Single amino acid substitution in Plasmodium yoelii erythrocyte ligand determines its localization and controls parasite virulence

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Citation
PNAS, 106(17), pp.7167-7172; 2009

Issue Date
2009-04-28

URL
http://hdl.handle.net/10069/22077

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Abstract

The major virulence determinant of the rodent malaria parasite, *Plasmodium yoelii*, has remained unresolved since the discovery of the lethal line in the 1970s. As virulence in this parasite correlates with the ability to invade different types of erythrocytes, we evaluated the potential role of the parasite erythrocyte binding ligand, PyEBL. We found one amino acid substitution in a domain responsible for intracellular trafficking between the lethal and non-lethal parasite lines, and, furthermore, that the intracellular localization of *Py*EBL was distinct between these lines. Genetic modification showed that this substitution was responsible not only for *Py*EBL localization, but also the erythrocyte-type invasion preference of the parasite, and subsequently its virulence in mice. This previously unrecognized mechanism for altering an invasion phenotype indicates that subtle alterations of a malaria parasite ligand can dramatically affect host-pathogen interactions and malaria virulence.

Key words: erythrocyte, invasion, ligand, malaria, *Plasmodium yoelii*

Introduction

The rodent malaria parasite *Plasmodium yoelii yoelii* has been widely studied in order to understand the interactions between the malaria parasite and the host-cell (1). The non-lethal 17X line mainly infects young erythrocytes (reticulocytes) whereas the lethal 17XL and YM lines infect a wide range of erythrocytes. These lines have previously been studied in order to identify the genetic determinants of virulence (2, 3). These differences in erythrocyte invasion preference suggest the possible involvement of a parasite ligand that recognizes erythrocyte surface receptors, however, the actual molecular basis of the observed invasion preference differences remains unclear.

Erythrocyte invasion by the malaria merozoite is a multistep process, initiated by reversible binding to the erythrocyte surface, followed by the establishment of a tight junction between the apical end of the merozoite and erythrocyte surface and the subsequent movement of the merozoite into the nascent parasitophorous vacuole. Each step involves specific interactions between parasite ligands and erythrocyte receptors. Among the ligands of malaria parasites, the best characterized is a type I integral transmembrane protein encoded by the *ebl* (erythrocyte-binding-like) gene family. Upon release from the micronemes, EBL proteins recognize erythrocyte receptors and initiate the formation of the tight junction.

The importance of EBL in malaria virulence is exemplified in the human malaria parasite *Plasmodium vivax* which uses an EBL ortholog, *Pv*DBP, to recognize the Duffy antigen on the erythrocyte surface. As the parasite is apparently unable to utilize an alternative invasion pathway, individuals in whom the Duffy antigen is not expressed on the erythrocyte surface are completely resistant to *P. vivax* (4, 5). Due to this dramatic association between the disruption of a host-pathogen interaction and protection against a malaria parasite, *Pv*DBP and the *Plasmodium falciparum* EBL ortholog, EBA-175, have been targeted for vaccine development (6).

EBL proteins possess two Cys-rich regions conserved among EBL orthologs. The N-terminal Cys-rich region named the DBL (Duffy-Binding-Like) domain or Region 2 (7) recognizes a specific erythrocyte surface receptor. The C-terminal Cys-rich region named the C-cys domain or Region 6 is located adjacent to the transmembrane domain and the number and location of Cys residues are well conserved among known *Plasmodium* species. Region 6 exhibits structural similarity to the KIX-binding domain of the coactivator CREB-binding protein (8) and has been proposed to be a protein trafficking signal for transportation to the micronemes (9). Here we report a single non-synonymous nucleotide substitution in the *pyebl* gene between lethal and non-lethal lines of *P. yoelii* and show the effect of this substitution on the intracellular localization of EBL, erythrocyte-type preference and
consequently virulence of \textit{P. yoelii}.

**Results**

In order to investigate differences in EBL between lethal and non-lethal \textit{P. yoelii} lines, we compared sequences from a variety of malaria parasite species and \textit{P. yoelii} lines 17X, 17XL, and YM. We found one non-synonymous nucleotide substitution in Region 6 between non-lethal 17X and lethal 17XL lines in the entire open reading frame (Fig. 1). The non-lethal 17XL line possesses 8 conserved Cys residues that form 4 disulfide bridges (8), whereas the lethal 17XL line possesses an Arg instead of Cys at the second Cys position. This substitution was also found in another lethal line “YM” (2), which originated independently from the 17X line during serial passage (3). All \textit{Plasmodium} EBL orthologs for which protein expression was validated possess 8 conserved Cys residues in this region, further indicating that these Cys residues play an important role (Fig. S1). Thus the observed substitution from Cys to Arg is likely to abolish the native conformation of Region 6.

**EBL localizes in the dense granules in \textit{P. yoelii} line 17XL.** We raised specific polyclonal and monoclonal antibodies against \textit{PyEBL} and performed Western blot analysis. The \textit{PyEBL} protein was detected as a 110-kDa band in both the 17X and 17XL lines (Fig. 2A). The intracellular localization of \textit{PyEBL} in both the 17X and 17XL lines was compared by indirect immunofluorescent assay (IFA) using specific antibodies against \textit{PyEBL} (Fig. S2). In the 17X line, \textit{PyEBL} localized to the apical end of each merozoite in the segmented schizont-stage parasite and individual merozoites where it colocalized with AMA1, a known microneme protein, under immunofluorescent microscopy (Fig. 2B). However, in the 17XL line \textit{PyEBL} did not colocalize with AMA1 at the apical end of merozoites and showed a more diffused but granular distribution in comparison with parasites of the 17X line (Fig. 2B). Diffused localization of \textit{PyEBL} was also observed in parasites of the YM line (Fig. S3). Immunoelectron microscopy revealed that \textit{PyEBL} localized in micronemes in the 17XL line as reported for \textit{P. falciparum} and \textit{Plasmodium knowlesi} (9, 10). In the 17XL line, however, \textit{PyEBL} localized not in the microneme, but in another microorganelle - the dense granules (12) (Fig. 2C, Fig. S4).

Since there appears to be only one copy of \textit{PyEBL} in the genomes of both lines (Fig. S5) and significant differences were not observed in the level of transcription and protein expression between the 17X and 17XL lines (Fig. 2A, Fig. S6), the location of EBL appears to be the most significant difference between them.

**Genetic replacement of Arg and Cys in Region 6 alters EBL localization.** To evaluate if the Arg substitution at the second Cys position is responsible for the altered trafficking of \textit{PyEBL}, we exchanged Cys and Arg in the 17X and 17XL lines by genetic modification (17X-CtoR and 17XL-RtoC). The parasites were also transfected with control constructs which do not alter the Region 6 amino acid sequence (17X-CtoC and 17XL-RtoR) (Fig. 3A). Each of the transgenic parasites were evaluated for the correct integration of the constructs to the \textit{pyebl} gene locus by specific PCR analysis followed by sequencing of the PCR-amplified products (not shown) and Southern blot analysis (Fig. 3B).

In the 17X line, replacement of Cys with Arg (17X-CtoR) altered the \textit{PyEBL} localization from an apical pattern to a non-apical diffused pattern and \textit{PyEBL} did not colocalize with AMA1. Furthermore, the replacement of Arg with Cys in the 17XL line (17XL-RtoC) altered \textit{PyEBL} localization from a non-apical diffused pattern to an apical pattern. Control parasites did not display altered \textit{PyEBL} localization (Fig. 4). These results confirm that the observed substitution from Cys to Arg is responsible for the altered localization of \textit{PyEBL} from micronemes to dense granules in the 17XL line.

**EBL localization alters erythrocyte-type preference and course of infection.** In order to determine if altered localization of \textit{PyEBL} affects erythrocyte-type invasion preference, infected erythrocytes were examined by microscopy and a selectivity index (SI) was obtained by calculating multiple parasite infection of single erythrocytes for each parasite line on day 3 post-infection in mice (13). We found that 17XL-RtoC predominantly invaded reticulocytes in the same way as the non-lethal 17X line. The SI of the 17XL line (2.38) was increased in 17XL-RtoC (~35; \(P < 0.001\)). On the other hand, 17X-CtoR was able to invade a variety of ages of erythrocytes including mature erythrocytes, comparable to the lethal 17XL line, with the SI of the 17XL line (16.78) reduced in 17X-CtoR (~4; \(P < 0.001\); Table. 1). These results demonstrate that the localization of \textit{PyEBL} is responsible for the erythrocyte-type preference of the parasite.

As erythrocyte-type preference frequently correlates with virulence in malaria parasites, we further analyzed the transgenic \textit{P. yoelii} parasites for differences in the course of infection and survival of parasite-infected mice. Mice infected with the 17XL-RtoC line developed significantly lower parasitemias compared with the parental 17XL and control 17XL-RtoR lines (Fig. 5A) with 100% survival (Fig. 5C), whereas all mice infected with 17XL and 17XL-RtoR lines died by day 7 (Fig. 5C). The pattern observed for the 17X line was identical to that observed for the non-lethal 17X line. Thus, trafficking of \textit{PyEBL} to the micronemes causes the virulence of the 17X line to be reduced to the same level as the non-lethal 17X line, suggesting that \textit{PyEBL} is a critical virulence determinant in the 17XL line. The parasitemia of mice infected with 17X-CtoC increased significantly compared to those infected with parental 17X and control 17X-CtoC lines during the acute phase of infection on days 4 to 5 (\(P < 0.001\)). However, the parasitemia did not reach the level observed for the lethal 17XL line, and reduced to the same level observed for the 17X and 17X-CtoC lines by day 9 (Fig. 5B). No parasites were detectable by microscopy at day 17 (not shown). This suggests that the 17X-CtoR line is able to invade a greater repertoire of erythrocyte types than 17X, but is unable to invade as many types as the 17XL line. This reduced capacity to invade multiple erythrocyte types compared to the 17XL line results in a non-lethal infection, in which all mice survive (Fig. 5C).

Thus, displacement of the EBL from micronemes was not sufficient to make this line fully lethal, suggesting the existence of other determinant(s).

**Discussion**
The results of this study indicate that replacement of Cys to Arg at the second Cys position of PyEBL. Region 6 is the major determinant of the difference between lethal and non-lethal lines of P. y. yoelii parasites. This substitution alters the intracellular organelle localization of PyEBL from the micronemes to the dense granules, and alters the erythrocyte-type invasion preference, course of infection and parasite virulence in the host.

The crystal structure of Region 6 of P. falciparum EBA-175 indicates that the second Cys residue forms a disulfide bridge with the fourth Cys residue in this region. Arg substitution of the second Cys residue in the P. yoelii 17XL line abolishes this disulfide bridge, and thus likely destroys the Region 6 structure which is critical for the trafficking of the protein to the micronemes. It is possible that incorrectly folded Region 6 would not allow the protein to be properly recognized by an (as yet uncharacterized) partner molecule responsible for the trafficking of the EBL protein to the micronemes (9). The mechanism involved in the trafficking of the mutated protein to the dense granules remains unresolved.

Using genetic modification, we have demonstrated that when PyEBL is trafficked to the microneme in the 17XL line genetic background, the erythrocyte-type invasion preference and the course of infection are comparable with those of the non-lethal 17X line. This indicates that the substitution of Cys to Arg is a major determinant of the lethal phenotype of the 17XL line. However, when PyEBL was not trafficked to the microneme in parasites with the 17X line genetic background, the course of infection was intermediate between the two parental lines, suggesting that although PyEBL is a critical determinant, other factor(s) are also involved in the lethal phenotype of the 17XL line. In P. falciparum, the expression of EBL appears to be co-operationally regulated with another Plasmodium ligand encoded by the rbl (reticulocyte-binding-like) multigene family that is composed of 6 members in P. falciparum and at least 14 members in P. yoelii (14 - 16), thus the P. yoelii rbl protein, Py235, is a potential candidate for such factor(s). Consistent with this hypothesis is the finding that when Py235 expression was suppressed, the course of infection of the lethal P. yoelii YM line was altered from a lethal pattern to an suppressed, the course of infection of the lethal hypothesis is the finding that when Py235 expression was a candidate for such factor(s). Consistent with this migration from the micronemes to the dense granules, and alters the erythrocyte-type invasion preference, course of infection and parasite virulence in the host.

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A Linkage Group Selection analysis conducted by Pattaradiokrat et al [submitted together with this manuscript] identified a chromosomal region which included the ebl gene locus as a major determinant in the multiplication rate differences between the lethal P. y. yoelii YM line and a non-lethal 33X line, supporting the role of the EBL protein in controlling virulence phenotypes. Consistent with our findings that another genetic factor may be involved, they also identified a further genomic region on P. y. yoelii chromosome 5 or 6 that showed weak association with multiplication rate.

As PyEBL localized in the dense granules is potentially non-functional, we attempted to disrupt the pyebl gene locus in both the 17X and 17XL lines (Fig. S7). However, repeated attempts failed to achieve this, despite the successful genomic integration of the control plasmid. This indicates PyEBL is essential for parasite survival even when it is not trafficked to the microneme. Two possible explanations for this may be; an undetectable amount of PyEBL may still localize in the micronemes and remain functional, or PyEBL is functional during erythrocyte invasion (or for another unknown critical role during the life cycle) even when localized in the dense granules. Although a subgroup of the dense granules, known as exosomes, were recently reported to secrete their contents immediately prior to schizont rupture (19), we found that PyEBL was not detected on the surface of released individual merozoites of 17XL parasites (Fig. S8). Thus, the identity of the PyEBL-containing dense granules and the timing of PyEBL secretion from them, if at all, in the 17XL line remain undetermined.

In summary, we have found that a single nucleotide substitution altered the intracellular localization of the malaria parasite ligand PyEBL, which in turn altered erythrocyte invasion preference, course of infection, and parasite virulence. The virulence-mediating mechanism described in this report furthers our understanding of parasite-host interactions, and has important implications for malaria vaccine design especially those based on PvDBP for P. vivax malaria.

Materials and Methods

Rodent malaria parasites. Plasmodium yoelii 17X, 17XL, and YM lines were maintained in BALB/c mice (Charles River Japan Inc., Japan). P. y. yoelii YM line was a kind gift from David Walliker, Edinburgh University, UK.

DNA and RNA isolation. Parasite genomic DNA (gDNA) was isolated from parasite-infected mouse blood using DNAzol BD reagent (Invitrogen, Carlsbad, CA). Parasite-infected blood was passed through one CF11 cellulose column to remove leukocytes and a schizont-enriched fraction was collected by differential centrifugation on a 50% Percoll™ solution (GE Healthcare, Buckinghamshire, UK). Total RNA was isolated from the schizont-enriched fraction using RNeasy Mini Kit (QIAGEN, Germany). Complementary DNA (cDNA) was synthesized using Omniscript reverse transcriptase (QIAGEN) with random hexamer after DNase treatment.

PCR amplification and sequencing of ebl genes. ebl genes were PCR-amplified from gDNA using KOD Plus DNA polymerase (TOYOBO, Japan) with specific primers for each ebl gene designed using the P. yoelii genome database (The Institute for Genomic Research website) and the P. chabaudi and P. berghei genome project' databases (The Sanger Centre website). ebl sequences were determined by direct sequencing using an ABI PRISM® 310 genetic analyzer (Applied Biosystems, Foster City,
CA) from PCR-amplified products. Sequences were aligned in CLUSTALW implemented in MacVector (ver. 9.0; Accelrys, Inc., San Diego, CA).

**Southern blot analysis.** Five micrograms of *P. yoelii* gDNA were digested with EcoRI, EcoRV, ClaI, BanI and NspI, and BanI and HpaI with appropriate buffer overnight. Digested gDNA was subjected to electrophoresis on 0.8% agarose gels, followed by alkaline transfer onto a Hybond-N+ PVDF membrane (GE Healthcare). Probes were firstly PCR-amplified with 5′–TAAATCTAAATGGAATCAGCAT–3′ and 5′–AGTTGGATGTTAAGTTCAAGTT–3′ primers for the *pyebl* region (Pr), cloned into pGEM-T Easy® plasmid (Promega, Madison, WI), digested from the plasmid, and then hybridized onto membranes. Probes were labeled with the AlkPhos Direct kit (GE Healthcare) and a chemiluminescent signal developed with CDP-star reagent (GE Healthcare) was recorded on RX-U film (FUJI-FILM, Japan).

**Recombinant proteins.** Expression plasmids were constructed based on the pEU-E01-G(TEV)-N2 vector (20) by inserting PCR products amplified from *P. yoelii* 17X gDNA using KOD Plus DNA polymerase with following primers: 5′–ggagaCTCGAGTTAATTTATTAAGAAGCATAAT CAAATTATTATTAATAGGAGGATTACTGAGG–3′ for regions 1 to 2 (R1-2; 28 – 787), 5′–ggagaCTCGAGTTAATTTATTAAGAAGCATAAT CAAATTATTATTAATAGGAGGATTACTGAGG–3′ for regions 3 to 5 (R3-5; 423 – 716), and 5′–ggagaCTCGAGTTAATTTATTAAGAAGCATAAT CAAATTATTATTAATAGGAGGATTACTGAGG–3′ for region 2 (R2; 113 – 436). Digested gDNA was subjected to electrophoresis on 0.8% agarose gels, followed by alkaline transfer onto a Hybond-N+ PVDF membrane (GE Healthcare). Probes were labeled with the AlkPhos Direct kit (GE Healthcare) and a chemiluminescent signal developed with CDP-star reagent (GE Healthcare) was recorded on RX-U film (FUJI-FILM, Japan).

**Immunofluorescence microscopy.** *P. yoelii*-infected mouse erythrocytes were smeared onto glass slides, air dried and stored at −80°C without fixation. Slides were thawed, acetone-fixed, preincubated with PBS containing 5% non-fat milk at 37°C for 30 min, incubated with mouse anti-EBL and rabbit anti-AMA1 sera at room temperature for one hour, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-(mouse IgG and IgM) antibody (Biosource Int., Camarillo, CA) and Alexa-546 conjugated goat anti-rabbit IgG antibody (Molecular Probes, Eugene, OR) at 37°C for 30 min. Parasite nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Differential interference contrast (DIC) and fluorescent images were obtained using a fluorescence microscope (BX50; Olympus, Japan) with a CCD digital camera (DC500; Leica, Germany), and processed using Adobe Photoshop CS (ver. 8.0; Adobe Systems Inc., San Jose, CA).

**Immunoelectron microscopy.** *P. yoelii*-infected mouse blood was fixed in 1% paraformaldehyde–0.1% glutaraldehyde in Hepes-buffered saline, and embedded in LR white resin (Polyscience, Inc., Warrington, PA). Sections were blocked for 30 min in PBS-milk-Tween 20, incubated overnight at 4°C in PBS-milk-Tween 20 containing mouse anti-EBL R1-6 serum, and then incubated for 1 hour in PBS-milk-Tween 20 containing goat anti-mouse IgG conjugated with gold particles (10 nm diameter; Janssen, Pisicaway, NJ). Sections were stained with 2% uranyl acetate in 50% methanol and examined by electron microscopy (JEM-1230; JEOL, Japan).

**Genetic modification of the *pyebl* gene locus.** Two basic plasmids, pPhDT3U-B12 and pHDEF1-mh-R12, were constructed. A DNA fragment encoding cyan fluorescent protein (CFP) was PCR-amplified from pECFP-C1 plasmid (Stratagene) using KOD Plus DNA polymerase with primers 5′–agaGCTAGCTAGGAAAGCCGAG–3′ (Nhel site is underlined) and 5′–ggacGTCAGGATCTCTCTCTCTCTCTATCCAGG–3′ (SalI site is underlined) and then hybridized onto membranes. The insert was then digested with Nhell and SalI, purified, and ligated into pRGDT-B12 (21) using the Nhell enzyme and ligated with Freund's adjuvant, and sacrificed for serum collection. To produce rabbit anti-EBL R3-5 serum, a female Japanese white rabbit was subcutaneously immunized 3 times with recombinant *P. yoelii* EBL R3-5 emulsified with Freund's adjuvant. To produce mouse anti-EBL monoclonal antibodies, the spleen was removed from a mouse immunized with recombinant *P. yoelii* EBL R1-6 and spleen cells were fused with a mouse myeloma cell line derived from a BALB/c mouse by conventional polyethylene glycol method. Supernatants of cultured hybridoma colonies were tested with recombinant *P. yoelii* EBL R1-6 by enzyme-linked immunosorbent assay and on *P. yoelii* 17 X blood smears by indirect immunofluorescent assay. Positive hybridoma colonies were selected and cloned by two rounds of limiting dilution. The epitope region of each monoclonal antibody was tested by Western blot with a panel of recombinant *P. yoelii* EBL proteins. Anti-AMA1 rabbit serum was a gift from Carole Long, National Institutes of Health, USA.
and SalI sites, yielding pRCDT-B12. pRCDT-B12 was digested with ClaI and XbaI and filled with an oligonucleotide linker comprising egatCTCGAGCCCCGGGt and ctgaCCCCGGGCTCGAGat to generate XhoI (underlined) and SmaI (bold) sites to yield pPbDT3U-B12. pHDEF1-mh (22) was digested with SmaI and ApaI to remove the 3' untranslated region of histidine rich protein 2, the ApaI cohesive end was blunted and a Gateway gene conversion cassette C1 (Invitrogen) was inserted. The XhoI site was destroyed by XhoI-digestion, filled-in using KOD Plus DNA polymerase, and self-ligated to yield pHDEF1-mh-R12.

To modify the pyebl gene locus, a DNA fragment encoding pYEBL region 6 to the stop codon was PCR-amplified from gDNA of the P. yoelii 17XL line with primers 5’–gGATCCCTCGAGAAAAATTCTACAGGTATATT–3总理 follow by cloning into the EcoRI and XbaI-digested plasmid pGEM-T Easy® (Promega). The insert was ligated into the NcoI and XhoI sites of pPbDT3U-B12 to yield pR6Cyt-B12. DNA fragments encoding pYEBL region 3 to the stop codon were PCR-amplified from cDNA of P. yoelii 17X and 17XL lines with primers 5’–atCTTCTCTTTAAAAATGCTACAGGTATATT–3’ and 5’–ccAGATCTCTTTAAAAATGCTACAGGTATATT–3’ (BgIII site is underlined). PCR products were then ligated into the SmaI site of pR6Cyt-B12, yielding pR6Cyt+t+R3Cyt(X)-B12 and pR6Cyt+R3Cyt(XL)-B12, respectively. pR6Cyt+R3Cyt(X)-B12 and pR6Cyt+t+R3Cyt(XL)-B12 were subjected to a BP recombination reaction with the donor vector pDONR™221 (Invitrogen) to produce the corresponding entry plasmids pENT.R6Cyt+t+R3Cyt(X) and pENT.R6Cyt+R3Cyt(XL). These entry plasmids were subjected to a LR recombination reaction (Invitrogen) according to the manufacturer's instructions with pHDEF1-mh-R12 to yield replacement constructs pYEYBL-R6Cyt+t+R3Cyt(X) and pYEYBL-R6Cyt+R3Cyt(XL), respectively.

P. yoelii schizont-enriched fraction was collected by differential centrifugation on 50% Histodenz in PBS and 20 µg of XhoI-digested transfection constructs were electroporated to 5 × 10⁶ of enriched schizonts using the Nucleofector™ device (Amxam Gmbh, Germany) with Human T cell solution under program U-33 (23). Transfected parasites were intravenously injected into 8-week-old BALB/c female mice, which were treated by intraperitoneal injection with 1 mg/kg of pyrimethamine daily. Before inoculation of 17X line parasites, mice were treated with phenylhydrazine to increase the reticulocyte population in the blood. Drug resistant parasites were cloned by limiting dilution. Integration of the transfection constructs was confirmed by PCR-amplification with a unique set of primers for the modified pyebl gene locus followed by sequencing, and Southern blot analysis.

Course of infection. To assess the course of infection of transgenic and wild type P. yoelii parasite lines, 1 × 10⁶ parasitized erythrocytes were injected intravenously into 8-week-old female BALB/c mice. Thin blood smears were made daily, stained with Giemsa’s solution, and parasitemias were recorded. Mouse survival was evaluated by the Kaplan-Meier method. Parasitemias of each group were compared by one-way ANOVA test and Tukey’s post test implemented in Prism4.0 (GraphPad Software, San Diego, CA).

Selectivity index. To compare erythrocyte preference between transgenic and wild type P. yoelii parasite lines, a selectivity index was calculated as follows; multiple-infected erythrocytes divided by the expected number of multiple-infected erythrocytes, which was calculated from the number of infected erythrocytes and parasitemia (13). When the preferred erythrocyte type is limited, the observed number of multiple-infected erythrocytes increases. More than 200 parasitized erythrocytes were examined on Giemsa-stained thin blood smears collected on day 3 post-inoculation. The selectivity index of each group was compared by one-way ANOVA test and Tukey’s post test implemented in Prism4.0.

ACKNOWLEDGMENTS. This work was supported in part by Grants-in-Aids for Scientific Research 19790308 (to HO) and 19590428 (to OK) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by JSPS-NUS Joint Research Program 07039011-000161 (to OK). We thank D. Walliker for P. yoelii 33X, 33XPr3 and YM lines; C. Long for anti-AMA1 rabbit serum; H. A. del Portillo for pHDEF1-mh; Y. Tanaka, K. Kameda and K. Oka, Ehime University for their expertise; N. Kangwanrangs for anti-PyMSP1-19 serum; and R. Cullerton for critical reading. Preliminary sequence data for P. berghei, P. chabaudi and P. vinckei were obtained from The Institute for Genomic Research (http://www.tigr.org). Nucleotide sequence data reported in this paper are available in the GenBank™/EMBL/DDJB databases under the accession numbers: AB430781 – AB430789. Animal experiments were carried out in compliance with the Guide for Animal Experimentation at Ehime University School of Medicine.

References

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**Figure 2. Western blot analysis and PyEBL localization in** *P. yoelii* **schizont by immunostaining.** (A) Western blot analysis with monoclonal antibodies (mAb) 5B10 (lane 1), mAb 1G10 (lanes 2) and mouse serum (lane 3) specific for PyEBL against purified *P. yoelii* schizont extracts. A 110-kDa band was detected in both 17X and 17XL lines with no significant difference in the protein expression level (arrowhead). This band was not detected by normal mouse serum (lane 4). Anti-AMA1 serum detected 66-kDa band in the similar level (lane 5). (B) *P. yoelii* schizonts were incubated with mAb 5B10 (α-EBL), rabbit anti-AMA1 serum (α-AMA1), and DAPI (blue) for nuclear staining. Schizonts labeled with anti-PyEBL (5B10) were stained with FITC secondary antibody (green). Anti-AMA1 were stained with Alexa-546 secondary antibody (red). Differential interference contrast (DIC) images are shown in the right-hand column. 17X line shows apical PyEBL signal co-localized with AMA1, but region 6 substituted 17XL line shows diffused staining that does not co-localize with AMA1. (C) Immunoelectron microscopy was carried out for resin-embedded *P. yoelii* 17X and 17XL lines with anti-PyEBL mouse serum and secondary antibody conjugated with gold particles. PyEBL was detected in the micronemes (arrowheads) of the 17X line, but in the 17XL line, it was located in the dense granules (arrows). N, Mn, DG and Rh indicate nucleus, microneme, dense granule and rhoptry.

**Figure 3. Amino acid replacement of PyEBL region 6 second cysteine location by targeted recombination.** (A) Schematic representation of the wild-type (WT) and modified (TG) *pyebl* gene loci. The replacement cassette (Insert) was inserted into the *pyebl* gene locus by double crossover recombination. In this schematic, the second Cys in Region 6 was replaced with Arg in the 17X line to generate 17X-CtoR. Other transgenic lines were generated in a similar fashion. ClaI restriction sites and the expected size of the DNA fragment following ClaI digestion are shown. "Pr" indicates a probe region used in Southern blot analysis. (B) Southern blot analysis of the *pyebl* gene locus in WT and transgenic parasite lines derived from *P. yoelii* 17X and 17XL. The absence of the 4.2-kb WT band and the presence of an 11.1-kb band indicate that the PyEBL locus was modified in all transgenic clones.

**Figure 4. Replacement of Cys to Arg in Region 6 altered subcellular localization of PyEBL.** Schizonts of transgenic parasite lines were incubated with mAb 5B10 (α-EBL), rabbit anti-AMA1 serum (α-AMA1), and DAPI (blue) for nuclear staining. DIC images are shown in the right-hand column. In 17X background, control (C to C)
shows apical PyEBL signal co-localized with AMA1, but replaced (C to R) shows 17XL pattern. Inversely, 17XL background control (R to R) shows diffused non-apical pattern, but replaced to cysteine (R to C) shows apical and co-localized with AMA1.

**Figure 5.** Effect of the alteration of pyebl gene loci on the course of infection and parasite virulence in mice. Mice were intravenously inoculated with $1 \times 10^6$ parasitized erythrocytes from wild type or transgenic parasite lines. (A) Parasitemia of 17XL-RtoC was dramatically reduced to the same level as that of the non-lethal 17X line. (B) Parasitemia of 17X-CtoR was significantly higher than parental 17X and control 17X-CtoC on days 4 and 5 ($P < 0.001$), the acute phase of infection, however the pattern observed is intermediate between the lethal 17XL and non-lethal 17X lines. Parasitemias are plotted using the geometric mean and standard deviation of log-transformed data from groups of 5 mice. (C) All mice infected with 17XL-RtoC survived whereas all mice infected with parental 17XL and control 17XL-RtoR lines died by day 7. All mice infected with 17X, 17X-CtoC and 17X-CtoR survived.

**Table legends**

**TABLE 1. Selectivity index of wild type and transgenic *Plasmodium yoelii* lines.**

<table>
<thead>
<tr>
<th>Parasite</th>
<th>n</th>
<th>Selectivity Index (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17X-CtoR #1</td>
<td>5</td>
<td>3.87 (1.86 - 5.32)</td>
</tr>
<tr>
<td>17X-CtoR #2</td>
<td>5</td>
<td>4.25 (2.38 - 7.97)</td>
</tr>
<tr>
<td>17X-CtoC</td>
<td>5</td>
<td>23.53 (16.49 - 36.00)</td>
</tr>
<tr>
<td>17X</td>
<td>5</td>
<td>16.78 (7.60 - 24.99)</td>
</tr>
<tr>
<td>17XL-RtoC #1</td>
<td>5</td>
<td>34.35 (29.18 - 38.05)</td>
</tr>
<tr>
<td>17XL-RtoC #2</td>
<td>5</td>
<td>35.99 (29.97 - 42.72)</td>
</tr>
<tr>
<td>17XL-RtoR</td>
<td>5</td>
<td>1.31 (0.57 - 2.13)</td>
</tr>
<tr>
<td>17XL</td>
<td>5</td>
<td>2.38 (1.58 - 3.75)</td>
</tr>
</tbody>
</table>

Selectivity indices were calculated from parasitized Giemsa stained thin blood films collected from each infection.
Supporting Online Material

Supplementary Materials and Methods

Quantification of ppyeb1 and ppyama1 transcripts. To quantify and compare ppyeb1 transcription levels between 17Xa and 17XL, real-time PCR was carried out under the following conditions; five BALB/c mice were infected with P. yoelii intraperitoneally and parasites were collected on day 5 by cardiac puncture. Total RNA was isolated from each mouse independently and cDNA was generated from the schizont enriched fraction. Real-time PCR was carried out using the QuantiTect™ SYBR® Green PCR Kit (QIAGEN) with 5’-TGAATCTTTCCAATTTTCCC-3’ and 5’-CCATGTCTTCGGTCAATGTG-3’ primers for ppyeb1 or with 5’-GAAAAGTGCATGTGTTCTGG-3’ and 5’-GAAAAGGTGCATGTTCTGG-3’ primers for ppyama1 using the LightCycler® system (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Copy number of transcripts was determined using a standard curve generated by serially diluted plasmids containing the target insert sequence.

SDS-PAGE and Western blot analysis. Parasite-infected blood was passed through a CF11 cellulose column to remove leukocytes and a schizont-enriched fraction was collected by differential centrifugation on a 50% Percoll solution. Schizont proteins were first extracted by repeated freezing-thaw cycles in phosphate buffered saline (PBS) containing protease inhibitors [PI; 1 µg/mL of leupeptin, 1 µg/mL of pepstatin A, 100 µM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride] and 1 mM EDTA (ethylenediaminetetraacetic acid), followed by extraction of the insoluble pellet in 1% Triton X-100 in PBS-PI. Triton X-100 soluble proteins, containing PyEBL and PyAMA1, were incubated with 6% β-mercaptoethanol at 100°C for 3 min, and subjected to electrophoresis on a 5–20% polyacrylamide gel (ATTO, Japan). Proteins were then transferred to a Immuno-Blot PVDF membrane (Bio-Rad, Hercules, CA), immunostained with mouse serum or monoclonal antibodies followed by horseradish peroxidase-conjugated goat anti-(mouse IgG Fc) antibody (The Jackson laboratory, Bar Harbor, ME), and visualized with Immobilon™ Western Chemiluminescent HRP substrate (Millipore, Billerica, MA). Images were captured on RX-U film.

Live staining of P. yoelii free merozoites. Parasitized erythrocytes collected from mice were suspended in RPMI1640 medium containing 20% fetal calf serum (complete medium) and incubated at 24°C for 13 hours for schizont maturation. Schizonts were then enriched by differential centrifugation on 50% HistoDenz (Sigma, St. Louis, MO) in PBS at 2,500 rpm for 12 min. Collected parasites were passed through a 29G syringe needle 5 times, suspended in 500 µl of RPMI1640 complete medium, and then incubated at 37°C for 2 min. Parasites were washed, incubated in RPMI1640 complete medium containing rabbit anti-PyEBL R3-5 and mouse anti-PyMSP1-19 sera at room temperature for 30 min, washed again, and smeared on glass slides. Air dried smears were fixed in ice-cold acetone, and incubated with Alexa-488 conjugated goat anti-rabbit IgG antibody (Molecular Probes) and Alexa-546 conjugated goat anti-mouse IgG antibody. Parasite nuclei were stained with DAPI. Images were captured as described above.

Knock-out strategy of the ppyeb1 gene locus. To knock-out the ppyeb1 gene locus, a DNA fragment from the 5’ side of ppyeb1 was PCR-amplified from gDNA of the P. yoelii 17XL line with primers 5’–ACCAAATGCTAGATTATTTA–3’ and 5’–AAATTTGATTCTCTCATTGATG–3’. PCR products were then ligated into Smal site of pR6Cyt-B12, yielding pR6Cyt+5U-B12, which was processed in a similar manner as described above to yield knock-out construct pYEBL-KO.

Supplementary Figure legends

Figure S1. Alignment of the deduced amino acid sequence of Plasmodium EBL region 6. Cys residues are highlighted and numbered. P. y. Plasmodium yoelii, PbDBPα1, β, and γ (M90466, M90694, and M90695, respectively), Plasmodium knowlesi Duffy Binding Proteins; PvdBP (DQ156512), Plasmodium vivax DBP; P/EB/A75 (M93397), P/JE/EBL (AB080796), Pb/BAEBL (AF332918), P/PE/BL (DQ100425), and P/E/BL1 (AF131999), Plasmodium falciparum EBLs. Nucleotide sequences for P. y. yoelii EBL 17XL line (AB430781), YM line (AB430783), 17XL line (AB430782), 33X line (AB430784), and 33XP3 line (AB430785), P. y. nigeriensis EBL (AB430786), P. berghei EBL (ANKA line, AB430787), P. chabaudi EBL (AB430788), and P. vinckeii EBL (AB430789) are deposited in DDBJ. The number and location of Cys residues in region 6 are conserved among 17 EBL proteins except P. yoelii 17XL and YM lines, and P/E/BL1. The transcription of P/E/BL1 has been shown to below, the transcription pattern throughout the asexual life cycle was distinct from other members, and protein expression has not been observed to date. P. yoelii 33X, 33XP3 and YM lines are kind gifts from D. Walliker. Preliminary sequence data for P. berghei, P. chabaudi and P. vinckeii were obtained from The Institute for Genomic Research (http://www.tigr.org). Nucleotide sequence data reported in this paper are available in the GenBank™/EMBL/DDBJ databases under the accession numbers: AB430781 – AB430789.
Figure S2. Recombinant EBL proteins and anti-PyEBL antibodies. (A) Schematic representation of a panel of glutathione S-transferase (GST)-fused PyEBL recombinant proteins used for immunization and/or epitope mapping of antibodies. SIG, predicted signal peptide sequence; TM, transmembrane region; cyt, cytoplasmic region; R1, R2, R3-5, and R6 indicate PyEBL regions 1 to 6. (B) Epitope mapping of antibodies. Western blot analysis showed that monoclonal antibodies (mAb) 1G10 and 5B10 reacted with GST-PyEBL R1-6 and GST-PyEBL R3-5, indicating that the epitope was located in PyEBL region R-5. Mouse anti-PyEBL R1-6 serum (Mo α-R1-6) reacted with all recombinant PyEBL proteins.

Figure S3. Subcellular localization of EBL in *Plasmodium yoelii* YM line. *P. yoelii* YM line schizonts were incubated with mAb 5B10 (α-EBL; FITC, green), rabbit anti-AMA1 serum (α-AMA1; Alexa-546, red), and DAPI (blue) for nuclear staining. Differential interference contrast (DIC) images are shown in the right-hand panel. EBL did not co-localize with AMA1 in the YM line, similar to the observation in 17XL line.

Figure S4. Further immunoelectron microscopy images. PyEBL was detected in the micronemes (arrowheads) in the 17X line, however, it was detected in the dense granules (arrows) in 17XL line. N, Mn, DG and Rh indicate nucleus, micronemes, dense granules and rhoptries.

Figure S5. *pyebl* is a single copy gene. (A) Schematic of the *pyebl* gene locus (not to scale). Restriction sites and the expected size of the DNA fragment by enzyme digestion are shown. “Probe” indicates the probe region used in Southern blot analysis. (B) Southern blot analysis of the *pyebl* gene locus in 17X and 17XL lines. Hybridization of *pyebl* probe detected only a single band from EcoRI- or EcoRV-digested DNA and only two bands from BanI/HpaI- or BanI/NspI-digested DNA. If the *P. yoelii* genome possesses more than a single copy of *pyebl*, additional bands are likely to be observed. Taken together with the fact that only a single copy of the *pyebl* gene can be found in the *P. yoelii* (17X line) genome database (5-fold coverage), this result strongly suggests that *pyebl* is a single copy gene in both the 17X and 17XL lines.

Figure S6. Quantitative comparison of PyEBL transcription. No significant difference between 17X and 17XL in the relative transcript number of *pyebl* against *ama1* quantified by real-time RT-PCR (n = 5). The bars indicate median values.
Figure S7. Knock-out strategy of the *pyebl* gene locus. Schematic of the wild-type (WT) and knock-out (KO) *pyebl* gene loci. The knock-out cassette (Insert) would be inserted into the *pyebl* gene locus by double crossover recombination and disrupts gene function. A total of 4 attempts to disrupt the *pyebl* gene locus were performed in 17X and 17XL lines (twice for each) without success. As a control, replacement plasmids, pYEBL-R6Cyt+R3Cyt(X) and pYEBL-R6Cyt+R3Cyt(XL), were simultaneously transfected and genomic integration of these plasmids was always obtained.

Figure S8. *PyEBL* was not expressed on the released merozoite surface. Live merozoites of *P. yoelii* 17X and 17XL lines were incubated with rabbit anti-*PyEBL* R3-5 serum (α-EBL) and mouse anti-*PyMSP1*-19 serum (α-MSP1), followed by secondary antibodies conjugated with fluorescent Alexa dyes and DAPI for nuclear staining. Differential interference contrast (DIC) images are shown in the right-hand column. MSP1 was detected on the merozoite surface, but EBL was not. Anti-*PyMSP1*-19 serum was produced by N. Kangwanrangsan, Ehime University.