs and vector control have been highly effective in preventing malaria, the ideal for long-term prevention would be an effective and widely-available vaccine. More than 40 *P. falciparum* malaria vaccine formulations have reached the clinical trial stages of

[4]Chloroquine is still effective in some regions of Central America

development[300]. Two of the most-developed are based on sporozoite-stage antigens; although natural immunity is not thought to rely on targeting sporozoites, the induction of a neutralising anti-sporozoite response could result in sterile immunity, which would be the gold-standard of efficacy for a vaccine. RTS-S/AS01E is a vaccine based on the *P. falciparum* circumsporozoite protein (PfCSP). However, phase III clinical trials on this vaccine indicated a only partial efficacy, which declined to below 20% protection within four years[244]. 'Leaky' vaccines that do not provide sufficient protection have the potential to drive evolution of more virulent vaccine-resistant parasites[15], so an alternative is desperately needed. A whole-sporozoite vaccine, which may evoke a broader, more effective immune response against pre-erythrocytic parasites, is another initiative which has recently been tested. Whilst some early results are promising, there is scepticism about whether the production of such a vaccine (which involves the dissection of individual infected mosquitoes) would be scalable[86].

A growing number of blood-stage *P. falciparum* antigens are being developed as potential vaccines. These include a range of merozoite surface proteins such as PfMSP1, PfMSP3 and PfAMA1, but as yet none have shown high efficacy in field trials[82, 242, 308, 329]. Sequence diversity in blood-stage antigens may represent a major driving force behind low reported rates of protection mediated by vaccines of this kind. Immunisation against one variant of a particular antigen results in allele-specific immunity, such that individuals are only protected against parasites displaying that particular antigen variant. This means that vaccines using polymorphic blood-stage antigens will need to include a diverse range of protein variants, or focus on functionally-important conserved epitopes, to induce cross-strain immunity[80, 256]. Transmission-blocking vaccines that target the sexual stages of the parasite could be a useful tool in the global eradication of malaria. Pfs25, an ookinete surface antigen, is thought to be a suitable vaccine antigen[126]. However vaccines of this type require almost complete coverage to be effective, and voluntary uptake stands to be low since vaccination does not directly protect the recipient. Vaccines based on multiple antigens, perhaps from multiple life-cycle stages, may induce a broader, more effective immune response and help prevent the emergence of vaccine-escaping *Plasmodium* strains[200]. With emerging drug resistance and no certainty of a cost-effective vaccine in the immediate future, it will be important to develop new strategies for controlling the incidence and impact of malaria. The rational design of such interventions will demand a deeper understanding of parasite biology at the molecular level[125].

1.1.9 Research resources

The study of the molecular biology of the malaria parasite has been greatly aided by a growing number of resources and data sets. Perhaps the most influential development in recent decades has been the sequencing of *Plasmodium* genomes. Genomes of *P. falciparum*[107], *P. vivax*[49], *P. knowlesi*[260], the rodent parasite *P. yoelii*[48] and *P. reichenowi*[255]- a chimpanzee parasite very closely related to *P. falciparum* - are published. Draft assemblies have been produced for many isolates, including those from the rodent parasites *P. berghei* and *P. chabaudi*[132], the macaque pathogen *P. cynomolgi*[323] and the avian parasite *P. gallinaceum*. Genome sequencing is becoming more routine, such that thousands of *P. falciparum* genomes[203, 225] and a smaller number of *P. vivax* genomes[52, 140, 218, 235] have now been analysed, allowing deeper insights into the genetic variation that exists in nature. Annotated sequences are freely available and are constantly being improved[254, 343]. Complementing these genomes, transcriptome data has been generated for a number of different species at different stages of their lifecycles[32, 189, 253, 289, 355], and large proteome data sets are also published[98, 187, 270].

We are now able to culture *Plasmodium* parasites in isolated RBCs, which provides an *in vitro* system to study phenotypes of blood stage parasites. The first attempts to grow *P. falciparum*, *P. vivax* and *P. malariae* in human blood were made over a century ago[21], but it was not until the 1970s that the continuous growth of a *P. falciparum* strain could be achieved[335]. Since then *P. knowlesi* and a number of additional strains of *P. falciparum* have been successfully grown in culture[228], though we still lack the capacity to sustain the growth of *P. vivax* along with many *P. falciparum* field isolates outside of a host. *P. falciparum* cultures have been used extensively as a source of parasites for a range of studies into processes such as gene expression, protein localisation and RBC invasion[330]. Genetically manipulating these *Plasmodium* parasites, particularly by knocking-out genes-of interest, allows the functions of candidate genes to be determined. For instance the roles of hypothesised RBC invasion ligands have been investigated using cultured *P. falciparum* parasites that have been genetically altered to be deficient in PfEBA175, PfMSP7 and a range of other proteins[77, 78, 159]. Systematic approaches that can facilitate stable genetic modifications in *P. falciparum*, *P. vivax*, *P. knowlesi, P. berghei* and *P. yoelii* have been developed, making the study of candidate genes possible in *in vitro* and *in vivo* systems [50, 228, 230, 265, 371].

Appropriate animal models of malaria are required to study the course of infections *in vivo* and represent a valuable research resource. *Plasmodium* species that naturally infect African rats have been adapted to grow in laboratory mice and now represent the most common animal models of human malaria. Model systems using *P. chabaudi*, *P. berghei* or *P. yoelii* parasites

have been used with some success to study *P. falciparum* protein orthologues, such as the prospective immune evasion ligand PfPMIF[9] and PfTRSP, a putative hepatocyte invasion ligand[180]. However these systems are somewhat limited in their utility to study proteins that do not have orthologues in rodent malaria parasites, or to examine aspects of human malaria pathology that are not accurately replicated in rodent models. The *P. berghei* mouse model of cerebral malaria has been widely criticised, as the hallmark human symptom of pRBC sequestration does not appear to be replicated in brain microvasculature, though its pathological effects are perhaps substituted by leukocyte sequestration[43]. This makes the model inappropriate for the study of cytoadhesive interactions and anti-cytoadhesion therapies, and it also appears to have had limited power in predicting the efficacy of human vaccines; for instance antibodies against PbCSP protected mice from infection[269], yet immunogenic, human vaccines based on PfCSP have not shown the same success[120, 244]. To overcome some of these issues, adapted model systems are being developed, such as mice infected with *P. berghei* parasites expressing *P. falciparum* proteins[119] and chimeric mouse models that can carry human erythrocytes to support the growth and study of *P. falciparum*[154]. For studies that require the closest possible proxy for human infection, *Aotus* monkeys can be used as a host for *P. falciparum* and *P. vivax*[139]. However, there is widespread ethical objection to the use of non-human primates in research, such that their use is restricted to very specialised facilities and for experiments on only the most promising therapeutic targets.

To complement the data generated about *Plasmodium* parasites, there has also been a focus on the biology of the human host and mosquito vector. Tens of thousands of human genomes have been sequenced to date and these data are continually enhancing our understanding of the host factors underlying susceptibility to malaria (see 1.1.5.2), and provide an insight into how host and pathogen interact to cause disease.

1.2 Protein-protein interactions in malaria

1.2.1 Parasites interact directly with their human host

To establish disease, pathogens interact extensively with their hosts. Systematic screening approaches have identified a myriad of potential interactions between viral proteins and those of their hosts[45, 68]. In fact, one study suggests that all ten major proteins of human influenza viruses make interactions with multiple host factors[303]. With much larger genomes, the host-pathogen interactomes for bacterial and protozoal diseases are not as well characterised, but still over 60 such interactions are known in Salmonellosis alone[297]. *Plasmodium*'s com-

plex life cycle means that it interacts with a broad range of human and vector environments. *Plasmodium* parasites invade a range of different cell types at different points in the life cycle, and these processes are known to involve a series of protein-protein interactions (PPIs).

1.2.1.1 RBC invasion

RBC invasion by the merozoite is the most comprehensively studied process where host and *Plasmodium* cells interact. PPIs occurring at around a 20-30nm membrane-membrane distance, are thought to mediate the initial recognition between the cells. As the primary component of the merozoite surface, PfMSP1 has been implicated in mediating this initial interaction, perhaps with the abundant Band 3 protein on the erythrocyte surface[116]. A number of other inter-cell receptor-ligand pairs have been identified as part of the merozoite-RBC recognition process. Amongst these, *P. falciparum* erythrocyte-binding-like (EBL) family proteins PfEBA175, PfEBL1 and PfEBA140 are thought to bind RBC surface glycophorins A, B and C respectively[201, 207, 306]. A family of reticulocyte binding-like homologue (RH) proteins also have known RBC ligands, with PfRH4 and PfRH5 interacting with CR1 and BSG respectively[64, 328]. There are thought to be multiple pathways by which the the merozoite can enter the RBC, such that there is a substantial degree of redundancy in *P. falciparum*'s invasion ligands. This allows the parasite to infect hosts regardless of polymorphisms in their RBC receptors[78]. The interaction between PfRH5 and BSG appears to be particularly fundamental to the invasion process, as blocking this interaction with antibodies or recombinant proteins completely inhibits invasion in a wide range of *P. falciparum* isolates *in vitro*[64].

1.2.1.2 pRBC/host interactions

PfEMP1 has at least 20 hypothesised ligands which facilitate the sequestration of pRBCs away from innate immune destruction[285]. These include a range of endothelial ligands including CD36, Thrombospondin, ICAM-1, SELP and EPCR, with some variants able to bind placental CSA[17, 23, 24, 279, 302, 338]. Certain PfEMP1 variants are known to mediate rosetting phenotypes via their interactions with CR1[284], AB blood group antigens[47], heparan sulphate-like molecules[54] or possibly CD36[134], which has also been implicated as the host ligand responsible for platelet-mediated pRBC clumping[259]. A range of known PfEMP1 ligands are present on the surface of leukocytes, such that pRBCs may interact with immune effector cells via direct PPIs. pRBC binding to macrophage CD36 has been suggested to lead to phagocytosis and parasite clearance [210], however there is conflicting evidence that this pRBC/CD36 interaction subverts the functioning of DCs and causes the suppression of

immunological memory[340].

1.2.1.3 Sporozoite/host interactions

A number of sporozoite proteins are implicated in the parasites' migration from the skin to the liver, and in the invasion of hepatocytes. The major sporozoite surface protein, PfCSP, as well as PfTRAP are thought to be important hepatocyte invasion ligands, both suggested to bind to highly sulphated heparan sulphate proteoglycans (HSPGs) on the hepatocyte surface[100, 280]. PfTRSP-depleted parasites are unable to invade hepatocytes[180] and antibodies against ligands including PfSPATR, PfSTARP and PfEMP3 inhibit hepatocyte invasion [96, 128, 180]. Despite this range of potential invasion ligands, the identification of hepatocyte receptors has proved difficult. A recent model for cellular invasion by *Toxoplasma gondii*, another intracellular Apicomplexan parasite, hypothesises that the parasites insert their own invasion receptors into the host membrane[25]. This phenomenon is also thought to occur in *P. falciparum*-mediated RBC invasion where the interaction between PfAMA1 and RBC-targeted PfRON2 is thought to be critical[183]. If *Plasmodium* sporozoites also behave in this way, this may account for why host receptors for cell traversal and hepatocyte invasion have not been characterised.

1.2.1.4 Identification of novel interactions can provide important insights into parasite biology

Of over 5,000 potential protein-coding genes identified from the *P. falciparum* genome[107], we understand the functions of surprisingly few. To understand the intricacies of how *Plasmodium* parasites manipulate their hosts and gain access to a range of cell types, it will be invaluable to identify the interacting proteins of pathogen and host. The identification of these interactions will help us to understand the disease process at a molecular level and could help elucidate new targets for therapeutics. The most interesting proteins in these respects are perhaps those at the cell surface of, and secreted from, the parasite as they make direct contact with host cells and are directly exposed to host proteins and immune effectors. For example, by identifying receptor-ligands pairs in cell invasion, we could design safe, specific antibody or small-molecule inhibitors to target these processes and prevent the parasite from accessing the host cell. Blocking PPIs involved in cell invasion has shown promise for the treatment of viral infections[72, 298] and is being investigated as a therapeutic target for malaria[316, 354]. Similarly, specific drugs that could rapidly prevent the adhesive properties of pRBCs could be a valuable clinical tool[348]. Vaccines too could be based on antigens

that participate in pathological host-pathogen PPIs, inducing long-lasting antibody responses that can block these interactions. An understanding of the interactions that allow the parasite to evade the immune system will also contribute to the development of successful vaccines, advising their rational design to elicit the most effective immune response in the host.

1.2.2 Interaction discovery

1.2.2.1 Studies on candidate proteins

The majority of the known interactions involved in RBC invasion have been discovered following the observation that isolated *Plasmodium* ligands bind to the RBC surface. The identity of the receptors for each of these ligands has been inferred or narrowed-down based on the sensitivity of the interactions to enzymatic treatment of the RBC, or by the inability of ligands to bind RBCs naturally lacking specific receptors[5]. For example, the first of these RBC-binding ligands to be discovered, PfEBA175, could not bind to neuraminidase-treated RBCs, which implicated a sialylated protein as the receptor[46]. PfEBA175 was also unable to bind M^kM^k, Tn or En(a-) RBCs which do not express glycophorin A (GYPA) on their surface, thus leading to the identification of GYPA as the receptor[250, 307].
Similarly, the PfEBA175-related proteins PfEBL1 and PfEBA140 were shown to bind specifically to erythrocytes displaying glycophorins B and C respectively[196, 207]. Likewise a recombinant protein corresponding to a conserved sequence block from PfMSP1 bound to the RBC surface, but could not bind to the surface of RBCs from spherocytosis patients[138], which lack one of a number of cytoskeletal proteins including Band 3 and spectrin. A segment of the Band 3 protein, believed to contain the PfMSP1 binding domain was subsequently shown to block the interaction between PfMSP1 and the RBC surface[116]. PfRH4 was shown to interact with the erythroctyte surface in a neuraminidase-resistant but trypsin and chymotrypsin-sensitive manner. This restricted the potential receptor for PfRH4 to only a handful of proteins, including CR1, antibodies against which were shown to block the binding of PfRH4 to the erythrocyte surface[328]. Similarly, some of the known pRBC/endothelial interactions were initially identified following the observation that pRBCs could bind to a number of cell lines displaying human endothelial surface proteins[299, 339]. Anti-CD36 antibodies were able to prevent this adhesion, leading to CD36's eventual identification as a PfEMP1 ligand[17, 20]. Affinity purification methods have also been applied to the discovery of PPIs involved in malaria. This involves creating an isolatable binding reagent based on a protein-of-interest, incubating this reagent with the potential ligand and demonstrating that

[5]Since RBCs are anucleate it has not yet been possible to engineer cells lacking receptors of interest

the ligand co-purifies with the binding reagent. For instance, the earliest evidence that SELP might be an endothelial receptor for PfEMP1 came from an experiment where PfEMP1-coated beads were incubated with recombinant SELP. SELP could be detected on the surface of these beads, but not negative control beads, by Western blotting[302].

1.2.2.2 Screening approaches

The cell-binding approaches described in Section 1.2.2.1 have relied upon prior knowledge and hypothesis-driven rationale on which to select candidate *Plasmodium* or host proteins for study. The identification of the full repertoire of host-pathogen receptor-ligand interactions will require exhaustive, high-throughput, unbiased screening approaches. In recent years computational analyses have been used to infer novel host-pathogen PPIs occurring in malaria. New candidate interactions have been predicted based on both structural similarities with experimentally-determined interactions and correlation of the gene expression data for host and parasite[278, 362]. These approaches often generate a large number of candidate interactions, which require experimental validation, and refinement is still needed to reduce the false positive and false negative discovery rates.

The yeast-two-hybrid (Y2H) system has been systematically applied to discover nearly 3000 candidate *Plasmodium-Plasmodium* PPIs[181], and has also been used to screen for PPIs occurring between *Plasmodium* proteins and those expressed in the human liver and brain[345]. Whilst Y2H screening has provided a wealth of candidate interactions, and has been used in combination with computational approaches to help define the *Plasmodium-Plasmodium* PPI network[275], it is again limited by high rates of false positives and negatives. The cloning steps used to generate bait and prey libraries mean that only fragments of much larger proteins are screened and that a proportion of proteins will not be represented in the screening library. By expressing *Plasmodium* proteins in a heterologous system such as yeast, it is likely that a proportion of the protein fragments will be not be folded in their native conformation. Improvements in the efficiency of recombinant protein production have enabled high-throughput screening between libraries of full-length proteins, which has led to the identification of two more erythrocyte-merozoite PPIs (see 1.2.3).

Another set of approaches has screened *Plasmodium* proteins against cells that have been transfected to display human receptors-of-interest on their surface. An expresssion-cloning screening approach where COS cells were transfected with endothelial ligands was used to discover ICAM1 as another receptor for pRBCs[24]. More recently a much larger scale microarray-based reverse transfection system has been used to screen PfEMP1 against over 2500 potential human cell surface receptors, leading to the identification of EPCR as a pRBC

receptor[338].

1.2.2.3 *In vitro* validation and kinetic analysis of interactions

Many of the methods used to identify interactions (discussed in 1.2.2.1) are equally appropriate for use in generating evidence to validate the occurrence of an interaction predicted by computational or *in vitro* screening approaches. For example, for an interaction involving an isolated or recombinant RBC receptor, it would be useful to demonstrate that its binding partner could interact with the RBC surface in a manner dependent on the presence and availability of the RBC receptor. Conversely, for interactions discovered between a *Plasmodium* ligand and the RBC surface, it would be useful to validate the identity of the expected RBC receptor by demonstrating that the interaction can take place between isolated proteins. In either case, a routine test of interaction specificity is to demonstrate that the interaction can be blocked by an agent known to bind to either of the interacting proteins (for example, a monoclonal antibody or known ligand, as described in many of the above references[20, 64, 116, 302]). Affinity purification studies are another common approach used to validate interactions. Such experiments can be designed to validate interactions between recombinant proteins (for example between PfMSRP proteins and PfMSP1[215]), but it is preferable to design these experiments such that one binding partner can be used to co-purify, or 'pull down', its interacting protein from its native source, such as a parasite lysate or RBC membrane preparation, as has been demonstrated for the interaction between PfMSP1 and PfMSP7[160].

A number of methods that measure biophysical properties of binding proteins can be used to validate interactions. Specific binding interactions usually display saturable binding kinetics and thus these methods can be adapted to estimate interaction affinities. Surface plasmon resonance (SPR) is a widely used biophysical analysis method that can be used to determine kinetic parameters of interactions occurring between isolated proteins. The technology uses light directed at a metal-coated surface, which is reflected back onto a detector. Some of the light causes electrons to resonate at the chip surface (these electrons are surface plasmons), which results in a loss of intensity of the reflected light at a particular angle of detection. The surface plasmons travel parallel to the metal surface so are sensitive to the presence of other molecules along their path. Thus the binding of molecules to the surface of the chip can be quantified by measuring the shift in the angle where the dip in intensity of the reflected light can be detected. PfRH4/CR1 and PfRH5/BSG binding have been studied in this way, and as such have provided useful, quantitative information about the affinity and half-lives of these interactions[64, 328, 347].

1.2.3 Identifying new interactions: Approaches in the Wright Lab

Whilst hypothesis-driven studies on candidate proteins and a growing number of screening approaches have yielded a myriad of candidate PPIs for further investigation, the identification of additional host receptors for *Plasmodium* proteins has been impeded by technical difficulties. The first of these challenges is to produce sufficient quantities of correctly folded recombinant *Plasmodium* proteins, particularly since the genome of *Plasmodium falciparum* is predominated by A-T base pairs[107], which can be problematic for cloning and protein expression. The next hurdle to identifying host-pathogen PPIs is the low sensitivity of interaction detection methods; extracellular protein-protein interactions are often transient and of low-affinity and therefore not readily detected by classical biochemical purification techniques, which usually require stringent washing steps. In the laboratory where the work described in this thesis was carried out, we aim to overcome these challenges using a specialised protein expression system and a high-throughput interaction screening platform.

1.2.3.1 A mammalian expression system for *Plasmodium* proteins

By developing a high-throughput mammalian expression system based on the transient transfection of cells from the Human Embryonic Kidney line (HEK293 cells), we have been able to express a library of 62 merozoite cell surface and secreted proteins[65, 369]. This has acted as a valuable resource both to discover novel interactions and to use in *in vitro* assays to help determine their function. The details of this expression system are described in Section 2.1, but one of its key features is the use of codon-optimisation to overcome the A-T nucleotide bias that impairs routine molecular biology procedures. This process makes *Plasmodium* protein ectodomains much more amenable to expression in mammalian cells. Using this system, proteins can be pentamerised via the inclusion of a cartilage oligometric matrix protein (COMP) sequence in expression constructs[333]. These pentameric proteins have further increased our capacity to discover and study PPIs by increasing the avidity and stability of the interactions in which they participate (Figure 1.4B).

1.2.3.2 AVEXIS

Avidity-based extracellular interaction screening, or AVEXIS, is a technique developed in the Wright laboratory as a sensitive, high-throughput method for the discovery of protein-protein interactions[40]. Ectodomain regions of receptor proteins are expressed in HEK293 cells, such that protein libraries can be systematically screened against each other. One ectodomain library can be presented as an ordered array of 'bait' proteins immobilised on microtitre plates.

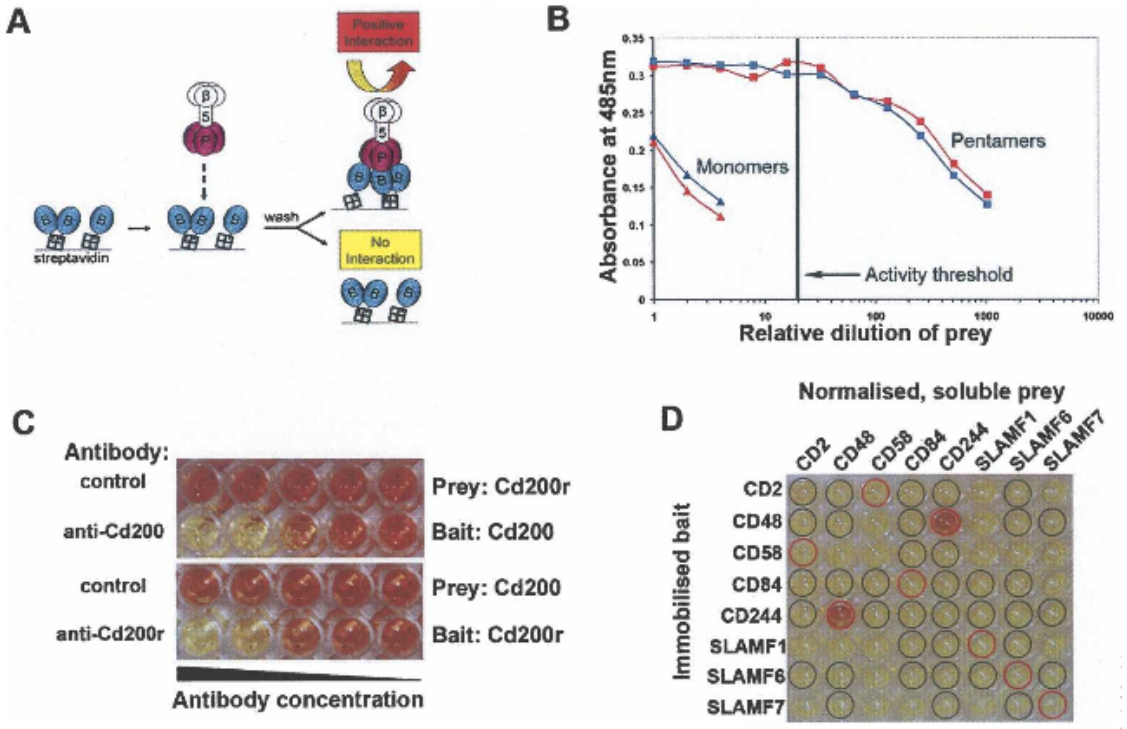


Figure 1.4: **AVEXIS as a highly sensitive interaction screening method**
Figure from *Large-scale screening for novel low-affinity extracellular protein interactions* by Bushell *et al.*, (2008)[40]. Reproduced with permission.
A. Diagramatic representation of the AVEXIS method. Biotinylated bait proteins (blue) are arrayed on the surface of a streptavidin-coated plate and incubated with pentameric prey proteins (red). Interacting prey proteins are captured by the immobilised baits and remain bound following a wash step. The β-lactamase activity of the prey hydrolyses nitrocefin, inducing a yellow-to-red colour change.
B. Pentamerisation of the prey proteins increases the avidity and stability of the Cd200/Cd200R interaction.
C. The specificity of Cd200/Cd200R interaction detection is shown, as an antibody against the bait protein can prevent the binding of the prey.
D. Example of an interaction screen: hits from the screen produce a red colour.

To detect interactions, these plates are probed with another library of pentamerised, enzyme-tagged ectodomain 'prey' proteins (Figure 1.4A). This approach has been used previously in this laboratory to identify receptor-ligand interactions involved in malaria. By screening a panel of merozoite proteins against a library of erythrocyte receptors, two novel interactions were identified and subsequently validated; SEMA7a was identified as a receptor for PfM-TRAP and BSG as a receptor for PfRH5[19, 64]. Both of these interactions are transient, with micromolar affinities calculated in SPR experiments; this demonstrates that AVEXIS is a

highly sensitive method, appropriate for the detection of low-affinity interactions.

1.3 Scope of this thesis

This thesis focuses on the use of existing methods and the development of novel methods for identifying host-pathogen protein interactions in malaria. In screening for interactions, *P. falciparum* merozoite surface protein 7 (PfMSP7) was identified as a potential ligand for human P-selectin (SELP) and the biochemistry of this interaction was characterised in detail. This work is summarised in three results chapters as follows:

Chapter 3: Identification and validation of an interaction between SELP and PfMSP7 This chapter describes the use of AVEXIS to test for novel interactions occurring between recombinant proteins from the *P. falciparum* merozoite and proteins from, but not restricted to, the human platelet. A novel interaction was identified between PfMSP7 and human SELP. These recombinant proteins were shown to be biochemically active and this interaction could also be observed in SPR experiments and in a flow cytometry-based assay developed as part of this project. Evidence is also presented that suggests that recombinant PfMSP7 oligomerises in solution, that this is a property of the protein's N-terminus, and that this oligomerisation might be important for its interaction with SELP.

Chapter 4: Biochemical investigations into the conservation and function of the interactions between *Plasmodium* MSP7s and SELP This chapter describes the biochemical and functional characterisation of the interactions between SELP, PfMSP7 and related proteins. AVEXIS was used to isolate the binding domains as the C-type lectin and/or EGF-like domains of SELP and the N-terminus of PfMSP7, and to screen more widely for interactions occurring between mammalian selectins and *Plasmodium* MSP7-family proteins. This revealed that SELP-binding is a conserved feature of multiple members of the *P. falciparum* MSP7 family. At least one *P. vivax* MSP7 protein also bound to SELP *in vitro* and the SELP/MSP7 interaction looked to be conserved in the *P. berghei* mouse model of infection. These data suggest that SELP-binding might be an important, previously unidentified, role for the PfMSP7 N-terminus and its numerous paralogues. The possibility that the PfMSP7/SELP interaction might play a role in RBC invasion was investigated and largely ruled-out. In *in vitro* binding experiments PfMSP7 could block the interaction that SELP makes with its known ligands, and can thus be implicated as having an immunomodulatory role.

Chapter 5: Development of a biochemical co-purification assay to detect interactions between *Plasmodium* merozoite proteins and human serum proteins This chapter describes the rational design of a biochemical co-purification procedure whereby recombinant *P. falciparum* merozoite proteins are immobilised on superparamagentic beads and used to isolate binding partners from normal human serum. The assay was optimised so as to balance maximal capture of interacting protein against minimal contamination with abundant non-specifically interacting serum proteins. The assay was shown to be very effective at detecting the high-affinity interaction between PfMSP3.4 and human IgM, which is an abundant serum component. The possibility that transient, low affinity interactions can also be detected by this approach was also demonstrated using the interaction between PfRH5 and BSG. A panel of over 50 merozoite proteins were screened for interactions with serum proteins using this method, though no novel interactions were identified.

Chapter 2

Materials and Methods

2.1 Protein production

2.1.1 Plasmid preparation

All plasmids used for protein expression were based on pTT3 which contains an ampicillin resistance marker for selection, an EBV origin of replication to allow amplification in HEK293E cells, and a series of promoter and leader elements to enhance expression[79] (Figure 2.1A). For the expression of mammalian proteins, the endogenous signal peptide was cloned into the vector, whereas the coding sequences of *Plasmodium* proteins were cloned downstream of the leader sequence of the mouse variable κ light chain (Figure 2.1B)[1]. *Plasmodium* protein sequences were codon-optimised for expression in mammalian cells using Life Technologies' GeneArt Service[240]. Since N-linked glycosylation is uncommon in *Plasmodium* (and prevalent in HEK cells), N-X-S/T glycosylation site-encoding motifs in *Plasmodium* constructs were mutated to N-X-A-encoding DNA, prior to gene synthesis.

[1] N-terminally truncated mammalian proteins were also cloned downstream of this leader sequence, without their endogenous signal peptide

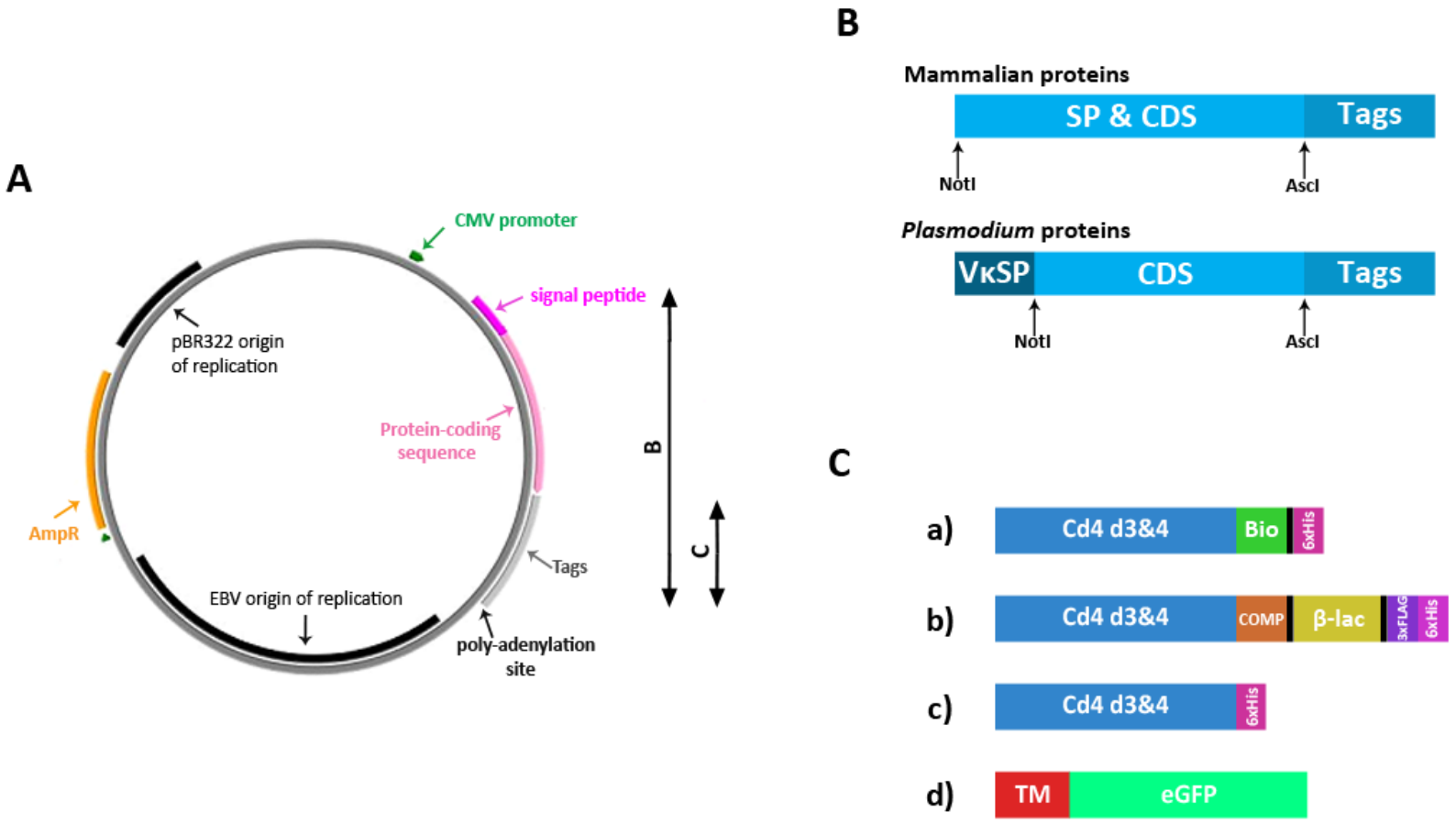


Figure 2.1: **Construction of plasmids for protein expression**
A. Generalised map of pTT3-based expression plasmids used in this work
B. Signal peptide (SP), protein ectodomain-coding sequence (CDS) and restriction-ligation cloning site arrangements for different expression plasmids
C. N-terminal tags on expression constructs included Cd4 expression tags, biotinylation sites (Bio), pentamerisation sequences (COMP), β-lactamase (β-lac) or eGFP reporters, and FLAG- or His-tags. Proteins destined for the cell surface were cloned upstream of the rat Cd200 transmembrane (TM) domain.

Depending on the intended use of the recombinant protein, a selection of different C-terminal tags were incorporated (Figure 2.1C). All secreted proteins were produced as fusions with rat Cd4 domains 3 & 4 (Cd4), which is likely to increase the proteins' solubility and hence expression, and also means that they could be detected via the use of a mouse anti-rat Cd4 OX68 monoclonal antibody[35]. For proteins that were to be biotinylated, an *Escherichia coli* BirA biotin ligase substrate motif was included[36, 40]. A six-histidine tag[141] and/or 3xFLAG® (Sigma) were also included for protein purification and detection. Pentamerisation of proteins was achieved via the inclusion of a pentamerisation domain from cartilage oligometric matrix protein (COMP)[333]. Pentameric proteins were also tagged with a β-lactamase reporter that could be used as a proxy to normalise the amount used in assays (as described in 2.3.1.2). To produce GFP-tagged proteins on the surface of cells, a transmembrane (TM) domain from rat Cd200 was included, along with an eGFP reporter[60].
All plasmid DNA sequences were validated by in-house capillary sequencing. Details of the expression plasmids used as part of this work can be found in Tables 2.4 & 2.5.

2.1.1.1 PCR

Several mammalian protein-coding sequence inserts were cloned from cDNA. PCR primers were designed so as to introduce NotI and AscI sites 5' of the signal peptide and 3' of transmembrane domain respectively. 1µg cDNA for human *SELE*, *SELL* and *SELPLG* (encoding PSGL1), as well as mouse *Selp* (Origene), were used as templates. The reaction was performed using 1U KOD Hot Start DNA polymerase (Novagen) in the manufacturer's buffer supplemented with 1.5mM $MgSO_4$, 1µM primers and 0.2 µM dNTPs. The thermocycler was programmed to carry out an initial five-minute denaturation step at 95°C followed by 25 cycles of denaturation for 15 seconds at 95°C, primer annealing at between 60 and 65°C for 30 seconds and elongation at 72°C for one minute. A final elongation step at 72°C was then carried out for five minutes before purifying the fragments using a QIAquick PCR Purification Kit (QIAGEN). In order to make truncated protein fragments of SELP and PfMSP7, similar PCR methods were applied using existing plasmids as templates. A list of primers can be found in Table 2.1 and details of the boundaries of the SELP and PfMSP7 fragments are provided in Tables 2.2 and 2.3.

2.1.1.2 Restriction-ligation cloning

New plasmids for these studies were constructed using restriction-ligation cloning methods. Vectors and inserts were digested for two hours at 37°C (most commonly using NotI and

AscI, NEB) and the resulting fragments were resolved by agarose gel electrophoresis. Bands were visualised under ultraviolet light and extracted using a QIAquick Gel Extraction Kit (QIAGEN). 20ng of vector and 60ng of insert were used in ligation reactions with T4 ligase (Roche) which were performed at 16°C for 3-12 hours. A 45-second 42°C heat shock was used to transform chemically-competent *E. coli* cells (Agilent) with the ligation products. Cells were plated out on LB-agar containing 100µg/mL ampicillin for selection. Positive clones were cultured overnight in liquid LB and plasmids were prepared for use at 1mg/mL using a PureLink HiPure Plasmid Maxiprep kit (Life Technologies). In-house capillary sequencing was used to verify the success of cloning new plasmids.

2.1.2 Protein expression using HEK293 cells

All recombinant proteins were produced in a mammalian expression system based on the transient transfection of cells from the Human Embryonic Kidney (HEK293) line. The majority of proteins were expressed using HEK293E cells which are stably transformed with Epstein Barr Virus Nuclear Antigen (EBNA1), so as to increase the number of plasmids maintained in transfected cells during protein production. HEK293F cells, which do not possess this EBNA1-based amplification system, were used for the expression of GFP-tagged recombinant proteins targeted to the cell surface.

2.1.2.1 Cell culture

HEK293 cells were maintained in suspension in 50mL Freestyle medium (Life Technologies) at 37°C, 70% humidity, 5% CO_2 and 120rpm orbital shaking. When HEK293E cells were grown, the medium was supplemented with 1% FCS (Life Technologies) and 50µg/mL G418 antibiotic (Sigma).

2.1.2.2 Transfection

24 hours prior to transfection, 50mL fresh medium was seeded with cells to give a final density of 2.5×10^5 cells/mL. For each transfection, 2mL of Freestyle medium, 25µg of expression plasmid and 50µg polyethylenimine (PEI) transfection regent were mixed and incubated together at room temperature for 10 minutes. The mixture was added to the 50mL culture which was then returned to the incubator. In order to produce biotinylated proteins, cells were seeded into Freestyle medium containing the same FCS and G418 supplements along with 100µM D-biotin (Sigma). During the transfection procedure, 2.5µg of a plasmid encoding a secreted

form of *E. coli* biotin ligase (BirA) was added alongside the expression plasmid. The BirA enzyme catalyses the addition of D-biotin to recombinant protein produced with a biotinylation site at their C-terminus (Figure 2.1C).

2.1.2.3 Collection

After four to six days of incubation post-transfection, secreted proteins were collected from the culture supernatant. Cellular material was removed following centrifugation at 3220g for 10 minutes and the resulting supernatant was filtered using 0.2µm filters. The filtered supernatants were stored at 4°C with 50µg/mL polymixin B antibiotic (Sigma). To remove free D-biotin, supernatants containing biotinylated proteins were transferred to 10kDa MWCO Snakeskin dialysis tubing (Thermo Scientific) then dialysed against 4.5L of HBS (0.14M NaCl, 10mM HEPES, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$) at 4°C. To ensure sufficient D-biotin removal, the HBS was replaced seven times over two days.

2.2 Protein purification and quantification

2.2.1 Immobilised metal ion affinity chromatography

6xHis tagged proteins were enriched from cell culture supernatants using 1mL HisTrap nickel columns and ÄKTAxpress purification apparatus (GE Healthcare). To decrease non-specific protein retention in the columns, 10mM imidazole and 200mM NaCl were included in the input protein sample. The HisTrap column was equilibrated with binding buffer (0.5M NaCl, 40mM imidazole, 20mM sodium phosphate buffer, pH7.4), loaded with the input protein, then washed with binding buffer. Elution buffer (0.5M NaCl, 0.4M imidazole, 20mM sodium phosphate buffer, pH7.4) was then flowed through the column and 0.5mL eluate fractions were collected by the apparatus. The ÄKTAxpress readout estimates the absorbance of the output samples at 280nm and was used to identify fractions with a high protein yield, which were then visualised and quality-checked using SDS-PAGE (see 2.2.4). The elution buffer was replaced with HBS during subsequent size-exclusion chromatography (see 2.2.2) or by dialysis (see 2.1.2.3) using 3.5kDa MWCO D-tube Dialysers (Novagen).

2.2.2 Size-exclusion chromatography

To remove protein aggregates from IMAC-purified proteins, fractions were further purified by size-exclusion chromatography (SEC), using a Superdex Tricorn 200 10/600 GL or Superdex

200 Increase 10/300 GL gel-filtration column (GE healthcare). Columns were connected to an ÄKTAxpress system which was used to pre-equilibrate the column with HBS buffer prior to the injection of the sample. After the protein was loaded onto the column, HBS buffer was pumped through the column at a flow rate of 1mL/min and 0.5mL fractions were collected once the void volume of the column was reached. Protein content of the eluting solution was inferred by the absorbance at 280nm, measured in real time by the ÄKTAxpress instrument.

2.2.3 Determination of protein concentration

The approximate total protein content of recombinant protein solutions was measured using their absorbance at 280nm, determined using a benchtop spectrophotometer or Nanodrop (Thermo). The extinction coefficients of proteins of interest were calculated *in silico* allowing the estimation of protein concentration using the Beer-Lambert law.

2.2.4 SDS-PAGE

NuPAGE SDS-PAGE reagents, including 4-12% pre-cast gradient gels (Life Technologies) were used for protein gels. All proteins were denatured proir to electrophoresis. Gels were stained with colloidal Coomassie blue G250 (Fisher) or SYPRO Orange (Sigma), according to the manufacturer's instructions. SYPRO-stained gels were visualised using a Typhoon phosphoimager.

2.2.5 Western blotting

Following SDS-PAGE, proteins were transferred from the gel to a PVDF (GE Healthcare) membrane. The transfer was carried out using NuPage transfer buffer (Life Technologies) supplemented with 10% methanol, an XCell II blot module (Novex) and 40V voltage for two hours at room temperature. To reduce non-specific streptavidin or antibody binding, the membrane was then blocked by incubation with HBS containing 2% BSA overnight at 4°C. To detect biotinylated proteins, the membrane was incubated in 25mL HBS containing 0.2% BSA and 25ng/mL streptavidin-HRP conjugate for one hour at room temperature. When rabbit polyclonal antibodies were used to detect proteins, 25mL HBS containing 2% BSA and 1µg/mL antibody were incubated together overnight at 4°C then incubated with the membrane for one hour at room temperature. The antibody-stained membranes were then transferred to 25mL HBS containing 200ng/mL HRP-conjugated anti-rabbit secondary antibody for one hour. All membranes were washed with HBST for at least one hour at room temperature. 1mL

SuperSignal West Pico enhanced chemiluminescent HRP substrate (Thermo) was applied to the membrane, which was then used to expose Hyperfilm (GE Healthcare).

2.2.6 Polyclonal antibody production and purification

His-tagged Cd4-tagged PfMSP7 was expressed and purified as described in 2.2.1. Approximately 1mg of the protein was sent to Cambridge Research Biochemicals and used to immunise one rabbit. 50mL of the harvest bleed was filtered through a 0.2µm filter then purified using a 1mL HiTrap Protein G HP column (GE healthcare) and the ÄKTAxpress apparatus. The Protein G column was equilibrated with 20mM sodium phosphate buffer (pH7.0), loaded with the input protein, then washed with sodium phosphate buffer. Bound antibody was eluted using 0.1M glycine HCl (pH2.7) buffer. 0.5mL fractions were collected and neutralised by the addition on 60µL 2M Tris-HCl (pH 9.0). The purified antibodies were dialysed against PBS and tested for reactivity against recombinant PfMSP7 and Cd4 domains 3 & 4 bait proteins by ELISA (see 2.3.1.1).

2.3 AVEXIS

Avidity-based extracellular interaction screening (AVEXIS) was performed as directed in Bushell *et al.* (2008), with some alterations[40]. In brief, 100µL bait proteins, normalised as described in 2.3.1.1, were captured on a 96-well streptavidin-coated plate, which was incubated at room temperature for one hour. The plate was then washed three times with HBST and once again with HBS. 100µL normalised prey protein was then added to the wells and incubated at room temperature for 90 minutes. Washes were performed as before and 60µL nitrocefin substrate was added to the wells. Absorbance at 485nm was used to quantify the colour changes after one hour, unless otherwise stated.

2.3.1 Standardisation

To standardise the amounts of cell culture supernatants to be used in AVEXIS, ELISAs were carried out on serial dilutions of bait proteins and the nitrocefin hydrolysis rates of serial dilutions of prey protein were also assessed[40]. If necessary, proteins were concentrated using Vivaspin 20 spin concentrators (Sartorius-stedim) or diluted with HBS containing 1% BSA.

2.3.1.1 ELISA

100µL two-fold serial dilutions of biotinylated proteins were captured on a streptavidin-coated plate (Nunc). After one hour the plate was washed three times with HBS containing 0.02% Tween (HBST), then once with HBS alone. OX68 is a mouse monoclonal antibody with a high affinity for the CD4 tag, and 100µL of a 1.4µg/mL solution was used as the primary antibody. After a further hour's incubation, the plate was washed again and 100µL of anti-mouse IgG coupled to alkaline phosphatase (Sigma A4656, 1:5000) was added to the wells. Following another hour's incubation and washing, 100µL of 1µg/µL phosphatase substrate (Sigma) was added. Substrate hydrolysis was assessed after 30 minutes by measuring absorbance at 405nm on a PHERAstar plus instrument (BMG Labtech).

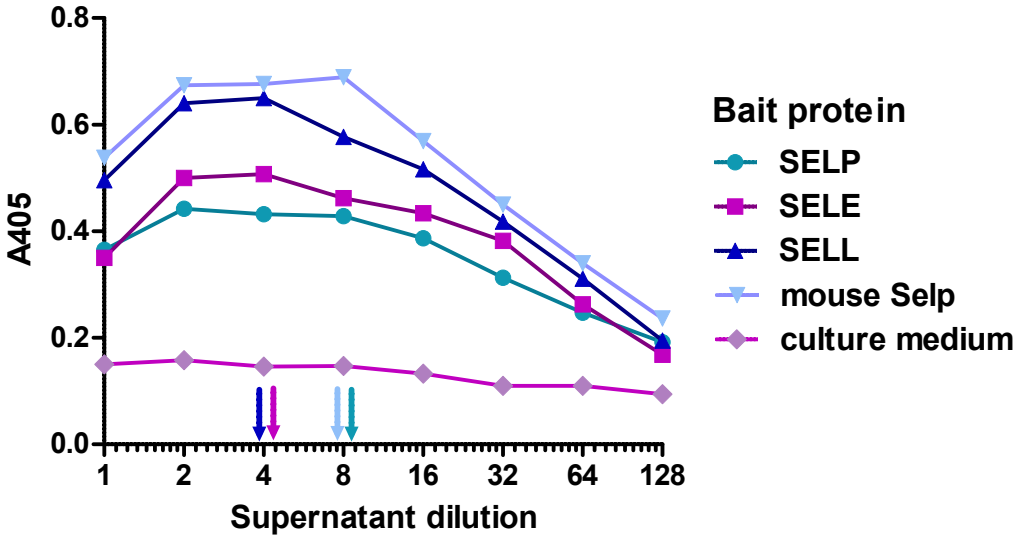


Figure 2.2: **Standardisation of bait proteins by ELISA**
Typical ELISA profiles for biotinylated bait protein supernatants. The arrows indicate the dilution of each protein that was used in subsequent AVEXIS-based assays. Serial dilutions of cell culture medium were used as a negative control, to demonstrate the signals observed were due to the presence of the transfected protein only.

The shapes of the ELISA curves were used to assess protein abundance. If the plate is saturated with biotinylated protein a consistent, high A405 at low dilution factors is expected, with this signal decreasing at higher dilutions of the protein (as gradually less bait is bound to the plate). In reality 'humped' ELISA profiles were often observed (Figure 2.2), for which there could be a number of explanations. Perhaps the most likely explanation when supernatants are used is that the dialysis process has not removed all the available biotin, such that free biotin competes

with biotinylated proteins for the strepatavidin, an effect which is not observed upon dilution as competition for biotin-binding sites decreases. For particularly highly-expressed or purified proteins the curves' shapes may reflect the 'over-crowding' of the plate at low dilutions, such that so much protein is captured on the plate that this impedes access of the OX68 antibody to the Cd4 tag (a manifestation of the prozone effect[42]). It might also be that other abundant proteins in the cell culture supernatant interfere with the specific capture of the biotinylated bait. For AVEXIS, bait proteins were used at the lowest dilution at which absorbance was maximised, so as to balance high levels of protein capture against over-crowding of the bait on the plate surface (indicated in Figure 2.2). Bait proteins that did not show a saturable ELISA signal were spin-concentrated then tested again by ELISA.

2.3.1.2 Nitrocefin hydrolysis assay

To assess their β-lactamase activity, 20µL serial dilutions of prey proteins were prepared in 96-well plates. The absorbance at 485nm was measured every minute for 15 minutes after the addition of 60µL 125µg/mL nitrocefin (Calbiochem) to each well. Prey proteins were used at the concentration where nitrocefin hydrolysis was saturated after ten minutes (Figure 2.3).

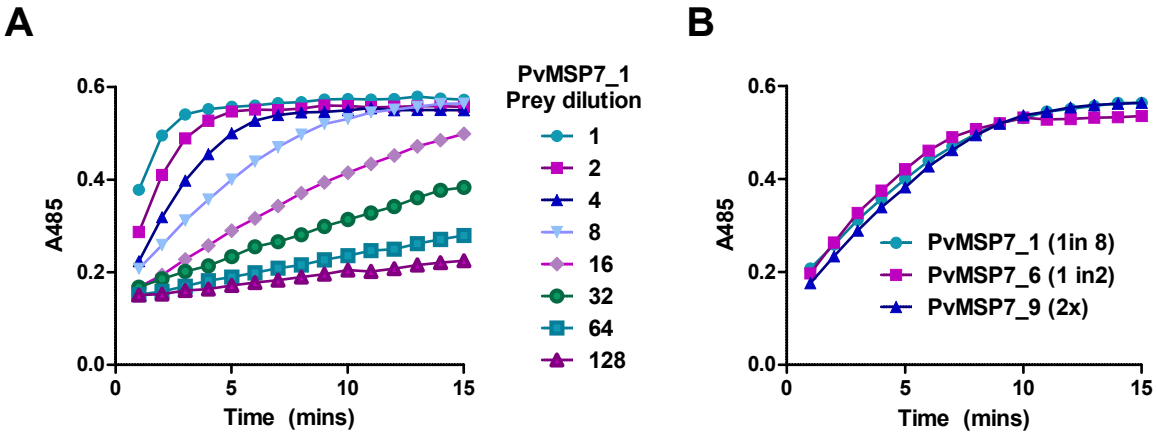


Figure 2.3: **Standardisation of prey protein by measuring nitrocefin hydrolysis**
A. A typical nitrocefin-hydrolysis assay for a single prey protein supernatant.
B. Nitrocefin hydrolysis activity of three *P. vivax* MSP7 protein supernatants at their chosen concentration for use in AVEXIS. The absorbance at 485nm saturates after approximately ten minutes.

2.3.1.3 Data presentation

AVEXIS data in this thesis is predominantly displayed as 485nm absorbance readings (A485). The nitrocefin substrate itself has a measurable A485, so to more clearly delineate binding signals from A485 measurements where no colour change occurs, a reference value was subtracted from each reading. This reference value, usually around between 0.05-0.2 was the mean A485 of the Cd4 bait controls for each prey protein.

2.4 SELP-ligand interaction blocking

2.4.1 sLe^X-SELP interaction blocking

To create a sLe^X-based binding reagent whose presence could be detected by measuring enzymatic activity, biotinylated sLe^X (Glycotech) was incubated with a streptavidin-alkaline phosphatase conjugate (strep-AP, Sigma). To determine the optimum ratio of the two components, a series of binding reagents were created by incubating a range of concentrations of sLe^X with a fixed amount of strep-AP for one hour[2]. The reagents were then used in an AVEXIS-like assay. SELP bait was immobilised on the surface of a 96-well strepatividin-coated plate and incubated with serial dilutions of the reagent. After one hour the plate was washed three times with HBST and once with HBS before 100µL 1mg/ml phosphatase substrate (Sigma) was added. To produce the most avid binding reagent from the sLe^X and strep-AP, it is important to maximise the amount of sLe^X bound to the AP-conjugate. However, if too much 'free' unconjugated sLe^X is present this could bind SELP independently of sLe^X-AP, decreasing the 405nm absorbance (A405) signal observed as a result of SELP binding. Therefore the ratio at highest sLe^X:strep-AP ratio (approximately 5:1) where A405 was still high was used in subsequent assays.

To determine whether PfMSP7 could block the interaction between SELP and sLe^X, SELP bait was immobilised on a 96-well strepatividin-coated plate and incubated with serial dilutions of purified pentameric PfMSP7 for one hour. The plate was washed three times with HBST and once with HBS. The wells were then incubated with sLe^X-AP binding reagent for one hour, washed and incubated with phosphatase substrate. The absorbance at 405nm was measured after 30 minutes and was used to assess the levels of sLe^X binding to SELP.

[2] each strep-AP molecule is expected to bind approximately two sLe^X-bio molecules

2.4.2 THP1 binding assay

THP1 cells were maintained in continuous culture at a density of 10^5-10^6 cells/mL in 50mL RPMI 1640 supplemented with 10% FBS and 2mM L-glutamine at 37°C and 5% CO_2. Saturating quantities of biotinylated proteins were used to coat the wells of a streptavidin-coated microtitre plate as described in 2.3. An anti-PSGL1 (LS-B2507 clone, LSBio) antibody was used as a positive control, to which cells should bind provided they have maintained their expression of PSGL1 receptors on their surface. These immobilised bait proteins were incubated with 10^4 THP1 cells diluted in HBS+1%BSA for one hour. Plates were washed gently by removing the liquid from the wells using a pipette and adding 150µL HBS. Four such washes were performed before counting the cells that remained adhered to the surface using a light microscope. To block interactions between SELP bait and THP1 cells, immobilised baits were incubated with CLB-thromb/6 anti-SELP antibodies (Santa Cruz biotechnology) or purified PfMSP7 pentameric prey each diluted in HBS+1% BSA for 90 minutes, after which plates were washed four times with HBS prior to the addition of cells.

2.5 Surface plasmon resonance

All SPR experiments were performed using a Biacore T100 instrument in combination with SA or CAP chips (GE Healthcare). Both chips use the biotin-streptavidin interaction to capture bait proteins onto their surface. When coating the SA chip, the surface was first 'activated' by three one-minute 30µL/min injection of a 1M NaCl/50mM NaOH solution. CAP chips couple biotinylated proteins onto their surface by means of a DNA-based intermediate, or CAPture reagent. To prepare the surface of the CAP chip for the immobilisation of biotinylated bait proteins, the manufacturer's regeneration solution was injected over the chip surface three times for 60 seconds at a flow rate of 20µL/min followed by a 60-second injection of the HBS running buffer and a five-minute 2µL/min injection of CAPture reagent. In each case, approximately 150 response units (RU), as measured using the Biacore T100 Control software, rat Cd4 domains 3&4 negative control bait was loaded into the first flow cell at a flow rate of 10µL/min. Molar equivalents of each bait protein were loaded into subsequent flow cells. After loading the flow cells, any remaining biotin-binding sites were saturated by injecting a 1nM biotin solution over the chip surface until no further binding responses were observed. All analyte proteins were purified by IMAC and subsequent SEC, and injected over the surface of the chip at a flow rate of 20µL/min for one minute. Binding responses were recorded and subsequently analysed using the Biacore T100 Evaluation software.

2.6 Flow cytometry

2.6.1 Labelling RBC surface proteins

To detect receptors present on the RBC surface, cells were washed, stained with monoclonal antibodies and a fluorescent secondary then analysed by flow cytometry. O-negative RBCs were prepared by centrifugation of 4mL whole blood and 10mL RPMI 1640 at 1800g for 5 minutes. The supernatant, and white blood cells from the top of the pellet, were removed and the remaining RBC diluted to 50% hematocrit with RPMI 1640. These RBCs were diluted 25-fold to 2% hematocrit in PBS containing 2% heat-inactivated FCS. 10µL, approximating to 10^6 cells, were stained on ice for 30 minutes using 1µg primary antibody in a total volume of 100µL PBS/FCS buffer. To remove unbound primary antibody, RBCs were twice pelleted by centrifugation at 450g for 3 minutes then resuspended in 100µL PBS/FCS. A 100µL 1:1000 dilution of Alexa 488-conjugated anti-mouse IgG1 secondary antibody (Abcam) was incubated with the cells on ice for 30 minutes. The washing steps were repeated and the cell pellet was resuspended in 250µL PBS prior to acquisition by flow cytometry using a BD FACSCalibur instrument. The Alexa488 was excited using the 488nm blue laser and its emission was detected using 530/30 filter. 20000 events were counted and the output was analysed using BD FACS Diva and FlowJo (TreeStar) analysis software.

2.6.2 Detecting protein-protein interactions on the surface of HEK cells

HEK293F cells were transfected (as described in 2.1.2.2) to express GFP-tagged receptors on their surface. Successful transfection was verified by fluorescence microscopy. 24 hours post-transfection, 1mL aliquots of 10^6 cells were incubated with 5µg pentameric FLAG-tagged reporter proteins for 1 hour at 4°C, with gentle orbital shaking. Cells were pelleted by centrifugation for five minutes at 200g and resuspended in an HBS buffer supplemented with 1% BSA and 1mM $CaCl_2$. This wash step was repeated before 5µg Cy3-conjugated anti-FLAG antibody (Sigma) was added and incubated at 4°C for one hour. To remove any unbound antibody, the cells were washed three times prior to flow cytometry. A BD LSR Fortessa instrument and FACS Diva software were used to record 10,000 events. The 488nm blue laser was used to excite eGFP, whose fluorescence was detected using a the 530/30 filter. Cy3 was excited via a 561nm yellow laser and fluorescence detected using a 582/15 band pass filter. FlowJo v10 (Tree Star) was used for further analyses. Where appropriate, cells were pre-incubated with 10µg mouse monoclonal IgG1 antibodies for one hour at 4°C, then washed as before, prior addition of the FLAG-tagged prey. These antibodies included the CLB-thromb/6

anti-P-selectin clone and the OX102 anti-rat Cd200R clone (BioLegend).

2.6.3 Platelet staining

Whole blood was isolated from healthy donors on the morning of the experiment. Platelet-rich plasma (PRP) was prepared by isolation of the supernatant following centrifugation of 2mL whole blood for six minutes at 200*g*. When optimising the platelet-staining protocol, 10µL blood or PRP was incubated with 20µL anti-SELP FITC-conjugated antibody or without 10µM ADP agonist in HBS buffer (total volume 50µL). Following a 30-minute incubation the mixture was fixed using 0.5mL formyl saline for ten minutes. A subset of samples were washed twice; the washing process involved centrifugation of fixed blood products for ten minutes at 1000*g*, after which they were resusupended in 0.5mL HBS. When staining platelets with FLAG-tagged reporter protein, 10µL PRP was incubated with 10µM ADP, 0.1-20µM reporter proteins in a total volume of 50µL for one hour, then fixed using 0.5mL formyl saline for ten minutes. Platelets were then washed and incubated with FITC-conjugated anti-FLAG antibody (Sigma) for 30 minutes prior to an additional wash step and analysis by flow cytometry. Stained platelets were examined using a FC500 flow cytometer (Beckman Coulter). FITC was excited using the 488nm blue laser and detected using the 525/40nm filter. 5000 platelets were counted and the data analysed using FlowJo.

2.7 Biochemical co-purifications

Avid merozoite protein reagents were created by conjugating biotinylated IMAC-purified proteins, or biotinylated proteins derived directly from dialysed transfected HEK293E cell-culture supernatant (prepared as described in 2.1.2.3), to streptavidin-coated superparamagnetic beads (Sigma). For each experiment, 100µL beads were washed three times with 1mL PBS using a magnet to isolate beads. Beads were resuspended in 1mL PBS and incubated with merozoite proteins for 30 minutes with rotation. To demonstrate that sufficient protein was provided to saturate the beads, ELISAs were performed on the supernatant as described in 2.3.1.1. If biotinylated protein could be detected then beads were deemed to be saturated. To remove unbound biotinylated protein, the beads were washed three times with 1mL PBS then resuspended in 100µL. Human serum (Sigma) was filtered through a 0.2µm filter before use to remove any aggregated protein before incubation with protein-coated beads for one hour at 4°C. Beads and their bound proteins were isolated using a magnet and washed five times with ice-cold PBS. Proteins remaining associated with the beads were eluted in 100µL 1% SDS for

five minutes. 25µL of the eluate was used in SDS-PAGE (see 2.2.4) and the gel was stained with SYPRO Orange (Sigma). See Figure 5.2 for a schematic representation of the method.

2.7.1 Mass spectrometry

SDS-PAGE-resolved elutants from co-purification assays were fixed with 40% methanol and 2% acetic acid for one hour and stained with a colloidal Coomassie (Sigma) overnight at 4°C. The background was cleared using 25% methanol for two hours then the gel was washed in water. Bands for analysis were isolated, then de-stained by incubation with an equal mixture of 50mM Ammonium Bicarbonate pH8.5 and acetonitrile (AmBic/CH_3CN) for 30 minutes at 37°C and 600rpm shaking. The AmBic/CH_3CN was replaced and incubation repeated until the blue colour of the stain was removed. De-staining was completed by incubating the gel pieces with 1mL CH_3CN for 30 minutes at 37°C, removing the liquid and allowing any remaining CH_3CN to evaporate. To digest any proteins in the bands, gel pieces were covered with 500µL AmBic containing 1µg/mL trypsin (Roche) and incubated for two hours at 37°C, then overnight at 25°C whilst shaking at 600rpm. The resulting peptides were then extracted from the surrounding liquid. Peptides were eluted from the gel by successive incubations of the gel pieces with a 50%CH_3CN/ 0.25% formic acid (Sigma) mixture. Pooled supernatants from each elution were dried completely to leave peptides, which were later resuspended in 40µL 0.5% formic acid prior to mass spectrometry, which was performed by the in-house mass spectrometry team. Peptides were analysed by LC-MS/MS on an Ultimate 3000 RSLCnano System (Dionex) coupled to a LTQ FT Ultra (Thermo Fisher) hybrid mass spectrometer. The raw mass spectrometry data was processed in Proteome Discoverer (V1.4) (Thermo Fisher) using Mascot v2.4 (Matrix Science) to assign protein sources for the detected peptides. The protein databases were a database of human proteins downloaded from Uniprot (as of February 2013) and a database of common contaminants. The reported protein/peptide list used a Mascot ion score cut-off of 30 with 0.05 as significance threshold.

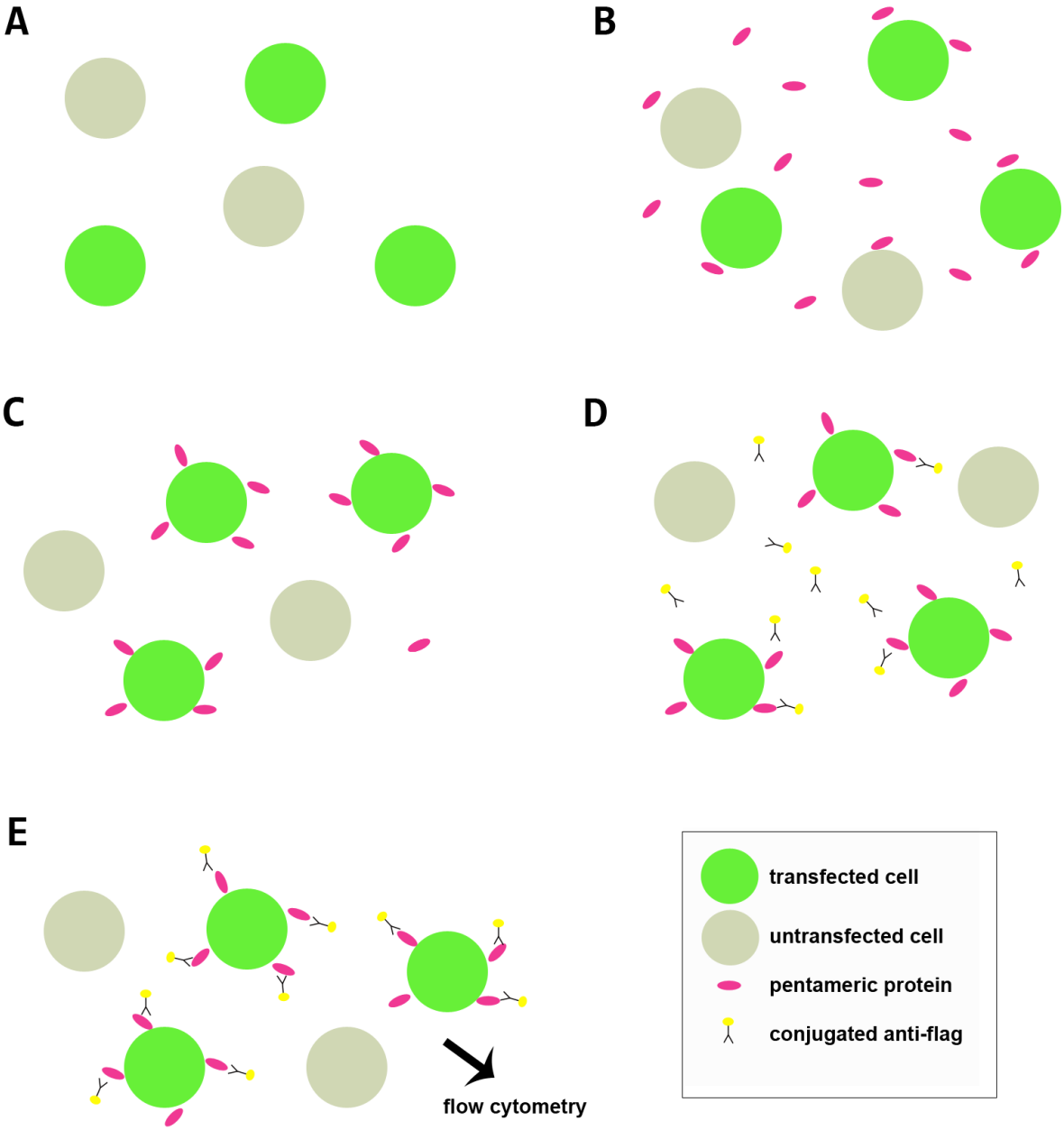


Figure 2.4: **HEK293F cell-surface binding assay**
A. Cells are transfected with recombinant receptor proteins with a cytoplasmic eGFP tag
B. Cells are incubated with FLAG-tagged pentameric *Plasmodium* proteins
C. Cells are washed to remove unbound pentamers
D. Cells are incubated with Cy3-conjugated anti-FLAG antibody.
E. Cells are washed to remove unbound antibody and are analysed by flow cytometry.

Primer name	Template	Sequence
SELP_F	SELP_BioLHis plasmid	TTTAAACTGCGGCCGCCACCATGGCCAACTGCCAGATCGCCATCC
SELP_EGF_F	SELP_BioLHis plasmid	TTTTTGCGGCCGCCACCTACACCGCCAGCTGCCAGGACATGA
SELP_CTL_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGCTGGCGGTGTAGCACAGGGCGTGC
SELP_EGF_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCCCGCACGTACTCGCACTCAGGGCCG
SELP_S3_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGCTGATGGCTTCACAGGTGGGCAGA
SELP_S6_R	SELP_BioLHis plasmid	TAGTAGGGCGCGCCGATGGCCTCGCACATGGCGGGGCTG
Mouse_SELP_F	cDNA	TTTAAACTGCGGCCGCCACCATGGCTGGCTGCCCAAAAGGTTCCT
Mouse_SELP_R	cDNA	TAGTAGGGCGCGCCACCCAAGTACGTCAGAGCTTCCTGG
SELE_F	cDNA	TTTAAACTGCGGCCGCCACCATGGTTGCTTCACAGTTTCTCTCAG
SELE_R	cDNA	TAGTAGGGCGCGCCGGGAATGTTGGACTCAGTGGGAGCT
SELL_F	cDNA	TTTAAACTGCGGCCGCCACCATGGTATTTCCATGGAAATGTCAGAG
SELL_R	cDNA	TAGTAGGGCGCGCCGGGGTTATAATCACCCTCCTTAATC
SELPLG_F	cDNA	TTTAAACTGCGGCCGCCACCATGGCTCTGCAACTCCTCCTGTTGC
SELPLG_R	cDNA	TAGTAGGGCGCGCCCTGCTTCACAGAGATGTGGTCTGGG
MSP7_Nterm_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCCACCCCCGTGAACAACGAAGAGGAC
MSP7_Nterm_R	MSP7_Bio plasmid	TAGTAGGGCGCGCCCTGGGCCTTCACTTTGGACAGCACG
$MSP7_{22}$_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCGAGCGAGACAGACACCAGAGCAAAGA
$MSP7_{19}$_F	MSP7_Bio plasmid	TGTCTGGTGCGGCCGCGGAAGTGCAGAAACCAGCCCAGGGCG
MSP7-Cterm_R	MSP7_Bio plasmid	TAGTAGGGCGCGCCCATGGTGTTCAGCAGATTGAAGATG

Table 2.1: **PCR primers**
Restriction sites are higlighted in red, sequences complimentary to pTT3-based plasmids in blue, and those annealing to cDNA in green.

SELP region	Amino acids	Sequence
'Full length'	1-771	MANC.....IQEA
SELP->CTL	1-162	MANC.....YTAS
SELP->EGF	1-208	MANC.....EYVR
SELP->S3	1-385	MANC.....EAIS
SELP->S6	1-570	MANC.....CEAI
SELP_EGF domain	159-208	YTAS.....EYVR

Table 2.2: **SELP protein fragment boundaries**
Truncated SELP proteins were produced comprising the regions indicated in Figure 4.2A using expression plasmids constructed using the primers detailed in Table 2.1. The amino acids from the endogenous SELP protein sequence (P16109 in Uniprot), alongside the four corresponding N-terminal and C-terminal amino acids for each construct, are shown here. The EGF domain was expressed downstream of the mouse variable κ light chain signal peptide. All proteins were expressed with a C-terminal rat Cd4 domains 3&4 tag.

PfMSP7 region	Amino acids	Sequence
'Full length'	28-351	TPVN.....LNTM
MSP7_N	28-176	TPVN.....VKAQ
$MSP7_{22}$	177-351	SETD.....LNTM
$MSP7_{19}$	195-351	EVQK.....LNTM

Table 2.3: **PfMSP7 protein fragment boundaries**
Expression plasmids for recombinant PfMSP7 protein fragments, as depicted in Figure 4.5A, were produced using the primers detailed in 2.1. The amino acids from the PfMSP7 protein sequence (PF3D7_1335100) and the four N-terminal and C-terminal PfMSP7 amino acids included in each construct are indicated here. All PfMSP7 constructs were expressed downstream of the mouse variable κ light chain signal peptide and included the C-terminal rat Cd4 domains 3&4 tag.

Plasmid name	Ectodomain	Accession number	Tags	Use	Constructor
SELP_BioLHis	HsSELP	P16109	Bio, His	AVEXIS, SPR	Dr Y. Sun
BSG_Bio	HsBSG	P35613-1	Bio	AVEXIS	Dr C. Crosnier
Cd4_Bio	RnCd4 domains 3&4	P05540 (partial)	Bio	AVEXIS, SPR, BCP	Dr S. J. Bartholdson
Platelet_BioLHis	HsPECAM1/HsAPLP2/HsCD59...	see Table 3.1	Bio, His	AVEXIS	Dr Y. Sun
MSP7_βlac	PfMSP7	PF3D7_1335100	COMP,β-lac	AVEXIS	Dr C. Crosnier
Merozoite_βlac	PfMSP1/PfMSP2/PfMSP4...	see Table 3.2	COMP,β-lac	AVEXIS	Dr C. Crosnier
MSP7_Bio	PfMSP7	PF3D7_1335100	Bio	AVEXIS	Dr C. Crosnier
MSP1_Bio	PfMSP1	PF3D7_0930300	Bio	AVEXIS	Dr C. Crosnier
SELP_βLFH	HsSELP	P16109	COMP,β-lac, FLAG, His	AVEXIS, FC	Dr Y. Sun
MSP7_His	PfMSP7	PF3D7_1335100	His	SEC, SPR	B. McDade
SELP_His	HsSELP	P16109	His	SEC, SPR	A. J. Perrin
Cd200_βLFH	RnCd200	A0A5D0	COMP,β-lac, FLAG, His	FC	Dr Y. Sun
Cd200R_TMGFP	RnCd200R	Q9ES58	TM, eGFP	FC	Dr G. J. Wright
MSP7_βLFH	PfMSP7	PF3D7_1335100	COMP,β-lac, FLAG, His	AVEXIS, FC	A. J. Perrin
SELP_TMGFP	HsSELP	P16109	TM, eGFP	FC	A. J. Perrin
MSP7_TMGFP	PfMSP7	PF3D7_1335100	TM, eGFP	FC	A. J. Perrin
SELP->CTL_BioLHis	HsSELP CTL domain	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->EGF_BioLHis	HsSELP CTL & EGF domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->S3_BioLHis	HsSELP CTL, EGF & 3xSCR domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP->S6_BioLHis	HsSELP CTL, EGF & 6xSCR domains	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
SELP_EGF_BioLHis	HsSELP EGF domain	P16109 (partial)	Bio, His	AVEXIS	A. J. Perrin
Cd200R_βlac	RnCd200R	Q9ES58	COMP,β-lac	AVEXIS	Dr K. M. Bushell
Cd200_BioLHis	RnCd200	A0A5D0	Bio, His	AVEXIS	M. Gallagher

Table 2.4: **Summary of expression plasmids used**
Proteins were produced with appropriate C-terminal tags, as described in Figure 2.1C. These proteins were then used in AVEXIS, surface plasmon resonance (SPR), Flow cytometry (FC), size-exclusion chromatography(SEC) and biochemical copurification (BCP) experiments. Plasmids are sorted according to the order in which they are first used in subsequent chapters. Accession numbers correspond to identifiers in Uniprot or PlasmoDB.

Plasmid name	Ectodomain	Accession number	Tags	Use	Constructor
MSP7_N_βLFH	PfMSP7 excluding PfMSP7$_{22}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7$_{22}$_βLFH	PfMSP7$_{22}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7$_{19}$_βLFH	PfMSP7$_{19}$	PF3D7_1335100(partial)	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
MSP7_N_His	PfMSP7 excluding PfMSP7$_{22}$	PF3D7_1335100(partial)	His	SEC	A. J. Perrin
MSP7$_{22}$_His	PfMSP7$_{22}$	PF3D7_1335100(partial)	His	SEC	A. J. Perrin
EFNB2_βLFH	HsEFNB2	Q2PDH7	COMP,β-lac, FLAG, His	FC	Dr Y. Sun
SELL_BioLHis	HsSELL	P14151	Bio, His	AVEXIS, SPR	A. J. Perrin
SELE_BioLHis	HsSELE	P16581	Bio, His	AVEXIS, SPR	A. J. Perrin
MSRPs_βLFH	PfMSRP1/2/3/4/5	PF3D7_1335000/4800/4600/4400/4300	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
Selp_BioLHis	MmSELP	Q01102	Bio, His	AVEXIS	A. J. Perrin
PbMSP7s_βLFH	PbMSP7/MSRP1/MSRP2	PBANKA_134910/920/900	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
PvMSP7s_βLFH	PvMSP7_1/6/9	PVX_082700/675/655	COMP,β-lac, FLAG, His	AVEXIS	A. J. Perrin
SELP R57H_BioLHis	HsSELP R57->H	rs7529463	Bio, His	AVEXIS	A. J. Perrin
SELP A156T_BioLHis	HsSELP A156->T	rs72712022	Bio, His	AVEXIS	A. J. Perrin
SELP A161P_BioLHis	HsSELP A161->P	rs142790885	Bio, His	AVEXIS	A. J. Perrin
SELP G179R_BioLHis	HsSELP G179->R	rs3917718	Bio, His	AVEXIS	A. J. Perrin
MSP7_COMPHis	PfMSP7	PF3D7_1335100	COMP, His	sLe^X	A. J. Perrin
MSP2_COMPHis	PfMSP2	PF3D7_0206800	COMP, His	sLe^X	A. J. Perrin
Cd4_BioLHis	RnCd4 domains 3&4	P05540 (partial)	Bio, His	BCP	Dr C. Crosnier
Merozoite_bait	PfAARP/PfAMA1/PfASP...	see Table 5.3&5.4	Bio +/- His	BCP	various
EBA175_His	PfEBA175	PF3D7_0731500	His	BSI	Dr M. Wanaguru
Rh5_His	PfRh5	PF3D7_0424100	His	BSI	Dr S. J. Bartholdson

Table 2.5: **Summary of expression plasmids used (continued)**
These proteins were primarily used in AVEXIS, but a some were also analysed by SPR, SEC, in a plate-based assay to demonstrate that PfMSP7 can block the interaction between sLe^X and SELP (sLe^X), in biochemical purifications (BCP) or back-scattering interferometry (BSI) experiments.

Chapter 3

Identification and validation of an interaction between SELP and PfMSP7

3.1 Introduction

This chapter describes the screening of *Plasmodium falciparum* merozoite cell surface and secreted proteins for interactions with human platelet proteins, and the subsequent identification and biochemical validation of an interaction between *P. falciparum* merozoite surface protein 7 (PfMSP7) and human P-selectin (SELP).

3.1.1 Rationale for screening

Extracellular host-pathogen protein-protein interactions are vital to the pathogenesis of disease and represent potential anti-malarial drug and vaccination targets (Section 1.2.1.4). High-throughput, unbiased screening approaches such as AVEXIS (Section 1.2.3) are powerful tools to identify novel interactions of this nature[40]. AVEXIS has had proven success with the discovery of, amongst others, the interaction between BSG and PfRH5, which is essential for parasite entry into RBCs and is thus an ideal and specific target for therapeutics[64]. This interaction was discovered via the screening of a library of recombinant *P. falciparum* merozoite cell surface and secreted proteins[65] against a panel of proteins from the RBC surface. In this work I screened a subset of the same merozoite protein library against proteins from the human platelet. A library of almost 200 recombinant platelet proteins had already been created in our laboratory, and within this library there are a large number of potential target proteins with which merozoite proteins could interact during disease. Platelets are implicated in both exacerbating disease and protecting the host, with reports that they are directly capa-

Platelet protein name	Uniprot Accession Number
PECAM1	P16284
SELP	P16109
APLP2	Q06481
BSG	P35613
CD59	P13987
ESAM1	Q96AP7
FURIN	P09958
GP6	Q9HCN6
ICAM2	P13598
PRNP	P04156
SCARF	Q14162
TMED1	Q13445
LAMP2	P13473
MET	P08581

Table 3.1: **Platelet bait proteins selected for AVEXIS screening**

ble of killing merozoites[211, 264] but are also responsible for the pathological clumping of pRBCs[259] and potentially contribute to the development of cerebral malaria[263]. Hence, detecting interactions between parasite and platelet proteins could contribute to our understanding of these processes at a molecular level. Many of the proteins in this platelet library are not restricted to the platelet surface, appearing on many other cell types with which *Plasmodium* parasites interact, for example endothelial cells and leukocytes. A large number are also released into the bloodstream as soluble proteins, many of which are important players in the immune response[346]. Hence interactions between proteins from the platelet and merozoite libraries could form part of a range of processes occurring in malaria, including cellular adhesion, platelet-mediated parasite killing and parasite-mediated immune evasion.

3.1.2 Selection of proteins for screening

For an initial screen I shortlisted high-expressing proteins from each library. These included 14 platelet bait proteins (see Table 3.1), each with evidence linking them to a role in immune functionality. For example, PECAM1, CD59, FURIN and PRNP have been implicated as regulators of T-cell maturation and functionality[146, 148, 168, 331], which *Plasmodium* parasites may suppress[224, 320]. PECAM1 and CD59 are also thought to affect B-cell development[148, 168] and thus have a role in the humoral immune response that protects the host from chronic or repeated infections, commonly observed with *P. falciparum*. APLP2 and

Merozoite protein name	PlasmoDB identifier
MSP1	PF3D7_0930300
MSP2	PF3D7_0206800
MSP4	PF3D7_0207000
MSP5	PF3D7_0206900
MSP10	PF3D7_0620400
Pf12	PF3D7_0612700
Pf38	PF3D7_0508000
ASP	PF3D7_0405900
AMA1	PF3D7_1133400
MTRAP	PF3D7_1028700
MSP3	PF3D7_1035400
MSP7	PF3D7_1335100
Pf41	PF3D7_0404900
Rh5	PF3D7_0424100
PF10_0323	PF3D7_1033200
AARP	PF3D7_0423400
MSP3.4	PF3D7_1035700
Pf12p	PF3D7_0612800
PF11_0373	PF3D7_1136200
PF14_0293	PF3D7_1431400

Table 3.2: **Merozoite prey proteins selected for AVEXIS screening**

LAMP2 are important regulators of antigen presentation[337, 373], FURIN and SCARF participate in signalling pathways that modulate the production of parasite-killing cytokines[305, 331], and CD59 has a well-characterised role in the complement cascade[168]. A subset of the selected proteins (PECAM1, SELP, ESAM, ICAM2, SCARF, MET) can also be found on the surface of endothelial cells, and of these PECAM1, SELP and ICAM2 have been associated with the recruitment of leukocytes to sites of inflammation[111, 148, 206]. Unsurprisingly for a group of platelet proteins, many also have known roles in haemostasis[7, 148, 152, 234], an important process to blood-dwelling pathogens[318]. I included BSG in the shortlist primarily to use as a positive control, known to interact with PfRH5[64]. However, BSG is also an interesting candidate to screen in its own right as it has been implicated as playing roles in cellular adhesion, leukocyte migration and RBC longevity[368], all of which could affect the survival of *Plasmodium* parasites in their host. The initial selection of merozoite prey proteins was more arbitrary; I chose 20 proteins from the library that were known to express at medium to high levels (see Table 3.2).

3.1.3 A brief introduction to SELP and PfMSP7

SELP, also known as GMP-140, PADGEM, CD62(P) and LECAM3, is a 140kDa glycoprotein expressed on the surface of activated platelets and endothelial cells. The protein is comprised of a C-type lectin domain, EGF-like domain and nine short consensus repeats as shown in Figure 4.2. Via its N-terminal C-type lectin domain, SELP binds to glycoprotein ligands such as leukocyte cell surface PSGL1 via sialyl Lewis-X (sLe^X) tetrasaccharide moieties on their surfaces. This property allows SELP to be an endothelial ligand to which leukocytes loosely adhere, or 'roll' in the early stages of inflammation. It thus contributes to the recruitment of leukocytes to vessel walls[71, 229].

The *PfMSP7* gene encodes a 40kDa precursor protein, and it is this full length protein that was used in the original screen. The precursor is believed to be proteolytically processed then exported from the merozoite. PfMSP7 interacts with PfMSP1 and a fragment from the C-terminus is retained on the merozoite surface, as part of the PfMSP1 complex (see Figure 3.1)[258]. The biological roles of these proteins, and those in their broader protein families, will be discussed in detail in Chapter 4.

3.2 Results

3.2.1 PfMSP7 and SELP interacted in an AVEXIS screen

To identify novel host-pathogen receptor-ligand interactions involved in the pathogenesis of malaria, I made use of the *P. falciparum* merozoite and human platelet protein expression constructs that were available in our laboratory. I shortlisted a panel of proteins from each library to screen against each other by AVEXIS, so as to identify interactions that merozoite proteins could be making with host immune effector targets. For this screen I produced 14 high-expressing human proteins with a known role in immune functionality as biotinylated 'baits' and 20 high-expressing merozoite proteins as pentameric β-lactamase-tagged 'preys'. Interactions were identified by observation of a colour change in the nitrocefin substrate; when a merozoite prey protein binds to one of the arrayed baits its β-lactamase enzyme cleaves the yellow substrate to produce a red binding signal, which can be quantified by measuring the solution's absorbance at 485nm (see 2.3 and Figure 1.4A). Of the merozoite prey proteins shortlisted for screening (see Table 3.2), I excluded three from the analysis; PfASP because it produced a binding-like signal when screened against the Cd4 negative control bait, and Pf12p and PF14_0293 as they did not produce any observable colour change when screened against the OX68 positive control bait. The remaining 17 merozoite proteins interacted with

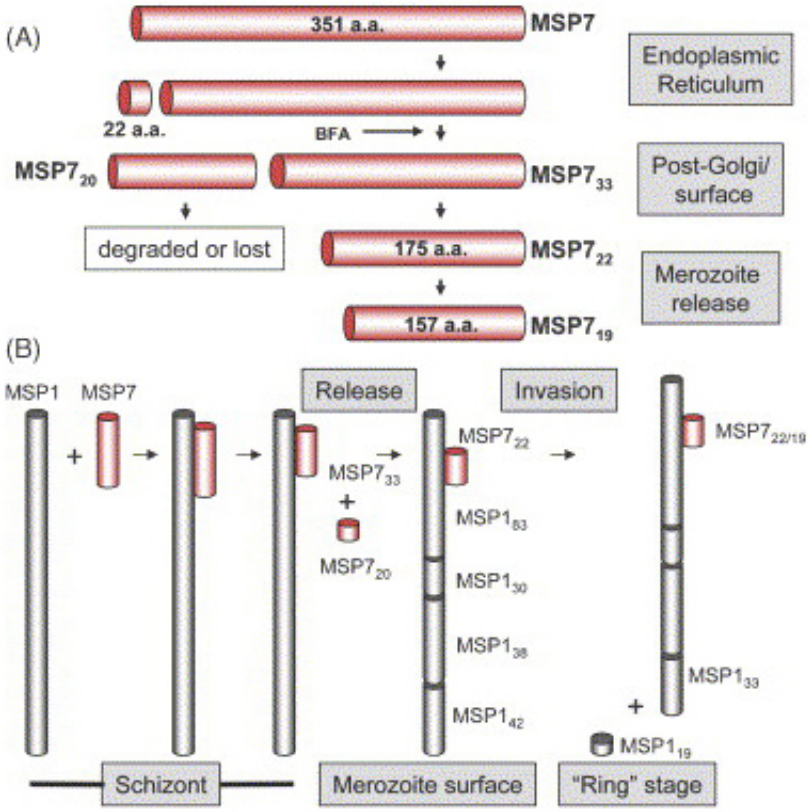


Figure 3.1: **PfMSP7 is proteolytically processed**
Figure from *Extensive proteolytic processing of the malaria parasite merozoite surface protein 7 during biosynthesis and parasite release from erythrocytes* by Pachebat *et al.* (2007)[258]. Reproduced with permission.
A. The PfMSP7 precursor protein is proteolytically processed in multiple steps. PfMSP7 is initially cleaved to release a 20kDa fragment from its N-terminus, then the 33kDa fragment remaining from the C-terminus of the precursor is N-terminally cleaved to leave a 22kDa fragment. This 22kDa fragment from the C-terminus of PfMSP7 is displayed on the surface of the merozoite as part of the MSP1 complex. In isolates containing glutamine at position 194 this fragment is cleaved again at the merozoite surface to leave a 19kDa fragment in this complex
B. The full-length PfMSP7 precursor protein is thought to associate with PfMSP1 soon after translation, after which PfMSP7 and PfMSP1 are processed concurrently.

the OX68 bait and not with the Cd4 tag bait, and were thereby judged to have been appropriately standardised for screening (Figure 3.2A). This original small-scale screen yielded two hits: the known interaction between PfRH5 and BSG (Figure 3.2C) and a potentially novel interaction between PfMSP7 and SELP (Figure 3.2B).

To further validate interactions discovered by AVEXIS we aim to demonstrate the interactions in both bait-prey orientations. PfMSP7 bait protein and SELP prey were seen to interact by AVEXIS, although the colour change observed was significantly slower than that seen when the assay was performed using in the reciprocal bait:prey orientation (where PfMSP7 is used as the prey, see Figure 3.3A). The PfMSP7 bait was not able to capture and retain sufficient β-lactamase-tagged SELP prey to saturate nitrocefin hydrolysis within one hour. Whist AVEXIS is not a quantitative assay, this result implies that the SELP prey/PfMSP7 bait interaction is not as strong as the PfMSP7 prey/SELP bait interaction. This could mean that the arrangement of proteins where SELP is pentamerised and PfMSP7 is arrayed on a surface is not optimal for the observation of the interaction[1]. SELP is thought to dimerise on the surface of platelets and endothelial cells, and this dimerisation has been shown to be important in increasing the avidity of the interactions SELP makes with its known ligands[16, 274]. It is possible that this SELP arrangement is replicated more accurately when the proteins are arrayed (as baits) as opposed to pentamerised via their COMP sequence (as preys).

3.2.2 The recombinant SELP and PfMSP7 proteins were biologically active

When working with recombinant proteins, especially those produced in heterologous expression systems, it is important to ensure that they are correctly folded so as to be biologically active. Fortunately, both SELP and PfMSP7 have previously-identified interacting partners which can be utilised to assay the functionality of our recombinant proteins. I was able to demonstrate that PfMSP7 prey binds to a recombinant PfMSP1 bait by AVEXIS (Figure 3.3A) and that SELP bait binds an alkaline phosphatase (AP)-conjugated sLe^X reagent[2] (Figure 3.3B), indicating that both recombinant proteins are functional and active. SELP was unable to bind to a recombinant PSGL1 in either bait:prey orientation, though this may be explained by a lack of essential post-translational modifications on the PSGL1 surface, since

[1] This is not an unusual phenomenon for AVEXIS. In fact in a large scale screen for interactions between zebrafish receptors only 56 of the 100 of heterophilic interactions identified could be observed in both bait-prey orientations[205]. These included a handful of known interactions, including those between Robo and Slit proteins involved in *Drosophila* development[34, 167].

[2] This sLe^X-AP reagent was created by saturating streptavidin-AP (Sigma) with biotinylated sLe^X (Glycotech)

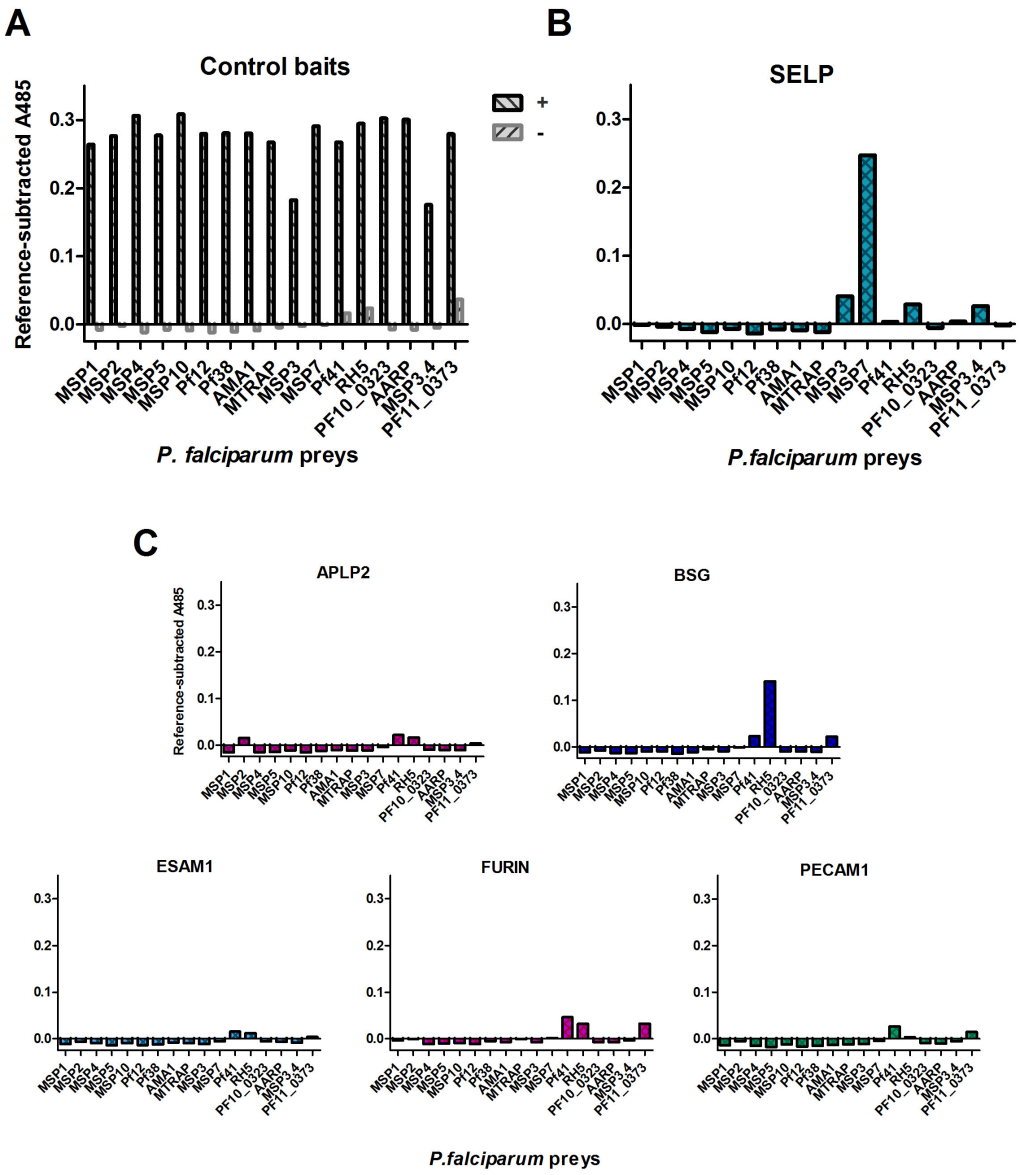


Figure 3.2: **SELP bait interacted with PfMSP7 prey**
All bars represent reference-subtracted AVEXIS signals as described in Section 2.3.1.3
A. AVEXIS signals when 17 *P. falciparum* merozoite prey proteins were screened against controls. Each prey protein included in the analysis produced a strong signal upon screening against positive control bait (OX68 antibody) and minimal signal when screened against the negative control (rat Cd4 tag region)
B. AVEXIS signals when SELP bait was screened against merozoite preys. SELP bait interacted with PfMSP7, but not with any other merozoite preys.
C. AVEXIS signals when additional platelet bait proteins were screened against the merozoite proteins. The signals observed for five of the 14 baits are shown. The only other significant signal observed was when BSG bait was incubated with PfRh5 prey. This is a known interaction.

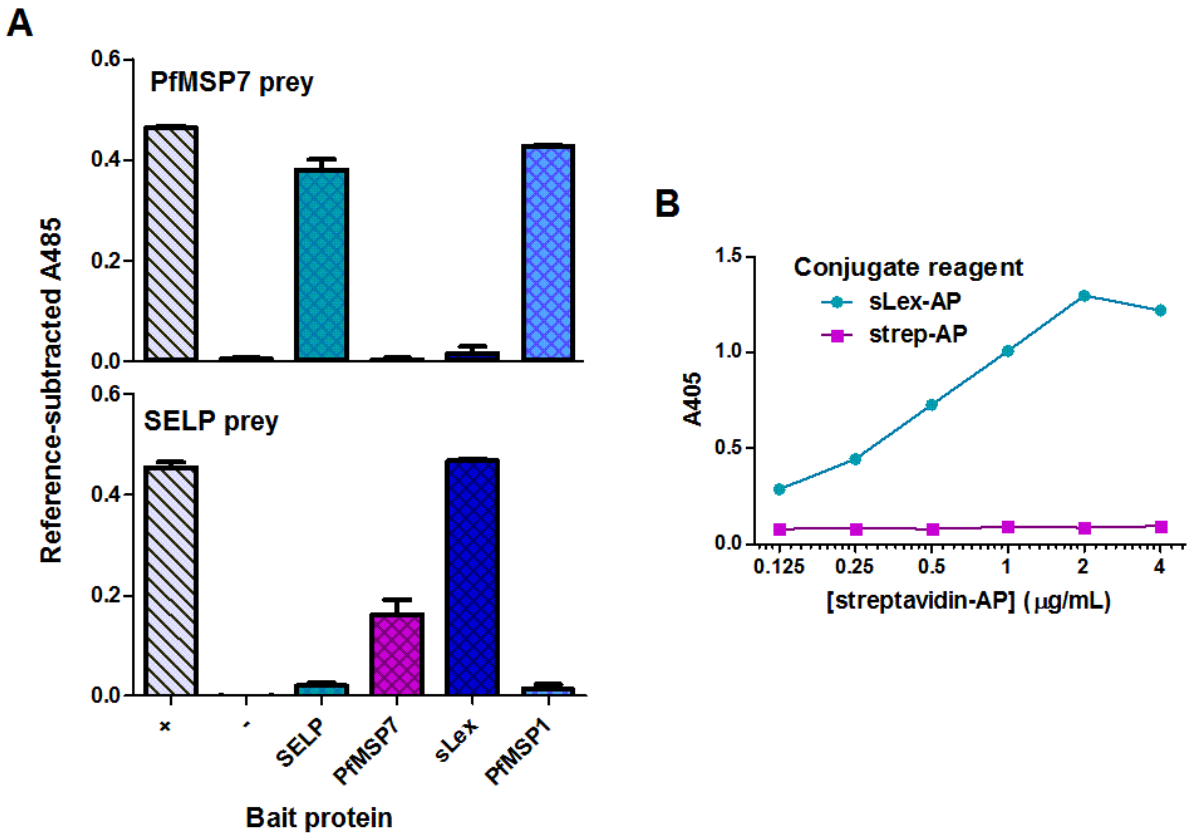


Figure 3.3: **Recombinant, soluble SELP and PfMSP7 preparations were biochemically active**

A. AVEXIS signals when PfMSP7 and SELP preys were screened against known ligands. PfMSP7 prey bound to PfMSP1, as well as SELP, bait. SELP prey bound to sLe^X and PfMSP7. Both prey proteins were also screened against OX68 positive control bait (+) rat Cd4 tag region negative control bait (-). Bars represent means +/- SD, n=3.

B. Alkaline phosphatase(AP) mediated substrate activity when streptavidin-AP conjugates were incubated with SELP bait. A sLe^X conjugate bound to SELP bait. The bait did not bind the streptavidin-AP used to create the conjugate.

co-transfection of a fucosyltransferase may be required to ensure that sLe^X is incorporated into the molecule[291]. It is also worth noting that both PfMSP7 and SELP proteins were expressed at high levels, which is in itself a good indicator that the proteins are correctly folded prior to secretion.

3.2.3 The SELP/PfMSP7 interaction could be blocked by an anti-SELP mAB

To provide further evidence for the correct folding of SELP, I tested the binding of the recombinant protein to a commerically-available anti-SELP monoclonal antibody (mAB). The antibody bound to recombinant SELP by ELISA. However the binding of this CLB-thromb/6 antibody was not affected by denaturation of the recombinant SELP bait (Figure 3.4B), indicating that the CLB-thromb/6-binding epitope is not conformation-sensitive. This means that the binding of the antibody cannot be used as an indicator of correct protein folding. To provide more evidence for the specificity of the SELP/PfMSP7 interaction, I showed that the binding of the antibody to immobilised SELP bait blocks the binding of PfMSP7 prey (Figure 3.4A). This result may indicate that there is some overlap between the binding sites for CLB-thromb/6 and PfMSP7 on SELP, and/or may result from the steric effect of the antibody, impeding access of PfMSP7 pentamers to their binding sites. Additionally, this result means that this particular antibody clone could be a useful reagent to block the interaction in further studies.

3.2.4 PfMSP7 bound to recombinant SELP at the cell surface

To demonstrate that PfMSP7 can bind to SELP at the cell surface, and to verify the interaction in a different experimental system, I developed a flow cytometry-based binding assay (described in Figure 2.4). By transfecting HEK293F cells with plasmids encoding protein ectodomains fused to a transmembrane domain and green fluorescent protein (GFP), I was able to over-express receptors at the cell surface with a cytoplasmic GFP tag (Figures 2.1C & 3.5A). I incubated the transfected cells with pentamerised, FLAG-tagged proteins and then with a Cy3-conjugated anti-FLAG antibody. I then used flow cytometery to distinguish fluorescent populations. I used the interaction between rat Cd200 and Cd200R proteins to optimise the experimental parameters of the assay, expressing Cd200R at the cell surface and incubating the cells with Cd200 pentamer. I observed a strong correlation between GFP signal and Cy3 fluorescence, indicating that Cd200 protein bound specifically to transfected cells, at levels

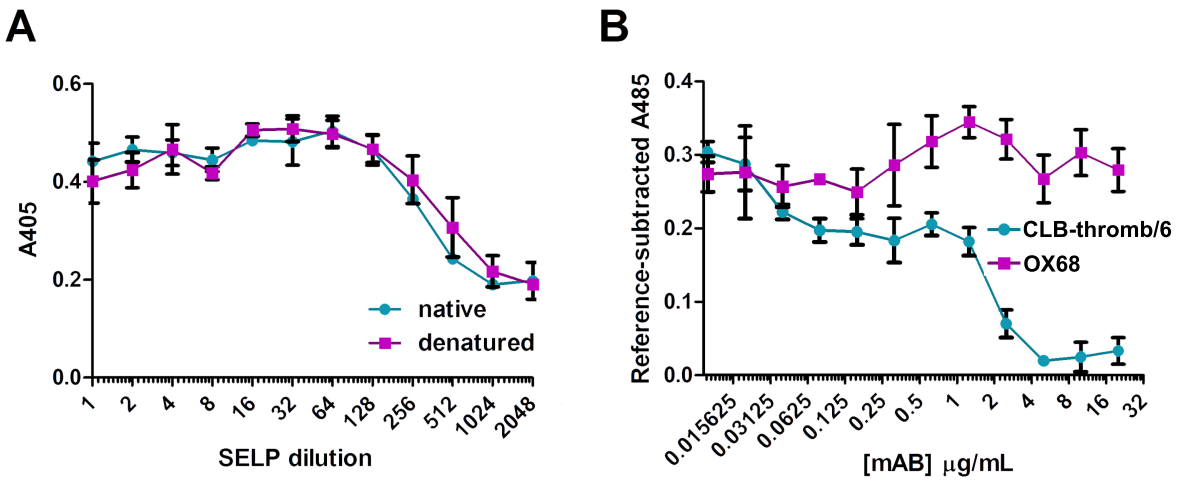


Figure 3.4: **An anti-SELP mAB blocked the SELP/PfMSP7 interaction**
A. ELISA using CLB-thromb/6 to detect recombinant SELP. The CLB-thromb/6 binding epitope on SELP is not conformation-sensitive, since boiling the protein for ten minutes with 1% SDS prior to capture did not affect the result.
B AVEXIS signals resulting from the interaction between SELP bait and PfMSP7 prey, blocked by incubating the bait protein with CLB-thromb/6, a mAB against SELP. The interaction was not blocked with OX68 mAB, which binds to the Cd4 tag region of SELP.
Error bars represent means +/- SD, n=3.

proportionate to the receptor concentration (Figure 3.5B). The interaction could be blocked by pre-incubating cells with OX102 anti-Cd200R monoclonal antibody, indicating that the Cd200 pentamers are binding to the cell surface specifically via their interaction with the transfected Cd200R.

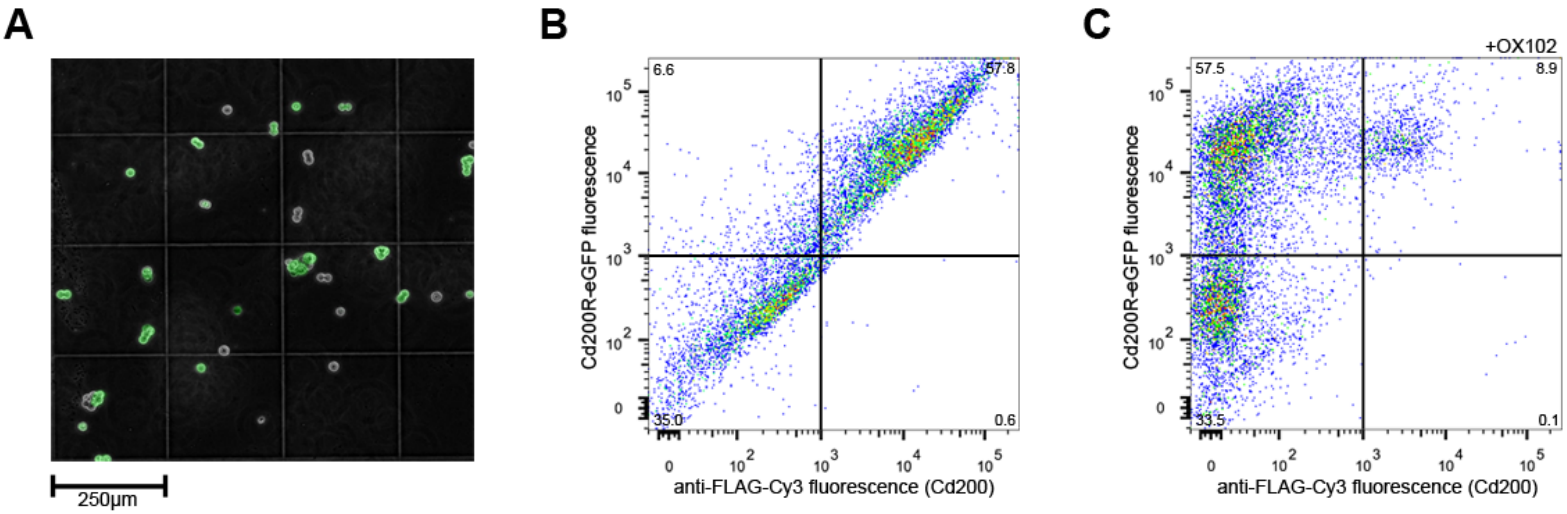


Figure 3.5: **Development of flow cytometry-based assay to measure protein binding to HEK cells**
A. Fluorescence microscopy on HEK293F cells 24 hours after transfection with a GFP-tagged Cd200R expression construct. Over half of the population of cells were fluorescent.
B. Flow cytometry dot-plots when Cd200R-eGFP transfected cells were incubated with Cd200. Cd200-FLAG pentamers bound specifically to Cd200R-eGFP transfected cells, as observed by the correlation of eGFP and Cy3 fluorescence intensity.
C. Flow cytometry dot-plots when OX102-blocked Cd200R-eGFP transfected cells were incubated with Cd200. The shift in the eGFP-positive cells from the double positive quadrant (top right) of the dot plot (in B) to the eGFP-positive quadrant (top left), shows that OX102 blocked the interaction between transfected cells and the Cd200 ligand.
5,000 events within forward and side-scatter parameters that facilitate counting of HEK cells are displayed on the above dot plots.

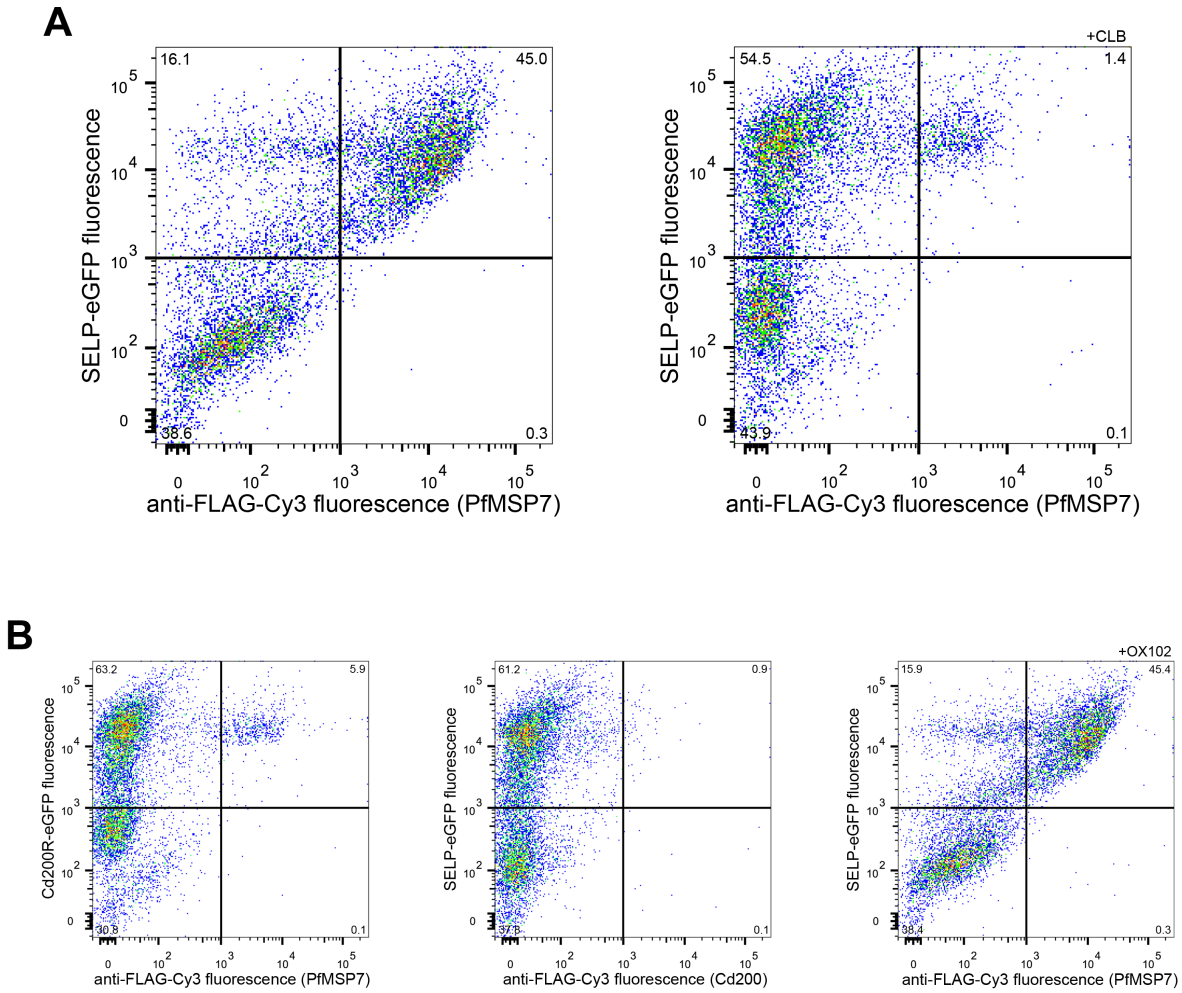


Figure 3.6: **PfMSP7 pentamers bound specifically to SELP at the cell surface**

A. Flow cytometry dot-plots when SELP-eGFP transfected cells were incubated with pentameric FLAG-tagged PfMSP7 bound to cells expressing GFP-tagged SELP on the cell surface. FLAG-tagged PfMSP7 interacted with eGFP-positive cells (left), but not those that were blocked by an anti-SELP mAB (right).

B. Dot plots of three negative controls. All negative control samples behaved as expected; Cd200R positive cells did not bind the PfMSP7 pentamers (left), SELP-transfected cells did not interact with Cd200 (centre) and mABs not directed against SELP could not block the SELP-PfMSP7 interaction (right).

5,000 events within forward and side-scatter parameters that facilitate counting of HEK cells are displayed.

Similarly, I could detect a specific interaction between SELP receptors and PfMSP7 pentamers (Figure 3.6). PfMSP7 prey did not bind to untransfected cells, or cells transfected with Cd200R-GFP. The CLB-thromb/6 anti-SELP monoclonal antibody that I earlier found to block the interaction (Section 3.2.3) could also block the interaction between the PfMSP7 and SELP-transfected cells. Antibodies against Cd4 or BSG (which is present on the HEK cell surface) did not block the interaction (Figure 3.6B). This further demonstrates that PfMSP7 bound to the cell surface specifically via its interaction with SELP. I was unable to express sufficient PfMSP7 at the cell surface to perform the assay using SELP prey.

3.2.5 Recombinant PfMSP7 formed metastable oligometric complexes in solution

To isolate monomeric PfMSP7 for use in surface plasmon resonance studies, I purified the 6xHis-tagged protein by immobilised metal ion affinity chromatography (IMAC) and then separated the elutant by size-exclusion chromatography (SEC). I observed high batch-to-batch variability in the elution profiles from each SEC experiment and observed multiple peaks in the majority of profiles (see Figure 3.7A). SDS-PAGE analysis on the eluted fractions confirmed that each peak contained only PfMSP7 proteins, indicating that PfMSP7 exists in multiple oligometric states (Figure 3.7B). The gel filtration column is calibrated using a series of globular protein standards, allowing the estimation of the molecular mass of proteins based on their elution volume. However, the volume at which a protein elutes is dependent on its size and conformation, not its molecular mass alone, such that elution volume more accurately reflects the hydrodynamic volume (V_{H}) or radius of gyration (R_{g}) of the eluting species[319]. In most cases the slowest-eluting fractions had a V_{H} equivalent to a globular protein with a molecular mass of at least 100kDa; if these proteins correspond to PfMSP7 monomers this would imply that PfMSP7 adopts a more extended conformation than globular proteins of an equivalent (~60kDa) mass. The earliest-eluting PfMSP7 fractions, with V_{H}s equivalent to a globular protein hundreds of kDa in mass, are certainly higher-order PfMSP7 complexes. The degree to which the PfMSP7 preparations oligomerise did not appear to be related to protein concentration or the length of time the protein had been stored.

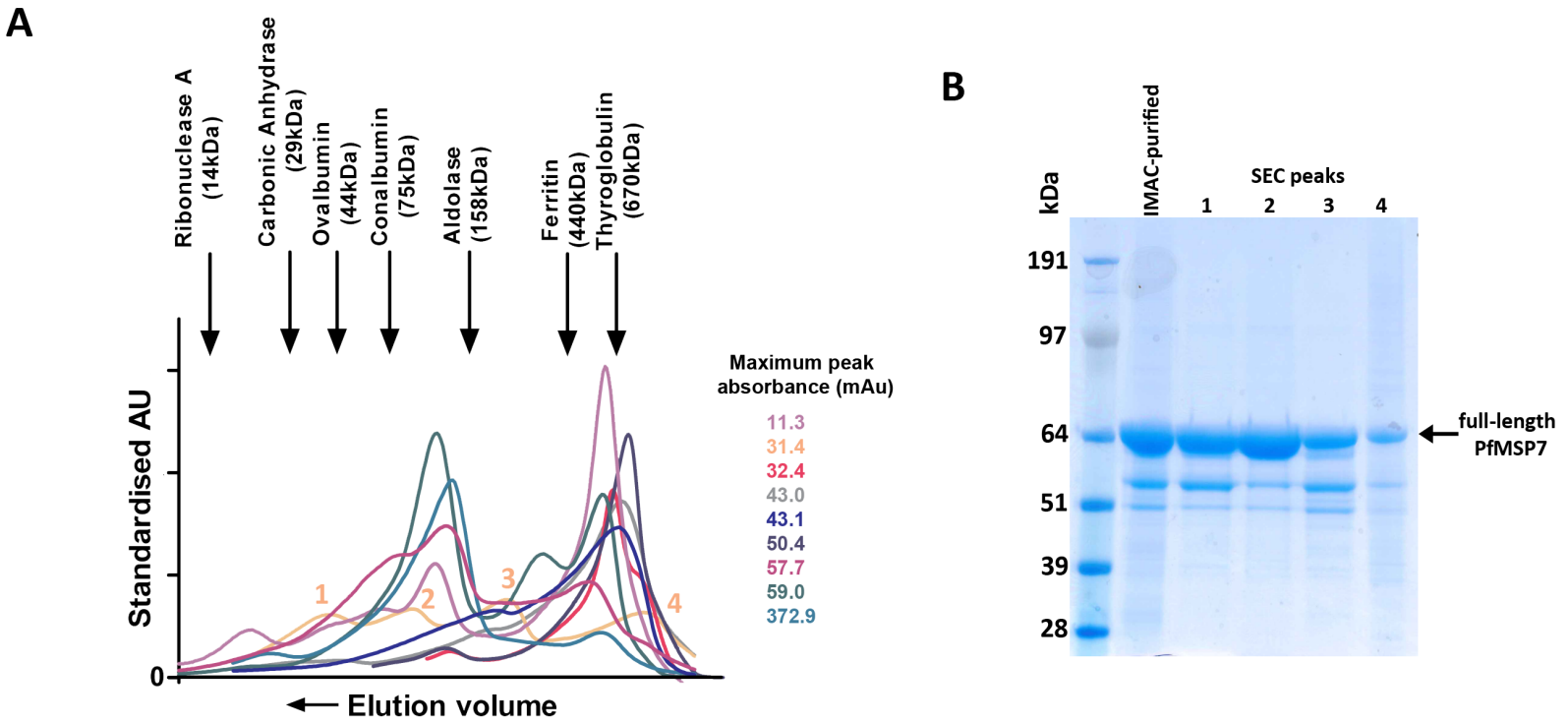


Figure 3.7: **PfMSP7 formed oligomers *in vitro***

A. Nine independent PfMSP7 gel filtration (SEC) profiles. The profiles were highly variable but reliably contained complexes containing multiple PfMSP7 molecules. As two different columns were used in the course of this work I have used the calibration data to convert elution volumes to an estimate of molecular mass, so as to more accurately compare profiles between columns. The traces shown are standardised for input protein amount by equalising the area under the curve for each protein. The recorded peak absorbance value for the highest peak in each trace is indicated.

B.SDS-PAGE analysis confirmed that each peak is comprised of PfMSP7 proteins. 15µL of the four 3µM SEC eluate fractions from the indicated peaks in A were resolved alongside 2µL of 25µM IMAC purified PfMSP7.

3.2.5.1 The flexible N-terminus of PfMSP7 was responsible for self-oligomerisation

PfMSP7 undergoes proteolytic cleavage to leave a 22 or 19kDa fragment from the C-terminus on the surface of the merozoite and fragments as part of the MSP1 complex, and N-terminal fragments that are thought to be degraded or lost from the merozoite (Figure 3.1)[158, 258]. To determine whether a particular portion of the molecule is responsible for the formation of metastable oligomers, I expressed and purified the 22kDa fragment of MSP7 ($MSP7_{22}$) and the remaining portion from the N-terminus (referred to hereafter as MSP7-N). and analysed both proteins by size-exclusion chromatography (SEC) (Figure 3.8A & C). Each of three independent PfMSP7-N preparations exhibited very different SEC elution profiles. This was similar to the behaviour I observed when analysing full-length PfMSP7 (Figure 3.7) and suggested that the N-terminus was the source of this potential oligomerisation activity and variability in the SEC elution traces. Corroborating this, three SEC profiles of $PfMSP7_{22}$ preparations were very similar to one another (Figure 3.8B & C). They showed a single predominant peak, which could theoretically correspond to a monomeric protein, and a smaller peak comprising oligometric protein, indicating that this C-terminal region is not the source of the variable oligomerisation behaviour observed in PfMSP7.

3.2.5.2 'Intrinsic disorder' in PfMSP7

Oligomerisation of *P. falciparum* merozoite surface proteins has been reported previously, with SEC elution profiles for PfMSP2, PfMSP3 and PfMSP3 fragments yielding multiple peaks with large predicated hydrodynamic radii[2, 118, 144]. It is thought that both PfMSP2 and PfMSP3 proteins self-assemble into amyloid like filaments, and it has been hypothesised that these assemblies promote binding to RBC surfaces prior to invasion[144, 372]. To investigate whether the same sort of oligometric self assembly may be occurring in PfMSP7, I looked for amino acid sequences that might promote this behaviour. I did not identify any regions of similarity to the leucine-zipper region or aggregation motif that are speculated to be involved in PfMSP3 self-assembly, or with the N-terminal region of PfMSP2 which is thought to be responsible for its oligomerisation[144, 366]. The formation of amyloid fibrils, similar in their structure to those seen in PfMSP2 and PfMSP3, is associated with flexible regions in component protein monomers[370]. By running the PfMSP7 amino acid sequence through the several algorithms that predict flexible or 'disordered' regions in protein structure, I identified that the PfMSP7 sequence is very likely to contain disordered regions, particularly towards its N-terminus (Figure 3.8D)[95]. The four algorithms PrDOS[147], Disprot[363], Disopred3[157] and IUPred[73] predict 44-75% of the amino acid residues comprising MSP7-N to be part

of disordered regions, whereas the percentage for $MSP7_{22}$ is only 18-22% (see Figure 6.1C). This suggests that the N-terminus of PfMSP7 is intrinsically disordered.

In recent years there has been growing recognition that a large proportion of proteins, particularly those in eukaryotes[350][3], lack the ordered, globular domain structures that are commonly associated with binding or catalytic functions[361]. These proteins are characterised by flexible structures, which have been shown to adopt more ordered conformations upon binding to their (often numerous) ligands. This property has been associated with the ability of proteins to perform multiple functions and to bind multiple targets, and could contribute to the tendency of PfMSP7 to self-associate[332].

3.2.6 PfMSP7 binding to SELP was observed using SPR

Surface plasmon resonance (SPR) is a leading method for determining the kinetic parameters of a protein-protein interaction. I attempted to use this approach to determine equilibrium binding measurements for the interaction between monomeric PfMSP7 and SELP. Upon injecting PfMSP7 analyte over immobilised SELP bait, I observed a clear binding response, indicating that the two proteins interact directly (Figure 3.9). Fitting the association curves to different binding models indicated that complex, multivalent binding of PfMSP7 to SELP took place, which was anticipated given the observed tendency for PfMSP7 to oligomerise (see 3.2.5). Where I observed multiple peaks in SEC elution profiles, I often observed significantly greater binding signals when PfMSP7 complexes of larger molecular mass (but equivalent amount of PfMSP7 molecules) were injected over SELP (Figure 3.9B). The binding of a larger complex will in itself increase the SPR signal which is inherently sensitive to the size of the binding analyte, though given the magnitude of this increase it could be speculated that these larger complexes are also binding more avidly. More detailed kinetic data are required to confirm this. In all cases where PfMSP7 species were injected, dissociation of PfMSP7 analyte appeared to be very slow, suggesting that this is a highly avid interaction (Figure 3.9).

Whilst these data validate the interaction between SELP and PfMSP7, their binding characteristics meant that the interaction was not particularly amenable to kinetic analysis. Firstly, binding did not saturate rapidly, such that a long injection time, and consequently a large volume of PfMSP7 analyte would be required to perform equilibrium binding analyses. Unfortunately I was unable to generate sufficient purified PfMSP7 analyte to perform these analyses.

[3] and as much as half of those in *Plasmodium* species [94]

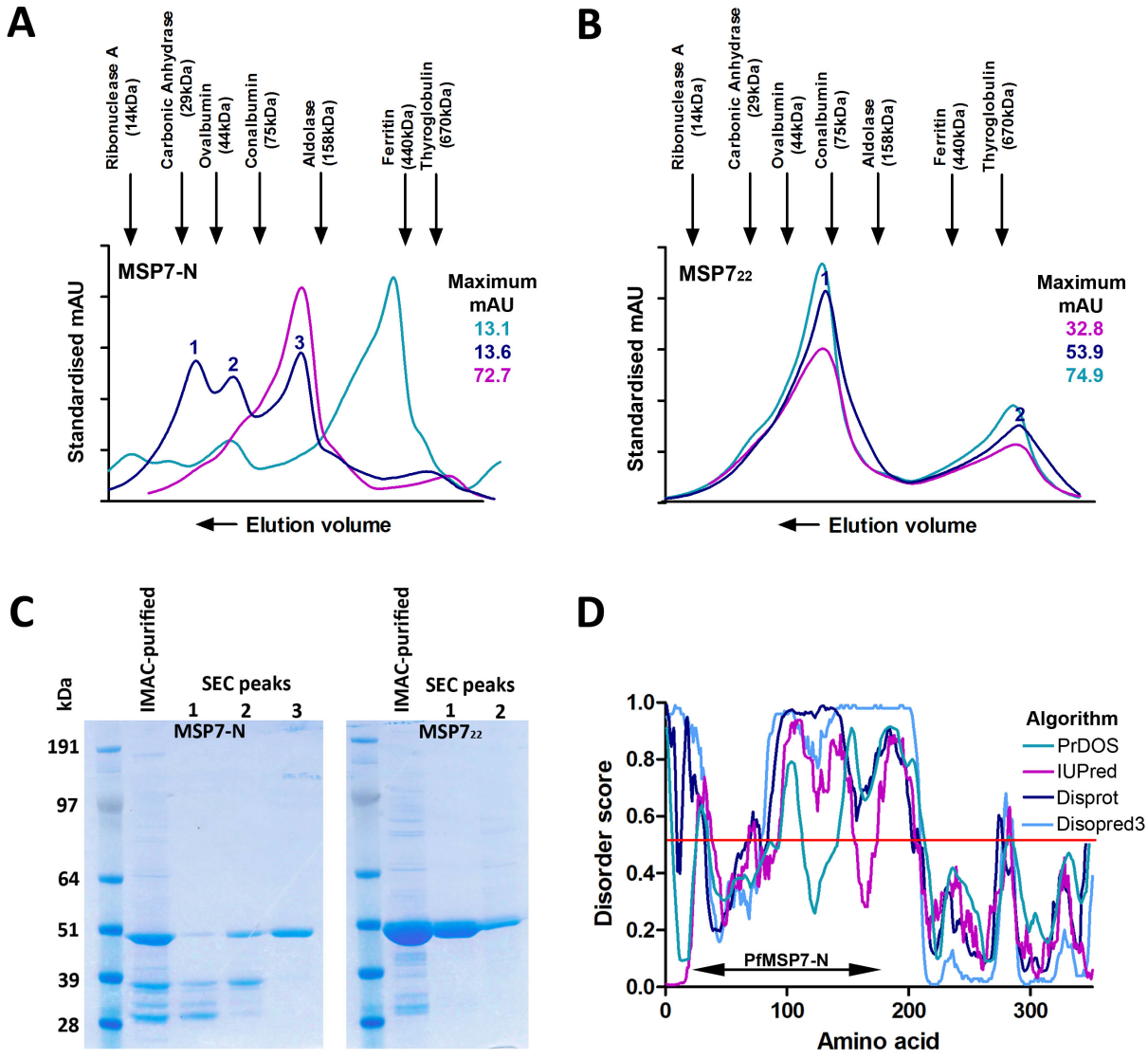


Figure 3.8: **PfMSP7-N showed similar oligomerisation behaviour to full-length PfMSP7**
A. SEC elution profiles from 3 independent PfMSP7-N preparations. As observed with full length PfMSP7 there is great batch-to-batch variabilty, with profiles showing multiple peaks.
B. SEC elution profiles from 3 independent PfMSP7$_{22}$ preparations. These profiles are much more consistent, with one predominant peak and a smaller peak containing higher order complexes.
C. SDS-PAGE analysis of each of the indicated Cd4 domain-tagged PfMSP7-N or PfMSP7$_{22}$ peaks confirms that each contains PfMSP7 fragments of the expected size. It is possible that the peak 1, with the smallest predicted V_H, from the PfMSP7-N SEC elutant did not contain PfMSP7 proteins.
D. Protein disorder scores predicted by four different algorithms IUPred, Protein disorder prediction system (PrDOS), Disprot and Disopred3. The N-terminal region of PfMSP7 is predicted to be significantly disordered, which may account for the formation of metastable oligomers in solution.

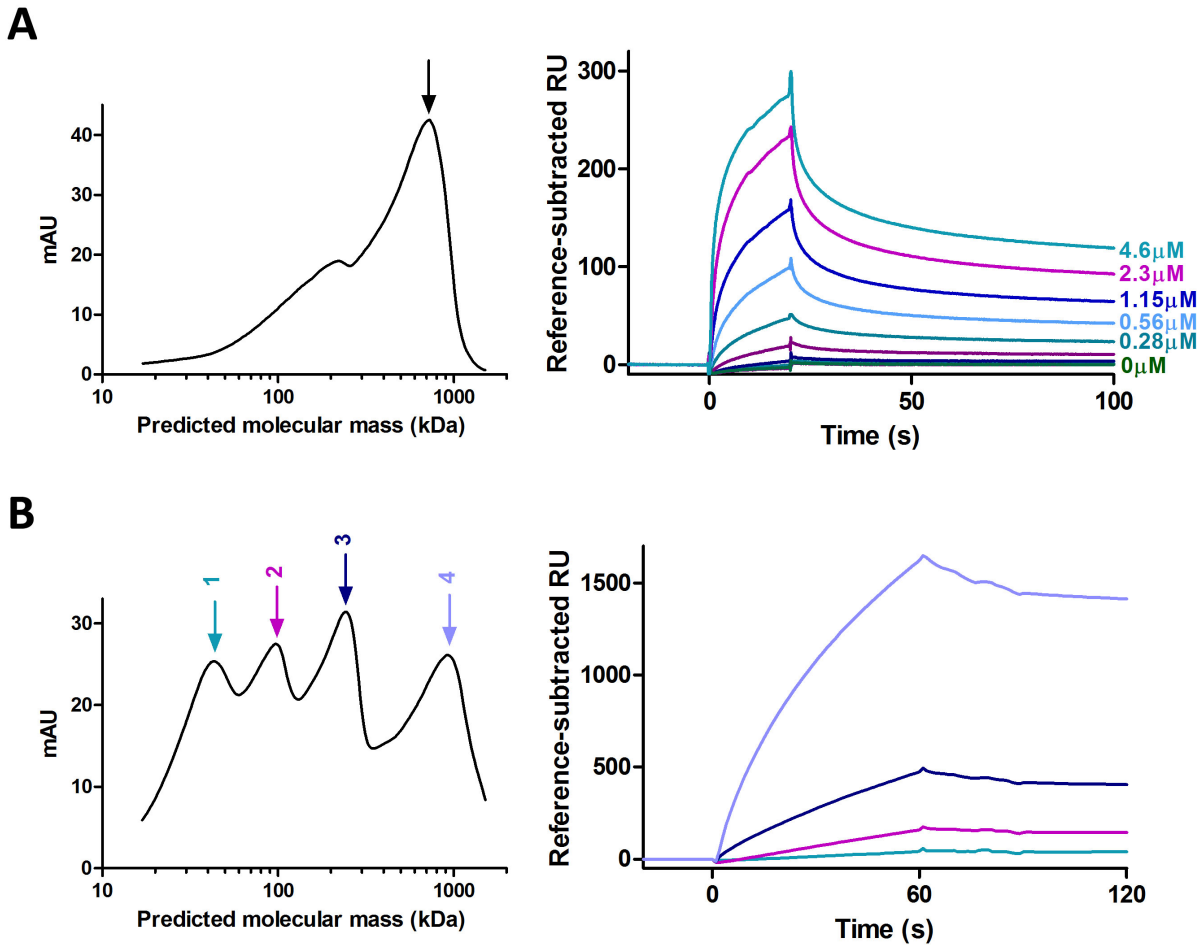


Figure 3.9: **PfMSP7 bound specifically to SELP in SPR experiments**
A. SPR signals when a range of PfMSP7 concentrations from a predominantly monodisperse SEC peak were injected over SELP bait. A representation of the SEC elution profile of the PfMSP7 analyte used in this particular experiment is shown on the left.
B. SPR signals when 3μM PfMSP7 protein from indicated SEC fractions was injected over SELP. Larger binding responses were observed when larger PfMSP7 complexes were injected.

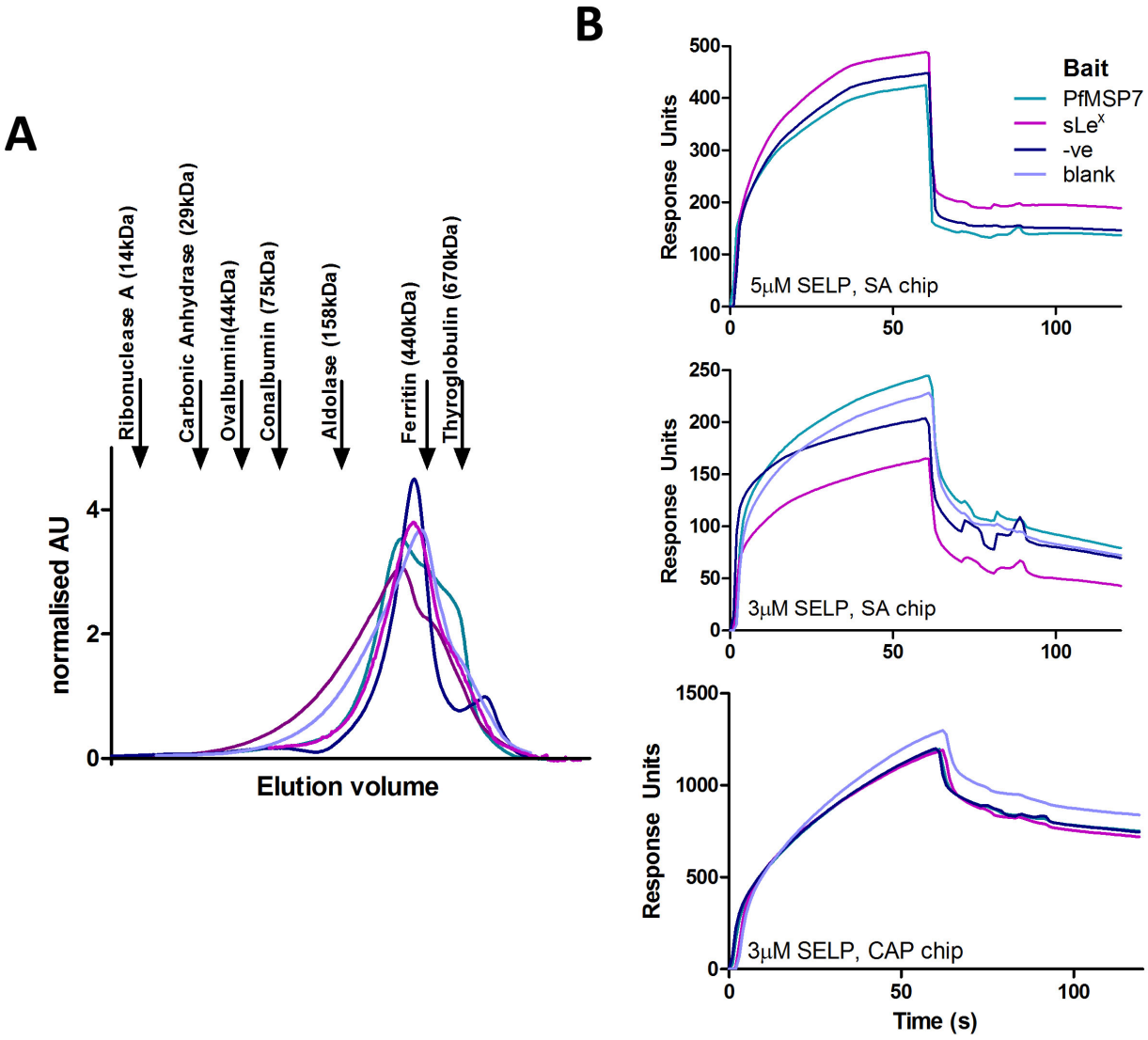


Figure 3.10: **Purified SELP bound to SPR chips**
A. SEC elution profiles from five independent SELP preparation. Predominantly broad, monodisperse peaks were observed.
B. SPR responses recorded when the indicated concentrations of SEC-purified SELP were injected over four baits for 60 seconds. In each case comparable and highly significant binding to positive controls (sLe^X tetrasaccharide), negative controls (rat Cd4 tag), PfMSP7 and even blank flow cells was evident.

Secondly the interaction was fairly resistant to washing with a range of regeneration solutions, including 0.2-1M NaOH, 1-3M Glycine HCl (pH1.5), 5M NaCl and 4M $MgCl_2$. This made it difficult to fully regenerate the SELP-coated surface, which adversely affected the reproducibility and reliability of successive analyte injections.

To overcome the difficulties in analysing multivalent PfMSP7 binding to SELP, I prepared SEC-purified SELP analyte to inject over immobilised PfMSP7. Encouragingly, the SEC elution profiles of SELP proteins were consistent between experiments, traces showing broad but monodisperse peaks (Figure 3.10A). However, injecting SELP analyte in SPR experiments proved problematic as it appeared to bind non-specifically in every flow cell (Figure 3.10B). This suggested that SELP was binding to the carboxymethylated dextran surface of the SPR chip. Selectins have been reported to bind dextrans, but only in a sulphated form[133], so it is currently unclear as to why this binding activity was observed.

3.3 Discussion

3.3.1 Multiple biochemical assays indicate that PfMSP7 and SELP interact, but oligomerisation of PfMSP7 may be required

The work described above shows that recombinant PfMSP7 and SELP proteins interact reproducibly in multiple assay systems (Figures 3.3, 3.9 & 3.6). Pentamerised PfMSP7 interacts with monomeric SELP in AVEXIS, with nitrocefin hydrolysis saturating in just minutes, significantly faster than all positive controls. Flow cytometry-based assays also show that PfMSP7 pentamers can bind specifically to SELP expressed at the cell surface. We can also see a large SPR signal when injecting purified PfMSP7 over SELP. In AVEXIS, PfMSP7 bait binding to SELP prey appears to be significantly weaker (Figure 3.3A), indicating that there may be a requirement for a higher-order spatial arrangement of either protein. SEC experiments show that recombinant PfMSP7 readily self-associates (Figure 3.7) and the higher-order oligomers appear to exhibit increased SELP-binding ability in SPR experiments (Figure 3.9B). Especially given the predicted flexibility of the N-terminus (Figure 3.8D), it is not unlikely that PfMSP7 proteins would oligomerise *in vivo*, and there is a precedent for doing so provided by PfMSP2 and PfMSP3[118, 366], whose oligomerisation is thought to increase the proteins' ability to participate in binding interactions[2, 144].

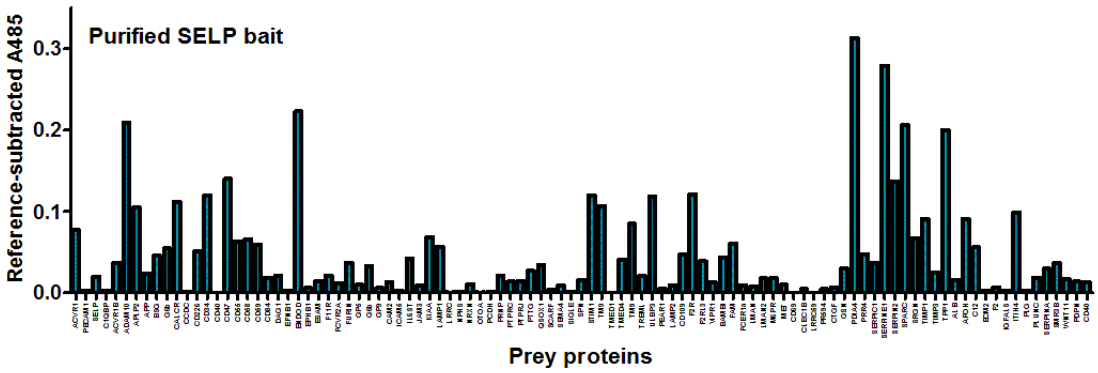


Figure 3.11: **Purified SELP bait interacted non-specifically in another AVEXIS screen** AVEXIS data from a platelet-platelet protein interaction screen showed that SELP bait interacts non-specifically with a range of platelet preys.
Data courtesy of Dr Yi Sun.

3.3.2 Recombinant SELP is prone to non-specific interactions, but multiple lines of evidence support the validity of its interaction with PfMSP7

In the course of this project, unrelated research within the group showed that purified recombinant SELP bait proteins exhibit 'promiscuous' binding behaviour in AVEXIS. In these experiments, purified recombinant bait proteins from a platelet protein library were screened against the same library expressed as purified prey proteins. A large proportion of the platelet prey proteins produced a binding signal when screened against SELP bait, which was amongst the 'noisiest' of the bait library (Figure 3.11). Similar results were observed in a microarray-based AVEXIS-like interaction screen using the same proteins[321].

These AVEXIS data, coupled with the observation that recombinant SELP binds to the supposedly inert SPR chip surface (Figure 3.10B), raised legitimate concerns about the meaningfulness of my biochemical data about the SELP-PfMSP7 interaction. In both instances where this promiscuous binding was observed the recombinant SELP protein was purified by IMAC, so it is possible that purification increases the propensity of SELP to bind non-specifically. In light of these concerning observations, I avoided using purified protein where possible and took measures to ensure that the SELP proteins used in all PfMSP7 interaction assays were correctly folded and functional, for instance by demonstrating that they bind to sLe^X (Figure 3.3). Even though these assays increase our confidence that the recombinant protein is folded and active, the possibility remains that a proportion of the proteins in each preparation are locally unfolded and responsible for non-specific interactions. To ultimately exclude the

possibility that the interaction we observe is the result of non-natively folded SELP proteins, I would ideally demonstrate that the interaction takes place between naturally-occurring proteins, which will be folded and post-translationally modified as they would be *in vivo*. The cell surface expression of SELP can be induced in platelets and endothelial cells, so I attempted to stain the surface of activated platelets and human umbilical vein endothelial cells (HUVECs) with FLAG-tagged PfMSP7 pentamers. Attempts to stain PMA-activated HUVECs with either anti-SELP antibodies or PfMSP7 were unsuccessful, both when using microscopy and flow cytometry as detection methods. It is possible that the surface expression level of SELP in this cell line was insufficient. The activation of platelets leads to a very significant increase in surface SELP, which I was able to detect by flow cytometry (Figure 3.12A). In initial attempts to stain platelets with PfMSP7, I incubated activated platelets with FLAG-tagged PfMSP7 pentamers followed by a fluorescently conjugated anti-FLAG monoclonal antibody. These experiments were ultimately unsuccessful, as the antibody itself bound the platelets at a level that was unaffected by the presence of PfMSP7, positive control or negative control pentamers (Figure 3.12B). Before pursuing *in vivo* experiments to investigate the function of the interaction, it will be important to improve this platelet-based assay or otherwise demonstrate that PfMSP7 can bind to native SELP. Data presented in Chapter 4 support there being an important, conserved role for this interaction in a number of *Plasmodium* species, and provide more evidence for the validity of the interaction discussed here.

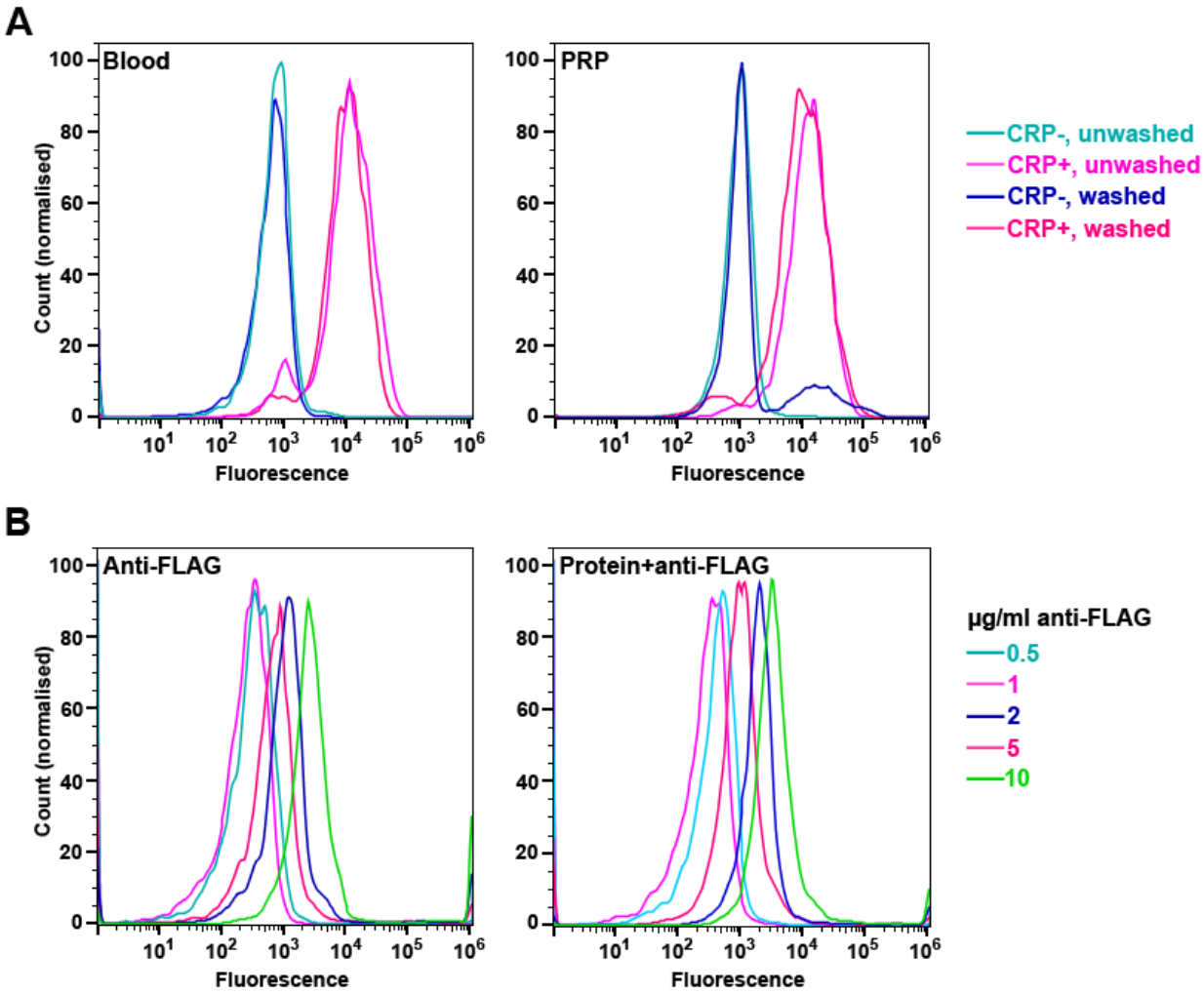


Figure 3.12: **Platelet staining assays were optimised but unsuccessful due to antibody binding to the platelet surface**

A. Flow cytometry histograms of FITC flourescence when platelets were incubated with conjugated anti-SELP antibody. SELP was detected on the surface of activated platelets within whole blood or platelet rich plasma (PRP). Washing the blood or PRP by centrifugation did not impair sample quality.

B. Histograms of FITC fluorescence when platelets were incubated with or without FLAG-tagged pentameric EphrinB2 positive control proteins and then with a FITC-conjugated anti-FLAG antibody. It was not possible to discern EphrinB2 binding to platelets(right) as the anti-FLAG antibody appeared to bind directly to platelets(left).

5,000 events within forward and side-scatter appropriate for counting platelets are displayed.

Chapter 4

Biochemical characterisation of the interactions between SELP and *Plasmodium* MSP7s

4.1 Introduction

Following the identification and validation of an *in vitro* interaction between human P-selectin (SELP) and *Plasmodium falciparum* merozoite surface protein 7 (PfMSP7), this chapter describes further biochemical characterisation of this interaction, and expands this characterisation to encompass the broader families of each protein.

4.1.1 The *Plasmodium* MSP7 family

4.1.1.1 MSP7s in a genomic context

The *Plasmodium MSP7* and *MSP7*-related protein (*MSRP*) genes have been defined by homology to the *PfMSP7* gene, first identified as the origin of the 22kDa MSP7 protein fragment found on the surface of the *P. falciparum* merozoite[215, 227, 257, 327]. These multi-gene families comprise variable numbers of protein-coding genes located very close to one another on one chromosome of each *Plasmodium* species with an available genome sequence. The genomes of rodent parasites *P. berghei*, *P.chabaudi* and *P. yoelii* contain three MSP7 family protein-coding genes, the genome of *P. falciparum* contains at least six and that of *P. vivax* contains eleven annotated *MSP7* genes (Figure 4.1)[108, 137, 158, 227]. The persistence of this large multi-gene family across the *Plasmodium* genus suggests that its members play an

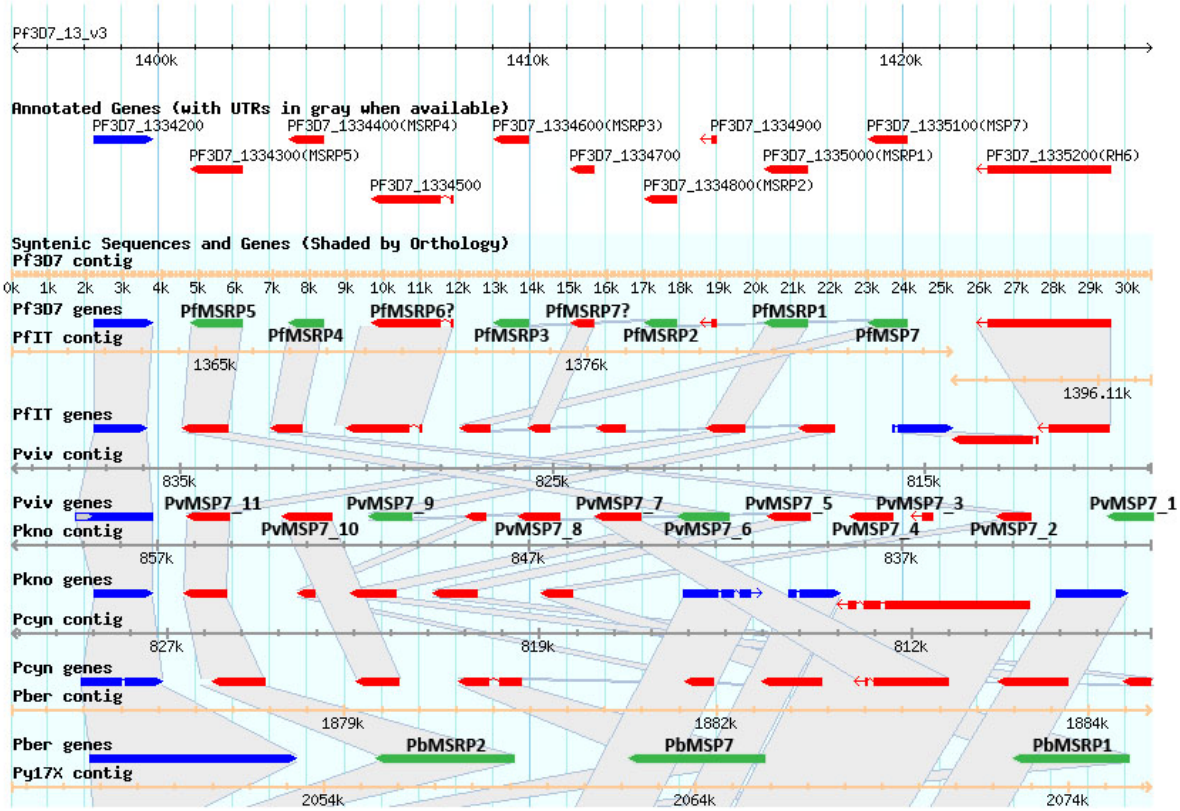


Figure 4.1: ***MSP7* gene organisation and nomencalture in different *Plasmodium* species**
Screenshot from PlasmoDB of the genomic locus containing the *MSP7* family. The *MSP7* genes in *P. falciparum*, *P. vivax* and *P. berghei* are annotated with the nomenclature used to refer to them in the text of this thesis. This nomenclature reflects each gene's description in PlasmoDB at the time of writing with the exceptions of *P. vivax MSP7*s and *P. falciparum*'s putative *MSRP6* and *MSRP7* genes, which are identified as part of this family in Garzón-Opsina *et al.* (2010)[108] and named as such in Heiber *et al.* (2013)[137]. The above labelling of the *P. vivax MSP7*s follows that used in Kadekoppala & Holder's 2010 review of the MSP7 gene family[158]. MSP7 proteins expressed as part of this work are coloured in green.

important biological role. There is currently no evidence at the DNA level to suggest functional divergence between *MSP7* paralogues, indicating that they might be redundant. The expansion in the number of *MSP7* genes in some species relative to others is therefore not fully understood, although it has been suggested that the number of family members has been fixed in each species at the optimal number or gene 'dosage' for the niche they each occupy[108]. Gene duplication events are thought to be responsible for the presence of multiple *MSP7*-family genes in each species. These genes share a similar structure and organisation, though sequence conservation is moderately low; in *P. falciparum* for instance, there is about 38 – 54% overall amino acid similarity between paralogues. Increased amino acid similarity is observed towards the C-termini of the MSP7-family proteins in all species, indicating that this region may be particularly important to the proteins' functionality[158, 227]. There is some evidence to suggest that the 3' end of the *PfMSP7* gene and the central regions of at least two *P. vivax MSP7* genes are under balancing selection, potentially to maintain antigenic diversity as a result of increased immune selection pressure[5, 110, 286, 326]. In contrast, most of the individual *MSP7*-family genes in *P. falciparum* and a number of *MSP7* genes in *P. vivax* are highly conserved, with few SNPs observed between isolates[109, 158, 286, 326]. Taken together the genetic information implies that the different MSP7-family proteins have the potential to carry out similar functions but are differentially exposed to selective pressures.

4.1.1.2 MSP7 protein expression

Transcripts for all MSP7-family proteins have been detected in blood-stage *P. falciparum* parasites, with a general increase in expression seen towards schizogony[160, 216]. However, only PfMSP7, PfMSRP1 and PfMSRP2 proteins have been detected experimentally. These three proteins have been detected by immunoflourescence in trophozoites[215], whilst PfMSP7 and PfMSRP1 have been detected in the detergent-resistant membrane fractions of schizonts[293]. Immunoblotting of trophozoite and schizont lysates detected PfMSP7 and PfMSRP2, but none of the other *P. falciparum* MSRP proteins[160]. Both PfMSP7 and PfMSRP2 have been shown to undergo proteolytic cleavage by subtilisin-like proteases[160, 173, 258, 304] (see Figure 3.1). Whilst the C-terminal fragments of PfMSP7 that are known to be associated with PfMSP1 on the the merozoite surface, the eventual destinations of most of the other PfMSP7 and PfMSRP2 fragments are currently unclear[304]. A 173 amino acid fragment from the N-terminus of PfMSP7 has been expressed with a GFP tag in *P. falciparum* parasites. The fragment could be visualised in the parasitophorous vaculolar space, indicating that this PfMSP7 fragment could be secreted from merozoites[158]. Although PfMSRP2 is thought to be processed in a similar manner as PfMSP7[160, 304] there is some debate as to whether

it associates with PfMSP1 or forms any part of the MSP1 complex[160]. A biochemical purification assay where GST-tagged PfMSP1 was incubated with recombinant PfMSPRP1 and PfMSRP2 indicated that both PfMSP7 family proteins bind to PfMSP1[215], but these interactions have not been shown to occur in parasites[160]. Antibodies against PfMSRP2 as well as N- and C-terminal regions of PfMSP7 have been detected in the serum of individuals living in malaria-endemic areas[158, 258, 349], indicating that these protein fragments are exposed to the immune system[1]. Although their native gene products have not been identified *in vitro* or *in vivo*, GFP-fusions of MSRP5 and putative MSRP6 and MSRP7 proteins have been detected outside of the merozoite, indicating that they are secreted[137].

Comparatively little is known about the destinations of *P. vivax* MSP7 proteins. At least eight are transcribed in late schizonts[32], and the protein with highest amino acid similarity to PfMSP7 (labelled as PvMSP7_2 in Figure 4.1) has been characterised to some extent; like PfMSP7 it is expressed and proteolytically processed, and at least one of the cleavage products is thought to localise to the merozoite surface in schizonts[227]. In rodent parasite *P. yeolii* all three MSP7 family proteins have been detected and are thought to bind PyMSP1. PyMSP7 and PyMSRP2 co-localise with PyMSP1 at the merozoite surface and are thought to induce a low level of protective immunity when administered as vaccines in mouse models[216]. Similarly *P. berghei* MSP7 localises with PbMSP1 at the merozoite surface, though immunoprecipitation experiments indicate that, unlike PfMSP7, PyMSP7 and PvMSP7_2, PbMSP7 does not undergo proteolytic processing[327].

4.1.1.3 PfMSP7 and PbMSP7 are associated with RBC invasion, but may perform additional functions

The *P. falciparum* MSP1 complex is comprised of four fragments of the PfMSP1 precursor protein and a peripherally-associated fragment of each of PfMSP6 and PfMSP7; the complex forms a major component of the thick, fibrillar coat of the merozoite and has hence been studied intensively as a target for vaccines and therapeutics. The MSP1 complex is known to be required for merozoites to invade erythrocytes. Antibodies against PfMSP7 can block invasion, either by preventing interactions that the MSP1 complex makes with the RBC surface or by preventing the maturation and/or shedding of the MSP1 complex[164, 357]. PfMSP7-knockout parasites still process PfMSP1 and present its peptides on the merozoite surface as normal. Similarly, knockout parasites are still able to survive and invade RBCs in *in vitro* and *in vivo* models, indicating that MSP7 itself is not essential. However, these knockout parasites

[1] Of particular note, naturally-acquired antibodies against PfMSP7 were shown to be protective against severe disease in a cohort of Tanzanian children[273].

do show a slight impairment in their invasion capacity when compared to wild-type. Assays using *in vitro P. falciparum* cultures show a 30% reduction in invasion when PfMSP7 is deleted[159] whilst *P. berghei* deletion mutants grew more slowly *in vivo* and demonstrated a preference for invading reticulocytes[117, 313, 327]. Adding to the evidence that PfMSP7 has an important, though not essential, role in RBC invasion, protein levels are seen up-regulated nearly eight-fold in W2mef strains switching to sialic acid-independent invasion[174]. *Plasmodium* parasites are known to enter RBCs via multiple invasion pathways so it is possible that PfMSP7 is involved in one or more of these. Based on its known localisation to the merozoite surface, it is reasonable to assign PfMSP7's role in invasion to its C-terminal 19 or 22kDa fragment within the MSP1 complex, although peptides corresponding to the N- and C-termini have been reported to bind to the RBC surface, potentially via an interaction with Band 3, and inhibit invasion by up to 50%[106].

Evidence from rodent infections implies that PbMSP7 may play an immunomodulatory role. *PbMSP7*-knockout parasites caused significantly fewer deaths in both a mouse model of chronic infection and an aged rat model. The delay in parasite growth and corresponding slower generation of anaemia in the *PbMSP7*-knockout infected mice was not sufficient to explain these results, leading the authors to suggest that the presence of PbMSP7 impaired the host's ability to respond effectively to the infection[117]. In mouse strains that are susceptible to experimental cerebral malaria (ECM) *PbMSP7*-knockout parasites were unable to induce the ECM phenotypes observed in mice infected with wild-type parasites, critically demonstrating a reduced ability to damage the blood-brain barrier. Again these results could not be explained by the growth impairment of the knockout parasites, indicating that the mechanism by which ECM is induced relies on the presence of the PbMSP7 protein[313]. The significance of the these results to human infections is currently unclear[2] but these data indicate that at least one MSP7 protein may possess functions that do not directly relate to RBC invasion alone.

4.1.1.4 The *P. falciparum* MSRPs have no known function

The presence of multiple MSP7 family proteins in *Plasmodium* species suggests that they might be functionally redundant. However, experiments in *P. falciparum* knockouts do not suggest that MSRPs can provide compensatory functions for PfMSP7's role in RBC invasion. MSRP transcript abundances remain comparable between wild type and PfMSP7 knockout lines, with the exception of PfMSRP5, whose transcription appears to be increased two-fold.

[2] especially given the differences in MSP7 processing and localisation between human- and mouse-infective *Plasmodium* species[215, 216, 227, 258, 327]

The consequence of this up-regulation is unclear, as the protein itself has not been detected at the RBC surface, nor elsewhere in parasite lysates[159]. All *P. falciparum* MSRPs can be individually deleted without any impairment of the parasites' ability to invade RBCs[160]. However, to date there have been no reported incidences of the generation of viable parasites of any species lacking all MSP7 family proteins.

4.1.2 The human selectins

4.1.2.1 The selectin proteins

Lectins are a group of carbohydrate-binding proteins, known for their roles in mediating cellular recognition and attachment events via their interactions with specific sugars. Selectins are classed among the C-type lectins, which were originally characterised by their calcium-dependent binding properties[75]. There are three genes encoding selectin proteins in humans. They are clustered on chromosome one and are thought to have arisen by gene duplication events occurring before the divergence of humans and mice[351]. Each of the proteins are structurally very similar, all type I membrane proteins sharing a conserved N-terminal C-type lectin and EGF-binding like domain then a variable number of short consensus repeats[156] (see Figure 4.2A). E-selectin (SELE) is expressed on endothelial cells whilst L-selectin (SELL) is expressed on the surface of leukocytes[26, 27, 324]. P-selectin (SELP) is localised to the alpha-granules of platelets and the Weibel-Palade bodies of endothelial cells. Upon activation, these bodies fuse with the membrane of their respective cell type, resulting in the rapid translocation of SELP to the surface membrane[209, 317]. All three selectins can be detected as soluble proteins in the bloodstream, a splice variant of SELP lacking a transmembrane domain being the source of much of this circulating protein[76, 237, 296]. Selectins exhibit calcium-dependent binding to a number of extracellular glycoproteins. This binding activity is mediated predominantly by the C-type lectin-like domain which interacts with sLe^X tetrasaccharides[87, 99]. Despite their shared ability to bind sLe^X sugars, the glycoprotein ligand-binding specificity of each selectin differs based on subtle structural differences between the full length proteins[186, 325]. Leukocyte P-selectin glycoprotein ligand 1 (PSGL1) is the best characterised SELP ligand[229, 239, 291, 292]. SELE can also bind PSGL1 but is thought to bind preferentially other leukocyte ligands to mediate rolling interactions[8, 365]. SELL has been shown to interact with PSGL1 with a much lower affinity and has a range of other mucin-like ligands[282, 314]. A low affinity interaction, in the millimolar range, is thought to occur between selectins and isolated sLe^X[268] but the interactions selectins make with their glycoprotein ligands are substantially stronger. It has been hypothesised that an

initial electrostatic interaction occurs between sLe^X and its binding site, following which the selectin molecule makes further contacts with the protein ligand, resulting in a higher affinity interaction[312]. The C-type lectin-like domain has been most heavily implicated in mediating binding behaviour in the selectins, as it is the region with which calcium ions, sLe^X and a range of small-molecule interaction inhibitors bind and it is structurally fairly isolated from the EGF-like domain[113, 123, 133, 312]. However, crystallographic evidence from the structures of SELP's EGF and C-type lectin-like domains in complex with a peptide from PSGL1 suggests that the EGF-like domain may play a role in high-affinity ligand binding[161, 312].

4.1.2.2 SELP-ligand interactions are important in inflammation and blood coagulation

Selectins have a well-characterised role as vascular adhesion molecules, each mediating interactions between leukocytes and endothelial cells[26, 112, 193]. Endothelial selectins and their ligands are necessary for the loose binding, or 'rolling', of leukocytes as they are recruited to vessel walls in the very early stages of inflammation[71, 190, 206, 239]. This is an important process in controlling infection, as evidenced by the recurrent, and often severe, bacterial infections suffered by selectin-deficient mice and patients with Type II Leukocyte Adhesion Deficiency, in which selectin ligands are sub-optimally glycosylated for binding to selectins[38, 89]. More recently it has been discovered that the leukocyte-endothelium interactions mediated by SELP might have a more profound effect on inflammation than by leukocyte recruitment alone. Firstly, it is thought that SELP selectively recruits pro-inflammatory Th1 (T-helper-1) cells in preference to Th2 cells, which can down-regulate inflammation[10, 30]. Secondly, leukocyte PSGL1 binding to endothelial SELP is also thought to trigger signalling pathways that prime cells for enhancing inflammatory responses. For instance this binding promotes phagocytosis in monocytes and leads to an enhanced production of a range of cytokines, including TNFα[83, 352], which is thought to be particularly important for parasite killing in the early stages of malaria[276]. Conversely, it is thought that circulating soluble SELP can prevent these interactions and help to limit the inflammatory response[76, 103, 358].

Roles for SELP in blood coagulation and haemodynamics have also been identified. SELP can induce monocytes to produce tissue factor[51], which both induces signalling in white blood cells and initiates the chain of interactions in the clotting cascade. Since this discovery, SELP has been characterised as a pro-coagulant molecule[7], the interaction between platelet SELP and PSGL1 being important in the formation of thrombi[90].

4.1.2.3 SELP is known to contribute to malarial pathology

As a key part of the inflammatory response, platelets and endothelia are activated which leads to raised levels of soluble and membrane-bound SELP in human and murine malarial hosts. Selectins on the surface of endothelial cells have been implicated in the sequestration of pRBCs. SELP and SELE have been independently identified as receptors for PfEMP1 on the pRBC surface and are thought to help the initial adhesion, or rolling, of pRBCs[241, 302]. These comparatively weak interactions are thought to aid pRBC sequestration by facilitating a stronger interaction between CD36 and PfEMP1[302]. pRBC rolling is reduced in a Selp knockout mouse[53] though no decrease in overall cytoadhesion is observed. Selp-deficient mice have been shown to be substantially less susceptible to ECM compared with wild-type control mice[59]. Complementing this observation, it has also been shown that Selp levels are increased in the brain vessels of mouse strains that are susceptible to ECM, whereas resistant BALB/c strains do not show this accumulation. Tissue specific Selp-knockout mice have been used to isolate endothelial (rather than platelet) Selp as the key contributor to the observed cerebral pathology; the incidence of ECM was decreased in mice deficient in endothelial Selp, whilst those lacking platelet Selp were not protected[59]. *Plasmodium* species' use of SELP as an endothelial receptor may explain these observations, though more recent research suggests that SELP can compromise the stability of the blood-brain barrier in certain situations[155], a state which is thought to precipitate cerebral malaria[233]. More research is needed to determine whether SELP has a pathological role in human malaria, and, if so, whether this is mediated by a direct host-pathogen interaction.

4.2 Results

4.2.1 Characterisation of PfMSP7 binding to selectins

4.2.1.1 The C-type lectin and EGF-like domains of SELP were required to bind PfMSP7

In Chapter 3, I described the identification of an interaction between SELP and PfMSP7. To identify the region of the SELP protein that interacts with PfMSP7, I made a series of biotinylated C-terminally truncated SELP proteins based on the protein's domain structure (Figure 4.2A). I then screened these baits against PfMSP7 prey using AVEXIS. All truncated proteins were able to bind to full length PfMSP7, except the smallest fragment which comprised only the C-type lectin (CTL) domain (Figure 4.2B).

The smallest binding fragment contained the CTL and EGF domains, indicating that both

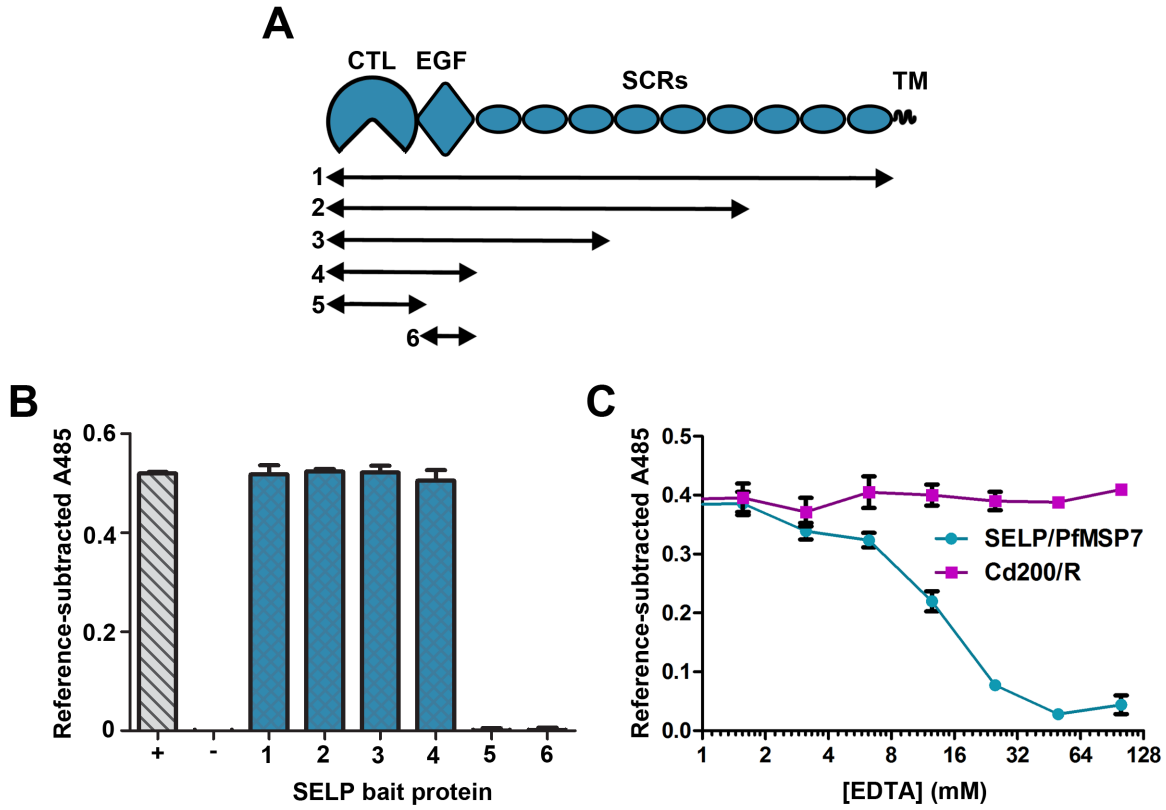


Figure 4.2: **The CTL and EGF domains of SELP were essential for PfMSP7 binding**
A. Schematic representation of the truncated SELP bait proteins used for domain mapping experiments, with C-terminal truncations made after the CTL, EGF, third short consensus repeat (SCR) or sixth SCR domain. The EGF domain, without the CTL domain, was also produced. Domain boundaries were determined using Pfam and are detailed in Table 2.2.
B. AVEXIS signals when truncated SELP baits were screened against PfMSP7 prey. All SELP bait protein fragments containing both the CTL and EGF domains bound PfMSP7 prey. PfMSP7 prey was also screened against OX68 positive control bait (+) rat Cd4 tag region negative control bait (-). Bars represent means +/- SD, n=3.
C. AVEXIS signals resulting from the interaction of SELP bait and PfMSP7 prey in the presence of EDTA. The interaction between SELP bait and PfMSP7 prey was blocked completely by around 30mM EDTA. The rat Cd200/Cd200R interaction was unaffected by EDTA. Error bars represent means +/- SD, n=3.

domains in combination, or the EGF domain alone, are minimally required for binding. To test whether the EGF domain alone could bind, I expressed the single domain as a bait protein and tested it for binding to PfMSP7 prey (Figure 4.2B). I saw no indication of binding, suggesting that both CTL and EGF domains are minimally required for binding, and that the binding site(s) for PfMSP7 is located within this region. The interactions SELP makes with its known ligands are typically dependent on the coordination of calcium by its CTL domain. To test whether PfMSP7 binding is also calcium-dependent, I incubated the proteins with varying concentrations of EDTA, a divalent cation chelator. By performing the AVEXIS-based binding assay in the presence of EDTA I observed that the interaction could be blocked by about 30mM EDTA, which is in line with published experiments where EDTA has been used to block SELP binding[172] (Figure 4.2C). This indicates that the conformation of the CTL-domain is important for PfMSP7 binding.

4.2.1.2 PfMSP7 bound to SELP and interacted weakly with SELL

SELP is one of the three human selectin proteins. These three selectins have a conserved structure, and a high degree of amino acid similarity within their shared domains (Figure 4.3A) so it is very plausible that PfMSP7 could bind to other selectins besides SELP. To determine whether this occurs, I sub-cloned SELL and SELE ectodomains from cDNA, expressed both as biotinylated bait proteins (Figure 4.3B) and screened them against PfMSP7 prey using AVEXIS (Figure 4.3C). I observed a degree of nitrocefin hydrolysis when SELL bait was used in the screen, indicating that some PfMSP7 prey was bound. Unlike when OX68 (positive control) and SELP baits were used, this nitrocefin hydrolysis did not saturate within one hour. This indicates that SELL can capture PfMSP7, but cannot capture as much of this prey protein as SELP can, implying that the interaction involving SELL is weaker. When I used all three selectins in SPR experiments with a PfMSP7 analyte, I only observed binding in the flow cells containing SELP (Figure 4.3D), which might indicate that PfMSP7/SELL binding phenomenon seen by AVEXIS is not biologically significant.

4.2.1.3 Naturally-occurring SNPs within SELP's binding domain did not affect binding to PfMSP7 without compromising protein function

Sequencing and genotyping have elucidated a number of SNPs occurring in the human *SELP* DNA sequence, and they have been catalogued in the dbSNP database. Using this resource, I identified four non-synonymous SNPs within SELP's CTL and EGF domains (highlighted in Figure 4.3A). Searching the 1000 human genomes data revealed that all of these variants are

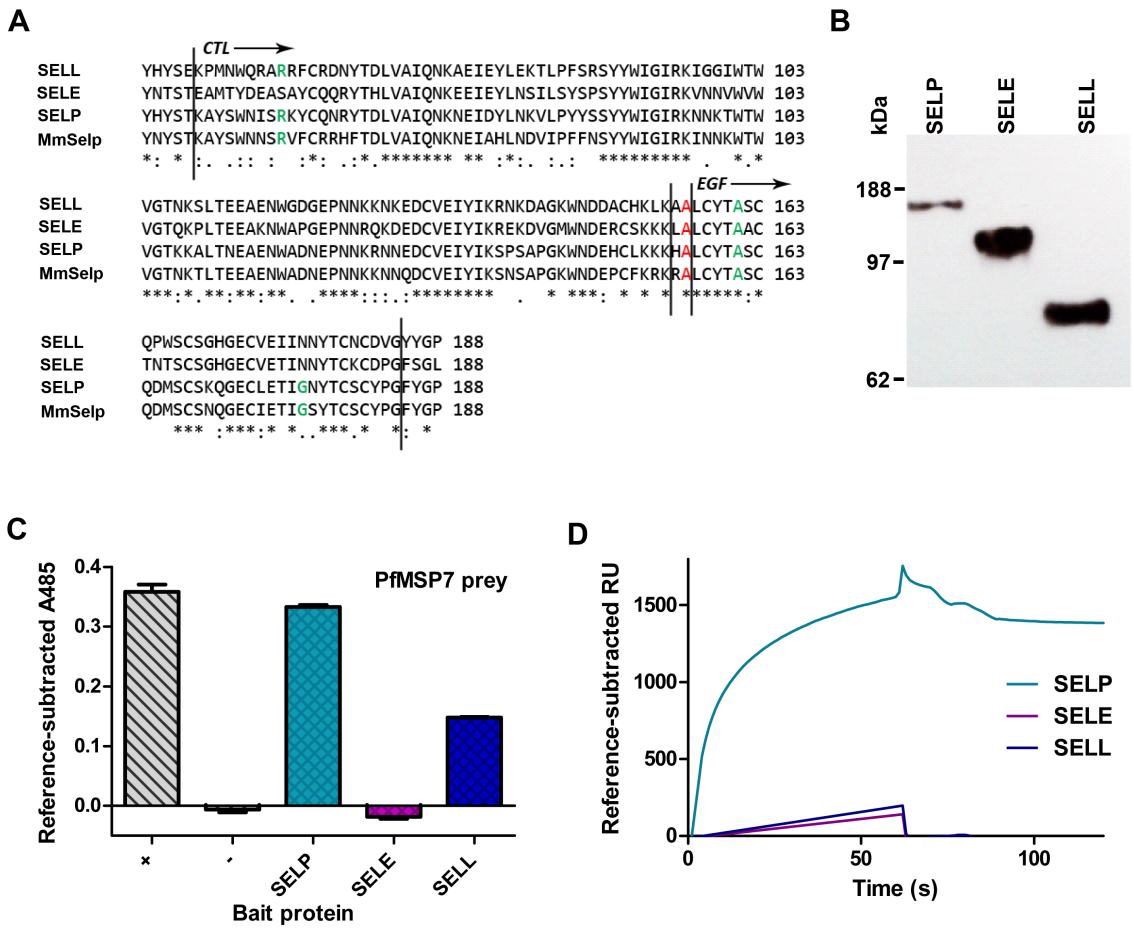


Figure 4.3: **Limited evidence for SELL and SELE binding to PfMPS7**
A. T-COFFEE amino acid sequence alignment of CTL and EGF domains of SELL, SELL, SELP and mouse Selp. Vertical lines indicate the boundaries of each domain as delineated by Pfam. Residues highlighted in red or green are those that are recorded in dbSNP as known variants in human SELP.
B. Selectin baits were successfully expressed and biotinylated. Single bands of expected sizes were observed by Western blotting using streptavidin-HRP.
C. PfMSP7 prey bound to SELP and SELL baits in AVEXIS assays. OX68 was the positive control bait (+) and the rat Cd4 tag region was the negative control bait (-). Bars represent means +/- SD, *n*=3.
D. PfMSP7 analyte did not bind SELL or SELL in SPR experiments.

very rare in all populations. I created expression constructs using site-directed mutagenesis and produced all four variants as bait proteins (Figure 4.4A, B). I then screened these variant proteins for binding to PfMSP7 prey. All variants bound indistinguishably from the wild-type SELP reference, with the exception of the A156T variant (Figure 4.4C). This variant did not bind the anti-SELP monoclonal antibody that was able to block the SELP-PfMSP7 interaction (Figure 4.4D). These results indicate either that A156, which lies at the boundary between the CTL and EGF-like domains, is an important part of the binding region for both the antibody and PfMSP7, or that mutation of this residue significantly affects the folding or conformation of the SELP protein. This particular residue is conserved in the protein sequences of all three human selectins and mouse Selp (Figure 4.4A, highlighted in red), perhaps suggesting that it has an important role in the correct functioning of the protein. To determine whether the A156 mutant was still a functional protein, I tested whether the mutant SELP prey protein could bind to immobilised sLe^X (Figure 4.4E). No sLe^X-binding activity could be detected using this mutant, thus indicating that the variant probably does not fold to form a protein that can carry out the normal *in vivo* binding roles of SELP.

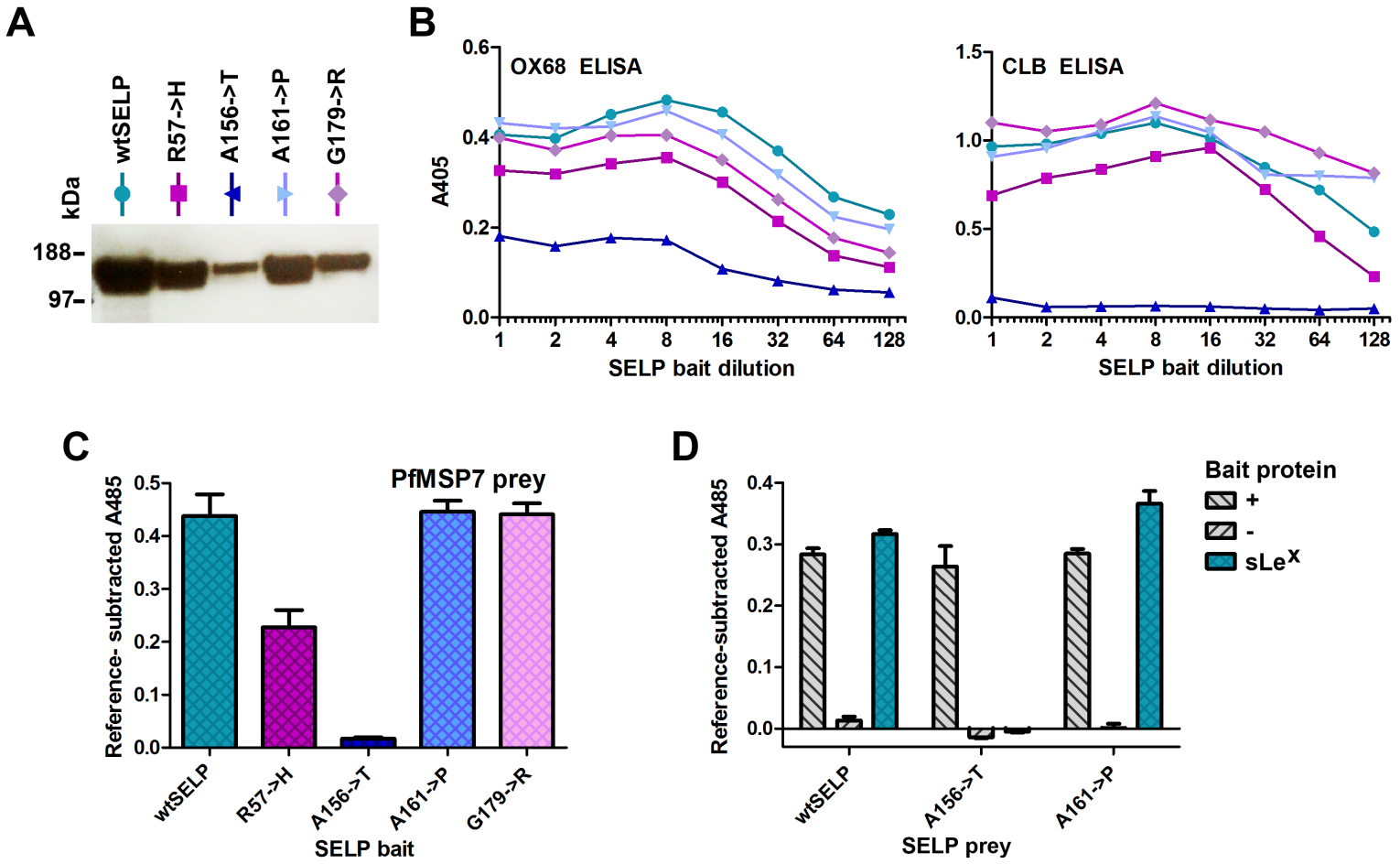


Figure 4.4: **Functional SELP variants retained binding to PfMSP7**

A. All variant baits were detected by Western blotting with streptavidin-HRP. The A156 variant had a noticeably lower expression level.

B. SELP variants were detected by ELISA. All proteins could be detected using OX68, an antibody that binds to the Cd4 tag. The A156T variants could not be detected by CLB-thromb/6, a mAB believed to bind at the interface between the C-type lectin and EGF-like domains of SELP[294].

C. All SELP baits, except the A156T variant, bound to PfMSP7 by AVEXIS.

D. A156T mutant SELP prey is unable to bind to sLe^X bait. Control baits were OX68 (+) and the rat Cd4 tag region (-). Bars represent means +/- SD, *n*=3.

4.2.2 Characterisation of SELP binding to *Plasmodium* MSP7-proteins

4.2.2.1 The N-terminus of PfMSP7 bound to SELP

To determine which fragment of the full-length PfMSP7 precursor protein binds to SELP, I expressed the merozoite surface-resident C-terminal fragments ($PfMSP7_{19}$ and $PfMSP7_{22}$) and the remaining N-terminal region of the protein (PfMSP7-N) as prey proteins to screen against full-length SELP (Figure 4.5A). PfMSP7-N but neither of the C-terminal PfMSP7 fragments bound to SELP. This suggests that the functions of the N- and C-termini are distinct; $PfMSP7_{22}$'s role in the MSP1 complex is separate from PfMSP7-N's SELP-binding function. Interestingly, all protein fragments showed some binding to PfMSP1 prey. $MSP7_{19}$ and $MSP7_{22}$ are thought to bind PfMSP1 within the MSP1 complex, but it is unclear whether the N-terminus of PfMSP7 associates with PfMSP1 outside of the merozoite cell, or solely during intracellular protein maturation. It is possible that the binding site in PfMSP7-N interacts only within the parasite with the unprocessed form of PfMSP1, since the N-terminus of PfMSP7 is not detected in the MSP1 complex.

4.2.2.2 SELP-binding was a characteristic of multiple members of the *P. falciparum* MSP7 family

MSP7 has at least five paralogues in *P. falciparum*, each with a similar overall structure but divergent sequence[158]. To determine whether these paralogues share a conserved SELP-binding function, I expressed them as prey proteins to screen against SELP bait (Figure 4.6A). PfMSRP2 and PfMSRP5 bound to SELP, though we cannot eliminate the possibility that the other PfMSRP proteins also bind; I could observe PfMSRP1-mediated nitrocefin hydrolysis after several hours of incubation with the substrate, indicating that a small amount of this prey was captured on SELP bait. The PfMSRP3 and PfMSRP4 prey proteins showed very low expression levels, and thus had to be significantly concentrated, which could impact their ability to bind SELP by AVEXIS. By concentrating cell culture supernatants, we concurrently increase the total protein concentration alongside that of the protein of interest. Thus the preys are incubated with the baits in a much more protein-rich environment, which could potentially block specific interactions from occurring as efficiently.

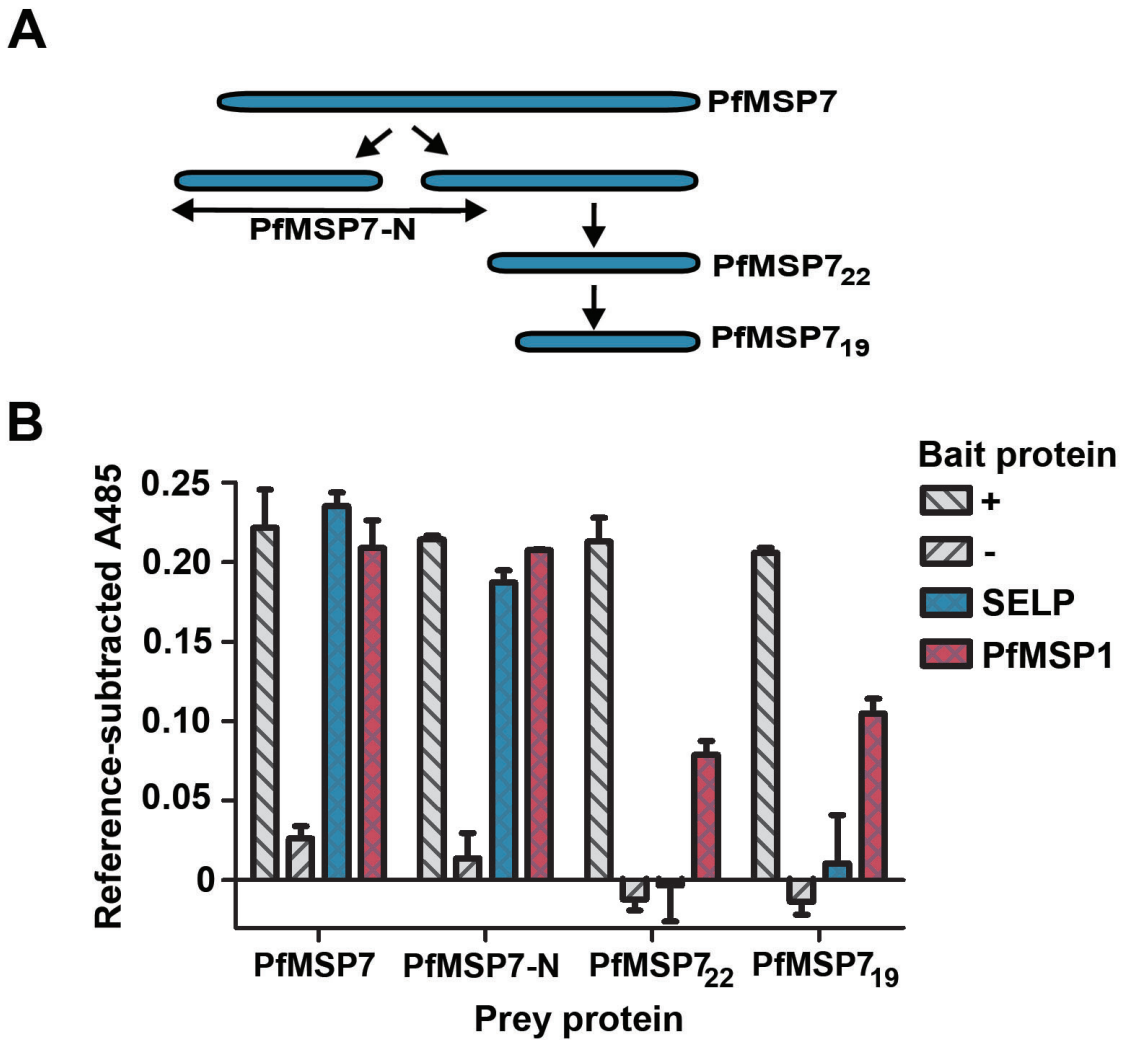


Figure 4.5: **The N-terminus of PfMSP7 bound to SELP**
A. Schematic representation of the truncations made to the full length PfMSP7 precursor. The boundaries of each protein fragment are detailed in Table 2.3. Transitions shown represent the proteolytic processing events believed to occur *in vivo*, detailed in Figure 3.1.
B. SELP bait bound the N-terminal, but not the C-terminal fragments of PfMSP7 by AVEXIS. All PfMSP7 fragment preys bound to PfMSP1 bait. OX68 was the positive control bait (+) and the rat Cd4 tag region was the negative control bait (-). Bars represent means +/- SD, n=3.

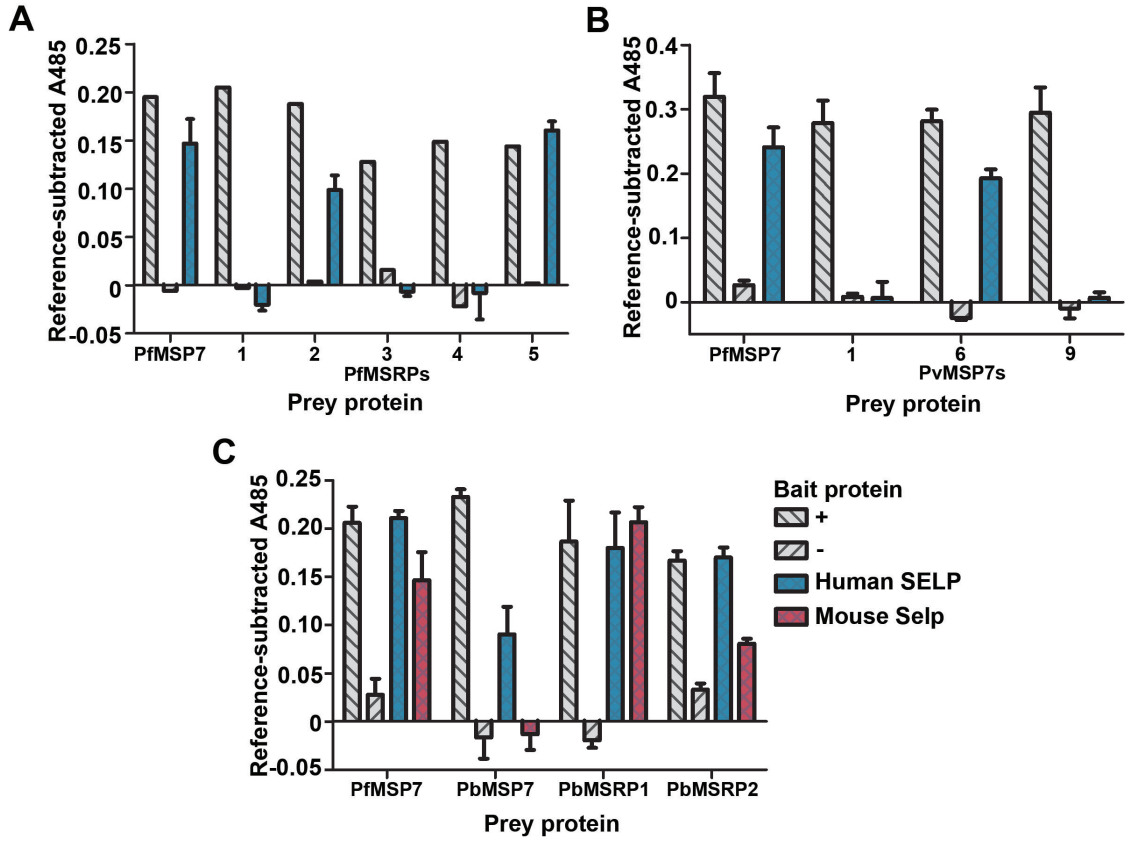


Figure 4.6: **SELP-binding is conserved in multiple *Plasmodium* MSP7s**
A. SELP bait was screened against six *P. falciparum* MSP7 family proteins. PfMSRP2 and PfMSRP5 preys, as well as PfMSP7, were able to interact with the SELP bait. SELP-bound PfMSRP2 prey was able to saturate nitrocefin hydrolysis more rapidly than PfMSP7.
B. Three of the *P. vivax* MSP7 proteins were screened against SELP. PvMSP7_6 (PVX_082675) prey bound to SELP bait.
C. *P. berghei* MSP7-family preys bound to human SELP and mouse Selp bait.
In each experiment, OX68 was used as the positive control bait (+) and the rat Cd4 tag region as the negative control bait (-). Bars represent means +/- SD, n=3.

4.2.2.3 The MSP7-SELP interaction was conserved across *Plasmodium* species

To determine whether SELP binding is a conserved feature of *Plasmodium* MSP7s, I expressed three of the eleven *Plasmodium vivax* MSP7s as prey proteins to test by AVEXIS for binding to SELP. PvMSP7_6 prey bound to SELP at sufficient levels to saturate nitrocefin hydrolysis within an hour, whilst no binding was evident when using PvMSP7_1 or PvMSP7_9 preys (Figure 4.6B). I also cloned and expressed the ectodomain of mouse Selp and screened it against the three MSP7 paralogues found in *Plasmodium berghei* (Figure 4.6C). Human SELP and mouse Selp bait bound sufficient PbMSRP1 prey to saturate nitrocefin hydrolysis within one hour, which is a good indication that this *P. berghei* protein interacts with mammalian P-selectin. Similarly, nitrocefin hydrolysis was saturated when I screened PbMSRP2 prey against human SELP. Nitrocefin hydrolysis was also observed, although it was not as rapid, when I used mouse Selp bait. This indicates that PbMSRP2 binds to both selectin baits but possibly does not bind to mouse Selp as strongly as it does to the human protein. However it is also possible that a lower proportion of the recombinant mouse Selp bait is correctly folded and fully functional. PbMSP7 prey expression levels were very low, and the cell culture supernatant was concentrated significantly to optimise and standardise prey activity. I did not observe any nitrocefin hydrolysis when screening PbMSP7 prey against mouse Selp bait, but saw a colour change when using human SELP bait. In summary, I found evidence that *P. berghei* MSRPs bind to selectin but I do not have enough evidence to suggest that PbMSP7 binds.

4.2.3 Investigating the influence of PfMSP7 on known binding interactions of SELP

4.2.3.1 PfMSP7 blocked the interaction between sLe^X and SELP

There is currently no defined biological role ascribed to the N-terminal fragments of PfMSP7. It is likely that PfMSP7's N-terminal fragment(s) are released into the blood stream upon pRBC lysis[158], where they could potentially interact with a myriad of host proteins. One hypothesis concerning the role of PfMSP7's N-terminus is that an interaction with SELP could act to prevent its normal role in the host's anti-parasite immune response. To start to investigate whether PfMSP7 could potentially modulate the normal binding functions of SELP, I tested whether PfMSP7 could block the interaction between immobilised SELP and a sLe^X-alkaline phosphatase conjugate. By incubating immobilised SELP with pentameric PfMSP7 and then introducing the sLe^X reagent, I was able to block the interaction between SELP and sLe^X

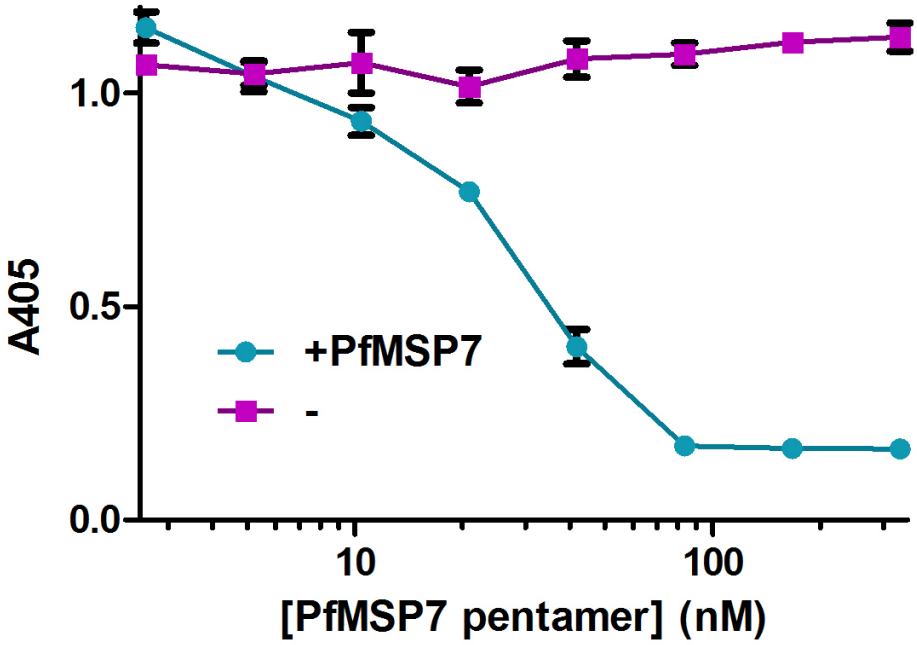


Figure 4.7: **PfMSP7 could block the SELP/sLe^X interaction**
The interaction between SELP bait and a sLe^X-alkaline phosphatase conjugate was detected using absorbance at 405nm produced by the activity of a phosphatase substrate. The interaction was blocked completely by incubating the SELP bait with 0.1µM PfMSP7 pentamers. Bars represent means +/- SD, *n*=3.

(Figure 4.7). This indicated that it is theoretically possible for PfMSP7 to interfere with the interactions that SELP makes with proteins involved in normal immune functionality.

4.2.3.2 PfMSP7 could block cellular adhesion to SELP

To further establish whether it would be theoretically possible for PfMSP7 to prevent the interactions by which leukocytes adhere to endothelium, I developed an assay based on monocytic cells binding a receptor coated-surface (see 2.4.2). I observed that THP1 cells could bind to both SELP and an anti-PSGL1 antibody (but not negative controls) immobilised on the surface of a microtitre plate. This showed that the cells had maintained expression of PSGL1 receptors in culture and also verifies that recombinant SELP is able to bind native ligands such as PSGL1 on leukocytes (Figure 4.8A).

To determine whether PfMSP7 could prevent the SELP-mediated binding of THP1 cells, I

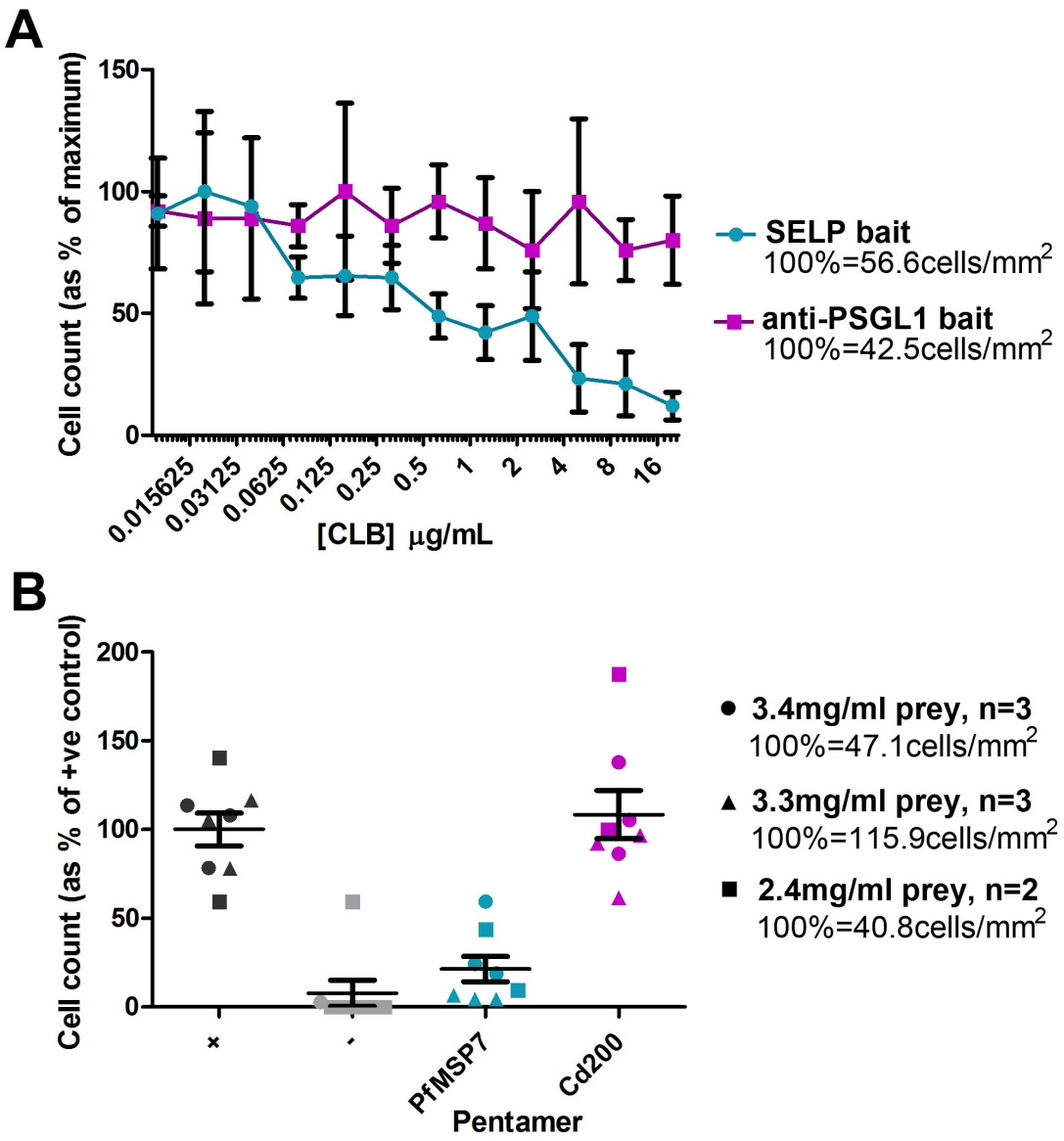


Figure 4.8: **PfMSP7 could block the adhesion of THP1 cells to SELP**
A. THP1 cells adhered specifically to immobilised SELP and anti-PSGL1 antibodies. The interaction between cells and SELP could be blocked by pre-incbating the bait proteins with the CLB-thromb/6 anti-SELP monoclonal antibody. THP1 binding via anti-PSGL1 was unaffected by CLB-thromb/6 antibody. Error bars represent means +/- SD, *n*=3.
B. Pre-incubation of SELP bait with PfMSP7 could prevent the binding of THP1 cells. As a positive control (+), cells were captured using SELP bait which was not subsequently incubated with any other recombinant prey. As a negative control (-) Cd200, to which the cells should not bind, was used as a bait protein. Cd200 pentamer (used instead of PfMSP7) could not block the interaction. This experiment was performed three times, each using an independent PfMSP7 prey preparation, once in duplicate and twice in triplicate. Error bars represent means +/- SD, *n*=8.

incubated the SELP receptors with serial dilutions of PfMSP7 prey[3] prior to the addition of cells. I observed a modest, incremental decrease in adhesion with increasing concentrations of PfMSP7 above approximately 2.5µM, but even 20µM PfMSP7 did not completely block the binding of cells. Using the highest concentrations of PfMSP7 possible (around 35µM or 7µM (700pmoles) of pentameric protein) from three independent preparations, I was able to block the interaction from occurring (Figure 4.8B). The requirement for such a high concentration of PfMSP7 pentamer to achieve a blockade of leukocyte binding is surprising, especially since a theoretical maximum of just 20pmoles of SELP protein can be immobilised in a single well. Significantly lower quantities of PfMSP7 (2pmoles of pentamer, effectively 10pmoles of PfMSP7 monomer) were required to block the interaction between SELP and sLe^X-AP (Figure 4.7). This discrepancy might reflect differences in the proportion of functional proteins in the preparations, such that a lower proportion of SELP and/or a higher proportion of the PfMSP7 used in Figure 4.7 participated in binding interactions compared to the preparations used in these experiments using THP1 cells. However the relative difficulty in blocking the interaction between SELP and THP1 cells might lie in part with the strength of the interactions involved; the interaction between individual $sLe^X$and SELP proteins is of relatively low affinity, with estimations ranging between 0.1 and 7.8mM [33, 171, 268], whereas the interaction between SELP and PSGL1 and the interaction between SELP and neutrophils are of substantially higher affinity, with calculated K_Ds of 3-320nM and 70nM respectively [63, 213, 342]. As a consequence it is likely to be more challenging to use PfMSP7 to block the interaction between SELP and THP1 cells than it is to block the interaction between SELP and isolated sLe^X. These *in vitro* data show that it is theoretically possible for PfMSP7 to prevent the SELP-mediated interactions between leukocytes and endothelia, but, given the high affinity of the SELP/PSGL1 interaction, this may require a high local concentration of PfMSP7 proteins.

4.3 Discussion

4.3.1 Further evidence that the PfMSP7-SELP interaction is biologically relevant

The data presented in this chapter further validate that recombinant PfMSP7 and SELP interact. The interacting domains on both proteins can be isolated (Figures 4.2A & 4.5) and the interaction can be replicated using variant SELP proteins, though PfMSP7 is unable to

[3] for 90 minutes, after which unbound protein removed by washing

bind to the potentially mis-folded A156T SELP variant[4] (Figure 4.4). Combined with the evidence that PfMSP7 prey cannot bind to SELP in the presence of EDTA (Figure 4.2B), which changes the conformation of the binding domain, this indicates that PfMSP7 can only bind to functionally active recombinant SELP. AVEXIS data indicate that SELP-binding is a conserved property of MSP7 proteins in multiple parasite species (Figure 4.6); these MSP7 proteins are diverse in sequence yet maintain the ability to bind SELP. Whilst this may not be the sole function of the MSP7 family, the ability of the proteins to bind SELP - an important adhesion molecule and component of the host immune system - may be part of the reason a repertoire has been conserved in *Plasmodium* parasite species. By demonstrating that PfMSP7 is able to block the interaction of SELP with sLe^X and, albeit less potently, with leukocytes (Figures 4.7 & 4.8), I have shown that there is a possible *in vivo* function for SELP/MSP7 interactions, whereby MSP7 prevents the binding of SELP to its human ligands (see 4.3.3).

4.3.2 The SELP-PfMSP7 interaction is unlikely to be involved in RBC invasion

The true test of the importance of this biochemical data will be if we can demonstrate an *in vivo* function for this interaction. Existing knowledge about PfMSP7 has implicated the protein in merozoites' entry into host RBCs[159, 164, 327]. It is unlikely that the interaction between SELP and PfMSP7 is involved in the invasion process for two main reasons. Firstly, SELP is not known to be present in the RBC proteome[262] and was not detected on the cell surface (Figure 4.9). Secondly, our data indicate that it is the N-terminus of PfMSP7 that is involved in SELP binding, and this part of the protein is not detected on the merozoite surface. To further investigate the role and localisation of PfMSP7, I purified His-tagged PfMSP7 to immunise rabbits and produce anti-PfMSP7 polyclonal antibodies. These antibodies bound specifically and sensitively to recombinant PfMSP7, as observed by ELISA and Western blot (Figure 4.10A). However, they were not able to replicate the invasion-blocking effect previously reported for anti-PfMSP7 polyclonal antibodies[164]. The levels of invasion observed when this antibody was added into an RBC invasion assay were 99.5% (+/- 3.345) of that observed when no antibody was added[5]. Despite being able use this antibody to detect very small quantities of recombinant PfMSP7 by Western blotting, I was unable to detect any PfMSP7 in the lysate from Percoll-purifed cultured *P. falciparum* schizonts (Figure 4.10B). This is surprising as PfMSP7 fragments are expected to be abundant on the surface of, and secreted from

[4] It is possible that this variant is a sequencing error in the database.

[5] This assay was performed by Dr Leyla Bustamante and is described in Theron *et al.*, (2010)[330]. The assay was performed in triplicate

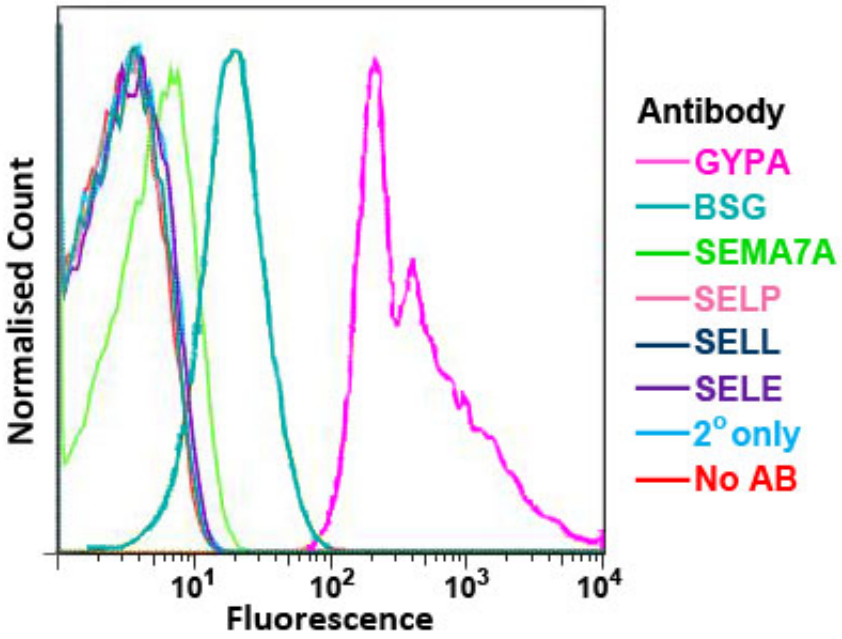


Figure 4.9: **Selectins were not detectable on the RBC surface**
A. Monoclonal antibodies against a range of RBC surface proteins and selectins with an Alexa488-conjugated secondary could not detect selectins on the RBC surface. Positive control proteins Glycophorin A (present at approximately 10^6 copies/cell), Basigin (10^4 copies/cell) and Sema7A (10^3 copies/cell) were all detected. 2000 events were counted.

schizont-stage parasites. However, I was also unable to detect PfEBA175 or PfMSP1 in this lysate using polyclonal antibodies in the same manner. This might indicate that this lysate is not a reliable source of intact merozoite proteins. Whilst our invasion assay results imply that PfMSP7 does not play an important role in RBC invasion, we cannot use these data to eliminate this possibility as we cannot be certain that the antibody is able to bind to PfMSP7 of parasite origin.

4.3.3 SELP-binding may have anti-inflammatory function

The binding of endothelial SELP to leukocyte PSGL1 is thought to enhance inflammation and promote phagocytic activity of immune effector cells. This process is potentially deleterious to the parasite as it results in the deployment of cytokines such as TNFα, which has known parasite-killing activity. An attractive hypothesis concerning the role of the SELP/PfMPS7 interaction is that the N-terminus of PfMSP7 acts as an anti-inflammatory mediator, binding SELP, blocking its interaction with PSGL1 and preventing the downstream enhancement of the anti-parasite immune response (see Figure 4.11). The evidence that PfMSP7 can block

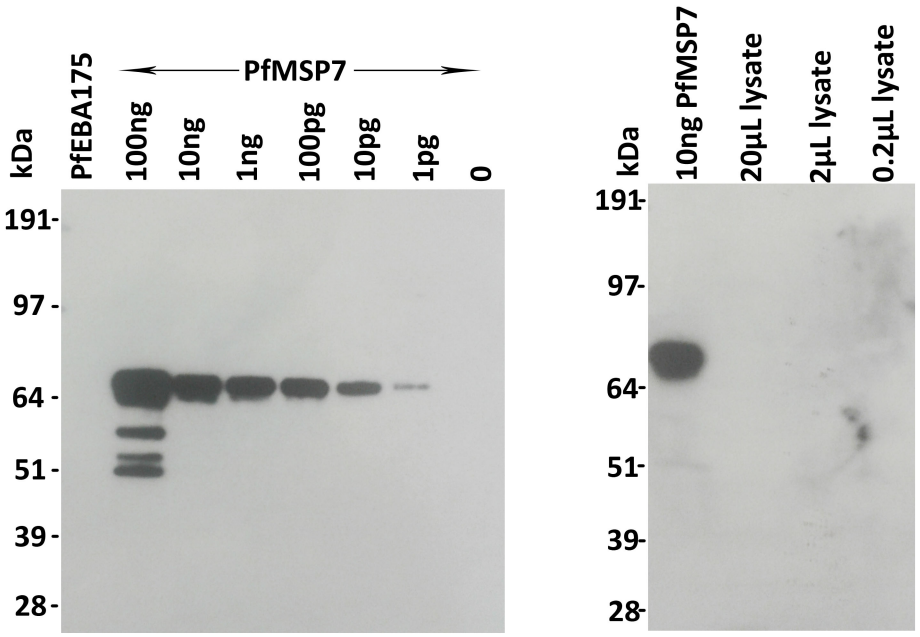


Figure 4.10: **PfMSP7 was not detected schizont lysate**
A. Polyclonal anti-PfMSP7 antibodies were used to detect even picogram quantities of PfMSP7. The antibodies were specific to PfMSP7 as no bands were observed when 100ng PfEBA175 was transferred to the membrane.
B. The same antibodies were not able to detect PfMSP7 fragments in Percoll-purified schizont lysate.

the interaction SELP makes with sLe^X and potentially leukocytes is an encouraging first step towards demonstrating that PfMSP7 has an anti-inflammatory role. We have collaborated with another research group who have developed an assay to measure SELP-dependent leukocyte rolling on endothelial-derived cells[74]. We hypothesised that by incubating the endothelial cells with PfMSP7 we would see a reduction in rolling when flowing leukocytes over the cell monolayer. In preliminary assays we did not observe a reduction in rolling, in fact both the PfMSP7 and Cd200 negative control seemed to increase rolling. The assay conditions are optimised for using antibodies to block protein interactions involved in rolling, and may need to be refined further for using pentameric proteins. I would like to revisit these assays in the future.

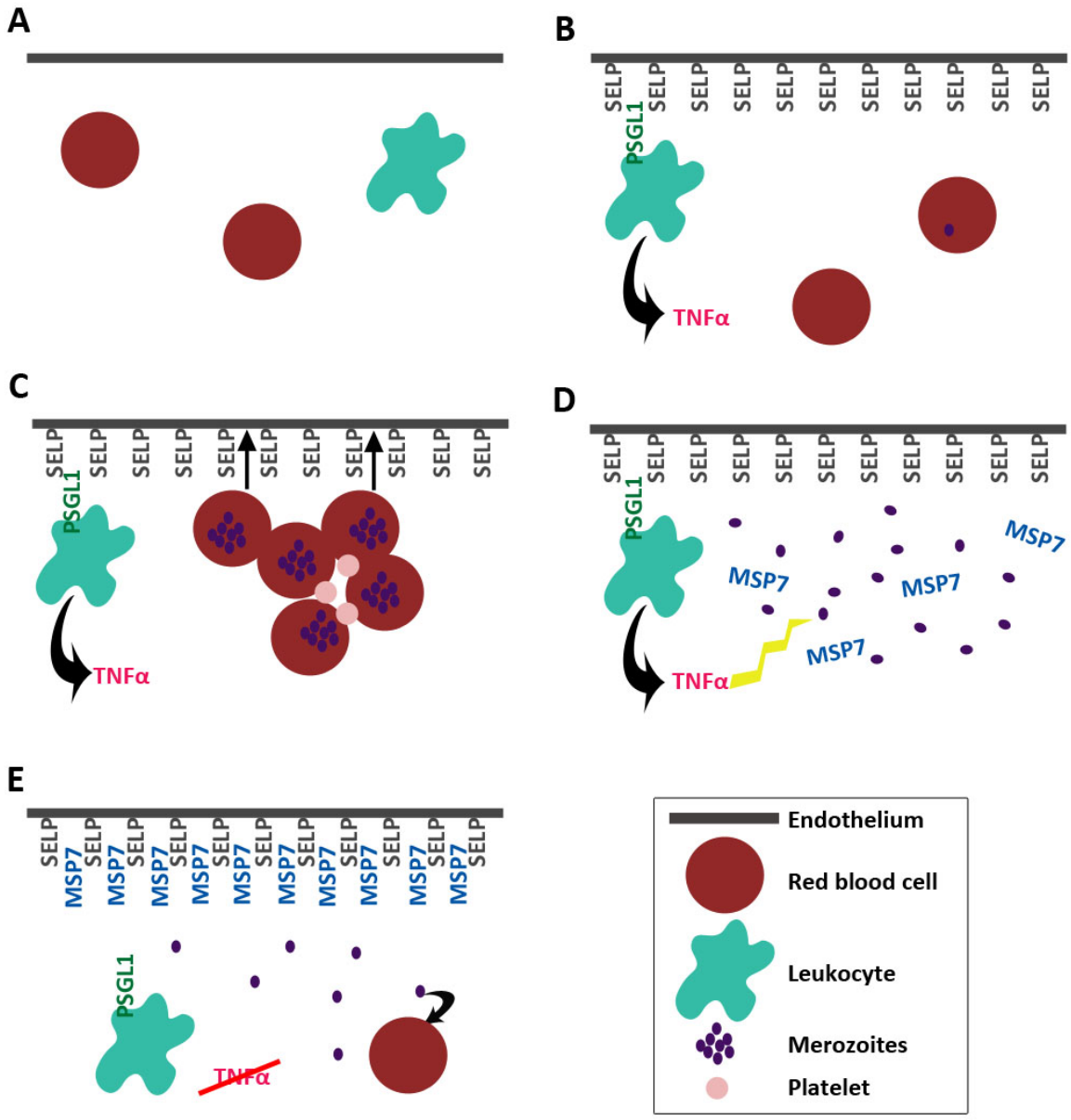


Figure 4.11: **Hypothesised anti-inflammatory activity of PfMSP7**
A. Prior to infection the endothelium is in a resting state.
B. Early in infection, the endothelium is activated and rapidly presents surface SELP. This results in the rolling adhesion of leukocytes via sLe^X-containing ligands such as PSGL1. This interaction can promote cytokine production and phagocytosis in leukocytes.
C. Aggregated pRBCs sequester on the endothelium, where they may be targeted by immune effectors.
D. The pRBCs rupture, releasing merozoites and PfMSP7 fragments into the blood stream. Free merozoites are vulnerable to the host immune response.
E. PfMSP7 N-terminal fragments bind endothelial SELP and prevent endothelium-leukocyte interactions. This dampens the immune response and allows more merozoites to successfully re-invade new RBCs.

4.3.4 Interactions between SELP and MSP7s could modulate a range of other cellular processes, including cytoadhesion.

Inflammation is not the only process in which an interaction between SELP and PfMSP7 could play a part. For instance, SELP is also an important player in blood coagulation[7, 51, 90]. Given the precedent set by blood-borne bacteria for pathogens producing anti-coagulant mediators to avoid being confined by a growing thrombus[318] it is possible that PfMPS7 could perform a role of this kind. The previous implication of Selp and PbMSP7 in the generation and severity of cerebral malaria in mouse models, such that the absence of either gene mitigates ECM, is particularly striking[59, 313]. It is possible that the binding of MSP7 proteins to SELP directly or indirectly contributes to blood-brain barrier damage, and/or affects the accumulation of leukocytes (in ECM) or RBCs in cerebral microvasculature. SELP is amongst the repertoire of endothelial receptors suggested to bind to PfEMP1 on the surface of the pRBC[302, 367]. This interaction is thought to enhance the sequestration of pRBCs but is not essential for the process to occur. By virtue of its potential binding to PfEMP1, SELP has also been implicated in platelet-mediated clumping of pRBCs[259]. It is plausible that MSP7s could affect either of these SELP-mediated adhesive processes, either by preventing them from occurring or by exacerbating them. In the hypothetical case whereby MSP7s enhance, rather than block, these cytoadhesive processes this phenomenon could contribute to explaining the deleterious effect of the presence of PbMSP7 on the outcome of infection for the host. The experimental work required to accurately define the function of the SELP/MSP7 interactions observed *in vitro* will be discussed in 6.3.2.

Chapter 5

Development of a biochemical co-purification assay to detect interactions between *Plasmodium* merozoite proteins and human serum proteins

5.1 Introduction

This chapter describes the development and application of a method using recombinant *P. falciparum* protein-coated paramagnetic beads to co-purify interacting proteins from human serum, with the aim of identifying novel, potentially immunomodulatory, interactions involved in the pathogenesis of malaria.

5.1.1 *Plasmodium* merozoites are exposed to human serum

During the blood stage of infection, *Plasmodium* parasites are predominantly intracellular and are thus largely shielded from the body's immune defences. Between cycles of erythrocyte invasion, however, free merozoites are exposed to immune effectors, perhaps more so than at any other point in the parasites' life cycle. *Plasmodium* parasites are highly adept at immune evasion, such that true sterile immunity is probably never achieved, even in highly exposed individuals[185]. With a multitude of mechanisms at work to eliminate the parasite from the blood, it is very likely that a number of *P. falciparum*'s 5,300 genes - the majority of which have as-yet undetermined functions - are involved in manipulating the immune system. One such example whereby a parasite-secreted molecule subverts the functioning of leukocytes has

been identified[320] and in this laboratory we have recently determined that certain proteins belonging to the PfMSP3 family are able to bind IgM, in another possibly immunomodulatory mechanism. We hypothesise that *Plasmodium* parasites possess a large repertoire of these defences and that merozoite proteins in particular play a key role in immune evasion.

Protein	Serum concentration (g/L)	Composition
Albumin	40	monomer
IgG	12	immunoglobulin
Transferrin	2.3	monomer
Fibrinogen	2	heterohexamer
IgA	2	immunoglobulin
Apolipoprotein A1	1.4	monomer
α2-macroglobulin	1.4	homotetramer
α1-antitrypsin	1.1	monomer
IgM	1	immunoglobulin
Complement C3	0.95	heterodimer
Haptoglobin	0.88	heterotetramer
Hemopexin	0.75	monomer
Apolipoprotein B	0.72	monomer
α1-acid glycoprotein	0.61	monomer
Antithrombin III	0.32	monomer
Apolipoprotein AII	0.3	monomer
Prealbumin	0.26	homotetramer
Ceruloplasmin	0.21	monomer
Complement C4	0.17	heterotrimer
Plaminogen	0.14	heterodimer
Fibronectin	0.11	homodimer
Other	0.038	n/a

Protein chain	Serum concentration (g/L)	kDa
Albumin	40	66
IgG γ chain	8	50
Ig light chains	4.8	25
Transferrin	2.3	80
IgA α chain	1.4	60
Apolipoprotein A1	1.4	28
α2-macroglobulin	1.4	190
α1-antitrypsin	1.1	52
Fibrinogen α chain	0.95	95
IgM μ chains	0.75	75
Hemopexin	0.75	60
Apolipoprotein B	0.72	250
α1-acid glycoprotein	0.61	41
Fibrinogen β chain	0.56	56
Complement C3 α chain	0.56	110
Fibrinogen γ chain	0.5	50
Complement C3 β chain	0.39	75
Antithrombin III	0.32	58
Apolipoprotein AII	0.3	17
Haptoglobin α chain	0.29	40
Prealbumin	0.26	16
Ceruloplasmin	0.21	132
Haptoglobin β chain	0.14	20 or 9
Fibronectin	0.11	230
Plamsinogen α chain	0.099	60
Complement C4 α chain	0.082	98
Complement C4 β chain	0.061	73
Plamsinogen β chain	0.041	25
Complement C4 γ chain	0.028	33
Other	0.038	n/a

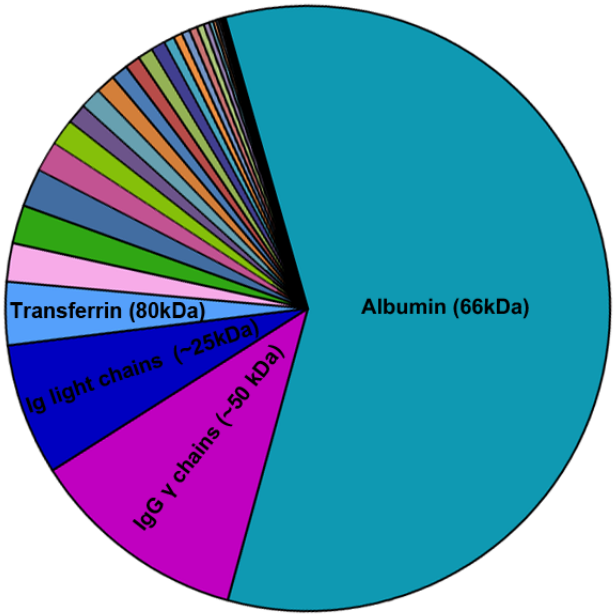


Figure 5.1: **Protein composition of human serum**
Breakdown by mass of the 21 most abundant proteins in human serum, which account for over 99% of the total protein content. The left hand table ranks the proteins by abundance in serum, presented as an average from a number of means of measurement[131]. The second table and pie chart break down of these proteins by chain, as they would appear in the denaturing SDS-PAGE step of the purification procedure.

5.1.2 A modified biochemical co-purification assay to discover novel merozoite-serum interactions

As a starting point to identify novel immunomodulatory mechanisms, I developed a screening approach for detecting interactions between merozoite proteins and human serum components. Human serum is a complex mixture of over 1000 proteins including antibodies, cytokines and complement proteins, as well as many others involved in homeostasis. It therefore represents an abundant (and readily obtainable) source of potential interacting partners to screen against merozoite proteins. In brief, the modified biochemical co-purification assay is a six-step process (Figure 5.2). The first step in this process involves creating a paramagnetic, multivalent *Plasmodium* protein reagent via the interaction of N-terminally biotinylated 'bait' proteins and streptavidin coated super-paramagnetic beads. Unbound proteins are removed by the isolation of the beads using a magnet. The beads are resuspended in buffer and re-isolated to wash. These reagents are then incubated with human serum and subsequently washed to remove proteins that have bound to the beads non-specifically. Proteins remaining bound to the beads are eluted, denatured and separated by SDS-PAGE for further analysis[1].

5.2 Assay development

5.2.1 Requirements of a sensitive assay

In order to recover sufficient material to identify a protein from an SDS-PAGE gel band using mass spectrometry, I aimed to pull down at least 100ng of protein from a single assay. To achieve this, the assay must fulfill three main criteria. In the first instance, sufficient serum protein must be bound to the merozoite protein-loaded beads following incubation. This means that interaction detection will be dependent on the successful capture of the merozoite protein on the bead and subsequently the strength of the interaction between the protein-coated bead and the serum component. Second, there must be sufficient serum protein left bound to the beads after washing. This will require a washing procedure that is sufficient to remove non-specifically bound proteins, but not so stringent as to remove the bound serum protein-of-interest. Finally, the elution process must release at least 100ng bound serum protein from the beads, which will be dependent on the previous two criteria, as well as the rigour of the elution process itself. In developing this assay I worked through each stage to optimise pro-

[1] For more details of the method see 2.7.

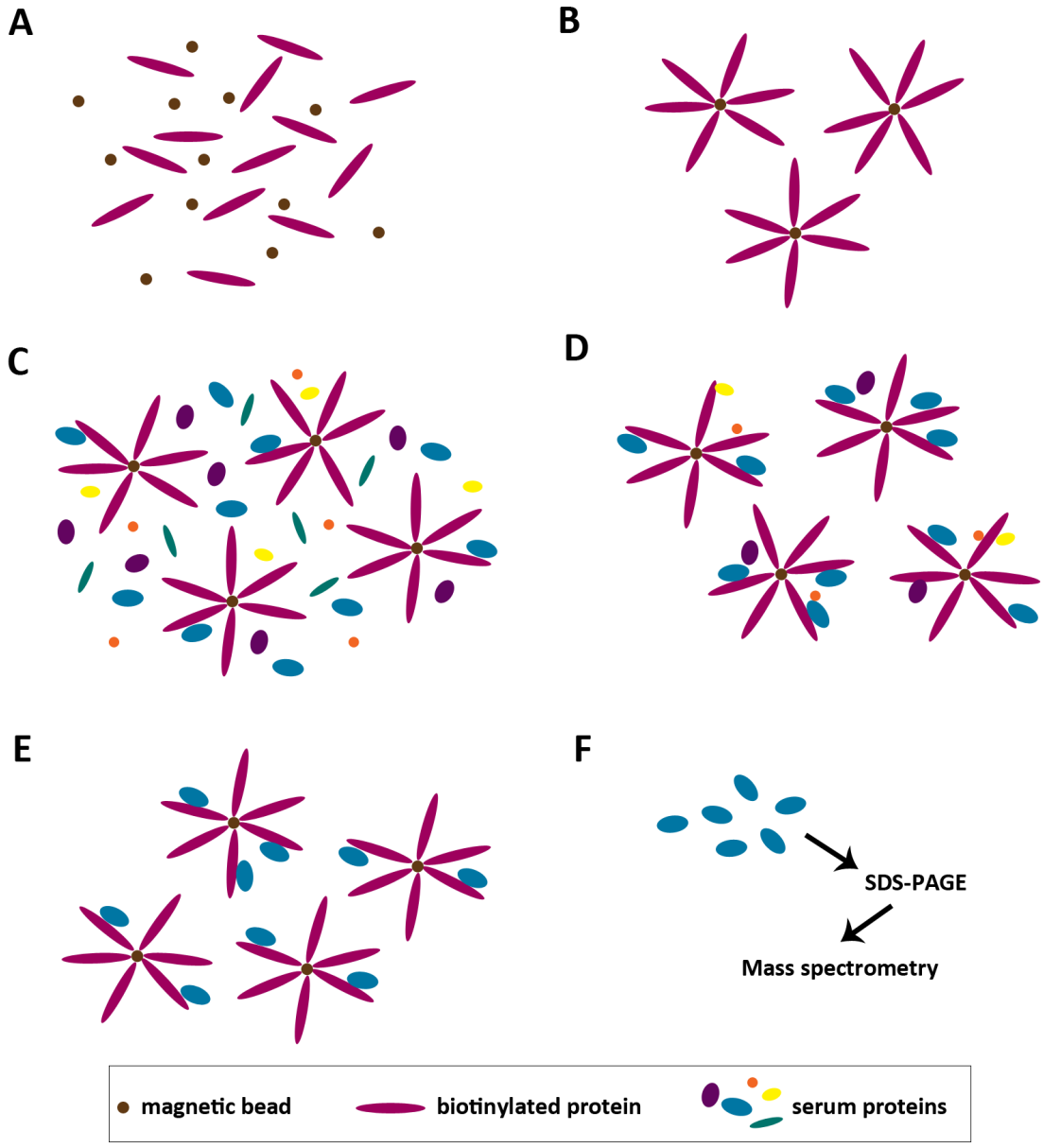


Figure 5.2: **A biochemical co-purification assay for the discovery of merozoite-serum protein interactions**
A. Biotinylated proteins are incubated with streptavidin-coated paramagnetic beads (not to scale).
B. Binding reagents are isolated using a magnet.
C. Binding reagents are incubated with human serum.
D. Binding reagents and their interacting proteins are isolated using a magnet.
E. Reagents are washed to remove loosely-bound proteins.
F. Interacting proteins are eluted from the beads, resolved by SDS-PAGE and identified by mass spectrometry.

cesses where protein losses could occur and to determine the theoretical range of interactions that could be detected.

5.2.2 Ensuring specific capture of biotinylated merozoite proteins

To create merozoite protein-conjugated 'bait' reagents on which to capture interacting serum proteins, I coupled biotin-tagged merozoite proteins to streptavidin-coated paramagnetic beads. To ensure that the beads were saturated with merozoite proteins, I incubated the beads with purified biotinylated proteins for 30 minutes, isolated the beads and performed ELISAs to detect biotinylated proteins (as described in Section 2.3.1.1) in the supernatant. If biotinylated protein was successfully captured on the beads, I observed a decrease in the amount of protein recovered from the supernatant when compared with an equivalent amount of input protein (Figure 5.3A). To achieve the highest sensitivity for the assay I would ideally capture the maximum amount of merozoite proteins on the beads[2]. Hence, it was important to ensure that sufficient amounts merozoite proteins were incubated with the beads to saturate their biotin-binding capacity. I demonstrated saturation of this binding by recovering biotinylated protein from the supernatant following the isolation of protein-coated beads from 30-minute incubations of beads and biotinylated proteins (Figure 5.3A). Before every biochemical co-purification experiment, these ELISA-based bead saturation assays were performed, so as to determine the amount of protein required to completely coat the bead surface. The interaction between biotin and streptavidin has a very high affinity[69], such that merozoite proteins bound to the beads via this interaction will not be removed by even very stringent wash steps. It was therefore important to confirm that the merozoite proteins were binding to the beads specifically via the biotin-streptavidin interaction, and not via weak, transient interactions that would not withstand the subsequent steps of the assay. To demonstrate this, I pre-incubated the beads with biotin prior to adding the biotinylated proteins. This should block the available sites for the biotinylated merozoite proteins to bind and hence prevent their capture. By performing ELISAs on the supernatant following isolation of the beads after their 30-minute incubation with biotinylated PfMSP3.4, I was able to demonstrate that saturating the beads with biotin could completely block subsequent capture of biotinylated protein, indicating that merozoite proteins are indeed being captured specifically via their biotin tag (Figure5.3B).

[2]In theory 100µL beads can bind 40pmoles of biotinylated protein, which, for example, would be 2µg of a 50kDa protein

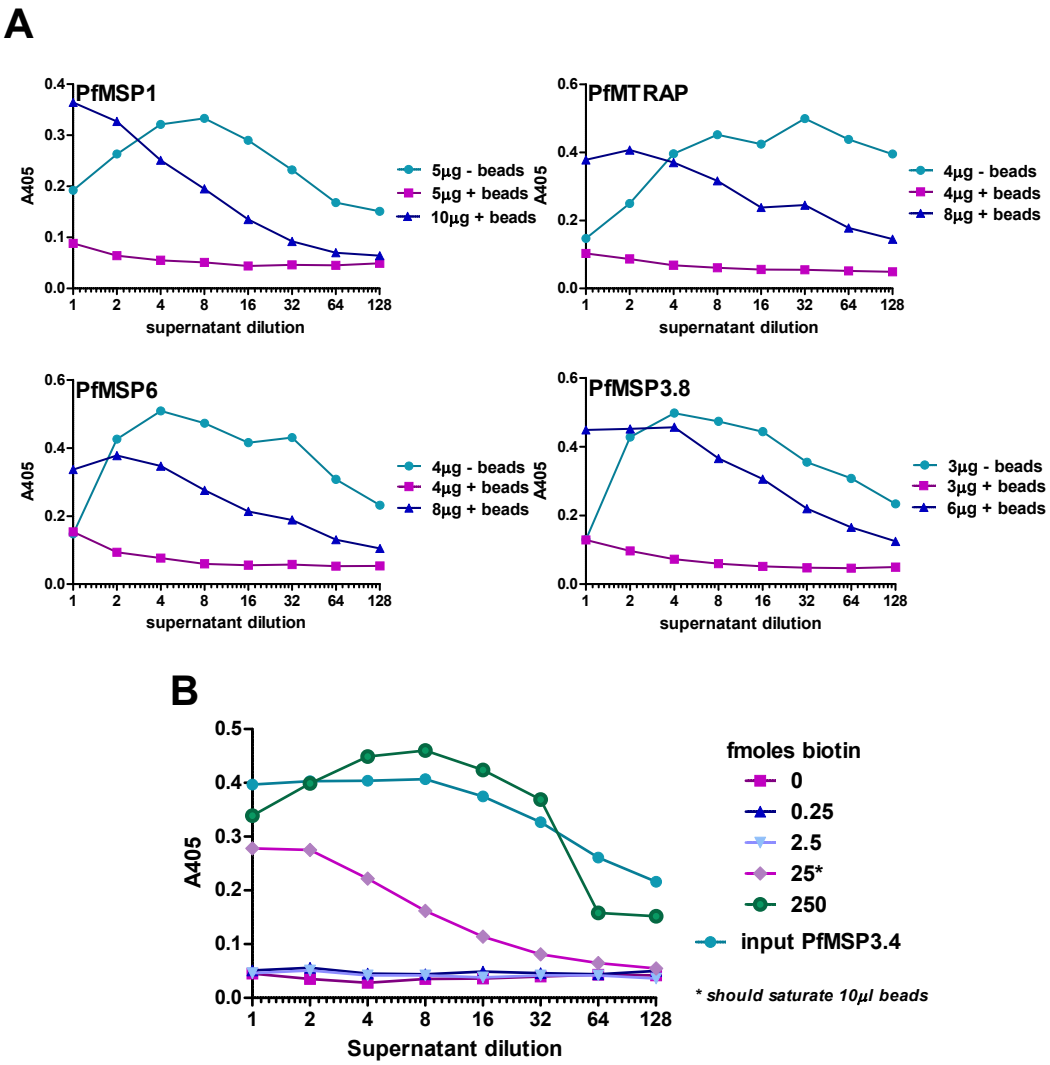


Figure 5.3: **Biotinylated merozoite proteins were specifically captured on paramagnetic beads**

A. ELISA-based analyses on the supernatants from the protein/bead incubations demonstrated saturable capture of biotinylated protein. The absorbance readings reflect the amount of biotinylated protein captured from the supernatant after 33µL beads were incubated with the indicated biotinylated proteins. Where beads had been incubated with insufficient biotinylated protein to saturate the bead surface, very little biotinylated protein remained in the supernatant, reflected in negligable turnover of phosphatase substrate and consequent low-level absorbance at 405nm (A405) in ELISAs (magenta squares). These signals can be compared to the cyan data series, which shows the ELISA A405 readings for the equivalent amount of each protein without incubation with the beads. Where beads had been incubated with an excess of biotinylated proteins, protein could be recovered from the supernatant following bead isolation, and A405 signals were observed by ELISA (blue triangles).

B. Capture of biotinylated PfMSP3.4 can be blocked by preincubation of beads with biotin. 20µL beads were incubated with biotin prior to incubation with 10µg PfMSP3.4. A405 readings reflect the amount of PfMSP3.4 that could be re-isolated from the beads (i.e. that which could not bind to the biotin-blocked beads).

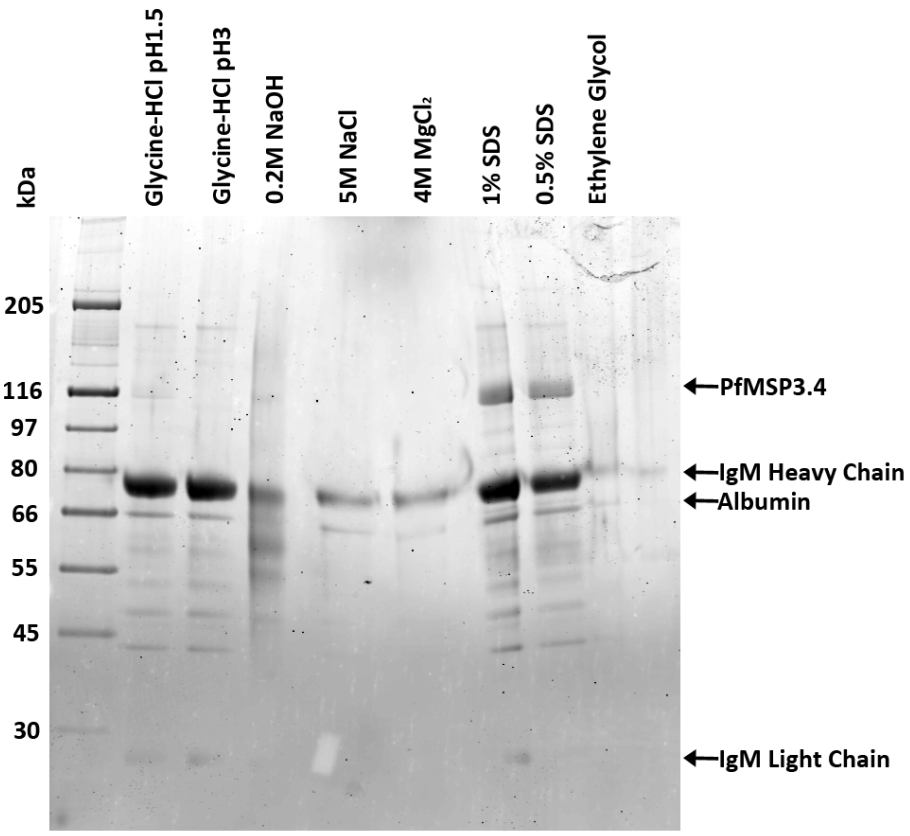


Figure 5.4: **Identification of appropriate elution buffers**
SDS-PAGE analysis of the eluate from biochemical co-purifications using a range of elution reagents indicated that Glycine-HCl or SDS solutions were appropriate elution buffers. Biochemical co-purifications were performed using 100µL PfMSP3.4-coated beads, 1mL serum and three 60-second washes. Proteins were eluted by five minute incubations with the indicated solutions.

5.2.3 Optimising elution efficiency

Using effective elution conditions, it should be possible to release a high proportion of bound protein from the beads. There are various types of solutions that can be used to disrupt protein-protein interactions including those with high salt concentrations, low or high pH and denaturing reagents. To test the compatibility of various elution buffers with the proposed biochemical co-purification method, I performed a series of preliminary experiments using the known interaction between PfMSP3.4 and human serum IgM (see Figure 5.4).

High salt solutions (5M NaCl and 4M $MgCl_2$) were not compatible with the assay, as they did not allow a particularly clear resolution of bands and also distorted the running of the surrounding lanes on the gel. Low pH and SDS solutions successfully brought down proteins

of the correct size to be the IgM heavy chain. SDS also brought down a band of the correct size to be the input PfMSP3.4, indicating that the denaturing conditions may be strong enough to dissociate the streptavidin-biotin interaction. However PfMSP3.4, like other members of the PfMSP3 family, is thought to oligomerise[118], so this band may also be PfMSP3.4 that is covalently associated to the beads via a PfMSP3.4 homotetrameric interaction, as opposed to via its biotin tag. Hence I chose SDS for the assay's elution buffer, as it returned the highest yield of PfMSP3.4-interacting protein.

5.2.4 Optimising washing steps

Serum is a complex matrix from which to isolate proteins, so it is important that the washing of the beads following incubation with serum is sufficiently stringent to remove non-specifically bound serum proteins. However, this needs to be balanced against washing away the interacting proteins and losing the beads themselves between wash steps, which will reduce the yield of any interacting proteins. To advise the number and type of wash steps, I performed a series of theoretical calculations. Assuming first-order dissociation kinetics during wash steps, mass (in g) of interacting protein bound to the beads declines in a time-dependent manner such that $mass(t) = mass(0) \times e^{-\lambda t}$, where $mass(t)$ is the mass of serum protein bound to the beads after t seconds of washing, $mass(0)$ is the mass of serum protein bound prior to washing and λ is the dissociation rate constant, which is inversely related to the interaction half life ($\lambda = \frac{\ln 2}{t_{\frac{1}{2}}}$). The mass of bound serum protein required prior to washing can therefore be found using the following equation:

$$mass(0) = \frac{mass(t)}{e^{-\lambda t}}$$

By applying this equation to a range of bead-serum protein interaction half-lives, I could estimate the $mass(0)$ required to ensure $mass(t) > 10^{-7}$, i.e. the mass of serum protein present prior to washing necessary to leave 100ng for elution, as a function of washing time (Figure 5.5A). These calculations indicate that this method cannot be expected to detect interactions that dissociate rapidly during washing; those with a half-life in the order of seconds are unlikely to be detectable, but the method has promise to detect those with a half-life in the order of minutes. As serum is a complex mixture of proteins, many of which are highly abundant (see Figure 5.1), multiple wash steps will be required to remove proteins that are bound non-specifically to the protein-coated beads. By iterative application of exponential decay calculations, I estimated $mass(0)$ required to isolate 100ng serum protein after a succession of

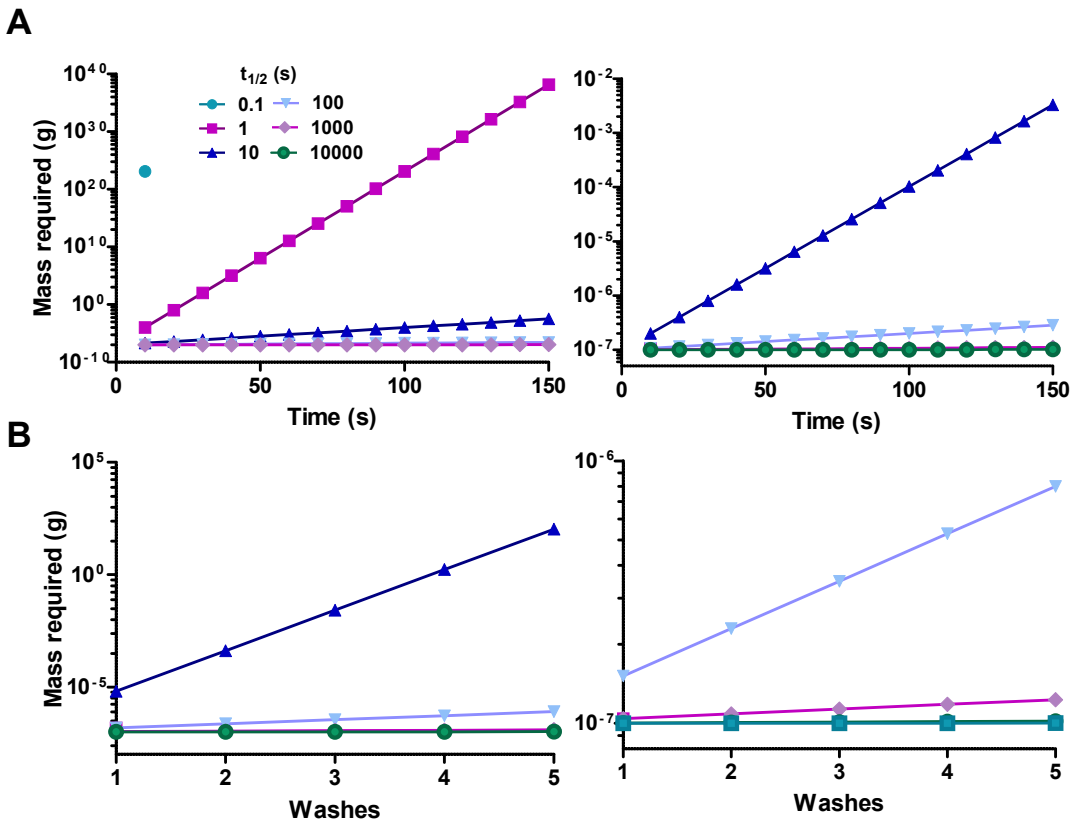


Figure 5.5: **Theoretical assessment of wash-step dependency of interaction detection**
A. The minimum required mass(0) - the amount of a 50kDa serum protein bound to the beads prior to washing to ensure that 100ng can be recovered from co-purifications using 100µL beads and 1mL serum - was plotted against as a function of washing time, assuming first-order dissociation kinetics, for a range of given interaction half-lives.
B. The minimum required mass(0) was plotted as a function of the number of successive 60-second wash steps.

60-second wash steps[3] (Figure 5.5B). These calculations indicate that the requirement for repeated washing will only slightly increase the threshold interaction half-life needed to ensure detection.

To investigate the effectiveness of washing for removing non-specifically bound proteins, it was necessary to perform some additional preliminary biochemical co-purification assays. I observed that the beads had a tendency to adhere to the walls of the polypropylene tubes in which they were being handled, so I tested whether transferring the resuspended beads to a

[3]Note that a washing time of less than 60s is impractical, as at least 45s are required for the magnetic separation of the beads for the supernatant

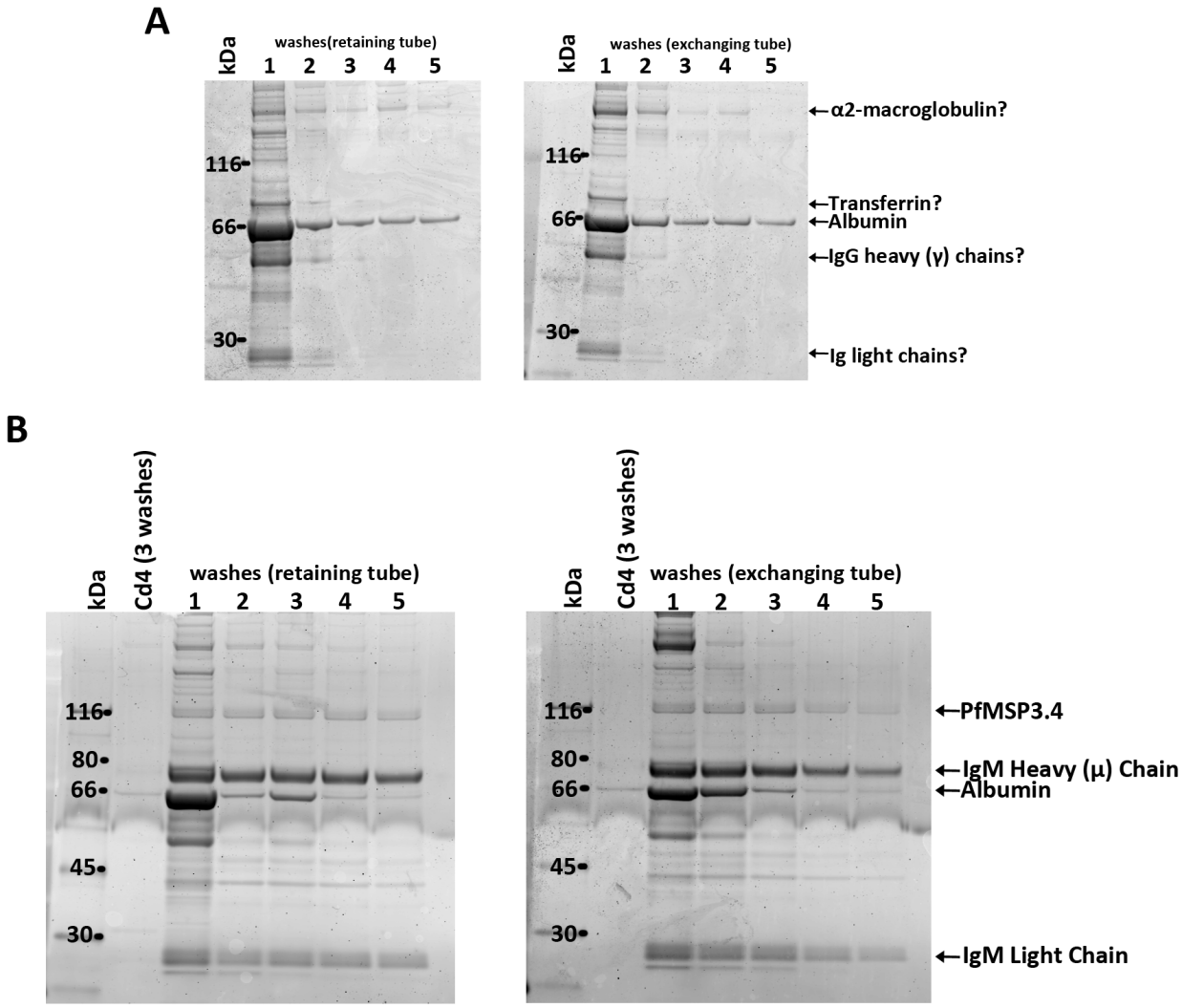


Figure 5.6: **Experimental optimisation of washing steps using negative and positive control bait proteins**

A. 100μL beads coated with the Cd4 tag region were used in preliminary co-purification assays with 1mL serum and the indicated number of 60-second wash steps. On the left-hand panel the same tube was used for all wash steps, whereas in the right-hand picture the beads were transferred to a fresh tube between each wash step. In either case, only albumin was recovered after three washes. The approximate locations of other common contaminating serum proteins, identified in Figure 5.1 are indicated on the right.

B. PfMSP3.4-coated beads were used in similar preliminary co-purification assays. At least two washes were required to remove contaminating serum proteins. IgM remained stably bound to the beads during washing, though IgM yields were slightly decreased after multiple washes when fresh tubes were used for each wash.

fresh tube between 60 second washes stood to increase the purity of eluted protein, without compromising the yield. To systematically determine the optimum number and type of wash steps, I performed a series of co-purifications using a rat Cd4 domains 3&4 bait, which we do not expect to bind serum proteins (Figure 5.6A), and the PfMSP3.4 bait which we know to bind IgM (Figure 5.6B). The results of these preliminary experiments demonstrated that:

- Even after five washes, it is not feasible to remove all non-specifically bound albumin protein, shown by the consistent presence of a 65kDa band in all lanes in Figure 5.6.
- There is no significant increase in the purity of the eluted IgM after four washes, shown in Figure 5.6B.
- Exchanging tubes between wash steps does not significantly impact the purity of the eluted IgM (Figure 5.6B).
- Losses of the interacting IgM are more noticeable when changing tube between washes. This is most easily observed by examining the light chain band in Figure 5.6B.
- Higher levels of non-specifically bound proteins are observed when performing the biochemical co-purification using PfMSP3.4-coated beads than the Cd4-coated beads (as seen by comparing Figure 5.6B with Figure 5.6A). This might mean that serum proteins have a greater propensity to interact with other proteins than with the beads themselves, or the comparatively small Cd4-tag region.
- Recurring contaminant bands likely represent the most abundant serum proteins, which are summarised in Figure 5.1. The hypothesised identities of some of these bands are indicated in Figure 5.6A.

Based on these observations, pull down assays were subsequently performed using four 60-second wash steps, without exchanging the tube between each.

5.2.5 Theoretical assessment of assay sensitivity

For a receptor-ligand interaction $R + L \rightleftharpoons RL$, at equilibrium

$$K_D = \frac{[R][L]}{[RL]}$$

And if a is the concentration of *Plasmodium* protein added into serum, and x the initial concentration of the ligand in serum, then

$$a = [RL] + [R]$$

and

$$x = [RL] + [L]$$

Combining these equations gives

$$K_D = \frac{(x - [RL])(a - [RL])}{[RL]}$$

where $[RL]$ can be determined from $mass(0)$ in the wash-step dependency calculations in Section 5.2.4. By applying this formula to a hypothetical experiment using 100µL beads and 1mL human serum, and involving a medium-sized (50kDa) serum protein, it is possible to approximate the ranges of interaction affinities and target protein abundances into which detectable interactions would fall (Figure 5.7). These rough calculations indicate that this method could detect interactions with a K_D in the micromolar range, provided the concentration of serum protein is in the micromolar, or even the high nanomolar range. Using these calculations we can speculate that an assay using 100µL beads and 1mL human serum would be well suited to the detection of interactions:

- with half-lives in the order of minutes, so as not to be lost during washing (Figure 5.5);
- with K_Ds in the micromolar range, or lower
- provided their serum binding partner is present at high nanomolar concentrations, or higher. At least 30 serum proteins can be expected to be present in this concentration range [131][4].

However, these calculations are highly simplified and make several assumptions, and are thereby likely to underestimate the potential this screening approach has to discover novel interactions. The calculations have been based on biochemical co-purification assays using 1mL of serum and 100µL of beads; by increasing the amount of beads used in the assay, the amount of serum protein that can be captured also increases so number of interacting serum proteins that we can hope to detect will rise[5]. These calculations also assume that proteins are interacting monovalently. In reality a number of serum proteins, including IgM, are multimeric and may thus interact with the merozoite protein-coated beads with a high avidity, increasing the sensitivity of the detection system. Perhaps the biggest flaw with these calculations is the

[4] As proteins are denatured prior to detection, the abundance of the particular interacting chain of multimeric proteins will also be important. See Figure 5.1.

[5] Taking into account the assumptions made in the above calculations, doubling the amount of beads would theoretically double the maximum detectable K_D for a given concentration of serum protein

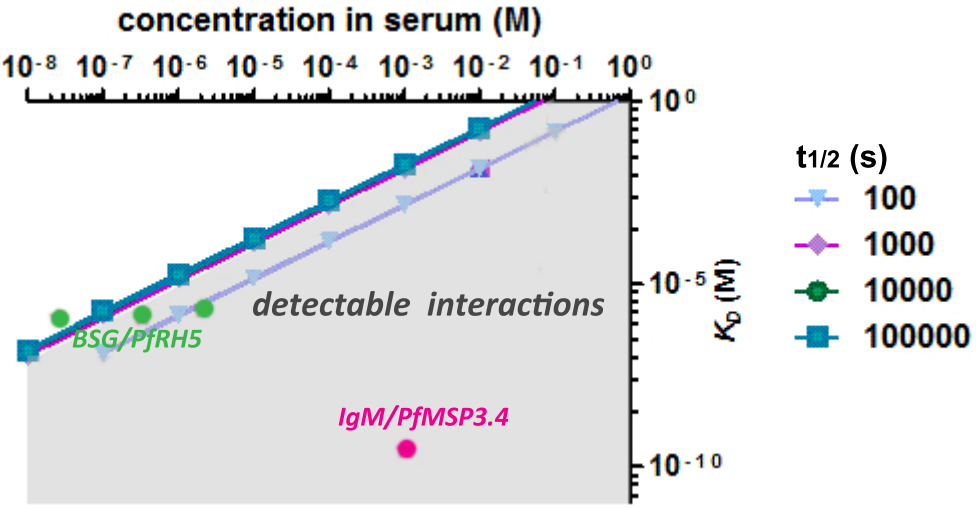


Figure 5.7: **Theoretical determination of the range of detectable interactions**
For given interaction half lives (during washing), the maximal K_D that would allow the recovery sufficient serum protein is shown as a function of the protein's original concentration in the serum. The interaction between serum IgM and PfMSP3.4 falls well within the predicted detectable range. The indicated BSG concentrations correspond to those used in the experiment depicted in Figure 5.10.

simplification that the proteins are interacting in free solution, when in fact they are interacting around the surface of a bead. This may significantly increase the effective half-life of the bead-serum protein interaction, as there is a very high local concentration of *Plasmodium* bait protein. This way, when an interacting serum protein dissociates from an individual *Plasmodium* bait it can readily re-associate with a neighbouring bait protein and remain bound to the bead surface for longer than would be expected for an interaction between free, monomeric proteins. This single simplification may mean that assuming exponential decay during wash steps massively underestimates the range of half-lives of detectable interactions, and also that lower-affinity interactions have a higher chance of detection than the calculations suggest.

5.3 Results

5.3.1 The optimised assay reproducibly detected the interaction between PfMSP3.4 and IgM

In developing the assay, I used the previously-identified interaction between PfMSP3.4 and serum IgM. In a biochemical co-purification assay using PfMSP3.4 bait, we would therefore expect to elute 72kDa and 25kDa proteins corresponding to the heavy and light chains of IgM. We might also expect to recover the 115kDa PfMSP3.4 protein. These three species were all observed by SDS-PAGE (Figure 5.6B). To support the assumption that the identity of bands are as expected, I ran purified IgM, biotinylated PfMSP3.4 and bovine serum albumin (BSA, which is almost identical in size to human serum albumin) alongside the eluate by SDS-PAGE (Figure 5.8); each band of the eluate was identical in size to their purified expected counterpart. To confirm the identity of the IgM heavy chain, I extracted peptides from the appropriate bands from PfMSP3.4 and PfMSP3.8 biochemical co-purification assays and submitted them for in-house tandem mass spectrometry analysis (LC-MS/MS). The most abundant peptides identified from these bands mapped to human IgM, indicating that this protocol can be used to accurately determine the identity of eluted interacting serum proteins (Tables 5.1 & 5.2).

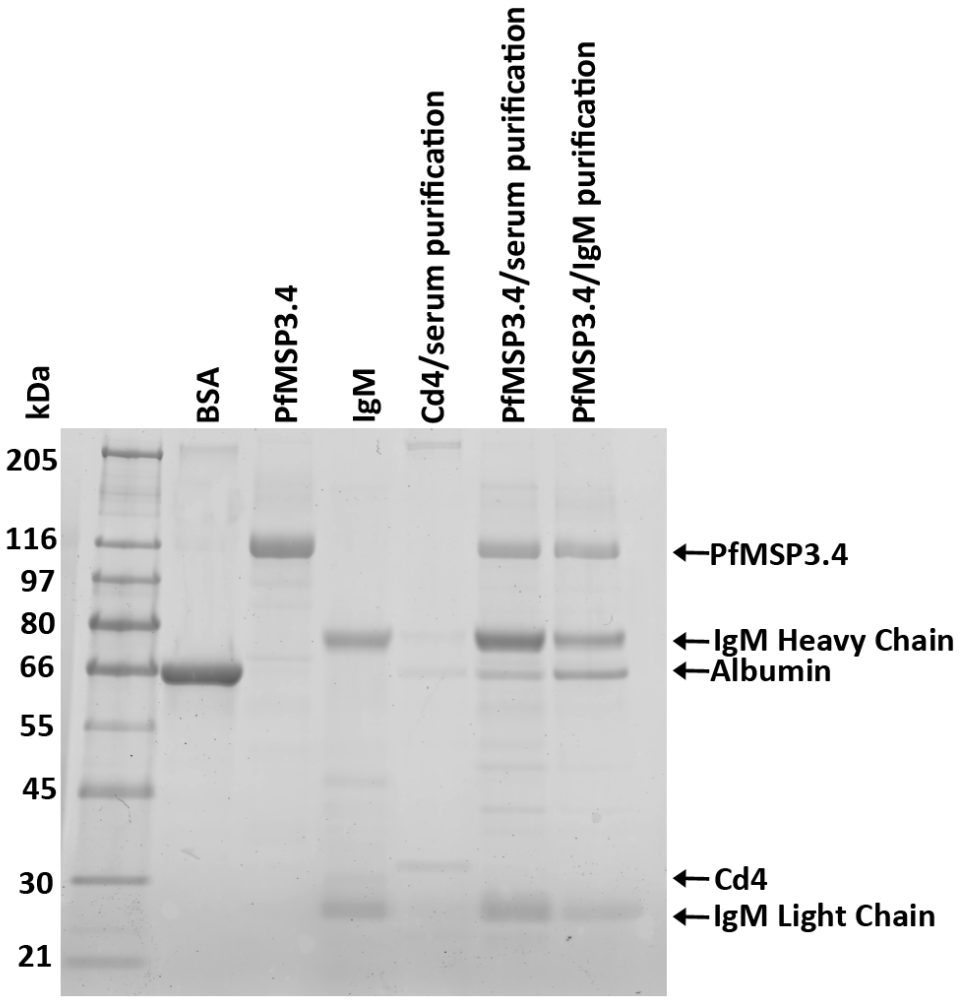


Figure 5.8: **Identification of IgM from PfMSP3.4 biochemical co-purification experiments** Purified BSA, biotinylated PfMSP3.4 and IgM were resolved by SDS-PAGE alongside eluates from serum biochemical co-purification experiments using Cd4- and PfMSP3.4-coated beads. The biochemical co-purification protocol was also performed using PfMSP3.4-coated beads, replacing serum with purified IgM in 5% BSA (far-right lane).

Protein	Example Uniprot ID	Unique peptides
IgM heavy chain constant region	P01871	54
Keratin	P35527	37
Complement C4	B0UZ83	27
Ig heavy chain variable region	P01765	24
Heat shock protein	P07900	6
RNA-binding protein	H3BPE7	6
Desmoplakin	P15924	6
IgG heavy chain constant region	P01857	4
Dermicidin	P81605	4
Serotransferrin	P02787	4
Arginase 1	P05089	3
Desmoglien 1	Q02413	1
Caspase 14	P31944	1
Coagulation factor V	P12259	1

Table 5.1: **Peptides identified by mass spectrometry from hypothesised IgM heavy chain band from PfMSP3.4 biochemical purification** In this experiment, the β chain of Complement C4 was a significant contaminant, which is perhaps unsurprising as it as an abundant component of human serum and is equivalent in mass to the IgM heavy chain (Figure 5.1).

Protein	Example Uniprot ID	Unique peptides
IgM heavy chain constant region	P01871	57
Ig heavy chain variable region	P10766	22
Keratin	P35527	8
IgG heavy chain constant region	P01857	3
Complement C4	B0UZ83	1

Table 5.2: **Peptides identified by mass spectrometry from hypothesised IgM heavy chain band from PfMSP3.8 biochemical purification**

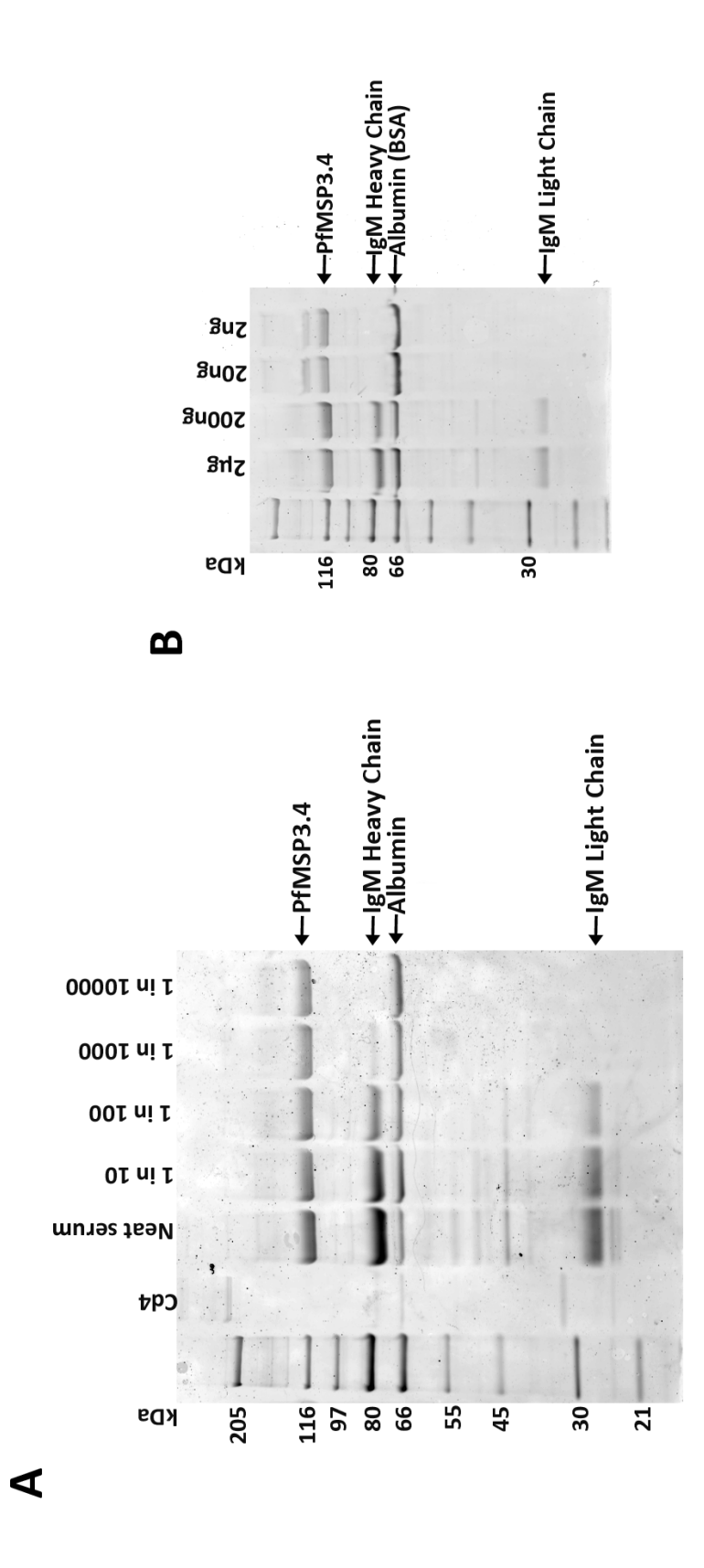


Figure 5.9: **PfMSP3.4 was used to detect IgM with high sensitivity**
A. SDS-PAGE analyses of the eluates from biochemical co-purifications performed using 1mL serial dilutions of serum indicate that 100µL PfMSP3.4-coated beads can purify IgM even when its serum concentration is diminished 1000-fold.
B. Similarly, biochemical co-purifications performed using purified IgM/BSA instead of serum indicated that 100µL PfMSP3.4-coated beads can purify IgM when only 200ng is present in a 1mL volume.

5.3.2 The PfMSP3.4 bait could be used to co-purify IgM with a very high sensitivity

To test the sensitivity of the optimised IgM co-purification assay, I performed it using serial dilutions of serum. I diluted the serum in PBS containing 5% BSA, such that the albumin content of the dilutions remained approximately constant, but the concentration of IgM was reduced (Figure 5.9A). The interaction between recombinant PfMSP3.4 (in its oligometric form) and IgM has an estimated K_D of 0.3nM, determined by SPR. According to the theoretical calculations in 5.2.5, this high-affinity means that the PfMSP3.4 should be able to purify IgM, even if the antibody is present at very low levels (Figure 5.7). IgM can still be detected when serum is diluted 1000-fold, when its expected concentration would be approximately 2µg/mL. I performed the same assay replacing serum with purified IgM diluted in PBS + 5% BSA (Figure 5.9B). Using the purified IgM it was possible to detect the interaction when only 200ng of IgM was added, approximating to a 1 in 10,000 dilution of serum. According to the manufacturers instructions, SYPRO staining can be used to detect as little as 4ng of protein in an SDS-PAGE band, so when 20ng was added to the biochemical co-purification assay, this is probably at the very limit of detection of the assay[6]. We might expect to be able to detect IgM at a 1 in 10,000 dilution of serum, when the IgM concentration is thought to be 200ng/mL, however IgM bands are not present for this dilution in Figure 5.9A. Despite this, IgM bands are seen in Figure 5.9B when 200ng purified IgM was present, indicating that the assay is a highly sensitive method to detect this interaction.

5.3.3 The biochemical co-purification assay was capable of detecting a low affinity interaction

IgM interacts with PfMSP3.4 with a high affinity, but the interactions we wish to identify may not be so strong. To experimentally determine whether the optimised assay is capable of detecting low affinity interactions, I tested it using the interaction between PfRH5 and BSG, which has a micromolar affinity[64]. Even under equilibrium binding conditions, the interaction between monomeric proteins has a half-life of only a few seconds[347], so if the assumption that exponential decay occurs during washing is correct, it may not be possible to detect the interaction (Figure 5.5). BSG is not a known component of human serum, so I added a range of concentrations of purified BSG into the serum before use in the assay using

[6] only 25µL of the 100µL elutant was analysed by SDS-PAGE

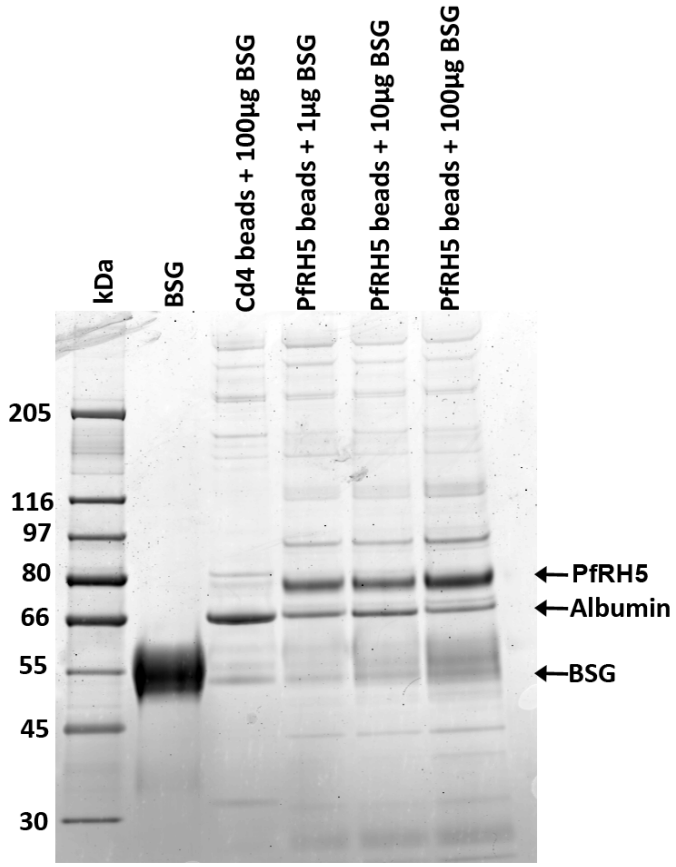


Figure 5.10: **PfRH5-coated beads could be used to co-purify BSG**
The biochemical co-purification assay was performed using 100µL PfRH5-coated beads in 1mL serum spiked with purified human BSG. When sufficient BSG was added, a enhancement of SYPRO staining is seen in the 55kDa region of the SDS-PAGE resolved eluate from co-purifications using PfRH5-coated beads when compared with that using Cd4-coated beads.

PfRH5 as the bait protein (Figure 5.10). Even if BSG remains bound to the PfRH5 beads following washing, these concentrations are at the very limits of the expected detection range for this interaction, given its micromolar K_D (indicated in Figure 5.7). High concentrations of BSG were required to be able to see an appropriately-sized band in the PfRH5 biochemical co-purification. When 100µg BSG was added into 1mL serum, there was a clear differential between the elution profiles when using Cd4- and PfRH5-coated beads, such that a 55kDa band corresponding to BSG was specifically observed in the PfRH5 lane. This implies that the stability of the BSG/bead interaction is higher than anticipated, and that the assay is potentially more sensitive than the above calculations suggest (as discussed in Section 5.2.5).

5.3.4 Serum interaction screens using purified merozoite baits

To screen for novel merozoite-serum interactions, I initially selected a range of merozoite proteins that are known to be exposed to the bloodstream. This shortlist included proteins known to be cleaved from the surface of the merozoite during the invasion process. PfMSP1, and hence the peripherally associated fragments of PfMSP6 and PfMSP7, as well as PfAMA1 are released as the parasite enters the host blood cells via the activity of the PfSUB2 [135]. PfMTRAP has also been implicated in RBC invasion and is potentially cleaved from the surface by a rhomboid protease upon the parasite's entry into the cell[12, 22]. PfRH5 was also included because it is of particular interest to vaccine research taking place within the laboratory, and elsewhere[41]. The elution profiles obtained appeared to contain a number of bands specific to the *Plasmodium* protein used to coat the beads. However, the majority of the bands were the correct size to be those of the input *Plasmodium* protein (indicated by grey circles in Figure 5.11). The elution profile when using PfAMA1 protein initially indicated that the protein was interacting with a serum component consisting of 75 and 25kDa fragments, but analysis of the protein size and re-sequencing of the expression plasmid revealed that the protein was in fact PfMSP3.8. We can therefore safely assume that these bands correspond to the heavy and light chains of IgM. The elution profile using PfRH5 bait also looked potentially interesting, as three unique bands were observed. SDS-PAGE analysis of the input recombinant PfRH5 showed that each of these bands were in fact fragments of the full-length PfRH5 precursor (Figure 5.11C). Proteolytic processing of recombinant PfRH5 has been previously reported[41, 277], so it is likely that we are observing a similar phenomenon in these preparations.

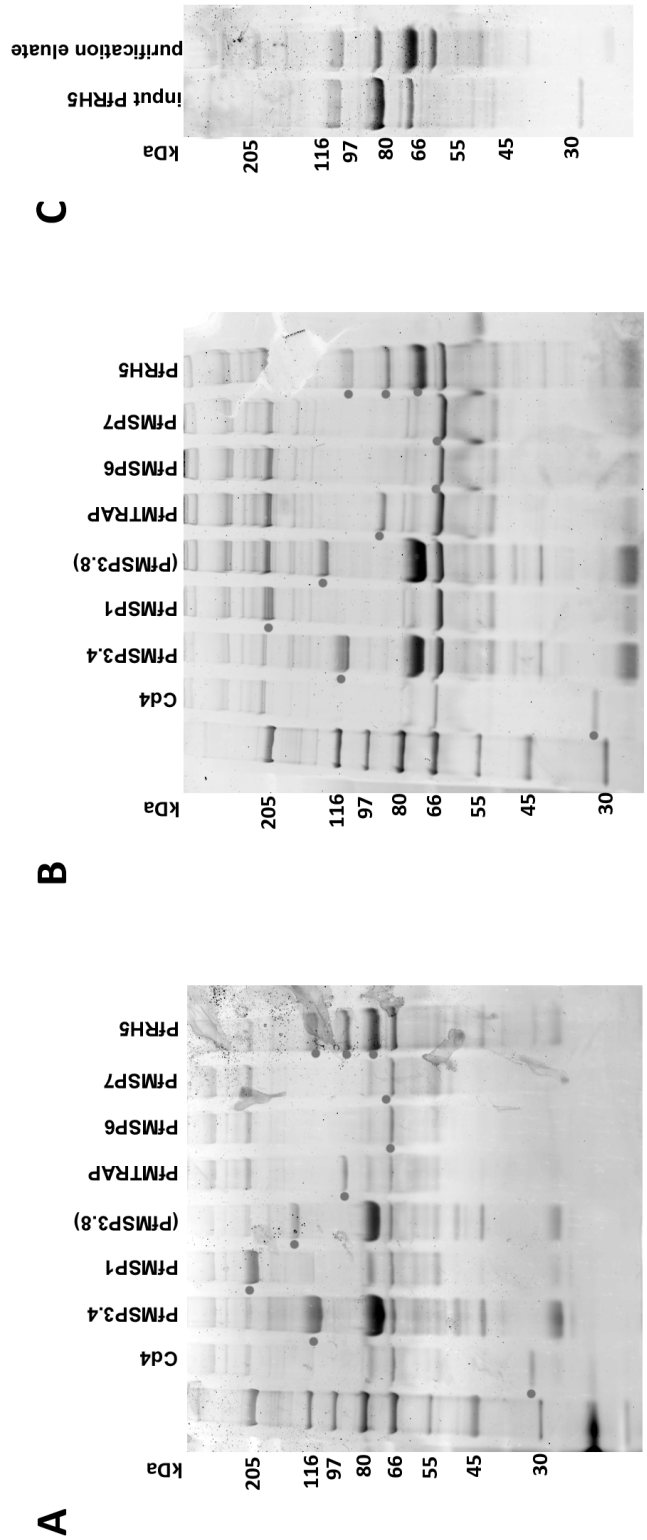


Figure 5.11: **An initial serum protein interaction screen identified no novel interactions**
A,B. SDS-PAGE elution profiles from biochemical co-purification assays using a range of serum-exposed *Plasmodium* baits. Grey circles to the left of bands indicate that the band corresponds to the input *Plasmodium* protein.
C. The PfRH5 input protein was resolved alongside the PfRH5 elution profile. PfRH5 appears to be proteolytically processed such that biotinylated species of 3 major lengths are used in the biochemical co-purification assay.

In two additional screens, all members of the PfMSP3 family and six members of the PfMSP7 family were loaded onto beads and incubated with human serum. These multi-gene families are enigmatic in that multiple members are preserved in parasite genomes, yet the reason for this is unclear. Most of the members of these families have no currently-identified function. We already know that PfMSP3.4 and PfMSP3.8 bind serum IgM in a potential immunomodulatory mechanism; however none of the other PfMSP3 family members appear to demonstrate the same antibody-binding behavior, nor did I reproducibly identify any other serum proteins to which PfMSP3 proteins could additionally bind (Figure 5.12). Recognising that the sensitivity of the assay may be limited by the amount of serum used, I repeated the assay using serum volumes up to 15mL. I also increased the bead volume to 200µL so as to expand the range of interactions that could potentially be discovered. Neither of these enhancements, individually or in combination, resulted in any novel bands in SDS-PAGE analyses of their eluates. Similarly, biochemical co-purification assays did not identify any serum protein binding partners for PfMSP7 proteins (Figure 5.13).

5.3.5 High-throughput screening of the merozoite protein library

In the screens described above, the major limitation on the throughput of the assay was the need to purify the recombinant biotinylated merozoite proteins with which to coat the beads. Although purified protein is preferable (as it can be more easily quantified and quality checked), it should be possible to use transfected HEK293E cell culture supernatant[7] as a direct source this biotinylated protein to coat the beads. To test whether this would be a viable approach, I selected a panel of eight merozoite protein baits for a pilot screen. I incubated 100µL beads with 1.5mL cell culture supernatant for 30 minutes, isolated the beads and performed ELISAs on the resulting supernatant. In most cases 1.5mL cell culture supernatant was insufficient to clearly saturate the binding capacity of 100µL beads, such that that no biotinylated protein could be detected following isolation of the beads. Where this occurred I re-incubated the partially coated beads with a further 1.5mL cell culture supernatant for 30 minutes and repeated the bead saturation ELISA. If I was still unable to observe saturation, I repeated the incubations with 1.5mL culture supernatant until unbound biotinylated protein could be detected by ELISA (Figure 5.14). Having demonstrated that bead saturation was possible without the use of purified proteins, I screened these proteins against human serum

[7] Dialysed as described in 2.1.2.3 to remove excess D-biotin

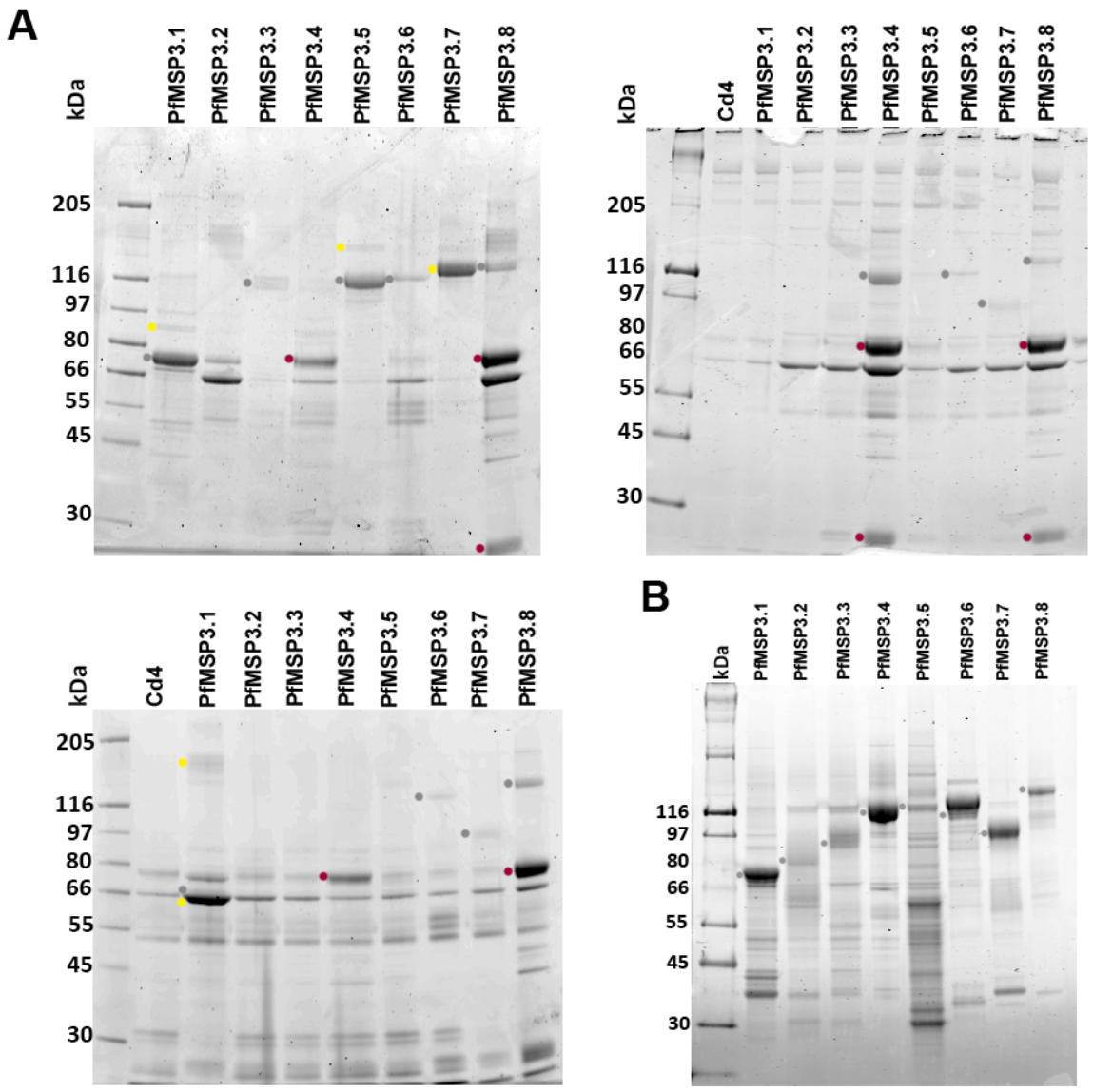


Figure 5.12: **PfMSP3-family serum biochemical co-purification screen revealed no novel interactions**

A SDS-PAGE elution profiles from biochemical co-purification assays using eight PfMSP3 family baits. Grey dots to the left of bands indicate that the band corresponds to the input *Plasmodium* protein. Pink dots indicate bands corresponding to the heavy or light chain of IgM, and yellow dots are placed to the left of bands that are potentially unique and do not correspond to the input bait protein or IgM. The three gels are a selection from six sets of biochemical co-purification experiments, each using a different batch of human serum.

B. SDS-PAGE analysis of the nickel-purified proteins incubated with the beads to produce the binding reagents. Grey dots indicate the expected protein sizes.

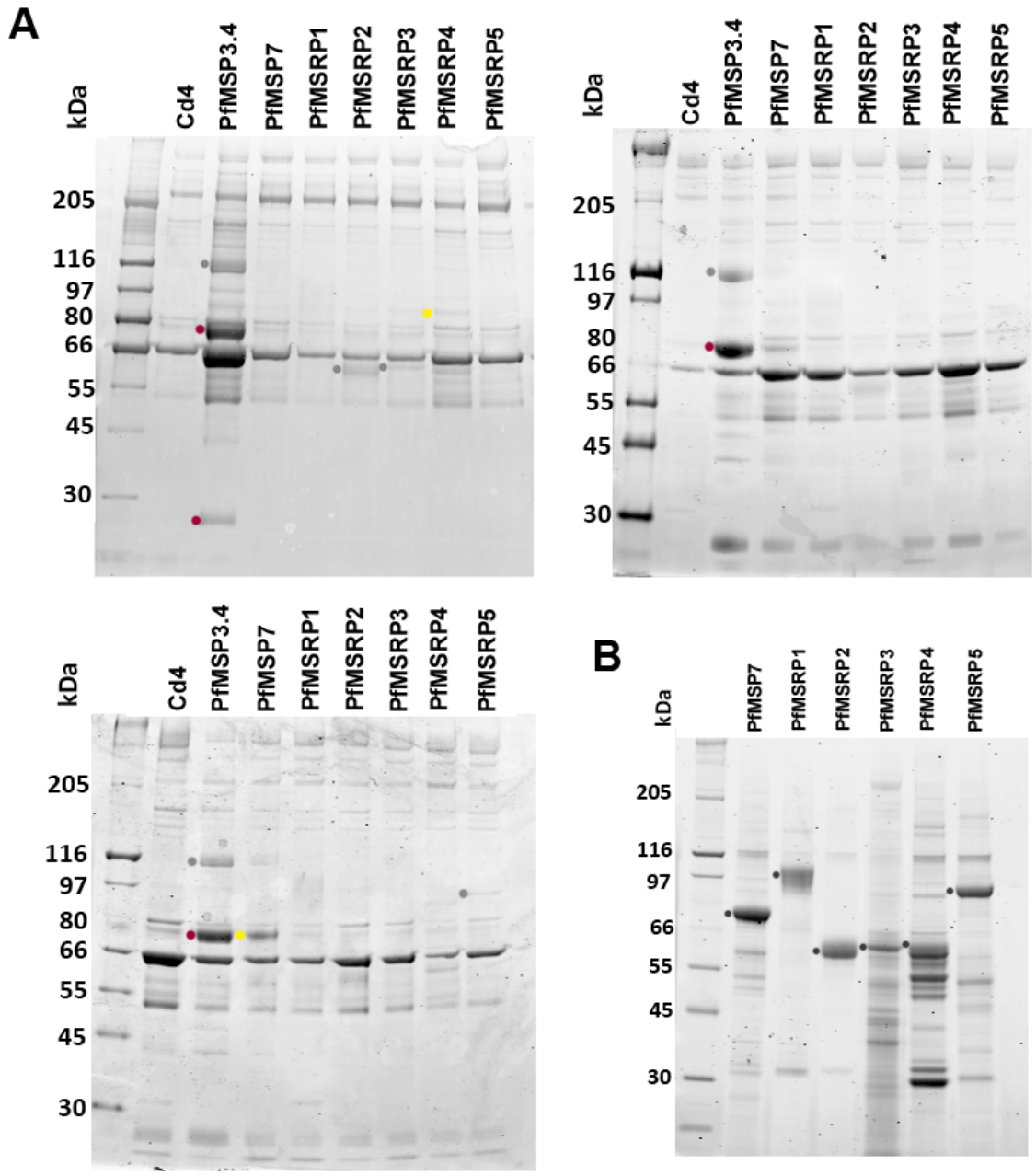


Figure 5.13: **PfMSP7-family serum biochemical co-purification screen revealed no novel interactions**

A SDS-PAGE elution profiles from biochemical co-purification assays using six PfMSP7 family baits. Grey dots to the left of bands indicate that the band corresponds to the input *Plasmodium* protein.

B. SDS-PAGE analysis of the nickel-purified proteins incubated with the beads to produce the binding reagents. Grey dots indicate the expected protein sizes.

as before, but without the bait protein purification step used originally. Encouragingly, the interaction between PfMSP3.4 and IgM was still readily detectable, as I reliably observed a clear band corresponding to the IgM heavy chain. A few bands appeared to be specific to the input bait protein, thus representing potential 'hits' in this screen. A proportion of these were of the correct size to be the bait protein itself, but a number of them were not. Of these, no single specific band was observed reproducibly in three independent screens. Following this pilot screen, I produced 32 additional proteins from the *P. falciparum* merozoite for to screen for interactions with human serum. I coated the beads by 3 successive 30-minute incubations with 1.5mL filtered, dialysed transfected cell culture supernatant, retaining the supernatant from the final incubation to confirm bead saturation by ELISA. I screened panels of up to ten proteins using 100µL beads and 1.5mL serum, and did so at least twice, using a different batch of serum on each occasion. I used these high throughput screens to select candidates for screening using 250µL beads and 5mL serum (Figure 5.17). None of the unique bands observed in Figures 5.15 & 5.16 were repeated or validated using this approach.

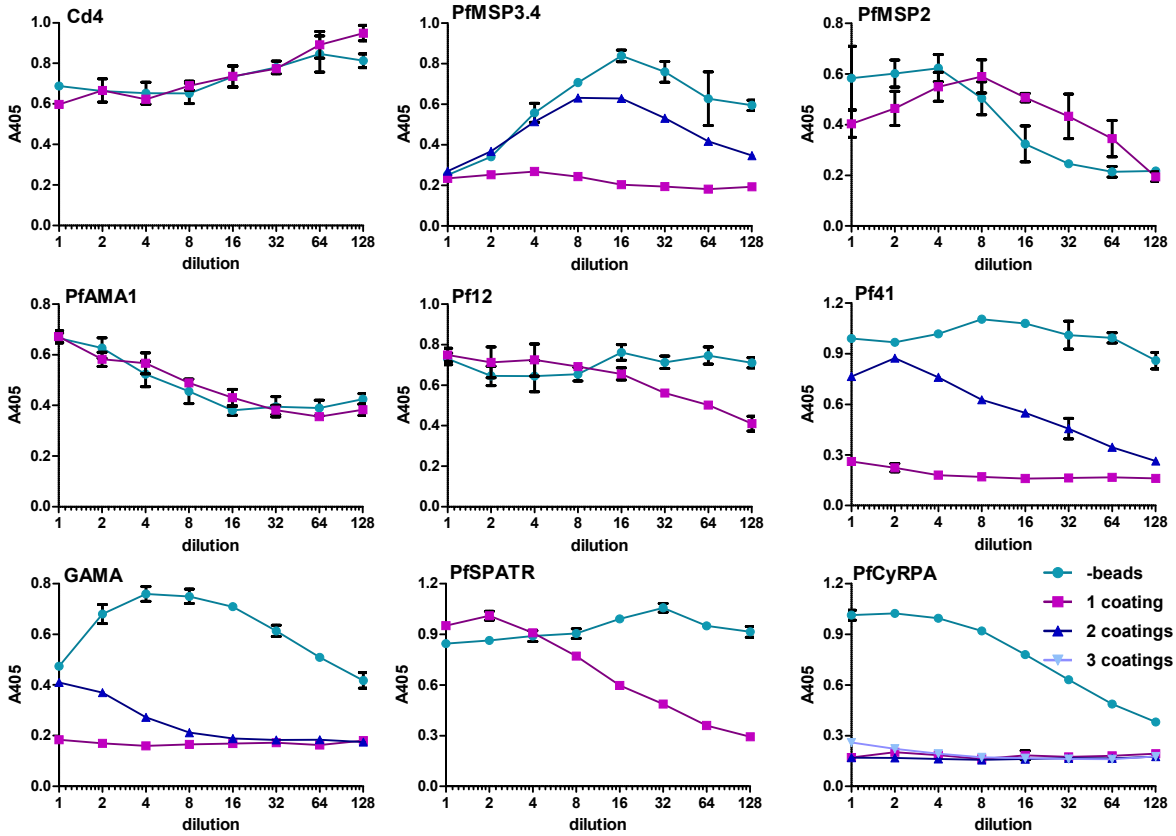


Figure 5.14: **Recombinant protein from HEK cell culture supernatant could saturate beads**

Bead-saturation ELISA measurements were performed on the supernatants following the isolation of 100µL beads after 30 minute incubations with 1.5mL filtered, dialysed HEK293 cell culture supernatants. The cyan data series shows the phosphatase substrate turnover, measured by absorbance at 405nm, resulting from the capture of biotinylated protein from the cell culture supernatant prior to its incubation with the beads. The remaining data series show the detection of biotinylated proteins following the indicated number of successive incubations of 1.5mL supernatants with 100µL beads. Error bars repesent the mean +/- SD, n=2.

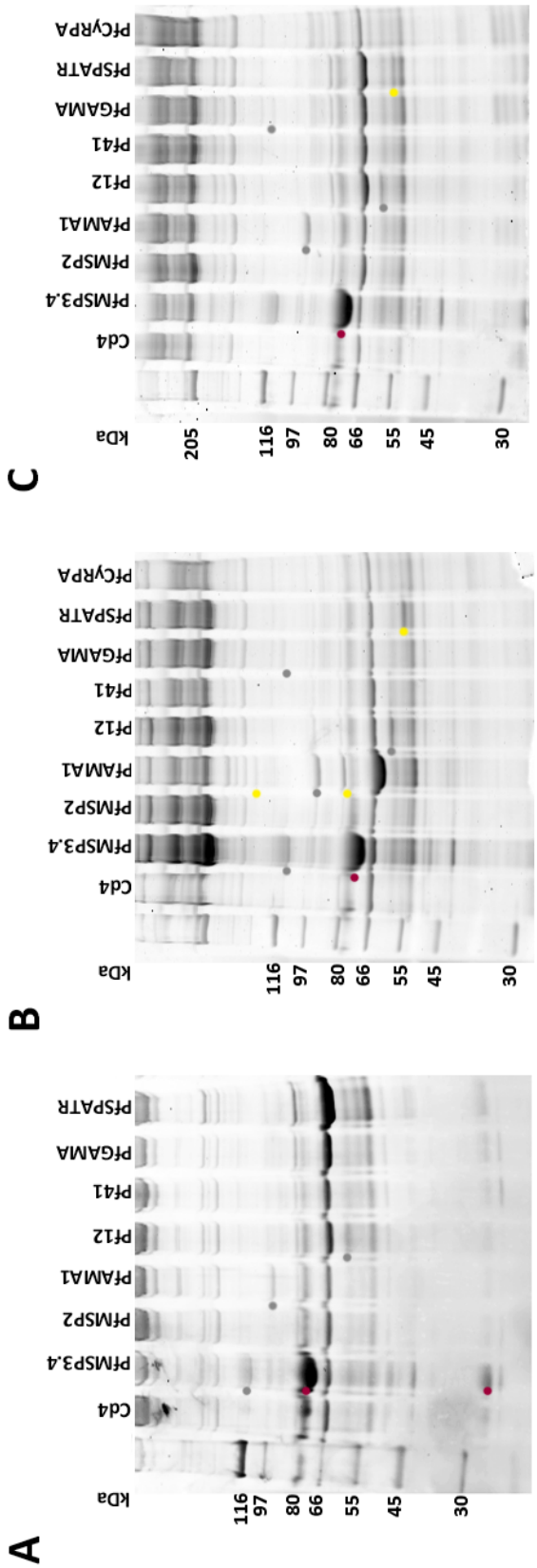


Figure 5.15: **A pilot high-throughput screen identified candidate *P. falciparum* proteins for further investigation**
Eight *P. falciparum* proteins were used to coat 100µL beads, which were used to probe 1.5mL human serum for potential host-pathogen interactions. The pink circles to the left of the indicated bands indicated the expected IgM chains purified using the PfMSP3.4 baits. Yellow circles to the left of bands indicate that they appeared specific to the bait protein used, and could hence represent hits in the screen. A subset of these are liable to be the bait proteins themselves (grey circles), for instance the PfAMA1-specific band around 90kDa, the Pf41-specific band around 60kDa, PfGAMA-specific band around 100kDa and the PfSPATR-specific band around 55kDa. Specific bands around 80 and 150kDa were seen when PfAMA1 bait was used in B, and significantly more albumin appeared to be detected when PfSPATR protein were used as a bait in A; this might indicate that more albumin interacted with PfSPATR-coated beads or that PfSPATR interacted with a serum protein of approximately 65kDa.

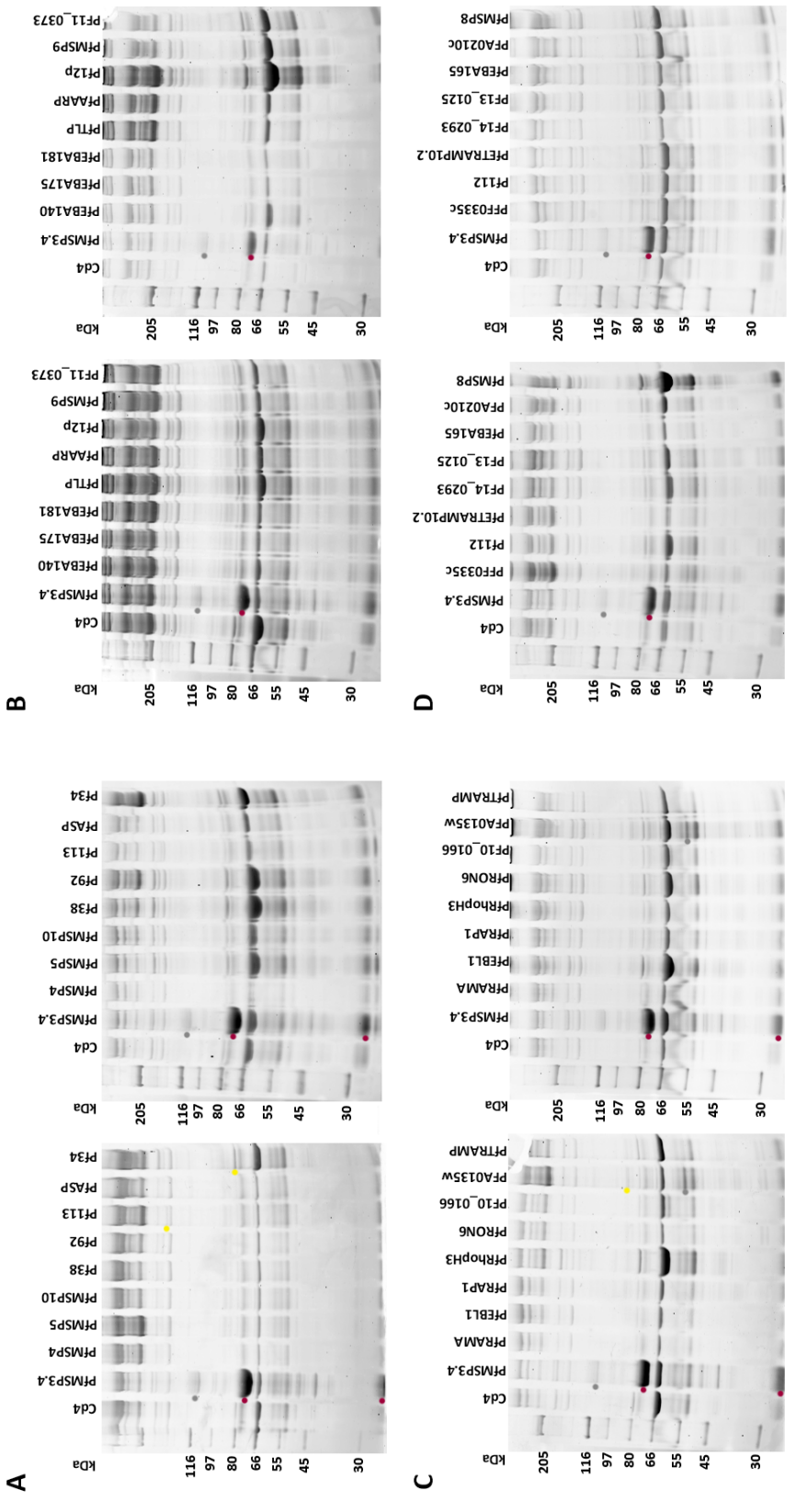


Figure 5.16: **Additional high-throughput interaction screens**
32 additional merozoite proteins were screened for interactions with human serum. Screens were performed in duplicate, using panels of eight proteins.

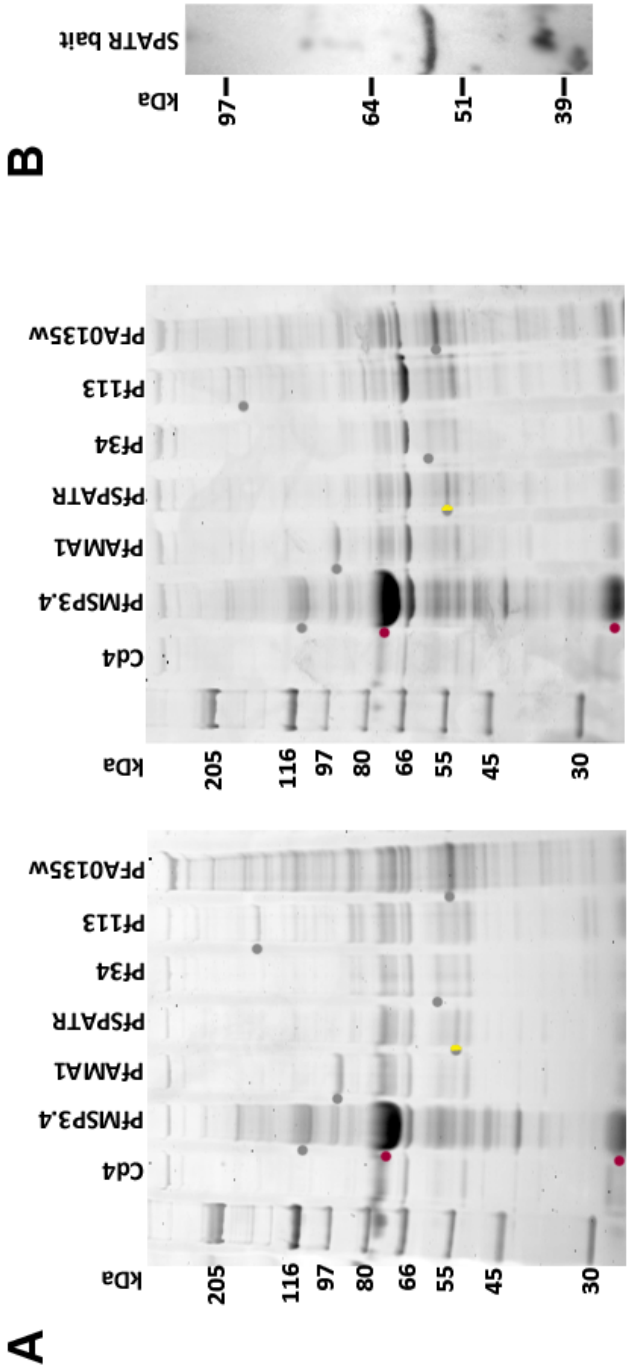


Figure 5.17: **Follow-up screening did not validate potential hits from high-throughput screening**
A Five *P. falciparum* proteins that potentially co-purified specific proteins from serum in earlier screens were screened, in duplicate, using 250µL beads and 5mL serum. Higher background was observed when PFA0335w was used as a bait protein, which could indicate that an interaction is occurring between PFA0335w and a human serum component. However, it is not possible from this information to speculate on an identity for a PFA0335w-interacting serum protein. Aside from those resulting from the PFA0335w co-purification, all unique bands observed were consistent in size with the respective bait proteins, indicated with a grey dot. The yellow-grey dot indicates a band that appeared to be significantly larger than the expected 49kDa size of the SPATR bait.
B Western blotting confirmed that the electrophoretic mobility of this band is consistent with being the input biotinylated SPATR protein.

5.4 Discussion

5.4.1 Strengths of the assay

This assay is capable of detecting interactions with a wide range of affinities, and can be combined with mass spectrometry to determine the identity of binding partners for *Plasmodium* proteins. Serum is an extremely protein-rich medium to sample for interactions (Figure 5.1), yet this assay is able to yield elution profiles with fairly low levels of contaminating proteins (Figures 5.6 & 5.8). Whilst the screens using 56 *P. falciparum* bait proteins did not lead to the identification of any novel interactions (Figures 5.11, 5.12, 5.13, 5.15, 5.16 & 5.17), there are still many more serum-exposed *Plasmodium* proteins that have yet to be screened. Hence this assay could be a useful tool for screening proteins for which there is a rational basis to assume that they might interact with a component of human serum. Serum is not the only matrix that can be screened using this approach; the method can be used to sample any other fluid. For instance I used SELP-coated beads to try to detect PfMSP7 fragments in parasite culture supernatant. In summary the assay has demonstrated potential for interaction detection, and could be used to discover novel interactions between recombinant proteins and partners in serum and beyond.

5.4.2 Potential improvements to the assay

Despite these successes, the assay is limited by the low abundances of certain serum proteins and the potentially transient nature of many host-pathogen interactions. However, there are a number of steps that could be taken to overcome these challenges.

- *Increase volumes:* Interaction detection capacity is certainly limited by the amount of serum protein that can be captured, either due to a low abundance of protein present in serum or a low interaction affinity that means that fewer molecules of interacting protein are bound to beads at equilibrium. As serum is abundantly available, it is very straightforward to increase its volume, although it is more cumbersome experimentally. Increasing the volume of beads has no negative effect on running the experiment, but is associated in a significantly increased cost. I performed the PfMSP3-family biochemical co-purification assay using 200µL beads and 15mL serum to see whether this would reveal any additional bands, but recovered very similar elution profiles to those presented in Figure 5.12.

- *Further increase the avidity and stability of interactions:* Coating *Plasmodium* bait pro-

***P. falciparum* bait**	**Accession number**	**Tags**	**Mass (kDa)**	**Plasmid constructor**
AARP	PF3D7_0423400	Bio	43.1	Dr C. Crosnier
AMA1	PF3D7_1133400	Bio	82.7	Dr C. Crosnier
ASP	PF3D7_0405900	Bio	103	Dr C. Crosnier
CyRPA	PF3D7_0423800	Bio, His	64.5	Dr Z. Zenonos
EBA140	PF3D7_1301600	Bio	152	Dr C. Crosnier
EBA165	PF3D7_0424300	Bio	178	Dr Z. Zenonos
EBA175	PF3D7_0731500	Bio	186	Dr C. Crosnier
EBA181	PF3D7_0102500	Bio	192	Dr C. Crosnier
EBL1	PF3D7_1371600	Bio	318	Dr C. Crosnier
ETRAMP10.2	PF3D7_1033200	Bio	26.5	Dr C. Crosnier
GAMA	PF3D7_0828800	Bio	103	Dr C. Crosnier
MSP1	PF3D7_0930300	Bio, His	216	A. J. Perrin
MSP2	PF3D7_0206800	Bio	45.9	Dr C. Crosnier
MSP3.1	PF3D7_1035400	Bio, His	62.4	A. J. Perrin
MSP3.2	PF3D7_1035500	Bio, His	65.5	A. J. Perrin
MSP3.3	PF3D7_1035600	Bio, His	70.5	A. J. Perrin
MSP3.4	PF3D7_1035700	Bio	100	Dr C. Crosnier
MSP3.4	PF3D7_1035700	Bio, His	102	A. J. Perrin
MSP3.5	PF3D7_1035800	Bio, His	104	A. J. Perrin
MSP3.6	PF3D7_1035900	Bio, His	87.8	A. J. Perrin
MSP3.7	PF3D7_1036000	Bio, His	68.7	A. J. Perrin
MSP3.8	PF3D7_1036300	Bio, His	110	A. J. Perrin
MSP4	PF3D7_0207000	Bio	49.1	Dr C. Crosnier
MSP5	PF3D7_0206900.1	Bio	50.1	Dr C. Crosnier
MSP7	PF3D7_1335100	Bio, His	63.1	A. J. Perrin
MSP8	PF3D7_0502400	Bio, His	89.0	Dr Z. Zenonos
MSP9	PF3D7_1228600	Bio	107	Dr C. Crosnier
MSP10	PF3D7_0620400	Bio	78.5	Dr C. Crosnier
MSRP1	PF3D7_1335000	Bio, His	65.4	A. J. Perrin
MSRP2	PF3D7_1334800	Bio, His	55.2	A. J. Perrin
MSRP3	PF3D7_1334600	Bio, His	57.4	A. J. Perrin
MSRP4	PF3D7_1334400	Bio, His	59.0	Dr Z. Zenonos
MSRP5	PF3D7_1334300	Bio, His	76.8	Dr Z. Zenonos
MTRAP	PF3D7_1028700	Bio	73.1	Dr C. Crosnier
Pf12	PF3D7_0612700	Bio	56.7	Dr C. Crosnier
Pf12p	PF3D7_0612800	Bio	61.4	Dr C. Crosnier
Pf34	PF3D7_0419700	Bio	57.0	Dr C. Crosnier
Pf38	PF3D7_0508000	Bio	58.8	Dr C. Crosnier
Pf41	PF3D7_0404900	Bio	63.6	Dr C. Crosnier
Pf92	PF3D7_1364100	Bio	110	Dr C. Crosnier
Pf112	PF3D7_1436300	Bio	133	Dr C. Crosnier
Pf113	PF3D7_1420700	Bio	130	Dr C. Crosnier

Table 5.3: ***P. falciparum* merozoite proteins screened against human serum**

P. falciparum bait	Accession number	Tags	Mass (kDa)	Plasmid constructor
PF10_0166	PF3D7_1017100	Bio, His	58.8	Dr Z. Zenonos
PF11_0373	PF3D7_1136200	Bio	94.4	Dr C. Crosnier
PF13_0125	PF3D7_1321900	Bio, His	76.9	Dr Z. Zenonos
PF14_0293	PF3D7_1431400	Bio	133	Dr C. Crosnier
PFA0135w	PF3D7_0102700	Bio, His	57.1	Dr Z. Zenonos
PFA0210c	PF3D7_0104200	Bio, His	76.1	Dr Z. Zenonos
PFF0335c	PF3D7_0606800	Bio	55.0	Dr C. Crosnier
RAMA	PF3D7_0707300	Bio	122	Dr C. Crosnier
RAP1	PF3D7_1410400	Bio	111	Dr C. Crosnier
RH5	PF3D7_0424100	Bio, His	85.2	A. J. Perrin
RhopH3	PF3D7_0905400	Bio	125	Dr C. Crosnier
RON6	PF3D7_0214900	Bio	133	Dr C. Crosnier
SPATR	PF3D7_0212600	Bio	49.7	Dr C. Crosnier
TLP	PF3D7_0616500	Bio	173	Dr C. Crosnier
TRAMP	PF3D7_1218000	Bio	56.0	Dr C. Crosnier

Table 5.4: ***P. falciparum* merozoite proteins screened against human serum (continued)**

teins onto paramagnetic beads has the dual purpose of facilitating the isolation of interacting proteins and increasing the stability of interactions with serum proteins. It may be possible to increase the strength of binding interactions further by changing the way that merozoite bait is arrayed on the surface of the bead. For instance, it might be possible to coat the bead surface with pentamerised proteins, so as to increase the number and density of receptors on the surface of the bead. Stabilisation of interactions could also be achieved by chemically cross-linking beads and interacting proteins prior to washing, although this might also compromise the purity of the eluate.

- *Improve quality of serum*: Serum was purchased as frozen, pooled isolates from a number of healthy donors. However, even before performing any assays with the serum, there was observable variation between the batches received. For instance, some batches were seen to contain large, visible protein aggregates upon thawing. I removed these aggregates by centrifugation and subsequent filtration, but in doing so could have removed an important source of serum protein that could potentially interact with one (or even many) of the merozoite baits. I expect that variability in the serum batches might account for differences in backgrounds of the serum screens, for example in Figures 5.11, 5.12 and 5.13. In the future it may be possible to improve the source of serum, such that steps can be taken to reduce protein aggregation and increase the consistency of screens.

- *Alter elution conditions*: The experiment shown in Figure 5.4 indicated that SDS or Glycine-HCl would be appropriate elution buffers for the assay. The main difference between these buffers was that SDS was able to elute PfMSP3.4 as well as IgM from PfMSP3.4/serum biochemical co-purification experiments. Knowing that PfMSP3.4 oligomerises *in vitro* I had hypothesised that the best elution process would bring down IgM and the PfMSP3.4 that was peripherally associated with the beads (i.e. not via the biotin-streptavidin interaction). However, given the appearance of input protein in the elution profiles of each screen (Figures 5.11, 5.12 and 5.13), I expect that the denaturing conditions used are able to dissociate the biotin-streptavidin interaction that binds the merozoite protein to the beads. By using an acidic elution buffer, such as pH 1.5 Glycine-HCl, the biotin-streptavidin interaction may remain intact and only serum proteins may be eluted. This would remove the input protein bands from the gels, making the presence of additional interesting bands more immediately identifiable.

- *Decrease stringency:* These experiments were designed with the intention of observing a single, unique band in a denaturing gel electrophoresis-based analysis of the eluate from washed beads. This way the protein-of interest could be specifically isolated from the gel and identified by tandem mass spectrometry. This is perhaps an unrealistically stringent expectation and, as mass spectrometry techniques advance, it might be possible to compare more of the eluate from a large numbers of biochemical co-purifications and identify human proteins that are specifically enriched when particular merozoite baits are used[261], without an SDS-PAGE step.

Chapter 6

General discussion

6.1 Summary of results

In this work I have employed a range of techniques using recombinant *Plasmodium* proteins to identify host-pathogen protein-protein interactions occurring in malaria. These have included the use of established methodologies such as AVEXIS and surface plasmon resonance, and the development of flow cytometry and biochemical purification approaches.

High-throughput screening using AVEXIS enabled the identification of an interaction between human SELP and PfMSP7, which I demonstrated in several experimental systems and characterised in detail, identifying the interacting regions of both recombinant proteins. I subsequently tested a range of PfMSP7 homologues and discovered that this SELP-binding property is a conserved feature of multiple members of the MSP7 family, across at least three *Plasmodium* species. Given the known role of SELP in human immunity, I have investigated the hypothesis that MSP7 proteins could have an important immunomodulatory role. The observation that PfMSP7 can block the interaction between SELP and sLe^X, and potentially SELP and leukocytes, *in vitro* provides encouragement that the interaction could modulate the normal *in vivo* interactions between SELP and its sLe^X-containing ligands.

Screening using other approaches did not lead to the identification of any new receptors for *Plasmodium* proteins.

6.2 Implications of SELP/MSP7 interactions

6.2.1 A possible mechanism underlying MSP7-knockout phenotypes

The molecular mechanisms underlying the observed reduction in pathology in PbMSP7-knockout parasites compared to their wild-type counterparts in rodent models are currently unknown. These data regarding the interactions between MSP7 proteins and SELP might contribute to explaining these observations. Gomez *et al.* demonstrated that rodents infected with PbMSP7-knockout parasites were somewhat protected from death and they hypothesised that an immunomodulatory function for PbMSP7 underlay this phenomenon[117]. It is possible that an interaction with Selp that prevents its recruitment and activation of leukocytes (as described in 4.11) could provide a molecular basis for these results.

In addition to the data that suggest an immunomodulatory function for PbMSP7, Spaccapelo *et al.* showed that PbMSP7-knockout parasites induced less cerebral pathology than wild type parasites in experimental cerebral malaria models[313]. This is particularly interesting when combined with the data that show that Selp-knockout mice are completely protected from cerebral malaria[53, 59]. Whilst there may be distinct mechanisms underlying these two observations[1], it is noteworthy that when either binding partner (PbMSP7 or Selp) are lacking from experimental malaria in an ECM-susceptible mouse, cerebral pathology is significantly reduced. In terms of a mechanism by which an interaction between Selp and PbMSP7 could interact to exacerbate ECM symptoms, it is tempting to suggest that PbMSP7 binds to Selp and prevents the adhesion of leukocytes in the mouse brain. However, leukocyte adhesion in the brain is not thought to be affected in infected Selp-knockout mice, which makes this mechanism less likely[53].

Endothelial SELP is thought to preferentially recruit Th1 cells which are associated with the production of pro-inflammatory cytokines, including TNF, IFNγ and lymphotoxin[10, 30], which have all been implicated in exacerbating cerebral malaria[6, 85, 105, 122, 169, 177, 287, 364]. With this is mind, a hypothesis that unifies the data discussed above on the pathology of mice infected with PbMSP7 knockout parasites would be that an interaction between Selp and PbMSP7 prevents the recruitment and activation of Th1 cells, dampening the inflammatory response so as to reduce circulating levels of cytokines that control infection but also exacerbate cerebral pathology.

It is important to note that I did not observe a Selp/PbMSP7 interaction using AVEXIS (Figure 4.6C), which makes it highly speculative to correlate the observed features of infections with

[1] For example increased blood-brain barrier stability in Selp-knockout mice may be responsible for their protection from ECM[155]

PbMSP7-knockout parasites with the parasites' ability to bind Selp. However, the data described in 4.2.2.2 and Figure 4.6C do not eliminate the possibility that PbMSP7 interacts with Selp; the levels of PbMSP7 expression were consistently very low, which made it difficult to ensure that sufficient amounts of sufficient-quality PbMSP7 protein were used in experiments.

6.2.2 Implications for vaccine development

Antibodies against PfMSP7 have recently been associated with protective immunity in a cohort of Tanzanian children[273]. This evidence, combined with the abundance of the MSP1 complex on the merozoite surface[115], data suggesting immunisation with *P. yoelii* MSRP2 protects mice, and the documented ability of anti-MSP7 antibodies to impair RBC invasion[164, 357], makes PfMSP7 an attractive vaccine antigen. When designing a vaccine based on any particular protein target, it is informative to determine the precise epitopes that confer protective immunity. More antibodies were detected against PfMSP7$_{22}$ than PfMSP7-N in S. E. Asian serum samples, suggesting that the humoral immune response against the PfMSP7 components that are present on the merozoite surface might be more dominant, though it is not known whether or which of these antibodies provided protection from malaria[349]. Since the work described in this thesis indicates that PfMSP7$_{22}$/PfMSP7$_{19}$ and the N-terminal fragments of PfMSP7 perform distinct functions, it will be important to determine the respective benefits to the host of immune responses against each PfMSP7 fragment. It is likely that antibodies against PfMSP7$_{22}$ can confer protection by preventing merozoite-RBC interactions or by inhibiting the shedding of the MSP1 complex[164, 357], but there is currently insufficient evidence to know whether anti-PfMSP7-N responses would be complementary or deleterious to the host. On one hand, *in vivo* evidence from mice infected with PbMSP7-knockout parasites indicates that the presence of MSP7 enhances pathology in the host[117, 313], such that antibodies that eliminate the function(s) of MSP7 might be expected to be protective. On the other hand, if PfMSP7's N-terminal fragment(s) play an anti-inflammatory role, which might limit immunopathology in the host, it might be advantageous to exclude these regions from protein-based vaccines so as to preserve this potentially beneficial function.

6.3 Summary of potential future experiments

6.3.1 Biochemical details of SELP/MSP7-family protein-protein interactions

Whilst the interaction between recombinant PfMSP7 and SELP has been demonstrated in multiple experiments, there are still some biochemical details of this interaction that remain to be clarified. The following experiments could help to do so:

1. *Further validate the interaction by demonstrating that PfMSP7 binds to native SELP*: Given our concerns about the non-specific interaction behaviour of recombinant SELP, it will be important to verify that the interaction can occur using native SELP. We could do this by re-designing the flow cytometry platelet-binding experiment (Figure 3.12) so as not to use the anti-FLAG antibody, potentially by using recombinant PfMSP7 directly fused to a fluorescent reporter protein.

2. *Determine the molecular basis of PfMSP7 oligomerisation:* We hypothesise that the *in vitro* assembly of PfMSP7 could enhance its binding to SELP. Having confirmed that oligomerisation is a property the SELP-binding N-terminus of PfMSP7 molecule, it would be informative to further narrow down the region(s) involved in oligomerisation using truncated PfMSP7 proteins in size-exclusion chromatography (SEC) experiments and/or by using synthetic peptides to prevent oligomerisation[144]. Techniques such as size exclusion chromatography with multi-angle light scattering (SEC-MALS)[290], X-ray solution scattering (SAXS)[272] or atomic force microscopy[309] could be used to determine the number and conformation of PfMSP7 monomers in each of these complexes, and in complex with SELP. Amino acid analysis of the SELP/PfMSP7 complex could also reveal the stoichiometry of the interaction[288]. This information could help us determine how the complexes form *in vitro* and whether complexes are likely to form *in vivo*.

3. *Accurately determine kinetic parameters of the SELP/PfMPS7 interaction using SPR:* Our initial SPR experiments suggest that larger PfMPS7 complexes bind more strongly to SELP. To separate the increase in SPR signal due to increased complex binding from that due to the increased size of the binding species, we would have to determine binding constants for each species. This would involve producing sufficient quantities of PfMSP7 to saturate binding to immobilised SELP, measuring the binding signal produced at a range of lower concentrations, creating an equilibrium binding curve and

using this to determine the K_D. Given the difficulties experienced in performing these analyses by SPR, it might be necessary to seek an alternative method to calculate biophysical parameters. Free-solution methods such as isothermal titration calorimetry[67] or back-scattering interferometry (discussed in 6.3.3.3) may be appropriate methods.

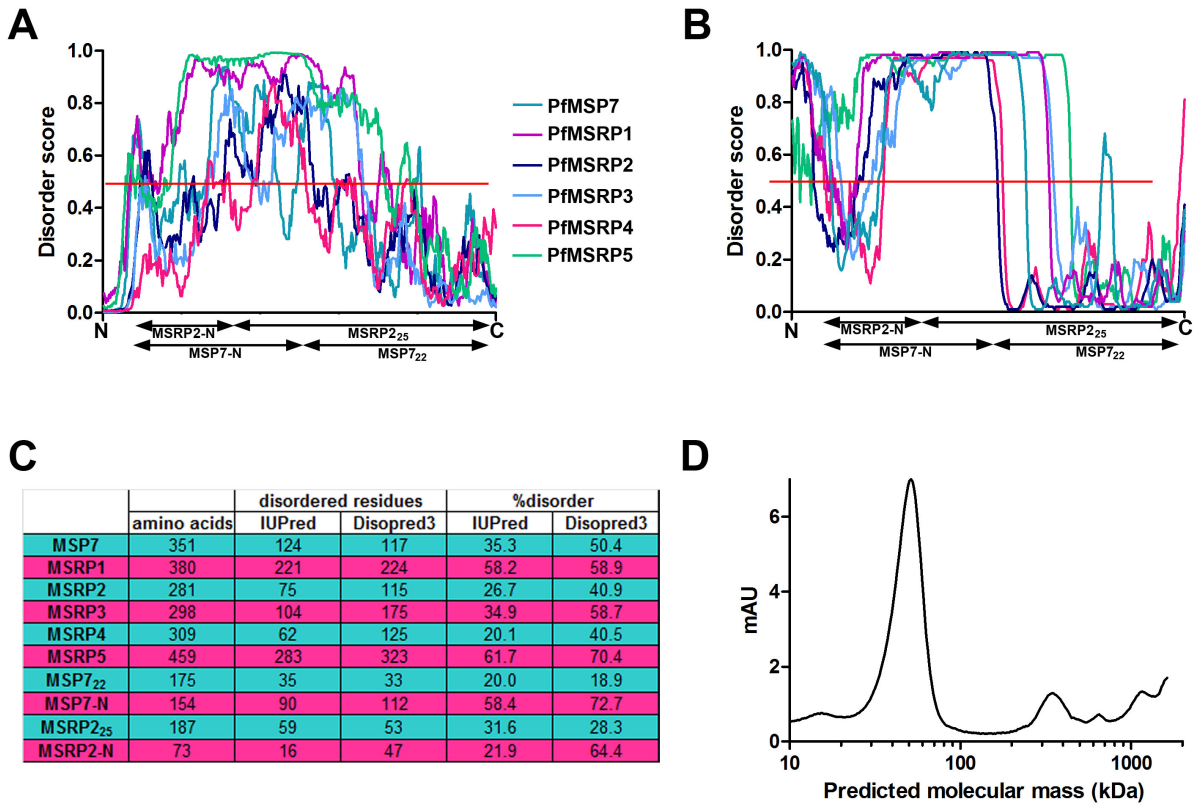
4. *Systematically characterise the oligomerisation and binding behaviour of the MSRPs:* AVEXIS data indicate that SELP-binding is not limited to PfMSP7. We could back up these data by observing the interactions by SPR or in the HEK-cell based flow cytometry assay (described in 2.6.2, 3.2.4 & Figure 2.4). It would be interesting to use SEC to determine whether the PfMSRPs also self-associate and whether this is necessary to promote their binding to selectins. If disordered regions in PfMSP7 are responsible for its oligomerisation, we might anticipate that the *P. falciparum* MSRP proteins would display similar behaviour, as all are predicated to be disordered, particularly at their N-termini (Figure 6.1A & B). Of the SELP-binding PfMSRP proteins, PfMSRP2 appears to be the least disordered (Figure 6.1A, B & C), and thus perhaps the most amenable to analysis by SPR. Early indications are that PfMSRP2 binding to SELP may indeed be easier to characterise, as an SEC-elution profile of purified material gave a monodisperse peak, with little evidence of significant oligomerisation (Figure 6.1D).

6.3.2 Determination of the function of the SELP/MSP7 interaction

Functional assays will be required to determine the potential *in vivo* role of the PfMSP7-SELP interaction. The following assays could be used to test our hypotheses as to the function of the interaction (introduced in 4.3):

1. *Leukocyte rolling assays*: Our initial attempts to use PfMSP7 in rolling assays[74] did not provide us with conclusive data to support or reject the hypothesis that PfMSP7 can modulate the rolling of leukocytes on endothelial cells. Having demonstrated that PSGL1-expressing THP1 cells can adhere to a SELP coated surface, and that PfMSP7 can be used to block this interaction, we hope to revisit and optimise these assays, as they represent a closer proxy for *in vivo* leukocyte-endothelial cell interactions than our existing plate-based assays.

2. *Inflammation assays:* We could test the hypothesis that PfMSP7 can behave as an anti-inflammatory molecule using Selp-dependent mouse model of inflammation. In one model system, inflammation is measured by counting blood and peritoneal neutrophils following injection of thioglycollate into the peritoneal cavity[206]. If PfMSP7-N can



		disordered residues		%disorder	
	amino acids	IUPred	Disopred3	IUPred	Disopred3
MSP7	351	124	117	35.3	50.4
MSRP1	380	221	224	58.2	58.9
MSRP2	281	75	115	26.7	40.9
MSRP3	298	104	175	34.9	58.7
MSRP4	309	62	125	20.1	40.5
MSRP5	459	283	323	61.7	70.4
MSP7$_{22}$	175	35	33	20.0	18.9
MSP7-N	154	90	112	58.4	72.7
MSRP2$_{25}$	187	59	53	31.6	28.3
MSRP2-N	73	16	47	21.9	64.4

Figure 6.1: **Predicted disorder is common to all *P. falciparum* MSP7-family proteins but lower in PfMSRP2**

A. IUPred prediction of disordered residues in the *P. falciparum* MSP7 family. Regions corresponding to PfMSP7$_{22}$, PfMSP7-N, the 25kDa C-terminal region of PfMSRP2 downstream of a validated SUB1 cleavage site (PfMSRP2$_{25}$) and the remaining portion (excluding the signal peptide) of its N-terminus (PfMSRP2-N) are indicated.

B. Disopred3 prediction of disordered residues in the *P. falciparum* MSP7-family

C. Summary of number and proportion of disordered residues predicted in *P. falciparum* MSP7-family, proteins.

D. SEC analysis of a single PfMSRP2 protein preparation gave a monodisperse peak. This might mean that SPR analyses using PfMSRP2 stand to be more successful than those using PfMSP7 (described in Chapter 3).

behave as an anti-inflammatory molecule, we might expect to see a reduced influx of neutrophils in mice that have been injected with PfMSP7 prior to thioglycollate introduction. This would constitute very convincing evidence that PfMSP7 has an immunomodulatory role

3. *Other adhesion assays:* To investigate whether PfMSP7 can affect other SELP-mediated adhesion events in disease it would be possible to introduce PfMSP7 into sequestration assays[101, 367] and RBC clumping assays[259]. We could test the hypothesis that PfMPS7 might modulate haemostasis by adding PfMSP7 into an appropriate assay to measure thrombus formation[281].

6.3.3 Expansion and improvement of screening for interactions occurring between *Plasmodium* proteins and human receptors

AVEXIS has been successful in identifying a number of host-pathogen interactions, including those between SELP and MSP7 proteins, that could contribute to our understanding of malarial pathology at the molecular level[19, 64]. However, this approach cannot be used exhaustively to screen all potential host receptors against our recombinant *P. falciparum* proteins; for instance there are many host proteins that we cannot produce recombinantly at sufficient levels, or with the appropriate post-translational modifications[2]. This makes it advantageous to screen for interactions using native sources of human receptor proteins, for instance in serum or on the surface of intact cells. Using receptors on the surface of intact cells has the particular advantage of conserving the native environment of the receptor proteins such that interactions involving protein complexes or proteins that traverse the membrane multiple times, can be studied.

The following approaches could potentially facilitate the identification of novel host-pathogen interactions that could in turn help to elucidate the molecular mechanisms involved in the pathogenesis of malaria:

1. *Screen more proteins for interaction with human serum:* In Chapter 5, I screened 56 *P. falciparum* proteins for interactions with human serum. There are many more serum-exposed *Plasmodium* proteins, including some included in our own existing protein expression libraries; I expressed at least 20 proteins that I did not screen, as their levels

[2]For example, we have been unable to produce recombinant Glycophorin A that is sufficiently sialylated to bind to PfEBA175. Recombinant PSGL1 was also unable to bind to SELP, which could very plausibly mean that HEK293E cells did not reproduce the post-translational addition of $sLe^X$to PSGL1 that occurs in leukocytes *in vivo*.

in cell culture supernatants were insufficient to saturate the protein-binding capacity of the paramagnetic beads. By scaling-up the production of these bait proteins, and enhancing their concentration by purification, it would be possible to screen more *P. falciparum* candidate bait proteins.

2. *Improve the sensitivity of the biochemical purification approach:* Despite screening a large number of proteins, the biochemical co-purification approach did not reveal any novel host-pathogen protein-protein interactions. This may be a consequence of the high stringency, and hence insufficient sensitivity, of this approach. By making the improvements identified in 5.4.2, and in particular by increasing the avidity and stability of interactions whilst decreasing the stringency of the detection method, the power of this method to detect novel interactions could be enhanced.

3. *Develop back-scattering interferometry (BSI) for the discovery and quantification of Plasmodium protein-host interactions*: BSI, described in Figure 6.2A, is a relatively new technique that can be used to measure binding events. In very recent years, the Bornhop laboratory has developed BSI as a highly sensitive method for studying biological interactions in free solution[3]. Various examples of simple protein-protein or protein-small molecule interactions have been studied using BSI[31] and it is also possible to use cell membrane preparations instead of isolated proteins in this system[13], which makes BSI an exciting new tool for studying interactions occurring at the cell surface. In the course of this work, we have been collaborating with the Bornhop laboratory to determine whether BSI can be used to study interactions occurring between our recombinant *P. falciparum* proteins and the RBC surface; preliminary data has indicated that BSI is a very sensitive method that can be used to accurately determine the kinetic parameters of interactions occurring between known *P. falciparum* invasion ligands and their receptors on intact human RBCs (Figure 6.2B). Thus, this method has great potential as a screening tool for the discovery of novel interactions between parasite and RBC surface proteins. Since BSI has also been applied to the detection of molecules in more complex matrices, including serum[175], it might also be possible to use this technique to screen our recombinant *Plasmodium* proteins against human serum; BSI could represent a highly sensitive detection method for serum-binding proteins, whose receptors could be subsequently be identified using biochemical purification and mass spectrometry approaches discussed in Chapter 5.

[3] In its earlier stages BSI used immobilised receptors on the surface of the chip, in a similar manner to SPR

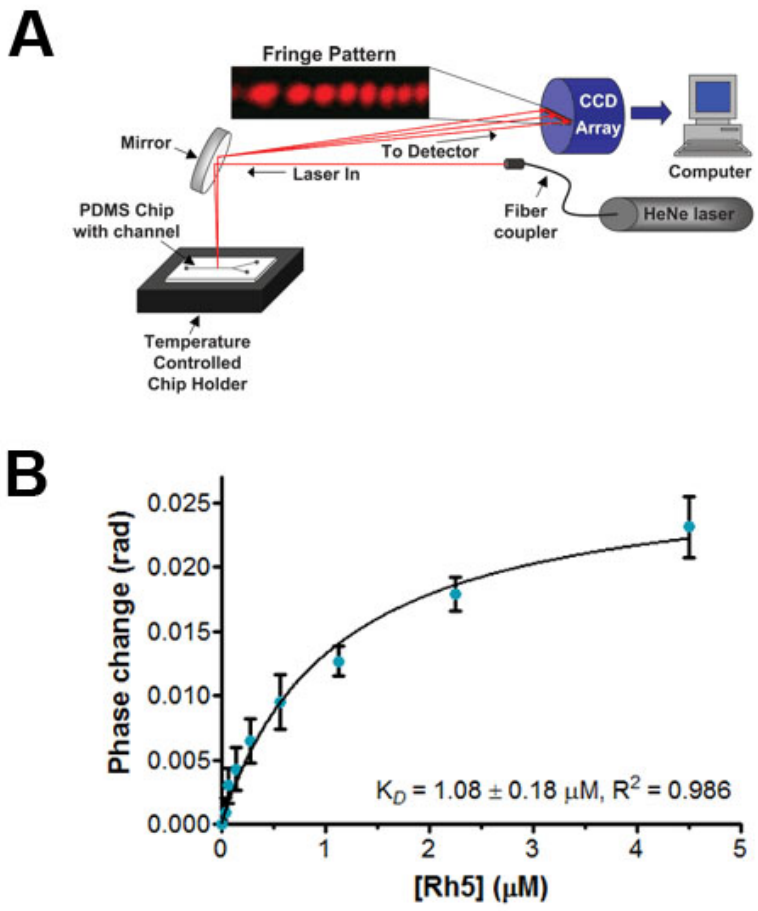


Figure 6.2: **BSI as a method to discover and measure protein-RBC interactions**

A. Schematic representation of the BSI methodology. In brief, a helium-neon laser beam is directed onto a microfluidic chip containing the sample. Photons take different paths through the matrix, being reflected in complex patterns from the walls of the channel such that they are out-of-phase (and thus interfere) upon their exit from the channel. This results in the formation of a visible interferometric fringe pattern. The fringe pattern can be recorded using a CCD camera, then analysed. An increase in the refractive index of the solution will proportionately decrease the speed at which the photons travel through the matrix, meaning that their phase upon exit from the channel will be different. Consequently, the waves interfere with each other differently and the position of the fringe pattern changes. BSI can be used to measure tiny shifts in the position of the fringes and infer a change in the refractive index of the solution. Biomolecular binding events can bring about profound changes to the organisation of molecules in solution, and hence cause refractive index changes. The formation of a new complex in the solution has been shown to induce measurable phase shifts in the fringe pattern detected by BSI, even at very low concentrations of the binding species. Figure from Bornhop *et al.*, (2007)[31]. Reproduced with permission from AAAS.

B. BSI was used to measure the interaction of purified recombinant PfRH5 and BSG receptors on the suface of intact RBCs. In brief, RBCs were incubated with a range of PfRH5 concentrations for one hour and the phase of the interference fringe pattern was measured using the BSI instrument. Phase change was calculated by subtracting this reading from that using negative control RBCs that had been pre-incubated with anti-BSG antibody, to prevent PfRH5 binding. These data allowed the plotting of a saturation binding curve and the estimation of the K_D at around 1µM, which is almost identical to that calculated for the interaction between recombinant PfRH5 and BSG by SPR by Crosnier *et al.*, (2011)[64]. Error bars represent mean +/- SEM, n=7. Data courtesy of Phoonthawee Saetear, Vanderbilt University.

6.4 Concluding remarks

Whilst the main result of this work is the discovery of a set of interactions occurring *in vitro* between human SELP and *Plasmodium* MSP7 proteins, I have also developed and optimised experimental techniques that can be used more broadly for the discovery and characterisation of protein-protein interactions. Going forward, the interactions between SELP and MSP7s require further validation and functional analyses so as to precisely define their role in the pathogenesis of malaria. Concurrently, the further use, development and refinement of interaction detection methodologies such as those discussed in this thesis could lead to the identification of additional host-pathogen interactions. The processes of validation, biochemical characterisation and functional analysis, as described for SELP/MSP7 interactions, could then contribute to our existing knowledge about the molecular details of the interactions *Plasmodium* parasites make with their hosts. These insights could contribute to the rational design of drugs and vaccinations, to combat the global burden of malaria.

Appendix A

Glossary

A.1 Abbreviations Used in this work

ADP: adenine diphosphate
AP: alkaline phosphatase
APC: antigen-presenting cell
AVEXIS: **Av**idity-based **ex**tracellular **i**nteraction **s**creening
BBB: blood-brain barrier
BSA: bovine serum albumin
BSI: Back-scattering interferometry
cDNA: complementary DNA
COS: A simian cell line, **C**V-1 in **O**rigin, and carrying the **S**V40 virus genetic material
CTL: C-type lectin
DC: dendritic cell
DNA: deoxyribonucleic acid
dNTPs: deoxyribonucleotide triphospates
EBV: Epstein-Barr Virus
ECM: experimental cerebral malaria
EDTA: ethylenediaminetetraacetic acid
ELISA: enzyme-linked immunosorbant assay
FACS: fluorescene-associated cell sorting
FCS: foetal calf serum
FITC: fluorescein isothiocyanate
FLAG: DYKDDDDK octapeptide tag
GPI: glycophosphatidylinositol

GST: glutathione S-transferase,
HBS: HEPES-buffered saline
HBST: HBS containing 0.2% Tween
HEK: Human Embryonic Kidney
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP: Horseradish peroxidase
HSPG: heparan sulphate proteoglycan
HUVEC: human umbilical vien endothelial cell
IMAC: immobilised metal ion affinity chromatography
ITN: insecticide-treated net
IRS: indoor residual spraying
LB: Luria broth
mAB: monoclonal antibody
MWCO: molecular weight cut-off
PAGE: polyacrylamide gel electrophoresis
PAMP: pathogen-associated molecular pattern
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PEI: polyethylenimine
PMA: Phorbol 12-myristate 13-acetate
PPI: protein-protein interaction
pRBC: parasitised red blood cell
PRP: platelet-rich plasma
PVDF: Polyvinylidene fluoride
RBC: red blood cell
SDS: sodium dodecyl sulphate
SEC: size-exclusion chromatography
sLe^X: Sialyl-$Lewis^X$
SMA: severe malarial anaemia
SNP: single nucleotide polymorphism
SPR: surface plasmon resonance
TM: transmembrane
WHO: World Health Organsiation
Y2H: yeast two-hybrid

A.2 Protein names

AMA1: Apical membrane antigen 1 (*P. falciparum*)
APLP2: Amyloid precursor-like protein 2 (*H.sapiens*)
ASP: Apical sushi protein (*P. falciparum*)
BirA: Biotin protein ligase (*E. coli*)
BSG: Basigin (*H.sapiens*)
CD: Cluster of differentiation protein (*H.sapiens, R.norvegicus)*
COMP: Cartilage oligomeric matrix protein (*H.sapiens*)
CR1: Complement receptor 1 (*H.sapiens*)
CSA: Chondroitin sulfate A (*H.sapiens*)
CSP: Circumsporozoite protein (*H.sapiens*)
EBA: Erythrocyte-binding antigen (*P. falciparum*)
EBL1: Erythrocyte binding ligand 1 (*P. falciparum*)
EBNA1: Epstein Barr Nuclear Antigen 1 (Epstein Barr virus)
EGF: Epidermal growth factor (*H.sapiens*)
EMP: Erythrocyte membrane protein (*P. falciparum*)
EPCR: Endothelial protein C receptor (*H.sapiens*)
ESAM: Endothelial cell-selective adhesion molecule (*H.sapiens*)
GFP: Green fluorescent protein (*A. victoria*)
GLURP: Glutamate rich protein (*P. falciparum*)
GYPA: Glycophorin A (*H.sapiens*)
ICAM: Intercellular adhesion molecule (*H.sapiens)*
IFN: Interferon (*H.sapiens)*
LAMP2: Lysosome-associated membrane protein 2 (*H.sapiens)*
MIF: Macrophage migration inhibitory factor (*H.sapiens)*
MSP: merozoite surface protein (*P. falciparum*, *P.vivax, P. berghei*)
MSRP: MSP7-related protein (*P. falciparum*, *P. berghei*)
MTRAP: merozoite TRAP-like protein (*P. falciparum*)
PECAM1: Platelet endothelial cell adhesion molecule 1 (*H.sapiens)*
PMIF: Plasmodium macrophage migration inhibitory factor (*P. falciparum*)
PSGL1: P-selectin glycoprotein ligand 1 (*H.sapiens)*
RH: Reticulocyte binding-like homologue (*P. falciparum*)
SCARF2: Scavenger receptor class F member 2 (*H.sapiens)*
SEA1: Schizont egress antigen 1 (*P. falciparum*)

SELE: E-selectin (*H.sapiens*)
SELL: L-selectin (*H.sapiens*)
SELP: P-selectin (*H.sapiens*, *M. musculus*)
SELPLG: P-selectin ligand (*H.sapiens*)
SEMA7a: Semaphorin 7a (*H.sapiens*)
SERA: Serine repeat antigen (*P. falciparum*)
SPATR: Sporozoite protein with an altered thrombospondin repeat (*P. falciparum*)
STARP: Sporozoite threonine-asparagine-rich protein (*P. falciparum*)
TLR: Toll-like receptor (*H.sapiens)*
TNF: Tumour necrosis factor (*H.sapiens)*
TRAP: Thrombospondin-related adhesive protein (*P. falciparum*, *P.berghei*)
TRSP: Thrombospondin-related sporozoite protein (*P. falciparum*)

A.3 Weblinks

1000 genomes sequencing database: www.1000genomes.org
dbSNP database: www.ncbi.nlm.nih.gov/SNP
Disopred3: Found at bioinf.cs.ucl.ac.uk/psipred
Disprot: www.disprot.org/pondr-fit.php
IUPred:iupred.enzim.hu
Pfam database: pfam.xfam.org
PlasmoDB: plasmodb.org/plasmo/
PrDOS:prdos.hgc.jp/cgi-bin/top.cgi
T-COFFEE multiple protein sequence alignment: www.ebi.ac.uk/Tools/msa/tcoffee
Uniprot: www.uniprot.org

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