# Differences in erythrocyte receptor specificity of different parts of the plasmodium falciparum reticulocyte binding protein homologue 2a

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1 **Differences in erythrocyte receptor specificity of different parts of the**

2 ***Plasmodium falciparum* Reticulocyte binding protein Homologue 2a**

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19      **Abstract**

20      The *P. falciparum* Reticulocyte-binding-like protein homologue (RH) and the

21      Erythrocyte Binding Like (EBL) protein families play an important role during

22      invasion, though their exact role is not clear. Both EBL and RH are thought to directly

23      bind different receptors on the surface of the erythrocyte and the binding properties

24      for a number of EBLs and RHs have been described. While PfRH1 and PfRH4, have

25      been shown to directly play a role in two alternative invasion pathways used by

26      merozoites, the function of PfRH2a and PfRH2b during invasion is less defined. Here

27      using monoclonal antibodies raised against a unique region of PfRH2a we show that

28      PfHR2a moves from the rhoptry neck to the moving junction during merozoite

29      invasion. Movement of PfRH2a to the junction is independent of the invasion

30      pathway used by the merozoite suggesting an additional function of the protein

31      independent of receptor binding. We furthermore show that PfRH2a is processed both

32      in the schizont as well as during invasion resulting in proteins with different

33      erythrocyte binding properties. Our findings suggest that PfRH2a and, most likly the

34      other members of the RH family, depending on their processing stage, can engage

35      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

60    parasite as well as erythrocyte proteins which are essential for the successful entry of

61    the merozoites into erythrocytes (12, 43).

62            Treatment with enzymes like neuraminidase, trypsin or chymotrypsin is

63    known to remove different receptors from the surface of erythrocytes and different

64    strains of *P. falciparum* have been shown to differ in their ability to invade these

65    treated erythrocytes (10, 14, 45, 50). This led to the suggestion that the ability of

66    parasite strains to differentially invade enzyme treated erythrocytes defines distinct

67    invasion pathways (18, 40, 45, 50). Two parasite encoded protein families termed

68    Erythrocyte Binding Like (EBL) proteins and Reticulocyte Binding-like Homologues

69    (RH) proteins have been shown to be involved in the differential recognition of

70    erythrocyte receptors and thereby define the invasion pathway utilized by a parasite

71    strain (1, 3, 6, 9, 17, 23, 25, 30, 33, 35, 36, 38, 39, 45). The EBLs are defined by a

72    conserved cysteine rich region termed Duffy binding like (DBL) domain that directly

73    mediates binding to erythrocyte receptors (7). In *Plasmodium falciparum*, six EBL

74    members have been identified, namely Erythrocyte binding antigen 175 (EBA175),

75    EBA140, EBA181, EBA165, EBL1 and MAEBL (1, 6, 25, 31, 33, 35, 53) with the

76    DBL domain of EBA175 recognizing the sialic acid component in glycophorin A (6,

77    13), that of EBA 140 binding to glycophorin C (30, 31, 33) and the DBL of EBA 181

78    binding to a so far uncharacterized sialoglycoprotein (20, 34). EBL1 has recently been

79    shown to bind to glycophorin B (32) and EBA165 is considered to be a pseudogene

80    (53). Unlike other EBLs, MAEBL does not have a DBL domain but contains

81    conserved domains, which are similar to AMA1 (5).

82

83    The RH was first discovered in rodent malaria parasite *P.yoelii* where they were

84    termed Py235 (15, 22). Subsequent work has though shown that RHs were found in

85    all the *Plasmodium* species so far analyzed (3, 15, 16, 21, 22, 24, 37-39, 52). In *P.*

86    *yoelii* PY235 is coded for by a multigene family (27) and was thought to be involved

87    in parasite virulence (15) as well as mediate a novel form of clonal phenotypic

88    variation (37). In *Plasmodium vivax,* homologues of Py235, the reticulocyte binding

89    proteins PvRBP1 and PvRBP2 were shown binding to reticulocytes, leading to the

90    suggestion that members of this protein family plays an important role in host cell

91    recognition [34]. In *Plasmodium falciparum*, six RH members have been identified,

92    they are PfRH1 (39), PfRH2a (38, 52), PfRH2b (38, 52), PfRH3 (46), PfRH4 (24, 45)

93    and PfRH5 (3, 42).

94

95    PfRH1 is the ortholog of PvRBP1 and binds to the sialic acid containing putative

96    erythrocyte receptor Y (4, 39, 50). The erythrocyte binding region of PfRH1 has been

97    identified and the antibodies raised against this region inhibits merozoite invasion

98    (17). Triglia *et al* has recently shown that RH1 protein undergoes a series of

99    proteolytic cleavage events that occur before and during entry into the erythrocyte and

100    further showed that the processed products along with EBA175 are important

101    components of the tight junction (51). PfRH2a and 2b have been identified by

102    comparative analyses with *Plasmodium vivax* reticulocyte binding protein 2 (PvRBP-

103    2) (38) and by gene knock out studies PfRH2a is shown to be involved in sialic acid

104    independent invasion pathway (10). Although antibodies against PfRH2a are able to

105    inhibit merozoite invasion (52), there is no evidence to date on erythrocyte binding

106    ability of PfRH2a. PfRH2b gene knock out studies showed that PfRH2b interacts with

107    the chymotrypsin sensitive erythrocyte receptor Z (4, 14). Recent findings provide

108    evidence suggesting that PfRH2 is naturally immunogenic and its antibodies are

109    associated with protection from malaria (41). Taylor *et al* described another member

110   of PfRH family, PfRH3 as a pseudogene (46). PfRH4 was identified as 220kDa

111   protein (24) and its differential expression was subsequently shown to be a major

112   factor in the invasion pathway switching of the parasite from a sialic acid dependent

113   to sialic acid independent pathway (18, 45). Recently PfRH4 was shown to bind to

114   erythrocytic receptor CR1 (47). PfRH5 lacking a transmembrane domain, with a

115   molecular weight of 63 kDa is the smallest member of RH and is found to be in the

116   tight junction during erythrocyte invasion (3, 42). While the erythrocyte binding

117   properties of PfRH1, PfRH4 and PfRH5 have been identified (3, 17, 19), only for

118   PfRH4 has complementary receptor 1 (CR1) been identified as the erythrocyte

119   receptor (47).

120

121   In this study we have focused on PfRH2a and provide evidence of its significant role

122   in invasion. Using monoclonal antibodies (mAb) against PfRH2a, we show that

123   PfRH2a colocalizes with PfRH1 in rhoptry neck of the merozoite. We furthermore

124   demonstrate that both in parasites using a sialic acid dependent as well as independent

125   invasion pathway, PfRH2a is found in the tight junction during merozoite invasion.

126   We show that PfRH2a undergoes proteolytic processing in schizonts and also during

127   invasion and that the different proteolytic forms display different erythrocyte binding

128   properties. Our findings demonstrate that PfRH2a plays an important role during

129   merozoite invasion and depending on the proteolytic stage is able to mediate different

130   ligand-receptor interactions, possibly at different times of the invasion process.

131

132   **Results**

133   **Two forms of PfRH2a are detected in schizont extract**

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134 As PfRH2a and PfRH2b share high sequence similarity except for the C-terminal

135 region, we raised monoclonal antibodies (mAb) against the unique region

136 corresponding to amino acids 2874 to 3052 of PfRH2a (**Fig 1A**). Screening of

137 hybridoma clones resulted in the identification of three antibody producing clones

138 (C22, C19 and C15). Using the mAb (C22), we have confirmed the previous results of

139 peak expression of PfRH2a at the schizont stage, of two parasite clones 3D7 and

140 W2mef (**Fig S1A**). Two forms of PfRH2a of approximately 360 and 270 kDa were

141 detected at the schizont stage of the parasite, with the 270 kDa form being more

142 abundant (**Fig 1B** and **S1C**). Western blot analysis of schizont extract from 3D7 and

143 3D7ΔPfRH2a, a parasite line in which PfRH2a had been disrupted (14), confirmed

144 that both the 360 as well as the 270 kDa were indeed due to the expression of PfRH2a

145 and not due to cross reactivity of the antibody with PfRH2b (Fig 1B and S1C). As

146 PfRH2a is predicted to be approximately 360 kDa in size it is likely that the ~360 kDa

147 protein detected in the western blot corresponds to the full length protein with the 270

148 kDa band representing a processed form of the protein. Proteolytic cleavage of PfRH1

149 has earlier been reported to be an important step during invasion, and results in a

150 number of specific products (51). Hence to determine whether PfRH2a undergo a

151 similar cleavage events, western blot was performed for schizont extract and culture

152 supernatant of 3D7, 3D7ΔPfRH2a and W2mef parasite line probed using C22 mAb.

153 As expected, the full length 360 and a processed 270 kDa protein was detected in both

154 schizont extracts from 3D7 and W2mef, while in the parasite supernatant an

155 additional 140 kDa protein band is consistently detected suggesting a secondary

156 processing of PfRH2a during invasion (Fig 1B). Taken together, this data is consistent

157 with the 360 kDa full length PfRH2a being proteolytically cleaved at the N-terminal

158 end of the protein, resulting in an approximately 270 kDa C-terminal fragment and

159  possibly an approximately 90 kDa N-terminal fragment (**Fig 5B**). During invasion a

160  second processing step removing a further 130 kDa N-terminal fragment occurs,

161  leaving a 140 kDa fragment detected by the mAb (Figure 1B and 5B). It is also

162  interesting to note that the 270 and 140 kDa bands appear as doublets in the culture

163  supernatant indicating the proteolytic removal of a second small fragment during the

164  invasion process (**Fig** 1 B, **C and D**). The 3D7 supernatant proteins incubated in the

165  presence or absence of protease inhibitor indicated no significant change in the

166  amount of the 140kDa fragment, indicating that this secondary cleavage event is not

167  due to the non-specific degradation of the 270 kDa or 360 kDa protein in the parasite

168  supernatant **(Fig S1B)**. Similar processing of RH2a was consistently observed using

169  the other RH2a mAbs C19 and C15 in 3D7 and 3D7ΔPfRH2a parasite strains (Fig 1C

170  and D) suggesting that the additional processing of RH2a could be a crucial event

171  during merozoite invasion.

172

173  **PfRH2a is located at rhoptries**

174  Previous studies have suggested that RH members are expressed either in the rhoptry

175  neck or at the apical prominence of different *P. falciparum* strains. To further

176  delineate the exact localization of PfRH2a, we performed immunofluorescence assay

177  (IFA) using fixed schizont smears. Using the mAb, PfRH2a showed a punctuate

178  pattern at the apical tip of the merozoite in schizonts and appears to co-localize with

179  PfRH1 in W2mef and 3D7 parasites, strongly suggesting that the RH2a is located at

180  the rhoptry neck (**Fig 2A** and **Fig S2**). The localization of RH2a with the mAb is

181  identical to that observed with the polyclonal antibody R2A9 raised against

182  PfRH2a/2b (R2A9 is raised against the amino acid stretch common to both

183  PfRH2a/2b) (**Fig 2B** and S2). As expected, there is a partial overlap with micronemal

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184  markers like AMA1 and EBA175 (**Fig 2C** and **2D** and **Fig S2**). This is consistent with

185  electron microscopy studies using polyclonal antibodies that react with both

186  PfRH2a/b, showing that they are located in the rhoptry neck of the merozoites (14).

187  Control experiments using the 3D7ΔPfRH2a parasites line showed no staining for

188  PfRH2a confirming the specificity of the mAb in IFA (Fig S2). These results confirm

189  that the monoclonal antibodies used in this study are highly specific for PfRH2a and

190  allow specific localization of the protein in the parasite.

191

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

193  It is clear from the above data that PfRH2a is expressed at the schizont stage of

194  various *P. falciparum* strains. However, there is limited data that supports a direct role

195  of PfRH2a in parasite invasion. Junction formation is linked to the irreversible

196  commitment of the merozoite to invasion and recent studies have shown that both

197  PfRH1 and PfRH5 are part of the junction suggesting a specific role of the RH

198  proteins during this part of the invasion process (3, 51). To test whether PfRH2a is

199  also located in the junction, invasion of merozoites was arrested by treating the

200  parasites with 0.1μM cytochalasin D. Cytochalasin is a cell permeable fungal toxin

201  that prevents cell ruffling and cell motility (8). It is found to be very effective in

202  causing actin depolymerization even at low concentration (54). The micronemal

203  markers AMA1 and EBA175 earlier shown to be localized in the tight junction, hence

204  we employed these proteins as markers for the tight junction. Immunofluorescent

205  assays using the junction-arrested merozoites showed that PfRH2a located at the tight

206  junction with EBA175 & AMA-1 in W2mef parasite clone (**Fig 3A**). *P. falciparum*

207  W2mef normally utilizes a sialic acid dependent invasion pathway, but upon selection

208  on neuraminidase treated erythrocytes is able to switch (W2mef/NM) to using a sialic

209    acid independent pathway (11). This switch in invasion pathway has been linked to

210    the activation of expression of PfRH4 (18, 45). As PfRH2a has been suggested to also

211    play a role in directing sialic acid independent invasion (10) we investigated whether

212    the location of PfRH2a changes in a parasite that uses a sialic acid independent

213    invasion pathway. In W2mef/NM, PfRH2a were located with junction markers

214    EBA175 and AMA1 in junction-arrested merozoites (**Fig 3B**). No apparent difference

215    in the location of PfRH2a was seen between W2mef and W2mef/NM junction-

216    arrested merozoites suggesting that the location of PfRH2a does not change in

217    response to the receptors utilized by the parasite. In the same way, RH2a located in

218    the tight junction of junction arrested 3D7 clone (**Fig 3C**). A similar assay was

219    performed in the absence of cytochalasin D to further study the localization of RH2a

220    in the moving junction with AMA1 in W2mef clone. PfRH2a was observed in the

221    initial junction formed just after the invasion process has started as well as all later

222    stages of the junction observed as the merozoite penetrates into erythrocyte (**Fig 4**).

223    Taken together, these observations indicate that RH2a localizes at the junction and

224    suggest that it could play an important role in helping merozoite to enter erythrocytes.

225

226    **Processing of PfRH2a changes erythrocyte receptor specificity**

227    The erythrocyte binding ability of PfRH1, PfRH4 and PfRH5 members have been

228    elucidated (3, 17, 19, 39, 42, 48). However, to date direct binding of PfRH2a to

229    erythrocytes has been unsuccessful (52). Here using the PfRH2a specific monoclonal

230    antibody, we show that PfRH2a binds to the erythrocyte. Erythrocyte Binding Assays

231    (EBA) performed using parasite supernatant obtained from 3D7, clearly showed that

232    the 360, 270 and 140 kDa proteins bind erythrocytes (**Fig 5A**). Comparing the relative

233    intensity of the 360 with 270 and 140 kDa bands in supernatant as well as the

234    erythrocyte bound protein fraction suggests that the 360 kDa protein is able to bind

235    erythrocytes more efficiently than the 270 and 140 kDa proteins (Fig 5A).

236

237    To determine the binding characteristics of PfRH2a in more detail, EBA using

238    enzyme treated erythrocytes was carried out using culture supernatant from *P.*

239    *falciparum* strains 3D7 and 3D7ΔPfRH2a. Erythrocytes were treated separately with

240    varying concentration of neuraminidase (Nm), chymotrypsin (Chymo) and trypsin

241    (Tryp) before each experiment. Treatment of erythrocytes with 25 or 50 mU of Nm

242    reduced the binding of the 360 kDa protein while little or no effect was seen on the

243    binding of the 270 and 140 kDa proteins (Fig 5A). However, binding of both the 360

244    as well as 270 kDa protein was highly sensitive to chymotrypsin treatment with

245    binding being abolished for both proteins, consistent with the previous genetic data

246    suggesting that PfRH2a interacts with a Chymo sensitive receptor (10, 14). In contrast

247    the 140 kDa protein appears to be moderately sensitive to Chymo treatment (Fig 5A).

248    Tryp treatment of erythrocyte completely abolished the binding of 370 kDa protein,

249    while the binding of both the 270 kDa as well as 140 kDa bands is not affected by

250    increasing concentration of Tryp. As a control the same western blot membranes were

251    re-probed for EBA175, which has been previously shown to bind to erythrocytes in a

252    Nm sensitive, Chymo resistant and Tryp resistant fashion (3, 48). This clearly showed

253    that the enzyme treatment had the expected effect, with binding of EBA175 being

254    reduced in NM treated erythrocytes, while being refractory to treatment with Chymo

255    and dose-dependent sensitivity to Tryp (Figure 5A). As expected no binding of

256    PfRH2a was detected with mAb C22 when using 3D7ΔRH2a culture supernatant for

257    EBA, while for control the same membrane were re-probed using EBA175, in which

258    the binding was identical to that seen in 3D7 supernatant (**Fig S3**). Taken together 360

259  kDa protein is sensitive to NM, Chymo and Tryp, While 270 kDa protein is sensitive

260  to Chymo and resistant to NM and Tryp, whereas 140 kDa protein is resistant to NM

261  and Tryp reasonably sensitive to Chymo.

262  **Discussion**

263  Reticulocyte binding like protein Homologues (RH) plays an important role in

264  invasion of erythrocytes in several *Plasmodium species*. However the precise role of

265  the RH in invasion is not yet clearly understood. In this study, using specific as well

266  as highly sensitive monoclonal antibodies we generated, we have focused on the

267  importance of *Plasmodium falciparum* RH2a protein. Previous studies focusing on the

268  genetic disruption of PfRH2a in both 3D7 and W2mef have shown that the role of this

269  protein is different in the two parasite strains (10, 14). While in the PfRH2a 3D7

270  knockout line no direct effect on the invasion capacity of the parasite was observed

271  (14), in the W2mef background PfRH2a appears to play a role in sialic acid

272  independent invasion (10). Using IFA we show that PfRH2a is located in the rhoptry

273  neck of both W2mef and 3D7 suggesting that the apparent different roles of PfRH2a

274  in these two parasite lines is not due to the differential location of PfRH2a. Additional

275  support for a role of PfRH2a in W2mef comes from our IFA data that shows very

276  clear colocalization of PfRH1 and PfRH2a at the rhoptry neck of merozoite support

277  previous assumption that both proteins are able to equally engage receptors on the

278  surface of the erythrocyte. A number of recent studies have shown that PfRH1 and

279  PfRH5 are directly located in the tight junction (3, 51). In line with these proteins, not

280  only being important in an initial host cell sensing but furthermore playing an active

281  role in the moving junction thereby mediating the movement of the merozoite into the

282  host erythrocyte. Our findings that PfRH2a does locate in the junction of W2mef,

283  W2mef/Nm and 3D7 is consistent with a role during the invasion process, despite the

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284     fact that genetic disruption and use of enzyme treated erythrocytes have not been able

285     to establish this previously (14, 52). Previously it has been shown that in the case of

286     PfRH1 not all the protein is translocated to the junction but rather that some of PfRH1

287     remains at the apical tip (51), In contrast, while we detect PfRH2a at the apical

288     location in free merozoites as well as those engaged in initial attachment and

289     reorientation, all PfRH2a appears to translocate into the junction with no protein

290     being left behind at the apical tip. It will be interesting to establish whether this is

291     indeed a difference between PfRH1 and PfRH2a or reflects a functional difference in

292     the reagents used. .

293     We show that PfRH2a is proteolytically cleaved with an initial processing step

294     occurring during schizont maturation resulting in the 360 kDa full length protein

295     being cleaved to a 270 kDa protein. This processing is followed by a second cleavage

296     of the 270 kDa protein to a 140 kDa fragment that occurs after schizont release,

297     possibly during the invasion. It is intriguing to speculate that the observed processed

298     doublet bands around 270 and 140 kDa in cell culture supernatant but not in the

299     schizoint extract could perhaps play an important role in the process of invasion.

300     Furthermore the protease inhibitor treatment of the culture supernatant indicate that

301     140 kDa processed product is not due to random degradation of the full length

302     protein. Our antibody does not allow us to establish whether the N-terminal fragments

303     produced by this processing (Fig 5B) are maintained in the merozoite and junction.

304     Sequential proteolytic cleavage of malaria invasion proteins is thought to be important

305     steps during merozoite invasion. As previously shown PfRH1 is also sequentially

306     processed with an initial cut occurring in the schizont followed by further cleavage

307     during invasion (17, 51) and it is interesting to note that the proposed sites of PfRH1

308     processing appear to be in similar locations to those observed for PfRH2a. This

309 strongly suggests that processing of all RH follows a conserved pathway during
310 merozoite maturation and invasion.

311 Enzyme treatment of erythrocytes has shown that different RH interact with
312 erythrocyte receptors that have different sensitivities to Nm, Chymo or Tryp. The
313 putative receptor Y for PfRH1 is Nm sensitive and Tryp/Chymo resistant (17), while
314 for PfRH2b the putative receptor Z is thought to be Chymo sensitive and Nm/Tryp
315 resistant (14). The receptor for PfRH4 has now been shown to be CR1 (47) and based
316 on the impact of PfRH2a disruption on invasion of enzyme treated erythrocytes
317 PfRH2a is thought to interact with a Chymo sensitive receptor (10, 14). Here we show
318 that the 360, 270 and 140 kDa fragments of PfRH2a are able to specifically bind to
319 erythrocytes, with the full-length protein binding stronger than the processed 270 and
320 140 kDa protein. Importantly, we demonstrate that processing of PfRH2a has a direct
321 impact on the binding specificity of the resulting proteins. The full length protein
322 binds to a receptor that is Nm, Chymo and Tryp sensitive while the 270 kDa fragment
323 binds to a receptor that is Nm/Tryp resistant and Chymo sensitive and the 140 kDa
324 fragment binds to receptor that is resistant to Nm/Tryp while fairly sensitive to
325 Chymo. This could suggest that PfRH2a contains two or more erythrocyte binding
326 domains, with the domain only found in the full length protein dominating over the
327 one coded for in the 270 and 140 kDa fragments. Alternatively, the results obtained
328 here could also be consistent with the 140 kDa region containing the binding domain
329 whose specificity is modified by other regions of the protein. Earlier it was suggested
330 that the N-terminal conserved regions of RH2a mediates sialic acid independent
331 invasion (10). However, our result shows that full length RH2a protein binds in a
332 sialic acid dependent manner while the 270 & 140 kDa processed form of the protein
333 bind in a sialic acid independent fashion. This suggests that the approximately 90 kDa

14

334 N-terminal region removed in the 270 kDa protein contains a sialic acid containing

335 receptor binding domain. The observation that the 270kDa processed form of PfRH2a

336 is the most abundant form of the protein could explain why the genetic approach (10)

337 was only able to identified the sialic acid dependent binding component of the

338 conserved region of PfRH2a/PfRH2b. The suggestion that RH contain more than one

339 binding domain has been proposed for PfRH2b by knock out studies with an N-

340 terminal domain mediating sialic acid independent binding and a C-terminal region

341 mediating the interaction to a Chymo and Tryp sensitive receptor (10). In PfRH2a the

342 removal of the N-terminal 90 kDa region from the full length protein removes the

343 binding to the Nm and Tryp sensitive receptor and the further removal of 130 kDa

344 region from the 270 kDa fragment removes the binding to chymo sensitive receptor.

345 Our work now provides a biological rational for the multiple processing steps

346 observed in the RH, with each processing step changing the binding potential of the

347 protein and thereby regulating the function at different points of the invasion pathway.

348 The observation that PfRH2a can be found in the junction of merozoites independent

349 of the invasion pathway used is intriguing. This could be solely due to the association

350 of unbound PfRH2a with another parasite ligand, like the interaction between the *P.*

351 *vivax* reticulocyte binding proteins PvRBP1and PvRBP2 (16), that leads to the protein

352 being translocated to the junction. Though alternatives, like an additional role in the

353 formation of a functional invasion complex or stabilizing the interaction with the

354 invasion motor are just some possible roles that this large protein could envisaged

355 playing a role in. Clearly, additional data is required to determine all the functions of

356 this protein.

357 In conclusion, we have provided compelling evidence that PfRH2a is an important

358 invasion ligand in *P. falciparum*. We show that PfRH2a is actively engaged in the

359 moving junction independent of the invasion pathway utilized by the parasite,

360 suggesting that PfRH2a has a role in merozoite invasion that is independent of

361 receptor binding. We furthermore, for the first time demonstrate direct binding of

362 PfRH2a to erythrocytes and show that the receptor specificity of the protein can be

363 changed by the proteolytic cleavage of the full length protein. Taken together these

364 findings suggest a finely regulated cascade of processing and binding interactions that

365 have a direct impact on the invasion process and highlights the complexity in

366 understanding the role of the RH family during merozoite invasion. In future it will

367 now be important to dissect how these processing steps change the function of the RH

368 during invasion.

369

370

371 **Materials and Methods**

372 *Plasmodium falciparum* **parasites and culture**

373 *Plasmodium falciparum* parasite strains 3D7, 3D7ΔRH2a,W2mef and W2mef/Nm

374 were cultured in fresh erythrocytes from the healthy human donors and RPMI 1640

375 supplemented with 5% albumax as described (49). For culturing W2mef parasites in

376 neuraminidase treated erythrocytes, the erythrocytes were treated with 10mU/ml of

377 neuraminidase (Roche) for 2 h at 37°C with constant rotation. Centrifuged for 3 times

378 5 min wash with incomplete RPMI 1640 (w/o albumax). W2mef parasites were

379 allowed to grow in neuraminidase treated erythrocytes. After 3-4 cycles of culturing,

380 the W2mef parasite adapts and remerges in neuraminidase treated erythrocytes (45).

381 This switched parasite is named W2mef/Nm parasite.

382

383    To maintain the developmental stages, synchronization was performed at the ring

384    stage parasites with 5% sorbitol (2, 29). Late schizont stage parasites were purified

385    using 70% Percoll centrifugation as described (28). For further synchronization, the

386    purified schizonts stage parasites were allowed to reinvade in fresh erythrocytes.

387    After 5 to 6 h of growth, the parasites were treated with 5% sorbitol, to remove the

388    remaining mature stage parasites.

389

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

391    The PfRH2a recombinant protein contains amino acids from 2874 to 3052. The PCR-

392    amplification was done using primer 5' GACCATATGataaaaagtaaactagaatct 3' and

393    5' AACCTCGAGggtattatcatcagtagtact 3' from *P. falciparum* 3D7 genomic DNA

394    (gDNA). The PCR products were digested with *Nde1* and *Xho*I and cloned into

395    expression vector pET24a (+) (Novagen) to generate a C-terminal His-tag. BL21

396    (DE3) (Stratagene) was used to express recombinant rPfRH2a. IPTG at a final

397    concentration of 1mM was added to cultures at $A_{600\ nm}$ of 0.6-0.8. Induced cultures

398    were allowed to grow 3 h at 37°C and then re-suspended in chilled lysis buffer 50mM

399    $NaH_2PO_4$, pH 8.0, 300mM NaCl, 5mM DTT with protease inhibitor cocktail, EDTA-

400    free (Roche) and lysed by sonication. The recombinant protein was purified under

401    native conditions using nickel-nitrilotriacetic acid-agarose (Ni-NTA) (Qiagen),

402    followed by ion-exchange chromatography using a MonoQ$^{TM}$ 5/50 GL column

403    (Amersham) and gel filtration chromatography using Superdex 75 Column

404    (Amersham) in PBS, pH 7.4, with a 180mM NaCl. The PfRH1 recombinant protein

405    has been previously described (Gao *et al.*, 2008).

406

407 **PfRH2a monoclonal antibody**

408 Purified recombinant RH2a protein was used to raise the monoclonal antibodies for

409 PfRH2a protein. The monoclonal antibodies were generated through BioGenes

410 (Germany). Eight different monoclonal antibody (mAb) producing clones generated

411 for RH2a were shown positive by enzyme-linked immunosorbent assay (ELISA)

412 (done by BioGenes). These 8 clones were further validated by western blot and

413 immunoflurescence (data not shown) and C22, C19 and C15 were highly sensitive

414 and specific RH2a mAbs.

415

416 **Parasite schizont extract, parasite culture supernatant preparation, protease**

417 **inhibitor treatment and western blotting**

418 The purified late schizont parasites were washed with incomplete RPMI by

419 centrifugation. The parasite pellet was dissolved in 1X PBS and stored at -20°C. The

420 parasite culture supernatant was prepared by allowing the percoll purified schizont

421 parasites to grow in complete medium in the absence of erythrocytes for 14-16 h at

422 37°C with constant agitation. After 16 h, the culture supernatant was obtained by

423 centrifugation and stored at -80°C. For protease inhibitor treatment, the culture

424 supernatant was incubated with/without the recommended concentration of protease

425 inhibitor cocktail (Roche). The samples were obtained at 1, 2, 4 and 16 h post

426 incubation and analyzed by western blotting. For western blotting, SDS loading buffer

427 was added to the extract or culture supernatants and heated at 95°C for 5 min. The

428 samples were separated by running in 6% (RH2a, and EBA175) and 12% (for actin)

429 SDS-PAGE and transferred onto nitrocellulose membrane (Bio Rad) for 2 h at 100V.

430 After the transfer, the membrane was blocked with 5% skimmed milk/PBST

431 (1XPBS/0.5% tween20) for 1 h and incubated with primary antibodies: RH2a-C22

432      (1:500), C19 (1:500) and C15 (1:500), α-rabbit PfEBA175 (1: 3000) (MR4), α-rabbit

433      actin (1:1000) (Sigma) overnight at 4°C. Followed by 3 times 5 min wash with PBST

434      and 1 h incubation with horseradish peroxidase (HRP) conjugated secondary antibody

435      at room temperature (RT). After washing the membrane 3 times with PBST,

436      membrane was developed using ECL plus western blotting detection system

437      (Amersham) according to manufacturer's instruction.

438

439      **Tight Junction Assay**

440      For junction arrested merozoites, the bursting schizonts stage parasite were added to

441      the complete media containing fresh erythrocytes and 0.1µM Cytochalasin D (Sigma).

442      And the parasites were incubated for 3 to 4 h and thin blood smear were made for the

443      Immunofluorescence assay (IFA). Similarly to capture the moving junction, viable

444      merozoites or bursting schizonts were allowed to grow and invade erythrocytes

445      briefly for 30 mins. Thin blood smears were made on the glass slide for IFA.

446

447      **Immunofluorescence assay**

448      Thin blood smears containing the late schizont stage of the parasites were prepared

449      and fixed with 100% acetone for 5 min, wrapped in aluminum foil and stored at -

450      20°C. When required, the slides were taken out and keep at RT for 5-10 min, blocked

451      with 3% BSA in 1X PBS for 30 min, then incubated for 1 h at 37°C with primary

452      antibodies: RH2a-C22 (1:200), C19 (1:200) and C15 (1:200), α-rabbit PfRH1 (1:500),

453      α-rabbit R2A9 (1:200), α-rabbit PfEBA175 (1:500) (MR4) or α-rabbit PfAMA1

454      (1:500). The slides were washed for 3 times 5 min with 1X PBS and then incubated

455      with secondary antibodies: Alexa flour 488 or 594 goat anti rabbit or goat anti mouse

456      antibodies (Molecular Probes) for 1 h at 37°C, followed by 3 times 5 min wash with

457     1X PBS. The slides were air-dried. Mounting medium containing DAPI (Vectashield)

458     was applied to the slide. Fluorescent images were captured using LSM 710, LSM 510

459     confocal microscopy (Carl Zeiss) and Olympus fluorescent microscopy.

460

461     **Erythrocyte binding assay (EBA)**

462     Erythrocytes were treated with different enzymes like neuraminidase (Roche), trypsin

463     (Sigma) and chymotrypsin (Sigma). The concentrations of the enzymes to treat the

464     erythrocytes were 25 and 50 mU/ml of neuraminidase, 0.1, 1.0 and 5 mg/ml of trypsin

465     and 2 mg/ml of chymotrypsin. Enzymes were added to the erythrocytes and kept at

466     37°C for 2 h with constant rotation. Trypsin and chymotrypsin treated erythrocytes

467     were washed with incomplete RPMI and the reaction was stopped by adding 1mg/ml

468     trypsin/chymotypsin inhibitor (Sigma) and kept at RT for 15 min with constant

469     rotation. Finally, the treated erythrocytes were washed for 3 times with incomplete

470     RPMI.

471     The culture supernatants from *P. falciparum* strains (3D7 & 3D7ΔPfRH2a) were

472     used. To 250μl of parasite culture supernatant, 100μl of packed erythrocytes or

473     enzyme treated erythrocytes and 50μl of FBS was added and made up to 600μl with

474     incomplete RPMI, incubated at 37°C for 2 h with constant rotation. After incubation,

475     the erythrocytes were spun through dibutyl pthalate (Sigma) oil to separate the

476     erythrocytes and the supernatant. The supernatant was removed by aspiration. The

477     proteins bound to the erythrocytes were eluted using 20 μl 0.5M NaCl by incubating

478     at RT for 10 min. Followed by centrifugation at 13,000 rpm for 2 min. The eluted

479     proteins were analyzed by western blotting.

480

481     **Acknowledgement**

20

486

**Ethics Statement**

488 This study was approved by the Institutional Review Board of Nanyang

489 Technological University. Informed written consent was obtained from all blood

490 donors.

491

492

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691

692

693    **Figure 1. Two forms of PfRH2a are expressed in schizont extract of different**

694    **parasite strains:**   **A.** Pictorial representation of the *P. falciparum* RH members

695    indicating, the signal peptides (red), RH homology region (blue), unique region

696    against which antibody was raised (green) and C-terminal transmembrane domain

697    (brown). The lengths of the proteins were represented by aminoacids (aa). **B.** Western

698    blots of schizont extracts and supernatant proteins of 3D7, 3D7ΔRH2a and W2mef

699    parasites were probed with mAb C22. Similar results were obtained in 3 other

700 independent experiments. Western blots of schizont extracts and supernatant proteins

701 of 3D7 and 3D7ΔRH2a were probed with (C) mAb C19 and (D) mAb C15. RH2a was

702 detected around 360 and 270 kDa in schizonts and an additional 140 kDa band was

703 observed in culture supernatants.

704

705 **Figure 2. Co-immunofluroscence for RH2a with rhoptry and micronemal**

706 **markers**. Immunofluroscence assay of a late schizont stage parasite stained for RH2a

707 using anti-RH2a mAb C22 and either co-stained with rhoptry neck marker (A) RH1

708 and (B) R2A9 (RH2a/2b) or micronemal markers, (C) AMA1 and (D) EBA175 in

709 W2mef. R2A9 is a rabbit polyclonal antibody, which recognize both RH2a and 2b.

710 Scale bars = 5 μM

711

712 **Figure 3. RH2a is localized at tight junction during invasion.** Co-localization of

713 RH2a proteins with EBA175 and AMA1 in junction arrested merozoites obtained by

714 cytochalasin D treatment, from W2mef (A) or W2mef/Nm (B) and 3D7 (C) parasites.

715 Arrows in the cartoon represents the proteins at the tight junction. Around 80 junction

716 arrested merozoites in random microscopic fields were observed to confirm the

717 localization of RH2a at the tight junction. Scale bars = 5 μM

718

719 **Figure 4. RH2a follows moving junction during invasion. A)** Immunofluorescence

720 images show RH2a following the progression of invasion. RH2a was co-stained with

721 AMA1. Merozoites progressing through different stages of invasion were shown. The

722 top row shows free merozoites, followed by attached merozoite, reoriented merozoite

723 where the apical end of the merozoite is facing the red blood cells, and finally three

724 rows were depicted where the moving junction has progressed. Scale bars = 5 μM

726 **Figure 5. Different processed forms of PfRH2a have different erythrocyte**

727 **binding activity. A)** Erythrocyte binding assay of PfRH2a showing difference in

728 binding of the 360, 270 and 140 kDa protein using 3D7 supernatant in untreated

729 erythrocytes (Un), and Neuraminidase (Nm - 25 and 50mU/ml), Chymotrypsin

730 (Chymo 2mg/ml) & Trypsin (Tryp - 0.1, 1 and 5mg/ml) treated erythrocytes. Bound

731 proteins eluted from the erythrocytes were separated by gel electrophoresis,

732 transferred and then probed using mAb C22. For control, the same blot was re-probed

733 with anti-EBA175. Similar results were obtained in two other independent

734 experiments. **B)** Model to illustrate the processing of PfRH2a before and during the

735 process of invasion. The full length proteins were colored black. The processed

736 products of PfRH2a detected by our mAb in schizonts and supernatants are colored

737 green. However our mAb against C terminal region was not able to detect the three

738 possible processing products (90, 130 and 220 kDa) shown in grey. We propose that

739 processing of PfRH2a changes the availability of binding sites at different times of the

740 invasion process, allowing multiple distinct contacts with their erythrocyte to enable

741 successful invasion.

742
743
744 **Supplementary Figure**

745 **Fig S1. Stage specific expression of RH2a and Protease Inhibitor Assay. (A)**

746 Western blots of Ring, Trophozite and Schizont extracts of 3D7 and W2mef parasites

747 probed with mcAb C22. Actin was used as the loading control. (**B**) Western blot

748 analysis of the protease inhibitor assay. The culture supernatant protein was incubated

749 with/without protease inhibitor and samples were collected at 1, 2, 4 and 16 h. S-

750 Supernatant protein (3D7), C- Control without protease inhibitor, PI- With protease

751 inhibitor. (**C**) 3D7 and 3D7ΔRH2a parasites schizont extract and supernatants were

752 run with High molecular weight marker and the same membrane was striped and

753 reprobed for EBA175 as loading control.

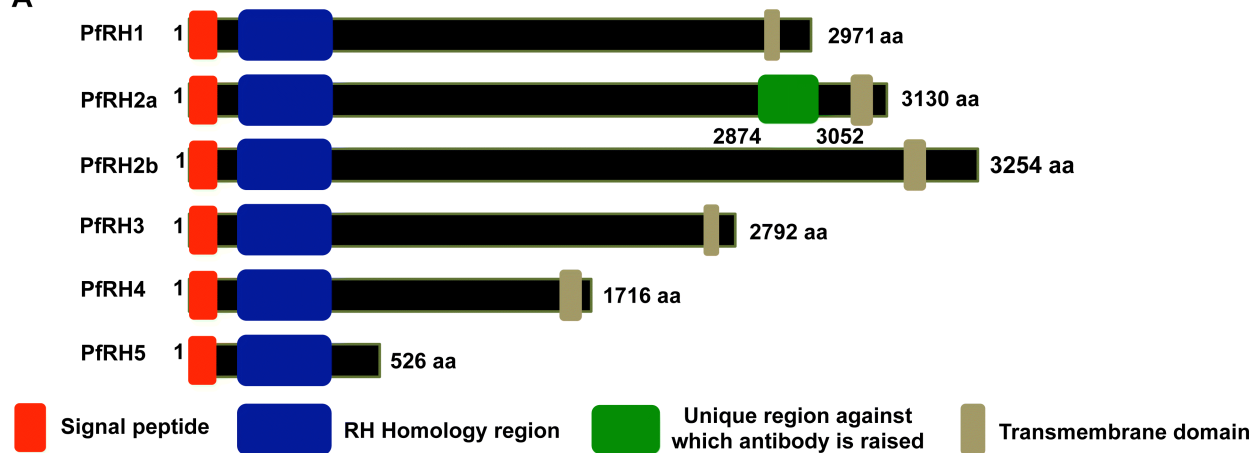754 **Fig. S2. Coimmunofluroscence for RH2a with rhoptry and micronemal markers**

755 Immunofluroscence assay of a late schizont stage parasite stained for RH2a using

756 anti-RH2a mcAb C22 (**A**), C19 (**B**), C15 (**C**) and either co-stained with rhoptry

757 marker RH1 and R2A9 and micronemal markers AMA1 or EBA175 in 3D7. The

758 parasite 3D7 RH2a KO was tested in parallel as a negative control.

759 **Fig S3. Erythrocyte binding assay in RH2a Knock out parasite.** Erythrocyte

760 binding assay of PfRH2a using 3D7 RH2a KO supernatant in untreated erythrocytes

761 (Un), and Neuraminidase (Nm - 25 and 50mU/ml), Chymotrypsin (Chymo - 1 and

762 2mg/ml) & Trypsin (Tryp - 0.1 and 1 mg/ml) treated erythrocytes. Bound proteins

763 eluted from the erythrocytes were separated by gel electrophoresis, transferred and

764 then probed using mcAb C22. For control, the same blot was re-probed with anti-
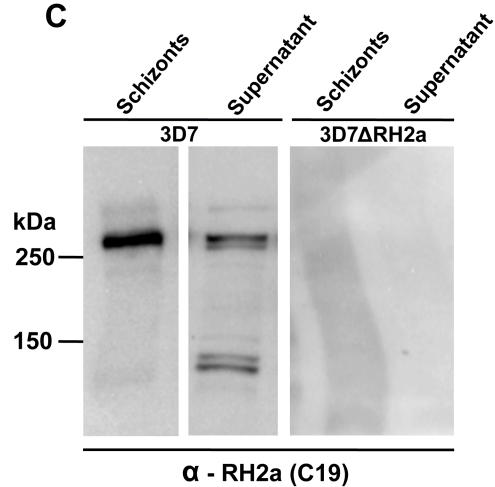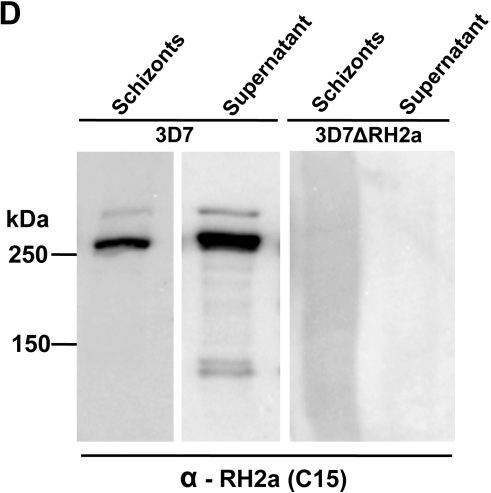
765 EBA175.

766

767

768
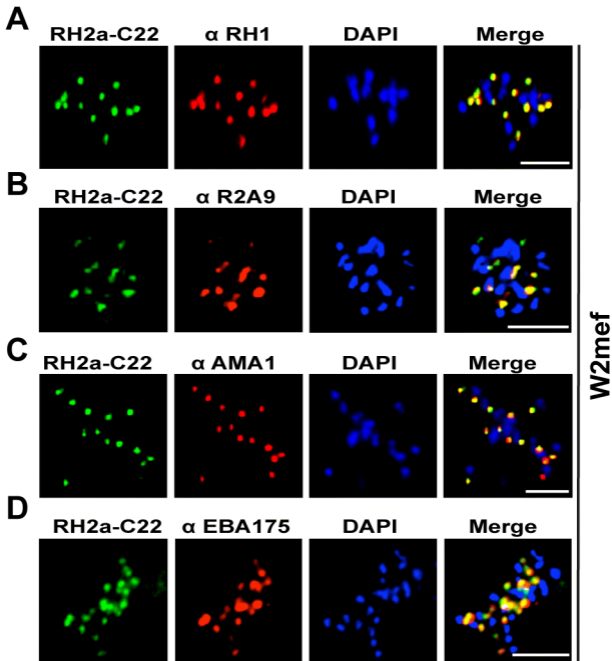
769

**Figure 1**



A

PfRH1  1 ▮▮ ——————————————▮—— 2971 aa

PfRH2a  1 ▮▮ ——————————▮▮—— 3130 aa
2874  3052

PfRH2b  1 ▮▮ ————————————————▮—— 3254 aa

PfRH3  1 ▮▮ ——————————▮—— 2792 aa

PfRH4  1 ▮▮ ————————▮—— 1716 aa

PfRH5  1 ▮▮ —— 526 aa

▮ Signal peptide   ▮ RH Homology region   ▮ Unique region against which antibody is raised   ▮ Transmembrane domain

B

α - RH2a (C22)

C

α - RH2a (C19)

D

α - RH2a (C15)

# Figure 2



| A | RH2a-C22 | α RH1 | DAPI | Merge |
| B | RH2a-C22 | α R2A9 | DAPI | Merge |
| C | RH2a-C22 | α AMA1 | DAPI | Merge |
| D | RH2a-C22 | α EBA175 | DAPI | Merge |

W2mef

# Figure 3

# Figure 4



**W2mef**

| RH2a-C22 | α AMA1 | DAPI | Bright Field | Merge | |
|----------|--------|------|--------------|-------|---|

Free Merozoites

Attachment

Reorientation

Moving Junction

# Figure 5

## A



α- RH2a

α- EBA175

## B