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Differences in Erythrocyte Receptor Specificity of Different Parts of the *Plasmodium falciparum* Reticulocyte Binding Protein Homologue 2a▽†

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The *Plasmodium falciparum* reticulocyte-binding-like protein homologue (RH) and erythrocyte binding-like (EBL) protein families play important roles during invasion, though their exact roles are not clear. Both EBL and RH proteins 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 *P. falciparum* RH1 (PfRH1) and PfRH4 have been shown to act directly in two alternative invasion pathways used by merozoites, the functions of PfRH2a and PfRH2b during invasion are less defined. Here, using monoclonal antibodies raised against a unique region of PfRH2a, we show that PfRH2a moves from the rhoptry neck to the moving junction during merozoite invasion. The movement of PfRH2a to the junction is independent of the invasion pathway used by the merozoite, suggesting an additional function of the protein that is independent of receptor binding. We further show that PfRH2a is processed both in the schizont and 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.

Malaria continues to be a serious public health problem, with nearly half of the world's population living in areas where malaria is endemic. The disease is caused by the cyclic infection and subsequent destruction of the host's erythrocytes by obligately intracellular protozoan parasites belonging to the genus *Plasmodium. 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 merozoite, 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 specialized apical organelles such as 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, 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 and erythrocyte proteins that are essential for the successful entry of the merozoites into erythrocytes (12, 43).

Treatment with enzymes such as neuraminidase (Nm), trypsin (Tryp), or chymotrypsin (Chymo) is known to remove different receptors from the surfaces of erythrocytes, and different strains of *P. falciparum* have been shown to differ in their abilities to invade these treated erythrocytes (10, 14, 45, 50). These findings led to the suggestion that the abilities of parasite strains to differentially invade enzyme-treated erythrocytes define distinct invasion pathways (18, 40, 45, 50). Two parasite-encoded protein families, termed erythrocyte binding-like (EBL) and reticulocyte-binding-like homologue (RH) proteins, have been shown to be involved in the differential recognition of erythrocyte receptors and thereby to 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 the Duffy binding-like (DBL) domain that directly mediates binding to erythrocyte receptors (7). In *Plasmodium falciparum*, six EBL members have been identified: erythrocyte binding antigen 175 (EBA175), EBA140, EBA181, EBA165, EBL1, and MAEBL (1, 6, 25, 31, 33, 35, 53). The DBL domain of EBA175 recognizes the sialic acid component in glycophorin A (6, 13); that of EBA140 binds to glycophorin C (30, 31, 33); and the DBL of EBA181 binds to an uncharacterized sialoglycoprotein (20, 34). EBL1 has recently been shown to bind to glycophorin 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 that are similar to AMA1 (5).

The RH proteins were first discovered in the rodent malaria

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parasite *Plasmodium yoelii*, where they were termed Py235 (15, 22). In subsequent work, though, RHs were found in all the *Plasmodium* species analyzed so far (3, 15, 16, 21, 22, 24, 37–39, 52). In *P. yoelii*, Py235 is encoded by a multigene family (27) and was thought to be involved in parasite virulence (15) and to mediate a novel form of clonal phenotypic variation (37). In *Plasmodium vivax*, homologues of Py235, *P. vivax* reticulocyte binding protein 1 (PvRBP1) and PvRBP2, were shown to bind to reticulocytes, leading to the suggestion that members of this protein family play an important role in host cell recognition (34). In *Plasmodium falciparum*, six RH members have been identified: *P. falciparum* RH1 (PfRH1) (39), PfRH2a (38, 52), PfRH2b (38, 52), PfRH3 (46), PfRH4 (24, 45), and PfRH5 (3, 42).

PfRH1 is the orthologue 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 inhibit merozoite invasion (17). Triglia et al. have recently shown that RH1 protein undergoes a series of proteolytic cleavage events before and during entry into the erythrocyte; they 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 PvRBP2 (38), and PfRH2a has been shown by gene knockout studies to be involved in a sialic acid-independent invasion pathway (10). Although antibodies against PfRH2a are able to inhibit merozoite invasion (52), there is no evidence to date on the erythrocyte binding ability of PfRH2a. PfRH2b gene knockout studies have shown that this protein interacts with the chymotrypsin-sensitive erythrocyte receptor Z (4, 14). Recent studies provide evidence suggesting that PfRH2 is naturally immunogenic and that its antibodies are associated with protection from malaria (41). Taylor et al. described another member of the PfRH family, PfRH3, as a pseudogene (46). PfRH4 was identified as a 220-kDa protein (24), and its differential expression was subsequently shown to be a major factor in the switching of the parasite from a sialic acid-dependent to a sialic acid-independent invasion pathway (18, 45). Recently, PfRH4 was shown to bind to the erythrocytic receptor CR1 (complementary receptor 1) (47). PfRH5, which lacks a transmembrane domain and has a molecular size of 63 kDa, is the smallest member of the RH protein family and has been found 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 CR1 been identified as the erythrocyte receptor (47).

In this study, we have focused on PfRH2a, and we provide evidence of its significant role in invasion. Using monoclonal antibodies (MAb) against PfRH2a, we show that PfRH2a colocalizes with PfRH1 in the rhoptry neck of the merozoite. We further demonstrate that both in parasites using a sialic acid-dependent invasion pathway and in those using a sialic acid-independent 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 in the invasion process.

MATERIALS AND METHODS

***Plasmodium falciparum* parasites and culture.** *Plasmodium falciparum* parasite strains 3D7, 3D7ΔRH2a, W2mef, and W2mef/Nm were cultured in fresh erythrocytes from healthy human donors and RPMI 1640 supplemented with 5% Albumax as described previously (49). For culture of W2mef parasites in neuraminidase-treated erythrocytes, the erythrocytes were treated with 10 mU/ml of neuraminidase (Roche) for 2 h at 37°C with constant rotation. The erythrocytes were then washed with incomplete RPMI 1640 (without Albumax) and then centrifuged for 5 min; these steps were repeated three times. W2mef parasites were allowed to grow in neuraminidase-treated erythrocytes. After 3 to 4 cycles of culturing, the W2mef parasite adapts and remerges in neuraminidase-treated erythrocytes (45). This switched parasite is named the W2mef/Nm parasite.

To maintain the developmental stages, synchronization of parasites was performed at the ring stage with 5% sorbitol (2, 29). Late-schizont-stage parasites were purified using 70% Percoll centrifugation as described previously (28). For further synchronization, the purified schizont-stage parasites were allowed to reinvade fresh erythrocytes. After 5 to 6 h of growth, the parasites were treated with 5% sorbitol in order to remove the remaining mature-stage parasites.

Cloning, expression, and purification of rPfRH2a in *Escherichia coli*. The recombinant PfRH2a protein (rPfRH2a) contains amino acids 2874 to 3052. PCR amplification was carried out using primers 5′-GACCATATGataaaaagtaa actagaatct-3′ and 5′-AACCTCGAGggtattatcatcagtagtact-3′ (lowercase letters indicate the PfRH2a gene sequence) from *P. falciparum* 3D7 genomic DNA (gDNA). The PCR products were digested with NdeI and XhoI and were cloned into expression vector pET24a(+) (Novagen) to generate a C-terminal His tag. BL21(DE3) (Stratagene) was used to express rPfRH2a. Isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was added to cultures at an A_{600} of 0.6 to 0.8. Induced cultures were allowed to grow for 3 h at 37°C, resuspended in chilled lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 5 mM dithiothreitol [DTT]) with an EDTA-free protease inhibitor cocktail (Roche), and lysed by sonication. The recombinant protein was purified under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen), followed by ion-exchange chromatography using a Mono Q 5/50 GL column (Amersham) and gel filtration chromatography using a Superdex 75 column (Amersham) in phosphate-buffered saline (PBS), pH 7.4, with 180 mM NaCl. The recombinant PfRH1 protein has been described previously (17).

MAb against PfRH2a. Purified recombinant RH2a protein was used to raise the monoclonal antibodies (MAb) against PfRH2a protein. The monoclonal antibodies were generated by BioGenes (Germany). Eight different MAb-producing clones generated for RH2a were shown to be positive by enzyme-linked immunosorbent assays (ELISA) (carried out by BioGenes). These 8 clones were further validated by Western blotting and immunofluorescence (data not shown), and C22, C19, and C15 were found to be highly sensitive and specific anti-RH2a MAb.

Preparation of the parasite schizont extract and parasite culture supernatant, protease inhibitor treatment, and Western blotting. Purified late-schizont-stage parasites were washed with incomplete RPMI by centrifugation. The parasite pellet was dissolved in 1× PBS and was 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 to 16 h at 37°C with constant agitation. After 16 h, the culture supernatant was obtained by centrifugation and was stored at −80°C. For protease inhibitor treatment, the culture supernatant was incubated with or without the recommended concentration of protease inhibitor cocktail (Roche). The samples were obtained at 1, 2, 4, and 16 h postincubation and were analyzed by Western blotting. For Western blotting, sodium dodecyl sulfate (SDS) loading buffer was added to the extract or culture supernatants, and the mixture was heated at 95°C for 5 min. The samples were separated by running in 6% (for RH2a and EBA175) or 12% (for actin) SDS-polyacrylamide gel electrophoresis (PAGE) gels and were transferred to a nitrocellulose membrane (Bio-Rad) for 2 h at 100 V. After the transfer, the membrane was blocked with 5% skim milk–PBST (1× PBS–0.5% Tween 20) for 1 h and was then incubated with the primary antibodies RH2a-C22 (dilution, 1:500), C19 (1:500), C15 (1:500), anti-rabbit (α-rabbit) PfEBA175 (1:3,000) (MR4), and α-rabbit actin (1:1,000) (Sigma) overnight at 4°C. This was followed by three washes, for 5 min each time, with PBST and 1 h of incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature (RT). After the membrane was washed 3 times with PBST, it was developed using the ECL Plus Western blotting detection system (Amersham) according to the manufacturer's instructions.

Tight-junction assay. For junction-arrested merozoites, bursting-schizont-stage parasites were added to complete medium containing fresh erythrocytes and 0.1 μM cytochalasin D (Sigma). The parasites were incubated for 3 to 4 h, and thin blood smears 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 min. Thin blood smears were made on glass slides 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 kept at RT for 5 to 10 min, blocked with 3% bovine serum albumin (BSA) in 1× PBS for 30 min, and then incubated for 1 h at 37°C with primary antibody RH2a-C22 (diluted 1:200), C19 (1:200), 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 3 times, for 5 min each time, with 1× PBS and were then incubated with secondary antibodies (Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-rabbit or goat anti-mouse antibodies [Molecular Probes]) for 1 h at 37°C, followed by 3 washes, for 5 min each time, with 1× PBS. The slides were air dried. A mounting medium (Vectashield) containing 4′,6-diamidino-2-phenylindole (DAPI) was applied to the slide. Fluorescent images were captured using LSM 710 and LSM 510 confocal microscopy (Carl Zeiss) and Olympus fluorescent microscopy.

Erythrocyte binding assays (EBA). Erythrocytes were treated with different enzymes, such as neuraminidase (Roche), trypsin (Sigma), and chymotrypsin (Sigma). The concentrations of the enzymes used 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. After the addition of enzymes to the erythrocytes, they were kept at 37°C for 2 h with constant rotation. Trypsin- and chymotrypsin-treated erythrocytes were washed with incomplete RPMI; the reaction was stopped by addition of 1 mg/ml trypsin/chymotrypsin inhibitor (Sigma); and erythrocytes were kept at RT for 15 min with constant rotation. Finally, the treated erythrocytes were washed 3 times with incomplete RPMI.

Culture supernatants from *P. falciparum* strains 3D7 and 3D7ΔPfRH2a were used. To 250 μl of the parasite culture supernatant, 100 μl of packed erythrocytes or enzyme-treated erythrocytes and 50 μl of fetal bovine serum (FBS) were added; the solution was made up to 600 μl with incomplete RPMI and was incubated at 37°C for 2 h with constant rotation. After incubation, the erythrocytes were spun through dibutyl phthalate (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 0.5 M NaCl by incubation at RT for 10 min, followed by centrifugation at 13,000 rpm for 2 min. The eluted proteins were analyzed by Western blotting.

RESULTS

Two forms of PfRH2a are detected in schizont extracts. Since 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 MAb C22, we have confirmed previous results showing peak expression of the PfRH2a proteins of two parasite clones, 3D7 and W2mef, at the schizont stage, (see Fig. S1A in the supplemental material). Two forms of PfRH2a, approximately 360- and 270-kDa proteins, were detected at the schizont stage of the parasite; the 270-kDa form was more abundant (Fig. 1B; see also Fig. S1C in the supplemental material). Western blot analysis of schizont extracts from 3D7 and 3D7ΔPfRH2a, a parasite line in which PfRH2a has been disrupted (14), confirmed that both the 360- and 270-kDa bands were indeed due to PfRH2a expression and not due to cross-reactivity of the antibody with PfRH2b (Fig. 1B; see also Fig. S1C). Since the molecular mass of PfRH2a is predicted to be approximately 360 kDa, it is likely that the ~360-kDa protein detected by Western blotting corresponds to the full-length protein and that the 270-kDa band represents a processed form of the protein. Proteolytic cleavage of PfRH1 has been reported previously to be an important step during invasion, resulting in a number of specific products (51). Hence, to determine whether PfRH2a undergoes similar cleavage events, Western blotting using MAb C22 to probe 3D7, 3D7ΔPfRH2a, and W2mef schizont extracts and culture supernatants was performed. As expected, the full-length 360-kDa and processed 270-kDa proteins were detected in schizont extracts from both 3D7 and W2mef, while in the parasite supernatants, an additional 140-kDa protein band was consistently detected, suggesting secondary processing of PfRH2a during invasion (Fig. 1B). Taken together, these data are consistent with the notion that the full-length 360-kDa PfRH2a protein is proteolytically cleaved at its N-terminal end, resulting in an approximately 270 kDa C-terminal fragment and possibly an N-terminal fragment of approximately 90 kDa (see 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 (Fig. 1B; see also Fig. 5B). It is also noteworthy 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 the protease inhibitor showed no significant change in the amount of the 140-kDa fragment, indicating that this secondary cleavage event is not due to nonspecific degradation of the 270-kDa or 360-kDa protein in the parasite supernatant (see Fig. S1B in the supplemental material). Similar processing of RH2a was consistently observed using the other anti-RH2a MAb C19 and C15 in the 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 family members are expressed either in the rhoptry neck or at the apical prominence in different *P. falciparum* strains. To further delineate the exact location of PfRH2a, we performed an immunofluorescence assay (IFA) using fixed schizont smears. Using MAb C22, PfRH2a showed a punctate pattern at the apical tip of the merozoite in schizonts and appeared to colocalize with PfRH1 in W2mef and 3D7 parasites, strongly suggesting that RH2a is located at the rhoptry neck (Fig. 2A; see also Fig. S2 in the supplemental material). The localization of RH2a with MAb C22 is identical to that observed with the polyclonal antibody R2A9, raised against the amino acid stretch common to PfRH2a and PfRH2b (Fig. 2B; see also Fig. S2). As expected, there is a partial overlap with micronemal markers such as AMA1 and EBA175 (Fig. 2C and D; see also Fig. S2). This is consistent with the results of electron microscopy studies using polyclonal antibodies that react with both PfRH2a and -2b, showing that they are located in the rhoptry necks of merozoites (14). Control experiments using the 3D7ΔPfRH2a parasite line showed no staining for PfRH2a, confirming the specificity of MAb C22 in the IFA (see Fig. S2 in the supplemental material). These results confirm that the monoclonal antibodies used in this study are highly specific for PfRH2a and allow the specific localization of the protein in the parasite.

PfRH2a is localized at the tight junction during invasion. It is clear from the data discussed above that PfRH2a is ex-

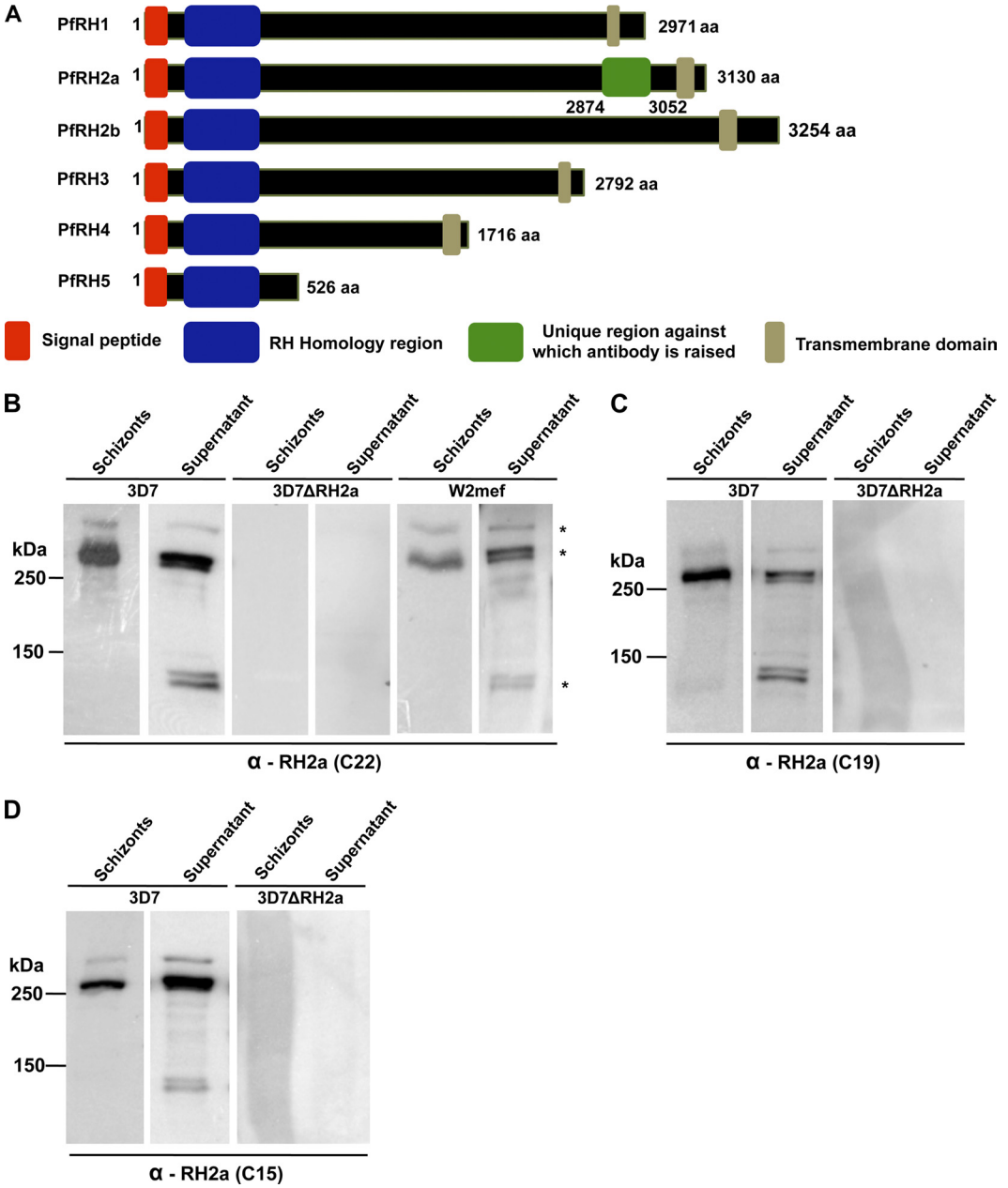

FIG. 1. Two forms of PfRH2a are expressed in the schizont extracts of different parasite strains. (A) Schematic representation of *P. falciparum* RH family members, indicating the signal peptides (red), the RH homology region (blue), the unique region against which antibodies were raised (green), and the C-terminal transmembrane domain (brown). The lengths of the proteins are given in amino acids (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. (C and D) Western blots of 3D7 and 3D7ΔRH2a schizont extracts and supernatant proteins were probed with MAb C19 (C) and C15 (D). RH2a was detected at ~360 and 270 kDa in schizonts, and an additional 140-kDa band was observed in culture supernatants.

pressed at the schizont stage in various *P. falciparum* strains. However, there are limited data supporting a direct role for 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 for 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 0.1 μM cytochalasin D. Cytochalasin is a cell-permeant fungal toxin that prevents cell ruffling and cell motility (8). It has been found to be very effective in causing actin depolymerization, even at low concentrations (54). The micronemal markers AMA1 and EBA175 have been shown previously to be localized in the tight junction (3, 51); hence, we employed these proteins as markers for the tight junction. Immunofluorescent

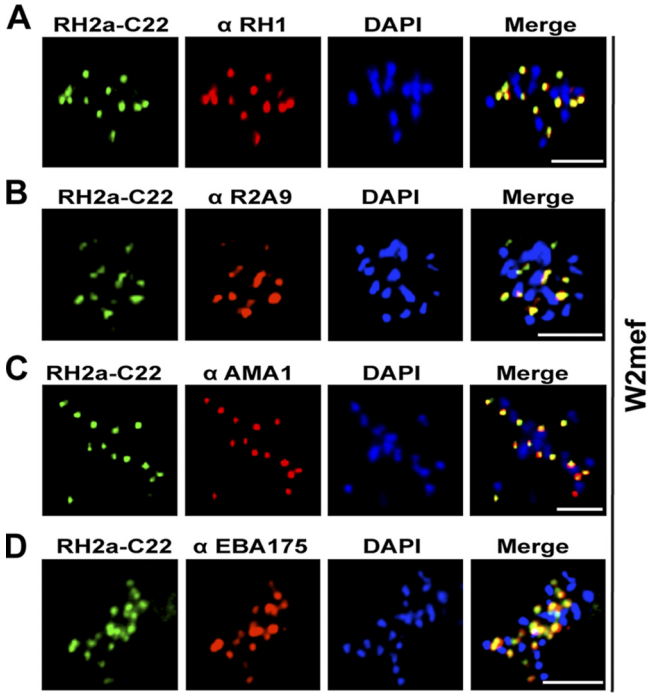

FIG. 2. Coimmunofluorescence for RH2a with rhoptry and micronemal markers. An immunofluorescence assay was performed on late-schizont-stage W2mef parasites stained for RH2a using the anti-RH2a MAb C22 and costained either with an antibody against the rhoptry neck marker RH1 (A), rabbit polyclonal antibody R2A9, recognizing both RH2a and RH2b (B), or an antibody against the micronemal marker AMA1 (C) or EBA175 (D). Bars, 5 μM.

assays using the junction-arrested merozoites showed that PfRH2a was located at the tight junction with EBA175 and AMA1 in the W2mef parasite clone (Fig. 3A). *P. falciparum* W2mef normally utilizes a sialic acid-dependent invasion pathway but upon selection on neuraminidase (Nm)-treated erythrocytes is able to switch (W2mef/Nm) to using a sialic acid-independent pathway (11). This switch in the invasion pathway has been linked to the activation of PfRH4 expression (18, 45). Since PfRH2a has also been suggested to 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 was 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 was located in the tight junction of the 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 the W2mef clone. PfRH2a was observed in the initial junction formed just after the invasion process had started, as well as at all later stages of the junction observed as the merozoite penetrated into the erythrocyte (Fig. 4). Taken together, these observations indicate that RH2a localizes at the junction and suggest that RH2a could play an important role in helping the merozoite to enter erythrocytes.

PfRH2a processing changes erythrocyte receptor specificity. The erythrocyte binding abilities of PfRH1, PfRH4, and PfRH5 have been elucidated previously (3, 17, 19, 39, 42, 48). To date, direct binding of PfRH2a to erythrocytes has not been demonstrated (52). Here, using a PfRH2a-specific monoclonal antibody, we show that PfRH2a binds to the erythrocyte. Erythrocyte binding assays (EBA) performed using parasite supernatants obtained from 3D7 showed clearly that the 360-, 270-, and 140-kDa proteins bind erythrocytes (Fig. 5A). Comparison of the relative intensity of the 360-kDa band with those of the 270- and 140-kDa bands in the 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 were carried out with culture supernatants from *P. falciparum* strains 3D7 and 3D7ΔPfRH2a. Erythrocytes were treated separately with varying concentrations of neuraminidase (Nm), chymotrypsin (Chymo), or 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, the binding of both the 360- and 270-kDa proteins was highly sensitive to chymotrypsin treatment—binding was 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 appeared to be moderately sensitive to Chymo treatment (Fig. 5A). Tryp treatment of erythrocytes completely abolished the binding of the 370-kDa protein, while the binding of the 270- and 140-kDa bands was not affected by increasing concentrations of Tryp. As a control, the same Western blot membranes were reprobed for EBA175, which has been shown previously to bind to erythrocytes in an Nm-sensitive, Chymo-resistant, and Tryp-resistant fashion (3, 48). This reprobing clearly showed that the enzyme treatments had the expected effects: binding by EBA175 was reduced in Nm-treated erythrocytes but was refractory to treatment with Chymo and showed dose-dependent sensitivity to Tryp (Fig. 5A). As expected, no binding of PfRH2a was detected with MAb C22 when the 3D7ΔRH2a culture supernatant was used for EBA, and when the same membranes were reprobed for EBA175 as a control, the binding was identical to that seen in the 3D7 supernatant (see Fig. S3 in the supplemental material). These results, taken together, show that the 360-kDa protein is sensitive to Nm, Chymo, and Tryp, while the 270-kDa protein is sensitive to Chymo and resistant to Nm and Tryp, and the 140-kDa protein is resistant to Nm and Tryp but reasonably sensitive to Chymo.

DISCUSSION

Reticulocyte-binding-like protein homologue (RH) proteins play an important role in the invasion of erythrocytes by several *Plasmodium* species. However, the precise role of the RH proteins in invasion is not yet clearly understood. In this study, using the specific and highly sensitive monoclonal antibodies that we generated, we have focused on the importance of the

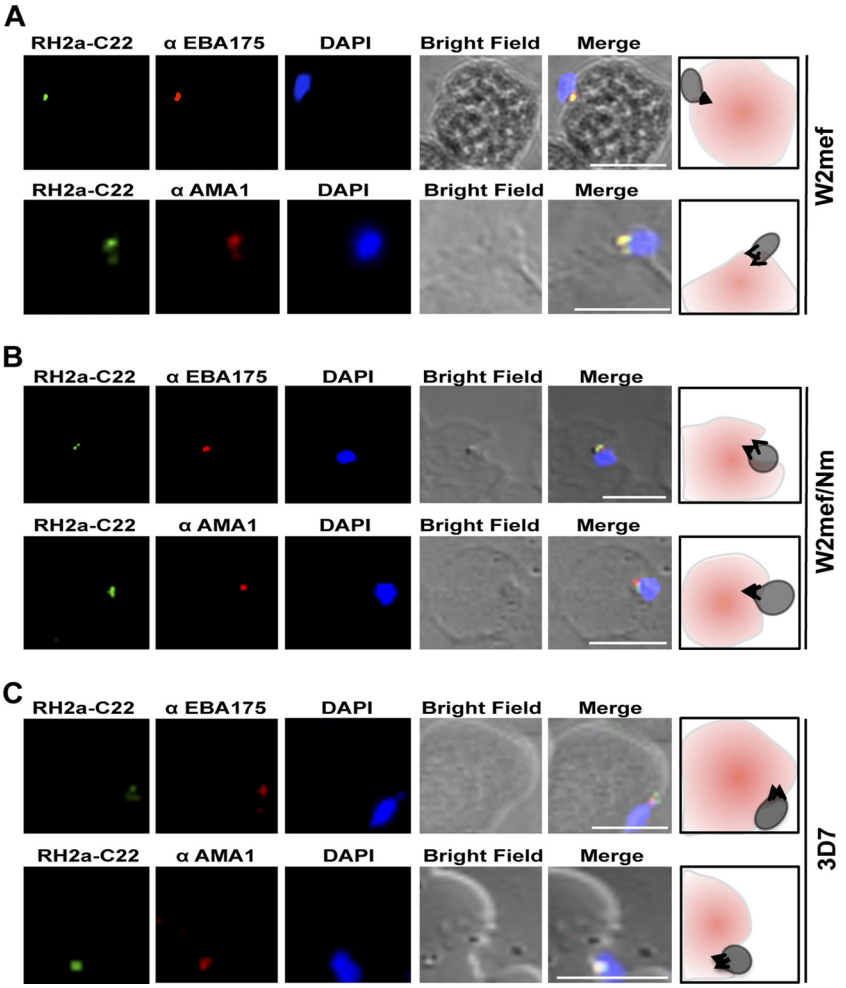

FIG. 3. RH2a is localized at the tight junction during invasion. Shown is the colocalization of RH2a proteins with EBA175 and AMA1 in junction-arrested merozoites, obtained by cytochalasin D treatment, from W2mef (A), W2mef/Nm (B), or 3D7 (C) parasites. Arrowheads in the cartoons represent the proteins at the tight junction. Approximately 80 junction-arrested merozoites in random microscopic fields were observed in order to confirm the localization of RH2a at the tight junction. Bars, 5 μM.

Plasmodium falciparum RH2a protein. Previous studies focusing on the genetic disruption of PfRH2a in both 3D7 and W2mef parasites have shown that the roles of this protein in the two parasite strains are different (10, 14). While no direct effect on the invasion capacity of the parasite was observed in the PfRH2a 3D7 knockout line (14), in the W2mef background PfRH2a appears to play a role in sialic acid-independent invasion (10). Using an IFA, we show that PfRH2a is located in the rhoptry neck in both W2mef and 3D7, suggesting that the apparently 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 showing very clear colocalization of PfRH1 and PfRH2a at the rhoptry neck of the merozoite and supporting the previous assumption that both proteins are equally able to 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 the notion that these proteins not only are important in initial host cell sensing but furthermore play an active role in the moving junction, thereby mediating the movement of the merozoite into the host erythrocyte. Our finding that PfRH2a does locate in the junction in W2mef, W2mef/Nm, and 3D7 parasites is consistent with a role during the invasion process, despite the fact that genetic disruption and the use of enzyme-treated erythrocytes have not been able to establish this previously (14, 52). Previously it has been shown for PfRH1 that not all the protein is translocated to the junction; some protein remains at the apical tip (51), In contrast, while we detect PfRH2a at the apical location in free merozoites as well as in those engaged in initial attachment and reorientation, all of the PfRH2a protein appears to translocate into the junction; no protein is left behind at the apical tip. It will be interesting to establish whether this result indeed reflects a difference between PfRH1 and PfRH2a or rather 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 cleavage of the full-length 360-kDa protein to a 270-kDa protein. This processing is followed by a second

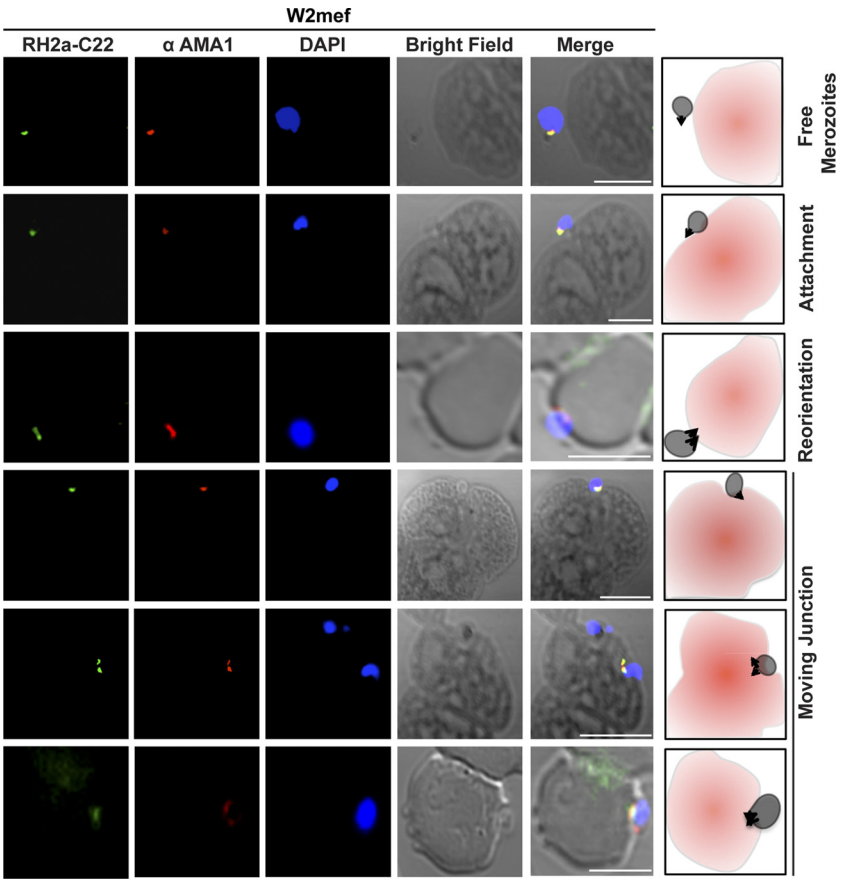

FIG. 4. RH2a follows the moving junction during invasion. RH2a was costained with AMA1. From top to bottom, immunofluorescence images show merozoites progressing through different stages of invasion: free merozoites, an attached merozoite, a reoriented merozoite whose apical end is facing the red blood cells, and the progression of the moving junction. Bars, 5 μM.

cleavage of the 270-kDa protein to a 140-kDa fragment, which occurs after schizont release, possibly during invasion. It is intriguing to speculate that the processed doublet bands observed around 270 and 140 kDa in cell culture supernatants but not in schizont extracts could perhaps play an important role in the process of invasion. Furthermore, treatment of the culture supernatant with a protease inhibitor indicates that the 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 the junction. Sequential proteolytic cleavage of malaria parasite invasion proteins is thought to constitute 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 noteworthy that the proposed sites of PfRH1 processing appear to be at locations similar to those observed for PfRH2a. This strongly suggests that the processing of all RH proteins follows a conserved pathway during merozoite maturation and invasion.

Enzyme treatment of erythrocytes has shown that different RH proteins 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 (Nm resistant and Tryp and Chymo sensitive) (47), and based on the impact of PfRH2a disruption on the 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 bind specifically to erythrocytes and that the full-length protein binds more strongly than the processed 270- and 140-kDa proteins. Importantly, we demonstrate that PfRH2a processing 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 a receptor that is resistant to Nm/Tryp but fairly sensitive to Chymo. This could suggest that PfRH2a contains two or more erythrocyte binding domains and that the domain found only in the full-length protein dominates over the domain in the 270- and 140-kDa fragments. Alternatively, the results obtained here could also be consistent with a hypothesis that the 140-kDa region contains the binding domain, whose specificity is modified by other regions of the protein. Previously it was suggested that the N-terminal conserved regions of RH2a mediate sialic acid-independent invasion (10).

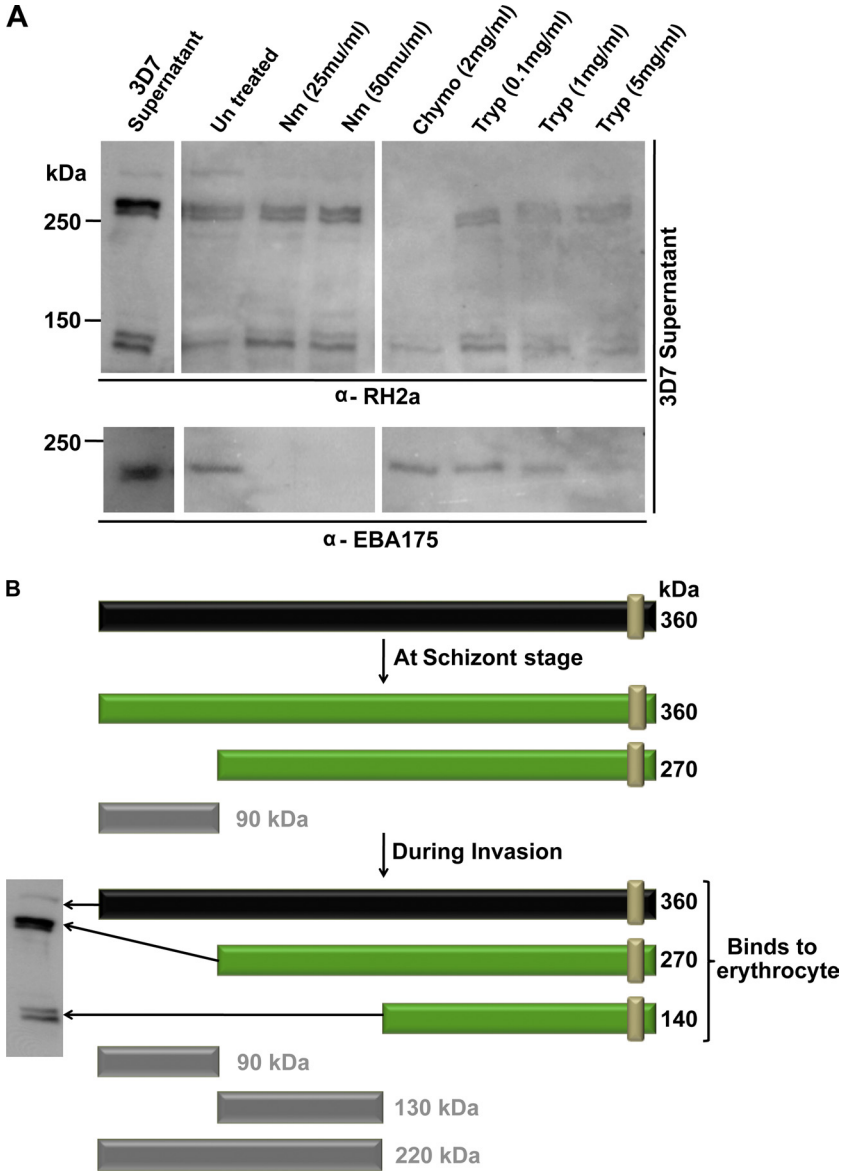

FIG. 5. Different processed forms of PfRH2a have different erythrocyte binding activities. (A) Erythrocyte binding assay of PfRH2a showing the difference in the binding of the 360-, 270-, and 140-kDa proteins using 3D7 supernatants with untreated erythrocytes and with erythrocytes treated with neuraminidase (Nm) (25 or 50 mU/ml), chymotrypsin (Chymo) (2 mg/ml), or trypsin (Tryp) (0.1, 1, or 5 mg/ml). Bound proteins eluted from the erythrocytes were separated by gel electrophoresis, transferred, and then probed using MAb C22. As a control, the same blot was reprobed with anti-EBA175. Similar results were obtained in two other independent experiments. (B) Model illustrating the processing of PfRH2a before and during the process of invasion. The full-length proteins are shown in black, and the processed products of PfRH2a detected by our MAb in schizonts and supernatants are shown in green. However, our MAb against the C-terminal region was not able to detect the three possible processing products (90, 130, and 220 kDa) shown in gray. We propose that the processing of PfRH2a changes the availability of binding sites at different times in the invasion process, allowing multiple distinct contacts with the erythrocyte to enable successful invasion.

However, our result shows that full-length RH2a protein binds in a sialic acid-dependent manner, while the 270- and 140-kDa processed forms of the protein bind in a sialic acid-independent manner. This suggests that the approximately 90 kDa N-terminal region removed in the 270-kDa protein includes a sialic acid-containing receptor binding domain. The observation that the 270-kDa processed form of PfRH2a is the most abundant form of the protein could explain why the genetic approach (10) was able to identify only the sialic acid-independent binding component of the conserved region of PfRH2a/PfRH2b. The possibility that RH proteins contain more than one binding domain has been proposed for PfRH2b by knockout studies showing an N-terminal domain mediating sialic acid-independent binding and a C-terminal region mediating the interaction with a Chymo- and Try- sensitive receptor (10). In PfRH2a, the removal of the N-terminal 90-kDa region from the full-length protein abolishes binding to the Nm- and Tryp-sensitive receptor, and the further removal of the 130-kDa region from the 270-kDa fragment abolishes binding to the Chymo-sensitive receptor. Our work now provides a biological

rationale for the multiple processing steps observed in the RH proteins, 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 independently of the invasion pathway used is intriguing. This could be due solely to the association of unbound PfRH2a with another parasite ligand, such as the interaction between the *P. vivax* reticulocyte binding proteins PvRBP1 and PvRBP2 (16), which leads to the translocation of the protein to the junction. Other alternatives, such as an additional role in the formation of a functional invasion complex or in the stabilization of the interaction with the invasion motor, could also be envisaged for this large protein. Clearly, additional data are required to determine all the functions of PfRH2a.

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 independently of the invasion pathway utilized by the parasite, suggesting that PfRH2a has a role in merozoite invasion that is independent of receptor binding. Furthermore, we demonstrate, for the first time, direct binding of PfRH2a to erythrocytes, and we show that the receptor specificity of the protein can be changed by 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 they highlight the complexity of the role of the RH protein family during merozoite invasion. It will now be important to dissect how these processing steps change the function of the RH proteins during invasion.

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

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