CHAPTER ONE – INTRODUCTION

1.1 Life cycle of *P. falciparum*

The life cycle of *P. falciparum* (Figure 1) alternates between an exogenous sexual phase (sporogony) in female *Anopheles* mosquitoes and an endogenous asexual phase (schizogony) in a human host (Bray and Garnham, 1982; Miller et al., 2002). Infection is initiated following the inoculation of sporozoites by an infected mosquito. While taking a blood meal, sporozoites are transmitted from the vector's salivary gland into the human bloodstream. Once in the blood, they migrate to the liver and invade hepatocytes. There they will remain dormant for approximately two weeks, during which time the parasites undergo a single round of schizogony. The hepatocytes will eventually rupture releasing thousands of merozoites, each capable of infecting an erythrocyte.

The invasion of erythrocytes by *P. falciparum* initiates the pathogenic phase of the lifecycle and involves a complex sequence of events. Inside the erythrocyte, the merozoite develops through a number of asexual junctures during a 48 hour cycle (Miller et al., 2002). The early parasitic stages are uninucleate and are referred to as ring forms. In the course of their development, they enlarge as feeding stages known as trophozoites. After a period of growth the trophozoites divide asexually, which culminates in the release of around 20 merozoites per mature parasite. The invasion cycle is repeated leading to an exponential increase in parasitaemia.

After several generations of asexual cycling, a small proportion of merozoites differentiate into male and female gametocytes (Dyer and Day, 2000; Miller et al., 2002). This event is essential for the transmission and survival of the parasite. The sexual cycle is completed when a female *Anopheles* mosquito ingests erythrocytes containing gametocytes during a blood meal. In the mid-gut, the gametocytes undergo further development

while the asexual parasites and erythrocytes are digested. Male and female gametes are extruded from the erythrocytes and fuse to form a diploid zygote. After fertilisation, the zygote becomes motile and is referred to as the ookinete.

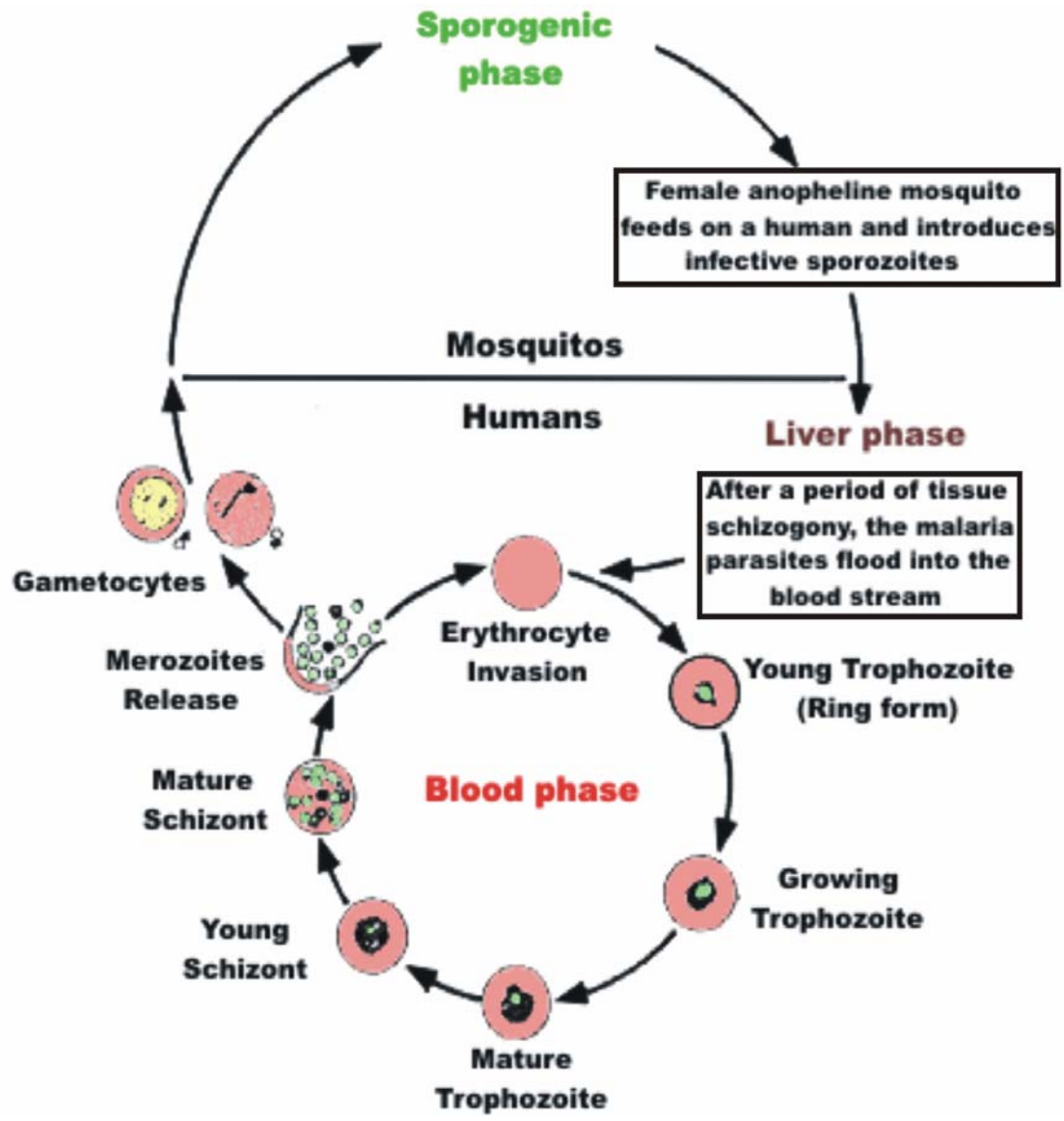

Figure 1- Life cycle of *Plasmodium falciparum* (WHO, 1991)

The ookinete penetrates the gut wall of the mosquito and forms a static oocyst on its outer surface. Sporogony ensues with the oocyst undergoing

repeated nuclear divisions giving rise to a number of haploid sporoblasts. As the oocyst ruptures, mature sporozoites are released and migrate to the salivary glands of the mosquito to complete the parasite's life cycle (Krettli and Miller, 2001).

1.2 Ultrastructure of the merozoite

Merozoites are ovoid-like cells (Figure 2) with dimensions of 1.6µm in length by 1.0µm in width. They have a characteristic conical projection or apical prominence at one end and a rounded surface at the basal end (Bannister et al., 2000). Merozoites contain a large nucleus situated basally as well as a single apicoplast and mitochondrion nearby. The region between the nucleus and apical end contains other organelles including endoplasmic reticulum, Golgi bodies and numerous free ribosomes. Surrounding the merozoite is a plasma membrane (PM) covered by a poorly defined filamentous coat. Coat proteins are anchored to the PM and initiate the first point of contact between the erythrocyte and merozoite during invasion. The coat is believed to be composed predominantly of the merozoite surface protein (MSP) 1 complex (Bannister et al., 2000). Two more membranes known as cisternae lie beneath the PM. The series of three membranes is termed the pellicle and are attached directly to each other with proteinaceous cross-bridges. Compared to other malaria species, the cytoskeleton of *P. falciparum* merozoites is relatively sparse; however it is important for maintaining the parasite's shape. At the merozoite's apex, three polar rings anchor the cisternae and a set of microtubules that extends underneath the parasite's PM (Bannister et al., 2000; Topolska et al., 2004). A number of proteins including actin, myosin and tubulin associate with the microtubules and together form the actomyosin motor (Kappe et al., 2004; Pinder et al., 2000). The motor is important for generating the force required for invading host cells. Merozoite movement into the erythrocyte manifests as a gliding action without any form of cellular deformation.

Merozoites house three types of specialised apical organelles, including rhoptries, micronemes and dense granules. The contents of these membranous vesicles are involved in the recognition, attachment and invasion of erythrocytes as well as the formation of the parasitophorous vacuole (PV) (Preiser et al., 2000; Soldati et al., 2004).

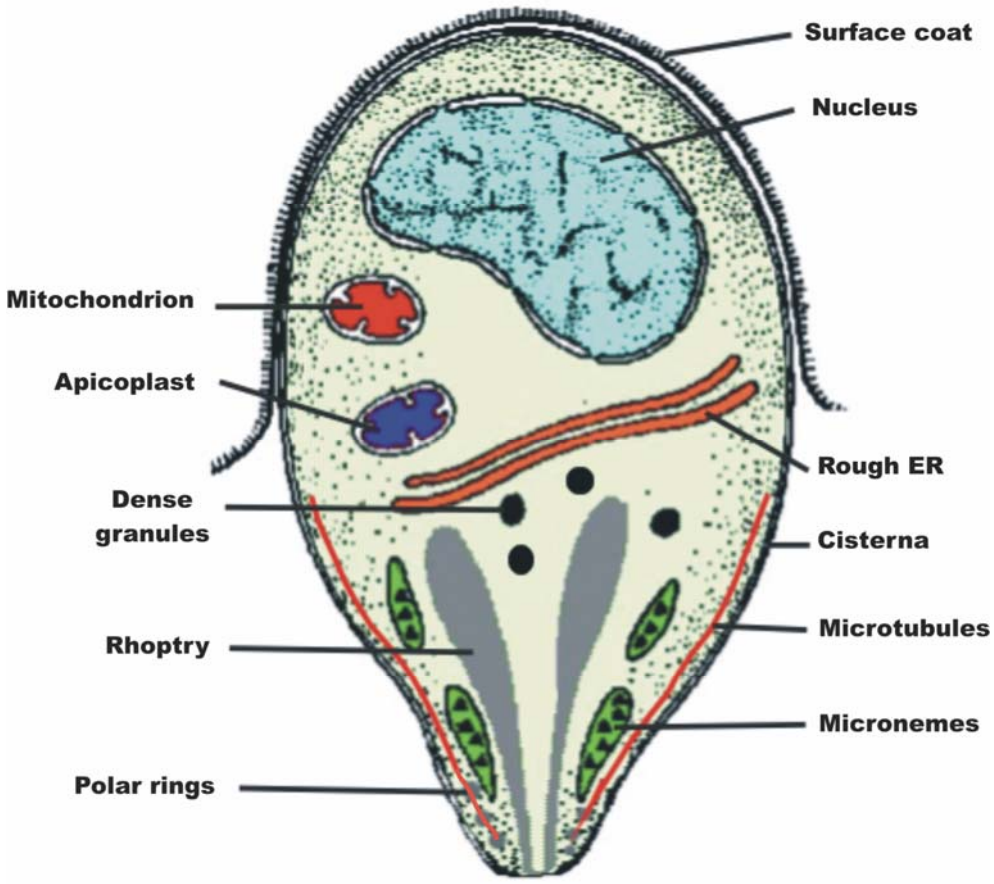

Figure 2- Ultrastructure of the *P. falciparum* merozoite

Morphology of the *P. falciparum* merozoite indicating major organelles and apical secretory vesicles. The filamentous coat, cisterna and sub-pellicular microtubules are also shown (adapted from: http://www-micro.msb.le.ac.uk/224/parasitology.html).

Rhoptries are the largest of the organelles and contain two parts: a dense bulbous body and a narrower rhoptry duct that converges just beneath the merozoite's PM. Both parts contain a mix of different proteins and complex lamellar structures (Preiser et al., 2000; Stewart et al., 1986). Evidence for

a lipid component stems from experiments utilising fluorescent lipid precursors (Mikkelsen et al., 1988). These probes localised to the rhoptries and upon erythrocyte invasion, were transferred to the PV membrane. Micronemes are smaller than rhoptries and are present in varying numbers. During the early stages of invasion, micronemes fuse with the rhoptry duct and are the first of the three organelles to release their contents. The third class of vesicles, dense granules, are packed granular structures located within the merozoite's cytoplasm. Following invasion, dense granules release their contents by exocytosis from the parasite's PM into the PV (Preiser et al., 2000).

1.3 Erythrocyte invasion

Erythrocyte invasion by malaria merozoites was initially detailed with studies using *P. knowlesi* (Aikawa et al., 1978; Bannister et al., 1975; Dvorak et al., 1975). The process in *P. falciparum* is similar and involves a complex sequence of events (Figure 3: **A-I**) which is completed within 30 seconds (Bannister and Dluzewski, 1990; O'Donnell and Blackman, 2005).

Erythrocyte invasion occurs when *P. falciparum* merozoites attach to the surface of the host cell. The initial contact occurs in a random fashion **(A)** and appears to be established by a weak interaction probably involving MSP 1 (Cowman et al., 2000). If invasion continues, the area of adhesion between the two cells tends to be maximised **(B)**, followed by reorientation of the parasite's apical end into close contact with the erythrocyte membrane **(C)**. This results in the formation of an irreversible junction between the two cells, often observed as a thickening of the erythrocyte membrane. The junctional zone is believed to be composed of erythrocyte skeletal proteins (Aikawa et al., 1981) and merozoite proteins such as apical membrane antigen 1 (AMA 1) (Narum and Thomas, 1994). Apical contact triggers the secretion of micronemes followed by the rhoptry contents. An increase in the parasite's intracellular calcium concentration

has been implicated in providing the major signal for this event (Docampo and Moreno, 1996; Scheibel et al., 1987; Wilson, 1990). Rhoptries and micronemes are responsible for discharging a protein cocktail rich in proteases and lipases (Blackman, 2000; Braun Breton and Pereira da Silva, 1993; Preiser et al., 2000). These molecules induce structural alterations to the erythrocyte membrane which essentially prepares the host surface for merozoite invasion. Important changes include the disruption and modification of erythrocyte skeletal components (Cooke et al., 2004b; Rangachari et al., 1986; Roggwiller et al., 1996) and decrease in cellular rigidity (Cooke et al., 2004b). In addition to the action of specific parasitic enzymes, mechanisms involving erythrocyte G protein receptor signaling may facilitate *P. falciparum* entry by remodeling the host membrane skeleton (Harrison et al., 2003). Lipid rafts also appear to be

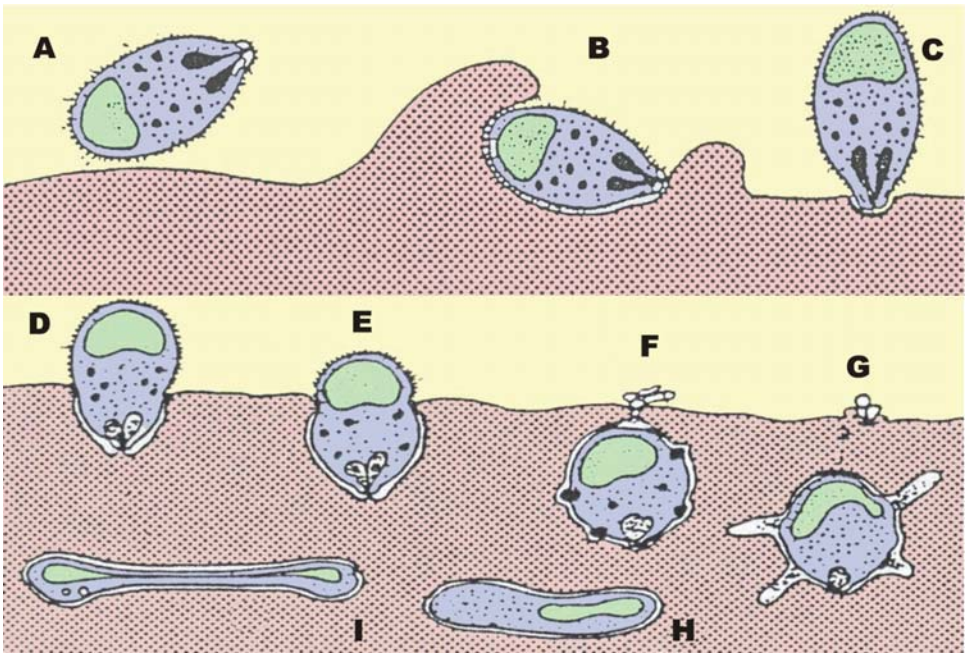

Figure 3- Diagrammatic representation of erythrocyte invasion by *Plasmodium knowlesi* (Bannister and Dluzewski, 1990)

Merozoite invasion is depicted from the initial attachment to the erythrocyte membrane (A), to visualization of major structural changes during early parasitic development (H and I). The process in *P. knowlesi* is perceived to be identical in *P. falciparum*. See text for details.

critical in parasite invasion (Samuel et al., 2001). These regions represent distinct micro-domains in cellular membranes, and have been implicated in a variety of sorting processes in cells (Haldar et al., 2001). Samuel and colleagues (2001) demonstrated that erythrocytes depleted of raft-cholesterol became resistant to invasion by *P. falciparum*. It appears that rafts play a role in the endovacuolation of parasites by contributing to biogenesis of the PV membrane (Haldar et al., 2001; Samuel et al., 2001).

Attachment and junction formation is followed by the invagination of the erythrocyte membrane and development of an invasion pit into which the merozoite glides **(D and E)** (Bannister and Dluzewski, 1990). Erythrocyte entry is an active process, which is dependent on the parasite's actomyosin motor (Pinder et al., 2000). During internalisation, the junctional zone moves over the merozoite as an annular ring while maintaining direct contact with the host surface at all times. Eventually, the moving junction between the two cells closes over and seals at the posterior end of the merozoite to form the PV **(F)** (Bannister and Dluzewski, 1990). At this point, the merozoite sheds its filamentous coat into the extracellular milieu **(G)**, a finding that probably corresponds with the proteolytic cleavage of MSP1, AMA1 and microneme proteins (Dowse and Soldati, 2004; Howell et al., 2005; Howell et al., 2003; O'Donnell and Blackman, 2005).

The final phase of invasion is characterised by a second secretory event, whereby the merozoite's dense granules discharge their contents into the PV **(G)** (Preiser et al., 2000). This is important for augmenting the surface area of the surrounding membrane to accommodate the growing size of the parasite. The exact origin and nature of the parasitophorous vacuole remains inconclusive but is likely a product of rhoptry secretions and lipid bilayer of the erythrocyte membrane (Mikkelsen et al., 1988; Topolska et al., 2004; Ward et al., 1993). Once in the erythrocyte, the parasite begins an active haemoglobin feeding process (Francis et al., 1997) and

proceeds with major structural changes during its asexual development (**H and I)** (Bannister et al., 2000).

1.4 Major merozoite proteins

1.4.1 Merozoite surface protein 1

Merozoite invasion takes place as a result of protein-protein interactions between the parasite's outer coat and erythrocyte membrane receptors. A number of proteins have been identified on the merozoite's surface (Topolska et al., 2004) (Table 1); one of these proteins, MSP1, is the most abundant and best-characterised. The MSP1 gene is localised on chromosome 9 of the *P. falciparum* genome and encodes a ~200 kilodalton (kDa) precursor protein. The protein is expressed in schizonts and is subject to proteolytic processing at the parasite's PM. Prior to merozoite release, MSP1 is cleaved into four different fragments known as $MSP1_{30}$, $MSP1_{38}$, $MSP1_{42}$ and $MSP1_{83}$. These fragments associate with the merozoite surface as a non-covalently bound membrane complex. At invasion, $MSP1_{42}$ is cleaved into two peptides; a 19kDa fragment remains bound to the parasite surface while a 33kDa fragment is shed together with the remaining components of the MSP1 complex (Blackman and Holder, 1992; Holder and Freeman, 1984; Holder et al., 1985; O'Donnell and Blackman, 2005). The function of MSP1 is not completely understood, but is a prerequisite for invasion (Fleck et al., 2003). MSP1 has been implicated in mediating low affinity contact between the parasite and erythrocyte membrane during the initial stages of invasion. Research has suggested that there are at least two protein-protein interactions involved. Intact MSP1 may contribute to the primary sequence of events, having been shown to bind the host surface in a glycophorin-dependent manner (Perkins and Rocco, 1988). Following the exposure of accessible MSP1 regions, a second binding event(s) takes place. Erythrocyte protein band 3 was identified as a crucial host receptor during invasion. Exofacial regions of the protein were shown to interact with native MSP_{42} and recombinant

Table 1- Surface proteins of *P. falciparum* merozoites

Protein	Characteristics	Gene locus[1]
MSP1	185-220kDa; GPI anchored protein; undergoes two proteolytic processing events; involved in the initial interaction of the merozoite with the erythrocyte	PFI1475w
MSP2	45-52kDa; GPI anchored protein; several protein forms differing in the number of central amino acid repeats; possible role in erythrocyte invasion	PFB0300c
MSP3	45-76kDa; non-covalently attached to merozoite surface; 4 heptad repeats with α-helical structure; involved in the growth of *P. falciparum* during blood stages	PF10_0345
MSP4; 5	40kDa; GPI anchored protein; conserved EGF-like domain	PFB0310c; PFB0305c
MSP6	36kDa; non-covalently attached to merozoite surface; exists in MSP1 complex	PF10_0346
MSP7	22kDa; non-covalently attached to merozoite surface; exists in MSP1 complex	PF13_0197
MSP8	98kDa; GPI anchored protein; Shares sequence similarity with MSP1 including two EGF-like domains; possible role in erythrocyte invasion	PFE0120c
MSP9; ABRA	110kDa; non-covalently attached to merozoite surface; contains repeating dimers of lysine and glutamic acid residues; Exhibits enzyme activity and possibly involved in protease action during parasite invasion	PFL1385c
MSP10	80kDa; non-covalently attached to merozoite surface; contains two EGF-like domains and like MSP1, there is evidence of proteolytic processing	PFF0995c

Major proteins identified on the surface of the merozoite (adapted from Topolska et al., 2004). [1]Gene locus obtained from the PlasmoDB database, version 4.4 (http://www.plasmdb.org). Abbreviations: ABRA, acidic basic repeat antigen; EGF, epidermal growth factor; GPI, glycosylphosphatidylinositol; kDa, kilodalton and MSP, merozoite surface protein.

$MSP1_{19}$, as well as more weakly with recombinant MSP_{38} (Goel et al., 2003). The importance of MSP_{19} involvement during merozoite invasion has been highlighted with immunological evidence. Antibodies specific for $MSP1_{19}$ have provided a degree of protection against *P. falciparum* blood

stages and MSP_{19} is currently a leading candidate for development of a human malaria vaccine (John et al., 2004; O'Donnell et al., 2001).

1.4.2 Rhoptry proteins

Immunofluorescence assays have been used to identify a number of rhoptry proteins (Table 2). These proteins share features including an N-terminal signal peptide and display conservation of gene sequences (Topolska et al., 2004). AMA1 was first isolated from *P. knowlesi* followed by the characterisation of homologues in *P. falciparum.* AMA1 in *P. falciparum* is processed in the micronemes to produce a 66kDa molecule, and then transported to the rhoptries (Topolska et al., 2004). Two N-terminal regions contain a total of 16 cysteine (cys) residues, and are suggested to play a conserved role across different parasite species. Upon schizont rupture, AMA1 is translocated onto the apical surface of the merozoite where it appears to function in parasite reorientation prior to invasion (Gaur et al., 2004).

Rhoptry proteins have been shown to exist in different non-covalent complexes. In *P. falciparum*, the low molecular weight complex contains three rhoptry-associated proteins (RAP), termed RAP-1 (80kDa), RAP-2 (42kDa) and RAP-3 (37kDa) (Preiser et al., 2000; Topolska et al., 2004). RAP-1 is expressed as a soluble protein, which is subjected to two proteolytic events during merozoite development. RAP-2 is a hydrophobic protein that is dependent on RAP-1 for trafficking to the rhoptries. The final member, RAP-3, shows significant sequence similarity to RAP-2 and does not appear to be essential for parasite survival. The precise function(s) of RAP proteins remain unclear, but evidence has implicated a role for the tri-molecular complex in merozoite attachment and invasion of erythrocytes. Three protein components (RhopH 1-3) in *P. falciparum* associate in a second, but higher molecular weight complex. The complex has been shown to bind human erythrocytes and inside-out vesicles prepared from a

Table 2- Rhoptry proteins identified in *P. falciparum* merozoites

Protein	Comments	Gene locus[1]
AMA 1	82/66kDa; transmembrane protein; subject to proteolytic processing; involved in merozoite invasion	PF11_0344
RAP 1; 2; 3	Proteins exist as low molecular weight complexes; potentially involved in merozoite attachment during invasion and/or release from schizonts	PF14_0102; PFE0080c; PFE0075c
RhopH 1; 2; 3	Proteins exist as a 140/130/110kDa tri-molecular weight complex possibly involved in interactions with the erythrocyte membrane during merozoite invasion	PFC0110w; PFI1445w; PFI0265c
MAEBL	Ligand domains are conserved and similar to AMA1; expressed in merozoites as well as sporozoites and possibly facilitates host cell invasion by binding specific membrane receptors	PF11_0468
*Pf*60 family	Identified as a multi-gene family; proteins may encode many functions including erythrocyte receptor recognition	[2]
*Pf*RH family	Transmembrane proteins that bind uncharacterised receptors on the erythrocyte membrane; family comprises homologues of *P. vivax* reticulocyte binding proteins, although no strong preference is displayed toward reticulocyte invasion	PDF0110w; PF13_0198; MAL13P1.176; PFD1150c; PFD1145c
gp76	Behaves like a serine protease; facilitates internalisation of the merozoite by degrading specific erythrocyte membrane proteins	[3]
*Pf*Rhop148	Transmembrane protein rich in asparagine; only rhoptry protein devoid of a signal sequence	PF13_0348

[1]Gene locus obtained from the PlasmoDB database, version 4.4 (http://www.plasmodb.org); [2]there are approximately 140 copies of *Pf*60 present in the genome of *P. falciparum*; [3]gp76 gene has not been cloned and its molecular identity remains obscure (Blackman, 2000). Abbreviations: AMA, apical membrane antigen; gp76, glycoprotein 76 kilodalton; MAEBL, *P. falciparum* erythrocyte binding ligand paralogue; RAP, rhoptry associated protein; *Pf*60, *P. falciparum* 60kDa rhoptry protein superfamily; PfRH, *P. falciparum* rhoptry protein homologue; PfRhop148, *P. falciparum* rhoptry protein 148 kilodalton and RhopH, rhoptry high molecular weight component. References are indicated in the text.

number of mammalian red cells. In particular, RhopH-3 is a glycosylphosphatidylinositol (GPI) anchored protein that has preferential binding for phospholipids found in the inner leaflet of the erythrocyte membrane. RhopH-3 is proposed to facilitate the formation of the PV during merozoite invasion (Preiser et al., 2000; Topolska et al., 2004).

MAEBL is a chimaeric rhoptry protein, being homologous with AMA1 and ligands of the Duffy-binding-like erythrocyte binding protein family. Two cys-rich domains, termed M1 and M2, are found at the protein's amino-terminal end and share significant identity with the first two sub-domains in AMA1. MAEBL also contains an evolutionary-distinct cys-rich domain followed by a transmembrane region and cytoplasmic tail at its C-terminal end. The protein localises to the rhoptries of merozoites and schizonts and is actively expressed on the surface of sporozoites as well as merozoites (Blair et al., 2002; Ghai et al., 2002; Kappe et al., 1998). MAEBL appears to play an important role during sporozoite entry into the mosquito salivary glands (Kariu et al., 2002). In addition, it exhibits erythrocyte binding activity and may function as an adhesion molecule during merozoite invasion (Fu et al., 2005; Ghai et al., 2002; Kappe et al., 1998). The study by Fu and colleagues demonstrated that MAEBL was specific for erythrocyte membrane proteins containing sialic acid residues. Furthermore, they revealed that MAEBL was not required for *P. falciparum* survival, but may rather function in mediating alternative invasion pathways (Fu et al., 2005). In contrast to their report, Ghai and colleagues previously demonstrated that MAEBL bound erythrocytes pre-treated with neuraminidase (Ghai et al., 2002). This finding suggested that erythrocyte invasion by MAEBL was independent of sialic acid residues.

A number of other rhoptry proteins have been identified in *P. falciparum*. A multi-gene family of 60kDa proteins has been postulated to be involved in host cell receptor recognition (Preiser et al., 2000; Topolska et al., 2004). These proteins are expressed in late schizonts and do not associate with

the merozoite membrane during invasion. The *P. falciparum* rhoptry protein homologue (*Pf*RH) family comprises four members, each containing a transmembrane, extracellular and cytoplasmic domain. *Pf*RH-3 is a transcribed pseudogene, whereas *Pf*RH-1 and 2 have been described as potential erythrocyte binding ligands (Topolska et al., 2004). The gp76 rhoptry protein is a serine protease cleaved from an 83kDa precursor protein by phospholipase C activity. The membrane bound GPI anchored protease is responsible for aiding merozoite invasion by modifying erythrocyte surface protein band 3 (Braun-Breton et al., 1988; Roggwiller et al., 1996). Additional proteins with varying molecular weights have also been described in *P. falciparum*: they include the 235kDa and 148kDa rhoptry proteins whose functions remain elusive, a 140kDa protein that exhibits protein-protein interaction with AMA1 and a potential 52kDa invasion protein (Preiser et al., 2000; Topolska et al., 2004).

1.4.3 Dense granule proteins

The contents of dense granules only play a role following merozoite invasion of the erythrocyte. The proteins contribute to the formation of the PV and probably function during early ring stage development (Preiser et al., 2000). Four molecules have been characterised from *P. falciparum* dense granules: the ring infected erythrocyte surface antigen (RESA), ring membrane antigen (RIMA) and two subtilisin-like proteases (*Pf*SUB).

RESA is released shortly after invasion into the PV, where it is translocated to the erythrocyte's membrane skeleton. The protein has been shown to bind a major host structural protein, spectrin, an association that may play a role in maintaining erythrocyte membrane stability during febrile episodes (Culvenor et al., 1991; Foley et al., 1991; Silva et al., 2005). RIMA is a small 14kDa protein, which has been localised to the membrane of ring-stage parasites (Trager et al., 1992). Its exact function remains unknown. *Pf*SUB-1 and 2 are both expressed in *P.*

falciparum asexual blood stages and show homology to a superfamily of serine proteases known as subtilases. *Pf*SUB-1 is an 82kDa protein that undergoes extensive post-translational processing during transport to the dense granules. The active form, known as p47, is shed from the merozoite suggesting a role during host cell invasion. *Pf*SUB-2 is a larger subtilisin-like protease and putative type-1 integral membrane protein. Like *Pf*SUB-1, it is also subject to post-translational processing. *Pf*SUB-1 and 2 have been implicated as merozoite surface sheddases responsible for the proteolytic cleavage of MSP1 during erythrocyte invasion (Blackman, 2000).

1.4.4 Microneme proteins and host-receptor interactions

The recognition of specific erythrocyte membrane receptors is a critical step for successful invasion by *P. falciparum* merozoites. Parasite-ligands involved in apical attachment and junction formation are located within the micronemes. These erythrocyte binding proteins (EBP) are found in a number of *Plasmodium* species, and are characterised by the presence of one or two Duffy-binding-like (DBL) domains (Adams et al., 2001). The Duffy-binding proteins (DBP) of *P. knowlesi* and *P. vivax* were the first DBL-EBP family members to have been identified. DBP interact specifically with the Duffy blood group antigen on the erythrocyte surface, thereby facilitating the invasion process in these parasites. In contrast to *P. knowlesi* and *P. vivax*, six EBP have been characterised in *P. falciparum*, which exhibit no dependency on the Duffy antigen for erythrocyte invasion. They include erythrocyte binding antigen (EBA)-175, JESEBL/ EBA-181, BAEBL/ EBA-140, MAEBL, PEBL/ EBA-165 and erythrocyte binding ligand-like (EBL) 1 protein (Adams et al., 2001; Cooke et al., 2004b; Gaur et al., 2004).

DBP and *P. falciparum* EBP share a number of conserved features (Figure 4) including a signal sequence, an N-terminal cys-rich DBL domain, a low

complexity intermediate region, a C-terminal cys-rich domain followed by a transmembrane sequence and cytoplasmic tail. Unlike *P. vivax* and *P. knowlesi*, the proteins in *P. falciparum* contain a DBL domain which has been duplicated to form an F1 and F2 region. The F2 region and DBL domain in *P. vivax* have been shown to bind specific erythrocyte receptors (Adams et al., 2001).

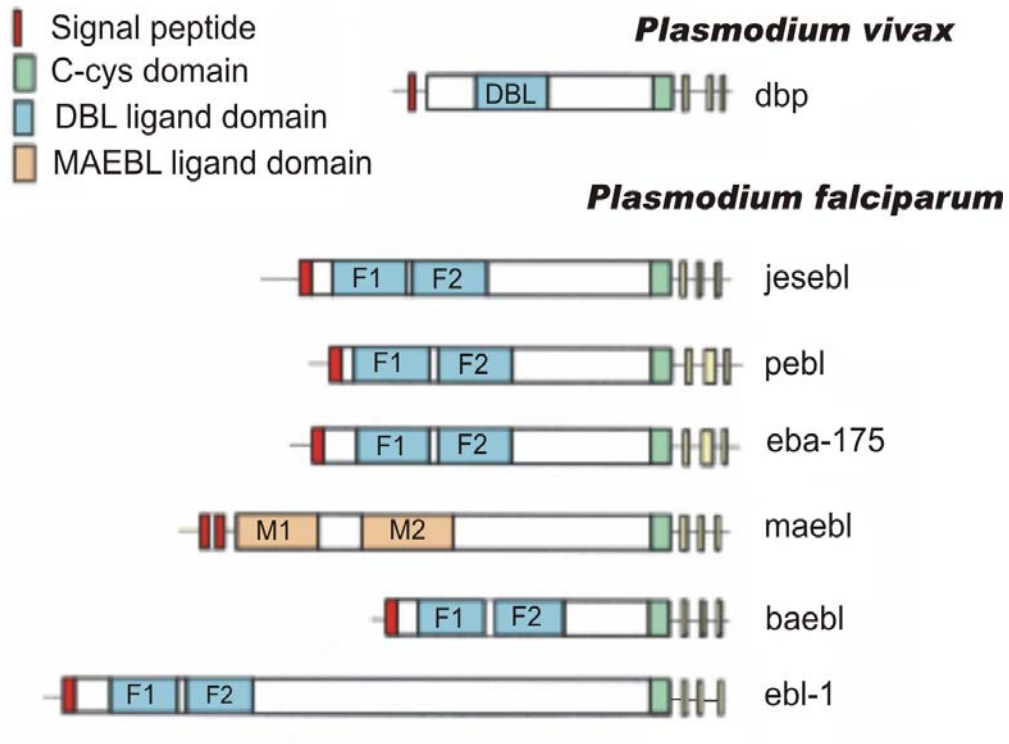

Figure 4- Diagram depicting the gene structure of *P. falciparum* and *P. vivax* erythrocyte binding proteins (Adams et al., 2001)

The genes have a conserved multi-exon structure with most of the open reading frame encoded by the first exon. The DBL ligand domain is duplicated in all *P. falciparum* genes except maebl, which contains two domains each corresponding to the first two sub-domains in AMA1. Unlike the common four-exon structure, the maebl gene consists of six exons and five introns. Conserved exons encode a signal sequence, erythrocyte binding domain (DBL, F1, F2, M1 and M2), c-terminal cys-rich domain, transmembrane domain and cytoplasmic tail. Abbreviations: C-cys, carboxyl cysteine-rich; DBL, Duffy-binding-like; dbp, Duffy binding protein; eba, erythrocyte binding antigen and ebl, erythrocyte binding ligand-like.

EBA-175 was the first and most important EBP to be identified and characterised in the *P. falciparum* micronemes (Adams et al., 2001; Camus and Hadley, 1985; Sim et al., 1990; Sim et al., 1992). The binding of EBA-175 to the erythrocyte membrane is dependent on sialic acid and peptide backbone of glycophorin A, a major surface sialoglycoprotein (Sim et al., 1994; Tolia et al., 2005). Genetic studies mapped the binding site to region II (F2) in the DBL domain. More importantly, the targeted disruption of EBA-175 led to an invasion phenotype independent of sialic acid (Duraisingh et al., 2003; Reed et al., 2000). Additional evidence has substantiated the importance of this ligand in erythrocyte invasion by *P. falciparum*. Antibodies against EBA-175 (Pandey et al., 2002) and structural analogues of sialic acid (Bharara et al., 2004) interfered with erythrocyte invasion, thus highlighting its crucial interaction with glycophorin A. The importance of EBA-175 C-terminal regions was also demonstrated. The cytoplasmic tail encodes vital information for its role during invasion by linking either directly or indirectly with the parasite's actomyosin motor. The C-cys region may function in protein sorting and targeting to the micronemes, while the transmembrane sequence plays a central role in anchoring invasion proteins to the merozoite's PM (Gilberger et al., 2003a; Sibley, 2004).

Four paralogues of EBA-175 were detected in the *P. falciparum* genome on the basis of sequence similarity. The characteristics of these proteins are summarised in table 3 (Adams et al., 2001). Ebl-1 was identified as a second member of the DBL-EBP family. It is encoded by a single-copy gene on chromosome 13 and is transcribed during the late schizont stage. Little data are available on EBL but it may potentially be involved in erythrocyte invasion efficiency (Peterson and Wellems, 2000). PEBL represents an additional member of the family, which is transcribed but not expressed at the protein level. pebl is therefore a pseudogene with no likely function in merozoite invasion (Topolska et al., 2004; Triglia et al., 2001).

When compared to EBA-175, BAEBL has shown clear differences in receptor specificity. A number of studies have implicated glycophorin C as the main erythrocyte surface receptor, but it appears that glycophorin D may also be involved (Lobo et al., 2003; Mayer et al., 2001; Narum et al., 2002). Investigations into polymorphisms present in regions F1 and F2 of BAEBL revealed differences in host-binding phenotype (Mayer et al., 2002). These variants indicated that single amino acid substitutions could alter BAEBL's interaction with host ligands, resulting in multiple invasion pathways.

Table 3- Characteristics of *P. falciparum* erythrocyte binding proteins

EBP	Predicted mass	Invasion pathway	Gene locus
EBA-175	175kDa	Sialic acid-dependent; binds glycophorin A; gene disruption results in switch to sialic acid-independent pathway	PF07_0128[1]
JESEBL	181kDa	Polymorphisms affect erythrocyte receptor specificity and dependency on sialic acid; host receptors unknown; gene disruption has no effect on invasion, but can be dependent on the parasite's genetic background	PFA0125c[1]
BAEBL	140kDa	Binds glycophorin C/D (sialic acid-dependent receptors); polymorphisms affect erythrocyte receptor specificity, which direct binding to unknown, sialic acid-independent receptors	MAL13P1.60[1]
EBL-1	304kDa	Receptor unknown	AF131999[2]
PEBL	Not translated	Pseudogene; gene disruption has no effect on invasion pathway	PFD1155w[1]

[1]Gene locus obtained from the PlasmoDB database, version 4.4 (http://www.plasmodb.org); [2]Gene locus obtained from the EMBL database (http://www.ebi.ac.uk/embl/). Abbreviations: EBA, erythrocyte binding antigen; EBL, erythrocyte binding ligand-like and EBP, erythrocyte binding protein. References are indicated in the text.

Like EBA-175 and BAEBL, JESEBL localises to the micronemes and is expressed at around the same time during schizogony. JESEBL was shown to bind a novel sialic acid-dependent receptor on erythrocytes and

to play a role during merozoite invasion (Gilberger et al., 2003b; Vera-Bravo et al., 2005). Furthermore, eight polymorphisms in the protein's DBL domains modulated JESEBL's erythrocyte binding patterns and receptor recognition (Mayer et al., 2004). It is speculated that polymorphisms in EBA-175 paralogues could contribute to differences in the relative virulence of parasite isolates, thus promoting survival advantage in genetically diverse human populations (Mayer et al., 2002; Mayer et al., 2004).

P. falciparum merozoites are not dependent on a single pathway of invasion, but possess the ability to recognise multiple receptors on the erythrocyte membrane. The concept of alternative invasion pathways was evident from studies that examined invasion in mutant erythrocytes and cells treated with proteases and other enzymes. These studies also uncovered additional uncharacterised receptors such as receptor E, X, Y and Z (Gaur et al., 2004; Gilberger et al., 2003b; Pasvol, 2003) (Figure 5).

Why *P. falciparum* evolved redundant invasion pathways is still a matter of debate. Firstly, it has been reported that erythrocyte surface proteins are highly polymorphic in the human population. Parasite lineages adopting multiple invasion strategies may guarantee successful invasion in a host susceptible to genetic diversity. Secondly, *P. falciparum* may evade host immune responses by evolving multiple ligands to offset the inhibition of particular invasion pathways (Gaur et al., 2004; Gilberger et al., 2003b; Mayer et al., 2004).

	Sialic acid-dependent (NS)	Sialic acid-independent (NR)
TS	EBA-175 ↔ glycophorin A BAEBL ↔ glycophorin C/D JESEBL ↔ receptor unknown	Ligand unknown ↔ receptor X BAEBL ↔ receptor unknown JESEBL ↔ receptor unknown
TR	ligand unknown ↔ glycophorin B JESEBL ↔ receptor E BAEBL ↔ receptor unknown *Pf*RH1 ↔ receptor Y	BAEBL ↔ receptor unknown JESEBL ↔ receptor unknown *Pf*RH2b ↔ receptor Z

Figure 5- Matrix depicting parasite ligand-host receptor invasion pathways *in P. falciparum*

P. falciparum parasites invade erythrocytes using multiple pathways and have the ability to switch between sialic acid dependent and independent routes. Polymorphisms in the DBL domains of BAEBL and JESEBL induce different receptor specificities. Erythrocytes treated with trypsin facilitate merozoite invasion via trypsin-resistant membrane molecules such as glycophorin B. *P. falciparum* is still capable of invading cells lacking glycophorin A and B, via unknown receptors. Treatment with neuraminidase removes sialic acid residues from the erythrocyte cell surface. The parasite ligand is indicated first in each quadrant, followed by its specific receptor. Abbreviations: NR, neuraminidase-resistant; NS, neuraminidase-sensitive; TR, trypsin-resistant and TS, trypsin-sensitive.

1.4.5 Merozoite proteases and invasion

A combination of studies has indicated that proteolytic activity is important during the invasion process of *P. falciparum* merozoites. Serine and cysteine protease inhibitors were shown to significantly inhibit erythrocyte entry as well as intra-erythrocytic parasite development (Blackman, 2000). Furthermore, investigations in the related apicomplexan *Toxoplasma gondii*, have modeled new roles for parasite-derived proteases in *P. falciparum*. Proteases are required for at least two steps during invasion: the processing of merozoite surface and apical organelle proteins, and the alteration of erythrocyte membrane proteins (Braun Breton and Pereira da Silva, 1993; Kim, 2004).

Protease activity is directed toward the maturation of proteins destined for the apical secretory organelles in merozoites. Microneme and rhoptry

proteins are processed during transit through the secretory pathway as well as upon release from the parasite. Microneme proteins with transmembrane domains undergo further processing shortly after transport to the merozoite PM. These proteins, including EBA-175, are routed to the posterior end of the parasite during invasion where they are cleaved from its surface. A family of serine proteases known as rhomboids has been implicated in shedding microneme proteins by way of intra-membranous proteolysis (Dowse and Soldati, 2004; Dowse et al., 2005; O'Donnell and Blackman, 2005). Protease activity is also required for the processing and shedding of other major proteins from the merozoite PM. Recently a multifunctional serine protease belonging to the subtilisin-like superfamily, was shown to cleave both AMA1 and MSP1 at juxtamembrane sites (Blackman, 2000; Howell et al., 2005; Howell et al., 2003).

Research indicates that proteolytic modification of the host erythrocyte membrane is a necessary step for merozoite invasion. The acidic basic repeat antigen (ABRA) or MSP-9 and gp76 are suspected serine proteases that fall outside the subtilisin-like family. The association of ABRA and gp76 with the merozoite surface has suggested a role during erythrocyte invasion. Both proteins display protease activity, which may be responsible for degrading erythrocyte membrane protein band 3. The disruption of band 3 during invasion may destabilise the membrane and facilitate merozoite invasion as well as PV formation (Kushwaha et al., 2002; Roggwiller et al., 1996). The serine repeat antigen (SERA) and related homologues represent another novel class of putative serine proteases in *P. falciparum* (Blackman, 2000). SERA interacts with the erythrocyte membrane by binding specific inner leaflet phospholipids. This association has been proposed to aid parasite invasion by disrupting the interaction between lipid bilayer and underlying skeleton (Perkins and Ziefer, 1994).

1.5 *P. falciparum* and the erythrocyte membrane

The parasitism of erythrocytes by *P. falciparum* is concomitant with significant morphological changes that affect cellular structure, antigenic character and function. During development, parasite-induced modifications occur in both the host lipid bilayer and underlying skeleton. Major changes include: marked alterations to the composition and organisation of phospholipids (Hsiao et al., 1991; Maguire et al., 1991; Schwartz et al., 1987); new transport pathways leading to increased permeability (Kirk, 2001); rearrangement and modification of endogenous membrane components and insertion of parasite proteins into the lipid bilayer and skeleton (Cooke et al., 2001). These observations indicate that *P. falciparum* must extensively modify the erythrocyte surface and intracellular environment to sustain its nutritional and physiological requirements.

1.5.1 Structure of the erythrocyte membrane

The modern concepts concerning the structure and organisation of biological membranes are accounted for in the fluid-mosaic model. Erythrocyte membranes consist of a lipid bilayer and a highly flexible membrane skeleton (Walensky et al., 2003) (Figure 6).

The lipid bilayer is composed primarily of phospholipids and cholesterol, with small amounts of glycolipids also present. Phosphatidylcholine and sphingomyelin are mainly found on the outer leaflet, while phosphatidylserine and phosphatidylethanolamine predominate on the inner half. The membrane provides a permeability barrier and is responsible for the basic structural integrity of the erythrocyte. Integral proteins such as glycophorin A and band 3 span the full width of the lipid bilayer. Glycophorin A accounts for the majority of surface charge while band 3 facilitates membrane transport and ionic balance. The erythrocyte skeleton is organised around a two-dimensional meshwork of peripheral

proteins that underlie the bilayer. Vertical interactions are established via associations between the skeletal network and integral proteins. Horizontal interactions are created by the cross-linking of spectrin tetramers and proteins of the junctional complex (JC) (Palek, 1995). Proteins of the skeletal network are essential for providing flexibility, mechanical strength and deformability. Erythrocytes are therefore able to maintain their biconcave shape and withstand the high shear forces encountered in the circulation.

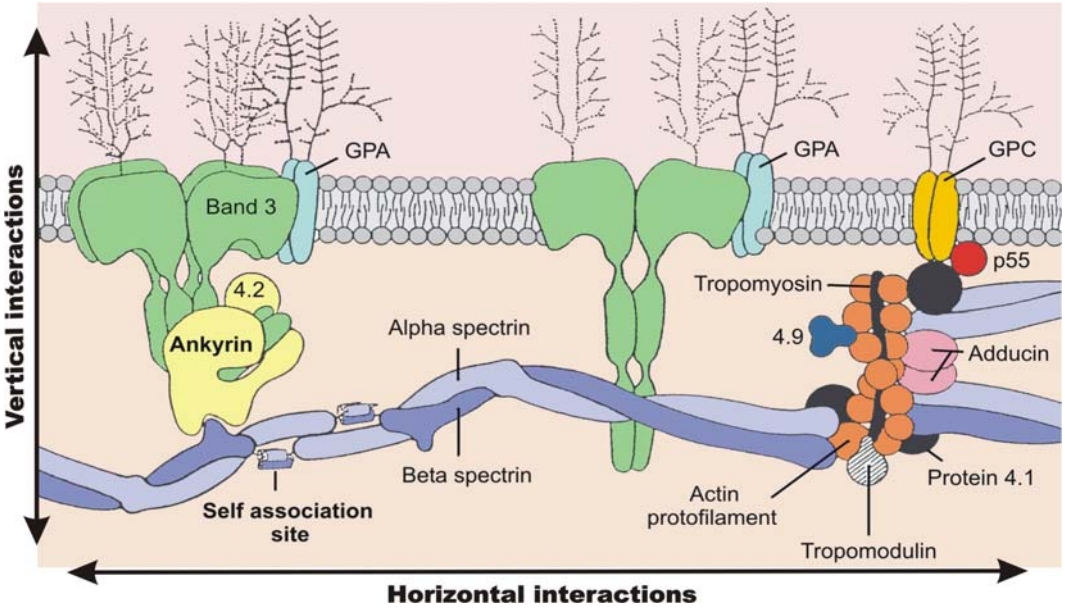

Figure 6- Schematic model of the erythrocyte membrane (Palek, 1995; Walensky et al., 2003)

Horizontal and vertical interactions of the membrane components are indicated. Abbreviations: GPA, glycophorin A and GPC, glycophorin C

Horizontal interactions

Spectrin is a major constituent of the erythrocyte membrane, comprising about 50-75 percent of the skeleton. The protein consists of two isomers, α and β spectrin, which intertwine around each other in an anti-parallel fashion. The respective genes are located on chromosomes 1 and 14 and encode proteins of 240kDa and 220kDa. Spectrin heterodimers associate head-to-head to form tetramers of approximately 200 nm in length, but are

capable of condensing to significantly smaller lengths during erythrocyte circulation (Gallagher and Forget, 1993).

Each spectrin chain is composed of repeating homologous domains that form triple helical segments of ~106 amino acids. α-Spectrin contains binding sites for calcium and may also interact with the JC, whereas β-spectrin associates with ankyrin and the JC. These junction points consist of actin and its accessory proteins, as well as key structural components such as protein 4.1 and p55 (Table 4) (Chishti, 1998; Gilligan and Bennett, 1993; Walensky et al., 2003).

Vertical interactions

The skeleton is linked to the erythrocyte membrane by two proteins, ankyrin and protein 4.1. Ankyrin is a globular protein of 210kDa that attaches β spectrin to the cytoplasmic domain of band 3. It contains three functional regions including an 89kDa band 3-binding domain, 62kDa spectrin-binding domain and a 55kDa regulatory domain (Peters and Lux, 1993). Band 3 is a major integral protein involved in the exchange of anions across the erythrocyte membrane. The protein encompasses two domains with different functions: a 40kDa domain anchors the skeleton to the lipid bilayer through interactions with ankyrin, and a 55kDa carboxyl-terminal region is responsible for chloride and bicarbonate transport (Tanner, 1997). Protein 4.2 is a 72kDa peripheral protein that associates with band 3. It is important for strengthening the link between membrane and skeleton by stabilising the band 3-ankyrin association (Walensky et al., 2003).

The erythrocyte skeleton is also anchored to the membrane by protein 4.1, via its interactions with band 3 and glycophorins C and D. Glycophorins consist of a family of five integral membrane glycoproteins, which covers the erythrocyte surface with an abundance of sialic acid residues (Cartron

et al., 1993; Fukuda, 1993). These sialo-moieties confer a large negative charge to the outer membrane, thus preventing adherence of erythrocytes to each other and to the endothelium of vessel walls (Walensky et al., 2003). Glycophorin A is the major sialoglycoprotein and is the principal receptor utilised by *P. falciparum* for invasion (Sim et al., 1994). Glycophorin C/D are alternative invasion receptors (Lobo et al., 2003; Mayer et al., 2001; Narum et al., 2002) and constitute the major

Table 4- Proteins of the junctional complex

Protein	Mass (kDa)[1]	Gene name	Function
Adducin	α Adducin: 103 β Adducin: 97	ADD1 ADD2	Binds, caps and cross-links actin protofilaments; enhances association of spectrin with actin
β actin	43	ACTB	Protofilaments comprise 12-18 actin monomers; binds spectrin and reinforces skeleton
Myosin	244[2]	-[6]	Binds protein 4.1 and contains actin activated ATPase activity; may function in an actomyosin contractile apparatus
Protein 4.1	78/80[3]	EPB41	Stabilises spectrin-actin interaction; binds band 3 and glycophorin C thereby linking the JC to the membrane
Protein 4.9/ Dematin	48/52[4]	EPB49	Involved in actin bundling and contributes to membrane stability
p55	55	MPP1	Binds the lipid bilayer, protein 4.1 and glycophorin C; may be involved in signal transduction
Tropomyosin	27/29[5]	-[6]	Stabilises actin protofilaments
Tropomodulin	43	TMOD	Functions as an actin-capping protein and may regulate actin filament length

JC, junctional complex [1]Masses were calculated from the proteins' mobility on sodium dodecyl sulphate polyacrylamide gels. [2]Erythrocyte myosin is composed of one heavy chain of 200kDa and two light chains of 19 and 25kDa. [3]Protein 4.1 is a doublet of 4.1a and 4.1b. The two isoforms have an apparent molecular weight difference of 2kDa, which results from the deamidation of 4.1b. [4]Dematin has two isoforms which associate to form a trimer containing two 48kDa subunits and one 52kDa subunit. [5]Tropomyosin is a heterodimer of 27 and 29kDa subunits. [6]The exact sequences of these genes have not been confirmed. References: (Gilligan and Bennett, 1993; Walensky et al., 2003)

membrane attachment sites for protein 4.1. Binding to protein 4.1 occurs either directly or indirectly via p55 (Hemming et al., 1994; Hemming et al., 1995; Marfatia et al., 1994; Marfatia et al., 1995). Evidence has also shown that protein 4.1 binds the cytoplasmic domain of band 3 (An et al., 1996; Hemming et al., 1995). However, this interaction does not appear to contribute significantly in linking the skeleton to the lipid bilayer in erythrocytes (Workman and Low, 1998).

1.5.2 Erythrocyte protein 4.1

Erythrocyte protein 4.1 (4.1R) is a major structural modulator of the cellular skeleton, and comprises approximately 5% of the protein content. 4.1R provides mechanical strength to the membrane through vertical interaction with glycophorin C/D and horizontal association with spectrin and actin (Conboy, 1993). 4.1R exists as a globular protein and contains four structural domains of 30, 16, 10 and 22/24kDa (Leto and Marchesi, 1984). The importance of 4.1R is demonstrated by a deficiency of the protein or mutation of its domains, which leads to disease characterised by haemolytic anaemia (Walensky et al., 2003).

The protein 4.1 gene

Molecular analyses of the 4.1R gene have revealed a complex set of protein isoforms in erythroid and non-erythroid cells. This apparent diversity is accounted for by alternative splicing of a single pre-mRNA molecule (Figure 7A), which is transcribed from the human EPB41 gene located on chromosome 1 (Conboy et al., 1986; Conboy et al., 1988; Conboy et al., 1991; Takakuwa, 2000; Tang et al., 1988; Tang et al., 1990; Walensky et al., 2003). Furthermore, the differential usage of two open reading frames generates two isoforms of different sizes. An upstream AUG directs the synthesis of a 135kDa non-erythroid isoform, which contains an amino-terminal extension of 209 amino acids. A downstream

AUG in exon 4 produces the 80kDa isoform (Figure 7B) found in mature erythrocytes (Baklouti et al., 1996; Conboy et al., 1991; Tang et al., 1990).

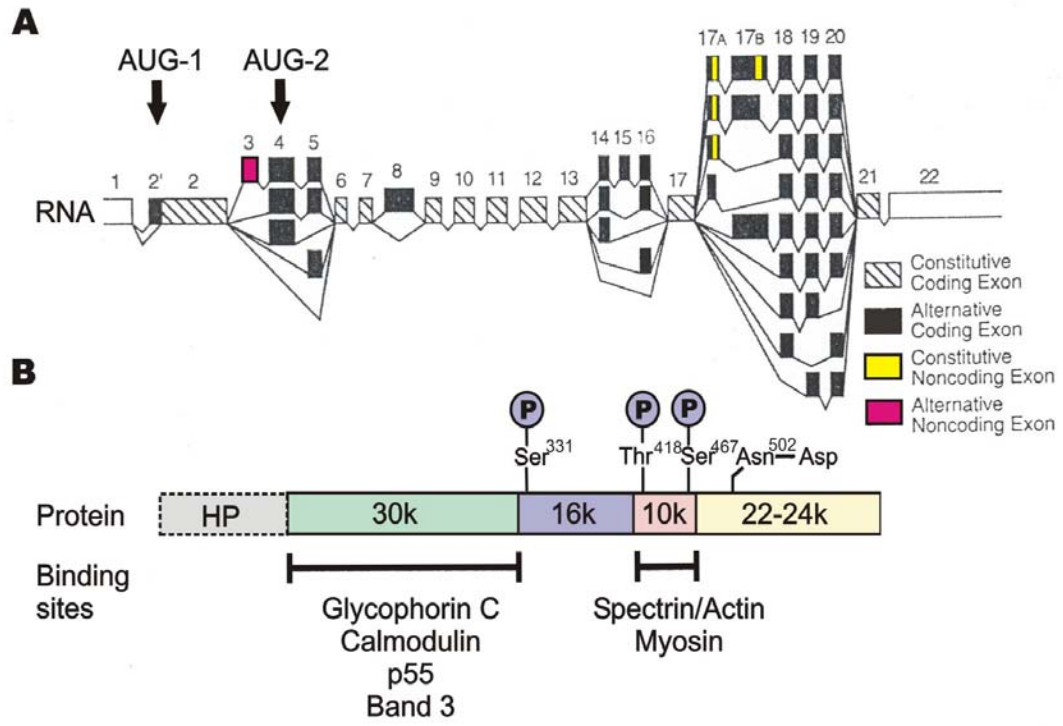

Figure 7- Schematic representation of the genetic and proteomic elements of 4.1R (Walensky et al., 2003)

A. Alternative splicing map of the protein 4.1 gene (EPB41). The gene contains at least 24 exons, which span over 200 kilobases in length. Expression is regulated by two translation initiation sites, AUG 1 and 2. 4.1R is translated from AUG-2 and includes exon 16, an erythroid-specific sequence essential for enhancing spectrin-actin association. AUG-1 orchestrates the synthesis of an additional 209 amino acids (HP), which is only observed in non-erythroid tissue. **B**. Map depicting structural domains of 4.1R. Residues subject to phosphorylation and protein binding sites for the 30kDa and 10kDa domains are indicated. The asparagine residue that is deamidated in mature erythrocytes is also indicated in the carboxyl-terminal domain (Conboy, 1993). Abbreviations: HP, headpiece and 4.1R, erythrocyte protein 4.1.

Structural domains in 4.1R

30kDa domain: The amino-terminal 30kDa domain consists of a hydrophobic amino acid sequence that displays homology to a group of proteins known as the Ezrin-Radixin-Moesin family (Conboy et al., 1986; Tsukita and Yonemura, 1997). These proteins facilitate interactions with

cellular membranes via a conserved 30kDa membrane binding domain (Tsukita and Yonemura, 1997). This domain in 4.1R has been shown to link the erythrocyte skeleton either directly or indirectly to the lipid bilayer and integral membrane proteins.

The 30kDa domain binds with low affinity to negatively charged phospholipids such as phosphatidylserine (An et al., 2001). Approximately 40% of 4.1R binds the cytoplasmic domain of glycophorin C/D (Hemming et al., 1995), which associates with p55 to form a ternary complex (Nunomura et al., 2000). About 20% of 4.1R interacts with another attachment site, band 3, which may function to control its association with ankyrin (An et al., 1996; Hemming et al., 1995). Taken together, the 30kDa domain of 4.1R acts as a critical regulator of binary protein-protein interactions in the erythrocyte skeleton. Evidence indicates that the calcium-dependent binding of 4.1R with calmodulin reduces the affinity of 4.1R interactions with band 3, glycophorin C/D and p55 (Marfatia et al., 1994; Marfatia et al., 1995; Nunomura et al., 2000). The 30kDa domain of 4.1R therefore modulates membrane stability and integrity by changing the nature of protein complexes in the erythrocyte skeleton.

16 and 22/24kDa domains: The 16kDa domain resides between the 30 and 10kDa domains. The amino acid sequence is characterised by a relatively low hydrophobicity and high proline content (Conboy et al., 1986). The function of this domain is poorly understood; phosphorylation of a serine residue at position 331 in this domain may modulate interactions with spectrin and band 3 (Conboy, 1993; Danilov et al., 1990; Ling et al., 1988). 4.1R resolves as a doublet of 80kDa (4.1a) and 78kDa (4.1b) on a denaturing polyacrylamide gel (Walensky et al., 2003). This is attributable to the deamidation of asparagine 502 in the C-terminal 22/24kDa domain. The conversion of 4.1Rb to 4.1Ra has been reported to coincide with erythrocyte aging, with greater quantities found in reticulocytes and mature cells respectively (Inaba et al., 1992). The 22/24kDa domain has been

shown to bind an immunophilin (FKBP13) enriched in erythrocyte membranes. The role of this interaction remains unclear (Walensky et al., 1998).

10kDa domain: The 10kDa domain is a highly charged region that demonstrates remarkable conservation in its primary sequence (Conboy et al., 1986; Winardi et al., 1995) Based on this information, the function of the 10kDa region is evolutionary conserved between different animal species and has been shown to promote ternary complex formation with spectrin and actin. 4.1R specifically generates high affinity β-spectrin-actin interactions that contribute to the maintenance of skeletal integrity (Correas et al., 1986). Another possible function for the 10kDa domain has been suggested; a study reported that 4.1R binds and regulates myosin activity *in vitro*, which may contribute to the regulation of an actomyosin contractile apparatus on the erythrocyte membrane (Fowler et al., 1985; Pasternack and Racusen, 1989). The formation of these 4.1R-protein interactions can be regulated by two means: phosphorylation of the 16 and 10kDa domains (Horne et al., 1990) and secondly through calcium and calmodulin binding (Tanaka et al., 1991).

1.5.3 Erythrocyte surface modifications

The virulence of *P. falciparum* is generally ascribed to dramatic alterations that the parasite induces to the surface of infected erythrocytes. During its asexual development, parasite-encoded proteins are transported to the surface of the host membrane (Cooke et al., 2001; Kirk, 2001). Some of these proteins render the erythrocyte adherent to a number of tissues; adherence to endothelial cells is referred to as cytoadherence, whereas adhesion to infected and non-infected erythrocytes is termed autoagglutination and rosetting respectively (Smith and Craig, 2004). The abnormal binding property of infected cells is an important factor that contributes to the survival and pathogenicity of *P. falciparum*.

Cytoadherence has been implicated in preventing the destruction of parasitised cells by side-stepping splenic clearance. Sequestration within the deep vasculature of various organs, not only obstructs blood flow, but underlies severe clinical conditions such as cerebral malaria. Numerous investigations have probed the molecular basis of adhesive interactions between infected erythrocytes and host tissues (Newbold et al., 1999; Smith and Craig, 2004).

Current evidence implicates a number of parasite proteins that act as ligands for binding (Smith and Craig, 2004). Their exposure on the erythrocyte's surface makes them prone to immune attack by the host organism. To establish chronic infection, many surface antigens are therefore structurally diverse. The ability of *P. falciparum* to express large, variant protein families constitutes the basis for the parasite's success at evading protective immunity. Adhesive ligands of *P. falciparum* are encoded by at least three gene families, known as *var*, *rif* and *stevor*. *Var* genes represent the largest family of highly polymorphic genes and express a key surface molecule known as *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) (Rasti et al., 2004).

PfEMP1

PfEMP1 is expressed on the surface of *P. falciparum*-infected erythrocytes, and resides in knob-like protrusions on the host membrane. *Pf*EMP1 is a product of *var* gene expression, a family of approximately 60 gene variants per parasite genome (Rasti et al., 2004). *Var* genes display a complex pattern of regulation, with only one gene selected and exclusively transcribed in each mature intra-erythrocytic parasite. Furthermore, they are harbored on the subtelomeric regions of most chromosomes and are prone to recombination events, duplications, as well as sequence alterations. Together, these factors ensure rapid antigenic variation and

increased receptor heterogeneity in the midst of host immune pressure (Flick and Chen, 2004; Gardner et al., 2002).

Var genes are characterised by a two-exon structure: the first exon encodes a variable extracellular domain, while the second exon encodes a more conserved intracellular region. The N-terminal segment of *Pf*EMP1 contains a variable number of DBL (α-ε) and cys-rich interdomain regions (α, β and γ) (Flick and Chen, 2004). Multiple tandem combinations of DBL and interdomain sequences are responsible for providing *Pf*EMP1 a distinct spectrum of binding function and resultant clinical disease outcomes.

*Pf*EMP1 domains possess adhesive properties and are capable of binding to several different host receptors. They interact with integrins, glycosaminoglycans, and proteoglycans as well as with proteins belonging to the immunoglobulin superfamily (Table 5) (Cooke et al., 2004b; Flick and Chen, 2004; Rasti et al., 2004). PfEMP1 associates most commonly with CD36, which appears to bind most, if not all parasites from infected patients. The wide range of *Pf*EMP1 receptors enables parasitised erythrocytes to adhere to different molecules expressed on host tissues. Cytoadherence to the endothelial walls of microvasculature can mediate severe pathology including the most lethal complication, cerebral malaria (Newbold et al., 1999; Serghides et al., 2003). Sequestration of parasites on placental syncytiotrophoblasts is mediated by adhesion to chondroitin sulphate A. The development of malaria infection during pregnancy may cause maternal morbidity and mortality, as well as contribute to an increased risk of low birth weight and mortality in the neonate (Rogerson and Brown, 1997). Rosetting is another form of adhesion, which involves the binding of two or more uninfected erythrocytes to a parasitised cell. *Pf*EMP1 plays a major role but other ligands are implicated. The physiological advantage of rosette formation remains unclear, however, a higher degree of rosetting has generally been associated with greater

disease severity (Smith and Craig, 2004). Autoagglutination between infected erythrocytes forms clumps, which are bridged by protein-protein interactions involving *Pf*EMP1 and CD36 on platelets. Like rosetting, autoagglutination in field isolates is associated with greater disease severity (Smith and Craig, 2004). Parasitised cells are also capable of binding monocytes and dendritic cells by interacting with CD36 and specific serum proteins. In so doing, the antigen presenting activity of these cells and their capacity to stimulate T-lymphocytes are down-regulated (Flick and Chen, 2004; Serghides et al., 2003).

Table 5- Receptor-ligand interactions between PfEMP1 and human host

Receptor location	Host receptor	
Endothelium	CD36 CD31 ICAM-1	CSA HS TSP
Erythrocytes	Antigens A and B, CR1/CD35, HS-like GAG	
Serum proteins	IgM/G	
Platelets	CD36	
Monocytes/ dendritic cells	CD36, IgM	
Placenta	CSA, HA, Non-immune IgM/G	

PfEMP1is a key adhesive antigen expressed on the surface of *P. falciparum* infected erythrocytes. The protein interacts with a wide array of receptors found in the human host. Abbreviations: CSA, chondroitin sulfate A; CR1, complement receptor 1; GAG, glycosaminoglycans; HA, hyaluronic acid; HS, heparin sulfate; ICAM-1, intercellular adhesion molecule-1; Ig, immunoglobulin; *Pf*EMP1, *P. falciparum* erythrocyte membrane protein 1 and TSP, thrombospondin. References are indicated in the text.

RIFINS and STEVORS

The repetitive interspersed family (*rif*) is the largest group of genes involved in antigenic variation. They are located in close association with *var* genes and are present in 149 copies per parasite genome (Gardner et al., 2002; Rasti et al., 2004). *Rif* genes are composed of two exons that encode phenotypically variable proteins known as RIFINS. Several

different RIFINS may localise on the erythrocyte membrane and are capable of inducing natural antibody responses in *P. falciparum* infection. Their variability and surface location indicate an important role in host-parasite interaction. More specifically, evidence suggests that RIFINS may be involved in rosetting and binding to CD31 receptors (Fernandez et al., 1999; Kyes et al., 1999).

Subtelomeric variable open reading frame (s*tevor*) is a third group of genes closely related to the *rif* multigene family. Like *rif*, *stevor* genes contain a two-exon structure and are located subtelomerically on all *P. falciparum* chromosomes (Blythe et al., 2004). The two families differ in that *stevors* have a significantly lower copy number and have been proposed to encode more conserved sequences among different parasite clones. The function(s) of STEVOR proteins remains unknown and there is no direct evidence indicating exposure on the erythrocyte surface. However, evidence indicates that STEVOR localises to submembranous structures known as Maurer's clefts. They may function during the transport of parasite proteins to the erythrocyte surface and have been suggested to protect Maurer's clefts from immune attack (Blythe et al., 2004).

Clag 9 and sequestrin

A gene at the end of chromosome 9, termed cytoadherence linked asexual gene 9 (*clag 9*), encodes a parasite ligand involved in cytoadherence (Holt et al., 1999). Targeted gene disruption indicated that the clag 9 protein is essential for adhesion to endothelial receptors such as CD36 (Gardiner et al., 2000; Holt et al., 1999; Trenholme et al., 2000). However, there is no evidence for the presence of the protein on *P. falciparum*-infected erythrocyte membranes (Smith and Craig, 2004). Sequestrin is another potential receptor for CD36. The protein is expressed as an approximately

270kDa surface polypeptide in asexual parasites (Ockenhouse et al., 1991). Its precise role in erythrocyte sequestration remains unclear.

Pfalhesins

P. falciparum alhesins (*Pf*alhesins) are modified band 3 proteins induced by developing intraerythrocytic parasites. Band 3 modifications include a marked reduction in mobility, increased aggregation and conformational changes resulting in antigenic variation on the erythrocyte surface (Parker et al., 2004; Winograd et al., 2005; Winograd and Sherman, 2004). *Pf*alhesins have been suggested to act as ligands for CD36 and possibly thrombospondin (Smith and Craig, 2004).

SURFIN

SURFINs are polymorphic antigens encoded by a family of surface-associated interspersed (*surf*) genes. These high molecular mass proteins are expressed on the surface of merozoites and parasitised erythrocyte membranes. Their exact functions need further clarification but evidence suggests a crucial role in parasite survival as well as host cell invasion (Winter et al., 2005).

1.5.4 Alterations to the erythrocyte skeleton

Once inside the erythrocyte, *P. falciparum* exports a variety of proteins into the erythrocyte cytoplasm. A number of these proteins associate with the membrane skeleton and appear to be essential for parasite survival (Table 6). Protein-protein interactions at the host-parasite interface generally involve highly charged *P. falciparum* polypeptides, which are characterised by sequences of low complexity and repeating motifs (Cooke et al., 2004b).

A major alteration to the parasitised erythrocyte membrane is the formation of electron-dense protrusions known as knobs. Knobs are rich in

parasite-derived proteins and serve as attachment points for sequestration of erythrocytes in tissue vascular beds. The formation of knobs is dependent on a major component, the knob-associated histidine-rich

Table 6- Major *P. falciparum* proteins that associate with the erythrocyte skeleton

Protein	Features
Knob-associated proteins	Knobs are comprised of parasite-derived and endogenous skeletal proteins. KAHRP and PfEMP1 associate with spectrin/actin/ankyrin and cluster at the knobs. Parasites that do not express KAHRP have undetectable knob structures. *Pf*EMP3 also localises at the knobs, but its function remains unknown.[1]
Giant proteins	*Pf*332 and *Pf*11-1 are extremely large proteins that associate with the erythrocyte membrane. Precise functions are unknown but may be involved in erythrocyte rupture and release of merozoites.[2]
Maurer's cleft protein	*Pf*SBP1 is an integral membrane protein of parasite-induced membranous structures known as Maurer's clefts. It associates with the erythrocyte skeleton and may be involved in protein trafficking.[3]
Falcipains	Cysteine proteases comprising four family members. Involved in haemoglobin degradation with falcipain-2 implicated in ankyrin/4.1R cleavage.[4]
Plasmepsins	Aspartic proteases residing primarily in the acidic food vacuole of the parasite. They consist of four active haemoglobinases. Plasmepsin II is also involved in spectrin/actin/4.1R degradation.[5]
Kinases	FEST is a serine-threonine kinase which associates with the host skeleton. May be responsible for the phosphorylation of parasite and erythrocyte proteins.[6]
Phosphoproteins	MESA binds 4.1R and may modulate p55 function. RESA associates with spectrin and may protect the membrane against thermal denaturation.[7]

Abbreviations: FEST, falciparum-exported serine-threonine kinase; KAHRP, knob-associated histidine-rich protein; MESA, mature parasite-infected erythrocyte surface antigen; *Pf*EMP, *P. falciparum* erythrocyte membrane protein; *Pf*SBP1, *P. falciparum* skeleton binding protein-1 and RESA, ring-infected surface antigen. References: [1]Cooke et al., 2004b; [2]Wiesner et al., 1998; [3]Blisnick et al., 2000; [4-7]indicated in the text.

protein (KAHRP). KAHRP is an 85-105kDa protein that binds a number of skeletal proteins including spectrin and ankyrin (Magowan et al., 2000; Oh et al., 2000; Pei et al., 2005). The cytoplasmic domain of *Pf*EMP1 also interacts with KAHRP as well as the spectrin-actin junction (Oh et al., 2000;

Waller et al., 2002). These interactions are crucial for anchoring *Pf*EMP1 to the knobs and thus promote *P. falciparum* virulence. The absence of KAHRP results in 'knobless' parasite strains, which leads to a weakened affinity between *Pf*EMP1 and endothelial receptors, as well as reduced cytoadherence (Crabb et al., 1997).

Proteases play an important role during the erythrocytic cycle of *P. falciparum*. Studies have demonstrated that merozoite exit from erythrocytes is susceptible to cysteine, aspartic and serine protease inhibitors. The application of different inhibitors to *P. falciparum* cultures prevented the hydrolysis of proteins associated with the PV and erythrocyte membrane. The order relating to the breakdown of the two barriers encapsulating the intracellular parasite is controversial (Salmon et al., 2001; Wickham et al., 2003). Although evidence proposed a role for proteases, the precise molecular events underlying parasite exit remains uncharacterised.

The identification of proteins involved in degrading specific host skeletal proteins has shed some light on the mechanisms governing erythrocyte rupture and merozoite escape. Falcipains are the best characterised family of cys proteases in *P. falciparum* (Rosenthal, 2004). Knockout experiments indicated that falcipain-1 is not essential for normal parasitic development (Sijwali et al., 2004). Falcipains-2 and 3 have been localised to the food vacuole and are presumably involved in the metabolism of haemoglobin (Rosenthal, 2004). After silencing falcipain 1 and 2 genes with their respective double stranded RNAs, Malhotra and colleagues revealed severe morphological abnormalities in parasites including substantial haemoglobin accumulation (Malhotra et al., 2002). To determine the role(s) of falcipain 1, 2 and 3 in erythrocytic stages of the parasite life cycle, Dasaradhi and colleagues treated asexual cultures with corresponding siRNAs. Their results indicated that the three proteases were required for normal parasite growth and survival. In addition, the

knockdown of falcipain 2 suggested a role in the release of merozoites by rupturing the erythrocyte membrane (Dasaradhi et al., 2005). These observations were strengthened by a previous study that demonstrated the cleavage of ankyrin and 4.1R using recombinant falcipain 2 (Dua et al., 2001). The proteolysis of these structural role-players affects the stability of erythrocyte membranes and provides a possible explanation for erythrocyte rupture. Plasmepsins represent another group of proteases, which may contribute to compromising skeletal integrity. They were originally described in initiating haemoglobin hydrolysis (Francis et al., 1997), but like falcipain-2, plasmepsin II displays multifunctional properties. Recombinant constructs were reported to selectively digest spectrin, actin as well as 4.1R from erythrocyte ghosts (Le Bonniec et al., 1999).

Phosphorylation of endogenous membrane proteins and the association of parasite-derived phosphoproteins (examples: MESA and RESA) with the erythrocyte skeleton are other important modifications of the *P. falciparum*-infected host cell (Cooke et al., 2004b). Alterations of the level of phosphorylation of skeletal proteins such as 4.1R, spectrin, band 3 and ankyrin have been reported (Chishti et al., 1994; Magowan et al., 1998; Magowan et al., 2000; Pasvol et al., 1993). These induced changes may decrease the mechanical stability of the membrane, thus imparting functional significance during merozoite invasion, parasitic development and erythrocyte rupture. The responsible kinases have not been fully characterised, but some have exhibited profiles relating to casein kinase (Chishti et al., 1994; Magowan et al., 1998). In addition, a *falciparum*-exported serine-threonine (FEST) kinase has been found associated with the erythrocyte skeleton. This suggests a role in the phosphorylation of host and/or parasite-encoded proteins (Kun et al., 1997).

P. falciparum and 4.1R interactions

P. falciparum requires 4.1R for optimal parasite growth and viability. Schulman and colleagues (1990) demonstrated normal invasion but markedly inhibited parasite growth in 4.1R-deficient erythrocytes. The authors suggested two mechanisms by which skeletal defects could disrupt the maturation of *P. falciparum*. Firstly, normal erythrocyte membrane proteins may be required for PV formation. Alternatively, normal host skeletal proteins might act as targets for protein-protein interactions, which in turn support parasite developmental processes (Schulman et al., 1990). The second option was supported by findings generated by Magowan and colleagues (1995). They reported reduced invasion and viability of MESA (+) *P. falciparum* strains in 4.1R-deficient erythrocytes when compared to normal cells. The accumulation of cytoplasmic MESA in the absence of 4.1R was found to be deleterious to parasite survival. A third study presented data that corroborated the importance of 4.1R for parasite invasion and intracellular growth (Chishti et al., 1996). In addition, these authors also demonstrated that ternary complex formation between 4.1R, p55 and glycophorin C may be required for normal development of *P. falciparum.*

4.1R associates with a number of *P. falciparum* proteins and the interaction between MESA and 4.1R is one of the best characterised associations. The precise function of MESA is unknown, but an increase in unbound protein levels results in parasite death through unknown mechanisms (Magowan et al., 1995). MESA utilises a short N-terminal sequence to bind a 51 amino acid region within the 30kDa domain of 4.1R. Binding studies revealed competition between MESA and p55 for 4.1R. This suggests an important role for MESA in the modulation of protein-protein interactions in the erythrocyte skeleton (Waller et al., 2003). Falcipain-2 is another parasite protein that interacts with 4.1R (Hanspal et al., 2002). Native and recombinant falcipain-2 was shown to cleave 4.1R immediately after lysine 437 in the 10kDa spectrin-actin binding domain.

The enzyme's specificity was confirmed using a short peptide inhibitor which spanned the cleavage site. Proteolytic activity was completely blocked and prevented membrane fragmentation of erythrocyte ghosts (Hanspal et al., 2002). These authors proposed a dual function for falcipain 2: the enzyme cleaves haemoglobin in the acidic food vacuole at the early trophozoite stage, whereas it degrades specific erythrocyte skeletal components at later stages during merozoite release. Finally, the phosphorylation of 4.1R is likely to signify an important event during *P. falciparum* growth (Chishti et al., 1994). Although the functional significance remains uncertain, the addition of phosphate groups to 4.1R structural domains reduces spectrin-actin association and membrane binding properties (Danilov et al., 1990; Horne et al., 1990; Ling et al., 1988). This may alter gross morphological characteristics of the erythrocyte during malaria blood-stages, including deformability, skeletal integrity and membrane permeability (Chishti et al., 1994; Magowan et al., 1998).

1.5.5 Transport pathways in Plasmodium-infected erythrocytes

P. falciparum faces huge metabolic demands during the asexual erythrocytic cycle. Parasites require essential nutrients from the extracellular milieu to survive and develop. At the same time, a large amount of metabolic waste is produced which needs to be eliminated. To resolve these complex issues, *P. falciparum* induces marked alterations in the transport properties of the erythrocyte membrane (Kirk, 2001). A number of mechanisms that control the flux of solutes across the infected host cell have been suggested. Firstly, a parasitophorous duct was proposed to connect the parasite directly with the extracellular environment. The duct would provide means for the uptake of macromolecules via endocytosis. Although there is evidence supporting this idea, controversy still surrounds the existence of direct transportation pathways (Kirk, 2001). Secondly, studies have demonstrated enhanced activity of endogenous host transporters. *P. falciparum* may increase the

rate of influx or efflux of substrates by directly or indirectly modulating erythrocyte membrane transport proteins (Kirk, 2001). Thirdly, new permeation pathways (NPP) are induced in the erythrocyte membrane (Kirk, 2001; Staines et al., 2004; Thomas and Lew, 2004). These pathways have broad specificity for a number of structurally unrelated solutes. Generally, they are selective for anions and electroneutral solutes (sugars, amino acids, peptides and nucleosides), but are still capable of transporting cations. Evidence suggests a number of functions for NPP including nutrient uptake, waste disposal, ionic balance and erythrocyte volume regulation. The characterisation of NPP has been advanced using techniques such as patch-clamp, however their molecular identity remains elusive (Kirk, 2001; Staines et al., 2004; Thomas and Lew, 2004). Finally, *P. falciparum* may insert parasite-encoded transporters into the erythrocyte membrane. Substrate-specific proteins that have been identified include hexose transporters, nucleoside transporters and ATPases, which are involved in metal ion homeostasis (Krishna et al., 2001).

It is well established that during the course of *P. falciparum* infection, proteins are exported to various cellular destinations including the erythrocyte cytosol and membrane. To achieve this, the parasite establishes a system of protein trafficking pathways that targets polypeptides to their cellular locations. Studies have revealed distinct membranous structures in the erythrocyte cytoplasm. The most prominent is a complex tubulovesicular network which appears to extend from the parasite's PV membrane toward the erythrocyte lipid bilayer (Cooke et al., 2004a; Foley and Tilley, 1998). This network may take part in the sorting of host and parasite components, but may also function in the trafficking of proteins to Maurer's clefts. Maurer's clefts are flattened lamellar structures that localise near the erythrocyte skeleton (Cooke et al., 2004a). Evidence suggests that clefts interact with specific skeletal proteins (Blisnick et al., 2000) and may possibly function as recruiting stations for parasite proteins

destined for the host membrane. Mobile vesicular elements have also been described as plausible conduits for protein trafficking, and may fuse directly with the erythrocyte bilayer (Cooke et al., 2004a; Foley and Tilley, 1998; Trelka et al., 2000). Alternatively, parasite proteins have been shown to be transported as membrane-free protein aggregates in the erythrocyte cytosol (Cooke et al., 2004a).

The trafficking of *P. falciparum* proteins in an infected erythrocyte is highlighted by models depicting multi-step processes. The first step proposes that proteins destined for export are transported via endoplasmic reticulum-Golgi stacks to the parasite's PV. Analyses of polypeptide sequences have revealed different secretory signals which control this event: these include classical hydrophobic N-terminal amino acids (examples: RIFIN and STEVOR), recessed N-terminal sequences (examples: MESA, RESA, KAHRP and *Pf*EMP3) and internal start-transfer signals (*Pf*EMP1) (Cooke et al., 2004a; Foley and Tilley, 1998). Transfer across the PV membrane is mediated by conserved amino acid motifs known as *Plasmodium* export element (Pexel) (Marti et al., 2004) and vacuolar transport signal (VTS) (Hiller et al., 2004) . Although sketchy, the membranous structures in the erythrocyte cytosol are implicated in trafficking proteins from the PV to different cellular locations (Cooke et al., 2004a; Foley and Tilley, 1998). The presence of Pexel and VTS, together with other signaling motifs, is responsible for directing protein trafficking and subsequent function of parasite proteins.

1.6 Phage display technology

The completion of the *P. falciparum* genome sequencing project will undoubtedly lead to a greater understanding of the parasite (Gardner et al., 2002). Our greatest challenge rests in deciphering the molecular intricacies of malaria pathogenesis. Determining the functions of novel gene products has emerged as an important area of scientific research. As

a result, sophisticated methods have been developed to identify and characterise proteins that contribute to parasite virulence (Florens et al., 2002).

The growing interest in protein-protein interactions has produced methodologies based on the combinatorial display of selected proteins or peptides on the surface of bacteriophage. Phage display involves the production and screening of random peptide sequences expressed on the surface of phage particles. It has therefore been possible to isolate ligands to various targets as well as studying binding specificities of diverse molecules (Benhar, 2001). Phage display technology thus provides a powerful tool for probing the molecular basis of host-parasite interaction.

1.6.1 Phage display libraries

Phage display was first introduced by George Smith in the mid 1980's (Smith, 1985). Exogenous peptide sequences were inserted into gene III of filamentous bacteriophage. Foreign peptides were displayed on the phage surface as fusions with the minor coat protein pIII. Subsequently, Parmley and Smith demonstrated the affinity purification or biopanning of fusion phage from a library comprising 10^8-fold excess of ordinary phage (Parmley and Smith, 1988). In 1990, three independent reports described the construction of random peptide libraries as well as the isolation of sequences displaying specific binding activity (Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990). These experiments set the stage for engineering libraries with a diverse array of molecules on their surfaces, including protein domains, entire proteins, antibody chains and complementary deoxyribonucleic acid (DNA) libraries (Burton, 1995). Since then, the field has developed into a wide-ranging area of novel applications and advances (Azzazy and Highsmith, 2002; Benhar, 2001).

Phage display libraries are produced by cloning a random mixture of oligonucleotide sequences in frame with one of the phage coat genes. Phage particles are therefore used as vehicles for expressing or displaying gene fusion products on their surfaces. This technology has the advantage of constructing millions of peptide combinations simultaneously, instead of engineering sequence variants one at a time (Smith and Petrenko, 1997). Bacteriophage fd is one of the most widely used phage display vectors. Foreign sequences may be fused with either the minor (pIII) or major (pVIII) coat protein. Methodologies based on other vectors have also been developed including phage λ heads and tail proteins, P4-Psu capsid protein, T7-10B capsid protein, MS2 coat protein as well asT4-Hoc/Soc surface proteins (Benhar, 2001). To select peptides with desired ligand specificity, a technique known as biopanning is used to enrich the respective phage particles from libraries of extremely high titres (Figure 8). After reaction with an immobilised ligand, unbound particles are removed by washing and bound phage eluted. The phage are propagated in *Escherichia coli* (*E. coli*) cells and several rounds of biopanning repeated to increase the quality of the interaction. This display system has two important attributes: firstly, the enrichment of clones is accomplished simply by amplification in bacteria; secondly, the amino acid sequence of the displayed peptide is easily ascertained by sequencing its encoded DNA in the phage particle (Smith and Petrenko, 1997).

1.6.2 Applications of phage display to *P. falciparum*

Significant progress has been made in the field of phage display and *P. falciparum* has not fallen short of its application. One of the most successful uses of the technology has been the isolation of recombinant antibodies and analyses of antibody responses to various antigens. Phage display antibodies were produced from variable single chain fragments derived from the sera of malaria-infected individuals (Sowa et al., 2001). Libraries were constructed and biopanned against three asexual parasite

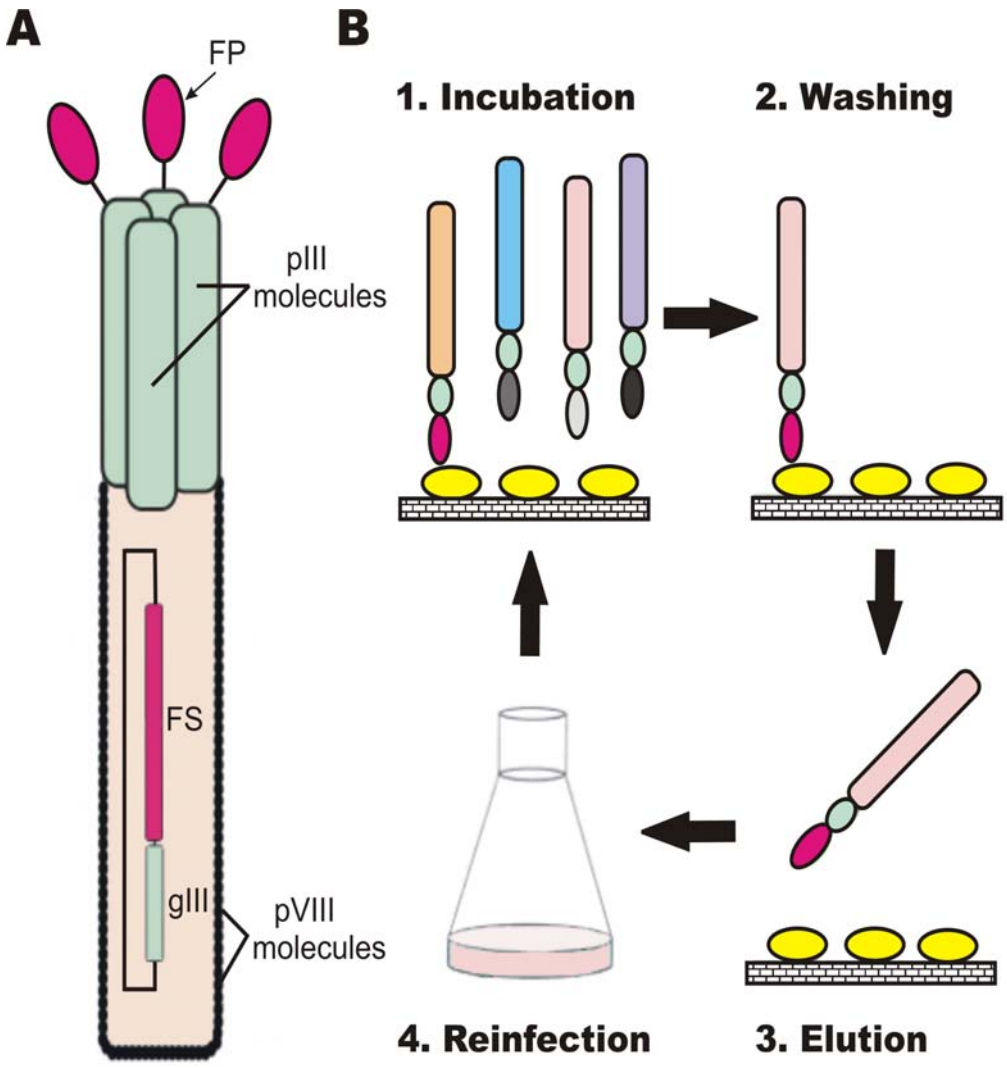

Figure 8- Principles of phage display technology (**A**: adapted from Azzazy and Highsmith, 2002; **B**: adapted from Watkins and Ouwehand, 2000)

A. Schematic representation of a filamentous phage displaying foreign peptides (FP) on its surface. The inner core of the particle is surrounded by major (pVIII) and minor coat proteins (pIII). A foreign oligonucleotide sequence (FS) has been cloned adjacent to the minor coat protein gene (gIII). Consequently, a pIII-FPfusion protein is expressed at the tip of the phage. **B**. Biopanning of a phage display library. The library is represented by four phage particles each expressing different peptide epitopes. It is first incubated with a ligand (yellow) immobilised on a solid support (**1**), followed by washing to remove unbound or weakly binding phage (**2**). The stringency of wash conditions may be altered using buffers with different ionic strength and pH. Ligand-specific phage are eluted under conditions that retain their infectivity (**3**). Strong alkaline or acidic buffers as well as urea and detergents may be used for this purpose. Eluted phage are amplified in bacteria for further rounds of biopanning (**4**). Finally, phage DNA is sequenced to determine the amino acid sequence of the interacting peptide.

proteins (Chappel et al., 2004; Fu et al., 1997; Sowa et al., 2001), which have received considerable attention for vaccine development. Chappel and colleagues revealed that protective responses against the circumsporozoite surface protein were restricted to a single heavy and light chain antibody pairing (Chappel et al., 2004). Sowa and colleagues reported the successful isolation of human antibodies specific for MSP1 from a malaria patient-derived phage display antibody library (Sowa et al., 2001). Antibody libraries generated from mice immunized with *P. chabaudii* AMA1 exposed a subset of antibody fragments specific for *P. falciparum* AMA1, a protein implicated in erythrocyte invasion (Fu et al., 1997). Recombinant human libraries have also been screened for binding activity against a *P. falciparum* sexual stage antigen by Roeffen and colleagues, who concluded that antibodies against *Pf*s48/45 could facilitate the development of transmission blocking vaccines (Roeffen et al., 2001).

The expression of random *P. falciparum* peptides on the surface of bacteriophage capsids is another important application of phage display. It allows the *in vitro* identification of peptides that bind a variety of ligands including antibodies. These peptides may be used to elucidate the structure and function of interacting ligands as well as to enhance the possibility of developing novel therapeutic agents. Libraries representing a random mixture of fragments from the circumsporozoite protein (de la Cruz et al., 1988; Greenwood et al., 1991), glutamate-rich protein (Theisen et al., 2001) and *P. falciparum* liver stage antigen 1 (Heal et al., 2000) contained immunogenic determinants characteristic of a potential peptide vaccine. In other studies, phage display peptides and antibodies targeting mimics of AMA1 were able to inhibit the invasion of erythrocytes by merozoites (Casey et al., 2004; Li et al., 2002). Furthermore, AMA1 libraries assisted in delineating epitope sequences involved in binding monoclonal antibodies (Coley et al., 2001).

In an effort to identify peptides reactive to the infected erythrocyte, a phage display peptide library was screened using *P. falciparum* infected host cells. These studies uncovered several peptides, one of which inhibited intracellular parasite growth (Eda et al., 2004). Antiserum against another peptide sequence elicited haemolysis of parasitised erythrocytes (Eda and Sherman, 2005).

The inhibition of *P. falciparum* development in the mosquito vector offers new means for curbing the malaria threat. To ensure successful transmission, parasites need to establish themselves in the mosquito's salivary glands. Epithelial layers of these glands as well as the midgut are two major barriers that have to be breached. Antibodies specific for surface proteins on the salivary glands interfered with sporozoite invasion (Ghosh et al., 2002). As a result, the identification of protein-protein interactions important for harboring *P. falciparum* in the mosquito may show great promise in blocking the spread of malaria. To identify peptides that interact with salivary and midgut surface molecules, phage display libraries were screened. Peptides expressed on the phage capsids contained multiple combinations of 12 amino acids. A molecule termed SM1 bound specifically to epithelial layers of both organs, and inhibited invasion by sporozoites and ookinetes (Ghosh et al., 2002). Furthermore, transgenic mosquitoes expressing SM1 are severely impaired in their ability to sustain parasite development (Jacobs-Lorena, 2003).

1.7 Research objectives

Numerous proteins encoded by *P. falciparum* have been shown to interact with the erythrocyte membrane. Despite recent advances, numerous questions remain unanswered concerning the dynamic interplay between host and parasite.

The use of conventional techniques has limited the identification of new protein-protein interactions. The inability to maintain a very high parasitaemia of *P. falciparum* in continuous culture has hindered means to purify parasite-derived proteins in large quantities. Furthermore, the presence of contaminating erythrocyte membrane proteins in *P. falciparum* protein extracts makes it extremely difficult to obtain preparations of sufficient purity. To circumvent these limitations, phage display technology was used for mapping novel interactions between parasite and erythrocyte membrane protein 4.1.

Principal objectives of this research included:

- The construction of *P. falciparum* cDNA phage display libraries and biopanning against purified human 4.1R
- The identification of parasite sequences that interact with 4.1R
- The expression and purification of recombinant 4.1R structural domains and 4.1R-specific parasite protein
- The evaluation of binding kinetics between the recombinant proteins and determination of the 4.1R domain responsible for the interaction