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The Malaria-Infected Red Blood Cell: Structural and Functional Changes

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ABSTRACT

The asexual stage of malaria parasites of the genus *Plasmodium* invade red blood cells of various species including humans. After parasite invasion, red blood cells progressively acquire a new set of properties and are converted into more typical, although still simpler, eukaryotic cells by the appearance of new structures in the red blood cell cytoplasm, and new proteins at the red blood cell membrane skeleton. The red blood cell undergoes striking morphological

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alterations and its rheological properties are considerably altered, manifesting as red blood cells with increased membrane rigidity, reduced deformability and increased adhesiveness for a number of other cells including the vascular endothelium. Elucidation of the structural changes in the red blood cell induced by parasite invasion and maturation and an understanding of the accompanying functional alterations have the ability to considerably extend our knowledge of structure–function relationships in the normal red blood cell. Furthermore, interference with these interactions may lead to previously unsuspected means of reducing parasite virulence and may lead to the development of novel antimalarial therapeutics.

ABBREVIATIONS

AARP	asparagine- and aspartate-rich protein
Ag332	antigen 332
ATP	adenosine triphosphate
ATS	acidic terminal segment
BgpA	blood group A antigen
BiP	binding protein
bp	base pairs
bporf	break point open reading frame
C32 Melanoma	C32 amelanotic melanoma cell line
CD	cluster determinant
CIDR	cysteine-rich interdomain region
CLAG	cytoadherence-linked asexual gene
CR1	complement receptor 1
CRA	circumsporozoite protein-related antigen
CRM	cysteine-rich motif
CSA	chondroitin sulphate A
CSP	circumsporozoite protein
DBL	Duffy binding ligand
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
Exp-1	exported protein-1
FEST	falciparum exported serine–threonine kinase
FIRA	falciparum interspersed repeat antigen
GAG	glycosaminoglycan
GARP	glutamic acid-rich protein
GBP	glycophorin-binding protein
Glu	glutamic acid
GPC	glycophorin C
GTP	guanosine triphosphate
HA	hyaluronic acid
HbA	haemoglobin A
HbAA	haemoglobin AA
HbAS	haemoglobin AS
HbS	haemoglobin S

HbSS	haemoglobin SS
HLA	human leucocyte antigen
HRP	histidine-rich protein
HS	heparan sulphate
HS-like GAG	heparan sulphate-like glycosaminoglycan
HUVEC	human umbilical vein endothelial cells
IC_{50}	50% inhibitory concentration
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon gamma
IgM	immunoglobulin M
IOV	inside-out vesicle
IP	iodinatable protein
KAHRP	knob-associated histidine-rich protein
kb	kilobase-pairs
K_d	dissociation constant
kDa	kilodalton
KP	knob protein
MDa	megadalton
MESA	mature-parasite-infected erythrocyte surface antigen
mRNA	messenger ribonucleic acid
MSP	merozoite surface protein
MW	molecular weight (mass)
orf	open reading frame
PCR	polymerase chain reaction
PECAM-1	platelet–endothelial cell adhesion molecule 1
PfAARP1	*Plasmodium falciparum* asparagine- and aspartate-rich protein-1
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
PfERC	*Plasmodium falciparum* endoplasmic reticulum-located calcium-binding protein
PfHRP	*Plasmodium falciparum* histidine-rich protein
Pfsbp1	*Plasmodium falciparum* skeleton binding protein 1
PRBC	parasitized red blood cell
RAP-1	rhoptry-associated protein-1
RESA	ring-infected erythrocyte surface antigen
rif	repetitive interspersed family
RSP	ring surface protein
SDS	sodium dodecyl sulphate
SHARP	small histidine- and alanine-rich protein
SSRBC	homozygous sickle red blood cell
stevor	sub-telomeric variable open reading frame
TM	thrombomodulin
TD	transmembrane domain
TR	transferrin receptor
TRAP	thrombospondin-related anonymous protein
TSP	thrombospondin
var	variant
VARC and VAR_{CD}	equivalent terms for the cytoplasmic domain of PfEMP1
VCAM-1	vascular cell adhesion molecule 1
ves	variant erythrocyte surface
VESA	variant erythrocyte surface antigen

1. INTRODUCTION

Malaria caused by protozoa of the genus *Plasmodium*, particularly *P. falciparum*, is the most serious and widespread parasitic disease of humans. Each year, several hundred million people become infected with malaria parasites and 2–3 million (predominantly young children) die as a result of the infection. The signs and symptoms of malaria are manifested during the part of the infection in which the asexual stage parasites invade red blood cells. This process is still not well understood but involves an ordered multi-step process, which ends with the parasite residing inside the red blood cell within a membrane-lined vacuole in the red blood cell cytoplasm, called the parasitophorous vacuole. The parasites mature and undergo nuclear division, over a period of time varying between 24 and 72 hours, depending on the species of parasite. The earliest intracellular form is called the ring stage because of its signet ring-like appearance when viewed on Giemsa-stained blood films. Subsequently, the parasite matures into the pigmented trophozoite stage and then to a multinucleate form known as a schizont (or meront or segmenter), which divides to produce a number of merozoites. Finally, at the time of red blood cell rupture, the merozoites are released and, in turn, can reinvade other red blood cells to continue the cycle.

The red blood cell has traditionally been viewed as a passive container that shields the parasite from host effector mechanisms such as antibody. We now recognize that, during the maturation of the intracellular parasite, a series of dramatic and extensive changes occurs in the structural and functional properties of the infected red blood cells. These changes have been most intensively studied in *P. falciparum* and include alteration of red blood cell morphology and changes in the membrane mechanical properties of the cell and the state of phosphorylation of membrane skeletal proteins. Strikingly, the infected cells become adhesive for a number of other cells, including other parasitized red blood cells, vascular endothelial cells, normal red blood cells, dendritic cells and platelets. These changes are crucial to the survival of the parasite and, in their absence, either the parasite dies or parasitized red blood cells are rapidly eliminated from the circulation. For example, it has been suggested that the ability of red blood cells infected with mature forms of *P. falciparum* to accumulate in the microvasculature of a variety of organs (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Silamut *et al.*, 1999) prevents parasitized red blood cells from destruction by the reticuloendothelial system and allows the microaerophilic parasite to mature in a relatively hypoxic environment in the deep vasculature. This in turn may be linked to the enhanced virulence shown by this species of parasite, although other factors such as induction of inflammatory cytokines undoubtedly play a part (Clark *et al.*, 1994, 1997; Udomsangpetch *et al.*, 1997).

It is likely that the various structural, morphological and functional changes occurring in the red blood cell are the result of export of parasite proteins into the red blood cell cytoplasm, where they interact with the cytoplasmic,

membrane skeletal and membrane components of the red blood cell. A number of reviews have examined various aspects of this issue over the years (Sherman, 1985; Tanabe, 1990a,b; Haynes, 1993; Ginsburg, 1994a,b; Cooke and Coppel, 1995; Foley and Tilley, 1995; Deitsch and Wellems, 1996; Oh *et al.*, 1997; Coppel *et al.*, 1998a,b). Several, such as that by Sherman (1985), still warrant careful reading. Recently there have been considerable advances in identifying the molecular players in phenomena such as red blood cell remodelling and cytoadherence, although knowledge of exact functional roles for many of these molecules is still missing. This review will consider a number of the key parasite molecules in turn, describe what is known of their interactions with other proteins, and indicate how these contribute to altered cellular function and the pathogenesis of malaria.

Before considering these parasite proteins, we will briefly review the structure of the normal red blood cell membrane skeleton. The red blood cell has become one of the pre-eminent systems for the analysis of structure–function relationships of biological membrane systems. It is probably the best understood eukaryotic cell in terms of the physical nature of the membrane skeleton and its relationship to the mechanical properties of the cell (Evans and Hochmuth, 1977; Mohandas *et al.*, 1984, 1992; Chasis and Mohandas, 1986; Mohandas, 1992; Mohandas and Chasis, 1993; Mohandas and Evans, 1994). The ordered arrangement of spectrin tetramers, their interconnection at the ternary complex with actin and protein 4.1, and the bonds to the overlying cell membrane via band 3 and glycophorin C (Figure 1A) provide the basis for the cell's ability to deform during repeated passage through the microcirculation during its 120 days' lifetime (Bennett, V., 1983; Gardner, K. and Bennett, 1989; Mohandas and Evans, 1994). The stability of the spectrin network is not only influenced by the primary sequence of the component proteins but can also be modulated by the levels of protein phosphorylation (Ling *et al.*, 1988; Manno *et al.*, 1995). This understanding of the relationship of the protein network to properties of the whole cell has been advanced by the study of pathological states such as inherited disorders of red blood cells including sickle cell disease, the thalassaemias and hereditary spherocytoses and ovalocytosis (Mohandas and Chasis, 1993; Mohandas and Evans, 1994). In these conditions, changes in haemoglobin structure, such as those in haemoglobin S for example, have led to altered cellular properties including changes in cell deformability and increased adhesiveness (Barabino *et al.*, 1987; Francis, 1991; Francis and Johnson, 1991; Morris *et al.*, 1993).

2. PARASITE PROTEINS EXPOSED TO THE RED BLOOD CELL MEMBRANE SKELETON

It is generally believed that almost all of the altered properties of parasitized red blood cells can be traced to the actions of a group of proteins of parasite

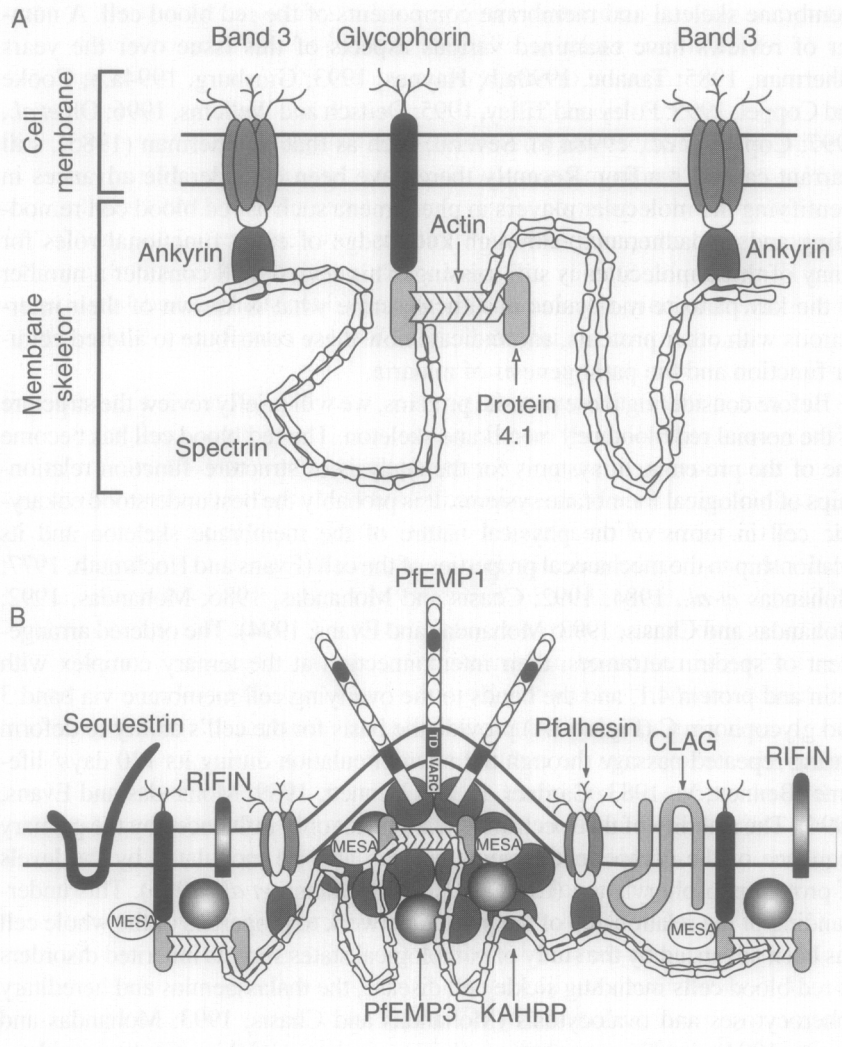

Figure 1 Schematic representation of the membrane skeleton of a red blood cell before (A) and after (B) invasion by *P. falciparum*, to indicate the changes that occur to the red blood cell as a result of infection. B depicts the typical knob structure at the infected red blood cell membrane formed by the interaction of parasite-encoded proteins such as KAHRP, PfEMP3 and MESA with the red blood cell membrane skeleton and the clustering of the major cytoadhesion ligand, PfEMP1, over the knob through interaction of its cytoplasmic tail (VARC) with KAHRP. Abbreviations are expanded on pp. 2–3.

origin that become associated with the red blood cell cytoplasm and the red blood cell membrane skeleton, either by deposition on the inner aspect of the membrane or by transient or more permanent insertion into the membrane and exposure on the red blood cell surface (Figure 1B). At present, our understanding of the number and character of the proteins that are exported to the red blood cell is far from complete. Both molecular and biochemical studies have addressed this question. Early studies attempted to isolate erythrocyte membranes and compare profiles of proteins between infected and uninfected cells. For example, Stanley and co-workers purified surface membranes by binding them to poly-L-lysine and then used silver staining and labelling to identify novel proteins in infected cells (Stanley and Reese, 1986). These studies identified at least six parasite-derived polypeptides (>240, 150, 55, 45, 35 and 20 kDa) that were associated with the infected red blood cell plasma membrane (Stanley and Reese, 1986). Interestingly, the authors suggested that four of these polypeptides (55, 45, 35 and 20 kDa) might be exposed on the surface of the infected red blood cell. Although not much was made of this observation at the time, intriguingly these protein sizes are very similar to those of the recently described products of the *rif* multi-gene family. Clearly, sensitivity of the labelling techniques or levels of expression must have been problematic, since we now know that there are more proteins associated with the red blood cell membrane than were described in this study. Alternative biochemical approaches to the identification of parasite proteins have used selective solubilization with a variety of detergents to examine membranes or to purify the electron-dense knob structures that appear on the surface of parasitized red blood cells (Chishti *et al.*, 1992; Rabilloud *et al.*, 1999). Parasite proteins that associate with the red blood cell membrane skeleton become insoluble in the non-ionic detergent Triton X-100, and this is often used as an operational definition of cytoskeletal association.

The advent of molecular cloning studies has enabled identification of a number of proteins that are located in the infected red blood cell (Tables 1 and 2). Typically such studies used antisera raised against recombinant proteins and immunolocalization by either light or electron microscopy, or both, to identify exported proteins. Kun and co-workers (1991) attempted to focus on this group of proteins by screening expression libraries with antisera made against membrane fractions. A number of proteins were identified including known exported proteins such as MESA,* but also novel sequences, some of which remain incompletely characterized at the time of writing this review. One of the novel sequences identified in this study proved to encode the exported serine–threonine kinase FEST (Kun *et al.*, 1997), so it may well be that the other sequences are also fragments encoding more, as yet unknown, exported proteins.

*Abbreviations used in the text, Tables and Figures are expanded on pp. 2–3.

Table 1 *P. falciparum* proteins associated with the red blood cell membrane skeleton and exposed on the surface of parasitized red blood cells.

Name[a]	Synonyms[a]	Molecular mass (kDa)	Comments[a]	References
PfEMP1	IP	265–285	Product of the *var* multi-gene family; mediates cytoadherence; often trypsin sensitive; clusters at knobs; antigenically variable; different forms can bind to different receptors; selection for cytoadherence selects for higher molecular mass PfEMP-1; not essential for growth *in vitro*	Leech *et al.*, 1984b; Howard, R.J., 1988; Howard, R.J. *et al.*, 1988; Magowan *et al.*, 1988; Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995; Cheng *et al.*, 1998; Newbold *et al.*, 1999
Rifins	Rosettins	35–45	Product of the *rif* multi-gene family; highly polymorphic; implicated in rosetting and antigenic variation	Cheng *et al.*, 1998; Fernandez *et al.*, 1999; Kyes *et al.*, 1999
Stevor		*c.* 30	Product of the *stevor* multi-gene family; highly polymorphic; believed to be a subfamily of the *rif* genes	Cheng *et al.*, 1998; Kyes *et al.*, 1999
Clag9		–	Member of the *clag* multi-gene family; complicated multi-exon structure; believed to be exposed on the red blood cell surface; apparently necessary for cytoadherence to the receptor CD36; not required for growth *in vitro*	Holt *et al.*, 1999; Gardiner *et al.*, 2000; Trenholme *et al.*, 2000
Sequestrin		–	–	Ockenhouse *et al.*, 1991b
TR		105	Putative receptor for transferrin	Haldar *et al.*, 1986; Rodriguez and Jungery, 1986

[a] Abbreviations are expanded on pp. 2–3.

Table 2 *P. falciparum* proteins associated with the red blood cell cytosol or membrane skeleton but not exposed on the surface of parasitized red blood cells.

Name[a]	Synonyms[a]	Molecular mass (kDa)	Comments[a]	References
KAHRP	HRPI, KP	80–109	Present at knobs; isolates lacking this protein do not have knob structures detectable by electron microscopy and do not cytoadhere under flow conditions; binds to spectrin, ankyrin, actin and the cytoplasmic tail of PfEMP1; not required for growth *in vitro*	Kilejian, 1979; Hadley *et al.*, 1983; Leech *et al.*, 1984a; Culvenor *et al.*, 1987; Sharma and Kilejian, 1987; Triglia *et al.*, 1987; Kilejian *et al.*, 1991; Crabb *et al.*, 1997a; Waller, K.L. *et al.*, 1999; Oh *et al.*, 2000
MESA	PfEMP2	250–300	Phosphoprotein; binds to protein 4.1; located at knobs; extensive repetitive regions; not required for growth *in vitro*	Coppel *et al.*, 1986, 1988; Howard, R.J. *et al.*, 1987, 1988; Lustigman *et al.*, 1990; Coppel, 1992
PfEMP3	Antigen 12A	315	Located both at knobs and elsewhere at the membrane; bound to the membrane skeleton; not essential for cytoadherence, extensive repetitive regions; not required for growth *in vitro*	Handunnetti *et al.*, 1992a; Pasloske *et al.*, 1993, 1994; Van Schravendijk *et al.*, 1993; Waterkeyn *et al.*, 2000
PfHRPII	SHARP		Controversial whether this protein is secreted from the intact red blood cell	Stahl *et al.*, 1985b; Howard, R.J. *et al.*, 1986; Wellems and Howard, 1986; Wellems *et al.*, 1987
Ag332		*c.* 2500	Giant protein; present in mature parasite stages; associated with the red blood cell membrane skeleton and at knobs; some evidence of exposure on the infected red blood cell surface; function or requirement for survival remains unknown	Mattei and Scherf, 1992; Mattei *et al.*, 1992; Hinterberg *et al.*, 1994b
41-2		29	Present in schizonts, possibly localized in the schizont membrane; associated with membranous structures in the red blood cell cytoplasm and with the red blood cell's membrane skeleton	Knapp *et al.*, 1991

Table 2 continued

Name[a]	Synonyms[a]	Molecular mass (kDa)	Comments[a]	References
RESA	Pf155	155	Phosphoprotein; present in ring-stage parasites; minor variability; binds to spectrin; increases thermal stability of the red blood cell	Coppel *et al.*, 1984b; Perlmann *et al.*, 1984; Favaloro *et al.*, 1986; Anders *et al.*, 1987b; Foley *et al.*, 1990, 1991, 1994; Culvenor *et al.*, 1991;
FEST	Pf255	210	Serine–threonine kinase associated with the membrane skeleton; suggested to be responsible for phosphorylation of MESA and RESA	Kun *et al.*, 1991, 1997
FIRA		300	Highly antigenic during infection, present in both immature ring and mature parasite stages	Stahl *et al.*, 1987
GBP	96tr	96–130	First described as located on the merozoite surface; now generally accepted that this was artefactual and it is in fact located in the cytoplasm of the infected red blood cell	Coppel *et al.*, 1984a; Ravetch *et al.*, 1985; Kochan *et al.*, 1986; Van Schravendijk *et al.*, 1987
46 kDa cleft protein		46	Poorly characterized; localized to Maurer's clefts in the infected red blood cell cytoplasm; may be the same as the recently described Pfsbp1	Hui and Siddiqui, 1988; Etzion and Perkins, 1989; Das *et al.*, 1994; Blisnick *et al.*, 2000
Rab		23	Present in all asexual blood stages of the parasite; marker for transport studies; localization of Rab6 suggests that the early and late Golgi apparatus are separate structures in *P. falciparum*	Decastro *et al.*, 1996; Van Wye *et al.*, 1996
Exp1	CRA	23	Integral membrane protein; localized to the periphery of the parasite	Coppel *et al.*, 1985; Hope *et al.*, 1985; Simmons *et al.*, 1987; Bianco *et al.*, 1988; Günther *et al.*, 1991
PfSar1p		23	GTP-binding protein involved in protein trafficking; localized at the periphery of the parasite and in discrete vesicles and in vesicles within the red blood cell cytoplasm	Albano *et al.*, 1999a

[a] Abbreviations are expanded on pp. 2–3.

We shall discuss a number of exported proteins in detail, but first we will make a few general points. Many of the exported proteins are large, ranging in size from 100 kDa to more than 2·5 MDa. These large proteins all contain extensive regions of low complexity sequence, often occurring in the form of tandemly repeated oligopeptides (Figure 2). The repeats are characteristically present in distinct regions, with each region composed of repeats of a particular sequence. Thus KAHRP has three repeat regions and MESA has six, whereas RESA has only two repeat regions. The repeats often contain charged residues, either positive or negative, so that the repeat regions are highly charged. A common motif is a dipeptide of glutamic acid, and antibodies raised to peptides containing Glu-Glu motifs have frequently been highly cross-reactive, reacting with a number of proteins including RESA, D260, Ag332 and Pf11.1 (Mattei *et al.*, 1989; Wåhlin *et al.*, 1992; Barnes *et al.*, 1995). These charged regions of low complexity sequence are associated with non-uniform binding of sodium dodecyl sulphate (SDS) and consequent anomalous migration in SDS–polyacrylamide gels. Typically, such proteins appear to be much larger than their predicted molecular mass (Coppel *et al.*, 1994). The repeat regions are typically predicted to be either alpha-helical, random coil or coiled coil.

Many other malaria proteins contain regions of repeated sequence, including a number of proteins of the merozoite surface (Anders *et al.*, 1987a, 1993; Anders and Smythe, 1989; Coppel *et al.*, 1994). Examples include integral membrane and peripheral membrane proteins such as MSP1, MSP2 and MSP3 and the S-antigens. It should be noted that, in the case of the merozoite proteins, the repeats are typically highly variable, differing in repeat length and sequence in different isolates (Anders *et al.*, 1993; Coppel *et al.*, 1994). In MSP2, there are estimated to be several hundred distinct alleles differing in repeat sequence from each other (Eisen *et al.*, 1998). In contrast, the exported proteins that are found inside the red blood cell generally show conservation of sequence, including the repeat regions, when sequences from different isolates are compared (Stahl *et al.*, 1987; Kun *et al.*, 1999). There are exceptions, for example, in the case of the 3′ repeats of KAHRP (Kant and Sharma, 1996; Hirawake *et al.*, 1997), but in general repeat conservation is very high. The only documented differences relate to numbers of repeat units, which may vary by one to several copies. Otherwise these regions are strikingly conserved. This suggests that the repeats may in fact have some sort of functional importance and in at least one case a definite protein-binding specificity has been documented (Waller, K.L. *et al.*, 1999). This contrasts with the group of exported proteins that are found on the exterior of the red blood cell, which are highly variable, suggesting that immunological pressure is driving the alteration in sequence (Hughes and Hughes, 1995; Newbold *et al.*, 1997a, 1999; Cheng *et al.*, 1998; Fernandez *et al.*, 1999; Newbold, 1999).

Many of the exported proteins are encoded by genes containing two exons. Examples include KAHRP, RESA, MESA, FIRA, GBP and PfEMP3. The

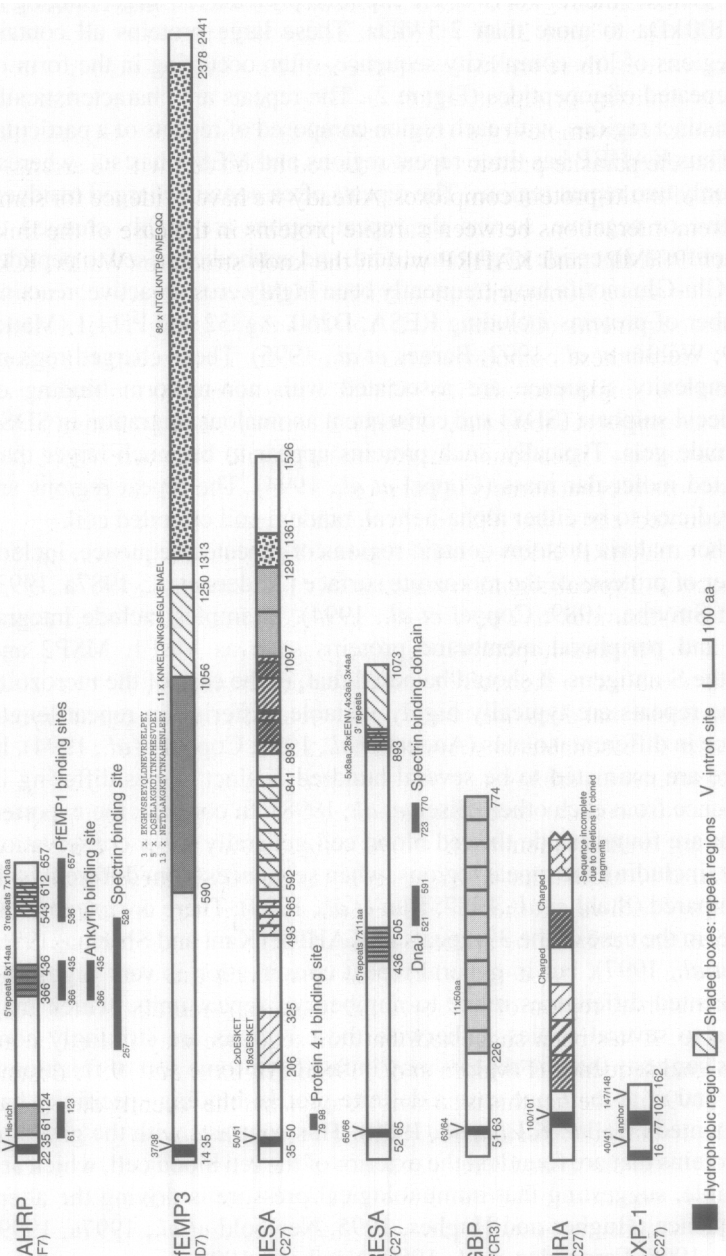

Figure 2 Schematic diagram of a number of *P. falciparum* proteins associated with the red blood cell cytoplasm and membrane skeleton. Hydrophobic and repeat regions are indicated. Abbreviations: aa, amino acids; other abbreviations are expanded on pp. 2–3.

first exon is typically short, in the range of 100–250 bp, and the second exon, which also contains the region of tandem repeats, is much larger, typically in the range of 2–6 kb (Figure 2). If one searches the malaria genome for genes with these properties, there are in fact a large number of two-exon genes containing low complexity or repeat sequences. It is likely that at least some of these are additional examples of exported proteins. This in turn suggests that it is likely that the parasite proteins within the red blood cell will be arranged in some form of multi-protein complexes. Already we have evidence for some protein–protein interactions between parasite proteins in the case of the linkages between PfEMP1 and KAHRP within the knob structure (Waller, K.L. *et al.*, 1999). Evidence from purification studies of the knob suggests that there are multiple components (Chishti *et al.*, 1992), although direct interactions between several of these components have not been demonstrated. Further, the absence of a single protein, KAHRP, is enough for this characteristic structure to disappear (Crabb *et al.*, 1997a). However, already a number of exported proteins have been found outside the knob and, with more to be discovered, it is possible that some quite complex structures will be found.

A feature noted early was that many of the exported proteins, such as MESA, RESA, KAHRP, FIRA and GBP, all lacked N-terminal hydrophobic signal sequences. Typical signal sequences of 13–15 hydrophobic residues located at the N-terminus had been found in proteins exported to the merozoite surface, in the parasitophorous vacuole and in the rhoptries, but not in these proteins exported to the red blood cell compartment. The red blood cell compartment is one that does not have an exact parallel in a typical eukaryotic cell, and it seems reasonable to suppose that the parasite requires additional trafficking machinery, perhaps a novel transport system, to place these proteins in their final cellular location. Exported proteins might then require some sort of tag to direct them to this novel export pathway or, alternatively, lack some retention signal that prevents their passage to the exterior along a default secretory pathway. If there are specific tags for transport, then there is no requirement that this tag be at the N-terminus, but it is intriguing to note that the N-terminus of these red blood cell-associated proteins is usually charged, with a short region of hydrophobic residues some 20–50 residues into the protein. It has been suggested that this sequence may indeed be an alternative signal sequence (Favaloro *et al.*, 1986; Triglia *et al.*, 1987; Braun-Breton *et al.*, 1990). Whether this is so is not known, but the recently developed techniques to transfect *P. falciparum* parasites (Van Dijk *et al.*, 1995; Wu *et al.*, 1995, 1996; Crabb and Cowman, 1996; Crabb *et al.*, 1997b) should now permit this question to be critically addressed. Once arrived within the red blood cell cytoplasm, these proteins assemble into multi-component complexes such as the knob structure. Presumably such assembly is driven by the presence of high-affinity binding domains specific for partner proteins (Table 3). The binding coefficients of such interactions described to date are

Table 3 Defined protein–protein interactions at the red blood cell membrane skeleton.

Protein partners[a]	Binding constants[b] (μM)	References
Spectrin and protein 4.1	0·1	Tyler *et al.*, 1980; Podgorski and Elbaum, 1985
Spectrin and ankyrin	0·1	Tyler *et al.*, 1980
Ankyrin and band 3	0·01	Bennett, V. and Stenbuck, 1980; Thevenin and Low, 1990
Protein 4.1 and p55	0·1	Nunomura *et al.*, 2000
Protein 4.1 and GPC	0·09	Nunomura *et al.*, 2000
p55 and GPC	1·6	Nunomura *et al.*, 2000
Protein 4.2 and ankyrin	0·1–0·4	Korsgren and Cohen, 1988
Protein 4.2 and band 3	0·2–0·8	Korsgren and Cohen, 1988
MESA and protein 4.1	0·63	Bennett, B.J. *et al.*, 1997
KAHRP and ankyrin	1·3–8·3	Magowan *et al.*, 2000
VAR_{CD} and F-actin	0·04	Oh *et al.*, 2000
VAR_{CD} and KAHRP	0·01	Oh *et al.*, 2000
VARC and KAHRP (K1A)	0·1	Waller, K.L. *et al.*, 1999
VARC and KAHRP (K2A)	3·3	Waller, K.L. *et al.*, 1999
VARC and KAHRP (K3)	13·0	Waller, K.L. *et al.*, 1999

[a] Abbreviations are expanded on pp. 2–3.
[b] Binding constants are dissociation constants (K_d) except for the MESA–protein 4.1 interaction, where the value is the IC_{50}.

generally of the order of 10^{-5} to 10^{-7}M, an affinity typical of interactions of host cell proteins of the normal red blood cell skeleton. We will now move on to discuss some of these parasite-encoded proteins in more detail.

2.1. Proteins Exposed on the Surface of Infected Red Blood Cells

2.1.1. *PfEMP1* (Plasmodium falciparum *Erythrocyte Membrane Protein 1)*

From the mid 1960s, evidence accumulated describing the appearance of new antigens on the surface of parasitized red blood cells (e.g., Brown and Brown, 1965; Langreth and Reese, 1979; Gruenberg and Sherman, 1983; Hommel *et al.*, 1983); however, whether these molecules were parasite derived or simply altered host proteins was not known. By metabolic labelling and lactoperoxidase-catalysed radio-iodination of monkey red blood cells parasitized by *P. knowlesi*, R.J. Howard and colleagues (1983) provided the first direct evidence that a molecule of parasite origin was exposed on the surface

of the parasitized cell. Using a similar biochemical approach, Leech and co-workers (1984b) later confirmed these findings using monkey red blood cells infected with *P. falciparum*. This high molecular weight (250–350 kDa) protein, now called PfEMP1, varies in size between different parasite lines and is antigenically highly variable. It is insoluble in Triton X-100 detergent, demonstrating a link to the red blood cell membrane skeleton, and frequently highly sensitive to proteases. The original definition of this molecule included the property of exquisite sensitivity to trypsin digestion (0·1 μg/mL), but PfEMP1 molecules that are resistant to trypsin have now been described (Chaiyaroj *et al.*, 1994a; Gardner, J.P. *et al.*, 1996; Smith *et al.*, 2000).

Although PfEMP1 had been associated with the phenomenon of cytoadherence for several years, defining more precisely its role in the process was hampered by the inability to identify the gene encoding this variant surface antigen. This situation changed spectacularly in 1995 when several groups simultaneously published papers describing a highly polymorphic family of genes, the *var* genes (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995), that encode PfEMP1. Initial estimates of the number of *var* genes per parasite were between 50 and 150 (Baruch *et al.*, 1995; Su *et al.*, 1995), but later studies suggest that the complement is generally 40–50 per haploid genome (Rubio *et al.*, 1996; Thompson *et al.*, 1997). The multiple copies of *var* are scattered throughout the genome and may be found on any chromosome and in either orientation (Rubio *et al.*, 1996; Fischer, K. *et al.*, 1997). They frequently occur in clusters and may be found centrally or in a subtelomeric location (Rubio *et al.*, 1996; Fischer, K. *et al.*, 1997; Hernandez-Rivas *et al.*, 1997), although their precise location in the genome varies between different parasite isolates. Although it is clear that *var* genes can be expressed irrespective of their position, it has been suggested that those in a subtelomeric position are more commonly expressed than those located centrally (Fischer, K. *et al.*, 1997). One interesting suggestion is that the subtelomeric location is prone to great variability and a high frequency of recombination, which may be part of the reason for the extreme variability of *var* genes (Rubio *et al.*, 1996; Fischer, K. *et al.*, 1997). The observation that there is quite a close relationship between some *var* genes in subtelomeric locations of different chromosomes supports this suggestion. These different genes vary in sequence, which results in antigenic heterogeneity and variability in binding specificity. It should be emphasized that different parasite isolates contain not only different numbers of *var* genes located in varying genomic locations but also different repertoires of sequences. Thus the total number of *var* gene sequences is of the order of thousands, perhaps as many as 10 000.

The primary structure of a number of *var* genes and their encoded proteins have now been determined. All the genes appear to have a similar general structure (Figure 3), comprising a long 5′ exon and a shorter 3′ exon. The 3′ exon is well conserved between different *var* genes (Bonnefoy *et al.*, 1997) and

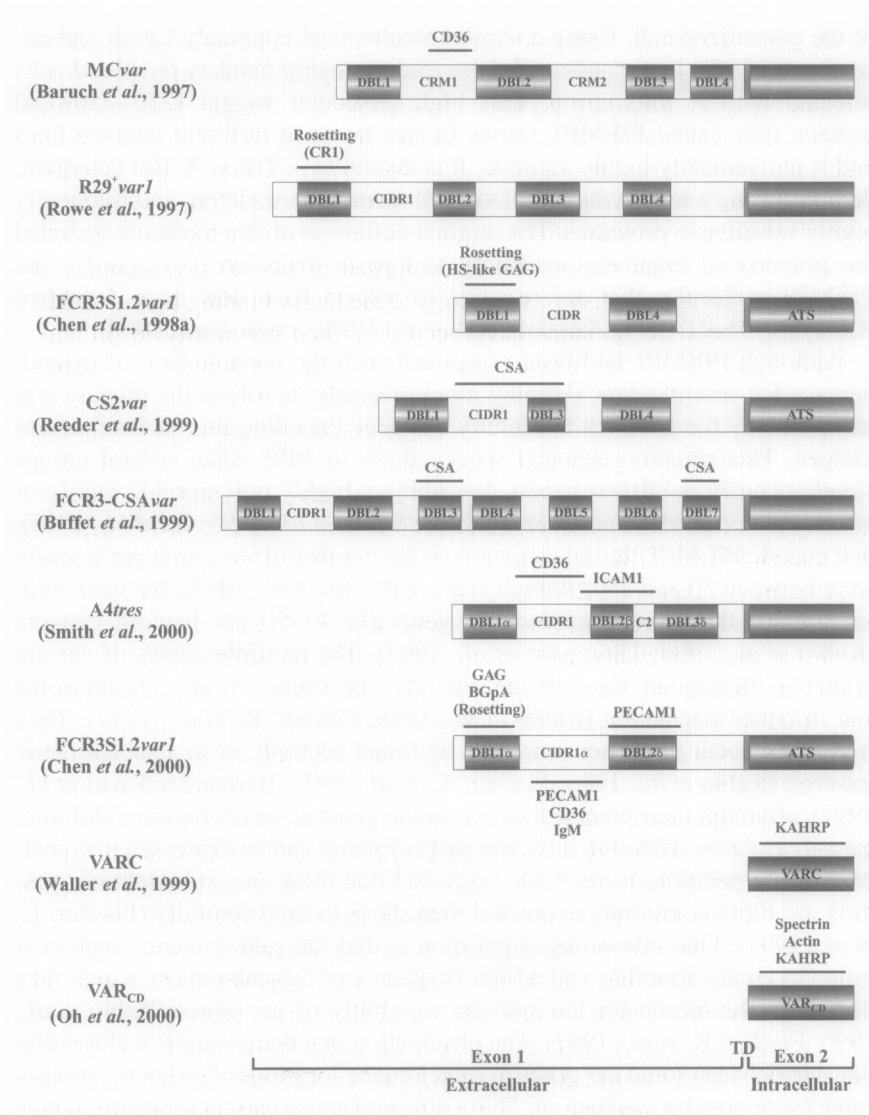

Figure 3 Structure of various cloned *var* genes from *P. falciparum* and defined functional domains in the PfEMP1 protein involved in cytoadhesion or rosetting. Specific domains of the molecule that interact with a number of endothelial cell-expressed adhesion molecules or components of the red blood cell membrane skeleton are shown where these have been determined. The terminology used to name the various structural domains of the molecule are those used by the original authors and the inconsistency demonstrates the confusing nomenclature that has developed. For example, CRM1, CIDR1 and CIDR1α are equivalent terms for the same cysteine-rich region of the molecule. Furthemore, the sequence of the DBL1 region between different *var* genes may not be identical. Abbreviations are expanded on pp. 2–3.

encodes the intracellular domain of the protein, which is rich in acidic residues. It shares homology with elements in the sequence of *Pf60*, a multi-gene family encoding quite disparate proteins (Bischoff *et al.*, 2000). This region is responsible for anchoring PfEMP1 to the membrane skeleton in infected red blood cells, particularly but not exclusively via the knob-associated histidine-rich protein, KAHRP (Waller, K.L. *et al.*, 1999; Oh *et al.*, 2000; Voigt *et al.*, 2000). Unlike the 3′ exon, the 5′ exon is extremely variable and encodes a variable extracellular region that is composed of two to seven copies of cysteine-rich domains that show homology to the Duffy binding ligand (DBL) of *P. vivax*. The DBL domains have been described previously in proteins involved in red blood cell invasion and bind to host red blood cell proteins such as the Duffy blood group antigen and glycophorin A (Adams, J.H. *et al.*, 1992; Sim *et al.*, 1994). The domains can be recognized by conservation of a number of residues, particularly cysteine, which occur in characteristic patterns. Otherwise, DBL-like domains vary greatly in sequence, although there are sub-patterns that allow recognition of DBL-1 domains from different parasites compared with DBL-4, for example. Different PfEMP1 sequences contain different numbers of DBL-like domains (Figure 3), but the significance of this is unclear. Different *var* gene sequences encode variant forms of PfEMP1 that differ in antigenicity and receptor specificity (Smith *et al.*, 1995). PfEMP1 is first synthesized by the late ring/early trophozoite stage and is transported to the red blood cell membrane where, by the late trophozoite stage, PfEMP1 is found in association with knobs and is exposed on the red blood cell surface.

Several studies have attempted to relate binding specificity to specific regions of the protein PfEMP1. Typical experimental approaches involve the expression of domains of the molecule either on the surface of COS or CHO cells and addition of receptors or cells in some form. Although such approaches ignore the potential co-operative effect of several domains, several specific binding domains have been localized (Figure 3). It appears that most or all PfEMP1 molecules contain a binding site for CD36 and this appears to reside in the cysteine-rich interdomain region (CIDR) of the molecule (Baruch *et al.*, 1997, 1999; Chen, Q. *et al.*, 2000). Additional receptors are bound by specific sequences present elsewhere in the protein and are limited to particular PfEMP1 sequences (Figure 3). Thus, the ability to bind to chondroitin sulphate A (CSA), for example, is manifested by only some isolates. Two PfEMP1 sequences from CSA-binding isolates have been reported to have this property (Buffet *et al.*, 1999; Reeder *et al.*, 1999). In one case the *PfEMP1* gene contained seven DBL-like domains (Buffet *et al.*, 1999), and in another case, only three (Reeder *et al.*, 1999) (see Figure 3). In the case of the larger protein, two isolated domains, DBL-3 and DBL-7, were capable of binding CSA, but only DBL-3 had the same spectrum of binding specificities as the parent parasite line. Accordingly it was concluded that DBL-3 was the CSA-binding region

(Buffet *et al.*, 1999). It is not yet known if the other PfEMP1 also uses DBL-3 for binding, and how similar the two binding domains may be. Human antibodies that develop after numerous pregnancies against CSA-binding variants of PfEMP1 cross-react with isolates from various locations around the world, suggesting a reasonable degree of sequence conservation (Fried *et al.*, 1998).

One of the great unknowns is the method by which *var* gene expression, and hence antigenic variation, is controlled. It has been suggested that only a single *var* gene is expressed per cell but that, within a population, several or many genes are switched on. It has been shown unequivocally that a single infected cell can bind to at least two endothelial cell-expressed receptors (Chaiyaroj *et al.*, 1994b), but this has been explained by the suggestion that a single PfEMP1 protein may have binding sites for two or more receptors (Gardner, J.P. *et al.*, 1996; Chen, Q. *et al.*, 2000). Currently, we view this question as unresolved but it has major implications for any model proposed for control of *var* gene expression. Clonal lines of parasites can change expression of PfEMP1 by some form of transcriptional control. The frequency by which cells can switch is estimated to be of the order of 2% per generation for one particular laboratory-adapted parasite line, although this is likely to be an unusually plastic clone (Roberts, D.J. *et al.*, 1992). Most cytoadherent parasites cultured *in vitro* are marked by the stability of the dominantly expressed PfEMP1 ligand. The total number of PfEMP1 molecules on the red blood cell surface is unknown but it is not believed to be an abundant molecule and the total may lie in the thousands. Finally, there is evidence that PfEMP1 transcription may be somewhat promiscuous with leaky transcription of many different *PfEMP1* genes early in the ring stage, followed by switching off transcription of all but the expressed *PfEMP1* gene as the parasite matures (Chen, Q. *et al.*, 1998b).

2.1.2. *Rifins*

In addition to the *var* gene products (PfEMP1), a second group of at least 12 radio-iodinatable proteins ranging from approximately 20 to 170 kDa can also be detected on the surface of parasitized red blood cells. Like PfEMP1, their expression is parasite stage-specific and they appear on the red blood cell surface about 14–16 hours after invasion, as the parasites develop into mature, pigmented trophozoites (Stanley and Reese, 1986; Helmby *et al.*, 1993; Fernandez *et al.*, 1999). Their precise role in adhesion is contentious, perhaps with the exception of adhesion to PECAM1 (CD31), which, in at least one parasite line, appears to be associated with the presence of one of these 35 kDa polypeptides (Fernandez *et al.*, 1999). Analysis of more than 20 different parasite lines, including both clinical isolates and laboratory-adapted cytoadherent lines and clones, has revealed that the most common and prominent of

these proteins occur in the 30–45 kDa size range. Other novel radio-iodinatable bands clearly distinct from *var* gene products can be detected in some lines, but these occur much less frequently and, with the exception of a strong 170 kDa band, are relatively weak in intensity. Further, this 170 kDa polypeptide is not recognized by immune sera that immunoprecipitate multiple bands in the 30–45 kDa cluster in the same parasite line, nor is it recognized by antisera raised against the highly conserved cytoplasmic tail of PfEMP1 by Western blotting (Fernandez *et al.*, 1999).

Until recently, this family of 30–45 kDa proteins had been collectively referred to as rosettins, since they were first identified in parasite lines that showed a high and stable propensity to form rosettes of red blood cells (Helmby *et al.*, 1993). They appear to be resistant to trypsin cleavage at concentrations sufficient to remove PfEMP1 from the red blood cell surface, a distinction that was exploited to demonstrate that these molecules were involved in rosetting. It is now clear that they are not exclusively linked to this phenotype since they are frequently present in non-rosetting lines. Furthermore, they have been detected in all clinical isolates that have been examined to date, suggesting that these antigens have some other, as yet unknown, primary function, which probably plays a much more critical role in parasite survival than rosetting (Fernandez *et al.*, 1999).

Weber (1988) described a repetitive gene sequence, *rif-1* (repetitive interspersed family), in *P. falciparum*, which he claimed was expressed. Although ignored at this time, the availability of the *P. falciparum* genome revealed that *rif*-like sequences were found near *var* genes in the subtelomeric regions of chromosomes (Cheng *et al.*, 1998; Gardner, M.J. *et al.*, 1998; Bowman *et al.*, 1999). These genes, now called rifins, were shown to encode the previously described rosettins, as antisera raised against the deduced amino acid sequences from multiple *rif* genes immunoprecipitated what is apparently the same set of radio-iodinatable proteins (Fernandez *et al.*, 1999; Kyes *et al.*, 1999). It has been suggested that there may be 200–500 *rif* genes per haploid genome (Fernandez *et al.*, 1999), which would in fact make it the largest gene family described to date in *Plasmodium*. The location of the protein and large size of this gene family, together with the observation that closely related clones of *P. falciparum* express different rifins on their surface, suggest that these are clonally variant polypeptides that may play an important role in antigenic variation and evasion of immune responses (Fernandez *et al.*, 1999; Kyes *et al.*, 1999).

A further multi-gene family that has recently been defined is the *stevor* family. Details of the *stevor* gene product are still scanty, but it has been suggested, based on sequence analysis, that *stevor* genes are in fact a subfamily of the *rif* genes (Cheng *et al.*, 1998). Opinion is still divided on this point, but it should be noted that *stevor* genes are found in the subtelomeric arrangement of polymorphic genes noted in several *P. falciparum* chromosomes (Gardner, M.J. *et al.*, 1998; Bowman *et al.*, 1999).

2.1.3. *Clag9*

The involvement in cytoadherence of a gene product encoded on *P. falciparum* chromosome 9 was first suggested by observations that loss of adherence of some clinical isolates and clones during culture *in vitro* was accompanied by overgrowth of parasites possessing a smaller form of this chromosome (Day *et al.*, 1993). Loss of cytoadherence was attributed to the apparent absence of PfEMP1 on the surface of the parasitized red blood cells. The story was, however, to become increasingly more complicated.

Deletions in chromosome 9 occur frequently and typically involve loss of up to 500 kb. Deletions occur subtelomerically at both ends of the chromosome, although the majority of the loss occurs from the right arm (Foote and Kemp, 1989; Shirley *et al.*, 1990). Analysis of a number of parasite lines allowed the cytoadherence-associated locus to be precisely mapped to an open reading frame on the right arm of the chromosome (Barnes *et al.*, 1994). Interestingly, examination of a number of non-cytoadherent clones revealed that the breakpoints for deletions from the right arm of chromosome 9 always occurred within a novel open reading frame (orf), which was called the breakpoint orf (bporf) (Bourke *et al.*, 1996). Some parasite clones have been maintained in culture for many years and, despite the acquisition of a shortened right arm on chromosome 9, have retained a stable adherence phenotype. Genetic analysis has revealed that in fact the bporf has been completely removed by an internal 15 kb deletion, while 55 kb of downstream sequence, which had been lost from a non-cytoadherent sibling clone C10, was retained. This observation indicated that this 55 kb region must encode a novel gene involved in cytoadherence that was distinct from PfEMP1 since no *var* gene was contained within this region of the genome (Bourke *et al.*, 1996). Partial sequencing of the entire 55 kb region led to the identification of a candidate gene located just downstream of bporf, which was dubbed the cytoadherence-linked asexual gene (*clag9*) (Holt *et al.*, 1999; Trenholme *et al.*, 2000).

The *clag* gene is approximately 7 kb and is a complex structure comprising at least nine exons. It is transcribed in mature-stage parasites and translated into a 220 kDa protein that is distinct from PfEMP1 and can be detected in parasitized cells by Western blotting. Its precise cellular localization remains to be determined; however, prediction of the structure of the protein *in silico* (i.e., predicted by computer modelling) from its hydrophobicity profile reveals four putative transmembrane domains, suggesting that it is membrane-associated and presumably could be exposed on the surface of the parasitized cell (Trenholme *et al.*, 2000). Preliminary immunofluorescence data support this hypothesis; however, at this stage its presence on the surface of the infected red blood cell must remain speculative. Nevertheless, recent evidence that adhesion of red blood cells parasitized by the parasite line 3D7, which exhibits a stable cytoadherence phenotype, was ablated when the *clag9* gene was knocked out

by transfection is compelling and confirms the essential role of *clag9* in cytoadherence, at least to CD36 (Trenholme *et al.*, 2000). Further, this same group have now confirmed their findings using an anti-sense approach (Gardiner *et al.*, 2000). Clearly, further work is required in order to determine the precise role in cytoadhesion of the clag9 protein to elucidate whether it is itself a cytoadherence ligand or whether it plays an indirect role in the adhesive process, perhaps by preventing surface expression of other adherence ligands such as PfEMP1. Further, the full range of receptors with which such gene products may interact on the surface of vascular endothelial cells remains to be determined. Recent data derived from the malaria genome sequencing project have revealed that the *clag9* gene is in fact part of a multi-gene family of homologous sequences found on a number of other chromosomes, although the nature and function of the gene products of these paralogues remain to be determined.

2.1.4. *Sequestrin*

Using anti-idiotype antibodies raised against the binding site of the anti-CD36 monoclonal antibody OKM8, Ockenhouse *et al.* (1991b) identified a novel trypsin-sensitive protein of *c.* 270 kDa in parasitized cells, which they termed sequestrin. Furthermore, the antibodies reacted with the surface of knobby parasitized cells and inhibited their ability to adhere to CD36 but did not bind to the surface of non-parasitized cells or to red blood cells infected with a knobless, non-cytoadherent parasite clone. No further characterization of this protein or the gene encoding it has ever been published and it is now widely argued that sequestrin is in fact PfEMP1. Interestingly, one recent paper describing the targeted knockout of the *clag9* gene (Trenholme *et al.*, 2000) referred to unpublished observations that these authors had knocked out the gene encoding sequestrin with no consequent reduction in the ability of the parasitized cells to adhere to CD36. Details of this protein and its function remain sketchy and we await further information.

2.2. Proteins Found in the Red Blood Cell Cytoplasm or on the Membrane Skeleton of Infected Red Blood Cells

2.2.1. *KAHRP (Knob-associated Histidine-rich Protein)*

Considerable information has been accumulated about this protein and it is now recognized to be of central importance in the changes occurring to the infected red blood cell, particularly with respect to the formation of the knob structure and its essential role in cytoadhesion. Although knobs had been described as early as 1966 (Trager *et al.*, 1966), little was known about the

biochemical composition of these structures until the work of Kilejian (Kilejian, 1979; Kilejian and Olson, 1979), who compared the stage-specific proteins of 'knobby' and 'knobless' lines of the parasite isolate FCR3. Parasites were biosynthetically labelled and the proteins separated by SDS–polyacrylamide chromatography. A labelled protein of *c.* 80 kDa was found in the knobby line but not in the knobless line. Fractions of infected cells enriched for red blood cell membranes were coincidentally enriched for the presence of this protein. In a later study, Kilejian (1979) demonstrated that this protein was strongly labelled when tritiated histidine was incorporated into the culture medium. Further, an antiserum to a histidine-rich protein found in *P. lophurae* appeared by immunoelectron microscopy to label knobs. We consider the protein identified in knobby parasites by these studies to be that now referred to as KAHRP.

This work was confirmed and extended by Hadley, Leech and others in a series of papers in the early to mid 1980s (Hadley *et al.*, 1983; Gritzmacher and Reese, 1984; Leech *et al.*, 1984a; Vernot-Hernandez and Heidrich, 1984, 1985). There was general agreement that knobby parasites produced a protein that, depending on the investigator and the parasite line examined, varied in molecular mass from 80 to 108 kDa. Leech *et al.* (1984a) used a method of differential detergent extraction to show that this protein was found in the Triton X-100 insoluble fraction, and this fraction was found by thin section electron microscopy to contain knobs. Extraction with 1% SDS led to the disappearance of this protein and the consequent disappearance of the knobs. The novel protein was synthesized from about the mid-ring stage and accumulated in infected red blood cell membranes during trophozoite and schizont stages (Vernot-Hernandez and Heidrich, 1984). Although Vernot-Hernandez and Heidrich (1985) also identified a novel protein of 92 kDa that was specific to knobby parasites, they concluded that it may not be the knob-forming material. This was based on trypsin and chymotrypsin digestion of purified red blood cell membranes, which led to the disappearance of this protein, while the knob structures were still discernible by electron microscopy. More puzzling were the results from their study of intact parasitized red blood cells treated with trypsin 10 hours after invasion, before the appearance of KAHRP and of knobs. In the subsequent development of these red blood cells, knobs formed normally, but no KAHRP could be detected. Vernot-Hernandez and Heidrich (1985) postulated that enzymatic treatment might destroy the locus of insertion or anchor point of KAHRP. There has been no reported attempt to replicate these results and they remain tantalizingly enigmatic. The size of KAHRP varies in different parasite lines; it was reported to be 92 kDa in Malayan Camp and 108 kDa in the St Lucia strain (Leech *et al.*, 1984a).

The molecular cloning of the gene encoding KAHRP set the stage for a revolution in our understanding of this protein and its role in the altered properties of the infected red blood cell (Kilejian *et al.*, 1986; Ardeshir *et al.*,

1987; Pologe *et al.*, 1987; Triglia *et al.*, 1987). The gene encoding KAHRP comprises two exons of the general structure outlined earlier. The extreme N-terminal sequence of the KAHRP protein is composed of predominantly basic residues with a hydrophobic core of 11 residues found at residue 22. The protein is highly charged and contains about 8% histidine, a histidine content considerably lower than that of HRPII and HRPIII, which is closer to 70% (Stahl *et al.*, 1985b; Wellems and Howard, 1986). There are three regions of repeat sequence in the protein. The first repeat region, called the 'histidine-rich region', occurs 24 residues into the beginning of the second exon and is composed of strings of polyhistidine varying in length from 6 to 11 residues. A tetrapeptide motif HQAP is repeated three times. The next repeat region, the so-called 5′ repeats, are composed of five copies of a 13–16 residue sequence based on a canonical sequence of SKKHKDNEDAESVK. The repeats are highly charged and, overall, basic. The 3′ repeats contain seven inexact copies of a 10-mer based on the canonical sequence SKEATKEAST. Human antibodies affinity-purified on recombinant protein, or experimental sera raised to the recombinant, recognized a protein of 80–100 kDa in knobby but not knobless parasites and localized the expressed protein to knobs (Ardeshir *et al.*, 1987; Culvenor *et al.*, 1987; Pologe *et al.*, 1987; Triglia *et al.*, 1987). The protein was present at the electron-dense knobs and was confined to the inside of the red blood cell membrane. It confirmed that knobless parasites did not synthesize any of this protein, a finding now explicable as being secondary to a chromosome breakage and gene deletion event (Pologe and Ravetch, 1986).

Comparative sequencing studies suggest that the gene encoding this region is relatively highly conserved. Many of the early reports of sequence differences appear to be the result of sequencing errors caused by the presence of areas very rich in AT. The one area of clear polymorphism is found in the 3′ repeats that vary in number and sequence (Triglia *et al.*, 1987; Kant and Sharma, 1996; Hirawake *et al.*, 1997). The number of repeats varies from three to seven copies and there are variations in repeat length as well. These variations are widespread and have been reported in isolates from India, Ghana and Honduras. Although the differences are not large, the highly charged nature of these sequences accounts for the close to 20 kDa differences between some isolates. The other repeat regions are much more strongly conserved. The reason for this may relate to the observation that the repeat regions partake in the interaction that anchors PfEMP1 at the knob (Waller *et al.*, 1999). Of the three repeats, the 3′ repeat interaction is of the lowest affinity (Table 3) and thus perturbation in the repeat number of this region would be least likely to affect the overall interaction.

The function of the KAHRP protein has been studied extensively. It takes part in a number of intermolecular interactions with host cell proteins including spectrin, actin and ankyrin, and with the parasite protein PfEMP1

(Kilejian *et al.*, 1991; Waller *et al.*, 1999; Magowan *et al.*, 2000; Oh *et al.*, 2000). The consequences of these interactions are to anchor PfEMP1 securely to the membrane skeleton and provide a stable structure that allows flowing parasitized red blood cells to cytoadhere and to resist subsequent detachment by the shear forces experienced in the dynamic environment of the circulation *in vivo*. KAHRP also appears to be essential for knob formation. These latter conclusions are based on an elegant set of experiments using parasites that had lost the capacity to express KAHRP by virtue of specific targeted disruption of the *KAHRP* gene (Crabb *et al.*, 1997a). In these studies, parasites of the 3D7 line were transfected with a vector in which the *KAHRP* gene was interrupted by insertion of a gene encoding a drug resistance marker. Repeated rounds of drug treatment selected transfected parasites that had undergone integration of the marker gene. The resulting parasites were cloned by limiting dilution and a number of cloned lines examined. These lines were shown to contain a *KAHRP* gene that had been disrupted and thus did not express the KAHRP protein. Electron microscopic studies showed that these parasites lacked knob structures, strongly suggesting that KAHRP expression is necessary for knob formation (Crabb *et al.*, 1997a). The technical limitations of the malaria transfection system prevented complementation studies being performed, so it remains a formal possibility that some other secondary change led to loss of knobs. With the development of additional selectable markers, it should become possible to perform complementation and prove the requirement for KAHRP in knob formation. When the knobless transgenic parasites were examined for their capacity to cytoadhere under static conditions, they showed complete retention of the ability to bind to CD36. However, when the parasites were exposed to shear flow in a flow chamber, the capacity of the *KAHRP* knockout lines to adhere to CD36 or to platelets was markedly diminished. This was manifested as both a decrease in the number of parasitized cells able to adhere from flow, and a higher level of detachment of previously adhering parasites at any particular shear stress (Crabb *et al.*, 1997a). As the shear stresses examined were selected as those likely to be encountered in post-capillary venules, where parasitized cells preferentially sequester *in vivo* (Chen, S., 1969), it was concluded that KAHRP had a role in enabling parasitized cells to cytoadhere in the dynamic flow environment of the vasculature *in vivo*. The localization of PfEMP1 in wild-type and *KAHRP* knockouts was examined. Although PfEMP1 was able to reach the surface of mutant parasites, it appeared to be present in more localized aggregations, manifested as a punctate pattern compared with the more uniform surface location of the parent line (Crabb *et al.*, 1997a). Whether these differences were secondary to the absence of knobs or whether KAHRP is involved in the trafficking of PfEMP1 to the red blood cell surface remains to be determined. KAHRP is generally considered to be an internal protein, not exposed on the surface of the cell. However, there is at least one report of antibodies to KAHRP exerting

an effect on intact parasitized cells (Carlson *et al.*, 1990b). In this study, a mononclonal antibody to KAHRP was observed to disrupt the formation of rosettes. Whether this was due to reactivity to KAHRP or cross-reactivity to some other protein is unknown, but it is certainly possible that rifins, for example, may contain sequences cross-reactive to KAHRP, which would explain these observations.

The observation that the absence of KAHRP led to a change in localization of PfEMP1 and a loss in efficiency of cytoadherence suggested some form of direct interaction between the two proteins. Previous mapping studies had identified an association between KAHRP and spectrin and actin, via a 271 residues region in the central region of KAHRP (Kilejian *et al.*, 1991). Thus the net effect of the interaction between KAHRP and PfEMP1 would be to anchor PfEMP1 indirectly to the red blood cell membrane skeleton. However the fact that PfEMP1 could still be detected on the surface of knobless parasitized cells and that cytoadherence still occurred, although to a lesser extent, suggested additional linkages to either other parasite proteins or host proteins, or both. Oh *et al.* (2000) recently identified an interaction between PfEMP1 and the spectrin–actin junction, particularly F-actin. They also noted a tendency for KAHRP to self-associate in structures that resembled knobs. In the case of the interaction of PfEMP1 with KAHRP, however, three distinct binding domains were identified and two of these have been mapped to repetitive regions of KAHRP, the histidine-rich region and the 5′ repeat region (Waller *et al.*, 1999). These two regions contain 63 and 70 residues, respectively. Such relatively short sequences make it likely that the binding motifs have a linear nature. Determination of the dissociation constants for the histidine-rich and 5′ repeats to the cytoplasmic domain of PfEMP1 gave values indicative of moderate affinity interactions (0·1 μM and 3·3 μM, respectively) (see Table 3). The third binding domain in the carboxyl terminal region of KAHRP that includes the 3′ repeats is of lower affinity (K_d = 13 μM) and the necessary experiments to map it to the repeat region have not been reported. The identification of multiple regions in KAHRP which bind to the cytoplasmic region of PfEMP1, termed VARC, contrasts with other studies focused on interactions between parasite proteins and host proteins of the red blood cell membrane skeleton. Both MESA and RESA have single binding regions for their cognate partners (Foley *et al.*, 1994; Bennett, B.J. *et al.*, 1997), as does MSP1 which is reported to bind to spectrin (Herrera *et al.*, 1993).

The histidine-rich repeats and the 5′ repeats contribute individually to the interaction between KAHRP and VARC at affinities comparable to the single binding domains identified in spectrin/protein 4.1, spectrin/ankyrin and MESA/protein 4.1 (see Table 3). Considering the data obtained for all the binding domains, its seems reasonable that the three regions may act co-operatively to result in an interaction of very high affinity. One caveat is that, in the absence of known three-dimensional structure for KAHRP, it is not certain whether it

is possible for all three regions of KAHRP to interact with a single PfEMP1 molecule. Studies involving nearly full-length KAHRP do indeed show an increased affinity with PfEMP1, with dissociation constants of 10 nM reported (Oh *et al.*, 2000). However, it is also possible that one or several of the binding regions could react with separate VARC molecules providing a cross-linking effect that would serve to anchor a number of PfEMP1 molecules in a compact space. This would provide a high density of PfEMP1 ectodomains at the knob and improve binding affinity for endothelial cells. Perhaps it is the loss of clustering of PfEMP1 in the absence of knobs that explains the loss of adherence of knobless infected red blood cells under flow conditions while their binding ability appears to be maintained in the absence of flow-induced haemodynamic stress. Alternatively, the weakened adhesive properties may be due to PfEMP1 being 'pulled out' of the membrane of infected red blood cells, owing to inadequate anchoring, when subjected to the physiological shear stresses that occur in the circulation *in vivo*.

As discussed earlier, a prominent feature of many malarial proteins is the presence of extensive regions of tandemly repeated sequence (Anders *et al.*, 1987a). It has been difficult to assign functional roles to these repeat regions. They are often the target of antibody-induced immunity in individuals living in endemic areas, and it has been suggested that they act as a form of immunological 'smoke screen', diverting the immune system to low affinity non-protective antibody responses (Anders, 1986; Coppel *et al.*, 1994). Occasionally, additional roles have been suggested. For example, the repeats of the circumsporozoite protein (CSP) have been proposed to play some role in the interaction of the sporozoite with hepatocytes (Aley *et al.*, 1986). However, more recently the binding site has been mapped to a non-repetitive sequence elsewhere in the CSP (Cerami *et al.*, 1992). Similarly, the binding site of a second sporozoite protein, TRAP, for hepatocytes has also been mapped to a region of non-repetitive sequence (Muller *et al.*, 1993). The binding domains of RESA, MESA and MSP1 mentioned above are all found in a non-repetitive sequence (Herrera *et al.*, 1993; Foley *et al.*, 1994; Bennett, B.J. *et al.*, 1997). Although the 271 residues spectrin/actin-binding region of KAHRP does in fact contain the 5′ repeats (Kilejian *et al.*, 1991), the interacting domain has not been mapped to a defined peptide sequence within this region. In contrast, both high affinity KAHRP binding domains for VARC identified in this study are mapped to defined repeat regions. The interaction of KAHRP with VARC is likely to have an electrostatic component since, at the pH of the infected red blood cell (Yayon *et al.*, 1984), the overall charges on the histidine-rich and 5′ repeats are positive (+7 and +11, respectively), whereas the overall charge on VARC is negative (–28). The importance of electrostatic forces has now been confirmed by the recent studies of Voigt *et al.* (2000), who have also provided some preliminary mapping of the binding sites on PfEMP1 for KAHRP.

2.2.2. *PfEMP3* (Plasmodium falciparum *Erythrocyte Membrane Protein 3)*

P. falciparum erythrocyte membrane protein 3 (PfEMP3) was first described in a series of papers by Pasloske and co-workers (Handunnetti *et al.*, 1992a; Pasloske *et al.*, 1993, 1994). These workers had characterized a rat monoclonal antibody called 12C11 that reacted with polypeptides of 44, 95, 117, 145 and 310 kDa, and localized to material in the parasite as well as antigen aggregates in the host cell cytoplasm that extended to the plasma membrane of the infected red blood cell. Screening of an expression library with this antibody identified a clone that encoded part of the PfEMP3 coding region. The complete coding region for PfEMP3 was not known until the determination of the sequence of chromosome 2 (Gardner, M.J. *et al.*, 1998). *PfEMP3* is a two-exon gene that encodes a polypeptide of 2441 residues with a predicted iso-elelectric point of 9·18 (see Figure 2). There are extensive series of repeat regions within the protein, particularly in the carboxyl-terminal half of the protein where 82 copies of a 13-mer based on the canonical repeat NTGLKNTP(S/N)EGQQ are found. There are two other extensive repeat regions, composed of units of 19 and 22 residues. There is a buried hydrophobic region of about 20 residues starting 15 residues in from the N-terminus of the protein. Antisera raised to the recombinant protein as well as the original monoclonal antibody showed that PfEMP3 is found at the erythrocyte membrane skeleton, both within and outside knob structures (Pasloske *et al.*, 1994). Its precise linkages to host proteins at the membrane skeleton and whether it is linked to PfEMP1 or KAHRP are not known. As the *PfEMP3* gene is located immediately adjacent to the *KAHRP* gene, but closer to the telomere, spontaneous deletion events that remove KAHRP will by necessity also lead to the complete deletion of PfEMP3. Thus which of these two proteins was the major contributor to the formation of the knob structure could not be ascertained until the availability of specific knockouts (Crabb *et al.*, 1997a). Targeted deletion of *PfEMP3* produced parasites that still had numbers of knobs detectable by transmission electron microscopy, although precise knob numbers and details of morphology were not determined (Waterkeyn *et al.*, 2000). Parasites that did not express PfEMP3 appeared to cytoadhere at levels similar to those found in the parental line in both static and flow-based assays, suggesting that this protein had no direct involvement in transport or anchoring of PfEMP1 (Waterkeyn *et al.*, 2000). Further phenotypic analysis will be needed to determine the role of PfEMP3 in parasite biology.

A curious phenotype was observed in a set of mutants in which integration had occurred in the 3′ end of the gene. A truncated form of PfEMP3, lacking the C-terminal 370–470 residues, was still expressed although, curiously, expression levels were considerably higher than that found in the parental line. In these over-expression mutants, cytoadherence was markedly reduced

and was concomitant with there being a reduced amount of PfEMP1 on the surface of the infected red blood cell. Instead, PfEMP1 was found to be accumulating in membrane-lined vesicles under the red blood cell membrane, a compartment in which PfEMP3 could also be found (Waterkeyn *et al.*, 2000). The authors concluded that this might be a compartment that is transiently present in normal cells, but the over-expression of PfEMP3 had led to accumulation of PfEMP1 under the surface and disruption of surface transfer. It did not appear to have affected the transport of KAHRP, which apparently moves by some other mechanism or is unsusceptible to the PfEMP3-induced blockade. The truncation of PfEMP3 did not affect its transport to the erythrocyte membrane but did interfere with its distribution on the underside of the membrane. An unresolved question is what transport processes occur after these proteins have reached these vesicles. PfEMP1 is found at knobs, with most of the protein exposed extracellularly, whereas PfEMP3 is located fairly uniformly throughout the cell, attached to the underside of the red blood cell membrane skeleton. Presumably there are further steps that traffic these proteins differently once they leave their shared location.

2.2.3. *MESA (Mature-parasite-infected Erythrocyte Surface Antigen)*

The mature-parasite-infected erythrocyte surface antigen (MESA) is a 250–300 kDa phosphoprotein (Coppel *et al.*, 1988; Howard, R.F. *et al.*, 1988) produced early in the trophozoite stage and found in association with the erythrocyte membrane skeleton (Coppel *et al.*, 1986; Howard, R.J. *et al.*, 1987). MESA interacts with the internal aspect of the host erythrocyte membrane and is not exposed on the external surface, although in late schizonts it becomes accessible to external surface-labelling reagents such as lactoperoxidase (Coppel *et al.*, 1988; Howard, R.J. *et al.*, 1988). A series of immunoprecipitation studies indicated that MESA co-precipitated with an 80 kDa phosphoprotein of host origin (Coppel *et al.*, 1988). Peptide mapping experiments suggested that this was protein 4.1 (Lustigman *et al.*, 1990). Further evidence for this interaction was provided by the observation that MESA was found in different locations in red blood cells that differed in their expression of protein 4.1 (Magowan *et al.*, 1995). In normal red blood cells, MESA is found at the periphery of the cell in association with the membrane skeleton. In contrast, in elliptocytes collected from individuals who did not express protein 4.1 in erythrocytes, MESA was found to be uniformly present in the red blood cell cytoplasm with no preference for the periphery (Magowan *et al.*, 1995). This implies that transport is a two-step process in which the protein first traffics to the red blood cell cytoplasm, followed by a second binding step to the membrane skeleton via a specific protein–protein interaction. Both MESA and protein 4.1 are phosphoproteins and in fact protein 4.1 becomes more

heavily phosphorylated in infected red blood cells (Coppel *et al.*, 1988; Lustigman *et al.*, 1990; Chishti *et al.*, 1994). MESA is phosphorylated at serine residues and there are several predicted sites that are good substrates for various kinases (Coppel *et al.*, 1988; Howard, R.F. *et al.*, 1988; Coppel, 1992). Neither MESA nor protein 4.1 influence phosphorylation of their partner protein as these two proteins are phosphorylated even in mutant cells that lack the cognate binding partner (Magowan *et al.*, 1998). The kinase involved in phosphorylating MESA has an inhibitor profile characteristic of casein kinases (Magowan *et al.*, 1998), and this profile is similar to that of the kinase implicated in protein 4.1 phosphorylation in cells infected with malaria (Chishti *et al.*, 1994). Curiously, in infected cells lacking KAHRP, both MESA and protein 4.1 appear to be phosphorylated by a different kinase with a different inhibitor profile (Magowan *et al.*, 1998). A further curious observation was that MESA in red blood cells deficient in protein 4.1 was found in the Triton X-100 insoluble fraction, even though by confocal microscopy it appeared to be free in the red blood cell cytosol. This suggests that either there is an association between some parts of the infected red blood cell cytosol, perhaps via the components of the novel transport system, or different populations of MESA exist in the red blood cell that are more readily detected by the differing methods.

Determination of the primary sequence of MESA revealed that it is encoded by 2 exons (Coppel, 1992). The MESA protein is heavily charged and contains 7 distinct repeat regions, which compose over 60% of the protein. The repeat regions vary in number among different isolates and in addition there are a number of scattered mutations in non-repetitive sequence (Kun *et al.*, 1999). Overall, however, the sequence is quite strongly conserved among isolates (Kun *et al.*, 1999). The predicted secondary structure suggests that MESA is a fibrillar protein and it shows similarity to a number of cytoskeletal and neurofilament proteins, including myosin, a protein that itself binds to protein 4.1. The protein 4.1 binding domain of MESA was subsequently mapped to a 19 residues sequence (DHLYSIRNYIECLRNAPYI) near the N-terminus of the molecule, a site different from the myosin homology region. This short region appears to be capable of forming an amphipathic helix, although whether this is important for binding is not currently known. Binding of MESA to the red blood cell membrane skeleton could be inhibited by addition of exogenous protein and the 50% inhibitory concentration (IC_{50}) of this interaction was 0·63 μM, indicative of a moderate affinity interaction.

A number of studies have attempted to define the function of MESA making use of a mutant parasite line that had undergone spontaneous deletion of the end of chromosome 5 that encodes the *MESA* gene (Petersen *et al.*, 1989; Magowan *et al.*, 1995). These studies indicated that MESA was not required for cytoadherence, as measured in static assays, for formation of knobs, red blood cell invasion, or for lysis. Growth rates did not differ markedly between

MESA+ and MESA− parasite lines, although this is a difficult property to measure accurately. A curious phenotype was detected, in which MESA− parasites were able to grow in red blood cells that were deficient in protein 4.1. MESA+ parasites could not grow in such cells, and it was noted that MESA had accumulated in the infected red blood cell cytoplasm in an abnormal location. Magowan *et al.* (1995) suggested that perhaps this accumulation of MESA in an abnormal location interfered with some important cellular process such as transport of either nutrients inwards or important parasite proteins outwards in these cells. Alternatively, it may be that some other protein, also deleted by the same chromosome breakage event, gives rise to this unusual phenotype. Such questions could be answered by the generation of a gene-targeted mutant for MESA.

2.2.4. *RESA (Ring-infected Erythrocyte Surface Antigen)*

RESA was one of the first well-characterized proteins found in the ring-stage parasite, where it was noted to be associated with the periphery of the infected red blood cell (Coppel *et al.*, 1984b), and exposed to the exterior after mild glutaraldehyde treatment (Perlmann *et al.*, 1984). Biosynthetic studies suggested that this protein was synthesized in mature stages of the parasite and deposited in vesicles from which it was transferred to the red blood cell at the time of invasion (Brown, G.V. *et al.*, 1985). RESA remains detectable in the red blood cell until about 18–24 hours after invasion, when it gradually disappears about the same time as MESA appears (Coppel *et al.*, 1988). At first it was suggested that RESA accumulated in micronemes (Brown, G.V. *et al.*, 1985), but subsequently it was shown that in fact RESA was present in a population of dense granules that were released once the invading merozoite had entered the red blood cell (Aikawa *et al.*, 1990; Culvenor *et al.*, 1991). RESA was then trafficked to the red blood cell membrane skeleton by a process that is still not understood. All of this was somewhat puzzling as there were numerous reports detailing the capacity of antibodies to RESA to inhibit growth of the parasite (Wåhlin *et al.*, 1984; Berzins *et al.*, 1986; Collins *et al.*, 1986; Carlson *et al.*, 1990b). At what stage were these antibodies coming into contact with their target, as RESA was apparently not exposed during the invasion process? This question remains unresolved and RESA has continued to be assessed for its capacity to induce host protective immunity, most recently in human clinical trials in Papua New Guinea (Saul *et al.*, 1999; Genton *et al.*, 2000).

RESA was shown to be encoded by a two-exon gene on chromosome 1 (Favaloro *et al.*, 1986; Corcoran *et al.*, 1987). The protein is highly charged and contains two blocks of repetitive sequence called the 5′ and 3′ repeats. The 5′ repeats are composed of degenerate 11-mers with a consensus

DDEHVEEPTVA, whereas the 3′ repeats are composed of 8-mer and 4-mer sequences EENVEHDA and EENV. There was no typical signal sequence identified, but rather a stretch of hydrophobic residues at positions 52–65 that corresponded to the end of the first exon. Sequence conservation between *RESA* genes in different isolates was remarkably high, differing at only 3 bases over a 1500 bp stretch (Cowman *et al.*, 1984; Favaloro *et al.*, 1986). Scattered single base changes occur at the 3′ end of the gene, and these mutations appear to exist in two alternative forms such that circulating strains can be divided into one or other group (Kun *et al.*, 1994). This is similar to the dimorphic families that have been reported for merozoite surface antigens such as MSP1 and MSP2 (Anders *et al.*, 1993). It was noted that the RESA protein contained a 70 residues region with a degree of homology (39%) to a domain of the *Escherichia coli* protein DnaJ (Bork *et al.*, 1992). DnaJ is a protein that acts as a molecular chaperone and the homology was to a region of DnaJ called the DnaJ motif, a region conserved among all known homologues. The degree of homology was similar to that found between bacterial and mammalian DnaJ homologues. This has led to the proposal that RESA may itself have some sort of chaperone function, perhaps while it is bound to the red blood cell membrane skeleton.

The observation that RESA binds to the internal face of the red blood cell membrane skeleton and that it is found in the Triton X-100 extract of parasitized cells supported the idea that there was a protein–protein interaction with components of the host cell. This was confirmed by experiments in which RESA found in culture supernatant was demonstrated to bind to inside-out vesicles (IOVs) derived from normal red blood cells of several species including mouse, rabbit, sheep and human (Foley *et al.*, 1991). A second parasite protein of 73 kDa was also shown to bind to IOVs, but its identity was not known. This suggested that the RESA receptor was a well-conserved molecule and protease experiments suggested that it was protein in nature (Foley *et al.*, 1991). Binding experiments with several purified red blood cell components showed that the protein bound by RESA was in fact spectrin (Foley *et al.*, 1991; Ruangjirachuporn *et al.*, 1991). Subsequent studies localized the spectrin binding domain of RESA to 48 residues of RESA located between two blocks of repeats (Foley *et al.*, 1994). The binding domain is distinct from, but near to, the DnaJ motif. It is proposed that RESA is a modular protein with the binding domain anchoring RESA at the membrane skeleton and the DnaJ motif engaged in chaperone-like activities (see below). The region of spectrin that contains the RESA binding domain has not yet been identified. Labelling studies using [γ-^{32}P]ATP showed that RESA in ring stages was phosphorylated at serine, but not in mature stages (Foley *et al.*, 1990). This was consistent with the view that RESA is phosphorylated after spectrin binding. It is not known whether the kinase responsible is of host or parasite origin and no data are available on the inhibitor profile of the kinase

involved or whether it is similar to that for the serine–threonine kinase involved in phosphorylation of MESA or protein 4.1.

Experiments with parasites that did not express RESA due to a chromosomal break showed that RESA is not required for red blood cell invasion, normal cellular growth, red blood cell lysis or cytoadherence. Experiments with nearly full-length recombinant RESA showed that binding of RESA to spectrin led to a degree of protection against heat-induced denaturation of spectrin (Da Silva *et al.*, 1994). Further, red blood cells infected with parasites lacking RESA were more susceptible to heat-induced fragmentation (Da Silva *et al.*, 1994). However, neither RESA nor anti-RESA antibodies added to resealed red blood cells before invasion seemed to inhibit the efficiency of this process, suggesting the stabilization role of RESA was not related to changing the kinetics of invasion (Da Silva *et al.*, 1994). The significance of this protection against spectrin denaturation *in vivo* is uncertain as the temperature used (50°C) was very much greater than that encountered in the bloodstream. However, there may be a number of destabilizing events, including temperature changes and the influx of calcium, that are associated with invasion.

A number of genes related to RESA have been identified, some of which contain the DnaJ motif (Cappai *et al.*, 1992; Vazeux *et al.*, 1993; Hinterberg *et al.*, 1994a; Gardner, M.J. *et al.*, 1998). The function of these various RESA-related proteins is currently unknown.

2.3. Other Less Well-Characterized Proteins in the Infected Red Blood Cell

Ag332 is a very large protein, estimated to be about 2·5 MDa, that is synthesized in young trophozoites and subsequently transported to the parasitized red blood cell, where it is found in association with large, flattened, vesicle-like structures called Maurer's clefts (Hinterberg *et al.*, 1994b). This transport process can be blocked by brefeldin A, a fungal metabolite that redistributes Golgi proteins to the endoplasmic reticulum (Hinterberg *et al.*, 1994b). The complete sequence of Ag332 is not yet available but it is known to be highly charged and to contain extensive blocks of repeats including many copies of the peptide VTEEI (Ahlborg *et al.*, 1991; Mattei *et al.*, 1992; Mattei and Scherf, 1992). This peptide is the target of Mab33G2, a monoclonal antibody that reacts with the surface of infected red blood cells and inhibits both red blood cell invasion and cytoadherence (Udomsangpetch *et al.*, 1986, 1989a). However, as antibodies to other regions of Ag332 have different reactivities and the monoclonal antibody is cross-reactive, the precise significance of these observations is unclear (Udomsangpetch *et al.*, 1989b). Such cross-reactivities bedevil the analysis of a number of proteins including D260, a 260 kDa protein that is found in the Triton X-100 insoluble fraction of

proteins and varies in molecular mass between different isolates (Barnes *et al.*, 1995). Antibodies to D260 can recognize a number of other proteins including RESA and it shares sequence motifs with Ag332. Although it has many of the immunochemical properties of a protein found in the infected red blood cell, indirect fluorescent antibody studies suggest that it may be found at the periphery of the parasite, although this is by no means certain (Barnes *et al.*, 1995).

P. falciparum exported serine–threonine kinase (FEST) was first identified by screening an expression library with rabbit antiserum raised against the membrane fraction of infected cells (Kun *et al.*, 1991). The gene encoding FEST is a two-exon gene and appears not to encode a typical signal sequence at the 5′ end of the first exon. Sequence analysis indicated that the encoded protein contained all the motifs that characterize serine–threonine kinases (Kun *et al.*, 1997). These motifs were more widely separated than in other kinases due to the presence of long asparagine-rich sequences. This has been found to occur quite commonly in many *P. falciparum* sequences of housekeeping proteins, and appears to occur at regions where the presence of such extraneous loops does not interfere with protein function (Bowman *et al.*, 1999). Antibodies to fragments of this gene reacted with a doublet of 210 and 220 kDa in biosynthetically labelled proteins from infected cells. Immunofluorescence studies revealed that FEST was present throughout the life cycle but most abundant during late-trophozoite and schizont stages. It was found both within the red blood cell cytoplasm and in association with the membrane skeleton, particularly at knobs. Within the cytoplasm, it was found associated with Golgi-like stacks. Consistent with this was the observation that FEST was found in both the Triton X-100 soluble and insoluble fractions. Although it is tempting to suggest that FEST may be responsible for phosphorylation of RESA, MESA, and the 46 kDa cleft protein (see below), as yet there has been no direct demonstration of kinase activity by FEST.

The glycophorin binding protein (GBP) was first identified during a random screening of expressing clones and characterized as a mature-stage protein of 120 kDa that was immunogenic during natural infection (Coppel *et al.*, 1984a). Subsequently it was also identified using an antiserum raised against two putative merozoite surface proteins with affinity for glycophorin A. The resultant gene had a two-exon structure and encoded a protein lacking a typical N-terminal hydrophobic signal sequence and composed predominantly of 50 residues repeating units (Ravetch *et al.*, 1985; Kochan *et al.*, 1986). These repeats were implicated as the binding domain for glycophorin A (Kochan *et al.*, 1986). More recent studies have questioned these initial findings and it is now generally accepted that the glycophorin binding was artefactual (Van Schravendijk *et al.*, 1987) and that the protein is not located on the merozoite surface, but rather in the cytoplasm of the infected red blood cell. The function of GBP is not known but it is used extensively as a marker for the red blood cell compartment in transport studies.

The falciparum interspersed repeat antigen (FIRA) is another large protein of *c.* 300 kDa that is present in all asexual stages (Stahl *et al.*, 1985a; Bianco *et al.*, 1988). It is located in the red blood cell cytoplasm in an irregular punctate pattern, which becomes more intense as the parasite matures until it forms a lattice around the merozoites of the schizont (Bianco *et al.*, 1988). The gene encoding FIRA is a two-exon structure with a large second exon encoding blocks of 13 degenerate hexapeptide repeats loosely based on the consensus sequence PVTTQE (Stahl *et al.*, 1987). This protein is extremely antigenic during natural infection and is recognized by a very high proportion of individuals from their earliest infections (Stahl *et al.*, 1985a). Its function is not known but it has been suggested that it may be involved in a network of cross-reacting proteins that tend to favour induction of low affinity antibodies during infection, the so-called 'smokescreen' effect (Anders, 1986; Stahl *et al.*, 1986).

P. falciparum asparagine- and aspartate-rich protein 1 (PfAARP1) is still incompletely characterized but apparently it is a large protein of more than 700 kDa encoded by an approximately 20 kb gene found on chromosome 12 (Barale *et al.*, 1997b). Structural features of this protein include nine repeat blocks rich in asparagine and aspartate residues and a PEST domain* that is found in rapidly degraded proteins. Computer analysis predicted that PfAARP1 has multiple transmembrane domains and at least five external loops. Antisera to the PfAARP1 protein reacted with the periphery of the infected red blood cell. Antibodies affinity-purified on a repeat peptide NNDDD reacted with the surface of unfixed cells (Barale *et al.*, 1997b). Although such a result may suggest that PfAARP1 is exposed on the surface, the use of antibodies to repeat regions is fraught with technical difficulties and the possibility of artefact. There is a large family of proteins rich in asparagine and it would be extremely difficult to ensure that such anti-repeat antibodies are specific to any single protein. The authors noted that there are at least two further proteins in the PfAARP family, called PfAARP2 and PfAARP3. PfAARP2 is a protein of 150 kDa that is first synthesized about 12 hours after invasion and is transported to the red blood cell cytoplasm where it is found in a vesicular pattern reminiscent of Maurer's clefts (Barale *et al.*, 1997a). The PfAARP2 protein can be solubilized by Triton X-100, suggesting that it has no direct association with the red blood cell membrane skeleton. There are no available data on the location of PfAARP3.

The protein encoded by the *41-2* gene is reported to have a mass of 29 kDa and to be localized on the schizont membrane, the internal surface of the infected red blood cell and membranous structures in the red blood cell cytoplasm (Knapp *et al.*, 1989). The gene encoding this protein differs from

*A domain of $\geq$12 amino acids containing at least one proline, one glutamic acid or aspartic acid and one serine or threonine residue.

many we have been discussing in that it is a single exon and does not encode any repeat region (Knapp *et al.*, 1989). There is an internal hydrophobic stretch of 11 residues near the N-terminus of the protein but it is shorter than that found in proteins such as GBP and FIRA.

The *P. falciparum* histidine-rich protein II (PfHRPII) is one of two low molecular mass histidine-rich proteins found in asexual stages (Wellems and Howard, 1986). The gene encoding this protein is a two-exon gene with the larger second exon containing a number of repeats that encode hexapeptides of sequence AHHAAD. The smaller protein PfHRPIII is encoded by a gene of quite similar overall structure and it is likely that the two genes resulted from a gene duplication event (Wellems and Howard, 1986). PfHRPII is a protein of *c.* 72 kDa that varies in size among isolates, based on the presence of differing numbers of repeats. The gene is not essential for growth *in vitro* or invasion, as laboratory isolates lacking this protein have been reported (Stahl *et al.*, 1985b). Immunofluorescence and immunoelectron microscopy studies localized PfHRPII to several cell compartments including the parasite cytoplasm, as discrete packets in the host erythrocyte cytoplasm and at the infected red blood cell membrane (Howard, R.J. *et al.*, 1986). The authors reported recovering approximately 50% of biosynthetically labelled PfHRPII from the cell-free supernatant of synchronized cultures at 2–24 hours and interpreted this as suggesting that the protein was secreted across several membranes (Howard, R.J. *et al.*, 1986).

PfSar1p is the *P. falciparum* homologue of a GTP-binding protein involved in trafficking proteins between the endoplasmic reticulum and the Golgi apparatus. PfSar1p shows 71% similarity to Sar1p from *Saccharomyces cerevisiae* (see Albano *et al.*, 1999a). Antibodies to PfSar1p recognized a protein of 23 kDa in immunoblots that was localized to the periphery of the parasite in discrete compartments, and that appeared distinct from the parasite endoplasmic reticulum (Albano *et al.*, 1999a). Intriguingly, PfSar1p was also found in structures in the cytoplasm of the infected red blood cell. This export was inhibited by treatment with brefeldin A. Surprisingly, there was no additional 5′ coding region in the *Sar1p* gene that might encode sequences involved in trafficking to the red blood cell, and its presence there is difficult to explain. Confirmatory experiments on the location of PfSar1p are needed but, on the basis of the paper by Albano *et al.* (1999a), there is now evidence that the parasite places components of the classical vesicle-mediated trafficking machinery inside the infected red blood cell.

The 46 kDa cleft protein is a polypeptide, identified by a several groups using various monoclonal antibodies, that has been localized to Maurer's clefts in the infected red blood cell cytoplasm (Hui and Siddiqui, 1988; Etzion and Perkins, 1989; Das *et al.*, 1994). Curiously, one of the antibodies was an anti-idiotypic reagent raised against a monoclonal antibody that reacted with the M blood group antigen on human glycophorin. The cleft protein is

synthesized in red blood cells infected with ring forms and trophozoites and transported to Maurer's clefts by a brefeldin A-sensitive process (Das *et al.*, 1994). Low temperature also blocks transport, suggesting that the Golgi transport process in parasites shares many features with that found in mammalian cells. A proportion of the cleft protein becomes phosphorylated and increases slightly in size (Das *et al.*, 1994). The site of phosphorylation is suggested to be in the clefts, or at least external to the parasite. The detergent solubility of this protein can vary, suggesting that some is more tightly membrane-bound (Etzion and Perkins, 1989), and there are unresolved differences in the reports on solubility in saponin or SDS (Hui and Siddiqui, 1988). Recently, Blisnick and co-workers (2000) described a novel 1·2 kb gene in *P. falciparum* located on chromosome 7, 8 or 9, which encodes a 48 kDa protein named *Plasmodium falciparum* skeleton binding protein 1 (Pfsbp1) that appears to be associated with Maurer's clefts. The protein product, encoded by a two-exon gene, appears to bind to a 35 kDa red blood cell cytoskeletal protein, whose precise identity has not yet been defined. The relationship of Pfsbp1 to the previously described 46 kDa cleft protein is not yet known, but should be readily addressable by appropriate immunoprecipitation and co-localization studies.

Exported protein 1 (exp-1), also known as the circumsporozoite-protein-related antigen, is a protein of 23 kDa that shares sequence elements with the repeat region of the circumsporozoite protein of sporozoites (Coppel *et al.*, 1985; Hope *et al.*, 1985; Simmons *et al.*, 1987). The protein is an integral membrane protein with the membrane anchor situated in the interior of the molecule (Coppel *et al.*, 1985). The protein is localized to the periphery of the parasite and in vesicles within the red blood cell cytoplasm (Coppel *et al.*, 1985; Simmons *et al.*, 1987; Bianco *et al.*, 1988). Electron microscopic studies suggested that exp-1 is associated with the parasitophorous vacuole membrane and with membranous vesicles in the red blood cell (Simmons *et al.*, 1987; Kara *et al.*, 1988, 1990). Protease studies demonstrated that the protein is inserted in the vesicle membrane in such a way that the N-terminus is within the lumen of the vesicle and the carboxy terminus protrudes into the red blood cell cytoplasm, where it is susceptible to the exogenous protease (Günther *et al.*, 1991). The authors conclude that, based on the uniform susceptibility of exp-1 to proteases throughout its transport, it must be trafficked across the various membranes by an alternating succession of membrane fusion and membrane budding events. Whether such a process applies to all exported proteins is unknown. Also unknown is whether this is an identical set of vesicles to those that contain the 46 kDa cleft protein (Hui and Siddiqui, 1988).

Finally, the glutamic acid-rich protein (GARP) appears to be a protein in search of identity. The gene encoding this protein is absolutely typical of those encoding exported proteins, being composed of two exons with the second exon considerably larger and encoding a number of repeat regions based on tri- and penta-peptides, rich in charged amino acids (Triglia *et al.*, 1988).

Overall, the protein is predicted to be composed of 26% glutamic acid. There is no predicted hydrophobic signal sequence at the extreme N-terminus of the protein. The gene is transcribed in asexual stages and GARP is commonly recognized by sera collected from individuals living in endemic areas (Triglia *et al.*, 1988). However, neither affinity-purified antibodies nor antibodies raised to recombinant proteins react with the protein by immunoblotting, immunoprecipitation, or immunofluorescence (Triglia *et al.*, 1988), perhaps because of its unusual highly charged composition.

3. TRAFFICKING OF EXPORTED PROTEINS

The functioning of the eukaryotic cell relies on newly synthesized polypeptides being transported to the appropriate location, whether it is within the cell cytoplasm or within an organelle, at the cell membrane, or in the external milieu. A complex and highly regulated process, involving many different proteins and organelles, operates to ensure this correct targeting (Pfeffer and Rothman, 1987; Rothman and Orci, 1992; Rothman, 1994; Waters and Pfeffer, 1999). Proteins destined for any of these compartments are synthesized in the cytoplasm by a single class of ribosomes. Information encoded within the polypeptide sequence or structure itself provides instructions to the targeting machinery. Proteins that are secreted or are being transported to the cell membrane move sequentially from their site of synthesis in the rough endoplasmic reticulum (ER) through the Golgi complex by direct targeting (Allan *et al.*, 2000) for post-translational processing, and then to secretory vesicles. The secretory vesicles eventually fuse with the plasma membrane upon receipt of a signal for exocytosis and the protein contents of the vesicles either stop there by virtue of specific sequences, or pass outside the cell (Pfeffer and Rothman, 1987).

The process of protein sorting commences when mRNA molecules move into the cytoplasm of the cell and contact unbound ribosomes. Once translation commences, if the protein is bound for eventual secretion or insertion in the cell membrane, the ribosome tightly attaches to the ER in such a way that the newly synthesized polypeptide chain passes into the lumen of the ER. This targeting of secretory proteins to the ER is due to the presence of a signal sequence of approximately 15–30 residues characteristically found at the amino terminus of the protein. Typically, the signal sequence consists of one or more positively charged residues followed by a continuous stretch of about 10–12 hydrophobic residues. These sequences are usually cleaved from the polypeptide chain within the lumen of the ER by a signal peptidase. The elucidation of the role of the signal sequence in targeting proteins for movement to the cell membrane or for secretion has come from recombinant DNA

experiments using chimeric gene constructs. A chimeric gene made up of a signal sequence (derived from β-lactamase) attached to the N-terminus of a protein that is not normally transported (α-globin) was made, cloned into an expression vector and transfected into cultured blood cells. The resulting protein was transported to the ER lumen, where the signal sequence was cleaved, exactly as if it had been a bona fide secreted protein (Lingappa *et al.*, 1984).

Once a polypeptide has entered the ER, it will proceed to the cell surface, unless specific signals within the protein sequence dictate its retention in an earlier compartment in the transport process, or redirection to other membrane-lined organelles. This 'bulk flow' of proteins to the cell surface passes through the cis-Golgi, the Golgi stack and the trans-Golgi before entering secretory vesicles and passing to the cell surface. Polypeptides that have been fully translocated to the lumen of the ER will be secreted, whereas those proteins that have their entry into the ER interrupted by the presence of stop-transfer or anchor sequences will be sorted to the cell surface (Pfeffer and Rothman, 1987). While in the Golgi system, polypeptides may undergo post-translational modifications such as N-linked glycosylation. Phosphorylation of N-linked oligosaccharides at mannose residues acts as a targeting signal to the lysosomes, and phosphorylation occurs in response to the presence of a particular domain structure present in the protein (Pfeffer and Rothman, 1987; Rothman and Orci, 1992).

Several protein motifs have been identified that will interfere with this default pathway. Proteins that have an N-terminal sequence of KDEL or, more generally, KXXX will become resident in the ER. This seems to occur by specific retrieval of such proteins from the Golgi system and their transport back to the ER (Rothman and Orci, 1992). Again, the importance of this sequence was elucidated by chimeric gene experiments in yeast in which the ER retention signal was added to the carboxyl terminus of a normally secreted protein (preproalpha factor fusion protein). After transfection, the resultant hybrid protein was retained in the ER (Dean and Pelham, 1990), but bore glycosylation changes typically added in the Golgi system, suggesting it had passed through that compartment before being returned to the ER. Sequences required for retention in the Golgi apparatus have also been defined by the construction of hybrid genes. In one experiment, the first of the three membrane-spanning domains of the E1 protein glycoprotein of avian coronavirus, a protein normally found in the Golgi complex, was used to replace the membrane-spanning domain of two surface proteins. This resulted in their retention in the Golgi system. Mutagenesis studies suggested that the important feature of the retention sequence appeared to be the uncharged polar residues which line one face of a predicted alpha helix (Swift and Machamer, 1991). Finally, targeting in polar cells to either the apical or basal portions of the cell appears to be in response to sequences found in the membrane-spanning and cytoplasmic domains of proteins (Weisz *et al.*, 1992). This

process of protein sorting occurs in all eukaryotic cells, but the model presented above appears to be insufficient to explain the protein sorting process in the malaria parasite.

The malaria parasite appears to possess protein transport machinery that must cater for a much more complicated problem in targeting than found in the typical eukaryotic cells we have just been considering. Thus, in addition to the normal targeting locations of the parasite cytoplasm, the parasite membrane and the exterior of the cell, the parasite targets proteins to a number of cellular locations outside the parasite cell membrane. These include the red blood cell cytoplasm, the red blood cell membrane skeleton, the red blood cell surface, membrane-lined vesicles within the red blood cell cytoplasm, and possibly outside the red blood cell itself. Consideration of the infected red blood cell structure suggests that the compartment corresponding to the location of secreted proteins in a typical eukaryotic cell is the parasitophorous vacuole. No direct analogue of the compartments in the infected red blood cell exists. Thus, there appears to be no available model of protein export in eukaryotic cells that could explain the sorting of proteins to these red blood cell locations outside the parasite boundaries, nor is it clear what structures or proteins, such as chaperones or transporters, might be required to accomplish this task.

The red blood cell is, of course, devoid of the machinery for the synthesis and transport of proteins, so the parasite must somehow provide the organellar system that will allow the proteins to reach locations that are external to the parasite. Electron microscopic studies suggest the presence of clefts, loops and a complex network of tubulovesicular membranes in the red blood cell cytoplasm (Elmendorf and Haldar, 1993). These membranes appear to contain enzymes and structural proteins similar to those found in the mammalian Golgi system (Elmendorf and Haldar, 1994). In addition, there appear to be components of ABC transporters associated with these membranes (Bozdech *et al.*, 1998). Additional structures reported in this location include proteinaceous packets (Howard, R.J. *et al.*, 1986) and, recently and most controversially, a duct-like structure that allows direct connection between the parasitophorous vacuole and the external milieu (Pouvelle *et al.*, 1991). The evolving consensus is that the tubulovesicular membranes may be involved in uptake of substances to the parasite, particularly lipid, but the structures involved in the outward transport of the proteins remain largely mysterious. Importantly, however, evidence exists that both the group of exported proteins and rhoptry proteins start the export pathway in a conventional manner (Haldar, 1998). For example, the fungal metabolite brefeldin A, which inhibits protein secretion in higher eukaryotes by disrupting the integrity of the Golgi apparatus, will disrupt the transport of proteins exported to the red blood cell such as GBP and the 45 kDa cleft protein, as well as transport of rhoptry proteins to the rhoptries (Howard, R.F. and Schmidt, 1995). Thus there is at least

one important similarity between the transport process used in infected red blood cells and higher eukaryotes.

In the previous section we discussed some of the extensive information about the primary amino acid sequence and cellular location of a large number of malaria proteins (Coppel *et al.*, 1994). In particular, many of the exported proteins lack typical signal sequences at their amino termini. The amino-terminal sequences of these proteins are studded with charged residues and lack a preponderance of hydrophobic residues such as phenylalanine, tyrosine, valine, leucine, or isoleucine, and instead contain many polar or hydrophilic residues such as serine or asparagine. The buried hydrophobic regions noted in proteins such as GBP, KAHRP and MESA are located approximately 20–60 residues from the N-terminus, and are hypothesized to signal export beyond the parasitophorous vacuole. For proteins that bind to the red blood cell membrane skeleton, additional cytoskeletal binding sequences, such as those described in RESA (Foley *et al.*, 1994) and MESA (Bennett, B.J. *et al.*, 1997), would guide proteins already in the red blood cell cytoplasm to these sites. Similarly, PfEMP-1 found at the red blood cell surface would have a membrane binding signal in addition to the red blood cell export signal (Baruch *et al.*, 1995). The way to test such hypotheses would be to take advantage of the new transfection technology (Waller, R.F. *et al.*, 2000) and introduce into parasites various chimeric constructs in which regions of transported proteins are added to proteins that are normally present in the cytoplasm. The location of these chimeric proteins could then be determined. It is expected that results of such experiments should be forthcoming in the not too distant future.

Beyond the question of what signals for transport may be encoded within the sequence of these proteins are the questions of what transport machinery the parasite uses to traffic proteins and what machinery it may place in the red blood cell? Also, how are the proteins able to cross so many membranes and spaces to reach their final locations? We do not yet possess a detailed understanding of the transport system but there is general agreement about at least some issues. The first of these is that the parasite possesses a conventional protein transport machinery including an ER. There are several lines of evidence for this including the identification of proteins that are components of the ER such as BiP (Peterson *et al.*, 1988) and PfERC (La Greca *et al.*, 1997) and many other proteins that are involved in vesicular transport pathways (reviewed by Albano *et al.*, 1999b). Furthermore, parasites are sensitive to the action of brefeldin A, a drug that disrupts the Golgi apparatus. When this drug is applied to cultures of parasites, several proteins are blocked from transport to their final destinations, including the rhoptry protein RAP-1 (Howard, R.F. *et al.*, 1998), the merozoite surface protein MSP1 (Wiser *et al.*, 1997), and the exported proteins GBP (Benting *et al.*, 1994) and Ag332 (Hinterberg *et al.*, 1994b). Wiser and colleagues (1997), working with *P. chabaudi*, confirmed

that many exported proteins are blocked from transport to the red blood cell when parasites are exposed to brefeldin A. However, they appear to accumulate in a different location from that of proteins such as MSP1. These authors suggested that there are two distinct transport compartments, one for proteins exported to the red blood cell and the other for proteins secreted to the parasitophorous vacuole, the rhoptries and the merozoite surface. These studies were performed using a confocal microscope and it is still questionable whether this instrument has sufficient resolving power to determine if these two locations are truly distinct compartments. To complicate matters further, at least one exported protein, KAHRP, is transported by a brefeldin A-insensitive pathway (Mattei *et al.*, 1999).

There are a number of different types of vesicles present in the red blood cell cytoplasm. Dye labelling studies by Pouvelle and co-workers (1994) suggested that many of these vesicles contain membrane derived from the parasitophorous vacuole. Electron microscopic studies by Trelka *et al.* (2000) revealed the presence of electron-dense vesicles, similar in appearance to mammalian secretory vesicles, in proximity to smooth tubulovesicular elements at the periphery of the parasite cytoplasm. These vesicles appeared to be coated and were found in the red blood cell, some being close to the parasitophorous vacuole membrane. The vesicles appeared to bind to, and fuse with, the red blood cell membrane, giving rise to cup-shaped electron-dense structures. An identical appearance had already been noted in studies on MESA transport (Coppel *et al.*, 1986). Treatment of mature parasites with aluminium tetrafluoride resulted in the accumulation of the vesicles with an electron-dense limiting membrane in the erythrocyte cytosol into multiple vesicle strings (Trelka *et al.*, 2000). As this reagent prevents coat shedding of vesicles, it suggests a process that is G-protein regulated. These vesicles appeared to be involved in the transport of parasite proteins PfEMP1 and PfEMP3 as they co-localized with the vesicles.

In addition to the problem of the transport of proteins to the red blood cell, there is also the issue of transport, predominantly of nutrients, in the other direction. New permeation pathways are elaborated by the parasite within the red blood cell and these have wide-ranging effects on permeability. A detailed discussion of this is beyond the scope of this review, but one may be found in the recent Novartis Foundation symposium on transport and trafficking in the malaria-infected erythrocyte (Bock and Cardew, 1999). As has been mentioned above, the tubulovesicular network has been implicated in transport of material to the interior of the parasite cell. One aspect of internal trafficking that has been completely ignored is the removal of parasite proteins from the red blood cell membrane skeleton. As mentioned, RESA is a protein found in dense granules in the merozoite that makes its way after invasion to the red blood cell membrane skeleton where it binds to spectrin. After about 18–22 hours, RESA disappears from the red blood cell membrane at about the time

that MESA appears (Coppel *et al.*, 1988). How does this happen? Is it detached and trafficked back to the parasite or is it selectively digested by proteases of host or parasite origin? We simply do not know. Certainly there appear to be one or more proteases active in the infected red blood cell cytoplasm, at least as judged by the apparent loss of spectrin during parasite development (Schrével *et al.*, 1990). Several studies have reported decrease in spectrin levels and other membrane skeleton components such as glycophorin in red blood cells infected by *P. berghei*, *P. chabaudi* and *P. lophurae* (see Weidekamm *et al.*, 1973; Konigk and Mirtsch, 1977; Sherman and Jones, 1979). However, this proteolysis of RESA would need to be selective, as there is no report of other exported proteins of parasite origin being affected. It would be interesting to know whether any other parasite proteins are also removed in this fashion, as it might be part of a previously unrecognized retrograde trafficking mechanism. However, it should be pointed out that few studies have been performed in a sufficiently quantitative manner for any strong conclusion to be drawn. Studies have noted the presence of uninfected red blood cells containing RESA in the circulation and this has been used to infer the existence of splenic removal of parasites from infected red blood cells (Angus *et al.*, 1997; Chotivanich *et al.*, 2000a). This suggests that the removal of RESA from the red blood cell membrane requires the presence of the parasite and could support the concept of a continuing trafficking process.

4. ALTERATIONS TO NATIVE RED BLOOD CELL PROTEINS DURING MALARIA INFECTION

Infection leads to several changes in antigenicity and arrangement of host red blood cell membrane proteins. The overall architecture of the red blood cell membrane skeleton does not appear to change, at least as revealed by whole cell mount electron microscopy, although electron-dense aggregates do appear (Taylor *et al.*, 1987a,b). Intramembranous particles (primarily due to glycophorins and tetramers of band 3) are specifically redistributed in the region of the knob (Allred *et al.*, 1986). This clustering of band 3 at knobs is also suggested by specific knob labelling using concanavalin A, a lectin that recognizes glycosylated band 3 (Sherman and Greenan, 1986). Band 3 undergoes several modifications to give rise to forms of >240 kDa and 65 kDa, which are more reactive with anti-band 3 autoantibodies (Winograd *et al.*, 1987). It has been reported that monoclonal antibodies that recognize band 3 only in parasitized cells (termed Pfalhesin) have also been prepared (Winograd and Sherman, 1989) and these are capable of blocking cytoadherence of infected red blood cells to C32 amelanotic melanoma cells or CD36, as are synthetic peptides based on the sequence of the altered band 3 (Crandall *et al.*, 1993).

These results appear to be contradicted by recent papers from the same group, which now highlight thrombospondin as the host receptor for modified band 3 (Lucas and Sherman, 1998; Eda *et al.*, 1999). It is not known precisely what processes lead to modification of band 3 but they are probably related to structural changes in the red blood cell secondary to malaria infection, since antibodies that react with altered band 3 on the surface of parasitized cells also react with the surface of sickle red blood cells and reduce their adhesiveness to cultured endothelial cells (Thevenin *et al.*, 1997). Levels of phosphorylation of red blood cell membrane skeletal proteins are also affected by malarial infection (Yuthavong and Limpaiboon, 1987; Murray and Perkins, 1989; Lustigman *et al.*, 1990; Chishti *et al.*, 1994). In red blood cells infected with *P. berghei*, there is a marked increase in phosphorylation of a 43 kDa red blood cell membrane skeletal protein, perhaps actin, and this increase correlates directly with filterability and inversely with the osmotic fragility of the infected cells (Yuthavong and Limpaiboon, 1987). In red blood cells infected with *P. falciparum* there is a marked increase in phosphorylation of protein 4.1, sometimes as much as tenfold, depending on the infecting parasite strain (Lustigman *et al.*, 1990; Chishti *et al.*, 1994). Since phosphorylation of protein 4.1 inhibits spectrin–actin interactions mediated by the protein (Ling *et al.*, 1988), one may expect that the parasite-induced phosphorylation would reduce membrane mechanical stability. This is accompanied by a lesser, but still measurable, increase in phosphorylation of band 3 (Chishti *et al.*, 1994).

5. ALTERATIONS IN CELLULAR PROPERTIES OF INFECTED RED BLOOD CELLS

Light and electron microscopic studies of infected red blood cells have identified a number of morphological changes within the red blood cell cytoplasm associated with infection. A number of names, such as Maurer's clefts and Schuffner's dots, have been given to these structures. Some of them can be seen by electron microscopy to be novel membranous structures that resemble Golgi stacks (Haldar, 1994). Their role is still controversial, but the balance of evidence suggests that they are most probably part of new pathways for nutrient transport by the parasite. The infected red blood cell becomes spherocytic with its surface punctuated by 5000–10 000 localized, electron-dense elevations of the red blood cell membrane called knobs (Aikawa, 1977; Aikawa and Miller, 1983; Gruenberg and Sherman, 1983). The knobs are located over the junctional complexes of the red blood cell (Chishti *et al.*, 1992; Oh *et al.*, 1997) and vary in size (70–150 nm) and density (10–70/μm^2), becoming smaller and more numerous as the parasite matures (Gruenberg *et al.*, 1983). Knobs appear to be required for parasitized red blood cells to cytoadhere

in vivo (Howard, R.J., 1988; Crabb *et al.*, 1997a) and are invariably found on infected red blood cells isolated directly from patients (Van Schravendijk *et al.*, 1991; Nakamura *et al.*, 1992). Further ultrastructural studies suggest that adhesion actually occurs between the parasite ligand PfEMP1 localized at knobs and the surface of the other cell (Van Schravendijk *et al.*, 1991; Nakamura *et al.*, 1992). Two recent papers have examined the knob structure using atomic force microscopy (Aikawa *et al.*, 1996; Nagao *et al.*, 2000). Interestingly, these studies revealed that knobs are in fact positively charged, with a membrane potential of +20 mV, when compared with the remainder of the red blood cell membrane which is negatively charged, and they are raised above the red blood cell membrane by 18–25 nm (Aikawa *et al.*, 1996). Although one study suggested that knobs were composed of two distinct sub-units (Aikawa *et al.*, 1996), this was not borne out in the second study (Nagao *et al.*, 2000), which demonstrated that the two sub-units structure was in fact an artefact of the technology used. The number of knobs is linearly related to the number of parasites infecting a particular cell (Nagao *et al.*, 2000) and, using this method of measurement, knob volume does not decrease with maturation of the parasite. It has been noted that there are a number of small electron-dense patches (30–65 nm) that are distinct from knobs in whole cell mounts of infected cells; however, the nature of these patches and their constituent molecules are not known (Taylor *et al.*, 1987b). Other species of *Plasmodium* have been reported to express knobs and these have been shown to be the site of cytoadherence (Kawai *et al.*, 1995).

6. RHEOLOGICAL CHANGES IN INFECTED RED BLOOD CELLS

Red blood cells are incredibly robust with uniquely adapted mechanical properties that enable them to circulate repeatedly up to half a million times during their 120 days' lifetime under the harsh extrinsic shear forces of the circulation *in vivo*. This is possible because red blood cells are highly deformable structures, which can undergo rapid and reversible shape changes repeatedly when exposed to haemodynamic shear. Normally biconcave discs in their 'resting' state, red blood cells deform to ellipses and align linearly in large vessels during arterial flow (Fischer, T.M. *et al.*, 1978). Furthermore, red blood cells can bend and fold to produce 'slipper' and other forms (Gaehtgens *et al.*, 1980) to enable these cells, which have diameters of approximately 8 μm, to traverse capillaries in the microcirculation with luminal diameters down to 3 μm or through the intraendothelial slits and basement membrane fenestrations in the spleen. The principle of cell deformability, and the wide array of methods available for its measurement, have been well reviewed in the past (Bull *et al.*, 1984; Stuart *et al.*, 1984; Stuart, 1985; Evans, 1989).

Fundamentally, there are three major determinants of red blood cell deformability: (i) the low viscosity of the cytoplasm (essentially just a solution of haemoglobin), (ii) the high surface area to cell volume ratio, and (iii) the highly visco-elastic membrane. Additionally, red blood cells are able to freely rotate their membrane around their cytoplasm in a 'tank-treading' fashion, which further facilitates transluminal passage by reducing hydraulic resistance (Bagge *et al.*, 1980; Gaehtgens *et al.*, 1980; Secomb and Skalak, 1982).

Invasion of red blood cells by malaria parasites has profound effects on all of these factors and as a consequence the rheological properties of parasitized cells are dramatically altered (Cranston *et al.*, 1984; Nash *et al.*, 1989; Paulitschke and Nash, 1993; Dondorp *et al.*, 2000). Compared with normal red blood cells, parasitized cells are more rigid, less deformable and, to a greater or lesser extent, more spherocytic. As the parasite matures, the cells' ability to circulate becomes increasingly impaired, and eventually they become completely immobilized in the microvasculature. The decreased deformability of parasitized cells is likely to impede the passage of these cells through the intraendothelial fenestrations in the spleen. It has been suggested that cytoadherence may have evolved in order to minimize exposure of rigid parasitized cells in the spleen and thus minimize splenic sequestration and their consequent destruction.

It is now almost 30 years since Miller and colleagues (1971, 1972) first demonstrated reduced deformability in monkey red blood cells parasitized by *P. knowlesi* and *P. coatneyi*, using relatively simple filtration techniques in which the rate of filtration of suspensions of parasitized cells through small diameter pores in filters was quantified. Flow rates were lowest for samples containing large numbers of mature parasites. Similar decreased filterability was reported for *P. falciparum*, using clinical isolates (Lee *et al.*, 1982). This decrease in filterability, however, was largely influenced by the presence of the rigid, spherical parasite itself, which may occupy as much as 90% of the total volume of the red blood cell. Furthermore, these filtration techniques were relatively insensitive and detected measurable differences only when the parasitaemia was high. This is frequently not the case for clinical isolates and methods for enrichment of parasitized cells would therefore be required which, themselves, may influence the results. Filtration times are also dominated by the presence of leucocytes, which must be removed from clinical samples before any meaningful result can be obtained (Chien *et al.*, 1983; Chan *et al.*, 1984). Cranston and colleagues (1984) were able to observe directly the flow behaviour of culture-derived parasitized cells in a rheoscope. They measured the extent of red blood cell elongation (length to width ratio) induced by graded levels of shear stress, the prevalence of tank-treading, and the time course for recovery of cell shape in synchronous cultures of parasitized cells and non-parasitized controls. A knobby line of *P. falciparum*, Indochina 1, and a knobless clonal line, D4, were examined. Red blood cells

containing young ring-stage parasites elongated less than controls and had a tendency to tumble, rather than align in the direction of flow. Moreover, the time taken to recover cell shape following cessation of applied shear stress was slower. The magnitude of these changes increased as the parasite matured, so that red blood cells infected with more mature, pigmented stages were even less deformable than ring forms and did not linearly align or tank-tread in flow. Knobless red blood cells were also relatively non-deformable, but quantitative data were not reported. This provided the first direct evidence that the extent of cellular modification was directly linked to the degree of parasite maturation. Although, in contrast to filtration, the rheoscope enabled individual cells to be observed directly, alterations in the mechanical properties of the cell membrane could not be precisely dissected from the influence of the parasite. Nash *et al.* (1989), however, addressed this problem by examining the membrane mechanical properties of individual cells using glass micropipettes. They aspirated individual red blood cells infected by both the uncharacterized cultured line WL and clinical isolates into micropipettes (3 μm diameter) and measured the time and pressure required for complete aspiration of the cell. They also demonstrated a loss of deformability that was greater for red blood cells infected with mature stages of parasites. Further, by measuring the increase in the length of a 'tongue' of red blood cell membrane aspirated into micropipettes with a much smaller diameter (*c.* 1·5 μm) at defined increasing pressures, they were able to calculate the shear elastic modulus for the cell membrane. Then, by simple geometry, they were able to estimate the surface area and volume of infected red blood cells. This was the first time that parasite-induced changes to the mechanical properties of the red blood cell membrane itself, which were not influenced by the presence of the parasite, had been quantified. There was some loss of deformability at the ring stage of infection, which was attributed to a reduction in the surface area to volume ratio and a slight rigidification of the cell membrane. Membrane rigidity was even higher for red blood cells containing mature forms, although no distinction was made between trophozoites and schizonts. The importance of particular parasite proteins in changing red blood cell properties has been suggested by Paulitschke and Nash (1993), who examined a series of knobby and knobless parasites. Although there was considerable inter-strain variation, a trend for increased membrane rigidity in knobby red blood cells was observed. A caveat to this study, however, was that the parasite lines tested came from a wide variety of different genetic backgrounds and from diverse geographical locations. This may explain the relatively high level of inter-strain variation and, moreover, may have masked detection of any subtle change that may have existed between different parasite lines.

Many cellular changes observed in the infected red blood cell cannot yet be related to specific molecular interactions. For example, the parasite exerts considerable oxidative stress on the red blood cell, which can contribute to

loss of membrane deformability, presumably by affecting a number of different proteins (Hunt and Stocker, 1990). Nor do we know what events result in the predictable lysis of the infected red blood cell about 48 hours after invasion, in the case of *P. falciparum*. It is reasonable to suppose that the reported decrease in the amount of red blood cell spectrin may be involved (Schrével *et al.*, 1990), as could alteration in the polarity and components of the red blood cell lipid bilayer.

Interestingly, some studies have shown that the deformability of non-parasitized red blood cells is also reduced during malaria infection (Lee *et al.*, 1982; Areekul and Yamarat, 1988) and appears to be related to an increase in the rigidity of the red blood cell membrane itself (Dondorp *et al.*, 2000). Furthermore, the degree of red blood cell rigidification appears to be greater in individuals with more severe disease, when measured by ektacytometry (Dondorp *et al.*, 1997). Others, however, have failed to confirm such observations by examining the rigidity of individual non-parasitized red blood cells from malaria cultures by micropipette analysis (Paulitschke and Nash, 1993). The mechanism by which the deformability of non-parasitized cells might be affected by malaria parasites is not known, but one possibility is the binding to the surface of non-parasitized red blood cells of exoantigens released by malaria parasites (Read *et al.*, 1990; Naumann *et al.*, 1991). When examined by immunofluorescence, a number of non-parasitized red blood cells from the peripheral blood of infected individuals also appeared to contain RESA in association with the red blood cell membrane skeleton (Angus *et al.*, 1997; Chotivanich *et al.*, 2000a). The presence of RESA in red blood cells that clearly did not contain parasites is difficult to explain, but could result from the selective removal of the parasite from some red blood cells in the spleen, followed by re-sealing and return of the red blood cell to the peripheral circulation. Although the deformability of such 'pitted' cells has not been quantified, it is reasonable to expect that rheological properties would be measurably altered. Clearly, more work is required to resolve this issue; however, the phenomenon could provide an explanation for the beneficial effects of exchange transfusion in individuals with severe malaria.

Changes in the rheological properties of uninfected red blood cells may also be implicated in the phenomenon of anaemia secondary to malaria infection. This is a common and severe complication of malaria, especially in young children, and is suggested to account for approximately 50% of malaria mortality in some endemic areas. The pathogenesis of this anaemia is not well understood and is almost certainly multifactorial (see Menendez *et al.*, 2000 and Wickramasinghe and Abdalla, 2000 for recent reviews). In addition to the obligatory lysis of parasitized red blood cells during schizont rupture, the greatest contributor to the reduction in haematocrit appears to be an accelerated destruction of uninfected red blood cells (Looareesuwan *et al.*, 1991; Salmon *et al.*, 1997). The mechanism by which uninfected red blood cells are

destroyed has not been fully elucidated; however, reduced cell deformability (Dondorp *et al.*, 2000), inversion of the membrane lipid bilayer (Joshi *et al.*, 1986) and increased red blood cell immunoglobulin binding resulting in premature phagocytosis or complement-mediated lysis (Waitumbi *et al.*, 2000) have all been suggested to play an important role. Clearly, further studies are needed to provide a convincing explanation for the pathogenesis of the anaemia during malaria.

7. ALTERED ADHESIVE PROPERTIES OF INFECTED RED BLOOD CELLS

Essentially, we can conveniently divide the altered adhesive properties of parasitized red blood cells into four distinct cytoadhesive phenotypes. Parasitized cells can adhere directly to the vascular endothelial cells (cytoadhesion), to uninfected red blood cells (rosetting), to other infected red blood cells (autoagglutination) and, most recently described, to dendritic cells (Urban *et al.*, 1999). Undoubtedly the most extensively studied of these is the interaction of infected red blood cells with the endothelial cells that line the vascular intima. As a consequence of this, red blood cells infected by mature parasites accumulate in the microvasculature and are notably absent from the peripheral circulation, a diagnostic feature of falciparum malaria. This phenomenon, known as sequestration, protects parasitized cells from entrapment and destruction in the spleen and maintains the microaerophilic parasites in a relatively hypoxic environment. While clearly beneficial for the parasite, sequestered red blood cells can perturb or completely obstruct blood flow in small diameter vessels of the microcirculation (Raventos-Suarez *et al.*, 1985), with serious vaso-occlusive consequences. Furthermore, high levels of inflammatory cytokines that are released locally at sites of sequestration can both increase the number of parasitized cells that accumulate or increase disease severity by a more generalized systemic effect of increased levels of circulating cytokines (Udomsangpetch *et al.*, 1997). The dogma that it is only red blood cells infected with mature stages of *P. falciparum* that are capable of cytoadherence has recently been challenged (Pouvelle *et al.*, 2000). These investigators examined parasite lines that had been selected *in vitro* for their ability to bind to CSA, or clinical isolates collected from a number of sources including the placentas of pregnant women. They noted significant numbers of red blood cells containing immature ring-stage parasites, which were capable of binding to cultured vascular endothelial cells derived from monkey brain. The adherent parasitized red blood cells had not matured sufficiently to express PfEMP1, but were shown to have two as yet uncharacterized polypeptides

of approximately 200 kDa and 40 kDa, designated ring surface protein-1 (RSP-1) and RSP-2 respectively, on the red blood cell surface. The receptor that these putative ligands recognized on the endothelial cell surface is also not certain but the interaction may involve heparan-like proteoglycans. Interestingly, as the parasites mature to trophozoites and PfEMP1 begins to appear on the red blood cell surface, the parasitized red blood cells switch to an exclusively CSA-binding phenotype and RSP-1 and RSP-2 disappear. Novel findings of this type undoubtedly require replication, and further studies will be needed to assess the significance of ring-stage adhesion in the pathogenesis of malaria. Again, however, this emphasizes the complexity of this ancient host–parasite relationship. The existence of rosettes and autoagglutinates in the circulation *in vivo* remains uncertain, although several studies have shown a correlation between both of these phenomena, when quantified in patients' blood *in vitro*, and the severity of clinical disease (Carlson *et al.*, 1990a; Treutiger *et al.*, 1992; Rowe *et al.*, 1995; Roberts *et al.*, 2000). Neither is their contribution to vascular obstruction well understood; however, both rosettes and autoagglutinates have been observed to form in flow-based adhesion assays that mimic the circulation *in vivo* using parasites taken directly from individuals with malaria (Cooke *et al.*, 1993). The force of interaction between uninfected and parasitized red blood cells in rosettes is at least five times higher than that between parasitized red blood cells and endothelial cells (see Table 4) when measured by single cell micromanipulation (Nash *et al.*, 1992a). Furthermore, rosettes from both laboratory-adapted parasite lines and clinical isolates can withstand disruption by physiologically relevant shear stresses applied using a rotational viscometer *in vitro* (Chotivanich *et al.*, 2000b).

7.1. Cytoadhesion

Because of the association of cytoadherence and sequestration with severe clinical syndromes such as cerebral malaria, where infected red blood cells preferentially sequester in the brain (MacPherson *et al.*, 1985; Pongponratn *et al.*, 1991; Silamut *et al.*, 1999), numerous studies have examined this phenomenon. Adhesion appears to be a critical process for maintenance of parasite virulence, as isolates that have lost the capacity to bind cause mild or inapparent infections in laboratory animals (Langreth and Peterson, 1985). Cytoadherence has been studied in a number of systems *in vitro* and infected red blood cells have been shown to be capable of adhering to at least 11 different receptors that are expressed on the surface of vascular endothelial cells or in the placenta, which differ in structure from members of the immunoglobulin super family and integrins to glycosamino- and proteoglycans (Figure 4 and Table 4). Although adhesion to some of these receptors

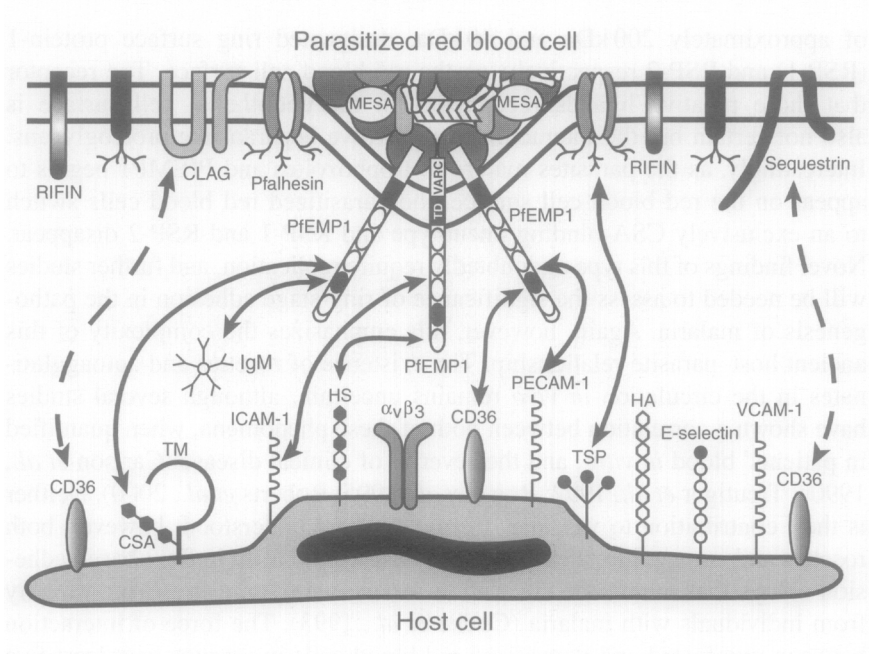

Figure 4 Schematic representation of the diverse array of molecules implicated in the adhesive interaction between red blood cells infected with *P. falciparum* and vascular endothelial cells or placental syncytiotrophoblasts (host cell). The figure shows a typical knob at the infected red blood cell membrane formed by the interaction of parasite-encoded proteins such as KAHRP, PfEMP3 and MESA with the red blood cell membrane skeleton. Solid arrows indicate interactions between specific receptors on the surface of the infected red blood cell and the host cell only where these have been unequivocally determined. Broken arrows indicate interactions for which there is currently less compelling evidence. References describing these interactions are provided in Table 3. Abbreviations: $\alpha_V\beta_3$, $\alpha_V\beta_3$ integrin; other abbreviations are expanded on pp. 2–3.

appears to be associated with particular forms of severe malaria (e.g., CSA and HA in placental malaria, or CD36 and ICAM-1 in the brain in the case of cerebral malaria), it is not, however, clear how relevant several of these interactions are *in vivo* (Cooke and Coppel, 1995). For example, the strength of binding of parasitized red blood cells to thrombospondin appears to be too low to allow formation of this interaction in normal post-capillary venules, where parasitized cells preferentially sequester (Cooke *et al.*, 1994). Similarly, adhesion of parasitized cells to hyaluronic acid (HA) appears to be critically shear-dependent (Table 5), so that high levels of adhesion occur only at shear stresses lower than those predicted to exist in post-capillary venules (Beeson *et al.*, 2000). This might indicate, however, that HA could be used only as a receptor for sequestration of parasitized cells in the placenta, where blood

Table 4 Receptor–ligand interactions implicated in cytoadherence and rosetting of red blood cells infected with *P. falciparum.*

Host receptor[a]	Parasite ligand[a]	Comments[a]	References
Cytoadherence			
CD36	PfEMP1	Most common binding phenotype of parasites; CIDR region of PfEMP1 appears to be involved; polymorphisms exist in CD36 in Africans, which appear to affect disease severity	Oquendo *et al.*, 1989; Baruch *et al.*, 1995, 1996; Chen, Q. *et al.*, 2000
ICAM-1 (CD54)	PfEMP1	Member of the immunoglobulin superfamily; rolling receptor for parasitized cells under flow; polymorphisms exist in ICAM-1 in Africans, which may influence adhesion and affect disease severity	Berendt *et al.*, 1989, 1992; Baruch *et al.*, 1996; Smith *et al.*, 2000
TSP	PfEMP1	Physiological role is in question due to low affinity of the binding under flow conditions	Roberts *et al.*, 1985; Sherwood *et al.*, 1987; Cooke *et al.*, 1994; Baruch *et al.*, 1996
TSP	Pfalhesin	Pfalhesin represents altered host red blood cell band 3, the anion transporter; previously reported to bind to CD36 but recently disputed	Crandall *et al.*, 1993, 1994; Lucas and Sherman, 1998; Eda *et al.*, 1999
Chondroitin-4-sulphate	PfEMP1	Found in association with thrombomodulin on the endothelial cell surface and on syncytiotrophoblasts of the placenta; appears to be important in malaria during pregnancy	Rogerson *et al.*, 1995; Fried and Duffy, 1996; Buffet *et al.*, 1999; Reeder *et al.*, 1999; Maubert *et al.*, 2000
HA	?	Appears to be important in malaria during pregnancy; low affinity binding under flow conditions	Beeson *et al.*, 2000
PECAM-1 (CD31)	PfEMP1	Appears to be an uncommon binding target for parasitized cells	Newbold *et al.*, 1997b; Treutiger *et al.*, 1997; Chen, Q. *et al.*, 2000
E-selectin (CD62E)	?	Appears to be an uncommon binding target for parasitized cells	Ockenhouse *et al.*, 1992

Table 4 continued

Host receptor[a]	Parasite ligand[a]	Comments[a]	References
VCAM-1 (CD106)	?	Member of the immunoglobulin superfamily; appears to be an uncommon binding receptor	Ockenhouse *et al.*, 1992; Newbold *et al.*, 1997b
CD36	Sequestrin	Obtained using an unusual approach with anti-idiotype reagents; role in cytoadhesion remains questionable	Ockenhouse *et al.*, 1991b
?	Clag9	Knockout of the *clag9* gene ablates binding of parasitized cells to CD36; precise role in adhesion remains unknown	Gardiner *et al.*, 2000; Trenholme *et al.*, 2000
$\alpha_v\beta_3$	?	First integrin receptor described for parasitized cells; remains to be independently confirmed or shown to be a receptor for clinical isolates	Siano *et al.*, 1998
Rosetting			
CR1 (CD35)	PfEMP1	CD35 polymorphisms exist in Africans, which may confer protection against detrimental effects of rosetting	Rowe, A. *et al.*, 1995
HS-like GAG	PfEMP1	Suggested to be heparan sulphate on red blood cell via GAG binding motifs on PfEMP1 DBL-1 domain; likely to be involved in heparin-sensitive rosetting	Chen, Q. *et al.*, 1998a, 2000; Barragan *et al.*, 2000a
CD36	PfEMP1	The level of CD36 present on older red blood cells is very low; the importance of this interaction is unknown	Handunnetti *et al.*, 1992b
Rosettins/rifins	?	Poorly defined, low molecular weight proteins implicated in rosetting and possibly adhesion to CD31	Helmby *et al.*, 1993; Chen, Q. *et al.*, 1998a; Cheng *et al.*, 1998; Fernandez *et al.*, 1999
ABO blood group antigens	PfEMP1	Appear to influence size rather than frequency of rosetting; blood group A appears to be particularly important	Carlson and Wählgren, 1992; Barragan *et al.*, 2000b; Chen, Q. *et al.*, 2000

[a] Abbreviations are expanded on pp. 2–3.

Table 5 Force required to detach red blood cells from other cells or purified receptors *in vitro*.

Red blood cells[a]	Cell/Receptor[a]	Force (pN)	References
RBC	HUVEC	4[b]	Nash *et al.*, 1992b; Rowland *et al.*, 1993
SSRBC	HUVEC	8[b]	Rowland *et al.*, 1993
PRBC	C32 melanoma	60[c]	Nash *et al.*, 1992b
PRBC	HUVEC	86[b,c]	Nash *et al.*, 1992b
PRBC	ICAM-1	Note d	–
PRBC	CD36	50[b]	Cooke *et al.*, 1994; Crabb *et al.*, 1997a
PRBC	CSA	42[b]	Cooke *et al.*, 1996
PRBC	TSP	Note e	–
PRBC	TM	50[b]	Rogerson *et al.*, 1997
PRBC	HA	14[b]	Beeson *et al.*, 2000
PRBC	Normal RBC	440[c]	Nash *et al.*, 1992a

[a] Abbreviations are expanded on pp. 2–3.
[b] Force calculated from the wall shear stress required to detach adherent red blood cells in a parallel-plate flow chamber.
[c] Force measured by single cell micropipette manipulation.
[d] Detachment force not quantified since PRBC continuously roll on this receptor under flow (Cooke *et al.*, 1994).
[e] PRBC do not adhere to TSP under flow conditions (Cooke *et al.*, 1994).

flow is slower than elsewhere in the body (Ramsey and Donner, 1980). The significance of the interaction with PECAM-1 (CD31) is also hard to gauge, as CD31 appears to be confined to areas of cell–cell contact between endothelial cells and to be absent from the luminal face to which parasitized cells adhere (Treutiger *et al.*, 1997). Although CD31 may redistribute to the luminal face under IFN-γ stimulation, both the timing and extent to which this happens during malaria infection are unknown.

For endothelial adhesion, the most common interaction appears to be between PfEMP1 and CD36, with studies suggesting that most if not all parasites can adhere to this receptor (Hasler *et al.*, 1990; Ho *et al.*, 1991; Ockenhouse *et al.*, 1991a, 1992; Cooke *et al.*, 1995; Newbold *et al.*, 1997b). The binding site on PfEMP1 has been localized within the CIDR (Baruch *et al.*, 1997) and it has been demonstrated that recombinant proteins from this region are capable of blocking and even reversing adherence of several isolates expressing antigenically distinct forms of PfEMP1 (Cooke *et al.*, 1998). It has proved difficult to detect CD36 on endothelial cells of the cerebral circulation, particularly in post-mortem studies of patients who have died of cerebral malaria (Turner *et al.*, 1994). In fact these individuals appear to be preferentially infected by parasites that adhere to both CD36 and ICAM-1, a

receptor readily identified in cerebral vessels. Chondroitin-4-sulphate appears to be present at high levels on the surface of syncytiotrophoblasts in the placenta (Maubert *et al.*, 2000) and isolates that recognize this receptor (but not CD36 or ICAM-1) appear to be preferentially involved in malaria during pregnancy (Rogerson *et al.*, 1995; Fried and Duffy, 1996; Maubert *et al.*, 2000). Such isolates may constitute a relatively restricted population, and development of strain-specific immunity to these may limit subsequent infection. This could explain why primigravidae are so much more susceptible to malaria than multigravidae.

One point of interest is the behaviour of parasitized red blood cells when they interact with different receptors under conditions of flow. When parasitized cells interact with CD36 they remain stationary, whereas they continuously roll over ICAM-1 (Cooke *et al.*, 1994). This is reminiscent of the interactions between activated white blood cells and the endothelium, although the receptors and their roles are clearly different. ICAM-1, for example, is an immobilizing receptor for white blood cells and a rolling receptor for parasitized red blood cells. Cytoadherence *in vivo* will most probably result from the sum of several interactions between parasitized cells and endothelial receptors, perhaps acting synergistically or in concert to determine the final pattern of adhesion (Cooke *et al.*, 1994; McCormick *et al.*, 1997; Newbold *et al.*, 1997b). Again, the complicated influence of upregulation of various endothelial-cell-expressed molecules by inflammatory cytokines released into the circulation in response to infection must also be taken into account.

7.2. Rosetting

A second form of adhesion is rosetting, the binding of two or more uninfected red blood cells around a single infected red blood cell (David *et al.*, 1988; Udomsangpetch *et al.*, 1989c). By transmission electron microscopy, the membranes of the infected red blood cell and surrounding uninfected red blood cells appear to be in close association (Udomsangpetch *et al.*, 1989c). Rosetting requires both calcium and magnesium (Carlson *et al.*, 1990a,b) and is inhibited by trypsin, heparin (Udomsangpetch *et al.*, 1991) — in some but not all strains — and, perhaps surprisingly, antibodies against KAHRP (Carlson *et al.*, 1990b). Rosetting is a property of only some strains of *P. falciparum*, and freshly collected field isolates can vary quite dramatically in the extent to which they rosette (Wahlgren *et al.*, 1994). The importance of rosetting in host–parasite relations has been under intense study. There is a good deal of controversy, but on balance it appears that rosetting parasites are responsible for more severe disease. Epidemiological studies in endemic areas have shown that severe clinical disease, such as cerebral malaria, is more common in individuals infected with strains capable of rosetting. Further, those

patients with antibodies capable of disrupting rosettes are found to suffer less severe clinical forms of malaria (Carlson *et al.*, 1990a; Ringwald *et al.*, 1993; Rowe, A. *et al.*, 1995). Contradictory results have been obtained in other epidemiological settings (al-Yaman *et al.*, 1995), and it may be that the variability in rosetting ability of the parasite, added to markedly different host factors such as HLA status, degree of endemicity of malaria, and presence of other infections, can give rise to different clinical outcomes. The molecules reported to mediate rosetting are also beginning to become increasingly diverse and complex (Figure 5). Both PfEMP1 and the rifins have been implicated as the parasite-encoded ligands responsible for rosetting (Helmby *et al.*, 1993; Rowe, J.A. *et al.*, 1997; Chen, Q. *et al.*, 1998a), although recent evidence suggests that PfEMP1 is the most likely candidate (Fernandez *et al.*, 1999; Barragan *et al.*, 2000b; Chen, Q. *et al.*, 2000). A number of counter receptors on the surface of red blood cells have been described to which PfEMP1 can

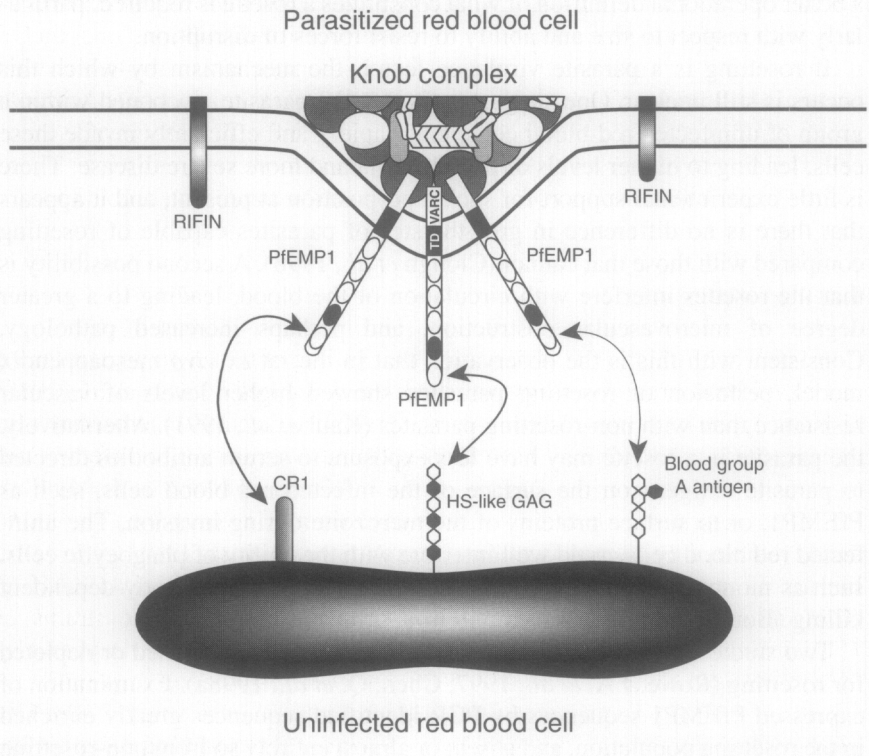

Figure 5 Schematic representation of the molecules implicated in the interaction between red blood cells infected with *P. falciparum* and non-parasitized cells (rosetting). Abbreviations are expanded on pp. 2–3.

bind, including complement receptor 1 (CR1) (Rowe, J.A. *et al.*, 1997), heparan sulphate or heparan sulphate-like glycosaminoglycans (Chen, Q. *et al.*, 1998a, 2000; Barragan *et al.*, 2000a,b), and the ABO blood group antigens, particularly blood group A (Carlson and Wählgren, 1992; Barragan *et al.*, 2000b; Chen, Q. *et al.*, 2000). The physical forces binding cells into a rosette have been measured using both dual micropipetting techniques (see Table 4) and viscometry, and, as stated above, are estimated to be at least five times higher than those involved in cytoadherence to endothelial cells (Nash *et al.*, 1992a; Chotivanich *et al.*, 2000b). Finally, there is evidence to suggest that some blood groups and thalassaemic red blood cells hinder rosette formation to the benefit of the patient (Carlson *et al.*, 1994). Rosetting has been observed in other malaria species that sequester, such as *P. chabaudi* and *P. fragile*, but it has also been described in *P. vivax* and *P. ovale*, which do not cause cerebral malaria and in general cause less serious disease (David *et al.*, 1988; Udomsangpetch *et al.*, 1991, 1995; Angus *et al.*, 1996; Lowe *et al.*, 1998). This still needs to be explained, and it may be that a better operational definition of what constitutes a rosette is required, particularly with respect to size and ability to resist forces of disruption.

If rosetting is a parasite virulence factor, the mechanism by which this occurs is still unclear. One suggestion is that the parasite, cocooned within a group of uninfected red blood cells, may rapidly and efficiently invade these cells, leading to higher levels of parasitaemia and more severe disease. There is little experimental support for such a proposition at present, and it appears that there is no difference in growth rates of parasites capable of rosetting compared with those that cannot (Clough *et al.*, 1998). A second possibility is that the rosettes interfere with circulation of the blood, leading to a greater degree of microvascular obstruction, and perhaps increased pathology. Consistent with this is the observation that in the rat *ex vivo* mesoappendix model, perfusion of rosetting parasites showed higher levels of vascular resistance than with non-rosetting parasites (Kaul *et al.*, 1991). Alternatively, the parasite in a rosette may have less exposure to serum antibodies directed to parasite antigens on the surface of the infected red blood cells, such as PfEMP1, or to surface proteins of the merozoite during invasion. The uninfected red blood cells could well interfere with the ability of phagocytic cells, such as monocytes, to destroy parasites by a process of antibody-dependent killing (Bouharoun-Tayoun *et al.*, 1990).

Two studies examined populations of parasites either enriched or depleted for rosetting (Rowe, J.A. *et al.*, 1997; Chen, Q. *et al.*, 1998a). Examination of expressed PfEMP1 sequences by PCR identified sequences greatly enriched in the rosetting population, and absent or almost entirely so from non-rosetting parasites. Expression of the DBL-1 regions of the identified genes produced proteins capable of binding to uninfected red blood cells. The two genes identified were not identical in sequence and their protein products bound to

different receptors, complement receptor 1 and glycosoaminoglycans (GAG), on an as yet unidentified proteoglycan, but at present presumed to be heparan sulphate. It is suggested that the binding to GAG is mediated by a number of basic GAG-binding motifs found scattered through the particular PfEMP1 sequence (Chen, Q. *et al.*, 1998a). These findings explain why it is that only certain isolates rosette, as these must be isolates that both contain and express a PfEMP1 capable of interacting with uninfected red blood cells. The relative frequency of rosetting suggests that more PfEMP1 sequences capable of rosetting will be identified; otherwise, it would mean that one or other of these two genes is expressed at a very high frequency. These observations also help to explain why some but not all rosetting strains could have their rosettes disrupted by the addition of heparin (Rogerson *et al.*, 1994; Wahlgren *et al.*, 1994). Presumably heparin-sensitive isolates are rosetting via a PfEMP1–GAG interaction. Further, since the highly variable PfEMP1 is involved in this interaction, it is possible that additional red blood cell receptors will be identified. It should be noted that specific proteins that also bind to heparan sulphate have been described in the sporozoite stage. It is not clear why it was necessary to evolve two such markedly different genes, unless this is related to the differing cellular locations of the two genes and differing requirements for transport. The relative importance of the various ligand–receptor combinations (see Figure 4), particularly in cases of severe malaria, is yet to be determined. The observation that CR1 polymorphisms are common in Africans is consistent with this rosetting interaction being common and associated with significant clinical disease and, hence, selective pressure (Rowe, J.A. *et al.*, 1997).

8. COMPARISON BETWEEN MALARIA AND *BABESIA* INFECTION OF RED BLOOD CELLS

Babesia bovis and *B. bigemina* are closely related intraerythrocytic protozoan parasites that infect cattle and cause bovine babesiosis. The pathogenesis and clinical picture of this disease bear striking resemblances to malaria in humans (Commins *et al.*, 1988; Wright *et al.*, 1988; Allred, 1995; Schetters and Eling, 1999), particularly when *B. bovis* and *P. falciparum* infections are compared with each other. Like malaria parasites, *Babesia* spp. are also members of the phylum Apicomplexa, and thus specialized for invasion and growth inside red blood cells. During their development inside bovine red blood cells, *Babesia* parasites also make a large number of modifications to the red blood cells, which inevitably affect their function. A major difference between *Plasmodium* and *Babesia*, however, is the time taken to complete the life cycle inside the red blood cell. For *B. bovis*, the cycle time is c. 15 hours, and thus it

can complete more than three life cycles during the time taken for *P. falciparum* to complete only one. The cellular modifications in bovine red blood cells infected with *Babesia*, therefore, occur much more rapidly and persist in the cell for a much shorter time than those that occur in red blood cells infected with *P. falciparum*. Although comparatively little is known at the molecular level about the precise nature or identity of the proteins that are involved in such modifications, their relative lability and the speed at which they must occur may indicate that these alterations are less complex than those that occur in malaria-infected red blood cells. *B. bovis* can also be cultured with ease *in vitro*, which, together with the relatively short doubling time, makes it an ideal model to explore parasite protein–red blood cell cytoskeleton interactions more thoroughly. Accumulation of parasitized red blood cells in microvasculature also accompanies *Babesia* infection (Hoyte, 1971), which frequently develops into severe and almost invariably fatal syndromes characterized by organ-specific sequestration, such as cerebral babesiosis (Callow and McGavin, 1963). Intimate interaction of parasitized cells with the vascular endothelium, and with each other (similar to rosetting), appears to be via 'stellate protrusions' of the infected red blood cell membrane (Wright, 1972; Aikawa *et al.*, 1985; Everitt *et al.*, 1986; O'Connor *et al.*, 1999). Although these have frequently been likened to the knobs of malaria-infected cells, they appear to be much larger projections (320 nm × 160 nm) than the knobs on *P. falciparum*-infected red blood cells (150 nm × 65 nm) and show much lower focal electron density (Aikawa *et al.*, 1985; O'Connor *et al.*, 1999).

B. bigemina, on the other hand, behaves quite differently and more resembles *P. vivax*. There is no evidence of sequestration at any stage of the infection (Callow and Johnston, 1963; Hoyte, 1971) and, in fatal cases, death usually results from anaemia. There is a marked difference between *B. bovis* and *B. bigemina* in their tendency to sequester in the brain (Callow and Johnston, 1963), which is a consistent finding that has been useful in diagnosis (Hoyte, 1971). In infected cattle, *B. bigemina* is rarely observed in brain capillaries (Callow and Johnston, 1963). Moreover, the virulence of *B. bovis*, but not that of *B. bigemina*, is reduced by repeated blood passage in splenectomized cattle and restored by passage in intact cattle or transmission by ticks. Compared with malaria, virtually nothing is known at the molecular level about the structural and functional alterations that occur in *Babesia*-infected red blood cells. The infected cells do demonstrate rheological abnormalities. For example, there is a profound reduction in whole cell deformability, as measured by rheoscope, and a reduction in the ability of the red blood cell membrane to tank-tread. The parasitized red blood cells also become abnormally adhesive for vascular endothelial cells (O'Connor *et al.*, 1999), although no specific adhesion molecule has yet been identified on the surface of the endothelial cells to mediate this process. However, a variant antigen

(VESA) appears to cluster over the knob-like protrusions on the surface of *Babesia*-infected red blood cells (O'Connor *et al.*, 1997, 1999) and is the product of the newly described *ves* multi-gene family (Allred *et al.*, 2000); this may be the cytoadherence ligand. Clearly more work is warranted on this parasite system since it offers great potential to serve as a much simpler and more amenable model, both *in vitro* and *in vivo*, for human malaria infection.

9. THE HOST–PARASITE RELATIONSHIP

The fact that *P. falciparum* manifests its mortality predominantly in young children ensures that it exerts extraordinary selective pressure on humans living in endemic areas. Thus it is no surprise to observe the presence of a number of phenotypes in humans that appear to confer resistance to malaria infection. These may manifest as individuals with more effective immune responses to malaria antigens or individuals with red blood cells that resist parasite invasion and growth by virtue of either haemoglobin or membrane protein mutations. Of those that act through changes in the red blood cell, the best known are probably the haemoglobinopathies such as sickle cell disease and the thalassaemias. The pathobiology of red blood cells in these conditions has been reviewed in detail (Evans and Hochmuth, 1977; Mohandas *et al.*, 1984, 1992; Chasis and Mohandas, 1986; Mohandas, 1992; Mohandas and Chasis, 1993; Mohandas and Evans, 1994) and description of the pathophysiology of these conditions is outside the scope of this current review. Many of these conditions induce rheological changes in the red blood cell that parallel those caused by malaria infection, and it is instructive to compare them.

Homozygous sickle cell disease is a devastating condition with protean clinical manifestations, most of which are undoubtedly the result of physical trapping of grossly mechanically impaired sickle red blood cells in the microvasculature leading to painful, vaso-occlusive crises with accumulative organ damage. In sickle cells, normal adult haemoglobin (HbA) is replaced by abnormal sickle haemoglobin (HbS), which can be inherited in either a heterozygous or homozygous state. In heterozygotes (sickle-cell trait), acquisition of only one copy of the *HbS* gene results in HbAS red blood cells that contain approximately equal proportions of HbA and HbS. Except under extreme conditions of low oxygen tension or oxidative stress, these individuals remain clinically unaffected by their condition. In fact, there is some advantage to acquisition of the trait in individuals living in areas endemic for malaria, since this condition offers relative protection against severe malaria. In these areas, malaria exerts a strong positive selective pressure on the sickle gene and is the primary reason it is maintained in the human gene pool. In contrast, homozygotes who inherit two copies of the sickle gene and whose

red blood cells contain only HbS (HbSS) bear the full brunt of this condition. Unlike HbA, HbS forms long rigid rods of polymer (nematic tactoids) upon deoxygenation, which leads to profound changes in red blood cell morphology. Furthermore, during repeated cycles of deoxygenation and reoxygenation, sickle cells become progressively dehydrated, most probably as a consequence of potassium loss via the Gardos channel (McGoron *et al.*, 2000), although other membrane transport pathways, including the KCl co-transporter, may also play a role (Joiner, 1993; Brugnara, 1997). Dehydration further exacerbates the reduction in cell deformability by increasing the intracellular haemoglobin concentration, which in turn dramatically increases the red blood cells' internal viscosity. The overall loss of cell deformability is also due in part to a marked decrease in the elasticity of the red blood cell membrane itself (Chien *et al.*, 1970, 1982; Nash *et al.*, 1984, 1986; Green *et al.*, 1988). While these changes occur to a substantial degree even when the cells are fully oxygenated, the degree of impairment is much greater upon deoxygenation, when they assume their characteristic sickle shape.

Although, like malaria-infected red blood cells, sickle cells have also been shown to be abnormally adhesive, the alteration occurs to a much lesser extent than in red blood cells parasitized by *P. falciparum*. In a direct comparison of adhesion of normal (HbAA), HbAS, HbSS and *P. falciparum*-infected red blood cells with cultured vascular endothelial cells under physiologically relevant flow conditions, the relative levels of adhesion were in the ratio of 1 : 1 : 3 : 1000 (Rowland *et al.*, 1993). Thus, it seems likely that the direct physical mechanical trapping of sickle cells in the small diameter vessels of the microcirculation, consequent upon their abnormal mechanical properties, is the primary event in the genesis of the vaso-occlusive pathology seen in sickle cell anaemia. In contrast, cell adhesion is the most likely key pathogenic event in malaria infection, with abnormal mechanics playing a secondary role. It has been suggested, however, that mechanical trapping of parasitized red blood cells in the bone marrow sinuses may exacerbate anaemia by inhibiting the release of new red blood cells into the circulation (Wickramasinghe *et al.*, 1987).

Interactions between host and parasite can become extremely complex and we do not yet understand many of these. For example, it is clear that the parasite requires a normal red blood cell membrane skeleton for parasite growth. Several groups have examined the capacity of red blood cells with an abnormal membrane skeleton to support the growth of *P. falciparum* in culture *in vitro* (Schulman *et al.*, 1990; Facer, 1995; Magowan *et al.*, 1995). Schulman and co-workers (1990) demonstrated that culture over two to three cycles resulted in diminished growth rates, which were proportional to the amount of spectrin in the red blood cell membrane skeleton. The reason for this is not clear but it may be related to some requirement for cytoskeletal components in the formation of a competent invasion complex on the merozoite surface. This

may explain the otherwise unexpected observation that MSP1 binds to spectrin (Herrera *et al.*, 1993). MSP1 is believed to be important for invasion and interacts with the outside of the red blood cell. Thus, the only time it comes into contact with spectrin is during red blood cell lysis before invasion. Spectrin-binding ability would be relevant only at this stage, perhaps by securing spectrin molecules to the surface of the merozoite. The most profound growth inhibition was noted in red blood cells deficient in protein 4.1, whereas red blood cells with abnormal band 3 proteins supported parasite growth as well as controls (Schulman *et al.*, 1990). The inability of parasites to grow in red blood cells deficient in protein 4.1 was confirmed by Magowan and co-workers (1995), who suggested that this growth failure was secondary to accumulation of MESA in the cytoplasm of the red blood cell because of the absence of its binding partner protein 4.1. Abnormalities of haemoglobin may have secondary effects on the integrity of the red blood cell membrane skeleton and this may also perturb the host–parasite relationship (Nagel and Roth, 1989; Yuan *et al.*, 1995). Examples include the apparent change in PfEMP1 accessibility to antibody in thalassaemic red blood cells, which appears to make parasites more susceptible to clearance by immune mechanisms (Luzzi *et al.*, 1991a,b). Transgenic mice with specific abnormalities of red blood cells have been examined for susceptibility to malaria infection (Shear, 1993; Shear *et al.*, 1993, 1998; Hood *et al.*, 1996). In general, these studies have confirmed the importance of abnormal haemoglobin in restricting parasite growth.

Another phenomenon in which the complex interplay of host and parasite factors is seen is that of cytoadherence, where it appears that modulating the level of cytoadherence can be of benefit to the host. *P. falciparum* parasites that do not cytoadhere cause milder disease than adherent strains (Langreth and Peterson, 1985). From this it could be argued that there may be a selective advantage for the host if it can decrease the level of adhesion. Accordingly, the structural genes encoding host receptors for PfEMP1 have been examined for evidence of polymorphism that may result from mutations that decrease the extent of sequestration (Fernandez-Reyes *et al.*, 1997; Adams *et al.*, 2000; Craig *et al.*, 2000; Smith *et al.*, 2000). There is a high frequency polymorphism in the human *ICAM-1* gene in a malaria-endemic population in Kilifi, Kenya. Studies *in vitro* showed that infected red blood cells bound less well to the mutant recombinant ICAM-1, termed ICAM-1^{Kilifi} (Adams *et al.*, 2000; Craig *et al.*, 2000). Paradoxically, however, individuals homozygous for this polymorphism, at least in this region of Africa, were twice as likely to develop the severe form of malaria known as cerebral malaria (Fernandez-Reyes *et al.*, 1997). The significance of this result is unclear but it challenges the contention that lowered levels of adhesion are beneficial for the host. However, these adhesion studies were performed using laboratory-adapted parasite lines, which may not accurately reflect those circulating in the field. Perhaps

in this particular area of Africa, where malaria transmission is high, compensatory mutations in PfEMP1 may have arisen that bind to ICAM-1^{Kilifi} with much higher avidity. Sequence analyses of African populations showed a surprisingly high frequency of mutations in the *CD36* gene that result in loss of expression of CD36 (Aitman *et al.*, 2000). Again, one might expect this to be protective against malaria, based on the widely professed importance of CD36 in cytoadherence. Surprisingly, however, individuals deficient in CD36 were in fact more susceptible to severe malaria, particularly cerebral malaria, than individuals expressing normal levels of wild-type CD36. Clearly more needs to be learnt about the importance of quantitative differences in adhesion levels in the causation of disease.

Finally, if cytoadherence really is a virulence factor, then preventing or reversing adhesion with anti-adhesive substances should significantly ameliorate the severity of the disease. Laboratory studies have identified potential anti-adherence reagents including recombinant fragments of PfEMP1 (Cooke *et al.*, 1998), but these have not been subjected to clinical trial. Further work over the next few years will undoubtedly improve our knowledge of the interaction between the malaria parasite and the host red blood cell. This in turn may suggest further strategies that could interfere with processes critical for parasite survival.

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