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Cooperativity between *Plasmodium falciparum* adhesive proteins for invasion into erythrocytes

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Abstract

Plasmodium falciparum is the most virulent of the *Plasmodium* species infective to humans. Different *P. falciparum* strains vary in their dependency on erythrocyte receptors for invasion and their ability to switch in their utilization of different receptor repertoires. Members of the Reticulocyte binding protein-like (RBL) family of invasion ligands are postulated to play a central role in defining ligand-receptor interactions, known as invasion pathways. Here we report the targeted gene disruption of *PfRh2b* and *PfRh2a* in W2mef, a parasite strain that is heavily dependent on sialic-acid receptors for invasion, and show that the PfRh2b ligand is functional in this parasite background. Like the parental line, parasites lacking either PfRh2a or PfRh2b can switch to a sialic acid-independent invasion pathway. However, both of the switched lines exhibit a reduced efficiency for invasion into sialic acid depleted cells, suggesting a role for both PfRh2b and PfRh2a in invasion via sialic acid-independent receptors. We also find a strong selective pressure for the reconstitution of PfRh2b expression at the expense of PfRh2a. Our results reveal the importance of genetic background in ligand-receptor usage by *P. falciparum* parasites, and suggest that the coordinate expression of PfRh2a, PfRh2b together mediate efficient sialic acid-independent erythrocyte invasion.

Keywords

Plasmodium falciparum; erythrocyte invasion; Reticulocyte binding protein; sialic acid; receptor

Introduction

The Apicomplexan parasite *Plasmodium falciparum* is the causative agent of the most severe form of human malaria and is responsible for much global morbidity and mortality (Greenwood *et al.*, 2008). Clinical symptoms of malaria occur when parasites proliferate within the bloodstream of infected individuals. During this asexual phase of *Plasmodium* development, invasive forms of the parasite called merozoites recognize, invade, and multiply rapidly within erythrocytes.

Erythrocyte invasion by *P. falciparum* is a complex process requiring the specific recognition of host receptors by parasite invasion ligands (Cowman & Crabb, 2006). These

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ligand-receptor interactions are referred to as invasion pathways. The utilization of alternative invasion pathways in different parasite strains provides a mechanism for immune evasion and/or adaptation to erythrocyte receptor polymorphism (Dolan *et al.*, 1990, Mitchell *et al.*, 1986, Hadley *et al.*, 1987).

One superfamily of parasite proteins involved in erythrocyte invasion is the Reticulocyte-binding-like (RBL) proteins that are postulated to play a major role in host selection. Most members of this family are large type-1 transmembrane proteins and recent evidence suggests that regions at the N-terminal ends of the ectodomain are involved in binding to host receptors (Gao *et al.*, 2008, Gaur *et al.*, 2007). Included in this superfamily are the *P. vivax* Reticulocyte-binding proteins (RBP)-1 and -2, *P. yoelii* Py235 proteins, and *P. falciparum* Reticulocyte-binding-like homologues (PfRh). Interestingly, PvRBP-1 and -2 engage a reticulocyte-specific receptor and may be responsible for targeting *P. vivax* parasites to reticulocytes (Galinski *et al.*, 1992). Similarly it has been shown that antibodies specific for Py235 can limit *P. yoelii* infections to reticulocytes (Freeman *et al.*, 1980), and that increased expression of Py235 is associated with an ability to invade a wider range of erythrocytes and virulence in *P. yoelii* (Iyer *et al.*, 2007).

P. falciparum expresses five RBL paralogs- PfRh1, PfRh2a, PfRh2b, PfRh4 and PfRh5-at the protein level, while PfRh3 is transcribed but not translated (Hayton *et al.*, 2008, Kaneko *et al.*, 2002, Rayner *et al.*, 2000, Rayner *et al.*, 2001, Triglia *et al.*, 2001, Taylor *et al.*, 2001). Different *P. falciparum* laboratory lines vary in their expression of members of the PfRh protein family (Duraisingh *et al.*, 2003b, Taylor *et al.*, 2002). These variant expression profiles are stable in continuous *in vitro* culture, but likely provide an opportunity for the parasite to either evade the immune system or attach to polymorphic receptors in the context of *in vivo* infection. N-acetylneuraminic acid is the only form of sialic acid found on the surfaces of human erythrocyte (Wang & Brand-Miller, 2003) and PfRh proteins are characterized by their ability to mediate merozoite invasion via sialic acid-dependent (PfRh1) or -independent (PfRh2b and PfRh4) receptors (Rayner *et al.*, 2001, Duraisingh *et al.*, 2003b, Triglia *et al.*, 2005).

Though some *P. falciparum* strains are reliant on sialic acid for erythrocyte entry, there exists some plasticity in the ability of a given parasite line to utilize different invasion pathways. Several parasite lines (W2mef/Dd2 and CSL2), but not all, have been demonstrated to be able to shift from a reliance on sialic acid-dependent invasion pathways to one of sialic acid-independence (Stubbs *et al.*, 2005, Dolan *et al.*, 1990). This shift in invasion pathway utilization is associated with the activation of PfRh4 expression (Stubbs *et al.*, 2005, Gaur *et al.*, 2006).

Here we report our analyses of PfRh function in the sialic acid-dependent parasite line W2mef. This parasite line expresses significant quantities of both the sialic acid-dependent RBL ligand *PfRh1* and sialic acid-independent RBL ligands, including PfRh2a and PfRh2b. Despite sharing over 7.5 kb of sequence PfRh2b, but not its paralog PfRh2a, has been shown to be functional in the sialic acid-independent parasite line 3D7 (Duraisingh *et al.*, 2003b). W2mef can switch towards sialic acid-independence invasion and we thus hypothesized that in addition to PfRh4, the sialic acid-independent ligand PfRh2b may play a role. Our results from targeted gene disruptions show that W2mef parasites deficient in *PfRh2b* utilize a new invasion pathway, demonstrating the functionality of a sialic acid-independent invasion ligand in sialic acid-dependent parasites. We also show that while neither PfRh2a nor PfRh2b is essential for phenotypic switching to sialic acid independent invasion, both of these ligands are required for efficient sialic acid-independent invasion. However, PfRh2b reconstitution at the expense of PfRh2a suggests a dominant role for this invasion ligand.

Results

The merozoite adhesive ligands PfRh2a and PfRh2b are dispensable in the *P. falciparum* W2mef parasite line

PfRh2b but not *PfRh2a* has previously been shown to be functional in the sialic acid-independent *P. falciparum* parasite line, 3D7 (Duraisingh et al., 2003b). Unlike 3D7, W2mef is a sialic acid-dependent *P. falciparum* parasite line that expresses *PfRh2a* and *PfRh2b* concurrently, as well as PfRh1 (Triglia et al., 2005). To identify a functional role for PfRh2a and PfRh2b in W2mef parasites, we targeted these genes for disruption using the pHTkΔrh plasmid for integration into the approximately 7.5 kb of 5' sequence that the two genes share, via double-crossover recombination (Figure 1A) (Duraisingh et al., 2003b). Recombinant parasites were obtained after selection with WR99210, and cloned by limiting dilution. Southern blots to distinguish between integration into PfRh2a and PfRh2b were performed using PrA and PrB, which correspond to the *PfRh2a* and *PfRh2b* 3' unique regions. These analyses confirmed that in one parasite line PfRh2a was disrupted by double crossover recombination while PfRh2b remained intact, and this clone was designated W2mefΔ2a (Figure 1B). In the other clone, integration into *PfRh2b* was observed by single crossover recombination via the 5' flank of pHTkΔrh, while PfRh2a remained intact, and this clone was designated W2mefΔ2b (Figure 1B). Successful disruption of *PfRh2a* and *PfRh2b* in *P. falciparum* W2mef demonstrates that their expression is not essential for parasite survival.

To determine whether genetic disruption of *PfRh2a* and *PfRh2b* abrogated protein expression, Western blots on synchronous W2mef, W2mefΔ2b, and W2mefΔ2a culture supernatants were performed. C-terminal-specific PfRh2b antibodies revealed the presence of the protein in W2mef and W2mefΔ2a, but not in W2mefΔ2b (Figure 1C). Antibodies specific to PfRh2a reveal the presence of the protein in parental W2mef and W2mefΔ2b, but not in W2mefΔ2a, corroborating our Southern data (Figure 1C). Disruption of one gene does not result in a compensatory change in the protein levels of the other gene.

PfRh2b is functional in the sialic acid-dependent parasite line W2mef

Parasite ligand-erythrocyte receptor interactions are defined by their sensitivities to enzymes, such as neuraminidase, chymotrypsin, and trypsin. These enzymes are specific in their activity and cleave only susceptible erythrocyte glycoproteins, leaving resistant receptors intact. The receptor for PfRh2b, known as Receptor Z, interacts with its cognate parasite ligand via sialic acid-independent/trypsin-resistant/chymotrypsin-sensitive means (Duraisingh et al., 2003b). The enzyme sensitivities of the receptor complementary to PfRh2a have not been determined.

Both W2mefΔ2a and W2mefΔ2b are similar to parental W2mef in their dependence on sialic acid-containing receptors (Table 1). Loss of *PfRh2a* does not affect W2mef invasion into chymotrypsin- or trypsin-treated erythrocytes (Table 1). These observations are in keeping with the absence of significant changes previously reported following disruption of *PfRh2a* in the 3D7 genetic background (Duraisingh et al., 2003b). Disruption of *PfRh2b* in W2mef resulted in significantly enhanced invasion into chymotrypsin- and low trypsin/chymotrypsin treated erythrocytes (Table 1), similar to that seen previously following loss of *PfRh2b* expression in 3D7. However, strain-specific differences were observed in the use of an invasion pathway revealed following high trypsin treatment of erythrocytes, with W2mefΔ2b exhibiting increased invasion into erythrocytes (Table 1), with no difference being observed in the 3D7Δ2b parasites (Duraisingh et al., 2003b). The shifted invasion profile of W2mefΔ2b parasites relative to W2mef is indicative of altered ligand-receptor

interactions and reveals a functional role for PfRh2b in the sialic acid-dependent *P. falciparum* line W2mef.

Neither PfRh2a nor PfRh2b are essential to phenotypic switching in W2mef parasites

Wild-type W2mef parasites possess the ability to switch from a sialic acid-dependent to -independent invasion pathway following selection on neuraminidase-treated/sialic acid-deficient erythrocytes. Activation of *PfRh4* is necessary for phenotypic switching, as W2mefΔRh4 parasites fail to grow on sialic acid-depleted erythrocytes (Stubbs *et al.*, 2005). *PfRh4* is not expressed in parental W2mef, but rather in sialic acid-independent strains, such as 7G8, 3D7, and HB3. We hypothesized that PfRh2b may also be required for phenotypic switching, given its similar independence on sialic acid for invasion. We thus proceeded to culture wild-type W2mef, W2mefΔ2a and W2mefΔ2b parasites on neuraminidase-treated (sialic-acid depleted) erythrocytes. Sialic acid-independent W2mef (W2mef/Nm) parasites emerge approximately ten days post-plating. We find that W2mef, W2mefΔ2a and W2mefΔ2b all possess the ability to switch to sialic acid-independent invasion to resulting in the parasite lines termed W2mef/Nm, W2mefΔ2a/Nm and W2mefΔ2b/Nm respectively.

The emergence of W2mefΔ2a/Nm and W2mefΔ2b/Nm parasites prompted us to characterize the molecular basis for the phenotypic switch. To this end, we employed Western blotting methods to assess PfRh2a and PfRh2b levels in switched wild-type and knockout parasite lines. With regard to switched W2mefΔ2a/Nm and W2mefΔ2b/Nm parasite lines, Western blot reveals that these parasites remain deficient in PfRh2a and PfRh2b respectively (Figure 2A). We carried out Southern blots to corroborate these results at the genetic level, finding that PfRh2a and PfRh2b remain disrupted in W2mefΔ2a/Nm and W2mefΔ2b/Nm, respectively (Figures 2B, 2C). We conclude that neither *PfRh2a* nor *PfRh2b* is essential to the phenotypic switching of W2mef to a sialic acid-independent invasion pathway.

We constructed a new plasmid, pHTkΔrh₂-BSD, by replacing the human DHFR gene of pHTkΔrh with the BSD gene. W2mefΔ2a parasites were transfected with this plasmid on several occasions for targeting of *PfRh2b*. Following negative selection, with ganciclovir and positive selection with blasticidin and WR99210, resistant parasites were obtained. However, Southern blots indicated that the PfRh2b remained intact, while the plasmid had integrated at a second site within the genome (data not shown).

PfRh2b is required for efficient sialic acid-independent invasion

We then tested to see whether the invasion phenotypes observed for W2mefΔ2b would be reflected in changes in the sialic acid-independent invasion of the parasite line, W2mefΔ2b/Nm. We find that the absence of *PfRh2b* in W2mefΔ2b/Nm results in increased efficiency of invasion into chymotrypsin and chymotrypsin/low trypsin-treated erythrocytes, similar to that seen for W2mefΔ2b when compared to either W2mef or W2mef/Nm (Figure 3).

In striking contrast to W2mef/Nm parasites, W2mefΔ2b/Nm parasites demonstrated a significant reduction in efficiency of sialic acid-independent invasion into neuraminidase-treated erythrocytes (Figure 3). This implies that while not essential, *PfRh2b* does affect the efficiency of sialic acid-independent invasion in switched parasites.

PfRh2a functions in sialic acid-independent invasion

We also determined the invasion profiles of W2mefΔ2a/Nm parasites. With regard to chymotrypsin- and chymotrypsin/low trypsin-sensitivity, W2mefΔ2a exhibits an invasion profile identical to that measured for both wild-type W2mef and W2mef/Nm, suggesting again that this ligand has no impact on protease-sensitive invasion (Figure 3). Surprisingly,

we find that the ability of W2mef Δ 2a to invade sialic acid-depleted erythrocytes is significantly reduced when compared to W2mef/Nm, similar to that determined for W2mef Δ 2b (Figure 3). This suggests that in the W2mef genetic background, PfRh2a plays a similar role to PfRh2b in sialic acid-independent invasion, and for the first time suggests a functional role for PfRh2a. This highlights the importance of genetic background, as disruption of PfRh2a does not result in increased sialic acid-dependence in the 3D7 genetic background (Duraisingh et al., 2003b).

Up-regulation of *PfRh4* in W2mef Δ 2a/Nm and W2mef Δ 2b/Nm parasite lines

PfRh4 has been shown to be activated in W2mef/Nm parasite, between 20 and 80-fold relative to wild-type (Gaur et al., 2006, Stubbs et al., 2005), and is essential for sialic acid-independent invasion in the W2mef parasite line. We thus quantified *PfRh4* expression in W2mef Δ 2a and W2mef Δ 2b clones. Quantitative-real time-polymerase chain reaction (qRT-PCR) was performed on cDNA transcribed from RNA harvested from synchronous schizont stage parasite lines (Figure). *PfRh4* expression in W2mef Δ 2a and W2mef Δ 2b parental lines is not significantly different to levels measured for wild-type W2mef. Transcription of *PfRh4* in W2mef/Nm, W2mef Δ 2a/Nm, and W2mef Δ 2b/Nm were all significantly increased when compared to wild-type W2mef (15.5-, 16.9- and 7.4-fold respectively, when controlled relative to AMA-1 expression)(Fig. S1). Activation of *PfRh4* in W2mef Δ 2a/Nm and W2mef Δ 2b/Nm parasites demonstrates that these sialic acid-independent members of the PfRh family can function independently of one another.

We also examined the levels of mRNA and protein expression of other invasion ligands of the PfRh and PfEBA families in the W2mef, W2mef Δ 2a, and W2mef Δ 2b and switched parasite lines (Fig. S1 and Fig. S2). We find no significant differences in the levels of expression of any of these other genes when comparing W2mef with the W2mef Δ 2a and W2mef Δ 2b lines, or when comparing the parental and switched parasite lines.

A strong selective force for *PfRh2b* reconstitution

We also obtained a clone, designated W2mef Δ 2bDXp, which has a region of the PfRh2b gene deleted by double-crossover recombination of the pHTk Δ rh plasmid. This was cloned again to give the parasite line W2mef Δ 2bDX. Western blots confirmed that this parasite line expresses PfRh2a but not PfRh2b (Figure 4B). Following cultivation on neuraminidase-treated cells, Western blots unexpectedly revealed expression of PfRh2b in the switched parasite lines while expression of PfRh2a was lost (Figure 4B). This parasite line was designated as W2mef Δ 2b $\rightarrow$ Nm. Double-crossover integration prevents restoration of the endogenous reading frame due to the loss of genetic material that follows the recombination event. We postulated that although pHTk Δ rh had integrated into genome via double-crossover homologous recombination, the 7.5 kb of 5' sequence shared between *PfRh2a* and *PfRh2b* may allow for genetic exchange. Transfer of pHTk Δ rh from *PfRh2b* to *PfRh2a* would lead to restoration of the *PfRh2b* reading frame and PfRh2b expression (Figure 4C). Enhanced ectopic/non-allelic recombination between *PfRh2a* and *PfRh2b* has previously been noted (Cortes, 2005). This hypothesis was confirmed by Southern blots performed with PrA and PrB—the *PfRh2a* locus is wild-type (9.7 kb) in W2mef Δ 2b/DX, but disrupted via double-crossover homologous recombination (7.8 kb) in W2mef Δ 2b $\rightarrow$ Nm (Figure 4D). The right panel, which probes the shared 5' domain (PrC), demonstrates that these parasites remain distinct from wild-type as double-crossover recombinants (Figure 4D). These data demonstrate that growth on sialic acid-deficient erythrocytes provides a selective pressure for *PfRh2b* reconstitution. Phenotypically, W2mef Δ 2b $\rightarrow$ Nm were indistinguishable from W2mef Δ 2a/Nm parasites, demonstrating reduced sialic acid-independent invasion. Similar results were obtained with the parental line, W2mef Δ 2bDXp.

Interestingly, we also observed reconstitution of *PfRh2b* at the expense of *PfRh2a* in W2mef Δ 2b/DX parasites following continuous long-term (~6 months) cultivation on normal, untreated red blood cells (data not shown), suggesting a physiological advantage for *PfRh2b*- over *PfRh2a*-expressing parasites that is more acute in the context of sialic acid-independent invasion.

Discussion

Here we report a genetic analysis of the roles of the *PfRh* merozoite ligands that play a major role in defining alternative invasion pathways in *P. falciparum* parasites. The parasite line W2mef was selected for study because (1) it can undergo phenotypic switching and (2) its larger repertoire of simultaneously expressed invasion ligands-*PfRh2a*, *PfRh2b* and *PfRh1*- mirrors that observed in a number of African field isolates from Kenya and Senegal (Jennings *et al.*, 2007, Nery *et al.*, 2006b). Interestingly, many Kenyan and Tanzanian isolates also show alternative expression of *PfRh* paralogs (Nery *et al.*, 2006b, Bei *et al.*, 2007), as observed previously in laboratory isolates (Triglia Duraisingh Taylor). Therefore, differences in *PfRh* expression profiles is a major feature of strain diversity.

Our results indicate that in the genetic background of W2mef, *PfRh2b* is required for invasion through chymotrypsin and chymotrypsin/low trypsin receptors (Table 1). We speculate that loss of either *PfRh2a* or *PfRh2b* allows for the redeployment and rearrangement of proteins that aggregate at the apical end of the invading merozoite (Figure 5B). This is the concept of the 'limited space' model, whereby parasite ligands compete for residence of the limited space found at the merozoite apex (Triglia *et al.*, 2005). Specifically, the vacancy left by the chymotrypsin-sensitive ligand, *PfRh2b*, permits occupancy by alternative invasion ligands— that bind to chymotrypsin-resistant receptors, such as EBA-175 and *PfRh1*.

We find differences between the phenotypes of the W2mef and 3D7 strains following disruption of *PfRh2b*. W2mef Δ 2b exhibits an enhanced ability to invade via trypsin-treated erythrocytes (Table 1), but no change is seen with 3D7 Δ 2b parasites (Duraisingh *et al.*, 2003b). These differences can be reconciled by the expression of different invasion ligand repertoires in each strain. Following disruption of *PfRh2b* in 3D7, a line that expresses low levels and does not utilize *PfRh1*, a shift is observed in dependence to alternative sialic acid-dependent ligands such as EBA-175 and EBA-140 that bind to glycophorins A and C (Camus & Hadley, 1985, Duraisingh *et al.*, 2003a, Sim *et al.*, 1994, Lobo *et al.*, 2003, Maier *et al.*, 2003). This interpretation is supported by antibody inhibition studies that show a greater dependence on EBA-175 and EBA-140 in 3D7 Δ 2b parasites (Baum *et al.*, 2005).

We find that the sialic acid-independent ligand *PfRh2b* is functional in the W2mef that is heavily dependent on sialic acid for invasion. This phenomenon of contradictory ligand expression and invasion pathway utilization has previously been observed with EBA-175, which is functional in both sialic acid-dependent and sialic acid-independent parasite lines (Duraisingh *et al.*, 2003a). A hierarchy for different invasion ligands has been proposed where certain ligands are dominant to others aggregated at the merozoite apex for invasion. However, our results suggest that dominance of an invasion ligand is not universal, as although *PfRh2b* is dominant in 3D7, it is not dominant in W2mef. Dominance *per se* is a function of epistatic interactions with other expressed invasion ligands. The repertoire of expressed ligands can vary dramatically between strains, while it appears that the invasion pathway of a given strain is measured as the sum of all expressed ligands. Although, it is likely that avidity of each ligand for its receptor is also function of the sequence of the ligand (Lobo *et al.*, 2006, Hayton *et al.*, 2008).

Invasion pathway utilization by W2mef is not static. Facultative expression of the PfRh4 ligand was found to be responsible for phenotypic switching between the use of sialic acid-dependent -independent invasion pathway for red cell entry (Soubes *et al.*, 1997, Dolan *et al.*, 1994) (Stubbs *et al.*, 2005, Gaur *et al.* 2006). Here, we show that the expressed ligands PfRh2a, PfRh2b work in co-operation with PfRh4 for efficient sialic acid-independent invasion (Figure 5B). PfRh4 expression is independent of PfRh2a and PfRh2b expression, although the latter appears to be coordinately expressed (Duraisingh *et al.*, 2003b). It is becoming increasingly clear the members of other merozoite protein families, such as the PfEBA and PfCLAG families, can also be variably expressed (Bei *et al.*, 2007, Nery *et al.*, 2006a, Cortes *et al.*, 2007, Taylor *et al.*, 2002). However, phenotypic switching at a high rate has only been observed for the PfRh4 ligand (Stubbs *et al.*, 2005).

A strong selective force for *PfRh2b* reconstitution was observed at the expense of *PfRh2a* (Figure 4). Further, our results suggest that while disruption of either *PfRh2a* or *PfRh2b* results in increased sialic acid-dependence in switched lines, only the deletion of *PfRh2b* results in increased dependence of the trypsin/chymotrypsin-sensitive receptors. This suggests that the divergent, C-terminal domain of PfRh2a and PfRh2b mediates invasion via protease-sensitive receptors, while a region at the N-terminal end of the ectodomain shared between PfRh2a and PfRh2b directs sialic acid-independent invasion (Figure 5A). Interestingly, erythrocyte binding domains have now been identified at the N-terminal ends of both PfRh4 and PfRh1, which confer binding to sialic acid-independent and -dependent receptors respectively (Gao *et al.*, 2008, Gaur *et al.*, 2007). This may suggest a general model for Plasmodium invasion ligands. For instance, with the CSP ligand, involved in sporozoite invasion of hepatocytes, an N-terminal domain binds to carbohydrates on the host surface, while the C-terminal region appears to be involved in protein-protein interactions (Coppi *et al.*, 2005). Similarly, with the merozoite ligand EBA-175, the DBL domain was found to bind in a sialic acid-dependent fashion, with a second sialic acid-independent binding domain mapping to a more C-terminal region (Kain *et al.*, 1993).

Then why is PfRh2a functional in W2mef but not 3D7? We postulate that this is due to the different set-points of expression of ligand repertoires in the two strains. In contrast to W2mef, 3D7 is stably reliant on sialic acid independent receptors for invasion. PfRh2a may therefore play a less prominent role in 3D7 in which the majority of ligands are sialic acid-independent. In this context, it is important to note that phenotypic switching is not a ubiquitous phenomenon. Like W2mef, the *P. falciparum* strains T994 and FCB-1 invade via sialic acid-dependent receptors, but these parasites are completely unable to switch to sialic acid-independent invasion. Intriguingly, both T994 and FCB-1 do not express either *PfRh2a* or *PfRh2b* and despite several attempts we have been unable to generate W2mef parasites with both PfRh2a and PfRh2b deleted. Phenotypic switching in parasite lines may be permissible only in the presence of a large and versatile repertoire of invasion ligands.

In conclusion, we have highlighted the importance of genetic background in determining specific invasion phenotypes and the ability to switch between them. The plasticity uncovered by this study in just a single *P. falciparum* strain reflects the resilience of *P. falciparum* to persist either in the face of immune response or in the need to utilize alternative receptors for invasion. In the future, it will be of great interest to study the deployment of invasion ligands in multiple parasite backgrounds from different geographical areas. This highlights the difficult task of designing effective vaccines and chemotherapies that can withstand rapid parasite adaptation and high levels of diversity.

Experimental Procedures

Parasites and plasmids

P. falciparum asexual stages were maintained *in vitro* in human O⁺ erythrocytes at a 4% hematocrit in RPMI 1640 (Sigma)-25 mM HEPES (EMD Biosciences) media supplemented with 0.5% Albumax II (Gibco). W2mef is a cloned line derived from the Indochina III/CDC strain. The plasmid used for targeting *PfRh2b* in W2mef was pHTkΔRh (Duraisingh et al., 2003b). Sorbitol-synchronized ring-stage parasites were transfected with 80 μg of purified pHTkΔrh plasmid DNA (Qiagen). Selection of stable transfectants with double-crossover recombination events were carried out as described previously (Duraisingh et al., 2002).

Parasite cloning

W2mef parasites and the recombinant parasite lines were cloned via limiting dilution in a 96-well round bottom microtiter plate at a dilution of 1, 3, or 9 parasites/well in 2×10^8 erythrocytes/ml. Growth medium was replenished every week with complete RPMI. Parasite growth was monitored after two weeks using DiffQuick-stained thin blood smears. Dilutions that achieved less than a 30% positivity rate were considered to have yielded clonal populations.

Southern probes and nucleic acids

Genomic DNA was harvested from synchronous, late stage parasite cultures using phenol-chloroform extraction. Southern blotting was carried using standard procedures. Three probes were amplified from parasite genomic DNA and were utilized to assess the status of *PfRh2a* and *PfRh2b*. Probe A (PrA), which is specific to the *PfRh2a* 3' unique region, was amplified using the primers 5'- GCGGATCCGAAAGAAAGAAAATCGAGTTAG and 5'- GCGAATTCTTAATTCTTTGATCGAGAAAAATTTTC. PrB, which is specific to the *PfRh2b* 3' unique region, was amplified using the primers 5'- GGACCCCTAGGCAACAACAAAGAAATATCCAAGAATTAG and 5'- GGACCATCGATTAAAAATATTTTCTTCATTTTCATCAAAC. PrC is complementary to sequences shared between the amino-termini of *PfRh2a* and *PfRh2b*. PrC was amplified using primers 5'- CCGCGGAGTCATGAGCATTTTGTGGACAATCAA and 5'- GTTAACCTCGTCATGTATATATCCAATAGAGTACATGT.

SDS-PAGE and Western blot analysis

Supernatants enriched in proteins required for merozoite invasion were obtained from synchronous parasite cultures. Proteins were resolved on 5% (EBA-175, PfRh2a, and PfRh2a) and 10% (SERA-5) SDS-PAGE gels. Proteins were then transferred to a 0.45 μm nitrocellulose membrane (Schleicher and Schuell) and probed with antibodies specific to PfRh2a (1:500), PfRh2b (1:500), EBA-175 (1:500), and SERA-5 (1:1000) (Triglia et al., 2005). Protein transfer to nitrocellulose membranes was performed according to standard protocols, and blots were visualized with a chemiluminescent detection system (ECL, Pierce).

Switching growth rate assay

W2mef, W2mefΔ2a, and W2mefΔ2b ring stage cultures were normalized to 0.5% parasitemia and 4% hematocrit. Erythrocyte pellets were washed several times in unsupplemented RPMI and resuspended in α-2-3,6,8-*Vibrio cholera* neuraminidase (66 mU/ml, Calbiochem) before being rocked gently for 1 hour at 37°C. After enzymatic treatment, erythrocyte pellets were washed several times in unsupplemented RPMI and resuspended to a final volume of 5 ml in Albumax II-containing RPMI medium. Parasitemia was determined upon microscopic examination of DiffQuick-stained thin blood smears every 48

hours. Cultures were regarded as neuraminidase-resistant when parasitemia reached 1% or greater.

Hypoxanthine invasion assays

The invasion assay was carried out essentially as described previously (Duraisingh et al., 2003b). Sorbitol-synchronized ring stage parasites were treated with α -2-3,6,8-*Vibrio cholera* neuraminidase (66.7 mU/ml, Calbiochem) and chymotrypsin (1 mg/ml, Worthington) to prevent reinvasion into donor erythrocytes. Analyses proceeded on receptor erythrocytes pre-treated with neuraminidase (66.7 mU/ml), chymotrypsin (1 mg/ml), or chymotrypsin (1 mg/ml)/ low trypsin (66.7 ug/ml, Sigma). Parasites were plated at a final parasitemia of 0.5-1% at 2% hematocrit. Following a 48-hour incubation to allow for reinvasion, parasites were exposed to ³H-hypoxanthine (1 uCi/well) for an additional 16 hours. Percent invasion into enzyme-treated erythrocytes was calculated relative to invasion of the same parasite line into untreated erythrocytes, which was set to 100%.

Quantitative-real time-polymerase chain reaction

Total RNA was harvested from late trophozoite, early schizont stage cultures using TRIzol isolation and the RNeasy kit (Qiagen). One ug of total RNA was twice-treated with DNase I and converted to cDNA either with or without reverse transcriptase using random hexamer primers. SYBR green (BioRad) incorporation was measured using the ABI 7300 System and the following PCR conditions: 95°C for 600 sec, 95°C for 15 sec, 45°C for 30 sec, 67°C for 28 sec, and 50°C for 28 sec. The primer sequences used for the detection of the invasion genes have been previously described (Stubbs et al., 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Disruption of *PfRh2a* and *PfRh2b* in *P. falciparum* W2mef

(A) The pHTKΔrh plasmid contains the positive selectable marker human *dihydrofolate reductase* (*hDHFR*), which is flanked by sequences homologous to the 5' regions of *PfRh2b* and *PfRh2a*. The negative selectable marker, thymidine kinase (TK), lies outside of the flanking regions. W2mefΔ2b and W2mefΔ2a are clonal lines that were disrupted for the *PfRh2b* and *PfRh2a* locus, respectively. *PfRh2a* and *PfRh2b* are depicted with their diagnostic restriction enzyme cut sites: *Swa*I (Sw), *Av* (*Ava*II), *Sac*I (S), *Pst*I (Ps) and *Pvu*II (P). (B) W2mef, W2mefΔ2b, and W2mefΔ2a gDNA was digested with *Sac*I, *Pvu*II, and *Ava*II and hybridized to *PrA* and *PrB*, which are specific to the *PfRh2a* and *PfRh2b* unique regions, respectively. *PfRh2b* was disrupted via single-crossover homologous recombination in W2mefΔ2b parasites, while *PfRh2a* expression was abrogated by a double-crossover homologous recombination event in W2mefΔ2a. (C) Supernatants harvested from W2mef, W2mefΔ2a, and W2mefΔ2b were probed with anti-*PfRh2a* and anti-*PfRh2b*. For the right panel, anti-EBA-175 was used as a loading control; in the left panel, SERA-5 was used.

Figure 2. Molecular characterization of W2mef, W2mefΔ2a and W2mefΔ2b parental and sialic acid-independent 'switched' parasite lines

(A) Western blots performed on synchronous cultured supernatants were probed with anti-PfRh2a, anti-PfRh2b, and anti-SERA-5. Following up-selection on neuraminidase-treated erythrocytes, W2mefΔ2b/Nm parasites remain PfRh2b deficient. SERA-5 was used as a loading control. (B) and (C) Genomic DNA harvested from 'parental' W2mef, W2mefΔ2a, W2mefΔ2b and 'switched' W2mef/Nm, W2mefΔ2a/Nm, W2mefΔ2b/Nm cultures was digested with *Ava*II, *Swa*I, and *Pvu*II. Digested DNA was hybridized with PrA and PrB, which correspond to the *PfRh2a* and *PfRh2b* unique regions, respectively. PrA fails to hybridize with wildtype PfRh2a in W2mefΔ2a/Nm, while PrB fails to hybridize with wildtype *PfRh2b* in the W2mefΔ2b/Nm.

Figure 3. Invasion pathway utilization in W2mef, W2mefΔ2a and W2mefΔ2b parental and sialic acid-independent 'switched' parental lines

Invasion efficiency of wild-type and PfRh2a-deficient parasites into neuraminidase, chymotrypsin, and chymotrypsin/low trypsin-treated erythrocytes is depicted. Invasion efficiency is the percent invasion calculated relative to invasion of untreated erythrocytes. Assays were performed a minimum of three times in triplicate. Error bars represent 95% confidence limits.

Figure 4. Reconstitution of *PfRh2b* in double-crossover recombinants

(A) Schematic depicting the selection of sialic acid-independent parasites from W2mefΔ2b/DX parasites to W2mefΔ2b→Nm. (B) Western blots performed on synchronous cultured supernatants probed with anti-PfRh2a, anti-PfRh2b, and anti-SERA-5 antibodies. (C) The proposed mechanism of *PfRh2b* reconstitution in W2mefΔ2b/DX parasites, with concomitant disruption of *PfRh2a*. (D) Southern blots were performed using PrA and PrB, which are probes specific to the *PfRh2a* and *PfRh2b* unique regions, respectively. Genomic DNA hybridized to PrA and PrB was digested with *Ava*II, *Sac*I, and *Pvu*II. PrC corresponds to the 5' domain that is shared between *PfRh2a* and *PfRh2b*. Genomic DNA hybridized to PrC was digested with *Pst*I and *Swa*I. Following up-selection on neuraminidase-treated erythrocytes or long-term culture on untreated cells, we observe reconstitution of *PfRh2b* in W2mefΔ2b/DX parasites, which occurs at the expense of *PfRh2a*.

Figure 5. A) Schematic depicting the proposed erythrocyte-binding domains of PfRh2a and PfRh2b

We propose that the shared amino-terminal domains act in concert to precipitate the most robust switch towards sialic acid-independence, while the PfRh2b unique region mediates binding via chymotrypsin-sensitive/trypsin-resistant means. Presence or absence of PfRh2a has no effect on protease-sensitive invasion phenotypes. **B. A cooperative space-filling model.** Following disruption of either *PfRh2a* or *PfRh2b* in *P. falciparum* W2mef, unknown ligands fill the vacancy left by the absence of the paralog. Relative to W2mef/Nm, an approximately 50% reduction in sialic acid-independence is seen in W2mefΔ2a/Nm and W2mefΔ2b/Nm. W2mefΔ2a expresses *PfRh2b* but not *PfRh2a*, while W2mefΔ2b/Nm is deficient in *PfRh2b* but not *PfRh2a*. Though both W2mefΔ2a/Nm and W2mefΔ2b/Nm express *PfRh4*, the most robust phenotypic switch is seen only in W2mef/Nm, which expresses *PfRh2a*, *PfRh2b*, and *PfRh4* concurrently. Si-Dep- sialic acid independent; Si-Indep- sialic acid-independent.

Table 1
Invasion efficiency of wild type W2mef and *PfRh2*-knockout parasites into enzyme-treated erythrocytes

Erythrocytes were treated with neuraminidase (66 mU/ml), chymotrypsin (1 mg/ml), low trypsin (66 ug/ml), high trypsin (1 mg/ml), or a combination of chymotrypsin (1 mg/ml)/low trypsin (66 ug/ml). Values represent percent invasion relative to untreated erythrocytes. Asterisks denote statistical significance by Student's t-test ($p < 0.05$). Experiments were performed a minimum of three times in triplicate. 95% confidence limits are shown.

Enzyme Treatment	Parasite		
	W2mef	W2mefΔ2a	W2mefΔ2b
Neuraminidase	3.5 ± 2.4	2.9 ± 1.2	5.4 ± 1.7
Chymotrypsin	56.7 ± 12.1	51.7 ± 11.0	91.1 ± 4.1**
Low Trypsin	71.2 ± 23.1	53.9 ± 13.2	77.3 ± 11.9
High Trypsin	35.2 ± 11.2	33.6 ± 3.7	49.6 ± 5.4**
Chymotrypsin/Low Trypsin	35.9 ± 9.6	32.3 ± 5.3	66.0 ± 16.0**