The Surface of the *Plasmodium falciparum*-infected Erythrocyte

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Abstract
In order to navigate its complex lifecycle, the malaria parasites must interact with a range of host cells. Examples of this are the invasion of hepatocytes by sporozoites and erythrocyte invasion by merozoites. This requirement for cell recognition brings with it the need to display cognate ligands on the parasite surface, and therefore the capacity of the host to develop defences against the infection. Even at a stage where the intracellular nature of erythrocyte development would appear to offer an opportunity for the parasite to be immunologically "silent", parasite-derived proteins are found on the surface of the infected erythrocyte. This review will discuss the proteins found on or associated with the surface of the infected erythrocyte and the resulting phenotypes.

Modifications Occurring to the Surface of Infected Erythrocytes

Plasmodium parasites have a complex life cycle that includes multiple stages of development both within a vertebrate and an anopheles mosquito host (for a recent review see Waters and Janse, 2004). Although *Plasmodium* are capable of establishing chronic infections in humans that can last as long as a year, survival of the parasite species is ultimately conditioned upon effective transmission between hosts. Indeed, the cycle of transmission requires a sexual replication cycle that is initiated in the vertebrate host but can only be completed in the mosquito. Thus, while malaria parasites replicate asexually in vertebrates to increase numbers and presumably enhance transmission success, they have evolved sophisticated mechanisms to ensure their transmission back to the mosquito. This review focuses on alterations occurring at the surface of infected erythrocytes with a special emphasis on *P. falciparum*, the most important *Plasmodium* species that infects humans. Reference is made to parallel mechanisms operating throughout the *Plasmodium* genus.

The initial period of parasite development in humans begins in the liver and last for approximately 7 to 10 days. This stage is not associated with disease but is an important period of growth and amplification that allows the parasite to overcome a transmission bottleneck due to the fact that few parasites are inoculated when a mosquito bites. While only a single parasite may infect a hepatocyte, 20-40,000 parasites are released at the end of the liver stage to infect erythrocytes.

The erythrocytic stage of parasite development produces a chronic infection that can last for over a year and during which disease can occur. In erythrocytes, *P. falciparum* grows, differentiates, and divides in a compartment within the erythrocyte cytoplasm called the parasitophorous vacuole. Asexual division of *P. falciparum* requires approximately 48 hrs. For each parasite that infects an erythrocyte approximately 10 to 24 are released to infect new red blood cells leading to amplification of the infection. Importantly, the number of infected erythrocytes (IE) is a significant risk factor for disease so that an important component of host immunity is directed at limiting parasite growth. Also within erythrocytes some parasites differentiate to sexual forms in a process that is still not completely understood. These gametocyte-infected erythrocytes are infective for mosquitoes and are responsible for completing the cycle of transmission the next time a mosquito feeds.

P. falciparum-infected erythrocytes display several dramatic morphological changes that affect membrane rigidity, surface antigenic character, and permeability. These changes are intimately connected to *Plasmodium* biology and involved in nutrient acquisition, the establishment of chronic infections, and the evolution of new adhesive properties displayed by some *Plasmodium* species. Parasite-induced modifications occur both to the erythrocyte cytoskeleton and the extracellular face of the membrane. Although sub-cellular modifications are critical to new adhesive properties exhibited by *P. falciparum*-infected erythrocytes these will only be briefly described here (for a review see Cooke *et al.*, 2001). Rather, the major focus of this review will be parasite proteins demonstrated or proposed to be surface-exposed on asexually-parasitised erythrocytes. Several excellent reviews on parasite proteins exported to the erythrocyte cytoskeleton have recently been written.

Until relatively recently the infected erythrocyte surface was regarded as having very few parasite-derived proteins on it. The earliest candidates were a modification of an existing erythrocyte protein, band 3 (Winograd and Sherman, 1989), and a biochemically defined, surface-labellable variant protein known as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Leech *et al.*, 1984). A combination of further biochemical characterisation and the advent of the genome sequence has provided a range of new IE surface candidates, turning a rather sparse molecular "landscape" into a potentially complex interface between parasite and host.

Before describing these different proteins and the evidence placing them at the erythrocyte surface it is constructive to briefly review our understanding of the

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natural immune response to *P. falciparum* infection and resulting expectations for surface-exposed parasite proteins. Protective immunity to *P. falciparum* is acquired slowly and only after repeated infections. The immunity that develops does not appear to ever provide complete protection from infection but does protect against disease and is still imprecisely understood. However, there is increasing evidence that natural malaria immunity is comprised of different elements including anti-disease and anti-parasite components that evolve with characteristic and distinct kinetics. In addition, there is evidence for clonal antigenic variation of antigens at the surface of infected erythrocytes that may allow parasites to establish chronic infections (for a review see Bull and Marsh, 2002). The variant antigens are highly immunodominant and antibodies that develop to them are typically strain-specific but appear to have a protective role against infection. Given the slow evolution of malaria immunity the parasite is highly successful at evading immunity. Strategies that a parasite might use to avoid immunity to surface-exposed proteins are to have these proteins belonging to large, diverse and varying protein families so that it takes an individual a long time to learn the different variants. Alternatively, parasite surface proteins may be exposed for only limited times or relatively inaccessible to antibody. Using these criteria we will review the list of potential surface-exposed parasite proteins beginning with the best-characterised example, PfEMP1.

PfEMP1

Evidence for the presence of neo-antigens on the IE surface was first suggested by Brown and Brown (Brown and Brown, 1965) in their seminal paper on antigenic variation in the primate malaria *Plasmodium knowlesi*. In the mid-1980's more direct molecular evidence was produced in two laboratories using radio-iodination of infected erythrocytes from primate (*P. knowlesi*; Howard *et al.*, 1983) and human (*P. falciparum*; Leech *et al.*, 1984) malarias. The latter identified a protein of variable molecular weight (200-350kDa) between different parasite lines that was Triton X-100 insoluble and sensitive to protease digestion of intact IE. Subsequent studies have demonstrated that this protease sensitivity is not universal (Chaiyaroj *et al.*, 1994; Gardner *et al.*, 1996) but this initial result supported the presence of this protein on the erythrocyte surface. This protein, termed *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), became the target for intense study over the next ten years but proved difficult to work with due to relatively low abundance and the paucity of specific immunological reagents. As often seen in science, the breakthrough, when it came, derived from the work of several groups in identifying the gene family encoding PfEMP1, namely the *var* genes (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The basis for this discovery came from several sources but mainly the production of a monoclonal antibody specific for a single PfEMP1, the identification of a candidate gene from a sequencing study (looking for the chloroquine-resitance gene!), the production of a phenotypically characterised parasite clone tree, and the development of antibodies to recombinant protein fragments of the genes.

The structure of the *var* gene matched the scientific expectations very well, with it being a multi-gene family with 50-60 copies per haploid genome and having a high degree of sequence divergence between different family members. This could not only explain the ability of parasites to switch antigenically, through differential expression of *var* genes, but also why sera from people infected by *P. falciparum* only agglutinated IEs from samples taken from earlier waves of parasitemia and not those from later peaks ((Brown and Brown, 1965; Hommel *et al.*, 1983)). By having a repertoire of surface-expressed PfEMP1s with little similarity and a mechanism of mutually exclusive gene expression, the parasite would be able to switch from one antigenic type to another in the face of host immune pressure. This form of immune evasion is believed to be an important factor in the establishment of chronic infection and presumably enhances transmission. Moreover, not only is the *var* repertoire of a single parasite genotype highly diverse but there is extensive diversity of *var* genes between different parasite genotypes. Estimates of *var* diversity based upon degenerate primers have demonstrated that there is little similarity in repertoires between different parasite genotypes (Fowler *et al.*, 2002; Kyes *et al.*, 1997). Thus, at the population level there is an incredible variety of sequence that may only be limited by the functional requirement of the protein to cytoadhere (see below). Strain-specific variation between *var* repertoires might explain why convalescent sera from children infected by *P. falciparum* agglutinate homologous parasites but frequently show little or no reactivity with heterologous parasites (Bull *et al.*, 1998).

Unlike many clonally variant antigens that appear to act only as immunological "smokescreens" or for which other functions have not yet been defined, PfEMP1 also encode binding properties. Around sixteen hours after merozoite invasion IE are able to adhere to a number of host receptors. In people suffering from malaria this can be seen as a sequestering of the parasites from the peripheral circulation into a number of microvascular sites around the body. Sequestration of infected erythrocytes has been recognised as a characteristic trait of *P. falciparum* infection for over 100 years and is a major pathogenic feature of disease. Perhaps the most famous example of how sequestration causes disease is cerebral malaria, which is associated with parasite adhesion to brain microvasculature and carries with it a high case-fatality (MacPherson *et al.*, 1985). However, sequestration appears to have an equally important role in pathogenesis that occurs in pregnant women (Fried and Duffy, 1996) and might impact malaria pathogenesis in multiple ways through other parasite-host cellular interactions discussed in the following sections.

PfEMP1 are key mediators in binding interactions between infected erythrocytes and host cells. While infected erythrocytes display a range of different binding properties (for reviews see (Cooke *et al.*, 2001; Craig and Scherf, 2001; Kyes *et al.*, 2001), individual parasites differ in their receptor specificity depending upon the expressed PfEMP1. An important question that is being addressed is how parasite receptor specificity influences parasite tropism for different tissues and cells and the impact on

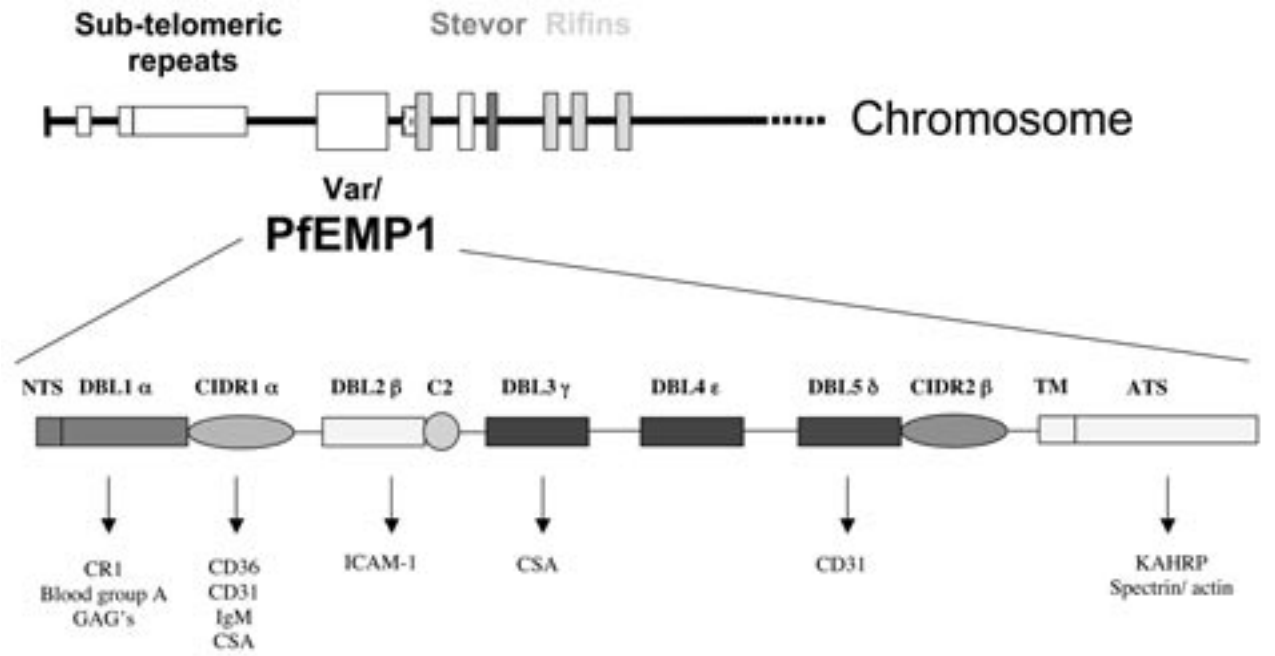

Figure 1. Schematic showing the general organisation of the sub-telomeric region of *P.falciparum* chromosomes (Bowman *et al.*, 1999; Gardner *et al.*, 1998) and structure of PfEMP1. At the top, left-hand side of the figure chromosomal elements are indicated for a telomeric repeat sequence followed by sub-telomeric repetitive elements (R-CG7, rep11, and rep20, respectively). Repetitive elements are followed by a *var* gene, R-FA3 repeat sequence and *rifl stevor* genes. Regions of PfEMP1 are indicated in the text except the transmembrane region (TM) and acidic terminal segment (ATS). The nomenclature for motifs within PfEMP1 indicates the position in the molecule (1-5) and the sequence type (α-ε). Note that the sequence types can be in different orders, except for DBL1α, which is always the N-terminal domain. The receptors for the various regions of PfEMP1 are indicated below the schematic.

disease. To begin to understand PfEMP1 function binding assays have been developed to examine recombinant proteins from these genes and sequence analyses have been performed (Smith *et al.*, 2000a; 2001; 2000b).

var genes are encoded in two exons. The first exon codes for the variable extracellular binding region and a transmembrane domain, while the second exon encodes a more conserved cytoplasmic tail. The PfEMP1 binding region is comprised of four different domains. These domains are the N-terminal segment (NTS), Duffy-binding-like (DBL) domain, the cysteine-rich interdomain region (CIDR), and the C2 domain. Both the DBL and CIDR domains have been demonstrated to possess adhesive properties and a system of adhesive domain classification has been developed to define their sequence relatedness. From a study of 20 different PfEMP1(Smith *et al.*, 2001), DBL domains grouped into five different sequence types: α, β, γ, δ, and ε. In contrast, CIDR domains grouped into three types: α, β, and γ. Within each adhesive domain classification there are a variety of different sequences, however, domains of a type share characteristic and distinctive amino acid features. As the number, location, and type of DBL and CIDR domains vary between PfEMP1 proteins, a nomenclature has been introduced that describes both the numeric position and sequence type of the domain (Figure 1).

Although PfEMP1 proteins are variably sized, an important concept that has emerged from sequence analysis is that the binding region is not created through a random assortment of domains. Rather adhesive domain sequence types tend to occupy characteristic positions in the protein and associate in favoured tandems. For instance, one tandem association is the DBLα and CIDR domains that form the semi-conserved head structure of almost all PfEMP1 (Chen *et al.*, 2000; Su *et al.*, 1995)(Figure 1). Other tandem combinations, DBLβ-C2 and DBLδ-CIDR are found internal to the head structure. Thus, adhesive domain sequence classification provides insight into PfEMP1 protein architecture and may have implications for binding function. At present, only limited numbers of PfEMP1 adhesive domains have been functionally characterised but already there are multiple examples in which different parasites adhesion traits have been shown to map to the same adhesive domain type. For instance, two different CSA-binding parasites used a DBLγ type domain to bind CSA even though the DBLγ sequences were distinct. One hypothesis that is being tested is the extent to which different PfEMP1 proteins that bind the same receptor use the same adhesive domain sequence type to bind. If there were functional constraints that caused like-binding domains to have a related structure and antigenicity this could have implications for vaccine design (Duffy *et al.*, 2001). Investigations have already begun to test for cross-reactive epitopes in different PfEMP1 domains (Gamain *et al.*, 2001). Our understanding of malaria pathogenesis

will also likely improve with further investigation of PfEMP1 function. For instance, small and large PfEMP1 have quite distinctive protein architectures and are comprised of different adhesive domain types. Large PfEMP1 may possess special adhesive characteristics responsible for a distinct spectrum of sequestration and disease outcomes.

From the beginning studies that established a surface localisation for PfEMP1 proteins using iodination and trypsin sensitivity to the development of serological reagents and functional assays, there are now multiple lines of evidence confirming PfEMP1s surface expression.

Rifins

The Rifins are comprised of two related but slightly distinctive multiple gene families, the *rifs* and the *stevors*, discussed separately below.

Rifs

Rifs were originally identified as a repetitive gene sequence (*rif*), which could be used to characterise different parasite lines due to the highly variable banding pattern seen on southern blots of genomic DNA from different lines of *P. falciparum* when probed with this sequence (Weber, 1988). The possibility that *rifs* encoded a set of surface proteins that were initially linked with rosetting (termed rosettins (Helmby *et al.*, 1993)), and subsequently with binding to CD31 (Fernandez *et al.*, 1999) was not recognised until the advent of the *P. falciparum* genome project (Gardner *et al.*, 1998). Sequences with similarity to the original *rif* gene were found as tandem arrays within the sub-telomeric regions. Initially it was not clear that they were expressed as they appeared to lack a proper 5′ region, but careful inspection of the DNA sequence revealed a small 5' exon. It now appears that *rif* genes have a two-exon structure, with the first exon coding for a predicted signal peptide and the second for a protein that is highly variable but contain stretches of relative amino acid conservation and conserved cysteine residues. The actual orientation of Rif proteins within the membrane is still debated because the second exon codes for more than one block of hydrophobic residues that could potentially act as a transmembrane domain. Thus, it is not known whether the bulk of the protein is exposed or whether Rif proteins make multiple passes through the membrane. However, most models predict that Rif proteins have at least one transmembrane domain near the end of the protein that is followed by a short, highly conserved cytoplasmic tail. Members of the *rif* gene constitute the largest gene family in *P. falciparum* discovered so far, with in excess of two hundred copies per genome.

The physical proximity of rif genes to *var* genes raised some early speculation that both gene families might be regulated in a co-ordinated fashion, but evidence to support this scenario remains to be established. A transcriptional analysis of *var* and *rif* expression indicated that *var* messages were expressed relatively early after erythrocyte invasion with a peak around 12h post-invasion while *rif* messages were limited to late ring and early trophozoite stages (Kyes *et al.*, 2000). Despite transcriptional differences, evidence has been presented that Rifs are co-expressed on the infected erythrocyte surface with PfEMP1 (Fernandez *et al.*, 1999; Kyes *et al.*, 1999). This evidence consists of similar criteria that were originally developed to establish the surface expression of PfEMP1. Thus, it has been demonstrated that Rif proteins are iodinatable and trypsin-sensitive albeit with a much higher trypsin requirement than PfEMP1. Unlike PfEMP1, there is no published evidence that antibodies raised to Rif recombinant proteins react with the infected erythrocyte surface although hyper-immune sera from adults in malaria endemic regions are able to immunoprecipitate the proteins indicating their natural immunogenecity (Fernandez *et al.*, 1999). It is curious that Rif proteins are predicted to act as clonal variant antigens, yet it has been difficult to establish their surface expression with antibodies. Equally elusive is the function of Rif proteins despite attempts to link their expression with specific adhesive phenotypes. However, the investment by *P. falciparum* in maintaining a large repertoire of these genes and the differential expression of *rifs* by parasite lines would indicate that they play an important role in the interaction between parasite and human host.

Stevor

Stevor genes are also found in close proximity to *var* and *rif* genes. Like *rif*, *Stevor* began as a multicopy probe for distinguishing *P. falciparum* isolates (Limpaiboon *et al.*, 1991) but was later recognized to have a two-exon gene structure in which the first exon is predicted to code for a signal peptide and the second for the protein (Cheng *et al.*, 1998). Within the protein, Rif and Stevor share some conserved cysteines and align with each other by Blast analysis but the two protein families have characteristic and distinct features including different numbers of conserved cysteines (Cheng *et al.*, 1998). Although the cytoplasmic domain of Stevors is relatively well conserved between different proteins it is distinct from that of Rif proteins. Like *rif*, *stevor* probes generate multiple bands on *P. falciparum* genomic DNA. However, unlike rif there is no published data indicating expression on the IE surface, although unpublished data suggest that *stevor* are transcribed in sexual and asexual stages and localised in the Maurer's clefts in the latter. Since no clear functions have been described for either *rif* or *stevor* gene products it is uncertain whether these protein families have similar or distinct activities.

KAHRP and CLAG9

Upon adaptation of *P. falciparum* to in vitro cultivation, many parasite cultures gradually lose the capacity to cytoadhere over a period of weeks (Udeinya *et al.*, 1983). Chromosomal deletions are frequently observed in *P. falciparum*, particularly during *in vitro* propagation. These genetic deletions are often associated with chromosome ends and can extend for as much as 500kb. While the deletion events appear to offer an *in vitro* growth advantage to the parasite outside of the context of a human host, non-cytoadherent parasite strains have proven a valuable resource to investigate the molecular requirements for infected erythrocyte cytoadherence.

The first chromosomal deletion to be characterised in molecular detail occurred in a telomere end of chromosome 2 and resulted in the loss of a protein called the knob-associated histidine-rich protein (KAHRP) (Pologe and Ravetch, 1986). KAHRP is transported by the parasite out of the parsitophorous vacuole and forms an association with the erythrocyte membrane cytoskeleton (Kilejian *et al.*, 1991; Waller *et al.*, 1999). This association leads to the production of knob-like membrane protuberances at the erythrocyte surface that are the point of contact between infected erythrocytes and endothelium. Although KAHRP is completely intracellular and not a parasite adhesion receptor it has been shown to be essential for knob formation and the binding of infected erythrocytes under conditions of flow (Crabb *et al.*, 1997).

Another common deletion in laboratory-adapted parasite isolates occurs on chromosome 9 (Barnes *et al.*, 1994; Day *et al.*, 1993; Shirley *et al.*, 1990). This deletion has been linked with the loss of cytoadherence to C32 melanoma cells (Barnes *et al.*, 1994), but not all cytoadherence (Chaiyaroj *et al.*, 1994). Initially it was thought that a gene encoding PfEMP1 might map within this region but subsequent detailed analysis of the locus showed that parasites with smaller deletions but still showing the loss of adherence retained the *var* gene at the end of chromosome 9 (Holt *et al.*, 1998). Sequencing of the deleted region revealed a complex structure consisting of nine exons, producing a mature transcript of around 7kb. This gene was called cytoadherence-linked asexual gene (*clag9*) (Trenholme *et al.*, 2000) and encodes a protein of about 220kDa that is associated with the IE membrane, but has not been proven to be on the surface. A number of transfection-based experiments have indicated that this gene is essential for adhesion to C32 via CD36, such that *clag9* knockout or antisense parasites lose the ability to bind to these cells (Gardiner *et al.*, 2000; Trenholme *et al.*, 2000). However it is not clear at what stage in the adhesion process clag9 acts and it is possible that the gene product is involved in the translocation of PfEMP1 from the cytoplasm to the IE surface, rather than being directly involved in adhesion.

It is perhaps surprising given the loss of phenotype seen in the *clag9* knockout that there are several *clag* genes in the *P. falciparum* genome. The genome-sequencing project has revealed at least five related sequences (Holt *et al.*, 1999), located close to the *var*/*rif*/*stevor* clusters at the end of the chromosomes. One of these, on chromosome 3, has been identified recently as encoding RhopH1, one of three proteins that make up the RhopH complex in the rhopteries of the malarial merozoite (Kaneko *et al.*, 2001). Further evidence for the involvement of *clag* paralogues in merozoite function has come from the discovery of two *clag*-related sequences in the rodent malaria *P.yoelii* (*pyrhoph1a* and *pyrhoph1a-p*). It appears that this family of sequences, now termed *rhoph1*/*clag*, mediate broader cell-cell interactions that was previously thought and it will be interesting to see at a structural level how *Plasmodium* has used this gene framework to address cell adhesion in multiple contexts.

Others

A number of other potential parasite ligands on the IE surface have been described as well as permeation pathway involving a voltage-dependent channel which allows the parasite to acquire the metabolites it needs to develop in what is essentially a metabolically inactive cell.

Band 3

The best characterised of this group of molecules is in fact a host protein that is modified by the parasite. Band 3 is a highly abundant erythrocyte surface protein that acts as an anion transporter. Modification of this protein is involved in the clearance of senescent erythrocytes, through the exposure of cryptic epitopes. Invasion by *P. falciparum* also causes modification to Band 3, which can be recognised by specific monoclonal antibodies (Crandall and Sherman, 1991). The basis of the modification is unknown but early experiments suggested that modified Band 3 was involved in adhesion to a range of cells via CD36. However, more recent data have implicated thrombospondin as the receptor (Eda *et al.*, 1999).

Sequestrin

Another potential parasite adhesion receptor is called Sequestrin (Ockenhouse *et al.*, 1991). Sequestrin was identified using anti-idiotype antibodies to an anti-CD36 monoclonal antibody that could block infected erythrocyte cytoadherence. The sera precipitated an approximately 270 kDa surface-iodinatable protein. Unpublished observations have described a gene for the Sequestrin protein but some doubts on the function of this protein are raised by a lack of an effect on cytoadherence in genetic knockout experiments (Trenholme *et al.*, 2000).

RSP1 and RSP2

One recent surprising result was the description of a new form of cytoadherence involving immature ring-stage forms of infected erythrocytes. It has been a long accepted dogma that immature forms of parasites circulate in the blood until approximately 16 hrs post-invasion when sequestration begins for more mature parasite forms. The new work investigating the stage-specific regulation of cytoadherence focused on mature parasites that had been selected to bind CSA but were simultaneously selected to express distinct adhesion ligands at the immature parasite stage. The fascinating observation was the ring-infected erythrocytes of parasites selected to bind CSA were able to adhere to some types of cultured endothelium and placenta tissue sections, but not via CSA (Pouvelle *et al.*, 2000). The binding appears to be mediated by two parasite-encoded proteins, RSP1 and 2, that are expressed on the erythrocyte surface shortly after parasite invasion. Adhesion of immature parasite forms appears to be mechanistically distinct from mature parasites since it occurs independently of knobs. In addition, there are still unanswered questions about the relationship of this binding phenotype to particular parasite adhesion traits. However, these results suggest that there may be non-circulating (cryptic) ring-infected erythrocyte subpopulations in malaria patients. Interestingly, a recent study by (Silamut *et al.*, 1999) was

the first to describe a cryptic population of ring-infected erythrocytes in the brain microvasculature of individuals who died from cerebral malaria.

"New Permeation Pathway" (NPP)

Other surface changes reflect nutritional requirements of the growing intraerythrocytic parasite. Erythrocytes are an interesting cell to parasitise because they are nearly metabolically inert and have been commonly referred to as "bags of haemoglobin". Despite their metabolic limitations, another protozoan parasite, *Babesia*, also invades and develops within erythrocytes during a period of its life cycle. During their development, *Plasmodium* parasites sample the contents of the erythrocyte cytoplasm and use haemoglobin as an important nutritional source. Although many of the amino acids required for parasite growth are supplied from haemoglobin metabolism, parasitised erythrocytes also acquire enhanced permeability characteristics that allow the uptake of factors from blood serum. This so-called "New Permeation Pathway" (NPP) (Desai *et al.*, 2000) is indicated by the increased uptake of many solutes including anions, sugars, purines, amino acids and organic cations and has been measured experimentally using a whole-cell voltage-clamp method on infected erythrocytes. Identifying the voltage-gated channel is a subject of intense molecular investigation, but it is not yet demonstrated whether it is caused by the insertion of parasite protein(s) in the erythrocyte membrane or modification of host red cell proteins.

Phenotypes Associated With the IE Surface

From the description above it can be seen that the major roles for the proteins on the surface of the IE are antigenic variation and adhesion. While all *Plasmodium* species that have been studied to date possess a capacity to vary surface antigenic character, it is not clear why an ability to cytoadhere has only developed for *P. falciparum* among the human malarias. However, this property appears a key determinant in the enhanced virulence of this species.

Clonal Antigenic Variation

At first glance it may appear curious that a parasite living within a erythrocyte, a cell that is relatively inaccessible to cellular immunity because it does not present peptide antigens through either the class I or class II antigen-presenting systems, would expose itself to antibody by exporting proteins to the erythrocyte surface. However, it has been pointed out that clonal antigenic variation is a common theme of different pathogens that rely on insect vectors to complete their life cycle (Kyes *et al.*, 2001). Clonal antigenic variation is probably an important factor that allows parasites to establish chronic infections and ensure transmission during periods when host to host contact is sporadic (e.g. during the dry season for malaria parasites). The importance of clonal antigenic variation to *Plasmodium* biology is demonstrated by the fact that it exists throughout the genus. Recently, several different large and diverse protein families expressed during the erythrocytic period of development have been described from several *Plasmodium* species. Although direct evidence for surface variant exposure of these proteins is still being established for all but *P. falciparum*, it is interesting that only some of these protein families are common while some species like *P. falciparum* appear to have evolved distinct and unique protein families. To date, no sequences sharing significant similarity to *var* or *rifin* genes have been reported from other *Plasmodium* species even though some, like *P. falciparum*, cytoadhere. It will be interesting to test for *var* and *rifin* genes in *P. falciparum*'s nearest evolutionary relatives. However, a gene coding for the variant surface antigen of *P. knowlesi* has been cloned and differs both in sequence and organisation from its *P. falciparum* counterpart (al-Khedery *et al.*, 1999). In addition a major gene family has been discovered in *P. vivax* called the *vir* genes which is also located within the sub-telomeric regions (del Portillo *et al.*, 2001). Subsequently genes related to *vir* were identified in the rodent malarias *P. chabaudi*, *P. yoelii*, and *P. berghei* (Carlton and Carucci,

Table 1. Host receptors for *P.falciparum*-infected erythrocytes.

Host Receptor	Cellular Target(s)	Parasite Ligand
Thrombospondin (Roberts *et al.*, 1985)	Endothelium	Modified Band 3 (Eda *et al.*, 1999) PfEMP1
CD36 (Barnwell *et al.*, 1989; Ockenhouse *et al.*, 1989b; Oquendo *et al.*, 1989)	Endothelium, Dendritic Cells (Urban *et al.*, 2001), Uninfected Erythrocytes (Handunnetti *et al.*, 1992), Platelet-bridged Infected Erythrocytes (Pain *et al.*, 2001)	CIDRα (Baruch *et al.*, 1996; Baruch *et al.*, 1997)
ICAM-1 (Berendt *et al.*, 1989)	Endothelium	DBLβC2 (Baruch *et al.*, 1996; Smith *et al.*, 2000a)
VCAM-1, E-selectin (Ockenhouse *et al.*, 1992), $\alpha_v\beta_3$ (Siano *et al.*, 1998)	Endothelium	
Chondroitin-4-sulfate (CSA) (Fried & Duffy, 1996; Rogerson *et al.*, 1995)	Placenta, Endothelium	DBLγ (Buffet *et al.*, 1999; Reeder *et al.*, 1999)
P-Selectin (Udomsangpetch *et al.*, 1997)	Endothelium	PfEMP1 (Senczuk *et al.*, 2001)
PECAM-1 (CD31) (Newbold *et al.*, 1997; Treutiger *et al.*, 1997)	Endothelium	CIDRα/ DBLδ (Chen *et al.*, 2000)
Hyaluronic acid (Beeson *et al.*, 2000), non-immune IgG (Flick *et al.*, 2001)	Placenta	
CR1 (Rowe *et al.*, 1997), HS-like GAG (Chen *et al.*, 1998), Blood Group A antigen (Carlson & Wahlgren, 1992)	Uninfected Erythrocytes	DBLα (Chen *et al.*, 1998; Rowe *et al.*, 1997)
IgM (Scholander *et al.*, 1996)	Uninfected Erythrocytes	CIDRα (Chen *et al.*, 2000)

2002; Janssen *et al.*, 2002). The telomeric location of variant gene families may have functional significance as recent work in *P. falciparum* has shown that telomere clusters are formed during mitosis, facilitating intergenic transfer and thereby the generation of genetic variation (Freitas-Junior *et al.*, 2000). The accumulating evidence that different *Plasmodium* species have independently evolved and developed distinct variant protein families to alter the surface antigenic profile of infected erythrocytes supports an important function for this adaptation.

Adhesion to Endothelium (Cytoadherence)

Erythrocytes infected with *P. falciparum* "disappear" from the circulation during their development in a process called sequestration. One of the major mechanisms associated with this event is adhesion to the endothelial cells lining small blood vessels, where the shear flow forces are sufficiently reduced to allow the relatively low avidity IE/ endothelial cell (EC) interactions to operate. A large number of endothelial receptors have been identified (see Table 1 for details) but not all of these are commonly used in patient isolates. A number of studies have attempted to correlate specific receptor usage with disease severity but these experiments are complicated by a wide range of confounders, not the least that the parasites available on admission to hospital (prior to treatment) may not accurately represent the sequestered mass.

An important and reproducible finding that has emerged from these investigations is that the host cell receptor CD36 is a major endothelial receptor for parasite cytoadherence both in terms of how frequently the receptor is utilised by different parasite isolates and the strength of binding. Indeed, most infections are characterised by parasites that bind CD36 to some extent (Newbold *et al.*, 1997), with the important exception of parasites sequestered in the placenta (below). In addition, infected erythrocytes adhere very avidly to CD36 such that they can stably bind under conditions of flow that mimic those in microvasculature while many other parasite-receptor interactions are only able to support rolling adhesion (Cooke *et al.*, 1994). Thus, the available evidence indicates that *P. falciparum* has made a significant investment into CD36 binding with the widespread cellular distribution of this receptor offering the parasite numerous different opportunities for host interactions. Roles for the parasite-CD36 interaction beyond sequestration are discussed below. By comparison to CD36, other receptor adhesion traits are less common or else support weaker binding. However, there is evidence that multiple receptor interactions can act cooperatively to anchor the infected erythrocyte (McCormick *et al.*, 1997).

In terms of parasite adhesion traits that may be involved in organ-specific sequestration this has generally been more difficult to investigate mainly because tissues that malarial researchers would like to study, such as brain, are not readily accessible. However, there is some evidence from sampling circulating blood and allowing parasites to mature *in vitro* that infected erythrocytes binding ICAM-1 are slightly increased in patients with cerebral malaria (Newbold *et al.*, 1997). In addition, cerebral sequestered parasites specifically co-localise to endothelium expressing ICAM-1 in post-mortem histological investigations (Turner *et al.*, 1994). An important area of future research is to further define receptors involved in cerebral sequestration in order to understand the pathological basis of this disease.

Recently, it was reported that isolates from patients with severe malaria were more likely to bind multiple receptors (Heddini *et al.*, 2001). Many of the receptor binding events described from severe isolates also participated in rosette formation between infected and uninfected erythrocytes (below), a property that had previously associated with severe disease. Thus, a combination of binding events acting synergistically may bring about severe disease.

Adhesion to Uninfected Erythrocytes (Rosetting)

Another form of adhesion is rosetting, the binding of two of more uninfected erythrocytes to an infected cell (Udomsangpetch *et al.*, 1989). While not all parasite isolates form rosettes and the degree of rosette formation can vary dramatically between isolates, higher rosetting rates have generally been associated with more severe disease (Carlson *et al.*, 1990; Rowe *et al.*, 1995) with some exceptions (al-Yaman *et al.*, 1995).

In a *P. falciparum* clonal lineage, rosetting was shown to be a clonally variant property of parasites that was eventually demonstrated to be mediated by specific PfEMP1 proteins (Chen *et al.*, 1998; Rowe *et al.*, 1997). The rosettins, now recognised as *rif* genes, were also initially believed to participate in rosette formation but direct evidence for this role is lacking. There are a number of receptors on the red cell surface that can participate in rosette formation including complement receptor 1 (CR1) (Rowe *et al.*, 1997), Heparan-sulfate (Chen *et al.*, 1998), and the ABO blood group (particularly group A) (Carlson and Wahlgren, 1992). In addition, rouleaux-forming serum proteins are also involved in rosetting (Treutiger *et al.*, 1999). Interestingly, CR1 polymorphisms are common in Africans suggesting the possibility that they may have a protective role against severe malaria (Moulds *et al.*, 2001).

Rosetting parasites differ in their receptor specificity. For instance, not all rosetting parasites bind CR1, parasites differ in their susceptibility to rosette disruption with heparin (Rogerson *et al.*, 1994), and parasites can form small or large rosettes. This variability highlights a limitation of correlative studies that tend to lump multifactorial binding phenotypes together or do not account for the possibility that binding properties may exist in different adhesive contexts on different PfEMP1. Thus, it may be a simplification to assume that all rosetting or all ICAM-1 binding parasites possess the same potential for disease.

Adhesion to Other Infected Erythrocytes (Autoagglutination)

Besides binding uninfected erythrocytes, a subset of parasites form autoagglutinates with other infected erythrocytes. Autoagglutination has also been shown to be a clonally variant property (Roberts *et al.*, 1992). Recently, it was demonstrated that autoagglutinate clumps were bridged by platelet cells (Pain *et al.*, 2001). One of the important receptors supporting platelet-mediated

clumping of infected erythrocytes is CD36. Since most parasites bind CD36 but do not form autoagglutinates, differences in parasite affinity or specificity for CD36 may be important for clump formation. Alternatively, there may be additional platelet-specific receptors required in combination to CD36 for autoagglutinates to form. Autoagglutination is observed in field isolates and associated with more severe disease (Roberts *et al.*, 2000).

Adhesion to Monocytes and Dendritic Cells

Infected erythrocytes also bind other cells in the blood circulation including monocytes and dendritic cells (Ockenhouse *et al.*, 1989a; Urban *et al.*, 1999). Again, CD36 has been demonstrated to be an important receptor bridging these interactions (Urban *et al.*, 2001). Infected erythrocyte adhesion to monocytes has been demonstrated to induce an oxidative burst (Ockenhouse *et al.*, 1989a). In contrast, infected erythrocyte adhesion to dendritic cells *in vitro* has been shown to down-modulate their antigen-presenting activity and their capacity to stimulate T cells (Urban *et al.*, 1999). Whether this same effect occurs during natural malaria infections in the cellular environment in which T cells are activated is unknown but because dendritic cells are crucial for inducing immune responses this effect could contribute to the immunosuppression typical of malaria infection. One of the functions of CD36 is to act as a receptor in the uptake of apoptotic cells. It has been hypothesised that infected erythrocytes binding to CD36 on dendritic cells may subvert an immodulatory pathway that evolved to prevent autoimmune disease (Urban *et al.*, 2001).

Adhesion to Placenta

The best understood system of organ-specific sequestration is the placenta due to the fact that sequestered parasites can be directly studied from this tissue after delivery. For a long time it had been recognised that women in Africa, even those with significant pre-existing malaria immunity, became susceptible to malaria infection during pregnancy. Malaria during pregnancy is associated with a massive accumulation of infected erythrocytes in the placenta, which can cause severe anaemia in mothers but also appears to be responsible for the development of low-birth weight babies at increased risk of death (Brabin, 1983; Walter *et al.*, 1982). The severity of malaria during pregnancy typically diminishes with successive pregnancies and is correlated with less sequestered parasites in the placentas of multi-gravid women and the acquisition of antibodies to placental-binding parasites (Fried *et al.*, 1998; Staalsoe *et al.*, 2001).

Fried and Duffy performed the seminal experiments investigating the binding properties of placental-sequestered parasites. The remarkable observation that they made was that unlike most infections, placenta sequestered parasites did not bind CD36 but rather these parasites had special affinity for chondroitin-sulfate A (CSA) (Fried and Duffy, 1996; Rogerson *et al.*, 1995). Interestingly, CSA-adherent parasites are rare in non-pregnant individuals. The model that emerged from these studies is that the placenta enriches for parasite binding variants that do not adhere well to microvasculature but have a special affinity for the placenta. Consequently, pregnant mothers become infected with parasite binding variants to which to which they have not previously developed immunity (for review see Beeson *et al.* (2001), Scherf *et al.* (2001)).

Although CSA-containing proteoglycans are widely distributed throughout the vascular endothelium, placental CSA has a unique chemistry with an especially low sulfate content to which infected erythrocytes adhere more avidly (Achur *et al.*, 2000; Alkhalil *et al.*, 2000). Indeed, the placental intervillous spaces contain a web-like matrix of the low sulfate proteoglycans which might "capture" infected erythrocytes and explain the mystery of why many placental sequestered infected erythocytes are not closely associated to cells. In addition, placental syncytiotrophoblasts are also known to bind infected erythrocytes. Thrombomodulin, a CSA-containing proteoglycan expressed by syncytiotrophoblast cells, has been shown to support parasite adhesion in vitro (Gysin *et al.*, 1997) and may be one of the sources for two cell-associated proteoglycans that were also characterised from placenta (Achur *et al.*, 2000).

Since the first binding descriptions it has been reported that placental-sequestered parasites can also bind hyaluronic acid (Beeson *et al.*, 2000) and non-immune immunoglobulins (Flick *et al.*, 2001), although there is still some controversy over whether hyaluronic acid is a receptor (Valiyaveettil *et al.*, 2001). Current efforts are directed at defining the relative importance of these different receptors for placental sequestration and other possible undefined placental sequestration receptors. Another major avenue of research is to understand a protective immune response that develops in pregnant mothers and prevents parasite adhesion to CSA. Remarkably, the anti-adhesion antibodies are pan-reactive and recognise placental sequestered parasites from all over the world (Fried *et al.*, 1998). This contrasts with typical anti-adhesion antibodies detected in children and adults that are typically highly strain-specific (Bull *et al.*, 1998). The evolution of broadly protective anti-adhesion antibodies in pregnant mothers is an encouraging sign and suggests that it may be possible to develop vaccines directed at parasite-encoded, variant erythrocyte surface proteins. Understanding the molecular basis and specificity of these antibodies is an important area of research (Duffy *et al.*, 2001).

Conclusions

This review summarises the evidence for molecules at the surface of infected erythrocytes. Our increased understanding of these proteins is an important goal because of their central importance to many essential features of the parasite biology including nutrient acquisition, establishment of chronic infection with greater opportunities for transmission, and pathogenesis. In addition to the molecules discussed there is biochemical evidence for additional surface proteins (Fernandez *et al.*, 1999; Howard, 1988). The Malaria Genome Sequencing Effort and post-genomic approaches may provide new opportunities to identify these molecules and thereby develop new strategies to combat this disease.

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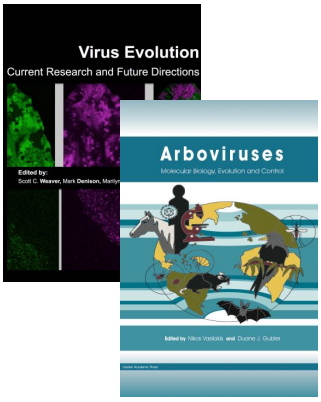

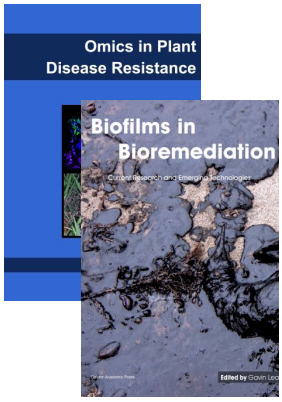

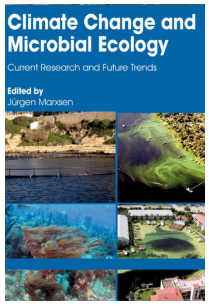

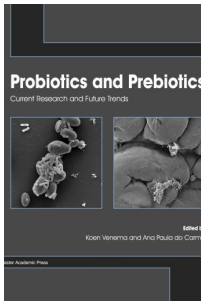

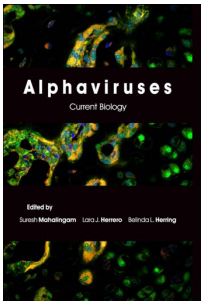

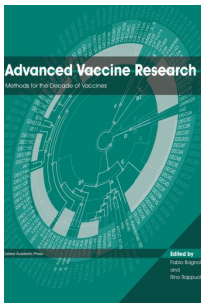

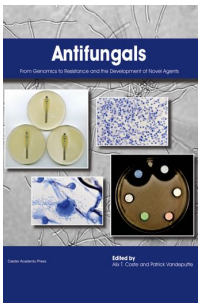

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