CHAPTER FOUR- DISCUSSION

The infection of human erythrocytes by *P. falciparum* is concomitant with major structural alterations to the erythrocyte membrane (Cooke et al., 2001; Cooke et al., 2004b). One of these changes has been highlighted by the association of parasite-encoded proteins with host skeletal components. Numerous *P. falciparum* proteins have been shown to interact with the erythrocyte membrane, but their precise roles remain poorly understood (Cooke et al., 2001; Cooke et al., 2004b). In this research, *P. falciparum* phage display libraries provided a powerful tool and novel approach for identifying new interactions between the parasite and erythrocyte membrane. A library was used for identifying proteins that demonstrated binding specificity towards host skeletal proteins including 4.1R and 4.1R/spectrin complexes. These findings thus provide functional insight into an important area of *P. falciparum* research, namely host-parasite interactions.

4.1 Utility of *P. falciparum* phage display libraries

The study of protein-protein interactions represents a major area of functional genomics research. Among the number of different strategies that have been adopted, phage display of protein or peptide libraries has emerged as a well established tool. Importantly, it provides a basis for the construction of protein interaction maps, which may be used in the discovery of new drugs and vaccine development.

Despite rapid progress in this field, a number of technical hurdles remain. The most important challenge when working with cDNA expression systems is the absence of control over the translation frame of the foreign sequences when subcloned into the host expression vector. As a consequence, a large number of clones are translated on alternative reading frames. Approximately two thirds of phage therefore display

unnatural proteins on their surfaces, which may be selected during biopanning as they may mimic the binding properties of the real ligand. Isolation of false-positives therefore represents a major drawback as their presence overshadows the selection of natural ligands and possibly rare transcripts, if present. The low titre of phage display libraries used in this study and small size of *P. falciparum* inserts may be viewed as another limitation (3.2.3 and 3.3.1). Generally, a robust phage display cDNA library should have a higher titre and the average length of the inserts should be longer. Despite these flaws, however, biopanning of the *P. falciparum* phage display library, sequencing and bioinformatic analyses identified a total of ten unique open reading frames that encoded proteins binding specifically to 4.1R (Table 7). Of the sixty clones analysed, two thirds were false positives while the remaining third displayed natural peptides. These proteins, however, do not necessarily represent all polypeptides that interact with 4.1R, as a single library is not representative of the entire parasite's genome. Representation may be improved by constructing several libraries from different parasite cultures, which may consist of either mixed asexual or synchronised stages. The genes expressed at each parasite developmental stage, the quality of isolated RNA and propensity for RNA degradation are factors which can influence the representation of rare transcripts in the cDNA library. The identification of phage that contain rare transcripts may be bettered by constructing libraries which are enriched in in-frame fusion phage (Ansuini et al., 2002). In addition, high throughput screening of phage libraries will increase the chances of finding displayed proteins encoded by rare mRNA molecules.

The *P. falciparum* phage display library created in this study is a unique tool that can be used to investigate a wide range of protein-protein interactions, as applied to other areas of malaria research. In the field of drug design, the library may be screened for peptides with the aim of developing pharmacological agents against specific host-parasite interactions. Compounds such as peptidomimetics are designed to interact

with selected proteins or receptors and in so doing, inhibit binding with their natural partner(s). Peptides that mimic epitopes on the surface of the parasite and infected host cells may act as potential targets for protective antibodies in vaccine development (Ladner et al., 2004; Manoutcharian et al., 2001). The disruption of specific host-parasite interactions using antibodies or peptide antagonists may effectively interfere with cellular functions in *P. falciparum*. This may curb the parasite's ability to invade and escape from host cells, as well as adversely affect its intracellular development.

4.2 *P. falciparum* proteins binding to 4.1R

4.2.1 Low complexity domains

The amino acid composition of the 4.1R binding sequences consists predominantly of charged residues at physiological pH, implying that the interaction of these proteins with 4.1R is most likely electrostatic in nature. Another prominent feature is the presence of low complexity (LC) sequences, which have been identified in the majority of 4.1R binding peptides. These domains are also found in varying proportions throughout the full-length sequences of the annotated *P. falciparum* proteins. EBA-175 (PF07_0128) and EBA-181 (PFA0125c) contain 3 and 13% LC domains respectively, while the predicted enzymes PFB0150c, MAL13P1.248 and PFI1570c contain 17, 70 and 7%. The proportions of LC regions in the five hypothetical proteins range from 18-85%.

Analyses of proteins in *P. falciparum* revealed that orthologous proteins in yeast and other parasitic eukaryotes can be significantly smaller in size. This finding has highlighted a remarkable feature of the *P. falciparum* proteome: a significant number of proteins are enriched with stretches of LC amino acids (Aravind et al., 2003). LC domains possess a large number of particular amino acids, most notably lysine, aspartic acid and glutamic acid as well homopolymeric stretches of asparagine. The

biological role of these domains remains unknown, but given their general amino acid composition, they may feature as unstructured coiled soluble elements which are orientated toward the surface of proteins (Aravind et al., 2003). Evidence indicates that LC regions have a role in protein-protein interactions (Liu et al., 2002). It appears that this function may also be extended to specific *P. falciparum* proteins, as evidence presented by the phage display data demonstrate that the majority of 4.1R binding peptides contain short runs of LC amino acids. It has also been hypothesised that LC domains are the target of non-productive antibody-induced immunity in individuals living in endemic areas, thereby facilitating immune evasion by the parasite (Anders, 1986; Coppel et al., 1983).

4.2.2 Coiled-coil domains

Bioinformatic analyses mapped 4.1R binding sequences to a common, negatively charged domain in four *P. falciparum* proteins (Table 9). These protein regions displayed homology to eukaryotic filament repeat motifs, including the short coiled-coil domain of myosin and coiled repeat pattern in intermediate neurofilaments. Coiled-coils are versatile folding motifs and represent important structural elements in fibrous proteins, such as microtubule motor proteins (kinesin and dynein), muscle motor protein (myosin) and skeletal proteins (nuclear lamins) (Burkhard et al., 2001; Lupas, 1996).

Myosin-like domains were predicted in EBA-181, PF14_0201 and PF11_0191 using PlasmoDB. These *P. falciparum* domains have a high probability of containing two short coiled-coil regions as determined by SMART and MultiCoil software analyses (Figure 23). The location of the 4.1R interacting peptides demonstrates that these predicted domains in specific parasite proteins have the ability to bind directly to 4.1R. Due to the high proportion of charged residues, the interactions are probably facilitated by contact between hydrophilic surfaces in the myosin-like

motifs and the erythrocyte skeletal protein. In support of these interactions, the 10kDa domain of 4.1R was shown to bind eukaryotic myosin and regulate its ATPase activity *in vitro* (Pasternack and Racusen, 1989).

4.2.3 Hypothetical proteins

Of the ten identified parasite proteins (Table 7), five were hypothetical, confirming that the proteome of *P. falciparum* is largely uncharacterised (Florens et al., 2002). One of the goals of bioinformatics is to predict the function of proteins from primary amino acid sequence information (Horrocks et al., 2000). Assigning putative functions to hypothetical proteins, in the absence of functional data, is difficult. However, *in vitro* association of the hypothetical *P. falciparum* proteins with the erythrocyte membrane has provided some insight into their biological roles. These proteins may modulate the interaction between 4.1R and other erythrocyte membrane proteins, since 4.1R enhances the binding affinities between spectrin and actin, as well as between glycophorin and p55 (Marfatia et al., 1994). Parasite proteins that interact with 4.1R may therefore influence normal 4.1R function, resulting in reduced protein interactions and structural alterations to the membrane skeleton. Further insight into the role of hypothetical proteins may also be gained by examining predicted domains of the proteins, although this discussion remains speculative in the absence of experimental data.

mRNA data indicate that PF14_0201, MAL6P.48 and PFE0570w are expressed in asexual parasite stages and gametocytes. Mass spectrometry data indicate that these proteins are expressed in sporozoites and trophozoites. Peptide fragments of MAL6P.48 have also been identified in merozoites and gametocytes, whereas additional fragments of PFE0570w have been identified in merozoites. These proteins have predicted transmembrane domains and presumably insert into the erythrocyte membrane during *P. falciparum* development, thereby

allowing the proteins to interact with 4.1R. In addition to binding 4.1R, PF14_0201 contains a peptide which may also interact with spectrin. The direct association between 4.1R and β-spectrin (Walensky et al., 2003) may allow the *P. falciparum* protein to interact with sequences derived from both skeletal proteins. This may dramatically impact on the integrity of the erythrocyte skeleton by inhibiting the assembly of spectrin-actin complexes. PFA0420w and PF11_0191 also have mRNA expression profiles in asexual parasite stages and gametocytes. Mass spectrometry data provides evidence for PF11_0191 expression in merozoites, trophozoites and gametocytes, whereas PFA0420w data indicate expression in merozoites only. These two proteins do not contain predicted transmembrane domains and therefore presumably bind directly to 4.1R via electrostatic interactions.

Based on their predicted domains and 4.1R binding peptide, PF11_0191 and PFE0570w may have dual functions in the malaria parasite. PF11_0191 contains a domain which shows significant alignment to small nucleolar RNA binding domains of pre-mRNA processing ribonucleoproteins. The function of this domain in PF11_0191 remains unknown, but may be involved in mRNA processing during *P. falciparum* development. Together with a 4.1R recognition sequence and peptide which localises at the erythrocyte membrane (mass spectrometry data), the protein probably associates with the erythrocyte skeleton by interacting specifically with 4.1R during asexual parasite growth and/or merozoite invasion/release.

PFE0570w contains domains predicted to be involved in protein-protein and protein-nucleic acid interactions. DED is a protein interaction module which is related in structure and sequence to the death and caspase recruitment domain. Several DED-containing proteins are involved in the regulation of apoptosis through their interactions with DED-containing caspases in humans (Pfam accession number: PF01335;

http://www.wustl.edu). Experimental data indicate that *P. falciparum* is also capable of undergoing programmed cell death and PFE0570w may be an active component of the parasite's apoptotic machinery (Deponte and Becker, 2004). The protein may function in mosquito midgut stages or erythrocytic stages to control parasite density. This hypothesis is based on the assumption that an uncontrolled proliferation of *P. falciparum* would lead to an early death of the host (Deponte and Becker, 2004). The MATH domain is necessary for receptor interaction and has been recognised as an important connection in establishing signaling complexes with tumor necrosis factor-1 receptor (Pfam accession number: PF00917). PFE0570w also contains a domain implicating it as a member of pseudouridylate synthases, which are central to modifying bases in RNA molecules (Pfam accession number: PF00849). The function of the zinc-finger region is unknown, but may be involved in interactions with proteins and DNA (Pfam accession number: PF01529). PFE0570w may therefore exist as a multi-functional protein in *P. falciparum:* the protein interaction cassettes (DED and MATH domains) and 4.1R recognition sequence indicate involvement in parasite signaling pathways and association with the erythrocyte membrane respectively; the zinc finger and pseudouridylate motif implicate a specific role in the manipulation of nucleic acid function.

4.2.4 Annotated enzymes

The identification of putative *P. falciparum* kinases, PFB0150c and MAL13P1.248, provides a potential mechanism of 4.1R and/or spectrin phosphorylation. Together with a host of other erythrocyte skeletal proteins (including ankyrin and band 3), the phosphorylation of 4.1R and spectrin influences protein-protein interactions which may lead to changes in membrane structure and function (Magowan et al., 1998). PFB0150c and MAL13P1.248 are expressed in merozoites and late asexual stages as indicated by their mRNA expression profiles. The discovery of new protein kinases may aid in understanding parasite invasion and release, as well as the development of *P. falciparum* within the erythrocyte.

Furthermore, kinases have become attractive targets for the formulation of anti-parasitic agents (Doerig, 2004; Florens et al., 2002).

PFI1570c is a predicted aminopeptidase which is reliant on zinc ions for normal catalytic function (Pfam accession number: PF02127). Mass spectrometry data indicate that the protein associates with the erythrocyte membrane, which is supported by the identification of a 4.1R/spectrin recognition sequence. Experimental evidence has recently shown that phage displaying a PFI1570c peptide binds purified spectrin (Personal communication, S. Lauterbach, 2006-03-12), but it is possible that it may associate with 4.1R as well. The protease may function by cleaving amino acids from the amino-terminal end of spectrin. Since 4.1R binds β-spectrin at its N-terminal end, the proteolysis event may disrupt spectrin-4.1R-actin interactions, thereby destabilizing the erythrocyte skeleton. Together with other *P. falciparum* proteases (Hanspal et al., 2002; Le Bonniec et al., 1999; Roggwiller et al., 1996), proteolytic breakdown of skeletal proteins results in erythrocyte lysis and thus could potentially provide a mechanism for merozoite release (Salmon et al., 2001; Wickham et al., 2003). PFI1570c may also function in the modulation of the intact host membrane skeleton, thus supporting parasite development and/or merozoite invasion.

4.2.5 Erythrocyte invasion proteins

EBA-175 and EBA-181 are essential DBL-EBP which localise to the micronemes of *P. falciparum* (Preiser et al., 2000). Upon invasion by merozoites, the release of these proteins has been linked to erythrocyte entry and junction formation (Chitnis and Blackman, 2000). However, the precise molecular mechanisms governing parasite invasion remain poorly understood. In recent years, it has become clear that alternative invasion pathways are mediated by a number of different molecules (Gaur et al., 2004; Pasvol, 2003). The host-parasite interface therefore represents an important opportunity for the development of therapeutics and vaccines, aimed at blocking *P. falciparum* invasion.

This research describes an interaction between the C-terminal cys-rich domain of EBA-175 and 4.1R. Specific binding was also demonstrated between the 10kDa domain of 4.1R and a myosin-like motif in EBA-181. The myosin-like motif is situated in an interdomain region between the C-terminal cys-rich domain and erythrocyte binding domains (Figure 4). Blot overlay (Figure 35) and histidine pull-down assays (Figure 36) were performed using a recombinant peptide from EBA-181 (6His-*Pf*J) encompassing the 4.1R recognition sequence. These experiments were designed to allow semi-quantitative assessment of the interaction between 6His-*Pf*J and recombinant 10kDa 4.1R domain. According to figure 37(A-C), the interaction profile was concentration dependent and saturable, thus confirming the specific nature of the association. The average Kd was determined by linear curve transformations and was found to be less than 1μM, indicating a relatively high affinity interaction between the two proteins. However, this method represents a poor means of accurately determining the Kd value. Alternative techniques such as calorimetry or biosensor assays may be exploited to obtain quantitative binding data. The reliability of the Kd value is likely to be affected by other factors. Expression of parasite and 4.1R proteins that contain 6His and GST tags may impact on the binding kinetics between the two proteins. Additional positive charges provided by the His tag and size of the GST protein (25kDa) may influence protein folding mechanisms and subsequent protein-protein interactions. In addition, steric hindrance caused by the GST tag may limit binding between 6His-*Pf*J and GST-10kDa.

The *in vitro* association of EBA-181 and EBA-175 with an underlying erythrocyte skeletal protein has important implications for *P. falciparum* invasion. 4.1R is a critical component of the erythrocyte skeleton. It functions by stabilising horizontal protein interactions and links the underlying skeleton to the lipid bilayer (Walensky et al., 2003). The conserved 10kDa domain of 4.1R interacts with a number of key proteins, and represents a pivotal point in the control of erythrocyte membrane

integrity. The domain facilitates the interaction between spectrin and actin, and in so doing maintains the deformability of the erythrocyte (Conboy, 1993). The 10kDa region was also shown to bind and regulate myosin activity (Pasternack and Racusen, 1989). Fowler and colleagues (1985) proposed that erythrocyte myosin could function together with actin and its associated proteins in an actomyosin contractile apparatus, raising the possibility of an ATP-dependent process responsible for regulating shape transformations in human erythrocytes. Cibert and colleagues (1999) explored a potential role for actomyosin complexes in the restoration of erythrocyte membrane skeletons, damaged by mechanical or chemical stress. Their model proposes that upon disruption of the skeletal network, cytosolic myosin is relocated to the erythrocyte bilayer where formation of an actomyosin complex initiates repair of the relevant area (Cibert et al., 1999). The repair process was suggested to involve a stable linkage of actin protofilaments by myosin filaments around the area of damage. The disruption of linkages between 4.1R, spectrin, actin and myosin therefore has serious consequences for the stability and repair of erythrocyte membranes. Damage inflicted by invading merozoites to the erythrocyte's protein and lipid bilayer, may initiate actomyosin repair processes. This would subsequently lead to the restoration of the damaged areas and provide a barrier for merozoite entry. *P. falciparum*, having sustained a long-standing association with human hosts, evolved complex evolutionary adaptations to ensure its own survival. It may be conceivable that specific *P. falciparum* proteins evolved to subvert host membrane repair. The interaction between EBA-181 and 10kDa domain of 4.1R may be such a mechanism, whereby EBA-181 blocks the binding of myosin to 4.1R. This prevents the activation of actomyosin repair machinery. As a result, the parasite modulates host cellular processes to guarantee successful invasion.

Binding of the *P. falciparum* invasion proteins, EBA-181 and EBA-175, to 4.1R may thus facilitate invasion and prevent repair of the damage to the

erythrocyte membrane, prior to parasite entry. Based on published findings from other groups and the EBA-181/4.1R interaction described in this work, the following highly speculative model of *P. falciparum* erythrocyte entry is proposed (Figure 38): Following initial attachment to the host cell surface, the merozoite reorients to bring its apical end in close contact with the host membrane (Gaur et al., 2004). An increase in the parasite's intracellular calcium concentration signals the secretion of the microneme and rhoptry contents onto the erythrocyte surface (Docampo and Moreno, 1996; McCallum-Deighton and Holder, 1992; Topolska et al., 2004). With the aid of a C-terminal transmembrane domain, DBL proteins are translocated from the micronemes onto the merozoite surface, where the DBL binding domains interact with erythrocyte receptors (Gilberger et al., 2003a). However, it is possible that alternative 3' splicing of messenger RNA (Singh et al., 2004) and/or post-translational proteolytic processing (Dowse and Soldati, 2004) results in DBL proteins devoid of this transmembrane domain. These proteins will then be free to interact with the erythrocyte skeleton by traversing pores in the host bilayer, created by proteases and lipases from the apical organelles (Cooke et al., 2004b; Preiser et al., 2000).

Microneme proteins, such as EBA-181 and EBA-175, may therefore serve multiple functions to mediate parasite entry into the erythrocyte: 1) the N-terminal DBL domains of the proteins initiate merozoite invasion by binding specific erythrocyte membrane receptors (Adams et al., 2001); 2) the interdomain and C-terminal regions may be involved in compromising the stability of the erythrocyte skeleton by destabilising spectrin-actin interactions as a consequence of binding to 4.1R, and 3) they may inhibit potential host membrane repair pathways.

The intimate interaction between *P. falciparum* and the erythrocyte membrane has provided fascinating insight into the cell biology of the malaria parasite. The potential functioning of the parasite proteins at the

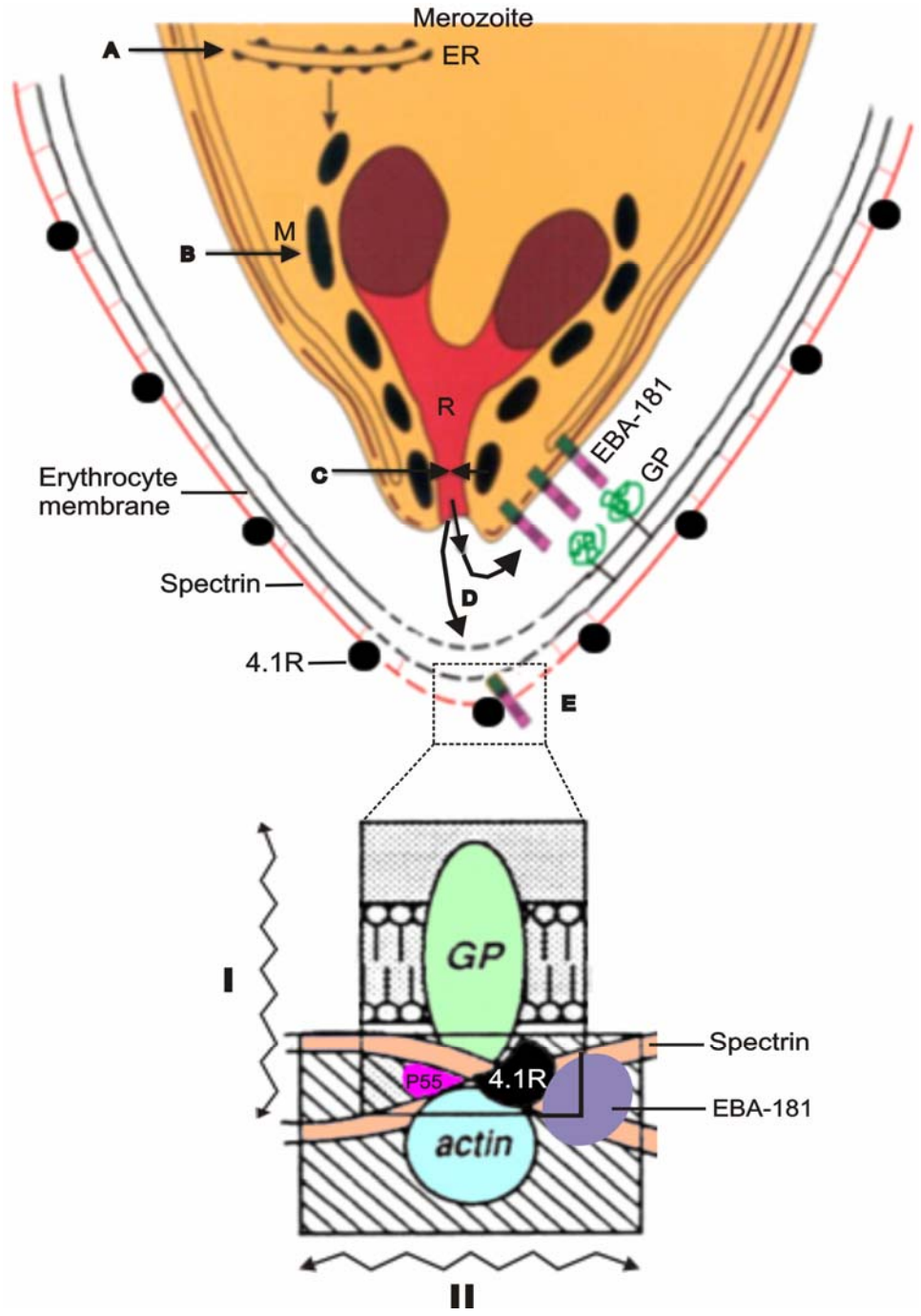

Figure 38- Proposed model of EBA-181 function during *P. falciparum* erythrocyte entry (Adapted from Palek, 1995 and Healer et al., 2002)

Once synthesised in the merozoite cytoplasm, EBA-181 enters the lumen of the endoplasmic reticulum (**ER**) (**A**), after which it is trafficked to the micronemes (**M**) (**B**). The protein is released into the rhoptry duct (**R**) upon increased calcium concentration in the parasite before invasion (**C**). Subsequently, EBA-181 is translocated onto the merozoite membrane where it interacts with glycophorin (**GP**), thus facilitating the entry process. A soluble form of EBA-181 may also move through pores in the host membrane (**D**) to the erythrocyte skeleton, where it binds 4.1R (**E**). This interaction may destabilise both vertical (**I**: GP-p55-4.1R) and horizontal (**II**: spectrin-actin-4.1R) protein interactions in the erythrocyte skeleton, thereby aiding the entry of a *P. falciparum* merozoite into the erythrocyte.

erythrocyte skeleton highlights the complex role of microneme invasion proteins.

4.3 Conclusion

The work presented here has focused on uncovering new protein-protein interactions between the malaria parasite and host erythrocyte membrane. A novel application of phage display technology was used whereby a *P. falciparum* expression library was constructed and biopanned against purified erythrocyte skeletal components. Sequencing and bioinformatic analyses enabled the identification of novel interactions between the parasite and 4.1R, as well as spectrin/4.1R complexes. These proteins may contribute to the modification of the erythrocyte membrane and in so doing, support the physiological requirements of *P. falciparum* during intra-erythrocytic development. Specifically, the interaction between 4.1R and proteins containing myosin and neurofilament-like sequences may emerge as a sustaining factor for parasite growth and survival. Further investigations revealed specific binding between the 10kDa domain of 4.1R and the myosin-like motif in EBA-181. This finding not only provides new insight into the mechanisms utilised by *P. falciparum* during erythrocyte entry, but potentially highlights the multi-functional role of malaria invasion proteins.

This research has generated data based on *in vitro* binding studies, and cannot be confidently extended to interactions *in vivo*. For this reason, verification of the 4.1R-parasite protein interactions *in vivo* offers an as yet unexplored avenue for future study. Co-precipitation, immuno-fluorescence and fluorescence resonance energy transfer assays can be performed to ascertain whether the parasite proteins of interest co-localise with 4.1R during the course of malaria infection. It would also be useful to determine whether parasite-host protein-protein interactions modulate the *in vitro* binding of 4.1R with spectrin and actin. In addition, accurate

quantitative data (including the dissociation constant) between 6His-*Pf*J and GST-10kDa may be obtained using either a calirometer or biosensor.

4.3.1 Closing remarks

The completion of the *P. falciparum* genome sequence has fundamentally changed the approach to malaria research and has shifted the focus more to the study of complex interactions of genes and proteins (Carucci, 2000). The elucidation of protein-protein interactions has emerged as an important proteomics tool and will undoubtedly lead to a better understanding of the parasite. In addition, the pursuit of agents that target such interactions is currently an active research field and may therefore contribute to the development of anti-malaria drugs and vaccines (Archakov et al., 2003).

Protein-protein interactions are extremely important in a wide range of biological processes. The identification of parasite proteins that associate with 4.1R may indeed represent novel targets for drug and vaccine design. Compounds that inhibit the association of the putative enzymes PFB0150c, MAL13P1.248 and PFI1570c with 4.1R may turn out to be crucial protein kinase and protease inhibitors. Traditional approaches target the active site of enzymes, however, drugs acting on such conserved regions may be prone to resistance by *P. falciparum* and may also bind similar domains in human proteins. Alternatively, these problems may be resolved by inhibiting enzyme-substrate complexes through drugs that target other regions where protein-protein interactions occur. The hypothetical proteins PF14_0201, MAL6P.48, PFE0570, PFA0420w and PF11_0191 may be good targets for inhibition as there are no similar proteins in humans.

The potential of *P. falciparum* invasion proteins as drug or vaccine targets has been considered in gene disruption and erythrocyte binding experiments (Bharara et al., 2004; Gilberger et al., 2003b; Pasvol, 2003;

Reed et al., 2000). Depending on the parasite's genetic background, loss of expression of EBA-175 and EBA-181 can reduce the ability of merozoites to invade human erythrocytes. However, multiple invasion pathways continue to confound the search for suitable vaccine targets. Despite this, the design of therapeutic agents that block protein-protein interactions between invasion proteins and host membrane receptors, as well as potential interactions between EBA-181 and the erythrocyte skeleton, may prove to be a feasible option.