<table>
<thead>
<tr>
<th>Page</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Erythrocyte binding ligands in malaria parasites: Intracellular</td>
</tr>
<tr>
<td></td>
<td>trafficking and parasite virulence.</td>
</tr>
<tr>
<td></td>
<td>Culleton, Richard; Kaneko, Osamu</td>
</tr>
<tr>
<td></td>
<td>Acta tropica, 114(3), pp.131-137; 2010</td>
</tr>
<tr>
<td></td>
<td>Copyright © 2009 Elsevier B.V. All rights reserved.</td>
</tr>
</tbody>
</table>

NAOSITE: Nagasaki University’s Academic Output SITE
Erythrocyte binding ligands in malaria parasites: Intracellular trafficking and parasite virulence
Richard Culleton and Osamu Kaneko

Department of Protozoology, Institute of Tropical Medicine (NEKKEN) and the Global Center of Excellence Program, Nagasaki University, Sakamoto, Nagasaki 852-8523, Japan

Corresponding author: Osamu Kaneko, Department of Protozoology, Institute of Tropical Medicine (NEKKEN), Nagasaki University, Sakamoto, Nagasaki 852-8523, Japan; Tel.: +81 95 819 7838; fax: +81 95 819 7838; e-mail: okaneko@nagasaki-u.ac.jp

Keywords: Erythrocyte; Invasion; Plasmodium; EBL; RBL; DARC

Abstract
The intracellular trafficking of an Erythrocyte Binding Like (EBL) ligand has recently been shown to dramatically affect the multiplication rate and virulence of the rodent malaria parasite Plasmodium yoelii yoelii. In this review, we describe the current understanding of the role of EBL and other erythrocyte binding ligands in erythrocyte invasion, and discuss the mechanisms by which they may control multiplication rates and virulence in malaria parasites.

1. Multiplication rate and virulence in malaria parasites

The multiplication rate of malaria parasites in the blood of the vertebrate host is often positively correlated with the severity of the disease they cause (Chotivanich et al., 2000). Such multiplication rates vary between species of parasites, and indeed, between strains of the same species (Yoeli et al., 1975; Dondorp et al., 2005; Reilly et al., 2007). Although many factors may influence the virulence of a parasite within a host, there is increasing evidence that such differences can be caused by parasite genetic polymorphism (Walliker et al., 1976; Rowe et al., 1997). Understanding the genetic basis of parasite virulence may pave the way for the design of anti-disease interventions such as novel drugs and vaccines.

In 1975 Yoeli et al. published a report describing the sudden acquisition of “virulence” in a line of the rodent malaria parasite Plasmodium yoelii yoelii (Yoeli et al., 1975). Prior to its sudden and dramatic change in character, the parasite line (17X) had exhibited a typical P. y. yoelii phenotype in laboratory mice, in which growth is restricted, almost exclusively, to very immature erythrocytes (reticulocytes), and which is characterized by a mild and chronic infection that is rarely fatal and often self-resolving. The parasite line in its original phenotypic state had been cryopreserved in the author’s laboratory following its arrival from a colleague at another institution. Upon reconstitution of the line, the parasite exhibited a dramatic alteration in phenotype; its growth remained restricted to reticulocytes for the first four days of the infection, at which point it invaded mature erythrocytes, attained parasitaemias in excess of 80%, and killed the host by the eighth day of infection. The new “virulent” line was subsequently typed by enzyme markers, which confirmed that it was almost certainly P. y. yoelii and not another contaminating species resulting from a laboratory error, and further tests showed that there were no confounding co-infections of bacteria or viruses that may have altered the virulence of the line. Given the sudden way the parasite had acquired its ability to invade and multiply within mature erythrocytes, and combined with the fact that the phenotype was stable through multiple passages in mice, and importantly, also through mosquitoes, the authors speculated that a genetic mutation may have been responsible for the observed alteration in virulence.

Genetic crossing experiments subsequently conducted by Walliker et al. clearly showed this to be the case (Walliker et al., 1976). Crosses were made between a clone of the virulent parasite (now named “YM”, an acronym of “Yoeli’s mouse”), and a genetically unrelated avirulent line (A/C). Of 56 clones of the resulting products of the cross, 34 were recombinants between the two parental types (detected by enzyme type and drug resistance markers) all of which, except one, could be unequivocally assigned either a virulent or avirulent phenotype comparable to the parental strains. The remaining clone exhibited an intermediate virulence phenotype which caused infections in mice in which mature erythrocytes were invaded, and peak parasitaemias of 75% were observed, but which did not result in the death of the host. The simple Mendelian fashion in which the virulence phenotype was inherited in the progeny of the cross suggested that the trait was, indeed, genetically controlled, and possibly by a single gene. The authors went on to discuss the feasibility of identifying the product of this gene and determining how it ultimately controls virulence.

Recent work using modern genotyping techniques has shown that the virulent YM parasite is indeed very probably isogenic with an avirulent parasite type derived from the original 17X isolate, supporting the suggestion that the sudden virulence increase was caused by a genetic mutation (Pattaradilokrat et al., 2008). Several clones were derived from the original 17X isolate, all except one of which appear to be of a single genotype. The virulent strain seems to have appeared independently at least three times from the original isolate, and is also known as 17XL, while the isogenic avirulent clones are known as 17X or 17XNL (Pattaradilokrat et al., 2008). This year, 34 years following Yoeli et al.’s initial observation, two reports appear to have finally identified the major genetic determinant of the multiplication rate differences between the virulent and avirulent P. y. yoelii lines. Otsuki et al. (2009) identified a single amino acid substitution in the P. yoelii Erythrocyte Binding Like (PyEBL) protein that was responsible for controlling the virulence differences between 17XL and 17XNL. At the same time, Pattaradilokrat et al. (2009) used Linkage Group Selection (LGS) (Culleton et al., 2005) to identify a region on P. y. yoelii chromosome 13 which was perfectly linked to the multiplication phenotype in the uncloned progeny of a cross between YM and 33X (a genetically unrelated avirulent P. y. yoelii clone). Following communication of Otsuki et al.’s results, they subsequently found that the gene encoding the P. y. yoelii gene...
PyEBL protein (pyebl) marked the trough of the selection valley observed following selection of the progeny for fast multiplying parasites. Combined, these two reports provide strong evidence that the major determinant of the virulence differences between these two parasites is controlled by pyebl. Otsuki et al. showed that a single amino acid substitution from a Cys (17XNL) to an Arg (17XL and YM) in PyEBL region 6 (R6) altered its localization within the merozoite from the micronemes to the dense granules. Based on these findings, along with the highly conserved nature of the Cys residues in EBL R6 between different malaria parasite species, the authors suggest that this region is a protein trafficking signal that controls transport of the protein to the micronemes. Thus, the substitution of the second Cys for an Arg abolishes the native conformation of region 6 by disrupting one of the four disulphide bonds formed between the eight Cys residues usually present in this region (Fig. 2) (Withers-Martinez et al., 2008). This subsequently leads to the incorrect trafficking of the protein to the dense granules rather than the micronemes, and dramatically increases parasite multiplication rate and virulence by allowing invasion and development within mature erythrocytes as well as reticulocytes. These results, along with a description of the EBL protein family, are discussed in more detail in the following sections.

2. Erythrocyte-Binding-Like (EBL) family

It has long been observed that individuals of west and central African origin are completely refractory to infection with *P. vivax*. It was subsequently shown that this protection is caused by the “Duffy-negative” phenotype characteristic of individuals from this region. The Duffy antigen/receptor for chemokines (DARC) is a transmembrane glycoprotein that is present on epithelial cells (Horuk et al., 1997), endothelial cells (Hadley et al., 1994), and erythrocytes. It is encoded by a single copy gene, of which there are four major alleles, FY*A, FY*B, FY*X, FY*Bnull (Zimmerman et al., 1999; Pruenter and Rot, 2006). Combinations of these alleles result in four common phenotypes, Fy(a-b-), Fy(a-b+), Fy(a-b+weak) and Fy(a-b-), FY*A and FY*B, by far the most common alleles among non-African populations, both encode functional proteins and differ from each other by a single amino acid change in the NH2 extracellular domain. The FY*Bnull allele carries a single nucleotide mutation which impairs promoter activity by disrupting a binding site for the h-GATA-1 erythroid transcription factor (Tourmamille et al., 1995). This results in the loss of DARC expression on erythrocytes, but does not affect expression in epithelial or endothelial cells. Individuals who are homozygous for this allele, thus express no DARC protein on the erythrocyte surface. In the late 1980s, a 135-140-kDa *P. vivax* molecule was identified as the ligand that binds to DARC (Haynes et al., 1988; Wertheimer and Barnwell, 1989). Using *P. knowlesi* monkey malaria parasites, the gene encoding *P. knowlesi* Duffy binding protein (PkDBP) was identified in 1990 (Adams et al., 1990) followed closely by the identification of the gene encoding *P. vivax* DBP (PvDBP) (Fang et al., 1991). Interestingly, in addition to the PkDBP (PKDBP-a) protein that specifically recognizes DARC, two paralogous molecules that recognize alternative erythrocyte receptors were also identified in *P. knowlesi*. In *P. falciparum*, a 175-kDa molecule (EBA-175) that recognizes sialic acid on glycophorin A was reported (CamU.S.A.nd Hadley, 1985) and the gene encoding the EBA-175 protein was subsequently sequenced (Sim et al., 1990). Later, the *P. falciparum* genome project revealed other actively expressed paralogs, BAEBL (EBA-140) and JESEBL (EBA-181), that bind to glycophrin C and an unknown receptor, respectively (Gaur et al., 2004). With their distinct binding specificity, such proteins provide alternative erythrocyte invasion pathways in *P. falciparum* and, probably, also in *P. knowlesi*. These erythrocyte binding proteins of *P. vivax*, *P. knowlesi*, and *P. falciparum* share similar protein architecture and their genes were termed the erythrocyte binding like (*ebl*) family (Adams et al., 2001; Gaur et al., 2004). Homologous genes have also been identified in other *Plasmodium* species including the rodent malaria parasites (Prasad et al., 2003).

The genes of the *ebl* family encode type I integral transmembrane proteins that possess two Cys-rich regions which are conserved among orthologs (Fig. 1). The N-terminal Cys-rich region (termed region 2 (R2)), which consists of one or two DBL (Duffy-Binding-Like) domains (Adams et al., 1992), is responsible for erythrocyte surface receptor recognition. Recombinant R2 of PvDBP (one DBL domain) and EBA-175 (two DBL domains) expressed on COS cells was shown to bind erythrocytes, indicating that this region is the binding domain of the EBL proteins (Chitnis and Miller, 1994; Sim et al., 1994). The binding specificity of EBL protein members have previously been reviewed in detail (Gaur et al., 2004).

3. Modification of EBL region 6 alters EBL localization, erythrocyte-type invasion preference, and, consequently, parasite virulence

The C-terminal Cys-rich region 6 (R6) (or the C-cys domain) is located adjacent to the transmembrane region with the number (eight) and location of Cys residues being highly conserved among known EBL proteins. The crystal structure of EBA-175 R6 reveals structural similarity to the KIX-binding domain of the coactivator CREB-binding protein, which can simultaneously bind to two independent proteins using two different hydrophobic interfaces. Due to this similarity, EBA-175 R6 has been proposed to possess the ability to bind two proteins (Withers-Martinez et al., 2008). An early hint of the function of R6 as a microneme trafficking signal came from an observation that the EBA-175 protein with R6, the transmembrane region, and the cytoplasmic tail removed was not trafficked to the microneme, which is the usual destination of EBL proteins (Gilberger et al., 2003). Furthermore, Treeck et al. (2006) showed that GFP fused with EBA-175 R6 is trafficked to the microneme, confirming that R6 is not only necessary, but also sufficient, for microneme trafficking.

EBL proteins are not confined to merozoites, as sporozoites, which invade mosquito salivary glands and mammalian hepatocytes, also possess an EBL-related protein termed MAEBL (Kappe et al., 1998; Kariu et al., 2002) MAEBL has similar protein structure to other EBLs, but possesses duplicated AMA-1-like domains (D1 and D2) instead of a DBL domain in R2. AMA-1-like domains also exhibit cell-binding abilities (Kappe et al., 1998; Ghai et al., 2002), thus the biological function of MAEBL is likely to be similar to the classic DBL domain possessing EBL proteins. MAEBL is known to localize in the micronemes of sporozoites and this, combined with the conservation of the number and position of the Cys residues in R6 with other EBLs, it seems likely that MAEBL R6 is responsible for
microneme trafficking in sporozoites. A hypothetical escortor protein was proposed to bind to EBL R6 and transport it from the Golgi apparatus to the micronemes in merozoites (Treeck et al., 2006). Such a mechanism (and possibly the escortor protein itself) may very well be conserved between merozoites and sporozoites.

During the characterization of *P. y. yoelii* EBL in order to evaluate a potential rodent malaria model for an EBL-based vaccine, Otsuki and colleagues observed that the second Cys residue in PyEBL R6 was substituted to Arg in the virulent line 17XL, but not in the avirulent line 17X (Fig. 2) (Otsuki et al., 2009). An immunofluorescent assay using a PyEBL-specific antibody revealed that the cellular location of the protein differed between the two lines. Immunoelectron microscopy revealed that PyEBL was trafficked to the dense granules, rather than the micronemes, of the 17XL line. This led them to hypothesize that the observed substitution in R6 may be responsible for the difference in PyEBL localization and potentially for the pathogenic differences between these two lines of *P. y. yoelii*, as modulation of an EBL gene in *P. falciparum* was previously shown to alter its erythrocyte invasion preference (Reed et al., 2000).

In order to evaluate this hypothesis, the PyEBL allelic type was genetically exchanged between the two lines. The replacement of Arg to Cys in the 17XL line (17XL-RtoC) altered the PyEBL localization from a non-apical diffused pattern to an apical pattern. In the 17X line, replacement of Cys to Arg (17X-CtoR) altered the localization from an apical pattern to a non-apical diffused pattern. These results confirmed that the substitution from Cys to Arg in R6 was responsible for the altered localization of PyEBL from the micronemes to the dense granules in the 17XL line. Furthermore, the 17XL-RtoC parasite predominantly invaded young erythrocytes in the same way as the avirulent 17X line, whereas the 17X-CtoR parasite was able to invade a variety of ages of erythrocytes including mature erythrocytes, comparable to the virulent 17XL line, thus demonstrating that the localization of PyEBL is responsible for the erythrocyte-type preference of *P. y. yoelii*.

Allelic replacement also affected parasite virulence. Mice infected with the 17XL-RtoC parasite developed significantly lower parasitemias compared with the parental 17XL line, and the infection was non-lethal, whereas mice infected with the 17XL lines inevitably died by day 7 (Fig. 3). The pattern observed for the 17XL-RtoC parasite was identical to that observed for the avirulent 17X line. Thus, the trafficking of PyEBL to the micronemes caused the virulence of the 17XL line to be reduced to the same level as the avirulent 17X line, suggesting that PyEBL is the critical virulence determinant of the 17XL line. The parasitemia of mice infected with the 17X-CtoR parasite increased significantly compared to that of mice infected with the parental 17X line during the acute phase of infection on days 4-5 post-inoculation. However, the parasitemia did not reach the level observed for the virulent 17XL line, and fell to a level comparable with the 17X line by day 9. This suggests that the 17X-CtoR parasite is able to invade a greater repertoire of erythrocyte types than the 17X line, but may be unable to invade as many types as the 17XL line. This reduced capacity to invade as great an erythrocyte-type repertoire as the 17XL line resulted in a non-lethal infection, in which all infected mice survived. Thus, displacement of PyEBL from the microneme was not sufficient to make this line fully lethal, suggesting the existence of other determinants(s). In support of this, Pattaradilokrat et al. also found evidence for a second determining factor in their whole genome LGS association study; two markers apparently unlinked to pyebl were also shown to be under multiplication rate selection, although they were unable to assign genomic locations to them for technical reasons.

4. Reticulocyte-Binding-Like (RBL) family; another key player in erythrocyte invasion

Micromere proteins are discharged early in the invasion of erythrocytes by merozoites, and are thought to play a part in the formation of the tight junction between the parasite and the host cell (Iyer et al., 2007a). Conversely, the dense granules only discharge their contents following successful invasion, and are thought to be involved in the modification of erythrocyte structure to aid parasite development (Culvenor et al., 1991). Just how the removal of an EBL protein from the micronemes and its relocation to the dense granules could allow a parasite to invade a larger repertoire of host erythrocytes is unclear.

In *P. falciparum*, EBL expression appears to be co-operationally regulated with another *Plasmodium* ligand encoded by the reticulocyte binding like (*rbl*) multigene family. For example, destruction of the EBA-175 gene locus was shown to increase the transcription level of one such RBL protein, PfRh4, resulting in the alteration of the parasite’s invasion phenotype (Stubbs et al., 2005). Members of the *rbl* family are also found in *P. y. yoelii*, comprising at least 14 genes termed *py235* encoding Py235 proteins (Iyer et al., 2007b). Thus Py235 is a potential candidate for the other factor that may be responsible for the difference in virulence between 17XL and the 17X-CtoR parasite. Consistent with this hypothesis is the fact that passive transfer of monoclonal antibodies specific for Py235 or immunization with purified Py235 protein restricted the invasion preference of the normally virulent *P. y. yoelii* YM line to reticulocytes (Freeman et al., 1980; Holder and Freeman, 1981). Further support is provided by work that showed that when *Py235* expression is suppressed, the course of infection of the virulent *P. y. yoelii* YM line is altered to an intermediate virulence similar to that observed with the 17X-CtoR parasite. Based on these observations, Otsuki and colleagues proposed that the removal of EBL from the micronemes may result in free space within this organelle that may be filled with other ligand(s), possibly *Py235*, which enables the parasite to invade a larger repertoire of erythrocyte types. As different *Py235* proteins may have different receptor-specificities, parasite invasion preference and the consequent course of infection may vary, depending on the *Py235* member that fills the free space in the micronemes made available by the absence of EBL. Such a switching mechanism for an erythrocyte invasion pathway has been previously proposed for *P. falciparum*, in which removal of one RBL protein, PfRh2b, was proposed to result in the switching of the invasion pathway by redeployment of pre-existing parasite ligands (Duraisingh et al., 2003; Baum et al., 2005). Detailed descriptions of the RBL family including the newly characterized member PfRH5 are described elsewhere (Gauer et al., 2004; Hayton et al., 2008; Rodriguez et al., 2008; Baum et al., 2009).
5. Further questions concerning the control of invasion phenotypes

Although a valuable addition to our understanding of the genetic determinants of multiplication rates and virulence in malaria parasites, the discovery of the influence of PyEBL on the phenotypic difference between avirulent and virulent *P. y. yoelli* parasites leaves many questions unanswered. For example, both *P. y. yoelli* 33X(Pr3) (a pyrimethamine resistant clone of *P. y. yoelli* 33X) and *P. yoelli nigeriensis* exhibit multiplication phenotypes that may be considered “intermediate” between the extremes of 17X and 17XL, yet do not possess the EBL R6 mutation characteristic of 17XL and YM (Fig. 2b) (Otsuki et al., 2009). Furthermore, slowly multiplying and avirulent *P. y. yoelli* parasites such as 33X, 17X and CU are often observed within mature erythrocytes 4 days post-inoculation of mice, but do not appear to be able to mature within them [Walliker et al., 1976 and R. Culleton (personal observation)]. There is also evidence for the dramatic influence of host immune status on the ability of the reticulocyte-restricted clones of *P. y. yoelli* to invade mature erythrocytes, with two reports describing the fast multiplication and lethal nature of 17XNL grown in immune-compromised mice (Fahey and Spitalny, 1984; Hisaeda et al., 2004). Interestingly, recent work has shown that variations in the multiplication rate and virulence of two *P. chabaudi adami* clones (DS and DK) are linked to differences in erythrocyte invasion preference (Gadsby et al., 2009). In this system, the erythrocyte invasion preference of the slowly multiplying, avirulent parasite did not appear to be for reticulocytes, but rather for an, as yet, unidentified sub-type of mature erythrocytes.

It has long been known that increases in rodent malaria parasite multiplication rate and virulence may occur as the result of extended serial passage through mice (MacKinnon and Read, 1999), and that this increased virulence may be “reset” by passing such selected lines through mosquitoes (although this does not always occur) (MacKinnon and Read, 2004). An increase in virulence was also observed for *Plasmodium knowlesi* infection of humans following serial passages conducted through the syphilis treatment of programme of the early 20 century (Garnham, 1966). This suggests that at least some aspects of the phenotypic variation in multiplication rates and virulence of malaria parasites are epigenetically controlled. It is also possible that changes at the genome level (such as deletions of genes involved in the sexual and sporogonic cycle) during serial passage may influence parasite virulence.

6. DARC: a potential receptor for PyEBL

Indirect evidence suggests that DARC expressed on the surface of mouse erythrocytes may be the receptor of PyEBL. When DARC-negative mouse erythrocytes were evaluated for their susceptibility to the *P. y. yoelli* 17X line, invasion of mature erythrocytes was dramatically reduced, but there was no alteration in invasion of reticulocytes when compared with DARC-intact erythrocytes. This suggests that although the *P. y. yoelli* 17X line does utilize DARC to invade erythrocytes, there are other receptor(s) on the reticulocyte surface which may be reduce in abundance and disappear altogether during erythrocyte maturation. Invasion into DARC-negative mature erythrocytes is Chymotrypsin-sensitive, but Trypsin-resistant, whereas DARC-negative reticulocyte invasion was abolished by treatment with Chymotrypsin or Trypsin, suggesting that the receptors on mature erythrocytes and reticulocytes are different (Swardson-Olver et al., 2002). An *in vitro* erythrocyte binding assay using radio-labeled parasite-derived protein revealed a 135 kDa erythrocyte binding protein, probably PyEBL, in the *P. y. yoelli* 17X line. This binding was sensitive to Chymotrypsin, but resistant to Trypsin treatments. The existence of Chymotrypsin-sensitive receptors on the erythrocyte surface would explain the inability of the 17X line to invade Chymotrypsin-treated mature erythrocytes (Ogun et al., 2000). An *in vitro* erythrocyte binding assay using a recombinant PyEBL DBL domain expressed on COS cells also showed that the erythrocyte surface receptor was Chymotrypsin-sensitive and Trypsin-resistant (Prasad et al., 2003).

Despite the fact that DARC is expressed on the surface of mature erythrocytes, the *P. y. yoelli* 17X line preferentially invades reticulocytes, even though PyEBL is correctly trafficked to the micronemes in this line. This invasion course is similar to *P. vivax*, which utilizes PvDBP to recognize DARC on erythrocytes, but also preferentially invades young erythrocytes. Galinski et al. (1992) identified two members of the *rbl* family in *P. vivax*, encoding reticulocyte binding protein-1 and -2 (PvRBP-1 and PvRBP-2), which specifically recognize young erythrocytes. They proposed a model in which PvRBP proteins reversibly attach to erythrocyte surface receptors in order to find favorable types of erythrocytes to invade before forming the irreversible tight junction using EBL proteins. Thus co-operational function of EBL and RBL proteins may not only be spatially, but also temporally hierarchical.

7. Is PyEBL still functional when localized to dense granules?

It appears likely that dense granule-localized PyEBL remains functional due to the following observations: In wild type mice, the parasitemia of the *P. y. yoelli* 17X line, in which PyEBL localizes in the dense granules, rapidly increases and causes host death by day 7 post-inoculation. However, when DARC knock-out mice (C56BL/6 background) were infected with the same line, parasitemia increased during the acute phase of infection on days 4-5 after inoculation, but did not exceed 10%, and mice subsequently resolved the infection (Akimitsu et al., 2004). Although Akimitsu et al. speculated that this observation was a result of the change in the immunological status of DARC-KO mice, given that there are no significant effects observed on the infection courses of the other rodent malaria parasite species, *P. berghei*, *P. vinckeii*, and *P. chabaudi*, we consider that this observation suggests that DARC still serves as a receptor for PyEBL released from the dense granule.

A subgroup of the dense granules, known as exonemes, were recently reported to discharge their contents immediately prior to schizont rupture (Yeoh et al., 2007), whereas classical dense granules are discharged into a newly formed parasitophorous vacuole space after or during invasion (Torii et al., 1989). Although Otsuki and colleagues failed to detect PyEBL on the surface of the released individual merozoite of 17XL parasites, these parasites were not naturally released (merozoites from artificially ruptured schizonts were analyzed), thus, the timing of the secretion of PyEBL from the dense granules in 17XL is still not fully determined.
Interestingly, even though attempts to disrupt the PyEBL gene locus in both 17X and 17XL failed, suggesting that the protein is essential, both lines are able to infect DARC-KO mice. This suggests that PyEBL is essential even when it cannot bind to DARC. As PyEBL R6 appears not to be functional (at least for trafficking to the micronemes) in the 17XL line, we speculate that other regions may possess essential functionality, for example the cytoplasmic tail may form an essential complex with other molecules. Alternatively, low affinity binding or even brief contact of PyEBL to molecules other than DARC may be important. Finally it is potentially possible that DARC is not the receptor for PyEBL.

8. Implications for \textit{P. vivax} virulence

Would a substitution from Cys to Arg in PvDBP R6 alter the invasion preference of \textit{P. vivax} from young erythrocytes to a wider range of human erythrocytes? It is reasonable to postulate that PvDBP with such a mutation in R6 would be trafficked to the dense granule instead of the micronemes. Then, if \textit{P. vivax} has the ability to express RBL member(s) that could recognize a greater repertoire of erythrocyte types, such a parasite would possess the potential for increased multiplication rate and hence greater virulence. This scenario cannot be ignored, as \textit{P. vivax} possesses 10 RBL genes on its genome (Carlton et al., 2008). We suggest that a PvDBP-based anti-\textit{P. vivax} vaccine should be carefully evaluated, taking such a possibility into account. An understanding of the expression pattern and binding specificity of other \textit{P. vivax} RBL proteins is, therefore, important.

Acknowledgments. This work was supported in part by Grants-in-Aids for Scientific Research 19590428 (to O.K.) and the Global COE Program, Nagasaki University, from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Japan Society for the Promotion of Science–National University of Singapore Joint Research Program 07039011-000161 (to O.K.), and a Japanese Ministry of Education, Culture, Sports, Science and Technology Special Coordination Fund for Promoting Science and Technology Tenure-Track Research Support Grant (to R.C.).

References


**Figure legends**

**Fig. 1.** Schematic representation of the domain architecture of *Plasmodium EBL* family proteins. DBL-EBP expressed at merozoite stage (Mz) and MAEBL expressed at sporozoite stage (Spz) are shown. D1 and D2 are domains similar to AMA-1. DBL, Duffy-Binding-Like domain; R6, region 6; TM, transmembrane region; S, predicted signal peptide sequence. Shaded box indicates non-globular region containing repeats.
Fig. 2. Predicted structure of PyEBL region 6 (R6). (a) Homology modeling of PyEBL R6 was done with Modeller 9v7 using EBA175 R6 (PDB ID: 2RJI) as a template and visualized using RasMol v2.7. Four predicted disulfide bridges (red bar; 1-4) were added using Adobe Photoshop (Sali and Blundell, 1993; Sayle and Milner-White, 1995). Central \(\alpha_3\) helices are surrounded by other helices (\(\alpha_1, \alpha_2, \) and \(\alpha_4\)). Note that \(\alpha_1\) may not be related to other elements. (b) Amino acid sequence comparison of \textit{Plasmodium} EBL region 6. PfEBA-175 (M93397), PfJESEBL (AB080796), PfBAEBL (AF332918), \textit{Plasmodium falciparum} EBLs; PfDBP (DQ156512), \textit{Plasmodium vivax} Duffy Binding Protein; PcyEBP, \textit{Plasmodium cynomolgi} EBP (Y11396); PkDBP-\(\alpha, \beta,\) and \(\gamma\) (M90466, M90694, and M90695, respectively), \textit{Plasmodium knowlesi} Duffy Binding Proteins; PyEBL-17X and -17XL, \textit{Plasmodium yoelii} EBL (AB430782 and AB430781, respectively); PEBL, \textit{P. berghei} EBL (ANKA line, AB430787); PchEBL, \textit{P. chabaudi} EBL (AB430788); and PviEBL, \textit{P. vinckei} EBL (AB430789). In the PfEBA-175 sequence, helices are highlighted in grey; residues involved in protein fold stabilization are highlighted in cyan and yellow for nonpolar and polar residues, respectively; all Cys residues are in red; and residues involved in the dimeric interaction are indicated by hash marks. Asterisks indicate the positions where amino acids are identical, and similar amino acids are indicated with colons or periods under the alignment. Disulfide bridges (red lines) are represented above the alignment. Scissors indicate the disulfide bridge that is abolished by substitution from Cys to Arg in \textit{P. y. yoelii} 17XL line. (For interpretation of the references to color in this figure legend, please see the online version of this article).
Fig. 3. Course of infection of various *Plasmodium yoelii*-derived lines. Three infection courses (1, lethal; 2, intermediate (non-lethal); 3, non-lethal) are based on experiments conducted by Otsuki et al. (2009), in which wild type (WT) BALB/c mice were intravenously inoculated with $1 \times 10^6$ parasitized erythrocytes with *P. y. yoelii* 17XL, 17X, 17XL-RtoC, or 17X-CtoR line parasites. Other data were categorized into these 3 groups, by reviewing the conditions and infection courses described in the following publications: DARC-KO mice (C57BL/6 background) were intravenously inoculated with $1 \times 10^5$ parasitized erythrocytes with *P. y. yoelii* 17XL and 17X (Akimitsu et al., 2004). Although overall infection of 17X line in DARC-KO mice (asterisk) is similar to that in WT mice (Akimitsu et al., 2004), the invasion of mature Duffy-negative erythrocytes by the 17X line of was dramatically reduced (Swardson-Olver et al., 2002). BALB/c mice were intravenously inoculated with $1 \times 10^7$ parasitized erythrocytes with *P. y. yoelii* YM and transgenic YM (YM-y235KD) in which Py235 transcripts were reduced by 10-fold (Iyer et al., 2007b). BALB/c mice were inoculated with *P. y. yoelii* YM and anti-Py235 antibodies were passively transferred when parasitemias reached 0.01-0.1% (YM α-Py235) (Freeman et al., 1980). C57BL/6 mice were intraperitoneally inoculated with $1 \times 10^6$ parasitized erythrocytes with *P. y. yoelii* 33X and 33X(Pr3), derived from the avirulent 33X line after ultraviolet light treatment of parasites (Walliker, 1981). All *P. y. yoelii* lines shown possess identical sequences at PyEBL region 6, except 17XL, 17X-CtoR, and YM lines (italicized) which possess an Arg residue instead of the second conserved Cys residue.

<table>
<thead>
<tr>
<th>WT mouse</th>
<th>DARC-KO mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>17XL</td>
<td>17XL</td>
</tr>
<tr>
<td>YM</td>
<td>YM-y235KD</td>
</tr>
<tr>
<td>17X-CtoR</td>
<td>YM α-Py235</td>
</tr>
<tr>
<td></td>
<td>33X(Pr3)</td>
</tr>
<tr>
<td>17X</td>
<td>17X*</td>
</tr>
<tr>
<td>17XL-RtoC</td>
<td>33X</td>
</tr>
</tbody>
</table>