The adhesion of malaria merozoite proteins to erythrocytes: a reflection of function?

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Introduction

The merozoites of malaria parasites are short-lived in the extracellular environment of the host and once they have burst from the infected erythrocyte, must quickly find a new red blood cell to invade. The invasion of a susceptible erythrocyte by a merozoite involves the completion of a number of rapid events before the parasite can safely reside again within a new host cell. The interaction with and entry into erythrocytes by malaria merozoites is a crucial, yet potentially vulnerable, event for parasite survival. However, since it is evident that malaria parasitism is highly successful in the hostile environment of its hosts, mechanisms to lessen this juncture of vulnerability have evolved during the establishment of the relationship between host and parasite. Through the definition of the molecular basis of invasion it may be possible, though, to discover and exploit weaknesses in the "defense strategy" of the parasite.

The observed steps of invasion can be broadly defined as (1) recognition of and attachment to the erythrocyte membrane by the merozoite, (2) positional reorientation of the merozoite while attached to the erythrocyte until the apical end of the parasite is oriented towards the host cell membrane and formation of a dense junction at the point of contact between the merozoite apical cone and the red blood cell membrane, and (3) the movement of the junction distally around the merozoite with simultaneous inward invagination of the erythrocyte membrane until the parasite sits inside the cell surrounded by the vestigal remnants of the host plasma membrane now termed the parasitophorous vacuole membrane (Dvorak *et al.*, 1975; Bannister *et al.*, 1975; Aikawa *et al.*, 1978; Miller *et al.*, 1979). A fourth event is essentially concerned with post-invasion activities and consists of resealing the erythrocyte membrane and the subsequent migration of dense bodies or microspheres to the parasite membrane where the contents of these vesicles are released into the space formed between the parasitophorous vacuole and the parasite membranes (Torii *et al.*, 1989).

The first and second events of invasion have been known for some time to have a degree of specificity associated with them. Merozoites of a given species of *Plasmodium* are capable of interacting only with erythrocytes from a limited range of species. However, even if there is some form of initial interaction between a merozoite and an erythrocyte of one species, invasion may not be able to be completed if secondary specificities are also not present. Presumably, this implies that, at the molecular level, more than one type of specific interaction between the parasite and erythrocyte must occur at the initiation of and during invasion. For example, the merozoite may attach initially but fail to reorient its position and then detach, or it may attach, reorient, but fail to form the crucial dense junction with the host cell membrane, at which point invasion does not proceed further. These experimentally dissected levels of specificity indicate that merozoite ligands interact with specific receptor molecules in the erythrocyte membrane to facilitate contact and to initiate the physically characterized events of invasion. The characterization of the parasite protein ligands and their corresponding erythrocyte receptors that participate in invasion and host cell recognition is a vital component in understanding the molecular dynamics of the invasion process. As well, the knowledge gained could help provide a rational base from which to generate insights that may aid the development of a vaccine capable of inducing immune responses that will effectively block invasion.

In recent years, since the initial and novel report of Camus and Hadley (1985) on the binding of a parasite antigen in *in vitro* culture supernatants to intact erythrocytes, progress towards the identification of merozoite ligand proteins potentially involved in host cell invasion has advanced considerably for the human malaria parasites *Plasmodium falciparum* and *P. vivax* and the model simian malaria *P. knowlesi*. Table I summarizes those merozoite proteins that have been found to bind to erythrocytes. This commentary will review these developments and the basis for an interpretation of function.

Table I. Summary of malarial merozoite adhesion proteins that bind to primate erythrocytes.

P. falciparum	*P. vivax*	*P. knowlesi* (*)
Pf200 (MSA-1,gp195,)	Pv250 (RBP-1&2)	Pk250 (MSP-1?)
Pf175 (EBA 175)	Pv210 (MSP-1,Pv200?)	Pk180/150 (**)
	Pv160/145 (RBP-3)	Pk160
	Pv140 (PvDAP-1)	Pk155
		Pk135 (PkDAP-1)
		Pk125 (***)
		Pk120 (***)

(*) The list refers to *P. knowlesi* culture supernatant proteins that have been found to bind to rhesus and other primate erythrocytes. Generally, only Pk250, Pk135, and Pk125 bind to human erythrocytes. All except Pk125 bind to rhesus monkey erythrocytes.

(**) PK180/150 is homologous to Pv160/145 or PvRBP-3 except it does not bind selectively to reticulocytes as does PvRBP-3.

(***) Pk125 and Pk120 are weak bands in fluorographs of human and rhesus monkey EBA, respectively. Although reported to be related to *P. knowlesi* Duffy binding ligand(s) (Adams *et al.*, 1990), the exact nature of these two proteins to each other and binding to the Duffy receptor need further clarification.

P. falciparum merozoite proteins that bind to erythrocytes

Two merozoite proteins have been reported in the literature to bind to human erythrocytes. Camus and Hadley (1985) first reported the binding to intact erythrocytes of a *P. falciparum* protein from *in vitro* culture supernatants in which the specificity of binding correlated with certain known requirements for the invasion of red blood cells by this parasite species (Perkins, 1981; Pasvol *et al.*, 1982). That is, it had been previously determined that erythrocyte membrane glycophorin, in particular, its sialic acid, was required for the invasion of erythrocytes by *P. falciparum* merozoites. This 175-kDa erythrocyte-binding protein, designated EBA 175, bound to human and aotus erythrocytes, which are invaded, but not mouse, rabbit, or rhesus monkey erythrocytes, which are not invaded. Furthermore, human erythrocytes treated with trypsin, which cleaves glycophorin from the erythrocyte membrane, or neuraminidase, which removes sialic acid, or human TN variant erythrocytes, which lack sialic acid on the o-linked oligosaccharides of glycophorin, did not bind the 175-kDa protein and were not invaded by *P. falciparum* merozoites. These and other experiments by these investigators led to the suggestion that this *P. falciparum* protein was involved in erythrocyte recognition and invasion.

The second *P. falciparum* merozoite protein that has been shown to bind in a specific manner to human and certain primate erythrocytes was first identified and its complete gene characterized a number of years ago (Holder and Freeman, 1982; Holder *et al.*, 1985). However, only recently was it shown that the intact merozoite surface protein, MSA-1 (gp195, pf200, p190) also binds to erythrocytes (Perkins and Rocco, 1988). Like EBA-175, the binding of MSA-1 to human erythrocytes is dependent upon the sialic acid of the glycophorin glycoproteins and bound only to those cells susceptible to infection by *P. falciparum*. Soluble glycophorin, a monoclonal antibody (mAb) reactive with a specific glycosylated domain of glycophorin, and a mAb to MSA-1 inhibited the binding of MSA-1 and immunoprecipitated the protein from culture supernatants.

A major question raised by these two sets of data and observations is the relationship, if any, between the 175-kDa protein and the approximately 195-205-kDa protein, MSA-1. Both are similar in relative mobility (Mr) as determined by SDS-PAGE analysis and appear to bind to the same or a similar receptor on erythrocytes. The gene for EBA-175 has been recently cloned (Sim *et al.*, 1990) and the deduced amino acid sequence is unrelated to that of MSA-1. Antibody to a 45-mer peptide of EBA-175 and antibodies from immune aotus serum purified on bound native or recombinant protein recognize the native protein (Orlandi *et al.*, 1990) and by indirect immunofluorescence (IFA) localize the protein to the apical region of merozoites as a small dot (Sim *et al.*, 1990). The deduced amino acid sequence of EBA-175 does not have a hydrophobic region typical for transmembrane anchoring, but prior data has suggested that this polypeptide is able to bind to the surfaces of both the merozoite and erythrocyte, thus leading to the hypothesis that it acts as a "bridge" between the merozoite and the red blood cell (Camus and Hadley, 1985). On the other hand, MSA-1 is located over the entire surface membrane of developing schizonts and the surface of mature merozoites and is probably attached to the membrane via a glycolipid anchor (Haldar *et al.*, 1985). Although the erythrocyte receptor appears to be similar for the two proteins, their different sites of localization and lack of any sequence similarity could

indicate the possibility that the two merozoite proteins operate at separate points to serve different but necessary functions during the events of invasion.

The other major question raised by the results of Perkins and Rocco (1988) on the adhesion of MSA-1 to red cells, which have been corroborated by one of us (JWB), concerns the nature of the actual molecular structure of MSA-1 on the surface of the *invasive* merozoite. Several studies with *P. falciparum* and also *P. knowlesi* have suggested that the large molecular weight form of MSA-1 is a "precursor" molecule that at or about the time of merozoite release from the infected red blood cell is proteolytically processed to form three fragments that remain attached to the merozoite membrane, presumably as a complex (Freeman and Holder, 1983; Holder and Freeman, 1984; David *et al.*, 1984). However, the form of MSA-1 that binds to erythrocytes is predominantly the intact high MW "precursor" polypeptide and not any of the smaller fragments that have been identified on the merozoite surface. It is well known that MSA-1 is prone to degradation by proteases present in the schizont and merozoite and some of the processing fragments are artifacts created during detergent extraction (David *et al.*, 1984). Some of the proteases are not inhibited or are only partially inhibited by common inhibitors of the cysteine and serine proteases. While several studies have indicated that MSA-1 exists only as processed fragments on the merozoite surface, some results with metabolically radiolabelled *P. falciparum* merozoites could suggest otherwise (Pirson and Perkins, 1985; Aikawa *et al.*, 1986). In fact, the processing could be the result of *in vitro* degradation. The identification of the processed fragments on the merozoite surface has been mostly by radioiodination of the merozoite surface. There is always the chance that the time and manipulations involved in the labelling procedure could allow for degradation of MSA-1 to occur, in which case, only fragments of MSA-1 would be visualized. The fact that merozoites after labelling with radioactive iodine are not invasive supports this possibility. Since the unprocessed form of MSA-1 of both *P. falciparum* (Perkins and Rocco, 1988; J. Barnwell, unpublished results) and *P. knowlesi* (J. Barnwell, unpublished results) can be precipitated from culture supernatants, this may well be the form of the protein on the surface of viable invasive merozoites. We believe this to be the case and the binding of MSA-1 to erythrocytes reflects a functional role in the initial attachment of merozoites to erythrocytes. This, however, would not necessarily mean the specific fragments identified on the merozoite surface are biologically insignificant. Protease activity appears to play a functional role in invasion (Hadley *et al.*, 1983a) and the specific fragments of MSA-1 may be products of this activity which could occur during the initial contact and reorientation of the merozoite on the erythrocyte surface. Several specific proteases have been described from merozoites (recently reviewed by Schrevel *et al.*, 1990) one of which may be activated to cleave MSA-1 that is bound to the erythrocyte surface (or, for that matter, could MSA-1 have some autoproteolytic activity?) Systematic binding and proteolysis could produce a gradient of contact to facilitate directionally oriented movement towards the apical pole. Such activity could also occur or be stimulated in the artificial serumless *in vitro* environment needed for ^{125}I-surface labelling. Obviously, further experimentation is needed to clarify these issues.

The Duffy adhesion proteins of *P. vivax* and *P. knowlesi* merozoites

P. vivax and the related simian malaria, *P. knowlesi* are distinct from *P. falciparum* in many ways, including the specificity of the mechanism for erythrocyte invasion. Although the mechanics of invasion may be similar between species, the fine specificity and molecular nature of the merozoite ligands and their erythrocyte receptors are quite dissimilar.

Invasion of human erythrocytes by the merozoites of either *P. vivax* or *P. knowlesi* is dependent upon a red blood cell membrane glycoprotein serologically defined by the Duffy blood group determinants. A *P. vivax* 135-140-kDa protein (Wertheimer and Barnwell, 1989) and a *P. knowlesi* 135 kDa protein (Haynes *et al.*, 1988; Wertheimer and Barnwell, 1989) present in culture supernatants have been identified and shown to bind specifically to the 43-kDa Duffy glycoprotein (Hadley *et al.*, 1983b) on human and other primate erythrocytes. The merozoite derived *D*uffy *a*dhesion *p*roteins (DAP) do not bind to phenotypically Duffy-negative erythrocytes. There is an inhibition of binding of the DAP when Duffy-positive erythrocytes are treated by a specific protease (chymotrypsin), or with anti-Duffy allotypic and mAb, or when the DAP-containing culture supernatants are preincubated with purified Duffy glycoprotein. The same treatments also inhibit *in vitro* invasion of human erythrocytes by *P. vivax* and *P. knowlesi* merozoites (Barnwell *et al.*, 1989; Miller *et al.*, 1975).

Antibody cross-reactivity has indicated the DAP of *P. vivax* and *P. knowlesi* are antigenically related (Wertheimer and Barnwell, 1989) and by immunofluorescence on unfixed merozoites appears to be located at the very apical prominence of the merozoite (Barnwell and Wertheimer, manuscript in preparation). However, SDS-PAGE analyses of the native proteins indicate there are some structural differences. The native *P. vivax* DAP, under reduced conditions, migrates as a broad band that appears to be a doublet; the *P. knowlesi* DAP migrates as a single sharp band. Under non-reducing conditions,

the native *P. knowlesi* DAP band remains a sharp band that does not show a change in Mr. In contrast, the native *P. vivax* DAP does show a change in Mr under non-reduced conditions of SDS-PAGE analysis. In fact, the suspected doublet becomes more apparent and both bands migrate at a faster Mr, and in addition, a third band of Mr 150 kDa which is not seen under reducing conditions becomes apparent. This raises the interesting possibility that perhaps the *P. vivax* DAP is a protein complex, since the 150-kDa protein band does not bind to Duffy-negative red cells.

Within the past year, the cloning of a gene for the 135-kDa *P. knowlesi* "Duffy receptor" (DR) (Adams *et al.*, 1990) and the presumptive gene for the *P. vivax* homologue (Fang *et al.*, 1991) have been reported. The former work concludes that in *P. knowlesi,* the cloned DR gene is part of a gene family and that the product(s) is located in the apical micronemes of the merozoite. Rabbit antisera to fusion proteins immunoprecipitated, in addition to two major bands of 135 and 138 kDa, a number of minor bands of 160, 155, and 120 from culture supernatants. The former bands were absorbed out with human Fy^{a-b+} erythrocytes and rhesus monkey erythrocytes respectively, suggesting cross-reactivity and perhaps expression of two gene family products. The 160- and 155-kDa proteins do bind to various primate erythrocytes, except for human red cells, but the bands in the anti-PkDR immunoprecipitates could just as well have been background or minor coprecipitates instead of being expressed representatives of the PkDR gene family.

The *P. vivax* DR gene, which was cloned using the *P. knowlesi* gene sequence as a probe, is closely related to the PkDR gene. Both genes have two cysteine-rich regions and an intervening proline-rich region. Interestingly, our data suggests that interchain disulphide bonds are absent in the native PkDAP and present in the native PvDAP. It has not yet been determined if antibody to the expressed product of the presumptive *P. vivax* DR gene immunoprecipitates PvDAP. If the presumptive PvDR gene is, in fact, proven to be the *DAP* gene, it is intriguing that PvDAP forms apparent interchain disulphide bonds, while the PkDAP may not.

P. vivax and the invasion of reticulocytes

P. vivax merozoites, in addition to being dependent upon the Duffy glycoprotein, and unlike either *P. falciparum* or *P. knowlesi,* are dependent upon the host red blood cell being a reticulocyte instead of a normocyte for invasion to be successful. It has been suggested that this very strong preference for invading reticulocytes may occur because *P. vivax* merozoites are incapable of invading normocytes due to a mature cytoskeletal protein network that makes the normocyte membrane less pliable, whereas, the reticulocyte membrane with its less mature cytoskeleton and more pliable membrane is more readily invaginated by the merozoites during invasion (Mons, 1990). This would mean that the merozoite would have to go about bouncing from cell to cell, attaching to and testing each cell at each contact, until it finds the cell with a membrane pliable enough for it to successfully invade. Given the normal range of reticulocytes in circulation, this could be between one to five hundred red blood cell contacts; a very inefficient way to survive in a potentially hostile environment. Thus, it would seem just as likely that *P. vivax* or any other reticulocyte preferring a malaria parasite such as *P. cynomolgi,* a simian malaria, or *P. berghei,* a rodent malaria, would have some mechanism to selectively discriminate between normocytes and reticulocytes.

We have recently identified and cloned the genes encoding two *P. vivax* merozoite proteins that could well be a factor(s) in a mechanism for selecting reticulocytes (Galinski *et al.*, 1991, submitted). Two proteins of approximately 250 kDa bind preferentially to reticulocytes of humans and other primates except for rhesus monkeys (*P. vivax* does not invade rhesus monkey erythrocytes, although they are Duffy-positive) and IFA analysis indicates these proteins are located at the apical portion of the merozoite. The deduced protein secondary structures and other general characteristics of these proteins are similar; however, the overall identity of the amino acid sequence between the two seems to be low. Whether the two proteins operate separately or as an associated complex in their interaction with a receptor on reticulocytes is uncertain at this time. Further analyses are needed to validate our thesis that the products of these two genes operate to aid *P. vivax* merozoites in the selective invasion of reticulocytes. It will be crucial to identify the reticulocyte receptor and certainly the binding domain(s) of these two *P. vivax* polypeptides we term as *r*eticulocyte *b*inding *p*roteins-1 and -2 (RBP-1 and RBP-2). It may be of some further interest to note that Southern blot analyses indicate that the *P. vivax* RBP-1 and RBP-2 genes only hybridize to DNA of *P. cynomolgi,* a simian malaria closely related to *P. vivax* that also invades primarily reticulocytes, and not to the DNA of *P. knowlesi* or *P. falciparum,* both of which do not preferentially invade reticulocytes.

Do the MSA-1 polypeptides of *P. vivax* and *P. knowlesi* bind to erythrocytes?

Two other high molecular weight proteins from *P. vivax* and *P. knowlesi* culture supernatants also specifically bind to human and primate erythrocytes. The *P. vivax* protein migrates with an Mr of

205-210 kDa, while the *P. knowlesi* protein is approximately 240-250 kDa, which was previously reported to migrate at 220-230 kDa (Wertheimer and Barnwell, 1989; Miller *et al.*, 1988). These MW are close in size to the MSP-1 polypeptides of *P. vivax* and *P. knowlesi* (Barnwell, 1986; del Portillo *et al.*, 1988; Epstein *et al.*, 1981) and it is tempting to speculate that the Pv210 and Pk250 erythrocyte-binding proteins possibly correspond to the MSP-1 polypeptides of these two species, especially since the analogous *P. falciparum* MSP-1 adheres to erythrocytes. Experiments to determine this possibility are in progress and some preliminary results are in support of this notion. However, further exploration of this question is needed before a definitive answer can be forthcoming.

It was previously reported that these two proteins, Pv210 and Pk250, did not bind to trypsinized red blood cells and the binding was either substantially reduced or absent when the erythrocytes were treated with chymotrypsin (Wertheimer and Barnwell, 1989). We have recently reexamined this data using other sources of the purified proteases and find that, in fact, trypsinization does not prevent the binding of these proteins but that the chymotrypsin treatment does. This is an important point, since trypsinization does not prevent the attachment of *P. knowlesi* (or *P. vivax*) merozoites to erythrocytes, whereas chymotrypsin treatment of the red cells does prevent the attachment of merozoites. This correlation now adds more credibility to the idea that these proteins could have a functional role in merozoite attachment to erythrocytes, whether or not they prove to be MSP-1.

Carbohydrate on receptors is not involved in the interaction of *P. vivax* and *P. knowlesi* merozoite adhesion ligands

Further differences between *P. falciparum* and *P. vivax* (and related malaria species) are also evident in the molecular nature of the interaction of the merozoite adhesion proteins with their erythrocyte receptor proteins. Both *P. falciparum* binding proteins identified to date, MSA-1 and EBA 175, bind to the sialic acid residues of the O-linked oligosaccharide chains of glycophorin and thus are similar to each other in this respect. However, none of the erythrocyte adhesion proteins of *P. vivax* or *P. knowlesi* appear to bind to carbohydrate moieties of erythrocyte glycoproteins. We have found that the treatment of erythrocytes with specific endoglycosidases such as neuraminidase, N-glycanase, O-glycanase, endoglycosidase H, or endo-β-galactosidase, did not have an effect on the binding of PvDAP, PkDAP, Pv210, Pk250, or the 250 kDa PvRBP-1- and RBP-2 (Barnwell and Wertheimer, 1991; Galinski *et al.*, 1991: manuscripts to be submitted). This was somewhat surprising for two reasons, especially in the case of the interaction of the DAP with the Duffy glycoprotein receptor. First, it is known that the Duffy glycoprotein is heavily glycosylated with N-linked oligosaccharides (Tanner *et al.*, 1988). N-glycanase completely removes carbohydrate from the peptide backbone of the 43-kDa glycoprotein, increasing its Mr to 28 kDa (Barnwell and Wertheimer, 1991, manuscript to be submitted). Secondly, it was recently reported that both fucoidan, a sulphated polysaccharide, and dextran sulphate, a glycosylaminoglycan, block the binding of PkDAP to human and rhesus monkey erythrocytes, selectively bind PkDAP in culture supernatants, and inhibit invasion of human Fy a^-b^+ erythrocytes and rhesus monkey erythrocytes (Dalton *et al.*, 1991). It was suggested from these results that these large polymers may mimic a similar negatively charged group, perhaps carbohydrate, on the Duffy glycoprotein receptor that binds to the merozoite DAP ligand. However, the observed interaction of the sulphated polysaccharrides with the PkDAP ligand could have alternative interpretations, especially, since we have shown that removal of the carbohydrate on the Duffy glycoprotein does not affect its interaction with PkDAP or PvDAP. Fucoidan or dextran sulphate may bind at a site on the DAP not relevant to the receptor-ligand interaction site but still block binding by either affecting DAP molecular conformation or through steric hindrance. Additionally, the data on the inhibition of merozoite invasion by these polymers could indicate some other action of these substances other than blocking DAP recognition of its receptor. Invasion of rhesus erythrocytes, unlike invasion of human erythrocytes by *P. knowlesi* merozoites, is independent of any interaction of PkDAP with the Duffy glycoprotein. Chymotrypsin treatment of rhesus monkey erythrocytes abolishes DAP binding, but has absolutely no effect on invasion, and yet the sulphated polysaccharides inhibited the invasion of rhesus monkey erythrocytes by *P. knowlesi*. In general, it seems that the binding of merozoite proteins to erythrocytes in *P. vivax* and *P. knowlesi* represents protein-protein interactions, whereas, *P. falciparum* proteins, so far, appear to be lectin-like interactions.

Concluding remarks

That some malarial proteins present in supernatants acquired from *in vitro* cultures of schizonts and released merozoites will bind to red blood cells has opened up new opportunities for identifying those parasite proteins that are possibly involved in erythrocyte recognition, specificity and the invasion mechanism. This binding, however, does not in itself offer *a priori* proof that these interactions can be interpret-

ed to have biological significance or relevance. Adjunctive assays, as well as the development of specific immunological reagents, the cloning of the respective parasite genes and identification and characterization of the erythrocyte receptors are needed to further define the functional significance and mechanistic roles of the various merozoite erythrocyte-binding proteins. In addition, the EBA as currently performed may not reveal some parasite proteins potentially involved in invasion, and some proteins are not consistently revealed depending upon their stability, the culture conditions employed and other varied technical factors that may differ between laboratories. Nevertheless, the experimental evidence discussed here suggests that most of the major erythrocyte binding proteins that have been identified probably perform some functional role in the invasion of erythrocytes by malaria merozoites.

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Malarial proteases: assignment of function to activity

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Introduction

As classical drug treatment of malaria becomes less efficient due to the appearance of resistant parasites, the development of new antimalarial agents is of importance. Enzymes involved in essential parasitic physiological pathways represent an interesting target, since their specific inhibition should block parasite growth.

Whether in prokaryotic or eukaryotic organisms, proteases have been shown to be involved in many important physiological events, such as protein maturation, gene regulation, metabolic pathway regulation, blood coagulation and immune reactions. Parasite proteases have been described as being involved in host protein degradation, protein processing, invasion of host cells and immune evasion. In this context, plasmodia proteolytic activities could be considered as targets for new drugs based on highly specific inhibitors.

The design of highly specific inhibitors depends on the determination of both the cleavage site and efficient peptidic substrates of a peptidase. For this reason, we will only focus on endopeptidases, from

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