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Various PfRH5 polymorphisms can support *Plasmodium falciparum* invasion into the erythrocytes of owl monkeys and rats

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ABSTRACT

Aotus nancymaae, the owl monkey, provides a useful laboratory model for research to develop drugs and vaccines against human falciparum malaria; however, many *Plasmodium falciparum* parasites are unable to invade *A. nancymaae* erythrocytes, rendering the parasites noninfective to the monkeys. In previous work, we identified a key polymorphism that determined the inheritance of erythrocyte invasion in a genetic cross of two *P. falciparum* clones that were virulent (GB4) or noninfective (7G8) to *A. nancymaae*. This polymorphism, an isoleucine-to-lysine polymorphism at position 204 (I204K) of the GB4 erythrocyte binding protein PfRH5, was nevertheless not found in several other *P. falciparum* lines that could also invade *A. nancymaae* erythrocytes. Alternative PfRH5 polymorphisms occur at different positions in these virulent parasites, and additional polymorphisms are found in *P. falciparum* parasites that cannot infect *A. nancymaae*. By allelic replacement methods, we have introduced the polymorphisms of these *A. nancymaae*-virulent or noninfective parasites at codons 204, 347, 358, 362, 410, and 429 of the endogenous *PfRH5* gene in the noninfective 7G8 line. 7G8 transformants expressing the polymorphisms of the *A. nancymaae*-virulent parasites show neuraminidase-sensitive (sialic acid-dependent) invasion into the monkey erythrocytes, whereas 7G8 transformants expressing the *PfRH5* alleles of noninfective parasites show little or no invasion of these erythrocytes. Parasites harboring PfRH5 polymorphisms 204K or 204R are also able to invade rat erythrocytes and are differentially sensitive to the removal of surface sialic acids by neuraminidase. These studies offer insights into the PfRH5 receptor-binding domain and interactions that support the invasion of various primate and rodent erythrocytes by *P. falciparum*.

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1. Introduction

Invasion of erythrocytes by malaria parasites is a multistep, complex process that involves a number of diverse and perhaps functionally redundant receptor-ligand interactions [1,2]. Invasion is initiated when a merozoite attaches to an erythrocyte [3–5]. The parasite repositions so the apical end is in direct contact with the erythrocyte surface and then secretes the contents of the rhoptries and micronemes, specialized apical organelles that store and release the molecules necessary for host cell invasion [6]. Parasite ligands in this released material bind to erythrocyte molecules, forming a tight junction between the host and parasite membrane surfaces. The tight junction develops into an annulus that supports entry of the parasite into the erythrocyte as the annulus is translocated by an actin-myosin motor from the apical to posterior ends of the merozoite [7]. After entry of the merozoite, the erythrocyte membrane seals, completing the process of invasion.

In *Plasmodium falciparum*, a number of important erythrocyte binding ligands occur in two families, the erythrocyte binding-like (EBL) molecules and the reticulocyte binding-like (RBL) molecules [1,2,8]. Members of the EBL family are orthologs of the Duffy binding protein of *Plasmodium vivax*, appear to be stored in the micronemes, and include erythrocyte binding antigen 175 (EBA-175), EBA-140 (also known as BAEBL), EBA-181 (or JESEBL) and EBL–1 [9–16]. *P. falciparum* members of the RBL family include five proteins of the rhoptries: PfRH1 (PfNBP1), PfRH2a, PfRH2b, PfRH4 and PfRH5 [17–25]. These proteins were originally identified by their homology to RBL members of other *Plasmodium* species, including the *P. vivax* reticulocyte binding proteins PvRBP1 and PvRBP [26] and Py235 proteins of the rodent malaria parasite *Plasmodium yoelii* that mediate erythrocyte selection and virulence [27].

Despite knowledge of several known ligand–erythrocyte receptor interactions, much about these interactions in erythrocyte invasion pathways remains to be understood. In *P. falciparum*, many interactions appear to be redundant and individually dispensable, offering an advantage that may promote parasite survival

Abbreviations: EBA, Erythrocyte Binding Antigen; EBL, Erythrocyte Binding-Like; RBL, Reticulocyte Binding-Like.

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when particular ligands or receptors are compromised, for example by host immune defenses or polymorphisms of the erythrocytes [28–30]. In some cases, members of the EBL and RBL families may function in cooperation [31]. Some but not all erythrocyte receptors for *P. falciparum* ligands have been identified: in the EBL family, EBA-175 binds sialic acid residues on the integral membrane protein glycophorin A [9,32,33], EBA-140 binds sialic acids on glycophorin C [12,34,35], and EBL-1 binds sialic acids on glycophorin B [16]. In studies of the RBL family, complement receptor 1 (CR1) and an isoform of basigin (BSG-S, the Ok blood group antigen, CD147) have been identified as human erythrocyte receptors for PfRH4 and PfRH5, respectively [36,37], and the binding domains of PfRH1, PfRH4 and PfRH5 have been mapped to N-terminal regions of their sequences [23–25,38,39].

Various *P. falciparum* lines are able to invade erythrocytes that lack receptors for specific PfEBL- or PfRH-proteins (because of mutations or after enzyme treatment in the laboratory). Conversely, knock-out parasites that no longer express individual PfEBL or PfRH proteins generally retain their ability to invade erythrocytes [2]. An important exception, however, has been the unsuccessful attempt in at least two laboratories to knock out the gene encoding PfRH5 [2,23]. Together with more recent evidence that invasion of human erythrocytes can be inhibited by a soluble pentamerized form of basigin, by anti-basigin monoclonal antibodies, or by reduction of basigin levels on the erythrocyte surface [37], these findings suggest that *P. falciparum* depends upon the PfRH5-basigin interaction for effective invasion of human erythrocytes. Unlike other members of the PfRH family, PfRH5 lacks a transmembrane domain; it instead interacts with a processed EGF-like protein (PfRipr) to form a complex that associates with an unidentified partner on the merozoite membrane (attempts to disrupt the *PfRipr* gene were also unsuccessful) [40].

The New World monkey *Aotus nancymaae* (owl monkey) is one of a limited number of primates susceptible to infection with human malaria parasites and available as a model for vaccine and drug development research. However, only some *P. falciparum* parasite lines are able to invade the erythrocytes of these monkeys and produce infections *in vivo*. Recently, using a laboratory genetic cross between the GB4 and 7G8 *P. falciparum* clones from Ghana and Brazil, we identified an I→K substitution at position 204 (I204K) of PfRH5 as an important GB4 determinant for the invasion of *A. nancymaae* red blood cells [23]. Further, in contrast to the finding that engineered absence of all glycans from human basigin did not alter PfRH5 binding [37], removal (by neuraminidase) of sialic acids from the *Aotus* erythrocytes abrogated the binding of I204K-containing PfRH5 as well as invasion of the GB4 parent and I204K-containing progeny [23]. Certain other *P. falciparum* parasites that carry PfRH5 polymorphisms different from I204K are also able to infect *A. nancymaae* [23–25,41]. Here, we report on investigations of these PfRH5 polymorphisms and interactions that support the invasion of various primate and rodent erythrocytes by *P. falciparum*.

2. Materials and methods

2.1. Parasites

The 7G8 *P. falciparum* clone, 7G8 × GB4 progeny clone LC12, and their transformants containing substitutions at PfRH5 positions 204 and 408, specifically $7G8^{204I/407I}$ ($7G8^{II}$ control transformant), $7G8^{204K/407V}$ ($7G8^{KV}$), $7G8^{204I/407V}$ ($7G8^{IV}$), $LC12^{204K/407V}$ ($LC12^{KV}$ control transformant), $LC12^{204I/407I}$ ($LC12^{II}$) and $LC12^{204K/407I}$ ($LC12^{KI}$) have been previously described [23]. The original 7G8 clone [42] does not invade *A. nancymaae* erythrocytes and is not infective to *A .nancymaae* owl monkeys, whereas the LC12 parasite progeny clone invades *A. nancymaae* erythrocytes and is highly virulent to owl monkeys [23]. *P. falciparum* parasites were cultivated using standard techniques [43,44]. Donor O^+ human blood was obtained from the Interstate Blood Bank (Memphis, TN).

2.2. Plasmid constructs

Using strategies similar to those for generation of the $7G8^{204I/407I}$, $7G8^{204K/407V}$, $7G8^{204I/407V}$, $LC12^{204K/407V}$, $LC12^{204I/407I}$, $LC12^{204K/407I}$ transformed lines [23], six constructs were designed to generate additional codon changes in the endogenous *PfRH5* gene of the 7G8 clone (Table 1). For this purpose, 1396-bp fragments of *PfRH5* were amplified by PCR from the genomic DNA of *P. falciparum* Dd2, Malayan Camp (MalCamp), Santa Lucia, and FVO parasites. Primers 5′ (TGCGGCCGCATGAAGACTATAAAAATGTGG) and 3′(ACTGCAGATGCTTTGTCTAATTAGAG) provided a *Not*I or *Pst*I site, respectively, for cloning into the pHD22Y vector [45]. The amplified fragments were incorporated into plasmids pHD22Y-$rh5^{Dd2}$, pHD22Y-$rh5^{MalCamp}$, pHD22Y-$rh5^{SantaLucia}$, and pHD22Y-$rh5^{FVO}$. A fifth construct, pHD22Y-$rh5^{429N}$, was generated using the site-directed mutagenesis kit QuikChange II, according to the manufacturer's instructions (Stratagene, La Jolla, CA).

During the course of our experiments, we noted that some plasmids consistently integrated into the target chromosome by single crossover events toward the 3′ of the 7G8 *PfRH5* gene, so that transfectants expressing all of the desired codon changes were difficult to obtain. This proved to be a particular problem for the case of the Palo Alto I204R polymorphism [23]. We therefore designed a sixth construct to express the amino acid sequence of the Palo Alto parasite but with codon adjustments, so that all codons downstream of I204R were with the nucleotide preferences of *Escherichia coli* instead of *P. falciparum* (pHD22Y-$rh5^{PaloAlto}$; GENEART, Toronto, Canada). Because the AT-rich *PfRH5* 3′ flanking sequence in the original design proved difficult to synthesize, this region of pHD22Y-$rh5^{PaloAlto}$ sequence was also modified so that it contains the 3′ flanking region of *Plasmodium berghei* dihydrofolate reductase-thymidilate synthase [46]. The resulting synthetic I204R *PfRH5* sequence, along with the dihydrofolate reductase-thymidilate synthase terminator, provided a 1.7-kb fragment that was cloned into pHD22Y using the *Not*I and *Pst*I sites. All other constructs in this study utilized the *PfRH5* endogenous 3′ flanking region. The plasmid constructs were confirmed by DNA sequencing.

2.3. Parasite transfections

Uninfected human erythrocytes were loaded with purified plasmid DNA by electroporation and mixed with mature-stage 7G8 parasites as described [47]. After 2 days, 5 nM WR99210 was applied to the cultures to select parasites carrying the transfected plasmids. After ~60 days of WR99210 pressure, homologous integration into endogenous *PfRH5* was detected by PCR. The transformed parasites were cryopreserved, confirmed by Southern blotting, and the presence of the desired codon polymorphisms in the allelic exchanges was verified by polymerase chain reaction (PCR) amplification and DNA sequencing.

2.4. Invasion assays

Mature-stage parasites were purified using the percoll/sorbitol method [48], washed three times, and adjusted to a final concentration of $1.0–1.5 \times 10^7$ parasitized erythrocytes/ml. Parasite suspension (100 μl) was added to 100 μl of target erythrocytes at 2×10^8 cells/ml in complete medium in a flat-bottomed 96-well plate, for an introduced parasitemia of approximately 5.0–7.5% [30]. The parasite suspension was also added to wells containing