<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Differences in erythrocyte receptor specificity of different parts of the plasmodium falciparum reticulocyte binding protein homologue 2a (Main article)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Karthigayan Gunalan; Gao, Xiaohong; Liew, Kingsley Jiin Liang; Preiser, Peter Rainer</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Karthigayan, G., Gao, X., Liew, K. J. L., &amp; Preiser, P. R. (2011). Differences in Erythrocyte Receptor Specificity of Different Parts of the Plasmodium falciparum Reticulocyte Binding Protein Homologue 2a. Infection and Immunity, 79(8), 3421-3430.</td>
</tr>
<tr>
<td><strong>Date</strong></td>
<td>2011</td>
</tr>
<tr>
<td><strong>URL</strong></td>
<td><a href="http://hdl.handle.net/10220/7305">http://hdl.handle.net/10220/7305</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>© 2011 American Society for Microbiology. This is the author created version of a work that has been peer reviewed and accepted for publication by Infection and Immunity, American Society for Microbiology. It incorporates referee’s comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [DOI: <a href="http://dx.doi.org/10.1128/IAI.00201-11">http://dx.doi.org/10.1128/IAI.00201-11</a>]</td>
</tr>
</tbody>
</table>
Differences in erythrocyte receptor specificity of different parts of the
*Plasmodium falciparum* Reticulocyte binding protein Homologue 2a
Karthigayan Gunalan, Xiaohong Gao, Kingsley JL Liew, Peter R Preiser*

School of Biological Sciences, Nanyang Technological University, 60 Nanyang
Drive, Singapore 637551, Singapore

*Corresponding author

Tel. +65 6316 2869
Fax. +65 6791 3856
prpreiser@ntu.edu.sg

Running title: *Plasmodium falciparum* merozoite invasion

Key words: Malaria, invasion, red blood cell binding, alternative invasion

pathways
Abstract

The *P. falciparum* Reticulocyte-binding-like protein homologue (RH) and the Erythrocyte Binding Like (EBL) protein families play an important role during invasion, though their exact role is not clear. Both EBL and RH are thought to directly bind different receptors on the surface of the erythrocyte and the binding properties for a number of EBLs and RHs have been described. While PfRH1 and PfRH4, have been shown to directly play a role in two alternative invasion pathways used by merozoites, the function of PfRH2a and PfRH2b during invasion is less defined. Here using monoclonal antibodies raised against a unique region of PfRH2a we show that PfHR2a moves from the rhoptry neck to the moving junction during merozoite invasion. Movement of PfRH2a to the junction is independent of the invasion pathway used by the merozoite suggesting an additional function of the protein independent of receptor binding. We furthermore show that PfRH2a is processed both in the schizont as well as during invasion resulting in proteins with different erythrocyte binding properties. Our findings suggest that PfRH2a and, most likely the other members of the RH family, depending on their processing stage, can engage different receptors at different stages of the invasion process.
Introduction

Malaria continues to be a serious public health problem with nearly half of the world’s population living in malaria endemic areas. The disease is caused by the cyclic infection and subsequent destruction of the host’s erythrocytes by obligate intracellular protozoan parasites belonging to the genus *Plasmodia*. *Plasmodium falciparum* is the most virulent of the four species infecting humans causing significant morbidity and mortality in millions of people each year. Invasion of the erythrocyte by the invasive form of the blood stage parasite, the merozoites is mediated by a complex set of interactions between different parasite ligands and erythrocyte receptors (9, 23, 36). The ligands utilized by the parasite during invasion are either expressed on the surface of the merozoite or discharged from the specialized apical organelles like rhoptries, micronemes and dense granules (9, 23, 36).

Merozoite invasion is a multistep event that begins with random attachment, when the merozoite forms a low affinity and reversible engagement with the erythrocyte. Subsequently, the merozoite reorients itself such that the apical end is in contact with the erythrocyte. Following the reorientation process a tight junction is formed and the rhoptry and micronemal proteins are discharged, indicating the irreversible commitment of the merozoite to invasion (23, 44). As invasion continues, the tight junction moves from the anterior to the posterior end of the merozoite. This movement of the merozoite into the erythrocyte involves a complex series of events driven by the parasite actin-myosin motor (26). In addition to the parasite motor, several parasite derived proteases are involved in the specific cleavage of a range of
parasite as well as erythrocyte proteins which are essential for the successful entry of
the merozoites into erythrocytes (12, 43).

Treatment with enzymes like neuraminidase, trypsin or chymotrypsin is
known to remove different receptors from the surface of erythrocytes and different
strains of *P. falciparum* have been shown to differ in their ability to invade these
treated erythrocytes (10, 14, 45, 50). This led to the suggestion that the ability of
parasite strains to differentially invade enzyme treated erythrocytes defines distinct
invasion pathways (18, 40, 45, 50). Two parasite encoded protein families termed
Erythrocyte Binding Like (EBL) proteins and Reticulocyte Binding-like Homologues
(RH) proteins have been shown to be involved in the differential recognition of
erythrocyte receptors and thereby define the invasion pathway utilized by a parasite
strain (1, 3, 6, 9, 17, 23, 25, 30, 33, 35, 36, 38, 39, 45). The EBLs are defined by a
conserved cysteine rich region termed Duffy binding like (DBL) domain that directly
mediates binding to erythrocyte receptors (7). In *Plasmodium falciparum*, six EBL
members have been identified, namely Erythrocyte binding antigen 175 (EBA175),
EBA140, EBA181, EBA165, EBL1 and MAEBL (1, 6, 25, 31, 33, 35, 53) with the
DBL domain of EBA175 recognizing the sialic acid component in glycoporphin A (6,
13), that of EBA 140 binding to glycoporphin C (30, 31, 33) and the DBL of EBA 181
binding to a so far uncharacterized sialoglycoprotein (20, 34). EBL1 has recently been
shown to bind to glycoporphin B (32) and EBA165 is considered to be a pseudogene
(53). Unlike other EBLs, MAEBL does not have a DBL domain but contains
conserved domains, which are similar to AMA1 (5).

The RH was first discovered in rodent malaria parasite *P. yoelii* where they were
termed Py235 (15, 22). Subsequent work has though shown that RHs were found in
all the *Plasmodium* species so far analyzed (3, 15, 16, 21, 22, 24, 37-39, 52). In *P. yoelii* PY235 is coded for by a multigene family (27) and was thought to be involved in parasite virulence (15) as well as mediate a novel form of clonal phenotypic variation (37). In *Plasmodium vivax*, homologues of Py235, the reticulocyte binding proteins PvRBP1 and PvRBP2 were shown binding to reticulocytes, leading to the suggestion that members of this protein family plays an important role in host cell recognition [34]. In *Plasmodium falciparum*, six RH members have been identified, they are PfRH1 (39), PfRH2a (38, 52), PfRH2b (38, 52), PfRH3 (46), PfRH4 (24, 45) and PfRH5 (3, 42).

PfRH1 is the ortholog of PvRBP1 and binds to the sialic acid containing putative erythrocyte receptor Y (4, 39, 50). The erythrocyte binding region of PfRH1 has been identified and the antibodies raised against this region inhibits merozoite invasion (17). Triglia *et al* has recently shown that RH1 protein undergoes a series of proteolytic cleavage events that occur before and during entry into the erythrocyte and further showed that the processed products along with EBA175 are important components of the tight junction (51). PfRH2a and 2b have been identified by comparative analyses with *Plasmodium vivax* reticulocyte binding protein 2 (PvRBP-2) (38) and by gene knock out studies PfRH2a is shown to be involved in sialic acid independent invasion pathway (10). Although antibodies against PfRH2a are able to inhibit merozoite invasion (52), there is no evidence to date on erythrocyte binding ability of PfRH2a. PfRH2b gene knock out studies showed that PfRH2b interacts with the chymotrypsin sensitive erythrocyte receptor Z (4, 14). Recent findings provide evidence suggesting that PfRH2 is naturally immunogenic and its antibodies are associated with protection from malaria (41). Taylor *et al* described another member
of PfRH family. PfRH3 as a pseudogene (46). PfRH4 was identified as 220kDa protein (24) and its differential expression was subsequently shown to be a major factor in the invasion pathway switching of the parasite from a sialic acid dependent to sialic acid independent pathway (18, 45). Recently PfRH4 was shown to bind to erythrocytic receptor CR1 (47). PfRH5 lacking a transmembrane domain, with a molecular weight of 63 kDa is the smallest member of RH and is found to be in the tight junction during erythrocyte invasion (3, 42). While the erythrocyte binding properties of PfRH1, PfRH4 and PfRH5 have been identified (3, 17, 19), only for PfRH4 has complementary receptor 1 (CR1) been identified as the erythrocyte receptor (47).

In this study we have focused on PfRH2a and provide evidence of its significant role in invasion. Using monoclonal antibodies (mAb) against PfRH2a, we show that PfRH2a colocalizes with PfRH1 in rhoptry neck of the merozoite. We furthermore demonstrate that both in parasites using a sialic acid dependent as well as independent invasion pathway, PfRH2a is found in the tight junction during merozoite invasion. We show that PfRH2a undergoes proteolytic processing in schizonts and also during invasion and that the different proteolytic forms display different erythrocyte binding properties. Our findings demonstrate that PfRH2a plays an important role during merozoite invasion and depending on the proteolytic stage is able to mediate different ligand-receptor interactions, possibly at different times of the invasion process.

Results

Two forms of PfRH2a are detected in schizont extract
As PfRH2a and PfRH2b share high sequence similarity except for the C-terminal region, we raised monoclonal antibodies (mAb) against the unique region corresponding to amino acids 2874 to 3052 of PfRH2a (Fig 1A). Screening of hybridoma clones resulted in the identification of three antibody producing clones (C22, C19 and C15). Using the mAb (C22), we have confirmed the previous results of peak expression of PfRH2a at the schizont stage, of two parasite clones 3D7 and W2mef (Fig S1A). Two forms of PfRH2a of approximately 360 and 270 kDa were detected at the schizont stage of the parasite, with the 270 kDa form being more abundant (Fig 1B and S1C). Western blot analysis of schizont extract from 3D7 and 3D7ΔPfRH2a, a parasite line in which PfRH2a had been disrupted (14), confirmed that both the 360 as well as the 270 kDa were indeed due to the expression of PfRH2a and not due to cross reactivity of the antibody with PfRH2b (Fig 1B and S1C). As PfRH2a is predicted to be approximately 360 kDa in size it is likely that the ~360 kDa protein detected in the western blot corresponds to the full length protein with the 270 kDa band representing a processed form of the protein. Proteolytic cleavage of PfRH1 has earlier been reported to be an important step during invasion, and results in a number of specific products (51). Hence to determine whether PfRH2a undergo a similar cleavage events, western blot was performed for schizont extract and culture supernatant of 3D7, 3D7ΔPfRH2a and W2mef parasite line probed using C22 mAb. As expected, the full length 360 and a processed 270 kDa protein was detected in both schizont extracts from 3D7 and W2mef, while in the parasite supernatant an additional 140 kDa protein band is consistently detected suggesting a secondary processing of PfRH2a during invasion (Fig 1B). Taken together, this data is consistent with the 360 kDa full length PfRH2a being proteolytically cleaved at the N-terminal end of the protein, resulting in an approximately 270 kDa C-terminal fragment and
possibly an approximately 90 kDa N-terminal fragment (Fig 5B). During invasion a second processing step removing a further 130 kDa N-terminal fragment occurs, leaving a 140 kDa fragment detected by the mAb (Figure 1B and 5B). It is also interesting to note that the 270 and 140 kDa bands appear as doublets in the culture supernatant indicating the proteolytic removal of a second small fragment during the invasion process (Fig 1B, C and D). The 3D7 supernatant proteins incubated in the presence or absence of protease inhibitor indicated no significant change in the amount of the 140kDa fragment, indicating that this secondary cleavage event is not due to the non-specific degradation of the 270 kDa or 360 kDa protein in the parasite supernatant (Fig S1B). Similar processing of RH2a was consistently observed using the other RH2a mAbs C19 and C15 in 3D7 and 3D7ΔPfRH2a parasite strains (Fig 1C and D) suggesting that the additional processing of RH2a could be a crucial event during merozoite invasion.

PfRH2a is located at rhoptries

Previous studies have suggested that RH members are expressed either in the rhoptry neck or at the apical prominence of different P. falciparum strains. To further delineate the exact localization of PfRH2a, we performed immunofluorescence assay (IFA) using fixed schizont smears. Using the mAb, PfRH2a showed a punctuate pattern at the apical tip of the merozoite in schizonts and appears to co-localize with PfRH1 in W2mef and 3D7 parasites, strongly suggesting that the RH2a is located at the rhoptry neck (Fig 2A and Fig S2). The localization of RH2a with the mAb is identical to that observed with the polyclonal antibody R2A9 raised against PfRH2a/2b (R2A9 is raised against the amino acid stretch common to both PfRH2a/2b) (Fig 2B and S2). As expected, there is a partial overlap with micronemal
markers like AMA1 and EBA175 (Fig 2C and 2D and Fig S2). This is consistent with electron microscopy studies using polyclonal antibodies that react with both PfRH2a/b, showing that they are located in the rhoptry neck of the merozoites (14). Control experiments using the 3D7ΔPfRH2a parasites line showed no staining for PfRH2a confirming the specificity of the mAb in IFA (Fig S2). These results confirm that the monoclonal antibodies used in this study are highly specific for PfRH2a and allow specific localization of the protein in the parasite.

**PfRH2a is localized at the tight junction during invasion**

It is clear from the above data that PfRH2a is expressed at the schizont stage of various *P. falciparum* strains. However, there is limited data that supports a direct role of PfRH2a in parasite invasion. Junction formation is linked to the irreversible commitment of the merozoite to invasion and recent studies have shown that both PfRH1 and PfRH5 are part of the junction suggesting a specific role of the RH proteins during this part of the invasion process (3, 51). To test whether PfRH2a is also located in the junction, invasion of merozoites was arrested by treating the parasites with 0.1µM cytochalasin D. Cytochalasin is a cell permeable fungal toxin that prevents cell ruffling and cell motility (8). It is found to be very effective in causing actin depolymerization even at low concentration (54). The micronemal markers AMA1 and EBA175 earlier shown to be localized in the tight junction, hence we employed these proteins as markers for the tight junction. Immunofluorescent assays using the junction-arrested merozoites showed that PfRH2a located at the tight junction with EBA175 & AMA-1 in W2mef parasite clone (Fig 3A). *P. falciparum* W2mef normally utilizes a sialic acid dependent invasion pathway, but upon selection on neuraminidase treated erythrocytes is able to switch (W2mef/NM) to using a sialic
acid independent pathway (11). This switch in invasion pathway has been linked to
the activation of expression of PfRH4 (18, 45). As PfRH2a has been suggested to also
play a role in directing sialic acid independent invasion (10) we investigated whether
the location of PfRH2a changes in a parasite that uses a sialic acid independent
invasion pathway. In W2mef/NM, PfRH2a were located with junction markers
EBA175 and AMA1 in junction-arrested merozoites (Fig 3B). No apparent difference
in the location of PfRH2a was seen between W2mef and W2mef/NM junction-
arrested merozoites suggesting that the location of PfRH2a does not change in
response to the receptors utilized by the parasite. In the same way, RH2a located in
the tight junction of junction arrested 3D7 clone (Fig 3C). A similar assay was
performed in the absence of cytochalasin D to further study the localization of RH2a
in the moving junction with AMA1 in W2mef clone. PfRH2a was observed in the
initial junction formed just after the invasion process has started as well as all later
stages of the junction observed as the merozoite penetrates into erythrocyte (Fig 4).
Taken together, these observations indicate that RH2a localizes at the junction and
suggest that it could play an important role in helping merozoite to enter erythrocytes.

Processing of PfRH2a changes erythrocyte receptor specificity

The erythrocyte binding ability of PfRH1, PfRH4 and PfRH5 members have been
elucidated (3, 17, 19, 39, 42, 48). However, to date direct binding of PfRH2a to
erythrocytes has been unsuccessful (52). Here using the PfRH2a specific monoclonal
antibody, we show that PfRH2a binds to the erythrocyte. Erythrocyte Binding Assays
(EBA) performed using parasite supernatant obtained from 3D7, clearly showed that
the 360, 270 and 140 kDa proteins bind erythrocytes (Fig 5A). Comparing the relative
intensity of the 360 with 270 and 140 kDa bands in supernatant as well as the
erythrocyte bound protein fraction suggests that the 360 kDa protein is able to bind erythrocytes more efficiently than the 270 and 140 kDa proteins (Fig 5A).

To determine the binding characteristics of PfRH2a in more detail, EBA using enzyme treated erythrocytes was carried out using culture supernatant from *P. falciparum* strains 3D7 and 3D7ΔPfRH2a. Erythrocytes were treated separately with varying concentration of neuraminidase (Nm), chymotrypsin (Chymo) and trypsin (Tryp) before each experiment. Treatment of erythrocytes with 25 or 50 mU of Nm reduced the binding of the 360 kDa protein while little or no effect was seen on the binding of the 270 and 140 kDa proteins (Fig 5A). However, binding of both the 360 as well as 270 kDa protein was highly sensitive to chymotrypsin treatment with binding being abolished for both proteins, consistent with the previous genetic data suggesting that PfRH2a interacts with a Chymo sensitive receptor (10, 14). In contrast the 140 kDa protein appears to be moderately sensitive to Chymo treatment (Fig 5A). Tryp treatment of erythrocyte completely abolished the binding of 370 kDa protein, while the binding of both the 270 kDa as well as 140 kDa bands is not affected by increasing concentration of Tryp. As a control the same western blot membranes were re-probed for EBA175, which has been previously shown to bind to erythrocytes in a Nm sensitive, Chymo resistant and Tryp resistant fashion (3, 48). This clearly showed that the enzyme treatment had the expected effect, with binding of EBA175 being reduced in NM treated erythrocytes, while being refractory to treatment with Chymo and dose-dependent sensitivity to Tryp (Figure 5A). As expected no binding of PfRH2a was detected with mAb C22 when using 3D7ΔRH2a culture supernatant for EBA, while for control the same membrane were re-probed using EBA175, in which the binding was identical to that seen in 3D7 supernatant (Fig S3).
kDa protein is sensitive to NM, Chymo and Tryp, while 270 kDa protein is sensitive to Chymo and resistant to NM and Tryp, whereas 140 kDa protein is resistant to NM and Tryp reasonably sensitive to Chymo.

Discussion

Reticulocyte binding like protein Homologues (RH) plays an important role in invasion of erythrocytes in several Plasmodium species. However, the precise role of the RH in invasion is not yet clearly understood. In this study, using specific as well as highly sensitive monoclonal antibodies we generated, we have focused on the importance of Plasmodium falciparum RH2a protein. Previous studies focusing on the genetic disruption of PfRH2a in both 3D7 and W2mef have shown that the role of this protein is different in the two parasite strains (10, 14). While in the PfRH2a 3D7 knockout line no direct effect on the invasion capacity of the parasite was observed (14), in the W2mef background PfRH2a appears to play a role in sialic acid independent invasion (10). Using IFA we show that PfRH2a is located in the rhoptry neck of both W2mef and 3D7 suggesting that the apparent different roles of PfRH2a in these two parasite lines are not due to the differential location of PfRH2a. Additional support for a role of PfRH2a in W2mef comes from our IFA data that shows very clear colocalization of PfRH1 and PfRH2a at the rhoptry neck of merozoite support previous assumption that both proteins are able to equally engage receptors on the surface of the erythrocyte. A number of recent studies have shown that PfRH1 and PfRH5 are directly located in the tight junction (3, 51). In line with these proteins, not only being important in an initial host cell sensing but furthermore playing an active role in the moving junction thereby mediating the movement of the merozoite into the host erythrocyte. Our findings that PfRH2a does locate in the junction of W2mef, W2mef/Nm and 3D7 is consistent with a role during the invasion process, despite the
fact that genetic disruption and use of enzyme treated erythrocytes have not been able to establish this previously (14, 52). Previously it has been shown that in the case of PfRH1 not all the protein is translocated to the junction but rather that some of PfRH1 remains at the apical tip (51). In contrast, while we detect PfRH2a at the apical location in free merozoites as well as those engaged in initial attachment and reorientation, all PfRH2a appears to translocate into the junction with no protein being left behind at the apical tip. It will be interesting to establish whether this is indeed a difference between PfRH1 and PfRH2a or reflects a functional difference in the reagents used.

We show that PfRH2a is proteolytically cleaved with an initial processing step occurring during schizont maturation resulting in the 360 kDa full length protein being cleaved to a 270 kDa protein. This processing is followed by a second cleavage of the 270 kDa protein to a 140 kDa fragment that occurs after schizont release, possibly during the invasion. It is intriguing to speculate that the observed processed doublet bands around 270 and 140 kDa in cell culture supernatant but not in the schizont extract could perhaps play an important role in the process of invasion. Furthermore the protease inhibitor treatment of the culture supernatant indicate that 140 kDa processed product is not due to random degradation of the full length protein. Our antibody does not allow us to establish whether the N-terminal fragments produced by this processing (Fig 5B) are maintained in the merozoite and junction. Sequential proteolytic cleavage of malaria invasion proteins is thought to be important steps during merozoite invasion. As previously shown PfRH1 is also sequentially processed with an initial cut occurring in the schizont followed by further cleavage during invasion (17, 51) and it is interesting to note that the proposed sites of PfRH1 processing appear to be in similar locations to those observed for PfRH2a. This
strongly suggests that processing of all RH follows a conserved pathway during merozoite maturation and invasion.

Enzyme treatment of erythrocytes has shown that different RH interact with erythrocyte receptors that have different sensitivities to Nm, Chymo or Tryp. The putative receptor Y for PfRH1 is Nm sensitive and Tryp/Chymo resistant (17), while for PfRH2b the putative receptor Z is thought to be Chymo sensitive and Nm/Tryp resistant (14). The receptor for PfRH4 has now been shown to be CR1 (47) and based on the impact of PfRH2a disruption on invasion of enzyme treated erythrocytes PfRH2a is thought to interact with a Chymo sensitive receptor (10, 14). Here we show that the 360, 270 and 140 kDa fragments of PfRH2a are able to specifically bind to erythrocytes, with the full-length protein binding stronger than the processed 270 and 140 kDa protein. Importantly, we demonstrate that processing of PfRH2a has a direct impact on the binding specificity of the resulting proteins. The full length protein binds to a receptor that is Nm, Chymo and Tryp sensitive while the 270 kDa fragment binds to a receptor that is Nm/Tryp resistant and Chymo sensitive and the 140 kDa fragment binds to receptor that is resistant to Nm/Tryp while fairly sensitive to Chymo. This could suggest that PfRH2a contains two or more erythrocyte binding domains, with the domain only found in the full length protein dominating over the one coded for in the 270 and 140 kDa fragments. Alternatively, the results obtained here could also be consistent with the 140 kDa region containing the binding domain whose specificity is modified by other regions of the protein. Earlier it was suggested that the N-terminal conserved regions of RH2a mediates sialic acid independent invasion (10). However, our result shows that full length RH2a protein binds in a sialic acid dependent manner while the 270 & 140 kDa processed form of the protein bind in a sialic acid independent fashion. This suggests that the approximately 90 kDa
N-terminal region removed in the 270 kDa protein contains a sialic acid containing receptor binding domain. The observation that the 270kDa processed form of PfRH2a is the most abundant form of the protein could explain why the genetic approach (10) was only able to identified the sialic acid dependent binding component of the conserved region of PfRH2a/PfRH2b. The suggestion that RH contain more than one binding domain has been proposed for PfRH2b by knock out studies with an N-terminal domain mediating sialic acid independent binding and a C-terminal region mediating the interaction to a Chymo and Tryp sensitive receptor (10). In PfRH2a the removal of the N-terminal 90 kDa region from the full length protein removes the binding to the Nm and Tryp sensitive receptor and the further removal of 130 kDa region from the 270 kDa fragment removes the binding to chymo sensitive receptor. Our work now provides a biological rational for the multiple processing steps observed in the RH, with each processing step changing the binding potential of the protein and thereby regulating the function at different points of the invasion pathway.

The observation that PfRH2a can be found in the junction of merozoites independent of the invasion pathway used is intriguing. This could be solely due to the association of unbound PfRH2a with another parasite ligand, like the interaction between the \( P. vivax \) reticulocyte binding proteins PvRBP1 and PvRBP2 (16), that leads to the protein being translocated to the junction. Though alternatives, like an additional role in the formation of a functional invasion complex or stabilizing the interaction with the invasion motor are just some possible roles that this large protein could envisaged playing a role in. Clearly, additional data is required to determine all the functions of this protein.
In conclusion, we have provided compelling evidence that PfRH2a is an important invasion ligand in *P. falciparum*. We show that PfRH2a is actively engaged in the moving junction independent of the invasion pathway utilized by the parasite, suggesting that PfRH2a has a role in merozoite invasion that is independent of receptor binding. We furthermore, for the first time demonstrate direct binding of PfRH2a to erythrocytes and show that the receptor specificity of the protein can be changed by the proteolytic cleavage of the full length protein. Taken together these findings suggest a finely regulated cascade of processing and binding interactions that have a direct impact on the invasion process and highlights the complexity in understanding the role of the RH family during merozoite invasion. In future it will now be important to dissect how these processing steps change the function of the RH during invasion.

Materials and Methods

*Plasmodium falciparum* parasites and culture

*Plasmodium falciparum* parasite strains 3D7, 3D7ΔRH2a, W2mef and W2mef/Nm were cultured in fresh erythrocytes from the healthy human donors and RPMI 1640 supplemented with 5% albumax as described (49). For culturing W2mef parasites in neuraminidase treated erythrocytes, the erythrocytes were treated with 10mU/ml of neuraminidase (Roche) for 2 h at 37°C with constant rotation. Centrifuged for 3 times 5 min wash with incomplete RPMI 1640 (w/o albumax). W2mef parasites were allowed to grow in neuraminidase treated erythrocytes. After 3-4 cycles of culturing, the W2mef parasite adapts and remerges in neuraminidase treated erythrocytes (45). This switched parasite is named W2mef/Nm parasite.
To maintain the developmental stages, synchronization was performed at the ring stage parasites with 5% sorbitol (2, 29). Late schizont stage parasites were purified using 70% Percoll centrifugation as described (28). For further synchronization, the purified schizonts stage parasites were allowed to reinvade in fresh erythrocytes. After 5 to 6 h of growth, the parasites were treated with 5% sorbitol, to remove the remaining mature stage parasites.

**Cloning, expression and purification of recombinant proteins rPfRH2a in *E.coli*.**

The PfRH2a recombinant protein contains amino acids from 2874 to 3052. The PCR-amplification was done using primer 5’ GACCATATGataaaaagtaaacagaatct 3’ and 5’ AACCTCGAGggtattatcatcagtagtact 3’ from *P. falciparum* 3D7 genomic DNA (gDNA). The PCR products were digested with NdeI and XhoI and cloned into expression vector pET24a (+) (Novagen) to generate a C-terminal His-tag. BL21 (DE3) (Stratagene) was used to express recombinant rPfRH2a. IPTG at a final concentration of 1mM was added to cultures at $A_{600\,\text{nm}}$ of 0.6-0.8. Induced cultures were allowed to grow 3 h at 37°C and then re-suspended in chilled lysis buffer 50mM NaH$_2$PO$_4$, pH 8.0, 300mM NaCl, 5mM DTT with protease inhibitor cocktail, EDTA-free (Roche) and lysed by sonication. The recombinant protein was purified under native conditions using nickel-nitrilotriacetic acid-agarose (Ni-NTA) (Qiagen), followed by ion-exchange chromatography using a MonoQ$^\text{TM}$ 5/50 GL column (Amersham) and gel filtration chromatography using Superdex 75 Column (Amersham) in PBS, pH 7.4, with a 180mM NaCl. The PfRH1 recombinant protein has been previously described (Gao *et al.*, 2008).
**PfRH2a monoclonal antibody**

Purified recombinant RH2a protein was used to raise the monoclonal antibodies for PfRH2a protein. The monoclonal antibodies were generated through BioGenes (Germany). Eight different monoclonal antibody (mAb) producing clones generated for RH2a were shown positive by enzyme-linked immunosorbent assay (ELISA) (done by BioGenes). These 8 clones were further validated by western blot and immunofluorescence (data not shown) and C22, C19 and C15 were highly sensitive and specific RH2a mAbs.

**Parasite schizont extract, parasite culture supernatant preparation, protease inhibitor treatment and western blotting**

The purified late schizont parasites were washed with incomplete RPMI by centrifugation. The parasite pellet was dissolved in 1X PBS and stored at -20°C. The parasite culture supernatant was prepared by allowing the percoll purified schizont parasites to grow in complete medium in the absence of erythrocytes for 14-16 h at 37°C with constant agitation. After 16 h, the culture supernatant was obtained by centrifugation and stored at -80°C. For protease inhibitor treatment, the culture supernatant was incubated with/without the recommended concentration of protease inhibitor cocktail (Roche). The samples were obtained at 1, 2, 4 and 16 h post incubation and analyzed by western blotting. For western blotting, SDS loading buffer was added to the extract or culture supernatants and heated at 95°C for 5 min. The samples were separated by running in 6% (RH2a, and EBA175) and 12% (for actin) SDS-PAGE and transferred onto nitrocellulose membrane (Bio Rad) for 2 h at 100V. After the transfer, the membrane was blocked with 5% skimmed milk/PBST (1XPBS/0.5% tween20) for 1 h and incubated with primary antibodies: RH2a-C22
(1:500), C19 (1:500) and C15 (1:500), α-rabbit PfEBA175 (1:3000) (MR4), α-rabbit actin (1:1000) (Sigma) overnight at 4°C. Followed by 3 times 5 min wash with PBST and 1 h incubation with horseradish peroxidase (HRP) conjugated secondary antibody at room temperature (RT). After washing the membrane 3 times with PBST, membrane was developed using ECL plus western blotting detection system (Amersham) according to manufacturer’s instruction.

**Tight Junction Assay**

For junction arrested merozoites, the bursting schizonts stage parasite were added to the complete media containing fresh erythrocytes and 0.1µM Cytochalasin D (Sigma). And the parasites were incubated for 3 to 4 h and thin blood smear were made for the Immunofluorescence assay (IFA). Similarly to capture the moving junction, viable merozoites or bursting schizonts were allowed to grow and invade erythrocytes briefly for 30 mins. Thin blood smears were made on the glass slide for IFA.

**Immunofluorescence assay**

Thin blood smears containing the late schizont stage of the parasites were prepared and fixed with 100% acetone for 5 min, wrapped in aluminum foil and stored at -20°C. When required, the slides were taken out and keep at RT for 5-10 min, blocked with 3% BSA in 1X PBS for 30 min, then incubated for 1 h at 37°C with primary antibodies: RH2a-C22 (1:200), C19 (1:200) and C15 (1:200), α-rabbit PfRH1 (1:500), α-rabbit R2A9 (1:200), α-rabbit PfEBA175 (1:500) (MR4) or α-rabbit PfAMA1 (1:500). The slides were washed for 3 times 5 min with 1X PBS and then incubated with secondary antibodies: Alexa flour 488 or 594 goat anti rabbit or goat anti mouse antibodies (Molecular Probes) for 1 h at 37°C, followed by 3 times 5 min wash with
1X PBS. The slides were air-dried. Mounting medium containing DAPI (Vectashield) was applied to the slide. Fluorescent images were captured using LSM 710, LSM 510 confocal microscopy (Carl Zeiss) and Olympus fluorescent microscopy.

**Erythrocyte binding assay (EBA)**

Erythrocytes were treated with different enzymes like neuraminidase (Roche), trypsin (Sigma) and chymotrypsin (Sigma). The concentrations of the enzymes to treat the erythrocytes were 25 and 50 mU/ml of neuraminidase, 0.1, 1.0 and 5 mg/ml of trypsin and 2 mg/ml of chymotrypsin. Enzymes were added to the erythrocytes and kept at 37°C for 2 h with constant rotation. Trypsin and chymotrypsin treated erythrocytes were washed with incomplete RPMI and the reaction was stopped by adding 1mg/ml trypsin/chymotrypsin inhibitor (Sigma) and kept at RT for 15 min with constant rotation. Finally, the treated erythrocytes were washed for 3 times with incomplete RPMI.

The culture supernatants from *P. falciparum* strains (3D7 & 3D7ΔPfRH2a) were used. To 250µl of parasite culture supernatant, 100µl of packed erythrocytes or enzyme treated erythrocytes and 50µl of FBS was added and made up to 600µl with incomplete RPMI, incubated at 37°C for 2 h with constant rotation. After incubation, the erythrocytes were spun through dibutyl pthalate (Sigma) oil to separate the erythrocytes and the supernatant. The supernatant was removed by aspiration. The proteins bound to the erythrocytes were eluted using 20 µl 0.5M NaCl by incubating at RT for 10 min. Followed by centrifugation at 13,000 rpm for 2 min. The eluted proteins were analyzed by western blotting.

**Acknowledgement**
We would like to thank Dr. Alan Cowman for providing us with the 3D7ΔPfRH2a knockout parasite and R2A9 antibody and Dr. Mike Blackman for providing us with Anti-Rabbit AMA1 antibody. This work was funded by the Biomedical Research Council Grant BMRC 08/1/22/19/581.

Ethics Statement

This study was approved by the Institutional Review Board of Nanyang Technological University. Informed written consent was obtained from all blood donors.

Reference


Figure 1. Two forms of PfRH2a are expressed in schizont extract of different parasite strains: A. Pictorial representation of the P. falciparum RH members indicating, the signal peptides (red), RH homology region (blue), unique region against which antibody was raised (green) and C-terminal transmembrane domain (brown). The lengths of the proteins were represented by aminoacids (aa). B. Western blots of schizont extracts and supernatant proteins of 3D7, 3D7ΔRH2a and W2mef parasites were probed with mAb C22. Similar results were obtained in 3 other
independent experiments. Western blots of schizont extracts and supernatant proteins of 3D7 and 3D7ΔRH2a were probed with (C) mAb C19 and (D) mAb C15. RH2a was detected around 360 and 270 kDa in schizonts and an additional 140 kDa band was observed in culture supernatants.

**Figure 2. Co-immunofluorescence for RH2a with rhoptry and micronemal markers.** Immunofluorescence assay of a late schizont stage parasite stained for RH2a using anti-RH2a mAb C22 and either co-stained with rhoptry neck marker (A) RH1 and (B) R2A9 (RH2a/2b) or micronemal markers, (C) AMA1 and (D) EBA175 in W2mef. R2A9 is a rabbit polyclonal antibody, which recognize both RH2a and 2b. Scale bars = 5 µM

**Figure 3. RH2a is localized at tight junction during invasion.** Co-localization of RH2a proteins with EBA175 and AMA1 in junction arrested merozoites obtained by cytochalasin D treatment, from W2mef (A) or W2mef/Nm (B) and 3D7 (C) parasites. Arrows in the cartoon represents the proteins at the tight junction. Around 80 junction arrested merozoites in random microscopic fields were observed to confirm the localization of RH2a at the tight junction. Scale bars = 5 µM

**Figure 4. RH2a follows moving junction during invasion.** A) Immunofluorescence images show RH2a following the progression of invasion. RH2a was co-stained with AMA1. Merozoites progressing through different stages of invasion were shown. The top row shows free merozoites, followed by attached merozoite, reoriented merozoite where the apical end of the merozoite is facing the red blood cells, and finally three rows were depicted where the moving junction has progressed. Scale bars = 5 µM

26
Figure 5. Different processed forms of PfRH2a have different erythrocyte binding activity. **A)** Erythrocyte binding assay of PfRH2a showing difference in binding of the 360, 270 and 140 kDa protein using 3D7 supernatant in untreated erythrocytes (Un), and Neuraminidase (Nm - 25 and 50mU/ml), Chymotrypsin (Chymo 2mg/ml) & Trypsin (Tryp - 0.1, 1 and 5mg/ml) treated erythrocytes. Bound proteins eluted from the erythrocytes were separated by gel electrophoresis, transferred and then probed using mAb C22. For control, the same blot was re-probed with anti-EBA175. Similar results were obtained in two other independent experiments. **B)** Model to illustrate the processing of PfRH2a before and during the process of invasion. The full length proteins were colored black. The processed products of PfRH2a detected by our mAb in schizonts and supernatants are colored green. However our mAb against C terminal region was not able to detect the three possible processing products (90, 130 and 220 kDa) shown in grey. We propose that processing of PfRH2a changes the availability of binding sites at different times of the invasion process, allowing multiple distinct contacts with their erythrocyte to enable successful invasion.

Supplementary Figure

**Fig S1. Stage specific expression of RH2a and Protease Inhibitor Assay.** (A) Western blots of Ring, Trophozite and Schizont extracts of 3D7 and W2mef parasites probed with mcAb C22. Actin was used as the loading control. **(B)** Western blot analysis of the protease inhibitor assay. The culture supernatant protein was incubated with/without protease inhibitor and samples were collected at 1, 2, 4 and 16 h. S-

Supernatant protein (3D7), C- Control without protease inhibitor, PI- With protease
inhibitor. (C) 3D7 and 3D7ΔRH2a parasites schizont extract and supernatants were
run with High molecular weight marker and the same membrane was stripped and
reprobed for EBA175 as loading control.

**Fig. S2. Coimmunofluorescence for RH2a with rhoptry and micronemal markers**

Immunofluorescence assay of a late schizont stage parasite stained for RH2a using
anti-RH2a mcAb C22 (A), C19 (B), C15 (C) and either co-stained with rhoptry
marker RH1 and R2A9 and micronemal markers AMA1 or EBA175 in 3D7. The
parasite 3D7 RH2a KO was tested in parallel as a negative control.

**Fig S3. Erythrocyte binding assay in RH2a Knock out parasite.** Erythrocyte
binding assay of PfRH2a using 3D7 RH2a KO supernatant in untreated erythrocytes
(Un), and Neuraminidase (Nm - 25 and 50mU/ml), Chymotrypsin (Chymo - 1 and
2mg/ml) & Trypsin (Tryp - 0.1 and 1 mg/ml) treated erythrocytes. Bound proteins
eluted from the erythrocytes were separated by gel electrophoresis, transferred and
then probed using mcAb C22. For control, the same blot was re-probed with anti-
EBA175.
Figure 4

<table>
<thead>
<tr>
<th>W2mef</th>
<th>RH2a-C22</th>
<th>α AMA1</th>
<th>DAPI</th>
<th>Bright Field</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free Merozoites</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
</tr>
<tr>
<td>Attachment</td>
<td><img src="image6" alt="Image" /></td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
<tr>
<td>Reorientation</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
<td><img src="image13" alt="Image" /></td>
<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>Moving Junction</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
</tr>
</tbody>
</table>
Figure 5

A

<table>
<thead>
<tr>
<th>3D7 Supernatant</th>
<th>Untreated</th>
<th>Nm (25 μM/ml)</th>
<th>Nm (50 μM/ml)</th>
<th>Chymo (2 μg/ml)</th>
<th>Tryp (0.4 mg/ml)</th>
<th>Tryp (1 mg/ml)</th>
</tr>
</thead>
</table>

α - RH2a

α - EBA175

B

At Schizont stage

During Invasion

Binds to erythrocyte

90 kDa

130 kDa

220 kDa