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Erythrocyte Binding Protein PfRH5 Polymorphisms Determine Species-Specific Pathways of Plasmodium falciparum Invasion

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Summary

Some human *Plasmodium falciparum* parasites, but not others, cause malaria in *Aotus* monkeys. To identify the basis for this variation, we crossed two clones that differ in *A. nancymaae* virulence and mapped inherited traits of infectivity to erythrocyte invasion by linkage analysis. A major pathway of invasion was linked to polymorphisms in a newly-identified erythrocyte binding protein, PfRH5, found in the apical region of merozoites. Polymorphisms of *PfRH5* from the *A. nancymaae*-virulent parent (GB4) transformed the non-virulent parent (7G8) to a virulent parasite. Conversely, replacements that removed these polymorphisms from *PfRH5* converted a virulent progeny clone (LC12) to a non-virulent parasite. A proteolytic fragment of PfRH5 from the infective parasites bound to *A. nancymaae* erythrocytes. Our results also suggest that PfRH5 is a parasite ligand for human infection, and that amino acid substitutions can cause its binding domain to recognize different human erythrocyte surface receptors.

Introduction

Clinical manifestations of malaria arise from infection of erythrocytes in the human bloodstream and the effects of these parasitized erythrocytes on critical host tissues and organs. *Plasmodium falciparum*, the greatest killer among the human malaria parasite species, is known for its ability to attack different organs with life-threatening conditions such as cerebral compromise and coma, severe anemia, pulmonary malaria, acidosis, renal failure, algid forms with shock and disseminated intravascular coagulation, and severe pathological effects on the mother and fetus in the malaria of pregnancy. Yet, much remains unclear about the processes by which the parasites enter the host erythrocytes and subsequently interact with host systems to cause these conditions.

P. falciparum parasites do not cause malaria in the majority of non-human primates. Limited episodes of malaria occur in splenectomized chimpanzees and, among monkeys, certain New World species are known to be susceptible to some *P. falciparum* parasites. Indeed, *Aotus*, the

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owl or night monkey, and *Saimiri*, the squirrel monkey, were frequently used to maintain *P. falciparum* lines prior to the advent of in vitro culture (Geiman and Meagher, 1967; Trager and Jensen, 1976); today these species are employed in drug and vaccine testing programs (Collins, 1994). However, only some *P. falciparum* lines infect *Aotus* and *Saimiri* whereas other lines do not, and the features of monkey malaria observed with these species can differ substantially from those of human *P. falciparum* malaria (Young et al., 1975; Heppner et al., 2001).

Malaria parasites can establish disease in the vertebrate host only if they are able to efficiently invade erythrocytes. In molecular terms, invasion requires a complex series of steps initiated by parasite recognition and attachment to the host erythrocyte membrane (Aikawa et al., 1978; Dvorak et al., 1975). Individual species and even strains of a particular species vary in the host receptors they use for these steps. *P. falciparum* parasites exhibit notable diversity in this regard, as they employ a number of alternative parasite ligand – host receptor combinations to invade human erythrocytes by different invasion pathways (reviewed by Iyer et al., 2007). Such ability to invade by various molecular pathways is likely an adaptation to human erythrocyte polymorphisms that have arisen with malaria in human evolution (Duraisingh et al., 2003; Mitchell et al., 1986; Perkins and Holt, 1988). Some *P. falciparum* parasites can switch to different pathways of invasion under selection pressure. For example, the Dd2 clone of *P. falciparum* undergoes an adaptive switch to non-sialic acid dependence when presented with neuraminidase-treated erythrocytes (Dolan et al., 1990); this phenotype change is associated with upregulation of PfRH4, a member of the reticulocyte-binding protein family (Gaur et al., 2006; Stubbs et al., 2005). In other studies, single amino acid changes in two *P. falciparum* ligands, JESEBL/EBA181 and BAEBL/EBA140, have each been shown to alter host receptor recognition and produce different erythrocyte binding patterns (Mayer et al., 2002; Mayer et al., 2004).

Ligand and receptor differences may also be involved in variation of *P. falciparum* infectivity to *Aotus*. One ligand with a possible role in the invasion of *Aotus* erythrocytes is PfEBA175, a binding protein that recognizes sialic acid residues of glycophorin A on the surface of human erythrocytes (Camus and Hadley, 1985; Sim et al., 1994). Studies have shown that erythrocyte sialic acid residues occur predominantly as *N*-glycolylneuraminic acid (Neu5Gc) in chimpanzees and Old World monkeys, whereas in humans and New World *Aotus* monkeys these residues occur only as *N*-acetylneuraminic acid (Neu5Ac), a precursor of Neu5Gc (Martin et al., 2005). Yet, despite the presence of correct sialic acid residues on *Aotus* erythrocytes and the ability of *P. falciparum* to switch to alternative invasion pathways, many *P. falciparum* lines are unable to infect these New World monkeys (Collins et al., 1997; Kaneko et al., 1999; Young et al., 1975).

In this report, we describe results from a genetic cross between a *P. falciparum* clone unable to infect *Aotus nancymaae* and a clone highly virulent to these monkeys. Linkage analysis of the progeny and DNA transfection studies demonstrate a key role of polymorphisms in a newly identified erythrocyte binding protein, PfRH5, for *P. falciparum* invasion of *A. nancymaae* erythrocytes. Our evidence also indicates that the PfRH5 protein containing these polymorphisms can bind to alternative receptors on human erythrocytes.

RESULTS

The 7G8×GB4 Cross

The GB4 parent of the cross is a clone from *P. falciparum* Ghana III (a gift from Bill Collins), a parasite line that produces highly virulent infections in various *Aotus* species both by mosquito-transmitted sporozoites and by direct inoculation of erythrocyte-stage parasites (Sullivan et al., 2003). Robust and highly reproducible infections were obtained with GB4 in

A. nancymaae (Figure 1A). In contrast, the 7G8 clone from the Brazil isolate IMTM 22 (Burkot et al., 1984) did not infect *A. nancymaae* monkeys on repeated attempts.

To cross 7G8 and GB4, we induced gametocytes from each parent in vitro, combined these sexual stages in equal numbers and fed them to about 1000 *Anopheles freeborni* and *Anopheles gambiae* mosquitoes. Tests of individual oocysts from the mosquito midguts on day 11 confirmed genetic recombination (data not shown). On day 15, approximately 300 infectious mosquitoes were fed on a splenectomized chimpanzee. Samples of parasitemic blood were collected from the chimpanzee on days 13, 16, 18, 19, 20 and 21 after mosquito feeding. Of more than 200 individual parasites cloned from these samples and typed by microsatellite analysis, we identified 28 progeny clones from 7G8 self-fertilization events, a single clone from GB4 selfing, and 32 unique haploid recombinant progeny clones (independent recombinants) for phenotype assessment and genetic linkage analysis.

Erythrocyte Invasion and *A. nancymaae* Infectivity by 7G8×GB4 Progeny Clones

In vitro, the 7G8 parent showed no invasion of *A. nancymaae* erythrocytes, consistent with its inability to infect the monkeys, whereas invasion by the virulent GB4 parent proved to be robust, 35% that of human erythrocytes (Figure 1B). The 32 independent recombinant progeny from the 7G8×GB4 cross varied in their ability to invade *A. nancymaae* erythrocytes. Some progeny were similar to the 7G8 parent, exhibiting little or no invasion of *A. nancymaae* erythrocytes. Other progeny invaded *A. nancymaae* erythrocytes at rates ranging from approximately 0.5× to 1.6× that of the GB4 parent (17% to 55% of the control rates in human erythrocytes; Figure 1B).

We also investigated the ability of 14 of the 32 progeny clones to infect splenectomized *A. nancymaae* monkeys. JB8, a progeny clone that had inherited the 7G8 parent's poor ability to invade *A. nancymaae* erythrocytes in vitro, failed to produce an observable infection with an inoculum of 1×10^7 parasites. Among the 13 remaining progeny, all of which were able to invade *A. nancymaae* erythrocytes in vitro, 10 clones produced patent monkey infections with marked differences in pre-patent periods: some progeny blood stages were detected within 10 days while others required 30–60 days to reach detectable parasitemia (Figure 1C). Clone KC2 gave an early peak that spontaneously remitted and was followed by a later peak before treatment. Clones KB8, JC3 and XF12 failed to produce detectable infections despite their ability to invade *A. nancymaae* erythrocytes in vitro (KB8 and JC3 were each tried in two animals and XF12 in one animal). This variability among the progeny was in contrast to the consistent appearance of parasitemia from the GB4 parent 19–20 days after inoculation in four different *A. nancymaae* (Figure 1A), suggesting that multiple inherited factors in the 7G8×GB4 progeny affect both virulence and time to parasitemia in *A. nancymaae*.

A Key Locus of *A. nancymaae* Erythrocyte Invasion Maps to *P. falciparum* Chromosome 4

We identified 285 polymorphic microsatellite markers that distinguish the 7G8 and GB4 parents across all 14 *P. falciparum* nuclear chromosomes and determined the inheritance of these markers in the 32 independent recombinant progeny. Recombination among these markers in the progeny indicated an average linkage separation of 2.2 cM and a map coverage of 628 cM over 14 linkage groups (corresponding to an average map unit distance of 36 kb/cM). Most genetic markers exhibited an approximately equal distribution of parental alleles in accord with random Mendelian inheritance (Figure 2A). However, highly skewed ratios of 7G8 and GB4 inheritance were evident in some regions, particularly at one end of chromosome 7, where every independent recombinant carried markers of this chromosome region from the 7G8 parent (Figure 2A, Table S1).

Associations between the invasion rates shown in Figure 1B and the markers from complete microsatellite maps of the 32 progeny were assessed by quantitative trait loci (QTL) analysis. Results from a primary scan identified a major locus for *A. nancymaae* erythrocyte invasion near the telomere of chromosome 4 with a log of odds (LOD) score of 11.8 (Figure 2B). A secondary scan to search for residual effects did not identify additional peaks with statistical significance (Figure S1).

We defined the boundaries of the principal QTL invasion locus more precisely with progeny clones carrying recombinant crossovers in the region of the peak from chromosome 4. One of these progeny clones, LA10, showed little or no invasion of *A. nancymaae* erythrocytes, indicating a position for the locus distal (right) of marker C4M30 on chromosome 4 (Figure 2C). Another clone, TF1, showed an *A. nancymaae* erythrocyte invasion rate comparable to that of the GB4 parent, indicating a locus position internal to a SNP (single nucleotide polymorphism) in the pseudogene *PEBL* (Figure 2C) (Triglia et al., 2001). Together, these results mapped the locus within a 13.7 kb region of chromosome 4. This region contains two predicted genes, *PfRH4* (PFD1150c) and *PfRH5* (PFD1145c), both of which are members of the reticulocyte-binding protein homolog (RH) family (Cowman and Crabb, 2006).

Distinct Coding Polymorphisms Occur in the *Aotus*-virulence Locus of GB4 Parasites

To evaluate potential roles of *PfRH4* and *PfRH5* in *A. nancymaae* erythrocyte invasion, we searched for an association of mRNA transcription levels or sequence polymorphisms with invasion by *P. falciparum* parasites from geographically distant regions with distinct genetic backgrounds (Wootton et al., 2002). No significant differences in the transcript levels of *PfRH4* or *PfRH5* were detected in synchronized 7G8 and GB4 schizont-stage parasites by microarray analysis (Figure S2). Further, subclones of the Dd2 parasite that express both *PfRH4* and *PEBL* at upregulated levels (Dd2/Nm) (Gaur et al., 2006) showed no change from Dd2's poor ability to invade *A. nancymaae* erythrocytes in vitro (data not shown).

Comparison of the 7G8 and GB4 sequences identified two unique non-synonymous polymorphisms in the GB4 *PfRH5* open reading frame (Table 1), whereas a single codon polymorphism in *PfRH4* (encoding Q438 in GB4 vs. K438 in 7G8) was found in other parasites whether or not they were invasive to *A. nancymaae* erythrocytes (data not shown). In light of these observations, we decided to focus on *PfRH5* as a possible determinant of *A. nancymaae* infectivity in the 7G8×GB4 cross.

Allelic modifications of *PfRH5* alter the ability of *P. falciparum* to invade *A. ancymaae* erythrocytes

The *PfRH5* gene occurs in a sub-telomeric region of *P. falciparum* chromosome 4 that lacks synteny with any of the rodent malaria parasite chromosomes and contains species-specific genes likely to be involved in host-parasite interactions (Kooij et al., 2005). Our genome database searches identified an ortholog of *PfRH5* only in the *P. falciparum*-like chimpanzee parasite *P. reichenowi*; we detected no orthologs in the human *P. vivax* parasite, the monkey parasite *P. knowlesi*, or the rodent parasites *P. berghei* and *P. yoelii*. PfRH5 is nevertheless conserved among *P. falciparum* lines and, as a member of the RH superfamily, it aligns with high similarity to the amino terminal regions of PfRH2a and PfRH2b (much larger 370 kDa and 383 kDa members of the RH family, respectively), the N-terminal regions of the *P. reichenowi* PrRH2b and the *P. yoelii* 235 proteins (Ogun and Holder, 1996; Rayner et al., 2000; 2004) (Figure 3).

To directly evaluate the role of amino acid substitutions in PfRH5 in *A. nancymaae* erythrocyte invasion, we modified the 7G8 *PfRH5* sequence to contain the I204K and I407V codons of the GB4 parasite. Following single site crossover and homologous integration of a transfected

episome carrying the GB4 sequence, modified 7G8 parasites were obtained that carry a full-length version of GB4 *PfRH5* upstream of the drug selection cassette and a 3' non-translated remnant of the original chromosomal 7G8 *PfRH5* sequence (Figure 4A). The appropriate allelic exchange in this line, $7G8^{KV}$, was confirmed by DNA restriction and Southern blotting (Figure 4B).

In assays with *A. nancymaae* erythrocytes, $7G8^{KV}$ parasites proved to have converted from the non-invasion 7G8 phenotype to invasion rates comparable to or better than the GB4 parent (42% of the control rates in human erythrocytes, Figure 4C; compare Figure 1B). Independent transformation of 7G8 parasites with the GB4 allele on a second occasion yielded similar results (data not shown). Control experiments, in which the transfection plasmid containing the native 7G8 *PfRH5* sequence was inserted by single site crossover into the chromosomal copy of 7G8 parasites ($7G8^{II}$ control), showed no significant change from the non-invasion phenotype (Figure 4C).

We performed further experiments to transform and test two progeny clones: LC12 that carries the GB4 *PfRH5* allele and invades *A. nancymaae* erythrocytes with high efficiency; and JB12 that carries the 7G8 *PfRH5* allele and does not invade *A. nancymaae* erythrocytes (Figure 1B). As expected, insertion of the transfection plasmid containing the GB4 *PfRH5* allele into the progeny clone LC12 (generating control $LC12^{KV}$ parasites) produced no significant change in *A. nancymaae* red cell invasion, whereas replacement of the LC12 *PfRH5* gene with the 7G8 allele resulted in a dramatic decrease of invasion efficiency ($LC12^{II}$ transformants; Figure 4C). In experiments with the non-invasive progeny clone JB12, we modified the *PfRH5* allele inherited from the 7G8 parent so that it contained the GB4 codons K204 and V407. The resulting parasites, $JB12^{KV}$, showed conversion to an invasion phenotype, which we measured as an increase from zero to about two-thirds of the ability of transformed $7G8^{KV}$ parasites to enter *A. nancymaae* erythrocytes (Figure 4C).

In these allelic exchange experiments, we also found that allelic crossovers occurred between the codons at positions 204 and 407 so that alternative combinations of parental *PfRH5* polymorphisms were obtained. 7G8 transformants lacking a change to codon K204 but containing codon V407 ($7G8^{IV}$) showed little change from the baseline non-invasion phenotype, suggesting a critical role for the K204 residue in *A. nancymaae* invasion by $7G8^{KV}$ parasites (Figure 4C). LC12 transformants containing codon K204 but lacking codon V407 ($LC12^{KI}$) retained substantial invasion rates, again consistent with an important effect of the charged K204 residue on *A. nancymaae* invasion relative to much less influence from the conservative I→V substitution at position 407 (Figure 4C).

We performed parallel experiments to replace the endogenous *PfRH5* allele in a *P. falciparum* clone, 3D7, which is unrelated to parasites of the 7G8×GB4 cross. In three separate transformation experiments, recovered parasites carrying the GB4 allele ($3D7^{KV}$) showed little change from their original non-invasion phenotype (Figure 4C). A possible explanation for this result is that, while *PfRH5* is a major determinant of *A. nancymaae* virulence in the 7G8×GB4 cross, different *P. falciparum* parasites may require manipulation of other determinants for *A. nancymaae* invasion.

Table 1 shows the distribution of non-synonymous polymorphisms in 7G8, GB4 and 16 additional parasite isolates with different infectivities to New World monkeys and their erythrocytes. Among the various polymorphisms, amino acid class differences stand out among the parasites able to invade *A. nancymaae* erythrocytes with efficiencies > 20%. These include the I204K/I407V substitutions in the GB4 parasite, the S197Y/Y358F substitutions of the Geneve parasite, the E48K/N347Y/K429N substitutions of Malayan Camp, the E48K/I204R/K429N substitutions in Palo Alto, the S197Y/Y358F/E362D substitutions in FVO and the

single N347D substitution of the Santa Lucia parasite (Table 1). Possible involvement of these different changes in monkey erythrocyte invasion by the PfRH5-mediated pathway remains to be examined.

Transformed 7G8^{KV} parasites are virulent to *A. nancymaae*

The ability of 7G8^{KV} transformants to efficiently invade *A. nancymaae* erythrocytes in vitro suggested they could also infect the monkeys in vivo. To test this possibility, we challenged each of six *A. nancymaae* monkeys with an inoculum of 1×10^7 7G8^{KV} parasites. A successful infection was detected in one animal on day 26, and the parasitemia was monitored until day 34 when it was drug cured (Figure 4D). Parasite samples recovered from this infection all expressed the I204K and I407V substitutions of the introduced GB4 allele, despite the absence of WR99210 drug pressure for >30 days (data not shown). The remaining five animals remained parasite negative until the 90 day limit of the experiment, at which time they were treated with mefloquine and retired from the study.

Substitution I204K directs PfRH5 binding to neuraminidase-sensitive receptors on *Aotus* and human erythrocytes

To investigate the erythrocyte binding and cellular localization of PfRH5 protein, we generated and validated rabbit antisera against a peptide from the PfRH5 sequence (predicted amino acids 314–329; overlined in Figure 3B). These antisera specifically detected single bands from *Escherichia coli* expressing transfected subsequences of *PfRH5*, but not from control *E. coli* cells (M_r 16K or 28K bands, Figure 5A). When tested against extracts of parasitized erythrocytes, the antisera detected a protein band with a M_r of 67K (Figure 5B), in the range expected from the predicted molecular weight of PfRH5 (~63 kDa). This band was present in preparations of erythrocytes parasitized by schizonts but not by rings or early trophozoites, in agreement with published *PfRH5* expression data (Le Roch et al., 2004).

We tested for associations of parasite infectivity with PfRH5 attachment to erythrocytes by standard binding assays with parasite culture supernatants (Camus and Hadley, 1985; Gaur et al., 2007). Salt-wash eluates from human erythrocytes that had been incubated with 7G8 or LC12 culture supernatants contained a strong M_r 28K band recognized by anti-PfRH5 sera (Figure 5C). The mobility of this band relative to the M^r 67K band from merozoites suggested it is a proteolytic fragment of the full length PfRH5 protein.

In assays with *A. nancymaae* erythrocytes, binding of the M_r 28K fragment was detected only for the parasite lines that were able to invade these cells. Anti-PfRH5 sera did not detect bound protein from culture supernatants of the 7G8 parasites that are unable to invade *A. nancymaae* erythrocytes, whereas the M_r 28K fragment was readily detected in the eluates after incubation with the LC12 supernatant (Figure 5C). Experiments with allelically-modified transfectants provided further evidence for a strict association between the PfRH5 fragment binding and PfRH5 polymorphisms as well as their ability to invade *A. nancymaae* erythrocytes. Among the 7G8 transformants, binding of the M_r 28K fragment was detected with the *A. nancymaae*-invasive 7G8^{KV} parasite culture supernatants but not with the non-invasive 7G8^{II} or 7G8^{IV} parasites (Figure 5D). Among the LC12 transformants, binding was detected with the invasive LC12^{KV} and LC12^{KI} parasites but not with the non-invasive LC12^{II} parasites (Figure 5D), again indicating a critical role for the position 204 I→K substitution in *A. nancymaae* receptor binding and invasion.

We also examined PfRH5 binding to human erythrocytes before and after treatment with neuraminidase to remove sialic acid residues from cell surface receptor molecules (Breuer et al., 1983). Whereas untreated human erythrocytes bound the M_r 28K fragment from the culture supernatants of all parasites in our experiments: 7G8, LC12 and the allelically-transformed

versions of these clones (Figure 5D), neuraminidase-treated human erythrocytes bound the M_r 28K fragment from 7G8 but not from LC12 (Figure 5E).

The inability of the LC12 PfRH5 fragment to bind neuraminidase-treated human erythrocytes suggested that binding might also be lost with neuraminidase treatment of *A. nancymaae* erythrocytes. Indeed, neuraminidase-treated *A. nancymaae* erythrocytes showed no binding of the M_r 28K fragment from culture supernatants of LC12 parasites (Figure 5E). To see if the sialic acid-mediated binding of the M_r 28K fragment was associated with invasion of *A. nancymaae* erythrocytes, we tested the ability of LC12 to invade neuraminidase-treated *A. nancymaae* erythrocytes. Results confirmed that sialic acid-deficient *A. nancymaae* erythrocytes were fully refractory to invasion (Figure S3). LC12 and 7G8 parasites remained invasive to neuraminidase-treated human erythrocytes (Figure S3), in agreement with previous observations that *P. falciparum* parasites can use various ligand-receptor combinations in different pathways to enter human erythrocytes (Iyer et al., 2007).

PfRH5 localizes to the apical end of merozoites

Immunofluorescence (IF) images of *P. falciparum* parasites probed with anti-PfRH5 sera showed signals from the apical region in developing merozoites of late schizonts and in mature individual merozoites released from parasitized erythrocytes (Figure 6A, B). PfRH5 signals were only faintly observed in the cytoplasm of late trophozoites (data not shown), and they were not detected at all in early trophozoite or ring stage parasites. We used dual-labeled indirect IF confocal laser scanning microscopy to determine the location of PfRH5 signals relative to those of other proteins known to reside in various organelles of the merozoite apical complex. PfRH5 signals at the apical end of merozoites were consistently observed near, but not overlapping with, signals from PfEBA175, a marker of merozoite micronemes (Adams et al., 1990) (Figure 6A). PfRH5 also localized near signals from PfRAP1, a protein of merozoite rhoptry organelles (Stowers et al., 1996) (Figure 6B). PfRH5 signals were identical in wild-type and allelically transformed parasites (data not shown).

Discussion

Our findings link polymorphisms in PfRH5, a newly identified erythrocyte binding protein, to switches of host receptor recognition by *P. falciparum* malaria parasites. In a genetic cross of two parasites that differ dramatically in their ability to infect *A. nancymaae* monkeys (7G8, GB4), inheritance of the *PfRH5* allele from one parent or the other in the haploid progeny proved to be the dominant determinant of infectivity. Transfection and allelic exchange results show that non-synonymous changes in just two codons were sufficient to convert the non-invasive parent (7G8) to a parasite ($7G8^{KV}$, carrying the equivalent of the GB4 parent allele) able to invade *A. nancymaae* erythrocytes and infect a monkey in vivo. Of these two polymorphisms, codon change I204K accounted for nearly all of the conversion in invasion phenotype. Furthermore, the invasion of *A. nancymaae* erythrocytes correlated completely with binding of a PfRH5 M_r 28 K fragment from parasites of the cross.

QTL analysis did not identify loci other than *PfRH5* that could be linked to *A. nancymaae* infectivity in 7G8×GB4 progeny recovered from the chimpanzee host. However, several observations suggest that additional genetic determinants may modulate the PfRH5 pathway for *A. nancymaae* erythrocyte invasion and infection of *A. nancymaae* monkeys, as: (1) different 7G8×GB4 progeny required less than 10 days to more than 50 days to produce patent *A. nancymaae* infections; (2) progeny KB8, JC3 and XF12 produced no infections in *A. nancymaae* even though these clones inherited the GB4 allele of *PfRH5* and invaded monkey erythrocytes well in vitro; (3) replacement of the 7G8 *PfRH5* allele by the GB4 allele in the JB12 progeny clone resulted in an *A. nancymaae* erythrocyte invasion rate about two-thirds of that observed for the same replacement in the 7G8 parent; and (4) non-invasive parasites of

the unrelated *P. falciparum* 3D7 clone remained unable to invade *A. nancymaae* erythrocytes after they were modified to express PfRH5 polymorphisms of the GB4 allele ($3D7^{KV}$ line). Detection of additional determinants involved in these variations may have been obscured by their multiplicity, interactions with partners, or variable effects among the different inherited genetic backgrounds of the 32 recombinant progeny in this study.

The region of highly skewed inheritance on chromosome 7 contains *PfEBA175*, the gene encoding a 175 kDa parasite protein that binds sialic acid residues of erythrocyte surface glycophorin A (Camus and Hadley, 1985; Sim et al., 1994). All 32 independent recombinants from the chimpanzee host inherited the *PfEBA175* allele and nearby genes on chromosome 7 from the 7G8 rather than the GB4 parent. In PfEBA175, the erythrocyte binding domain is defined by a 616 amino acid region (Sim et al., 1994), which differs in the 7G8 and GB4 parasites by nine amino acid residues at polymorphic sites reported previously (data not shown; Liang and Sim, 1997). As the *PfEBA175* gene is proposed to be a determinant of primate specificity (Martin et al., 2005), it is possible that inheritance of the 7G8 allele *vs*. the GB4 allele reflects upon the function of the PfEBA175 protein in primate sialic acid binding.

PfRH5 is the smallest member of the RH family. Its 63 kDa amino acid sequence aligns most closely with that of PrRH5, followed by the N-terminal regions of the 370 kDa PfRH2a and 383 kDa PfRH2b proteins (Rayner et al., 2000). PfRH5 therefore lacks regions corresponding to more than 300 kDa of peptide sequence in the body and C-terminal regions of PfRH2a and PfRH2b, including a predicted transmembrane domain characteristic of other family members. These alignments may help to define binding domains of PfRH2a, PfRH2b and other RH proteins. Our attempts and those of others to disrupt the *PfRH5* gene have been unsuccessful (data not shown; Cowman and Crabb, 2006), in marked contrast to the experimental knock-outs achieved for other members of the RH family as well as various *P. falciparum EBA* genes (Cowman and Crabb, 2006). *PfRH5* may therefore have a vital role in the ability of merozoites to recognize and enter various human and non-human erythrocytes. The location of PfRH5 at the apical end of the merozoite presumably makes the protein readily available for processing, discharge and binding with host cell proteins that function in the invasion of erythrocytes.

The ability of parasites in the 7G8×GB4 cross to infect *A. nancymaae* correlates fully with the binding of a M_r 28K fragment from PfRH5 molecules containing a positively charged lysine residue at position 204. Binding of this fragment may reflect a ligand-receptor function of PfRH5 or its processed forms for parasite entry into the *A. nancymaae* cells. Neuraminidase removal of negatively charged sialic acid residues from the surface of the monkey erythrocytes completely abrogated binding of the M_r 28K fragment and blocked invasion of the parasites in this work. These results suggest that an ionic bond between the positively charged lysine and a negatively charged sialic acid may promote an important ligand-receptor interaction.

Our results also show that human erythrocytes treated with neuraminidase to remove sialic acid residues can bind the M_r 28K fragment of PfRH5 when it contains I204 but not when it contains K204. Non-treated human erythrocytes, however, can bind both forms of the fragment. We note that different polymorphisms are also present in the PfRH5 sequences of other *P. falciparum* lines that are able to infect *A. nancymaae* erythrocytes (Table 1), and many of these polymorphisms involve amino acid class changes, including an I204R substitution in the Palo Alto PfRH5 that may have a functional effect similar to the I204K substitution in GB4. Considering the importance of sialic acid residues to the binding of PfRH5 domains containing K204 but not I204, we suggest that PfRH5 polymorphisms may enable *P. falciparum* parasites to switch their ligand-receptor interactions and adapt to different erythrocyte surface molecules. Point mutations in the sequences of two other *P. falciparum* ligands, JESEBL/EBA181 and BAEBL/EBA140, also alter their molecular specificity for different receptors on erythrocytes (Mayer et al., 2002;Mayer et al., 2004). These and other polymorphisms provide

an important adaptive ability in the various pathways *P. falciparum* uses to infect different human erythrocytes. The utility of these pathways is severely restricted in the non-natural host cells of *A. nancymaae*, accounting for the dependence of LC12 and other 7G8×GB4 parasites on PfRH5 as a single ligand for invasion.

Parasites that infect across species barriers are reported from a number of primate malarias. For example, *P. knowlesi* parasites that naturally infect monkeys have produced outbreaks of malaria in human populations (Cox-Singh et al., 2008). The close relationship between macaque *P. cynomolgi* and human *P. vivax* parasites (Cornejo and Escalante, 2006) points to the existence of shared reservoirs of infection before a recent evolutionary divergence, and the near identity of *P. simium* to *P. vivax* may reflect an even more recent host switch back to monkeys from humans (Cornejo and Escalante, 2006). In the present case, there is no evidence that *Aotus* monkeys carry a reservoir of *P. falciparum* parasites in South America (Collins, 1994). Some of the *P. falciparum* strains able to infect *A. nancymaae* in our study were isolated in Africa (GB4, Ghana; Palo Alto, Uganda) where these parasites had not been under selective pressure to adapt to *Aotus* monkeys. It seems likely that the ability of these parasites to invade *A. nancymaae* erythrocytes derives from the diversity of ligand-receptor interactions exploited by *P. falciparum* in its various pathways to invade human erythrocytes.

Experimental Procedures

Animal Studies

All animal studies were carried out in compliance with National Institutes of Health guidelines under an Animal Care and Use Committee-approved protocol. *P. falciparum* in *A. nancymaae* is virulent and can be fatal. Monkeys developing a parasitemia of 5% were promptly cured of their infection with 25 mg/kg mefloquine, as were monkeys showing any signs of dehydration, lethargy, decreased appetite or diarrhea, or anemia with a hematocrit below 25%. *A. nancymaae* monkeys were either naïve to *P. falciparum* or had a history of infection more than 6 months before entry in the study. All monkeys were splenectomized. Infections were initiated by the intravenous inoculation of 1×10^7 parasitized erythrocytes from human cell cultures.

The 7G8×GB4 Cross

7G8 and GB4 gametocytes were induced in-vitro as described (Ifediba and Vanderberg, 1981), grown to maturity, and fed through stretched Parafilm membranes to *An. freeborni* and *An. gambiae* mosquitoes using a membrane feeder. A splenectomized chimpanzee was infected with sporozoites by the natural biting of ~300 mosquitoes on day 15 (Walliker et al., 1987). Twenty-one days after sporozoite inoculation, the parasitemia in the chimpanzee reached 0.2%, at which time a final blood sample was collected and the asymptomatic infection cured with 15 mg/kg mefloquine. Independent recombinant progeny clones were cloned by limiting dilution, either directly from chimpanzee blood or from parasite lines adapted to culture in human erythrocytes.

Invasion Assays

Erythrocyte invasion assays were performed as described (Dolan et al., 1990). Briefly, erythrocytes infected with mature stage parasites were obtained by gelatin floatation or percoll/sorbitol purification and adjusted to a final concentration of 1×10^7 parasitized erythrocytes/ml. One hundred µl of the parasite suspension were added to 100 µl of target erythrocytes at 2×10^8 cells/ml in complete medium and incubated at 37°C for 12–18 hr. Human and rhesus erythrocytes were included as positive and negative controls, respectively. Invasion was determined by counting the number of ring-infected erythrocytes and expressed as a percent

of the invasion counts observed for human erythrocytes. Each assay was performed in triplicate, on at least three independent occasions.

Generation of the 7G8×GB4 Linkage Map and QTL Analysis

Progeny clones were genotyped using polymorphic microsatellites (Su et al., 1999). Additional primers were designed for areas with low coverage or to narrow informative meiosis events from chromosome sequence data for the 3D7 *P. falciparum* parasite (www.plasmodb.org) (Bahl et al., 2003). The segregation data for all heterozygous loci are provided in Table S1. These data were used to construct a genetic map using the MAPMAKER program and QTL analysis was carried out using the Pseudomarker program as described (Sen and Churchill, 2001; Ferdig et al., 2004).

Allelic Exchange at the *PfRH5* Locus

Allelic exchange plasmids were designed to contain a 1396 bp fragment from the *PfRH5* sequence of GB4 or 7G8. DNA fragments were amplified with primers containing *Not* I (5'-TGCGGCCGCATGAAGACTATAAAAATGTGG-3') and *Pst* I (5'-ACTGCAGATGCTTTGTCTAATTAGAG-3) restriction sites. These sites facilitated cloning into the pHD22Y vector containing the human dihydrofolate reductase (*hDHFR*) selectable marker (Fidock and Wellems, 1997). Uninfected human erythrocytes were loaded with plasmid DNA by electroporation and supplied to parasite cultures as described (Deitsch et al., 2001). Parasite populations containing integrated plasmid were identified after approximately 60 days by diagnostic PCR.

Production and Verification of Antisera

A multiple antigen peptide (MAP4) directed against a PfRH5 peptide sequence (LIKCIKNHENDFNKIC) was synthesized by Bio-Synthesis Inc., Lewisville, TX. Anti-peptide sera were produced by immunizing a rabbit with 250 μg peptide in CFA followed by 250 μg peptide in IFA on days 21, 42 and 63 (Spring Valley Laboratories, Inc.).

Two fragments of *PfRH5* were amplified from a synthetic bacteria codon-adjusted sequence (a gift from David Garboczi), encoding either a 16 or 28 kDa fragment of PfRH5 (amino acids 304–430 and 304–526). These fragments were cloned into the pNAN T7 expression vector (Su et al., 2006) and expressed in BL21 cells (Stratagene, La Jolla, CA) induced with 0.5 mM IPTG (isopropyl-β-d-thiogalactopyranoside) for 2 hr.

Immunoblot Analysis and Immunofluorescence (IFA) Microscopy

Sorbitol synchronized ring, trophozoite and schizont stages were lysed with 0.15% saponin in PBS, pelleted, and immediately frozen. Thawed pellets containing 5×10^6 parasitized erythrocytes were resuspended in sample buffer containing 250 mM sucrose, 20 mM HEPES, protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO), and 1x NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA). After separation by electrophoresis (NuPAGE® Novex system, 4–12% Bis-Tris Gels, Invitrogen), the proteins from the pellets were transferred to a nitrocellulose membrane (Invitrogen). Membranes were blocked overnight in 5% skimmed milk and incubated for 1 hr with rabbit anti-PfRH5 sera (1:250 dilution in PBS), or with rabbit anti-human spectrin to confirm sample loading (Sigma-Aldrich) and processed as described (Gaur et al., 2006).

For IFA, schizont preparations were fixed either with chilled acetone or 1% formaldehyde for 5 min at room temperature, incubated in blocking buffer (PBS with 0.1% Triton X-100, 2.5 mg/ml goat serum) for 5 min and then probed for 1 hr at 37°C with rabbit anti-PfRH5 sera plus either rat anti-PfEBA175 sera or mouse anti-PfRAP1 sera (Stowers et al., 1996) (1:50 dilution

in blocking buffer). Samples were processed and microscopic images were collected and analysed as described (Gaur et al., 2007).

Erythrocyte Binding Assay

Erythrocyte binding assays were performed as described (Gaur et al., 2007).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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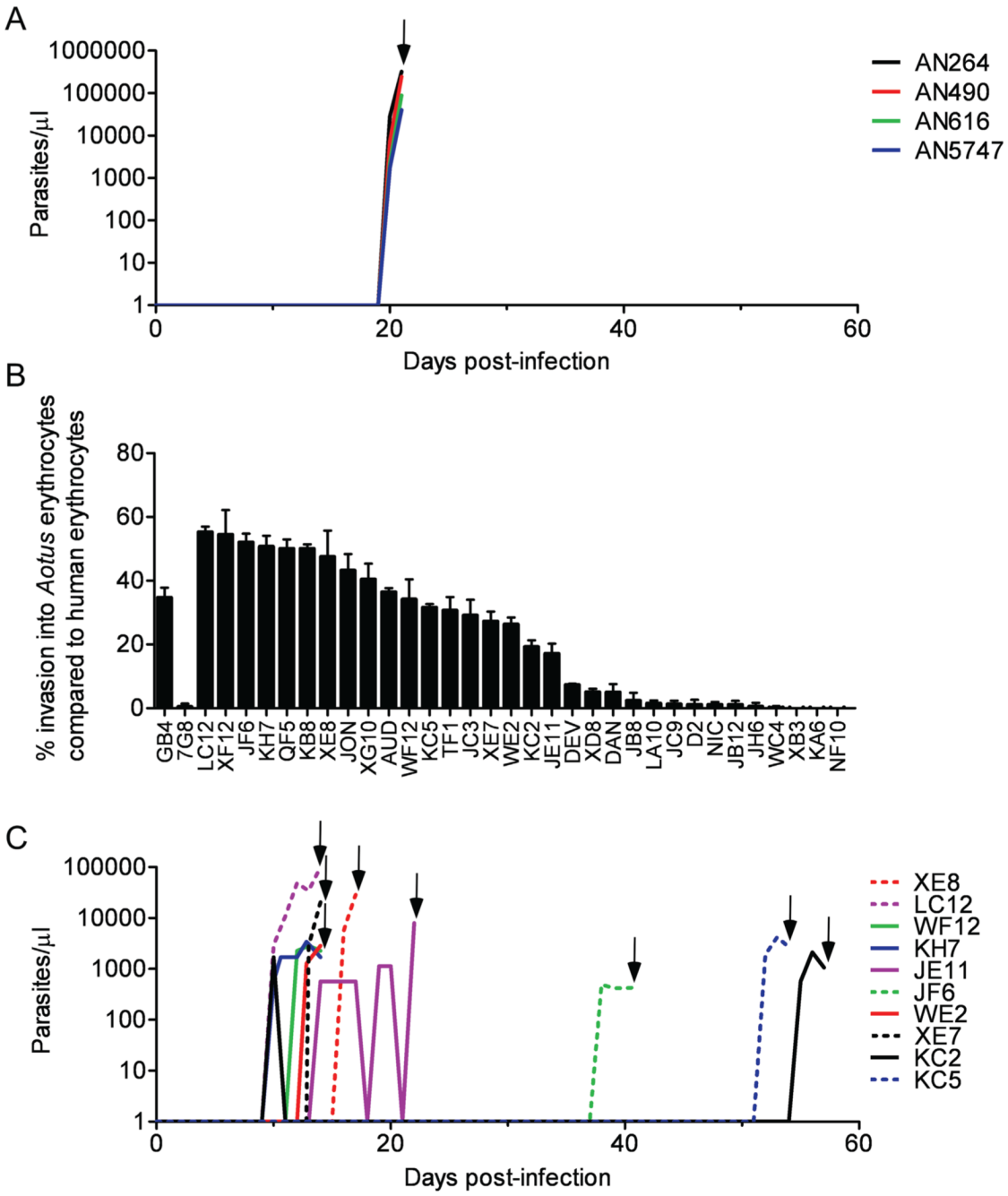

FIGURE 1. *P. falciparum* parasite infectivities to *Aotus* monkeys and to their erythrocytes in vitro

A) Parasitemias obtained in four *A. nancymaae* monkeys after intravenous inoculation of 1×10^7 GB4-parasitized erythrocytes. All infections were cured with 25 mg/kg mefloquine (arrow). B) *A. nancymaae* erythrocyte invasion rates by the parents and 32 independent recombinant progeny from the *P. falciparum* 7G8×GB4 cross. Percentage values are relative to rates of invasion into human control erythrocytes ± SEM. C) Infectivity of 10 7G8×GB4 progeny clones to *A. nancymaae* monkeys. All infections were cured with 25 mg/kg mefloquine (arrows).

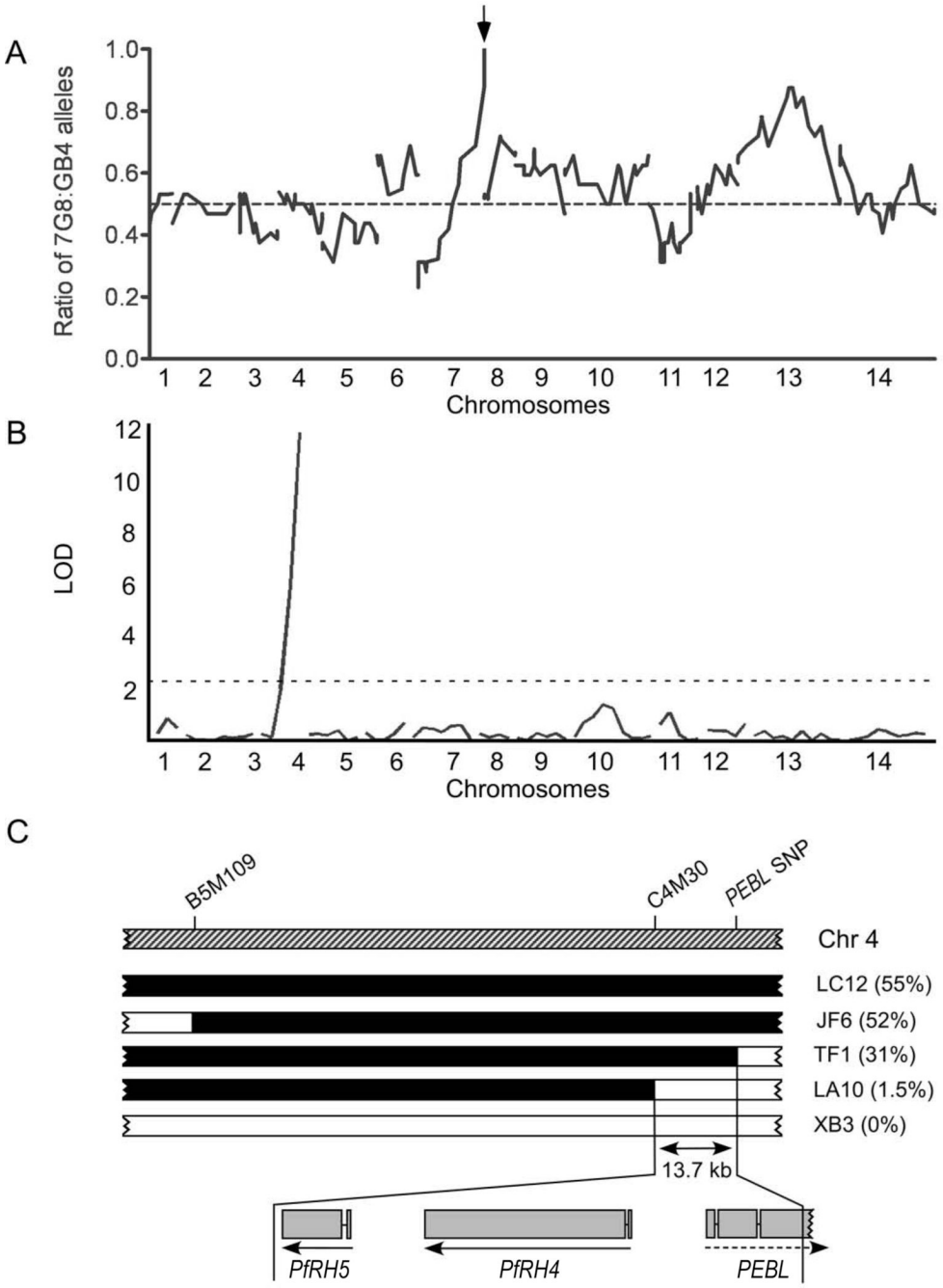

FIGURE 2. Identification of candidate genes for the *A. nancymaae* invasion phenotype

A) Genome-wide plot of the ratios of 7G8:GB4 markers among the 32 independent recombinants. A peak containing *PfEBA175* (arrow) identifies a region of chromosome 7 that is severely skewed in favor of the 7G8 parent. A dashed line indicates the 0.5 ratio expected from random assortment. B) Logarithm of odds (LOD) scores from the primary scan for quantitative trait loci (QTL) associated with *A. nancymaae* erythrocyte invasion. The peak with LOD of 11.8 maps to the sub-telomeric region of chromosome 4. A dashed line represents the significance threshold (0.05). C) Map of the chromosome region linked to *A. nancymaae* invasion phenotype. *A. nancymaae* erythrocyte invasion phenotypes (%'s in brackets) are shown for five progeny clones carrying different chromosome segments from each of the

parents in the mapped region (chr 4, chromosome 4; solid black segments, GB4; open segments, 7G8). The *Aotus* invasion locus maps to a 13.7 kb sequence defined by the microsatellite marker C4M30 and a single nucleotide polymorphism (SNP) in the pseudogene *PEBL*. The exons and transcription directions of the *PfRH4* and *PfRH5* genes are indicated by connected gray bars and arrows, respectively, while the reading frame direction of the pseudogene *PEBL* is shown with a dashed arrow. Comparisons of the genomic and cDNA sequences of the 7G8 *PfRH5* gene, including 5'- and 3'-rapid amplification of cDNA ends (RACE) sequencing, confirmed the two exon structure documented in PlasmoDB (www.plasmodb.org).

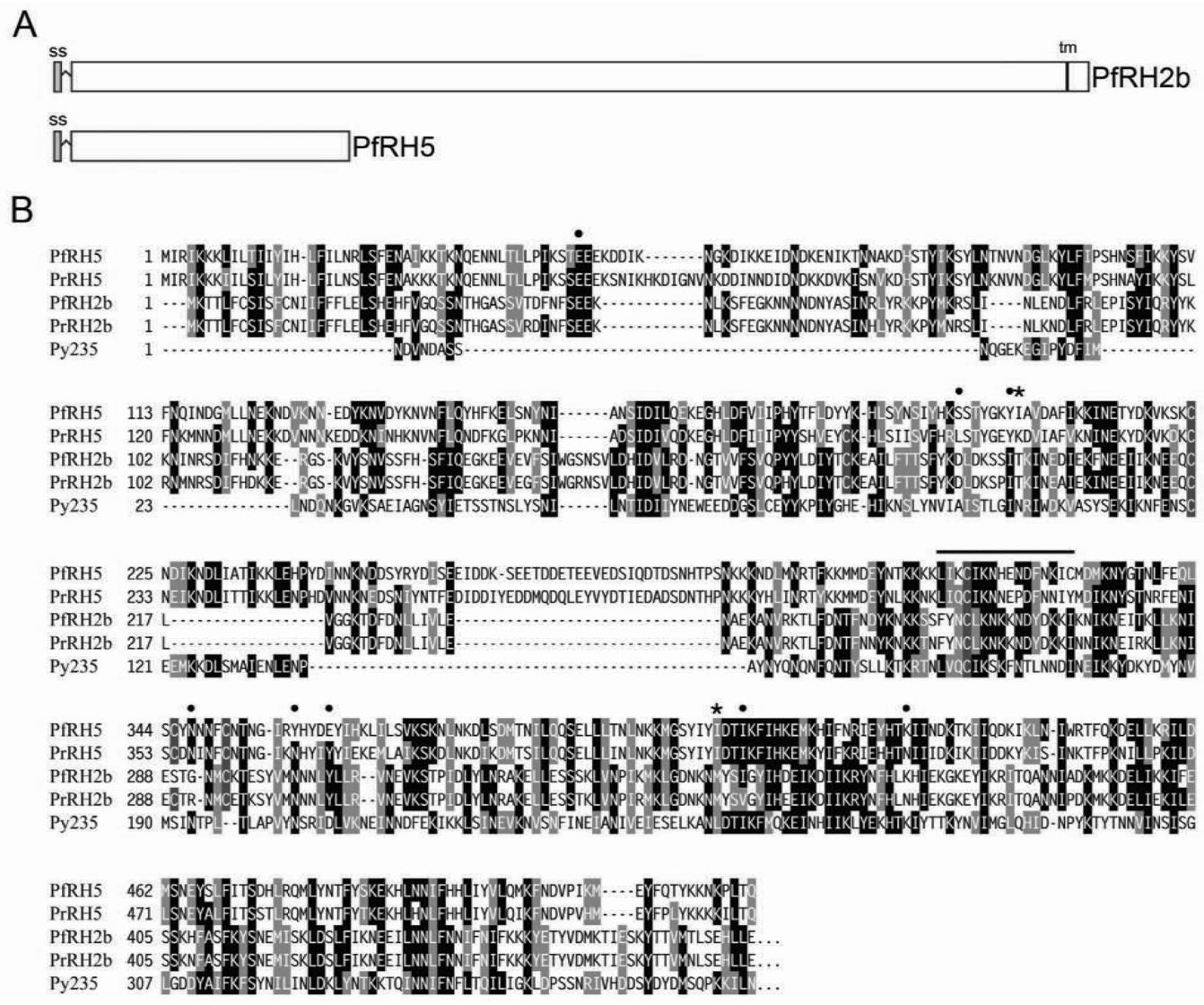

FIGURE 3. Schematic representation and sequence alignment of PfRH5 with members of the RH superfamily

A) Organization of PfRH5 and PfRH2b. ss, putative signal sequence (gray box); tm, transmembrane region (black box). PfRH5 has 526 predicted amino acids and an estimated molecular weight of 63 kDa. B) Alignments of predicted RH5 and homologous N-terminal RH sequences from *P. falciparum*, *P. reichenowi* and *P. yoelii*. Conserved residues are shaded black and semi-conserved regions shaded grey. PfRH5 carries cysteine residues (purple highlight) at four of the five positions where there are conserved or semi-conserved cysteines in the other sequences (185, 224, 317, 345, 351). Amino acids 1–24 of PfRH5 constitute a possible signal peptide (SignalP 3.0 program; Bendtsen et al., 2004). Asterisks mark positions 204 and 407 where polymorphisms occur between the 7G8 and GB4 clones; positions of differences in other isolates (Table 1) are marked with closed circles. Lysine occurs in position 204 of PfRH5 in GB4, but not in other *P. falciparum* lines of our survey (Table 1); this residue is also encoded at the equivalent codon position in the *P. reichenowi PrRH5* sequence and may represent a persisting ancestral codon. Amino acids of the synthetic MAP4 peptide are overlined. Sequences: PfRH5, (7G8 clone, accession number EU433391), PfRH2b (amino acids 1–375, AF312917), PrRH2b (1–473, Y166677), PrRH5 (EF142858), Py235 (partial

sequence, 1–464, XM_719883); Pf, *P. falciparum*; Pr, *P. reichenowi*, Py, *P. yoelii*. Sequence homology searches were performed using tblastn and alignments were generated with T-Coffee software (Notredame et al., 2000).

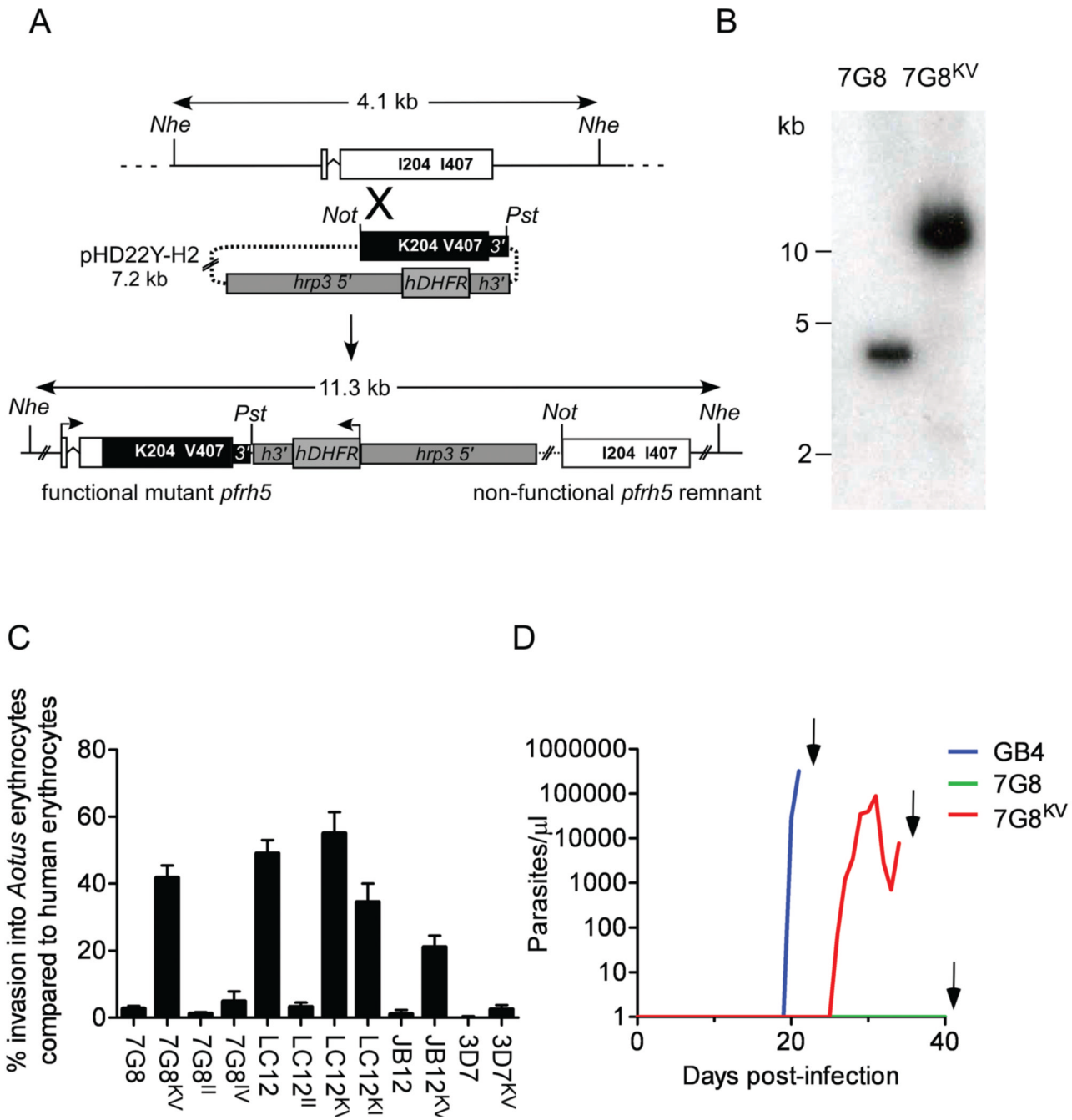

FIGURE 4. Allelic modification of *PfRH5* and confirmation of its role in erythrocyte invasion

A) Schematic of the strategy to generate a 7G8^{KV} parasite by homologous recombination. A portion of the GB4 *PfRH5* gene containing the K204 and V407 codons along with 0.2 kb of 3' flanking sequence replaces its counterpart in the expressed endogenous allele. The *hDHFR* (human dihydrofolate reductase) drug selection marker is located downstream of the modified *PfRH5* allele. Abbreviations: *3'*, 3' UTR of *PfRH5; hrp3 5'*, promoter region from *P. falciparum* histidine-rich protein 3; *h3'*, 3' UTR of *P. falciparum* histidine-rich protein 2; *Nhe*, *Nhe* I; *Not*, *Not* I; *Pst*, *Pst* I. B) Southern blot verification of the 11.3 kb *Nhe* I band resulting from homologous integration in the *PfRH5* locus. Genomic DNAs were isolated from untransformed 7G8 parasites and from transformed 7G8^{KV} parasites containing the GB4

PfRH5 allele. C) Invasion rates of untransformed and transformed *P. falciparum* lines into *A. nancymaae* erythrocytes ± SEM. D) Results in three naïve *A. nancymaae* monkeys after intravenous inoculation of bloodstream forms of GB4 (blue line), 7G8 (green line) or *PfRH5*-modified 7G8KV parasites (red line). All animals were splenectomized prior to infection and cured with 25 mg/kg mefloquine (arrows).

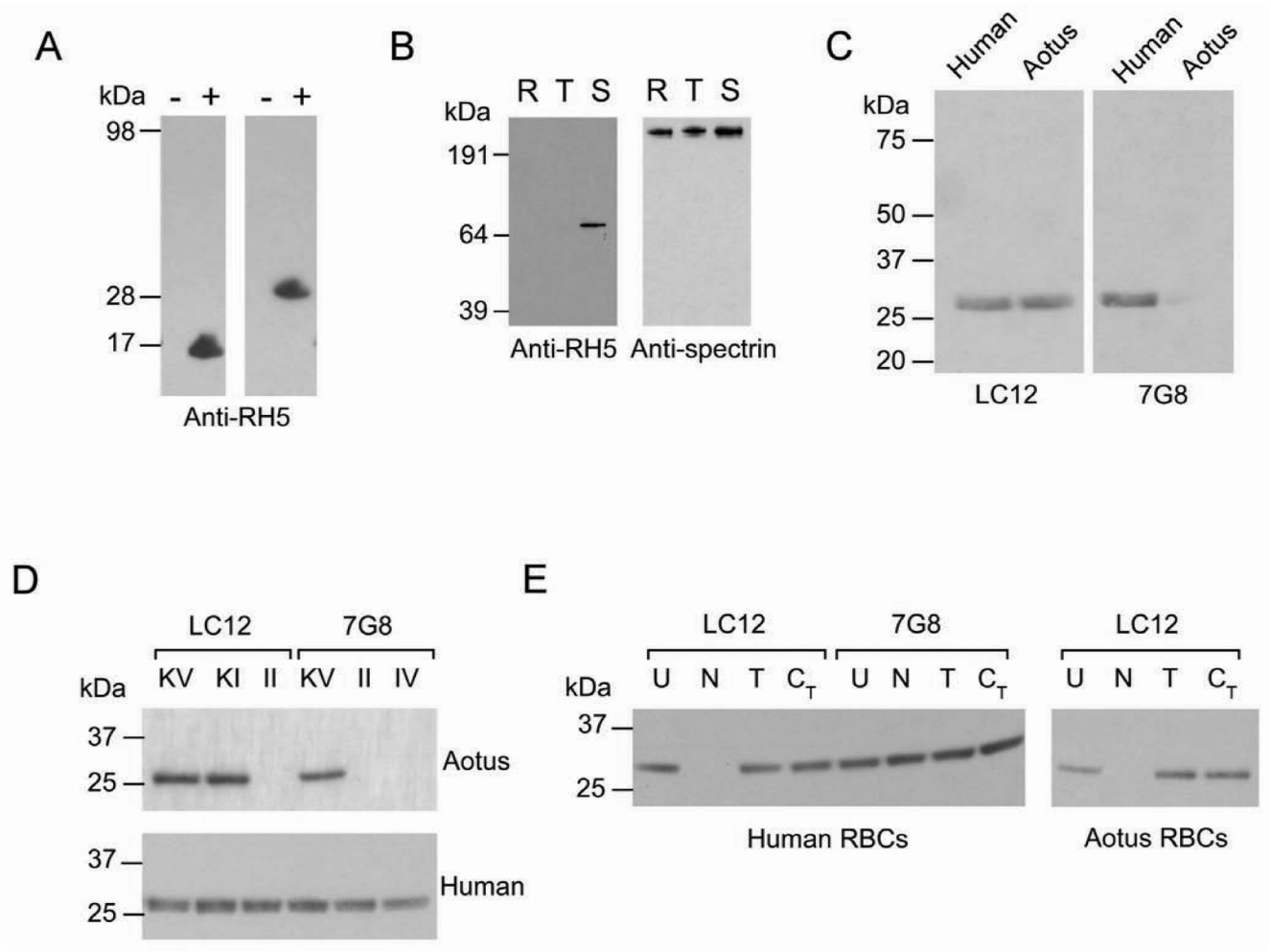

FIGURE 5. Immunoblot detection of PfRH5 and binding of a Mr 28K fragment to human and *A. nancymaae* erythrocytes

A) Rabbit antisera raised against a 16 amino acid peptide recognize 16 and 28 kDa fragments of PfRH5 in *Escherichia coli*. Polypeptides of the appropriate size are present in induced (+) but not uninduced (−) cells. B) Immunoblot analysis of 7G8 parasite extracts from synchronized ring stages (R), early trophozoites (T) and mature schizonts (S). A M_r 67K band is evident in the 7G8 schizont preparation probed by anti-PfRH5 peptide antisera. The signals from repeat probing of the blot with antibodies against human spectrin, an abundant red cell protein present in parasite extracts, verify that protein from comparable numbers of cells was present in each lane. C) The M_r 28K PfRH5 fragment of LC12 parasites binds to *A. nancymaae* as well as human erythrocytes, consistent with the ability of LC12 to infect both types of cells, while the PfRH5 fragment from 7G8 parent parasites only binds human erythrocytes. D) The M_r 28K PfRH5 fragment from parasites transformed to express K204 ($LC12^{KV}$, $LC12^{KI}$, $7G8^{KV}$), but not I204 ($LC12^{II}$, $7G8^{II}$, $7G8^{IV}$) binds to *A. nancymaae* erythrocytes. Human erythrocytes bind the PfRH5 fragment from all parasites. E) The human erythrocyte receptor for the PfRH5 fragment of 7G8 parasites is neuraminidase-, trypsin-and chymotrypsin-resistant, while both the human and *A. nancymaae* erythrocyte receptor for the PfRH5 fragment of LC12 parasites is trypsin- and chymotrypsin-resistant, but neuraminidase-sensitive. U, untreated; N, neuraminidase; T, trypsin; C_T, chymotrypsin.

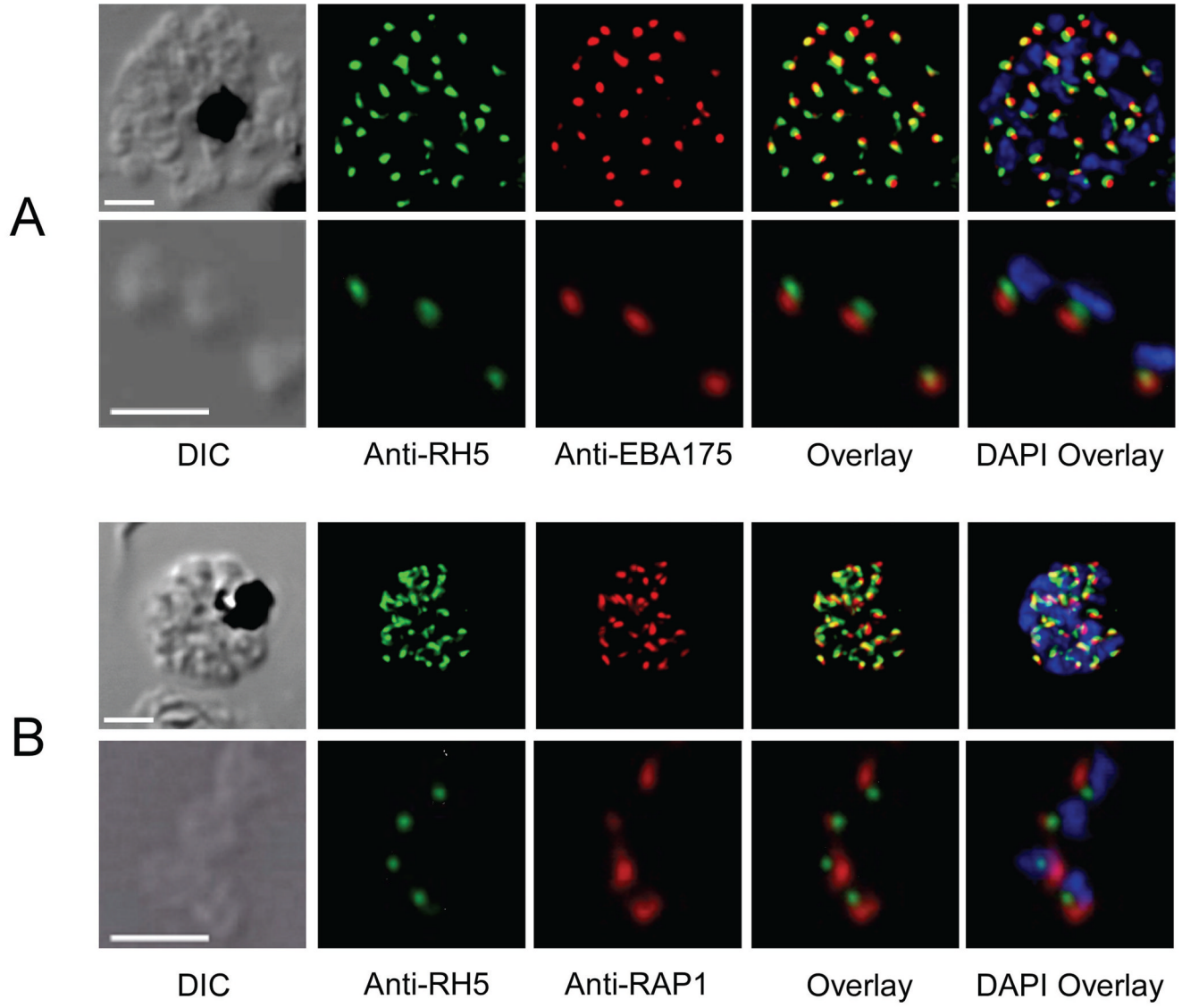

Figure 6. PfRH5 localization in schizonts and merozoites

A) Immunoconfocal microscopy showing the localization of PfRH5 relative to PfEBA175, a microneme marker. Signals from mature segmented schizonts are shown in the top panes; signals from free merozoites are shown in the bottom panes. B) Immunoconfocal microscopy showing the localization of PfRH5 relative to PfRAP1, a rhoptry marker. Signals from mature schizonts are shown in the top panes; signals from free merozoites are shown in the bottom panes. Scale bar, 2 μm.

TABLE 1

Position of amino acid polymorphisms in PfRH5 from parasites with various *A. nancymaae* erythrocyte invasion phenotypes.

Parasite	48	197	203	204	347	358	362	407	410	429	*A. nancymaae* invasion (% ± SEM)[a]
3D7	E	S	**C**	I	N	Y	E	I	I	K	0 ± 0.3
Tm284	E	S	**C**	I	N	Y	E	I	**M**	K	0 ± 0.7
7G8	E	S	Y	I	N	Y	E	I	I	K	0.4 ± 0.2
Haiti	E	S	Y	I	N	Y	E	I	I	K	0.4 ± 1.0
Mont S1[b]	E	**Y**	Y	I	N	Y	E	I	I	K	1.9 ± 0.0
JAV	E	S	Y	I	N	Y	E	I	I	K	2.4 ± 2.0
Ecu110	E	S	Y	I	N	Y	E	I	I	K	3.0 ± 1.6
ECP	E	S	Y	I	N	Y	E	I	I	K	3.7 ± 2.1
Dd2	E	S	**C**	I	N	Y	E	I	**M**	K	3.4 ± 3.4
Liberia II	E	S	Y	I	N	Y	E	I	I	K	3.8 ± 2.6
D10	E	S	Y	I	N	Y	E	I	I	K	4.7 ± 2.2
HB3	E	S	Y	I	N	Y	E	I	I	K	4.8 ± 0.2
Mal Camp	**K**	S	Y	I	**Y**	Y	E	I	I	**N**	22.6 ± 5.9
Geneve[c]	E	**Y**	Y	I	N	**F**	E	I	I	K	26.4 ± 0.8
GB4[d,e]	E	S	Y	**K**	N	Y	E	**V**	I	K	34.7 ± 3.2
Santa Lucia	E	S	Y	I	**D**	Y	E	I	I	K	46.5 ± 8.0
Palo Alto	**K**	S	Y	**R**	N	Y	E	I	I	**N**	46.8 ± 3.3
FVO	E	**Y**	Y	I	N	**F**	**D**	I	I	K	58.5 ± 1.8

[a] Merozoite invasion rates of *A. nancymaae* erythrocytes are shown as a percent of the invasion into human erythrocytes ± SEM.

[b] Infects *A. lemurinus griseimembra* (Collins et al., 1997).

[c] Grows in *Saimiri sciureus* (Fajfar-Whetstone et al., 1987).

[d] Previously shown to infect *A. lemurinus griseimembra*, *A. nancymaae* and *A. vociferans* (Sullivan et al., 2003).

[e] In addition to open reading frame polymorphisms, *PfRH5* in GB4 contains a microsatellite TA-repeat in its intron that is 17 nucleotides shorter than in 7G8.