A *Plasmodium falciparum* Homologue of *Plasmodium vivax* Reticulocyte Binding Protein (PvRBP1) Defines a Trypsin-resistant Erythrocyte Invasion Pathway

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Abstract

Invasion of erythrocytes by *Plasmodium* merozoites is an intricate process involving multiple receptor-ligand interactions. The glycophorins and an unknown trypsin sensitive factor are all erythrocyte receptors used during invasion by the major human pathogen *Plasmodium falciparum*. However, only one erythrocyte receptor, Glycophorin A, has a well-established cognate parasite ligand, the merozoite protein erythrocyte binding antigen-175 (EBA-175). The involvement of several other parasite proteins during invasion have been proposed, but no direct evidence links them with a specific invasion pathway. Here we report the identification and characterization of *P. falciparum* normocyte binding protein 1 (PfNBP1), an ortholog of *Plasmodium vivax* reticulocyte binding protein-1. PfNBP1 binds to a sialic acid dependent trypsin-resistant receptor on the erythrocyte surface that appears to be distinct from known invasion receptors. Antibodies against PfNBP1 can inhibit invasion of trypsinized erythrocytes and two *P. falciparum* strains that express truncated PfNBP1 are unable to invade trypsinized erythrocytes. One of these strain, 7G8, also does not invade Glycophorin B–negative erythrocytes. PfNBP1 therefore defines a novel trypsin-resistant invasion pathway and adds a level of complexity to current models for *P. falciparum* erythrocyte invasion.

Key words: glycophorin • malaria • merozoite • erythrocyte • reticulocyte

Introduction

The clinical symptoms and pathology associated with malaria occur during the asexual erythrocytic phase of the *Plasmodium* life cycle. *Plasmodium* merozoites, ovoid cells with an apical prominence at one end, invade erythrocytes, wherein they multiply and eventually rupture their host cell, releasing newly formed merozoites to initiate a new round of invasion. Erythrocyte invasion is a complex and dynamic process, involving both a reversible attachment and reorientation phase and an irreversible commitment and invasion phase. Yet although erythrocyte invasion has been extensively described at the ultrastructural level, the molecular details and the individual receptor–ligand interactions involved at each step are only beginning to be understood.

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In the case of the major human malaria pathogen, *Plasmodium falciparum,* several erythrocyte invasion receptors have been identified. The relative importance of each receptor is clearly strain dependent. Whether cultured extensively in vitro or collected directly from patients in the field, isolates can differ markedly in which receptors they are able to use (1, 2). Certain strains are able to switch between different pathways in a genetically stable manner, suggesting that *P. falciparum* can functionally adapt depending upon the available host cell receptor(s) (3). Treatment of erythrocytes with neuraminidase reduces or eliminates invasion in many *P. falciparum* strains. The closely related Glycophorins A and B and the minor glycophorins, C and D, have all been proposed to contribute to sialic-acid dependent invasion (4–7). Some strains invade erythrocytes that lack glycophorins by using a sialic acid–independent, trypsin-sensitive receptor(s), but extensive searching using well characterized mutant RBCs has not revealed the identity of this receptor, which is often referred to as Receptor

X (8–10). Receptors other than the glycophorins and Receptor X may also be involved in invasion.

On the parasite side, many *P. falciparum* proteins have been proposed to be important for invasion, largely because of where and when they are expressed in the merozoite. However, Glycophorin A is the only known erythrocyte invasion receptor to have a clearly defined parasite ligand, erythrocyte binding antigen-175 (EBA-175),* a *P. falciparum* homologue of the human malaria *Plasmodium vivax* and simian malaria *Plasmodium knowlesi* Duffy binding proteins (11–13). A specific domain of EBA-175 has been shown to bind Glycophorin A in vitro, requiring both sialic acid and the peptide backbone for efficient adhesion (14). Despite a high degree of homology between Glycophorins A and B, EBA-175 does not mediate binding to Glycophorin B, nor is it the ligand for the sialic acid–independent Receptor X (1, 15, 16). More recently, a paralogue of EBA-175, termed BAEBL, has been proposed to bind to Glycophorins C or D (17). Many *P. falciparum* ligands clearly remain to be identified.

Merozoites of the other major human malaria, *P. vivax*, have an additional level of complexity in their invasion process, as unlike *P. falciparum*, they only invade reticulocytes, a developmentally immature minor subpopulation of the erythrocyte pool. The *P. vivax* reticulocyte binding proteins, PvRBP1 and PvRBP2, are expressed apically in merozoites as a complex, bind specifically to reticulocytes, and have been proposed to select reticulocytes for invasion (18–20). Two homologs of PvRBP2 have recently been identified in *P. falciparum*, and are believed to be functionally important for targeting all RBCs for invasion by this species (21, 22). Additionally, a family of 235 kD rhoptry proteins of the rodent malaria *Plasmodium yoelii*, are related to PvRBP2 (20, 23). These proteins adhere to mouse erythrocytes and monoclonal antibodies raised against them can also inhibit invasion (24, 25).

Here we present the identification and characterization of a *P. falciparum* ortholog of PvRBP1, normocyte binding protein-1 (PfNBP1). The PfNBP1 protein is expressed at the invasive apical end of merozoites and appears to form a complex with at least one of the *P. falciparum* PvRBP2 homologs. PfNBP1 binds to erythrocytes in a sialic acid–dependent but trypsin-resistant manner and does not appear to bind to either Glycophorin A, B, C, or D, or Receptor X. Specific antisera against PfNBP1 and the *P. falciparum* PvRBP2 homologs inhibit merozoite invasion into trypsinised erythrocytes. Furthermore, two *P. falciparum* strains that express truncated PfNBP1 are unable to invade trypsinised cells. We therefore propose that PfNBP1 and the *P. falciparum* homologs of PvRBP2 are involved in a trypsin-resistant invasion pathway. These findings expand our knowledge of *P. falciparum* invasion and introduce another potential target for receptor-mediated blockade and vaccine-induced interventions against this important human malaria parasite species.

Abbreviations used in this paper: DBL, Duffy binding like; EBA, erythrocyte binding assay; EBA-175, erythrocyte binding antigen-175; PfNBP, *Plasmodium falciparum* normocyte binding protein; PvRBP, *Plasmodium vivax* reticulocyte binding protein; TMD, transmembrane domain; ORF, open reading frame.

Materials and Methods

Parasites: Culture and Strains. The FVO strain of *P. falciparum* was obtained from a parasite line adapted to *Aotus* monkeys and established in in vitro culture in human O^+ erythrocytes (26, 27). *P. falciparum* strains 7G8, HB3, and Dd2 were obtained from the Malaria Research and Reference Reagent Resource Center, strain 3D7 from WRAIR, and were cultured in human O^+ erythrocytes.

Nucleic Acid and Sequence Analysis. Databank BLAST-based searches were conducted at the Sanger Center internet site at http://www.sanger.ac.uk and at PlasmoDB at http://plasmodb.org. Sequence data for *P. falciparum* chromosome 4 was obtained from http://www.sanger.ac.uk/Projects/P_falciparum/. Sequencing of *P. falciparum* chromosome 4 was accomplished as part of the Malaria Genome Project with support by The Wellcome Trust. *P. falciparum* genomic DNA (gDNA) was prepared as described (18). RNA was prepared from mature schizonts using ToTALLY RNA Isolation Kit (Ambion) and treated with RNAase-free DNAase (Ambion). cDNA was synthesized and purified using a 5′ RACE kit (GIBCO BRL) and the gene-specific primer 5′-GGATATCAATAAATTTGGCAG-3′. Nested PCR was performed using the provided Abridged Anchor primer as a forward primer and the gene specific primer in the primary reaction, then forward primer 5′-GCAAAGGTGGATTTTCTGC-3′ and reverse primer 5′-CGAATGGATTCTAATCATTTCCAG-3′ in the secondary reaction. PCR was performed using EXPAND High Fidelity Kit (Roche) or Pfu Turbo Polymerase (Stratagene) and products cloned using a TA cloning kit (Invitrogen) or a PCR-Script AMP cloning kit (Stratagene). DNA sequencing was performed using a BigDye Terminator Cycle Sequencing v2.0 Ready Reaction Kit (ABI Prism) on a 3100 Genetic Analyzer (ABI Prism). Gene and deduced protein sequences were analyzed and compared using the MacVector 6.5.3 DNA/Protein analysis module (Oxford Molecular). Amino acid alignments were performed using CLUSTAL X (28). Phylogenetic and molecular evolutionary analyses of PfNBP1 and other members of the PvRBP-Like (RBL) superfamily was accomplished using MEGA version 2.1 (29).

Antisera, Immunoblots, and Immunofluorescence. A rabbit antiserum was raised against a GST fusion protein using the pGEX system (Amersham Pharmacia Biotech). A GST fusion protein was made by amplifying *P. falciparum* FVO gDNA, with primers 5′-GACGATATATACCATGCTGAT-3′ and 5′-TTGTGATATATCTTGAAGG-3′. Fusion protein was purified from *Escherichia coli* using standard methodologies on glutathione-sepharose 4B (Amersham Pharmacia Biotech) and injected into New Zealand White rabbits using Freund's complete and incomplete adjuvants. EBA-175 antiserum was obtained from the Malaria Research and Reference Reagent Resource Center. For immunoblots, protein samples made by boiling partially purified late segmented schizonts in reducing SDS-PAGE sample buffer were run on 6% polyacrylamide gels (37.5:1 acrylamide:bis), transferred to nitrocellulose membranes (Schleicher & Schuell), probed with primary antisera, and developed with an enhanced chemoluminescence (ECL) kit (Amersham Pharmacia Biotech). The anti-Glycophorin A and B antiserum was obtained from Sigma-Aldrich. Immunofluorescence assays (IFAs) were performed on

air-dried thin smears of *P. falciparum* (FVO strain) infected erythrocytes, with secondary antibodies from Zymed laboratories (FITC anti–rabbit) or Jackson ImmunoResearch Laboratories (Cy3 anti–mouse). Both secondary antibodies had been cross-adsorbed against sera from other species to minimize cross-reactivity. Images were merged using Adobe Photoshop 5.0 software.

Biosynthetic Radiolabeling and Immunoprecipitation. Biosynthetic radiolabeling of intraerythrocytic parasites with [^{35}S]methionine/cysteine (ICN) was performed at 100 μCi/ml during in vitro culture. Labeled schizont-stage parasites were lysed on ice for 30 min in PBS with 1% NP-40 and standard protease inhibitors added, with occasional vortexing. Lysates were cleared by centrifugation. Immunoprecipitations were performed as described (18).

Erythrocyte Binding Assays. Schizont stage parasites were purified by centrifugation on 40%/60%/70%/80% Percoll/sorbitol gradients, as described. (30). To make culture supernatants, purified schizonts were placed back into in vitro culture at 2.5×10^7 parasites/ml and allowed to rupture overnight. Cells were harvested by centrifugation and supernatants stored at −80°C. To perform erythrocyte binding assays (EBAs), culture supernatant was rotated with the appropriate erythrocytes at room temperature for 2 h. Cells were separated from the supernatant by centrifugation through a Dow Corning 550 fluid cushion, and bound material was separated from cells by incubation in an equal volume of 5× RPMI at room temperature. Released material was harvested by centrifugation and analyzed by immunoblotting. Cryopreserved S-s-U- human erythrocytes and control cells were obtained from Dr. Marion E. Reid at the New York Blood Center, New York, NY.

Reticulocytes were enriched as previously described (31). For enzymatic treatment, erythrocytes were treated at 1×10^8 cells/ml in RPMI with 1 mg/ml trypsin (Calbiochem), 0.025U/ml neuraminidase (Vibrio cholerae; Roche), or 0.5 mg/ml Pronase (Calbiochem) at 37°C for 1 h. Cells treated with trypsin were then washed and treated with 0.5 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) for 15 min at room temperature.

Invasion Assays. Schizont stage parasites were purified as above, and placed into in vitro culture with the appropriate target erythrocytes. Ring stage parasites were counted following a 12–20 h incubation. All invasion assays were repeated between two and ten times, and at least 1,000 erythrocytes counted per assay. If invasion rates were low, as when enzyme treated cells were used, up to 4,000 erythrocytes were counted in each assay. IgG was purified using Affi-Gel Protein A and MAPS II binding reagents (Bio-Rad Laboratories). After elution from the Protein A column, IgG was dialyzed against RPMI and added to invasion assays at concentrations as described in the text.

Results

Identification of PfNBP1, a Homologue of PvRBP1. Members of the interspecies PvRBP family are high MW proteins that share a low level of amino acid homology and structural features, most notably a short exon 1 encoding a signal sequence followed by a large exon 2 encoding the bulk of the protein and a single predicted transmembrane domain (TMD) close to the COOH terminus (18, 20, 21, 32). Antibodies recognizing the NH_2-terminal region and a central domain of PvRBP1 produce immunofluorescence patterns at the merozoite apical pole in mature schizonts and free merozoites of *P. knowlesi* and *P. falciparum* similar to those seen in *P. vivax* (data not shown), suggesting the presence of related proteins in these two species. TBLASTN searching of the *P. falciparum* genome databases with selected fragments or the complete PvRBP1 protein sequence identified several open reading frames (ORFs). While most corresponded to previously known homologs of PvRBP2 or spurious hits, one ORF on chromosome 4 (current PlasmoDB ORF 003009) consistently scored higher (referred to henceforth as *Pfnbp1*, as discussed below). There is a short exon and a potential intron of 98 bp with consensus intron splice sites upstream of this large ORF. In keeping with members of the PvRBP family, the short exon 1 encodes a potential signal sequence. PCR on *P. falciparum* FVO strain reverse transcribed RNA with primers flanking the predicted intron amplified a smaller band than amplified from gDNA (Fig. 1 A). Cloning and sequencing of the PCR products confirmed the predicted intron boundaries (Fig. 1 B).

The exon 2 ORF identified in the *P. falciparum* (3D7 strain) database extends for 8.3 kb but does not contain a COOH-terminal TMD. However, a second ORF of 627

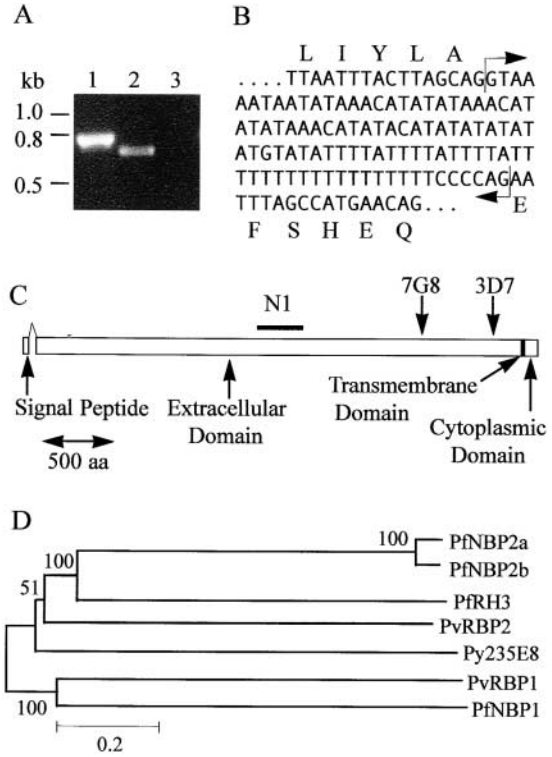

Figure 1. *Pfnbp1* shares features with members of the *Pvrbp* gene family. (A) PCR using primers flanking the putative *Pfnbp1* intron. Template was either *P. falciparum* FVO gDNA (lane 1), cDNA from *P. falciparum* FVO RNA (lane 2), or reverse transcription reaction performed in absence of reverse transcriptase (lane 3). (B) Sequence of the *Pfnbp1* intervening sequence and consensus splice sites (arrowed). (C) Diagram showing gene structure and encoded protein features of *Pfnbp1* shared by other PvRBP super family members. The position of the fragment used to raise antiserum (N1) is also noted. Arrows denote approximate positions of potential frameshift mutations in 3D7 and 7G8 strains. (D) Phylogenetic relationships of the known members of the RBL superfamily as determined by Neighbor-Joining following a Poisson model with pairwise deletion.

bp in another frame immediately downstream of the exon 2 sequence does contain a predicted TMD. Cloning and sequence analyses of a 2-kb fragment spanning this region from several *P. falciparum* strains (FVO, HB3, Dd2, and Malayan Camp K^-) indicate that in these strains, the two coding regions are contiguous as one ORF. Therefore, the gene is structurally consistent with other members of the PvRBP/Py235 family with an exon 2 of ~8.9 kb (Fig. 1 C). Thus, the 3D7 strain has a single nucleotide insertion in a polyadenine sequence that causes premature termination of the *Pfnbp1* transcript (termination site shown in Fig. 1 C). Other than this mutation, all strains had a very high level of sequence identity over the regions sequenced, with the exception of a series of repeated His-Asn residues near the predicted TMD, the number of which differs among the strains (GenBank/EMBL/DDBJ accession nos. AF411929–AF411933).

Importantly, the *Pfnbp1* locus in most strains shares a common gene and deduced protein structure with both *P. vivax rbps,* the *P. yoelii p235* genes, and the *P. falciparum rbp2* homologs (Fig. 1 C). Analysis of CLUSTAL W alignments of the predicted PfNBP1 amino acid sequence with the other members supports this familial relationship, with an overall level of similarity of 30–35% (identity plus conservative substitutions). In *P. vivax*, PvRBP1 and PvRBP2 are distantly related but do not share any regions of >22% identity (18, 20). The *P. yoelii* 235 kD and the previously identified *P. falciparum* PfRBP2-homologs consistently group in phylogenetic analyses with PvRBP2 rather than PvRBP1 (21, 22). In contrast, BLASTP searching with the new *P. falciparum* protein consistently identifies PvRBP1 as the top scoring entry. Construction of phylogenetic trees based on CLUSTAL X alignments and, most importantly, either Neighbor-Joining (Fig. 1 D) or Maximum Parsimony analyses supports such an orthologous relationship. PfNBP1 always groups with PvRBP1, whereas the PfRBP2-Hs, the pseudogene PfRH3 (33), and Py p235 always form a second grouping. For this reason, we have chosen to call this protein PfNBP1, for *P. f*alciparum Normocyte Binding Protein 1, as was originally proposed for orthologs of the PvRBPs (19), and to conform to the nomenclature for the PvRBPs, with 1 as a subscript to indicate the closer relationship with PvRBP1 than PvRBP2. To follow this nomenclature, we propose that the *P. falciparum* RBP2 homologs, which we initially called PfRB2-Ha and –Hb (21), should be renamed PfNBP2a and PfNBP2b and we will use this format for the rest of the paper.

PfNBP1 Is Located at the Invasive Apical End of Merozoites. A polyclonal PfNBP1 rabbit antiserum was raised against a polypeptide (N1) encoded by a fragment of the *Pfnbp1* exon 2 (Fig. 1 B). The N1 antiserum showed a staining pattern by immunofluorescence assay (IFA) on late schizonts that localized to the apical end of merozoites (Fig. 2 A). Although some discrete focal staining was visible, on the whole, staining was more widely distributed across the apex of the merozoite. In several instances, cap-like staining patterns over the conoid apical process of the merozoite were observed (arrowed, Fig. 2 A), similar to that described for PvRBP1 and PvRBP2 (18). By comparison, antisera against several regions of PfNBP2a and PfNBP2b, including the S3 antiserum against a portion of the shared region of these proteins, localize discretely to the apical end of merozoites with usually one, but occasionally two, foci in each merozoite (21; Fig. 2 A).

The PfNBP2a and PfNBP2b distribution has some hallmarks of localization to the paired rhoptry organelles (characterized by a consistent double dot pattern), but their patterns are most similar to that of the *P. falciparum* rhoptry protein Pf240, described as single foci occasionally resolving into double dots (34). Costaining of late schizonts with the S3 rabbit antiserum and a mAb recognizing the Pf240 protein showed very similar overlap of staining patterns, whereas the Pf240 protein staining only partially overlapped with a rabbit antiserum raised against EBA-175 (Fig. 2 B, and data not shown). The Pf240 specific mAb local-

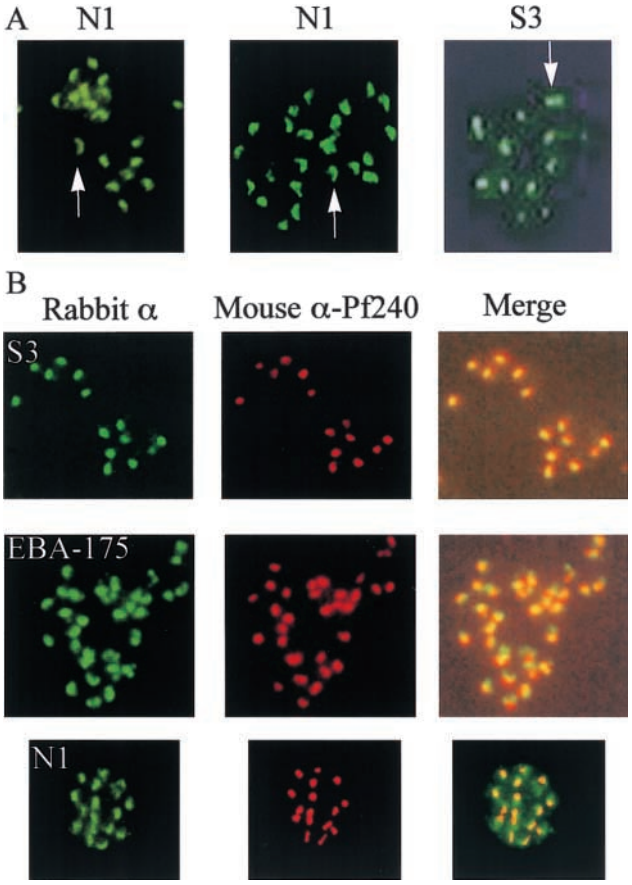

Figure 2. PfNBP1 is expressed at the apical end of merozoites. (A) Immunofluorescence assays performed on *P. falciparum* FVO infected erythrocytes, using anti-PfNBP1 (N1) or anti-PfNBP2a and b antisera (S3). S3 antiserum gives an occasional double dot in a single merozoite (arrowed). N1 staining is broader but defined, with the frequent appearance of apical cap staining (arrowed). (B) Costaining performed with mouse mAb (24C6 4F12), which recognizes Pf240, an antigen that localizes to the neck of the paired rhoptry organelles. Rabbit antisera against PfNBP2a and b (S3), the microneme protein EBA-175 (175), or PfNBP1 (N1) were coincubated with this mouse mAb, and FITC anti–rabbit IgG (green fields) and Cy3 anti–mouse IgG (red fields) secondary antibodies were used for detection.

ized the antigen by immuno-electron microscopy to the "neck" of the rhoptries where the peduncles join in a common duct immediately below the apical end of the merozoite (34). The precise colocalization of S3 staining with the Pf240 mAb suggests that PfNBP2a and 2b may also localize there. In contrast, EBA-175 is located in the micronemes (35), which are at the apical pole but quite distinct from the rhoptries. As expected given its staining pattern, the PfNBP1 antiserum shows only partial overlap with the Pf240 mAb. Thus, whereas PfNBP2a and PfNBP2b may be restricted to the neck of the rhoptries, PfNBP1 may be partially located there but is also clearly present in a broader apical localization.

The P. falciparum PvRBP Homologs Form a Complex. A 390-kD PfNBP1 band is immunoprecipitated with the N1 antisera from ^{35}S-labeled FVO strain schizont extracts (Fig. 3 A, top arrow). This antiserum also immunoprecipitates a smaller protein doublet (Fig. 3 A, bottom arrow), at ~260 kD and 250 kD, a position at which no PfNBP1 fragments are routinely seen in immunoblots (see below). An identical protein doublet is immunoprecipitated by an antiserum against the PfNBP2a and b shared region (S3), and an antiserum against the unique COOH terminus of PfNBP2b (B). This result suggests that PfNBP1 forms a complex with at least PfNBP2b, which is consistent with the known associative properties of the *P. vivax* PvRBP1 and PvRBP2 proteins (18, 19). In contrast, an antiserum raised against the unique COOH termini of PfNBP2a (A) did not immunoprecipitate any specific bands (Fig. 3 A). Cleavage of the PfNBP2a during the immunoprecipitation experiment, despite the presence of protease inhibitors, may remove the COOH-terminal epitopes against which this antiserum was raised. Indeed, the 260/250 kD doublet immunoprecipitated by the S3 and B antisera is smaller than the full length product recognized by these antisera by immunoblot analysis. Immunoprecipitations with S3 and B antisera do not reveal a high MW band corresponding to PfNBP1, but this may be because binding of these antisera disrupts complex formation.

A Second P. falciparum Strain Expresses a Truncated PfNBP1. The N1 antiserum was used in immunoblot analysis of late segmented schizonts extracts from several *P. falciparum* strains. In most of the strains examined, FVO, Dd2, and Malayan Camp K$^-$ (Fig. 3 B), and ItG2 and FCR3 (data not shown), a major high MW protein of ~390 kD was detected, which is slightly larger than the calculated PfNBP1 MW of 360 kD and the same size as the high MW band immunoprecipitated by this antiserum from labeled parasite extracts. A protein band at 220 kD was occasionally detected in these samples with the N1 antiserum (Fig. 3 B). Preliminary evidence indicates that this band represents a proteolytic cleavage fragment (data not shown). The 3D7 strain PfNBP1 protein (~350 kD) is slightly smaller than its counterpart in other strains, as predicted given the single nucleotide insertion in the 3D7 *Pfnbp1* gene that causes premature termination of this protein (Fig. 3 B). Strain 7G8 also expresses a truncated PfNBP1 product of ~310 kD. Importantly, PCR amplification of overlapping ~2 kb fragments spanning the *Pfnbp1* gene from several strains including 7G8 showed no significant differences in fragment size between isolates, indicating the absence of large scale deletions in the *Pfnbp1* gene in 7G8. The observed size of the 7G8 PfNBP1 is consistent with a mutation ~2.3 kb from the 3′ end of the gene causing a truncation of the PfNBP1 product (Fig. 1 C). By contrast, there were no size differences in PfNBP2a or PfNBP2b in any of these strains (Fig. 3 C).

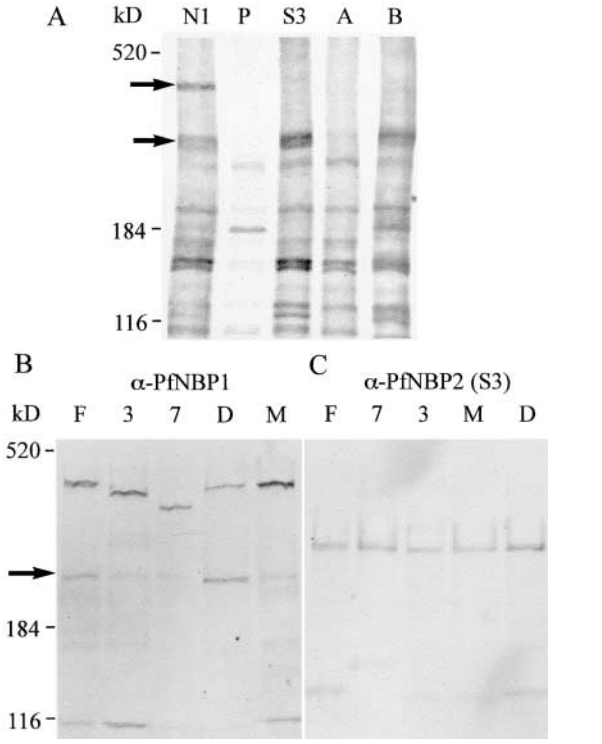

Figure 3. PfNBP1 is a large MW protein that coimmunoprecipitates with PfNBP2b and varies in size between isolates. (A) ^{35}S-labeled FVO schizont extracts immunoprecipitated with anti-PfNBP1 antisera (N1), preimmune antisera (P), or antiserum against shared (S3) or unique (A and B) regions of PfNBP2a and b, respectively. Anti-PfNBP1 antiserum (N1) immunoprecipitates a 390-kD band (top arrow) and also a 250/260 kD doublet (bottom arrow). The doublet is also immunoprecipitated by antisera raised against a region shared by PfNBP2a and b (S3) and the unique region of PfNBP2b (B). (B) Extracts from late schizont stage synchronized *P. falciparum* cultures were separated by SDS PAGE, transferred to nitrocellulose and probed with antisera raised against a fragment of PfNBP1 (N1). Culture strains used were FVO (F), 3D7 (3), 7G8 (7), Dd2 (D), and MC K$^-$ (M). The size of the PfNBP1 product differs between strains. (C) Extracts as in B were probed with an antiserum against a region shared by PfNBP2a and b (S3), showing no size difference between strains.

PfNBP1 Binds to Erythrocytes. Given their location at the invasive apical end of the merozoite and homology with known RBC binding proteins from other *Plasmodium* species, we performed EBAs to determine whether the PfNBPs bound to RBC. [^{35}S]methionine/cysteine labeled spent merozoite supernatants routinely contained one major RBC binding protein (data not shown), which we confirmed corresponds to EBA-175 (36, 37). Other smaller polypeptides also bound the RBCs, but none were in the 300 kD or greater size range as expected for the PfNBPs.

However, when samples were immunoblotted with anti-PfNBP1 antiserum, a fragment of PfNBP1 of ~220 kD was visible in the supernatant that bound equally well to both unfractionated RBC and blood enriched for reticulocytes (Fig. 4 A, middle arrow). A smaller fragment of PfNBP1, also visible in the supernatant (Fig. 4 A, bottom arrow) did not bind to the RBCs. The 220-kD PfNBP1 fragment detected in culture supernatants is the same size as a PfNBP1 fragment identified in whole parasite extracts (Fig. 3 B). The larger, faint band detected by the N1 antiserum (Fig. 4 A, top arrow) is due to cross-reaction of the N1 antiserum with an unrelated protein released from RBCs upon elution with high salt, as it was also visible in mock EBAs (M) performed without culture supernatants.

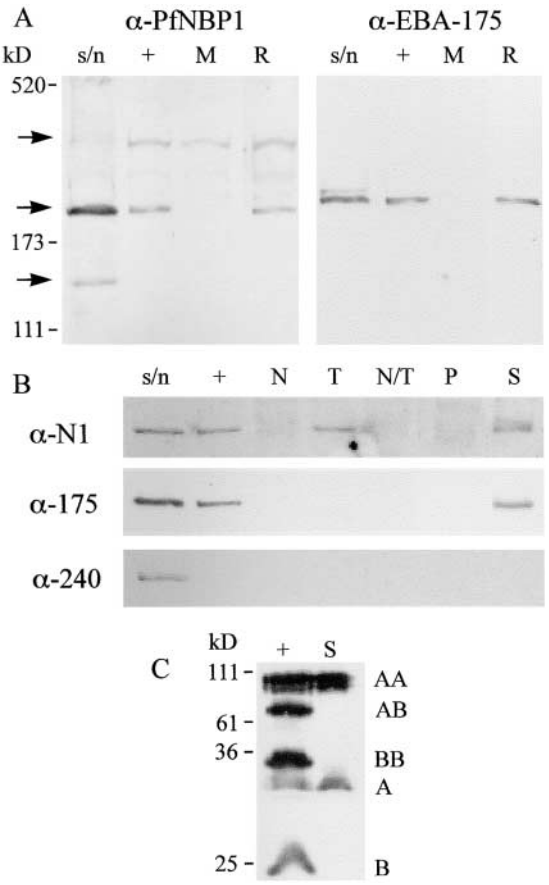

Figure 4. A fragment of PfNBP1 binds to a trypsin-sensitive receptor on the erythrocyte surface distinct from Glycophorin B. (A) EBAs using spent culture supernatants from *P. falciparum* FVO strain parasites. Bound material eluted from erythrocytes was immunoblotted with anti-PfNBP1 or anti-EBA-175 antisera. Lanes are starting supernatant (s/n), material bound to normal erythrocytes (+), material released from erythrocytes in the absence of culture supernatant (M), and material bound to erythrocytes enriched to ~10% reticulocytes (R). (B) EBAs using untreated erythrocytes (+); erythrocytes treated with neuraminidase (N), trypsin (T), both enzymes (N/T) or Pronase (P); or S-s-U$^-$ erythrocytes (S). Eluted material was immunoblotted with either PfNBP1 or EBA-175 antisera, or the mouse Pf240 mAb. (C) Erythrocyte ghosts from a Glycophorin B–positive donor (+) or a Glycophorin B–negative (S-s-U$^-$) donor (S) immunoblotted with an antiserum recognizing Glycophorin A and B monomers, homodimers, and heterodimers.

RBCs were treated with enzymes in several independent experiments to further characterize the erythrocyte receptor recognized by PfNBP1. PfNBP1 binding was prevented by treatment with neuraminidase (N), indicating that adhesion is dependent on sialic acid residues on the RBC surface, but was unaffected by trypsin (T) treatment (Fig. 4 B). In contrast, EBA-175 binding was affected by both neuraminidase and trypsin treatment, confirming that trypsin treatment had been sufficient to cleave Glycophorin A residues (Fig. 4 B). Pf240 is detectable in culture supernatants but does not bind RBCs (Fig. 4 B). PfNBP1 cannot therefore be binding to Glycophorins A, C, or D, which are all trypsin sensitive, or Receptor X, which is neuraminidase resistant, yet PfNBP1 clearly binds to some proteinaceous receptor, as adhesion was inhibited by treatment with Pronase (P) (Fig. 3 B).

The only known invasion receptor that would fit this profile is Glycophorin B, which is modified with sialic acid residues, but in contrast to Glycophorins A, C, and D, is not cleaved by trypsin. We therefore performed EBAs using erythrocytes from donors that lack Glycophorin B (S-s-U$^-$). Surprisingly, PfNBP1 was still able to bind to these cells (Fig. 4 B). This result was confirmed using several different culture supernatant preparations. The S-s-U$^-$ cells were confirmed to be Glycophorin B negative by immunoblotting erythrocyte ghosts with an antiserum that recognizes both Glycophorins A and B (Fig. 4 C). The PfNBP1 receptor is clearly trypsin resistant and neuraminidase sensitive, but does not appear to be one of the previously characterized invasion receptors. We refer to it henceforth as Receptor Y.

P. falciparum Strains that Express Truncated PfNBP1 Are Unable to Invade Trypsinized Erythrocytes. It is well established that *P. falciparum* strains differ in their invasion phenotypes. The two *P. falciparum* strains that express truncated PfNBP1 proteins, 3D7 and 7G8, still express the majority of the PfNBP1 protein yet lack the TMD and cytoplasmic domain. This type of mutation is very similar to the recently reported partial disruption of EBA-175, which resulted in a protein lacking its COOH terminus. This protein was still expressed and localized correctly, but the resulting parasite had a severely debilitated ability to invade RBCs. (15). We therefore performed invasion assays with five strains of *P. falciparum*, comparing invasion rates into untreated erythrocytes versus erythrocytes treated with neuraminidase, trypsin, and neuraminidase plus trypsin to define the invasion phenotype of each strain (Table I). Invasion of trypsin-treated cells is always substantially lower than that of untreated cells, but of the two strains expressing truncated PfNBP1, 7G8 was completely unable to invade trypsinized cells, and 3D7 did at a extremely reduced level. FVO, HB3, and Malayan Camp K$^-$, all of which express full-length PfNBP1, are readily able to invade trypsinized cells. By contrast, invasion of neuraminidase-treated cells showed no correlation with the presence or absence of *Pfnbp1* mutations. Thus, two strains isolated at quite different times and places and with two different mutations in the *Pfnbp1* gene, are both unable to invade

Table I. *Plasmodium falciparum Strains Expressing Truncated PfNBP1 Are Unable to Invade Trypsinized RBCs*

Pf strain	Neura-minidase	Trypsin	Neura./Tryp	S-s-U$^-$	Tryp/S-s-U$^-$
FVO	4 ± 1	30 ± 7	2 ± 1	79 ± 4	0
HB3	69 ± 19	38 ± 17	1 ± 1	77 ± 1	6 ± 4
MC K$^-$	16 ± 6	31 ± 4	7 ± 3	ND	ND
3D7	30 ± 7	5 ± 5	0	73 ± 6	2 ± 2
7G8	47 ± 6	2 ± 1	0	3 ± 3	0

Purified *P. falciparum* schizonts from strains listed were incubated with erythrocytes treated with neuraminidase, trypsin, or both (Neura./Tryp). Glycophorin B-negative cells (S-s-U$^-$) were also used. Invasion rates are expressed as a percentage of the invasion into untreated erythrocytes, with standard error.

trypsinized cells. This implies a role for PfNBP1 in a trypsin resistant invasion pathway, consistent with our previous findings that the PfNBP1 receptor is trypsin resistant.

Trypsinized erythrocytes had been previously proposed to have only Glycophorin B as a potential invasion receptor, as strains that are able to invade trypsinised erythrocytes are unable to invade trypsinized S-s-U$^-$ erythrocytes, where Glycophorin B is absent (1). Indeed, our experiments showed similar results with both FVO and HB3, which can invade trypsinized cells, but are unable to invade trypsinized S-s-U$^-$ cells (Table I). However, there are other interpretations of these results, which will be discussed further below. Surprisingly, 7G8 was completely unable to invade untreated S-s-U$^-$ cells, in two separate experiments and when other strains cultured in parallel invaded S-s-U$^-$ cells efficiently (Table I). 7G8 therefore appears to be dependent on Glycophorin B for invasion, a phenotype not previously reported for a *P. falciparum* strain.

Antibodies Raised against PfNBP1, PfNBP2a, and PfNBP2b Inhibit Erythrocyte Invasion. To confirm a role for PfNBP1 in a trypsin resistant invasion pathway, we performed invasion inhibition assays with IgG purified from the PfNBP1 and the PfNBP2a and PfNBP2b antisera, using a range of different *P. falciparum* strains and erythrocytes that had been modified with different enzyme treatments. None of the IgG had any affect on invasion of untreated cells when strains were used that were able to invade trypsinized erythrocytes (FVO, HB3, and MC K$^-$; Table II). However, if the targeted RBCs were first trypsinized, the IgG all had a marked effect on invasion rates at a concentration of 1 mg/ml, although the impact of different IgGs differed somewhat between strains. For example, 1 mg/ml of anti-PfNBP1 IgGs reduced invasion by 31 to 55% depending on the strain (Table II). Although we have been unable to show that either PfNBP2a or PfNBP2b bind to erythrocytes, antibodies raised against a region shared by both molecules (S3) or regions unique to each (A or B) all inhibited invasion of trypsinized cells. Anti-PfNBP1 and PfNBP2 IgG do not impact the sialic acid-independent, Receptor X pathway, as they had no effect on invasion of HB3 merozoites into neuraminidase-treated cells (data not shown). The PfNBPs clearly play a role in a trypsin-resistant invasion pathway.

Unlike all other strains tested, invasion of 3D7 parasites into untreated erythrocytes is inhibited by α-PfNBP1, α-PfNBP2a, and α-PfNBP2b IgG. Furthermore, when α-PfNBP1 IgG and α-PfNBP2 IgG were mixed we observed a greater level of inhibition of invasion than with either IgG alone, supporting a cooperative model of invasion (data not shown). Other researchers had noted that α-PfNBP2 IgG can inhibit 3D7 invasion (22), but the antiserum in that case was not specific to either PfNBP2a or PfNBP2b. We show clearly here that all three PvRBP homologs (PfNBP1, PfNBP2a, and PfNBP2b) are involved in invasion.

Discussion

We have identified a *P. falciparum* ortholog of the *P. vivax* reticulocyte binding protein, PvRBP1, and present evidence that this protein, PfNBP1, is the parasite ligand for an unknown, trypsin-resistant invasion receptor, Receptor Y. PfNBP1 binds to erythrocytes, and this adhesion is dependent on a neuraminidase-sensitive, trypsin-resistant

Table II. *Antisera Against Plasmodium falciparum Normocyte Binding Proteins Inhibit Merozoite Invasion of Trypsinized RBCs*

PF strain	Treatment	Prebleed	NBP1	NBP2 (S3)	NBP2a	NBP2b
FVO	None	106 ± 7	102 ± 15	107 ± 25	114 ± 4	104 ± 7
FVO	Trypsin	107 ± 7	45 ± 2	65 ± 2	65 ± 1	68 ± 12
HB3	None	94 ± 2	100 ± 1	110 ± 13	104 ± 17	95 ± 1
HB3	Trypsin	96 ± 3	69 ± 17	45 ± 15	66 ± 14	26 ± 10
MC K$^-$	None	118 ± 18	94 ± 5	139 ± 7	ND	ND
MC K$^-$	Trypsin	122 ± 7	60 ± 12	43 ± 6	ND	ND
3D78	None	95 ± 7	50 ± 1	51 ± 3	57 ± 7	70 ± 6

Purified schizonts were incubated with untreated erythrocyte or trypsin-treated erthrocytes (T) in the presence of purified IgG. IgG was added at 1 mg/ml and invasion rates are expressed as a percentage of invasion in the absence of any IgG, with standard error.

factor distinct from Glycophorin B on the erythrocyte surface. An antiserum against PfNBP1 inhibits *P. falciparum* invasion of erythrocytes via a trypsin-resistant pathway, and *P. falciparum* strains expressing truncated PfNBP1 are unable to invade trypsinized erythrocytes.

Immunoprecipitation data further suggests that PfNBP1 and PfNBP2b may form a complex, as was shown for the *P. vivax* reticulocyte binding proteins (18, 19). The *P. vivax* RBP1 and RBP2 proteins can be coimmunoprecipitated using both ^{35}S-labeled culture supernatants or in vitro translated fragments of each protein (18, 19; unpublished data). The formation of a similar protein complex in *P. falciparum* could explain the inhibitory effect of PfNBP2 antisera on invasion despite the lack of detectable adhesion of PfNBP2 to RBCs (Table II). Specific IgG raised against either shared or unique regions of PfNBP2a and 2b inhibit invasion of trypsinized cells by FVO, HB3, and MC K$^-$ parasites, arguing strongly that both are involved in the same trypsin-resistant pathway as PfNBP1. Further evidence of cooperativity between PfNBP1 and the PfNBP2s is shown by a greater effect on invasion when IgGs against these proteins are mixed than either IgG alone (data not shown). The functional significance of a potential PfNBP1-PfNBP2 complex and the relative contributions of PfNBP2a and 2b are details that remain to be established.

The PvRBP Superfamily. Gene structure, amino acid homology, phylogeny modeling, and erythrocyte binding analyses all support the proposal that the *P. vivax* RBPs, the *P. falciparum* NBPs, and the *P. yoelii* p235 rhoptry proteins form a super-family of related molecules (20–23, 33). While PvRBP1 and PvRBP2 share several features, they are not highly conserved at the amino acid level and, in fact, were originally viewed as two distinct proteins rather than as paralogues (18). We show here via phylogenetic and other analyses that PfNBP1 and PfNBP2a/2b are in fact respective homologs of these two proteins. Further, the reported *P. yoelii* p235 rhoptry proteins are evolutionarily related to PvRBP-2 and the PfNBP2 proteins. The data corroborate the fact that these proteins comprise a superfamily of two distinct subfamilies, PvRBP1-like molecules and PvRBP2-like molecules. In line with this, there is one other *P. falciparum* gene that is a paralogue to *Pfnbp1* and several simian malarias also have distinct PvRBP1-like and PvRBP2-like homologs (unpublished data). Although a family of PvRBP2-like molecules is known to play a role in invasion in the rodent malaria, *P. yoelii*, it is not yet known if a PvRBP1 ortholog or homologs exists in this distantly related species of *Plasmodium*. The situation in *P. yoelii* is somewhat unique given the large size of the p235 family and that each merozoite from a single schizont may express a different member of the family (38). At this stage, no such extensive family appears to exist in *P. falciparum*.

The binding specificities of different members of the PvRBP superfamily clearly differ. PfNBP1 has no preference to bind reticulocytes, whereas the PvRBPs were initially identified by their reticulocyte binding properties (18). Similarly, PfNBP1 binding is dependent on sialic acid, whereas binding of the *P. yoelii* p235 family is unaffected by neuraminidase (39). This is not unexpected given the evolutionary distance between these *Plasmodium* species and the different niches of parasitism they exploit in RBC lineage, and is akin to the erythrocyte binding-like (EBL) or, more appropriately termed, Duffy binding-like (DBL) invasion ligand super-family (13, 40). The *P. vivax* and *P. knowlesi* Duffy binding proteins bind to the peptide backbone of the Duffy glycoprotein (41, 42), while *P. falciparum* EBA-175 ligand primarily recognizes sialic acid residues on Glycophorin A (14), yet are still classified together, along with other paralogues, in the DBL family by structure and sequence relatedness (13, 40). As a parallel to the DBL classification, the PvRBPs and their respective homologs might best be referred to as the reticulocyte binding-like (RBL) superfamily.

The Nature of Receptor Y. Although we have established that the receptor recognized by PfNBP1 is a protein that contains sialic acid residues and is resistant to trypsin, no immediate candidates for the receptor on RBC surface are evident. Of the major rbc sialoglycoproteins, the glycophorins, only Glycophorin B is trypsin resistant, and PfNBP1 is able to bind erythrocytes that lack Glycophorin B. The presence of a second, neuraminidase-sensitive, trypsin-resistant receptor is unexpected, but not entirely unprecedented. The Malayan Camp K$^-$ strain is able to invade, albeit at a lower level, trypsinised M^{k}M^k cells, which through mutation lack both Glycophorin A and B and through trypsinization lack Receptor X and Glycophorins C and D as well (9). The invasion pathway used by MC K$^-$ parasites in this case is neuraminidase sensitive and trypsin resistant, just like Receptor Y. Given the extreme rarity of M^{k}M^k cells, the possibility of this unknown receptor matching Receptor Y could not be tested.

Although the identity of Receptor Y is unknown, the invasion data infers that Receptor Y and Glycophorin B may function in similar ways or at similar points during invasion. We and others have noted that strains that are able to invade trypsinised erythrocytes are unable to invade trypsinised S-s-U$^-$ erythrocytes. One explanation for this is that Receptor Y is necessary but not sufficient for invasion using a common trypsin-resistant pathway. Hence, strains with *Pfnbp1* mutations unable to utilize Receptor Y are unable to invade trypsinised cells and antibodies raised against PfNBP1 can inhibit the invasion of trypsinised cells (PfNBP1 is necessary for invasion using this pathway), but strains that posses functional PfNBP1 are unable to invade using it alone, as in trypsinised S-s-U$^-$ cells (PfNBP1 is not sufficient for invasion).

Implications for Models of Erythrocyte Invasion. If PfNBP1 contributes to invasion via a trypsin-resistant pathway, does this constitute a distinct, alternative invasion route? The phrase "alternative" pathways may not be the most helpful to our understanding of invasion. As others have mentioned (1), invasion pathways could operate in series or in parallel. That is, they could function only independently of one another or they may routinely function together but can be forced to function in isolation when host cells are enzymatically manipulated or selection pressure is

applied. An integrated model appears more likely. Where the number of available receptors has been restricted by enzymatic modification, invasion is almost always less efficient than invasion of untreated cells, arguing against strictly independent alternative invasion pathways. Furthermore, two strains that are able to invade using the same "alternative" pathway can do so at quite different efficiencies, implying that the ability to exploit a particular pathway is not always the result of the simple presence or absence of a single parasite ligand.

We think it likely that PfNBP1 and the trypsin-resistant pathway routinely play a specific role during the invasion of untreated erythrocytes and only inefficiently and in extremis function in isolation. Although IgG raised against PfNBP1, PfNBP2a, and PfNBP2b all had no affect on the invasion of untreated cells by FVO, HB3, and MC parasites, they did affect the invasion of 3D7 parasites into untreated cells, clearly implying a role for PfNBP1 in invasion of untreated erythrocytes in this strain. A broadly similar result was seen previously with a single PfNBP2 antisera, where invasion was affected in 3D7 parasites but not another strain, D10 (22). In that instance it was proposed that this difference might result from sequence divergence between the *PfNBP2* genes in the different strains, although few such differences were detected (22). In this study we found several different antisera were effective against a range of different strains, making the former explanation unlikely. Given the mutation in the *Pfnbp1* gene observed in 3D7, the expressed PfNBP1 product may be partially functional, but sufficiently compromised, to be relatively easily inhibited by antibodies, whereas in other strains with a fully functional PfNBP1, the trypsin-resistant pathway may be much more difficult to inhibit (perhaps because of the overlapping roles of Receptor Y and Glycophorin B discussed above). A disruption of the *Pfnbp1* gene causing the complete absence of PfNBP1, if such a phenotype were viable, would be the test for the role of this ligand in the invasion of untreated cells. Extensive selection of transformants with a *Pfnbp1* disruption plasmid in our laboratory has not produced any disruptants, suggesting that the gene may be recalcitrant to this type of genetic manipulation (data not shown).

If PfNBP1 does play a role in the invasion of erythrocytes, what is that role? EBA-175, through its familial relationship to the Duffy binding proteins of *P. vivax* and *P. knowlesi*, has been implicated in the formation of the electron-dense tight junction between the apical end of the merozoite and the erythrocyte membrane, a relatively late and irreversible step in the invasion cascade (43). A location of PfNBP1 at the apical surface of the merozoite could imply that it may function just before EBA-175, which is sequestered in the micronemes (35) and must be released from this internal location before interaction with the erythrocyte surface is possible. One model, based previously on invasion properties of *P. vivax,* proposed PvRBP1 and PvRBP2 function early in the invasion cascade, selecting reticulocytes for invasion and triggering the release of the contents of the micronemes which includes the *P. vivax* homologue of EBA-175, the Duffy binding protein (18, 19). Unlike *P. vivax, P. falciparum* shows no striking preference for subpopulations of erythrocytes. Nevertheless, in parallel with the hypothesis about the function(s) of the PvRBPs, PfNBP1 and the trypsin-resistant pathway may also play a role in this early step by recognizing Receptor Y and potentially inducing a signal to begin the invasion cascade, through release of EBA-175 and its paralogues.

Erythrocyte invasion by *P. falciparum* merozoites is a complex and incompletely understood process. Any vaccine designed to prevent erythrocyte invasion must reflect this complicated reality and include the ligands for all potential invasion pathways. Deployment of a vaccine of limited inclusiveness will risk the application of strong selection pressures, allowing parasite populations to switch from one pathway to another and to varying degrees escape the induced immunity. As the ligand for a trypsin-resistant pathway, PfNBP1, and perhaps its partners PfNBP2a and 2b, clearly merit further study in regards to function and certainly consideration as candidates for inclusion in any invasion blocking vaccine.

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