

The cytoplasmic domain of the *Plasmodium falciparum* ligand EBA-175 is essential for invasion but not protein trafficking

Tim-Wolf Gilberger, Jennifer K. Thompson, Michael B. Reed, Robert T. Good, and Alan F. Cowman

The Walter and Eliza Hall Institute of Medical Research, Melbourne 3050, Australia

The invasion of host cells by the malaria parasite *Plasmodium falciparum* requires specific protein–protein interactions between parasite and host receptors and an intracellular translocation machinery to power the process. The transmembrane erythrocyte binding protein-175 (EBA-175) and thrombospondin-related anonymous protein (TRAP) play central roles in this process. EBA-175 binds to glycophorin A on human erythrocytes during the invasion process, linking the parasite to the surface of the host cell. In this report, we show that the cytoplasmic do-

main of EBA-175 encodes crucial information for its role in merozoite invasion, and that trafficking of this protein is independent of this domain. Further, we show that the cytoplasmic domain of TRAP, a protein that is not expressed in merozoites but is essential for invasion of liver cells by the sporozoite stage, can substitute for the cytoplasmic domain of EBA-175. These results show that the parasite uses the same components of its cellular machinery for invasion regardless of the host cell type and invasive form.

Introduction

The intracellular parasite *Plasmodium falciparum* causes the most severe form of malaria in humans and is responsible for over two million deaths each year. Transmission of this protozoan parasite occurs during feeding of the Anopheles mosquito when sporozoite forms enter the human circulation and invade liver cells. Merozoites develop within the liver cells and are released into the blood where they invade erythrocytes. A crucial step for the survival of the parasite is a fast and efficient invasion process by both the sporozoite and merozoite into their target cells, and this involves recognition, adhesion, and active invasion of the respective host cell (Aikawa et al., 1978; Barnwell and Galinski, 1998; Dubremetz et al., 1998). After initial attachment of the parasite to the surface of the target cell, the intruder establishes a tight junction between its apical end and the host cell membrane. This tight junction progressively moves toward the posterior of the invading parasite as it enters the target cell. The process is independent of the host cell, and appears to be driven by intracellular translocation machinery involving transmembrane proteins and myosin motor modules (Dubremetz et al., 1998; Pinder et al., 1998).

The invasion process into host cells involves proteins located in specialized exocytic organelles (micronemes, rhoptries, and granula) that define the electron-dense apical pole of the invasive form of all Apicomplexa (Aikawa et al., 1978; Dubremetz and Schwartzman, 1993; Carruthers and Sibley, 1997). These proteins are targeted to their subcellular location, and this is mediated by either specific amino acid motifs or interaction with an escorter protein (Baldi et al., 2000; Di Cristina et al., 2000; Hoppe et al., 2000; Reiss et al., 2001; Cerede et al., 2002; Meissner et al., 2002; Ngo et al., 2003). The secretion of proteins stored in these organelles is essential for the invasion process. A group of micronemal proteins involved in merozoite and sporozoite invasion are each defined by an adhesive extracellular domain, a transmembrane region, and a cytoplasmic tail (Sultan et al., 1997; Sibley et al., 1998; Adams et al., 2001; Michon et al., 2002). These proteins are differentially expressed during the parasite life cycle (Rogers et al., 1992b; Blair et al., 2002). For example, in *Plasmodia*, the thrombospondin-related anonymous protein (TRAP)* is not expressed in the asexual life cycle, but is an essential protein for sporozoite invasion (Sultan et al., 1997). TRAP-deficient sporozoites are unable to invade mosquito salivary gland cells or liver cells and do not show any gliding motility. It has been proposed that the

Address correspondence to Alan F. Cowman, The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Melbourne 3050, Australia. Tel.: 61-3-9345 2555. Fax: 61-3-9347 0852. E-mail: cowman@wehi.edu.au
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*Abbreviation used in this paper: TRAP, thrombospondin-related anonymous protein.

cytoplasmic domain of TRAP plays a role in connecting the parasite actin–myosin machinery with the external substrate (Kappe et al., 1999). It is interesting to note that besides TRAP, just one other protein—the circumsporozoite protein—has been identified as playing a crucial role in the invasion of hepatocytes by sporozoites (Menard et al., 1997).

In contrast, merozoites, the invasive form of the *Plasmodia* asexual life cycle, use an array of adhesive transmembrane proteins similar to TRAP (Sim et al., 1994; Reed et al., 2000a; Duraisingh et al., 2003). For instance, members of the erythrocyte binding-like superfamily can provide the merozoite with high affinity binding ligands for a range of receptors on the surface of the erythrocyte (Adams et al., 2001). This multiplicity provides the genetic basis for ligand diversity and different host cell receptor specificity. The relative importance and usage of the ligands for invasion of merozoites is strain dependent (Hadley et al., 1987; Okoyeh et al., 1999; Duraisingh et al., 2003). EBA-175 is the ligand for glycophorin A, the dominant glycoprotein on the surface of the erythrocyte (Sim et al., 1994). The interaction of this ligand with its receptor defines one invasion pathway for merozoites and is dependent on sialic acid moieties on the receptor (Camus and Hadley, 1985). Disruption of this receptor–ligand interaction by either modifying the surface of the erythrocytes or by gene disruption has shown that the merozoite can use other invasion pathways (Dolan et al., 1990; Reed et al., 2000a; Duraisingh et al., 2003). Loss of function of EBA-175 in both sialic acid–dependent and –independent *P. falciparum* strains results in a decrease in invasion of chymotrypsin-treated erythrocytes (Duraisingh et al., 2003). This is due to the inability of these Δ EBA-175 parasites to use the chymotrypsin-resistant receptor glycophorin A. Additionally, in a sialic acid–dependent strain, disruption of the EBA-175/glycophorin A pathway manifests itself in a dramatic increase of invasion into neuraminidase-treated erythrocytes. The receptor interaction of EBA-175 is mediated by two adhesive modules (called F1/F2) located in the extracellular domain (Adams et al., 1992); however, nothing is known about the role of the cytoplasmic domain.

In this paper, we address the role of the cytoplasmic domain of EBA-175 in merozoite invasion via the glycophorin A–dependent pathway of human erythrocytes. We show that trafficking of EBA-175 is independent of its cytoplasmic domain. Further, we show that the cytoplasmic domain of EBA-175 is essential for a functional EBA-175/glycophorin A pathway and can be substituted by the cytoplasmic domain of TRAP. This suggests that the invasion of sporozoite and merozoite forms of *Plasmodia* into liver cells and erythrocytes, respectively, use the same cellular machinery.

Results

Sequence comparison and mutation of the cytoplasmic domains of EBA-175

Micronemal proteins identified so far in *P. falciparum* and other apicomplexa have an NH₂-terminal signal peptide and a single transmembrane domain with a short cytoplasmic tail of ~50 amino acids at the COOH terminus (Di Cristina et al., 2000; Adams et al., 2001). The cytoplasmic domains do not display any overall homology, but have common fea-

tures in that they are rich in acidic amino acids (15–24%) and have tyrosine-based motifs that may function in trafficking of these proteins to the micronemes of the apical complex (Fig. 1 A). The TRAP protein is localized within the micronemes of the mosquito sporozoite stage (Rogers et al., 1992a), whereas EBA-175 is expressed in the blood-stage merozoites and is also localized in micronemes (Sim et al., 1992). Both of these proteins function in invasion; however, the target cell of the sporozoite is liver cells, whereas merozoites invade erythrocytes.

To analyze the role of the EBA-175 cytoplasmic tail in trafficking to the micronemes and in invasion of erythrocytes, we constructed transgenic *P. falciparum* that expressed specific truncated, substituted, or mutated forms of this protein. This was done using the transfection vector pHH1 into which 1.1 kb of the 3' end of the *EBA-175* gene had been cloned. This *EBA-175* fragment was amplified from cDNA to remove the 3' introns located in this region (Reed et al., 2000a). The introns were removed to stabilize the transfection plasmid as previous constructs containing the introns underwent frequent deletions.

Nine transfection plasmids based on pHH1 were constructed that would introduce specific alterations by allelic replacement into the 3' end of the *EBA-175* gene (Fig. 1 B; Triglia et al., 1998). As a control, we used pHH1-3'R that would replace the 3' end of the endogenous *EBA-175* gene with cDNA coding for the same region. This would express normal EBA-175 protein, and these parasites would serve as a comparison for all transfectants. To determine if the cytoplasmic domain of TRAP could substitute for the same region of EBA-175, we constructed pHH1-175TRAP that would replace the 3' end of *EBA-175* and insert 114 bps of the *TRAP* gene. Additionally, we used the plasmid pHH1- Δ 230 that would allow deletion of the 3' cysteine-rich region, transmembrane, and cytoplasmic domains. This transfection plasmid has been used previously to produce a parasite line with a truncated EBA-175 that disrupts the function of this protein (Reed et al., 2000a). Importantly, transfection and integration of these plasmids into the endogenous *EBA-175* gene by allelic replacement would allow expression of the altered EBA-175 protein under the control of the endogenous promoter (Triglia et al., 1998).

The 10 pHH1-based plasmids were transfected into W2mef parasites, and additionally, the plasmids pHH1-3'R and pHH1- Δ tail were transfected into the 3D7 parasite line. The transfected parasites were selected for integration via a single crossover recombination event to derive: W2mef3'R, W2mefY₁₄₁₉, W2mefY₁₄₆₄, W2mefYY, W2mefEDD, W2mef Δ 15, W2mefY Δ 15, W2mefTRAP, W2mef Δ tail, W2mef Δ 230, 3D73'R, and 3D7 Δ tail (Fig. 1, C and D). To confirm that the plasmids had integrated, genomic DNA from the parental line W2mef and 3D7 (as well as the 11 transfectants) was probed with an *EBA-175* fragment in Southern hybridization experiments (Fig. 1 D). The structure of the integration events in the transfected parasite lines was confirmed by additional restriction enzyme mapping experiments (Fig. 1, C and D; unpublished data). The *EBA-175* gene of each transfected parasite line was further analyzed by PCR and sequencing to confirm that the 3' end

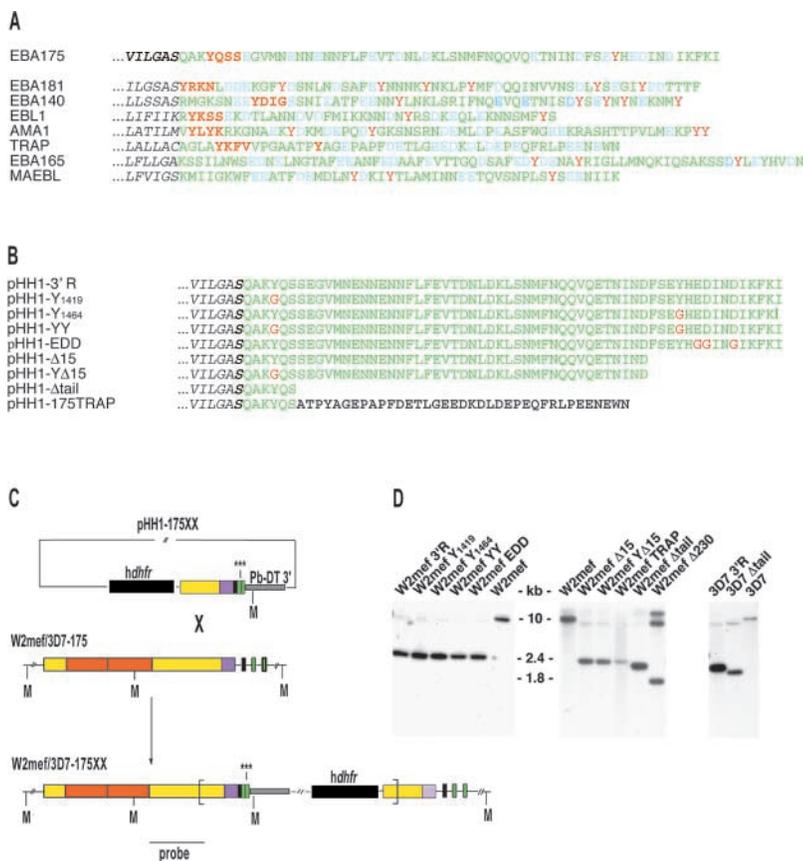


Figure 1. Sequence alignment of the cytoplasmic domain of microneme proteins and the EBA-175 mutations used in this paper. Also shown is the EBA-175 allelic replacement and Southern blot analysis. (A) Comparison of the microneme proteins EBA-175, EBA-181 (JESEBL), EBA-140 (BAEBL), EBL1, AMA1, TRAP, EBA165 (PAEBL), and MAEBL. The COOH-terminal amino acids of the highly conserved transmembrane domain are in black italics, tyrosine motifs and tyrosine residues are indicated by red letters, acidic amino acids are highlighted in blue. (B) Sequences of the constructs used in this paper to modify the cytoplasmic domain of EBA-175. COOH-terminal amino acids of the transmembrane domain are in black italics, and amino acid substitutions are in red. In gray is the sequence of TRAP fused to EBA-175 backbone. (C) Schematic representation of the 3' replacement of the EBA-175 gene by single crossover recombination of pHH1 constructs in the EBA-175 locus. The positive selection cassette (*hDHFR*) of the pHH1 vector is represented by the black box. An ~1.1-kb fragment of the COOH terminus with the introduced mutation (indicated by asterisks) was cloned in the pHH1 constructs in the EBA-175 locus. The positive selection cassette (*hDHFR*) of the pHH1 vector is represented by the black box. An ~1.1-kb fragment of the COOH terminus with the introduced mutation (indicated by asterisks) was cloned in the pHH1 constructs including the 3' cysteine rich region (purple), the transmembrane domain (black), and the mutated cytoplasmic domain (green). This fragment is flanked by the 3' UTR of the *P. berghei* dihydrofolate reductase gene (gray) in the pHH1 vector. Crosses refer to the regions where recombination events were expected. The intron/exon structure of the endogenous EBA-175 gene is shown. The red boxes indicate the adhesive F1/F2 ectodomains of the endogenous EBA-175.

The MfeI (M) restriction sites are marked and the position of the EBA-175 probe used for Southern analysis is indicated. (D) Southern blot analysis of genomic DNA (MfeI restricted) of W2mef and transgenic EBA-175 mutant parasites reveals that the plasmid has integrated into the EBA-175 gene. Variable numbers of plasmid copies have integrated into each transgenic parasite. The position of the probe used in the Southern hybridization leads to large fragments of 8.1 and 11 kb that differ in intensity depending on the number of plasmids integrated. The 2.4-kb band is indicative of the integration of the plasmid via single recombination into the 3' end of the EBA-175 gene. Importantly, the endogenous EBA-175 hybridizing band is 10 kb, and is different in the parasite lines where the 3' end of EBA-175 has been replaced. Sizes of the hybridizing bands are shown in kb.

had been replaced and that the expected mutations, deletion, or insertion were encoded within the gene.

Expression of mutant EBA-175 in the transfected parasites

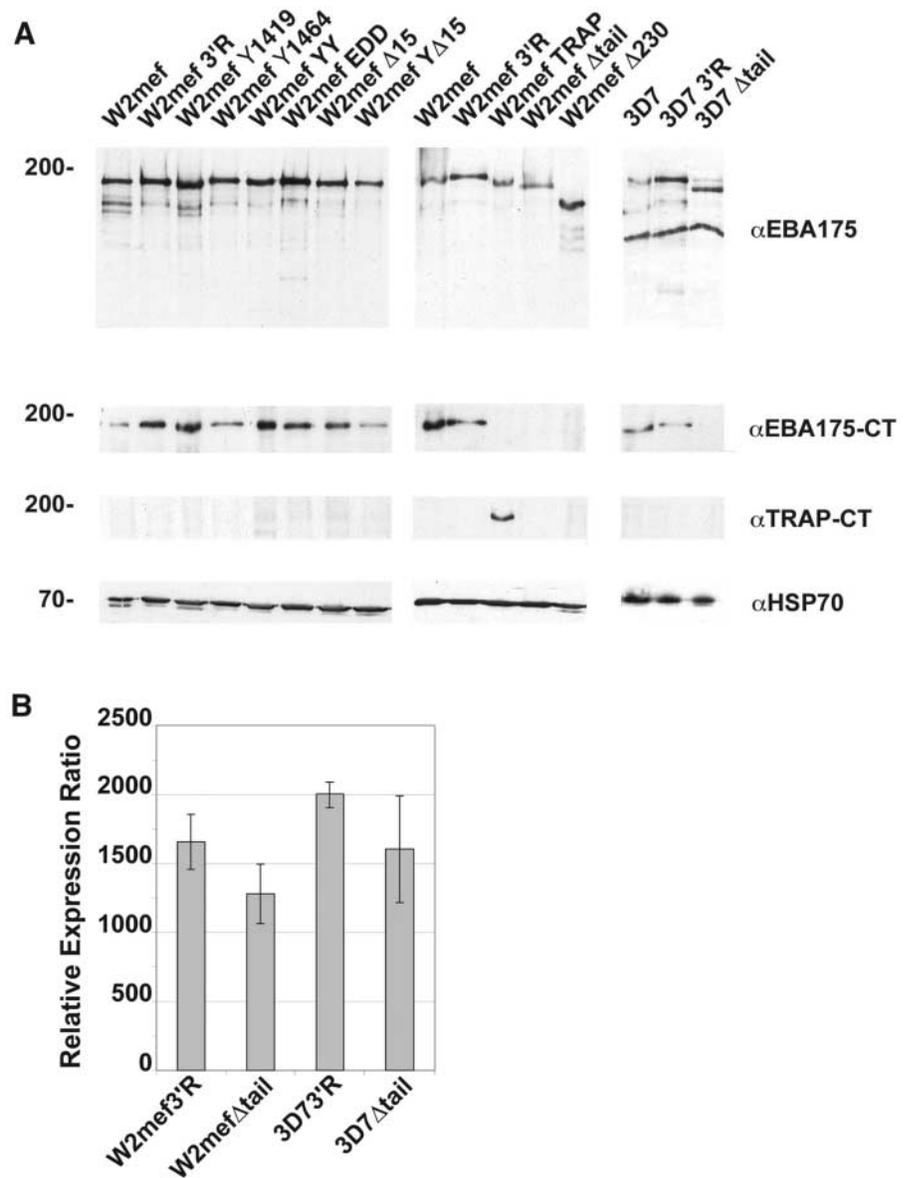
To confirm that the EBA-175 protein was expressed, we analyzed parasites in schizont stages of parental lines W2mef and 3D7, as well as the transfectants W2mef3'R, W2mefY₁₄₁₉, W2mefY₁₄₆₄, W2mefYY, W2mefEDD, W2mefΔ15, W2mefYΔ15, W2mefTRAP, W2mefΔtail, W2mefΔ230, 3D7Δtail, and 3D73'R parasites by Western blots with anti-EBA175, anti-EBA175-CT, anti-TRAP-CT, and also anti-HSP70 antibodies as a loading control (Fig. 2 A). Anti-EBA175 antibodies detect the ectodomain of the protein, and all of the parasite lines show expression of the appropriately sized protein. Additionally, in 3D7 and in 3D7-derived transgenic parasites, a strong (~100 kD) cross-reactive protein is detected. The W2mefΔtail, 3D7Δtail, and W2mefΔ230 parasites express a protein smaller than that observed for W2mef consistent with expression of the truncated protein. Antibodies specific to the cytoplasmic tail of EBA-175 detect the protein in W2mef, and all of the transfectants except for W2mefTRAP, W2mefΔtail,

W2mefΔ230, and 3D7Δtail. This shows that these transfected parasite lines express EBA-175 that lacks the cytoplasmic domain. The anti-EBA175-CT antibodies detect EBA-175 in W2mefYΔ15 and W2mefΔ15 despite the deletion of 15 amino acids. This is not surprising, as both of these truncated proteins retain 49 amino acids of the 64-amino acid cytoplasmic tail to which the antibody was raised.

To test if W2mefTRAP expresses a chimeric EBA-175 protein containing the TRAP cytoplasmic domain, we used anti-TRAP-CT antibodies in immunoblot experiments with the same schizont preparations used above (Fig. 2 A). This antibody detects a protein in W2mefTRAP with the identical size as that observed with anti-EBA175 antibodies; however, no signal is obtained with any of the other parasite lines tested.

The level of expression of the different mutant and chimeric EBA-175 proteins in each of the transgenic parasites was approximately the same as judged by multiple Western blots using independent schizont preparations (Fig. 2; unpublished data). However, to further investigate the level of transcription of EBA-175 in W2mefΔtail and 3D7Δtail parasites, W2mef3'R, 3D73'R, and parasite transcripts of the EBA-175 gene were quantified by real-time RT-PCR and compared with the relevant transfection controls

Figure 2. Immunoblot analysis of transgenic parasites expressing different EBA-175 mutant proteins. Proteins from synchronized parasite cultures were separated by SDS-PAGE on a 6% gel under reducing conditions. Proteins were detected with anti-EBA175, anti-EBA175-CT, anti-TRAP-CT, and anti-HSP70. Approximately equal signal was obtained with the anti-HSP70 antibodies, suggesting that each lane was loaded equally. In 3D7-derived parasite pellets, an additional cross-reactive band of ~ 100 kD is detected by anti-EBA-175 antibodies. (B) Bar graph of the relative expression ratio of *EBA-175* in late schizont stages of selected parasites. This ratio was calculated by dividing the average of three EBA175 assays with the average of the actin and histone 2B signals. The SD represents the sum of the *EBA-175* and housekeeping SDs. The averages are calculated and the relative expression ratio is obtained by dividing the *EBA-175* value with a mean of each control.



W2mef3'R and 3D73'R (Fig. 2 B). No significant differences between W2mef3'R, W2mefΔtail, 3D73'R, and 3D7Δtail could be detected.

The cytoplasmic domain of EBA-175 is not required for localization of the protein to the micronemes

Previously, it has been shown that the cytoplasmic tail of type 1 transmembrane proteins in Apicomplexa can contain sorting signals that are essential for correct subcellular localization (for review see Joiner and Roos, 2002). To determine if the mutant EBA-175 proteins were correctly localized to the micronemes, we used immunofluorescence to test colocalization with other microneme proteins including EBA-181 (Gilberger et al., 2003) and EBA-140. Using the parasite line W2mefΔ230, in which EBA-175 is expressed without the 3' cysteine-rich region, transmembrane, and cytoplasmic domain, we showed that this truncation leads to incorrect localization of truncated EBA-175 (Fig. 3). Although still detectable in schizonts (Reed et al., 2000a), it does not colocal-

ize with the microneme marker EBA-181 and importantly is not detectable in merozoites, as it appears to be released into the supernatant on schizont rupture (Fig. 3). This was confirmed by colocalization with a second microneme marker EBA-140 (unpublished data). Previously, it has been suggested that the same truncated EBA-175 may be localized to micronemes in schizont stages; however, no additional microneme markers were available at that time to confirm this result, and immuno-localization in free merozoites was not performed (Kaneko et al., 2000; Reed et al., 2000a).

In *Toxoplasma gondii*, it has been shown that tyrosine-based motifs are required for targeting of proteins to the micronemes and rhoptries (Di Cristina et al., 2000; Hoppe et al., 2000). To test the role of the two tyrosine residues (Y-1419 and Y-1464) in microneme targeting of EBA-175, each was mutated in transfectant parasite lines W2mefY₁₄₁₉ and W2mefY₁₄₆₄. To rule out the requirement of only one of the two Tyr residues, both were mutated in W2mefYY-transfected parasites. Immunofluorescence of free merozoites from

W2mefY₁₄₁₉, W2mefY₁₄₆₄, and W2mefYY parasites with anti-EBA-175 and anti-EBA-181 antibodies shows colocalization, confirming that the mutant EBA-175 proteins are located within the micronemes of these transfected parasites (Fig. 3).

To test the role of charged amino acid residues in EBA-175 trafficking, the amino acid residues E-1466, D-1467, and D-1470 were mutated in W2mefEDD using the transfection plasmid pHH1-EDD. Immunofluorescence of EBA-175 in W2mefEDD showed colocalization with EBA-181 in free merozoites, demonstrating that it was located within the micronemes (Fig. 3). To further investigate the possible synergistic role for trafficking of the acidic acid cluster and the tyrosines, we constructed the parasites W2mefΔ15 and W2mefYΔ15. These parasites express a truncated EBA-175

without the COOH-terminal acidic acid cluster EDD and without either one or both tyrosines. Immunofluorescence experiments with these transgenic parasites showed the same subcellular localization of mutated EBA-175 and EBA-181 in free merozoites, confirming that they are both present in micronemes (Fig. 3).

Finally, to determine if the cytoplasmic tail is required for trafficking of EBA-175 to the micronemes at all, we constructed transfected parasites W2mefΔtail and 3D7Δtail that express truncated forms of the protein. Although these parasites express EBA-175 without a cytoplasmic domain, this truncated protein is still trafficked to the micronemes (Fig. 3). Therefore, the cytoplasmic domain of EBA-175 is not required for localization of the protein to micronemes in *P. falciparum*.

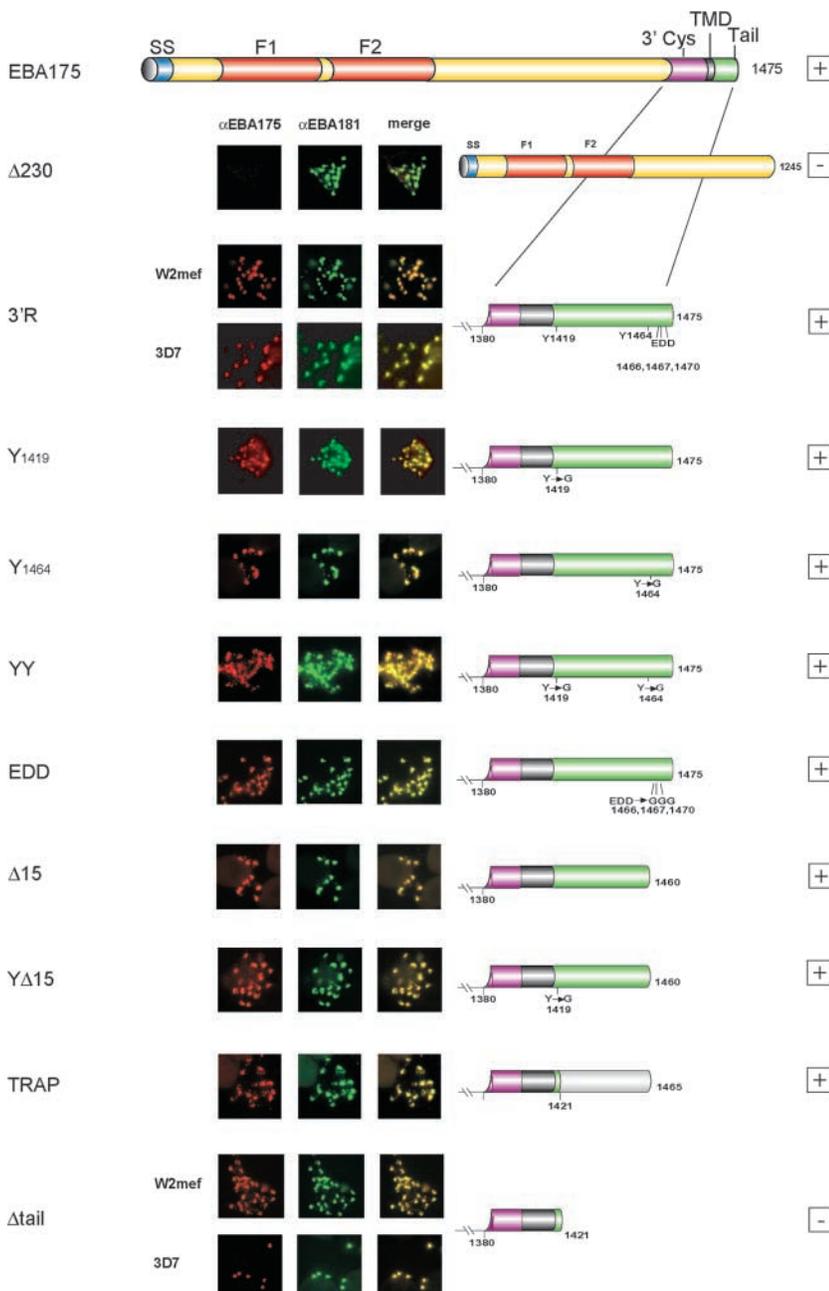


Figure 3. Immunolocalization of EBA-175 mutant proteins in transgenic parasites. The names on the left refer to the W2mef parasite lines expressing mutant EBA-175 proteins. The structure of EBA-175 and mutant proteins are schematically shown. Free merozoites were incubated with anti-EBA175 and anti-EBA181, followed by FITC-labeled anti-mouse and rhodamine-labeled anti-rabbit antibodies. To precisely visualize the localization of mutant EBA-175 with respect to the microneme protein EBA-181, the two fluorescence photomicrographs were merged. The function of the mutant EBA-175 was measured in each parasite line as shown in Fig. 4 A. + refers to absence of a switch in invasion demonstrating EBA-175 function is retained, whereas - signifies a switch in invasion phenotype and loss of function for EBA-175.

The cytoplasmic domain of EBA-175 is required for function in merozoite invasion

W2mef invades neuraminidase-treated erythrocytes very inefficiently because it is reliant on sialic acid-dependent invasion (Dolan et al., 1990), and EBA-175 is an important ligand in this process (Duraisingh et al., 2003). Additionally, the loss of function of EBA-175 can be directly measured in W2mef by a decrease in invasion of chymotrypsin-treated erythrocytes due to the inability of these parasite lines to use the chymotrypsin-resistant erythrocyte receptor glycoporphin A (Duraisingh et al., 2003).

The parasite 3D7 can invade via sialic acid-independent receptors on the erythrocyte, and disruption of the *EBA-175* gene does not cause a major shift in use of different receptors as has been described for W2mef, suggesting that it is not required for a dominant invasion pathway in this particular parasite line. However, EBA-175 is functional, and this has been demonstrated by a significant decrease in the ability of Δ EBA-175 3D7 parasites to invade chymotrypsin-treated erythrocytes (Duraisingh et al., 2003).

These parameters provided a measure of EBA-175 function in merozoite invasion and allowed us to test the functionality of mutant EBA-175 ligands in transgenic parasites. To determine the effect of the different mutations in the EBA-175 cytoplasmic domain on the function of this protein in merozoite invasion, we first tested the ability of the transgenic parasites to invade neuraminidase-treated erythrocytes (Reed et al., 2000a; Duraisingh et al., 2003). The loss of EBA-175 function in transgenic parasites that resulted in an increased invasion of neuraminidase-treated erythrocytes was confirmed by testing their ability to invade chymotrypsin-treated cells.

W2mefY1419, W2mefY1464, W2mefYY, W2mefEDD, W2mef Δ 15, and W2mefY Δ 15 invaded neuraminidase-treated erythrocytes as inefficiently as parental W2mef and the transfection control W2mef3'R, suggesting that EBA-175 was fully functional (Fig. 4 A). This suggested that these specific amino acid residues are not required for the function of EBA-175 in *P. falciparum*. This was in contrast to the loss of function of EBA-175 as a result of deletion of 54 amino acids from the cytoplasmic tail. W2mef Δ tail parasites that express a truncated form of EBA-175 lacking the cytoplasmic domain invade neuraminidase-treated erythrocytes efficiently (77%), whereas parental parasites W2mef and the transfection control (W2mef3'R) invade at \sim 16% compared with the invasion of untreated RBCs (Fig. 4 A). The loss of function of the truncated EBA-175 proteins was verified by demonstration that W2mef Δ tail parasites invade chymotrypsin-treated erythrocytes significantly less than W2mef and W2mef3'R parasites (Fig. 4 B). This switch in invasion phenotype was analogous to that seen for the W2mef Δ 230 parasites (Fig. 4, A and B; Reed et al., 2000a; Duraisingh et al., 2003). The baseline level for reinvasion between mutant EBA-175 parasites and wild-type parental lines is higher compared with that described previously (Duraisingh et al., 2003). This is likely to be a reflection of the altered quantification methods and assay conditions used in the independent studies; however, importantly, the magnitude of the differences is similar in this paper compared

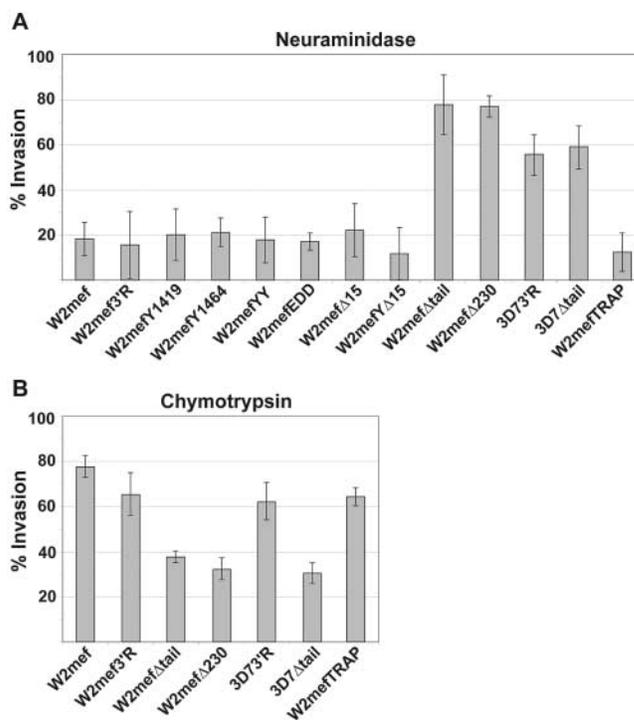


Figure 4. The deletion of the cytoplasmic domain of EBA-175 leads to a switch in the invasion phenotype. The TRAP cytoplasmic tail can reconstitute the function of the same region in EBA-175 for merozoite invasion. The ability to invade neuraminidase-treated erythrocytes is indicative of a switch in invasion to sialic acid independence and loss of EBA-175 function and this loss of function was confirmed directly by measurement of the ability to invade chymotrypsin-treated erythrocytes. RBCs were treated with neuraminidase (A) or chymotrypsin (B) as described in Materials and Methods before testing in merozoite invasion assays. Figures shown are the percentage of invasion compared with untreated erythrocytes. Error bars correspond to standard deviation. The data obtained with neuraminidase-treated erythrocytes were from four independent experiments for W2mef, W2mef3'R and W2mef Δ tail but only two independent experiments for W2mefTRAP. All experiments were performed in triplicate. The data obtained in panel B with chymotrypsin-treated erythrocytes represent one experiment done in triplicate. A second independent experiment has been performed and the results obtained are essentially the same as described here.

with previous data (Reed et al., 2000a; Duraisingh et al., 2003).

To determine the effect of the loss of the EBA-175 cytoplasmic tail in parasites such as 3D7, which invade primarily using nonsialic acid processes, we tested the ability of 3D73'R and 3D7 Δ tail to invade neuraminidase- and chymotrypsin-treated erythrocytes (Fig. 4, A and B). As expected, there was no significant difference between 3D73'R, expressing full length EBA-175, and 3D7 Δ tail in their ability to invade neuraminidase-treated erythrocytes. This is in agreement with our previous results comparing 3D7 and Δ EBA-175 3D7 parasites (Duraisingh et al., 2003). However, 3D7 Δ tail invaded chymotrypsin-treated erythrocytes significantly less than 3D73'R parasites, consistent with the loss of function of the EBA-175 protein as a result of truncation of the cytoplasmic tail (Fig. 4 B). This suggests that the cytoplasmic domain of EBA-175 is essential for the function of this protein in parasite lines

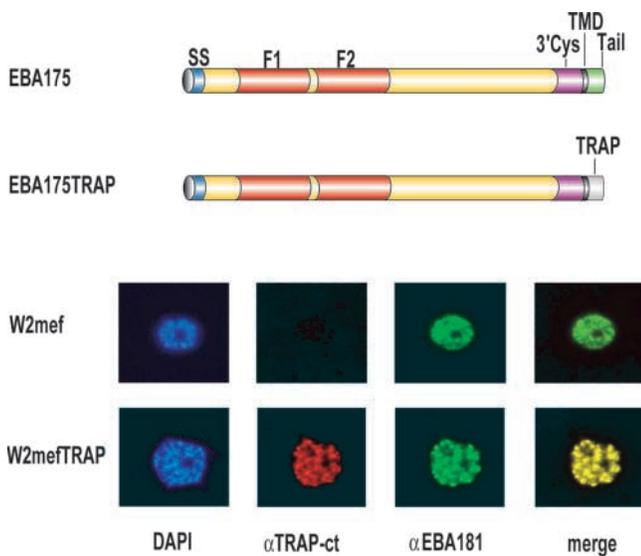


Figure 5. Expression and localization of EBA-175TRAP in transgenic parasites. Immunofluorescence assays using anti-TRAP-CT, anti-EBA-175 and anti-EBA-181 antibodies with parasite lines W2mef and W2mefTRAP are shown. Structural features of EBA-175 compared with the chimeric protein EBA-175TRAP. Nuclei are stained with DAPI in the first panel of each row. EBA-175 colocalizes with the microneme protein EBA-181 in W2mef parasites. EBA-175TRAP is not expressed in W2mef parasites but is expressed in W2mefTRAP transgenic parasites. It colocalizes with the micronemal protein EBA-181. The EBA-175 and EBA-181 patterns are merged to show colocalization in the last panel of each row.

that invade via sialic acid-independent or -dependent pathways. Importantly, the loss of function in W2mef Δ tail and 3D7 Δ tail parasites was not the result of incorrect trafficking or lower expression, but rather the inability of the truncated protein to participate in the invasion process.

The cytoplasmic domain of EBA-175 can be substituted by TRAP

EBA-175 functions in merozoite invasion of erythrocytes (Sim et al., 1994; Reed et al., 2000a), whereas TRAP is required for invasion of sporozoites into liver cells (Sultan et al., 1997). To test if the cytoplasmic tail of the TRAP protein was able to substitute for the same region in EBA-175, we replaced the COOH-terminal 54 amino acids with the last 37 amino acids of TRAP (Fig. 1 B). As expected, no fluorescence was detectable with anti-TRAP antibodies in W2mef. However, anti-TRAP-CT detected the EBA-175TRAP chimeric protein in W2mefTRAP parasites, and this protein showed colocalization with the micronemal marker EBA-181 in schizonts and merozoites (Fig. 3 and Fig. 5).

To test the functionality of the chimeric EBA-175TRAP protein in the transgenic cell line, we tested their ability to invade neuraminidase- and chymotrypsin-treated erythrocytes. Importantly, W2mefTRAP parasites invade neuraminidase- and chymotrypsin-treated erythrocytes at approximately the same efficiency as W2mef and W2mef3'R, conclusively demonstrating that the EBA-175TRAP chimera is fully functional (Fig. 4, A and B). Therefore, the cytoplasmic tail of TRAP can be substituted for the same domain from EBA-175 in merozoite invasion.

Discussion

The life cycle of *Plasmodia* is complex, requiring invasion of different cell types in the host and the mosquito vector. Recognition of the cell by the sporozoite and merozoite during the invasion process occurs by binding of specific ligands expressed in each life cycle stage to their cognate receptor on the target cell. The TRAP protein is essential for gliding motility and invasion of sporozoites into liver cells (Sultan et al., 1997; Kappe et al., 1999), whereas EBA-175 functions in merozoite invasion of erythrocytes using glycophorin A as receptor (Sim et al., 1994). The cytoplasmic tail of TRAP and EBA-175 show no obvious homology except a degenerate tyrosine-based motif and a high content of acidic amino acids (Di Cristina et al., 2000). It has been suggested that this region plays an important role in trafficking of the proteins to the micronemes, and interaction with the microfilament system via an actin-myosin motor to drive entry of the parasite into the host cell during the invasion process (Kappe et al., 1999; Bergman et al., 2003). Here, we have shown that the cytoplasmic tail of EBA-175 is required for the role of this protein in merozoite invasion and that the same region of the sporozoite TRAP protein is functionally equivalent. The functional homology of the EBA-175 cytoplasmic tail with that of TRAP suggests that the protein-protein interactions involved in linking these proteins to the sporozoite and merozoite molecular machinery for invasion into such different host cells as hepatocytes and erythrocytes are the same for the parasite life cycle stages.

Specificity of host cell target for sporozoites and merozoites presumably resides in the specific ligand(s) expressed in each life cycle stage. For example, merozoites express the ligands EBA-175 and EBA-140, and these bind glycophorin A (Sim et al., 1994) and C (Maier et al., 2003; Mayer et al., 2001; Thompson et al., 2001). In contrast, the TRAP protein in sporozoites binds to receptors on hepatocytes, probably via heparin sulfate or certain sulfated glycoconjugates (Holt et al., 1990; Cerami et al., 1992). After initial receptor binding of the ligands, they presumably participate in movement of the tight junction between the host cell and merozoite or sporozoite by binding of the cytoplasmic tail either directly or via an adaptor protein to an actin-myosin based motor system (Barnwell and Galinski, 1998; Pinder et al., 1998; Opitz and Soldati, 2002). The ability to substitute the cytoplasmic tail of TRAP for that of EBA-175 suggests that both are able to bind to the same adaptor protein required for merozoite invasion via glycophorin A on the erythrocyte.

Analysis of TRAP cytoplasmic tail function in the mouse malaria *Plasmodium berghei* (*Pb*TRAP) has shown that specific alterations can block infectivity of hepatic cells and in addition, drastically modify gliding motility (Kappe et al., 1999). It has been suggested that the *Pb*TRAP tail is bifunctional, with the proximal part required for protein translocation along the cortical microfilaments, whereas the distal region contains the signal for release of TRAP at the posterior end, probably by proteolytic cleavage. A penultimate tryptophan residue and a group of acidic amino acid residues present near the COOH terminus of the *Pb*TRAP tail have been shown to be critical for invasion and normal gliding movement (Kappe et al., 1999). The tryptophan residue is

conserved in a number of putative TRAP paralogues and orthologues that function in sporozoites and tachyzoites from Apicomplexan parasites; however, it is not present in EBA-175 or any of its paralogues known to be involved in merozoite invasion of erythrocytes (Fig. 1 A). Additionally, the acidic residues at the COOH terminus of EBA-175 are not required for normal function in merozoite invasion, and these results suggest differences in the role of the EBA-175 cytoplasmic tail compared with that suggested for TRAP. The EBA-175 tail may be involved in protein translocation along the cortical microfilaments, probably via an adaptor protein similar to that envisaged for TRAP, but it is likely that release at the posterior pole of the sporozoite or merozoite for the two proteins may involve different processes (Sultan et al., 1997; Kappe et al., 1999). It would be interesting to test the interchangeability of the cytoplasmic domains for sporozoite invasion by generating a parasite line with a chimeric TRAP/EBA175 protein, either with or without an additional penultimate tryptophan.

The ability to substitute the EBA-175 cytoplasmic tail with the equivalent region of TRAP would imply amino acid conservation. However, there is no obvious homology in this region between EBA-175 and TRAP, suggesting that the functional determinants are dependent on the tertiary structure of the cytoplasmic domains. This is consistent with the lack of amino acid homology between the EBA-175 cytoplasmic tail and its paralogues, such as EBA140 and EBA-181 (Fig. 1 A; Gilberger et al., 2003; Maier et al., 2003). The main feature of the cytoplasmic domain of these proteins is their highly charged nature, and it is likely that this is a key element in interaction with a putative adaptor protein and the merozoite invasion machinery. To further analyze sequence requirements for the proposed interaction, it would be of great interest to test if a charged mutant and/or random cytoplasmic domain of approximately the same size could functionally substitute the endogenous EBA-175 domain.

The general importance of the cytoplasmic domain is demonstrated by the deletion of the cytoplasmic domain of EBA-175 in either W2mef or 3D7 (W2mef Δ tail and 3D7 Δ tail) parasites that express functionally inactive forms of this ligand. The reported phenotype of these parasite lines was not due to lower expression of the mutant protein, as previously described for certain circumsporozoite protein mutants in *P. berghei* (Thathy et al., 2002), nor was it a result of incorrect trafficking as the protein was localized to the micronemes (Di Cristina et al., 2000). The EBA-175 Δ tail mutants show a complete loss of EBA-175 function in both sialic acid-dependent and -independent parasites.

The importance of specific amino acid motifs in the cytoplasmic tail of type 1 transmembrane proteins for post-secretory targeting to their subcellular location has been intensively studied in higher eukaryotes. These proteins enter the secretory pathway with their NH₂-terminal signal sequence that cotranslationally direct the proteins into the ER. Type 1 transmembrane proteins can have tyrosine-based motifs in their cytoplasmic tail required for post-Golgi sorting by binding to adaptor protein complexes that are involved in vesicular transport (Bonifacino and Dell'Angelica, 1999). In contrast to this detailed knowl-

edge, very little is known about the molecular mechanisms of protein trafficking in Apicomplexa. It has been shown in *T. gondii* that tyrosine-based motifs and an acidic amino acid motif are also required for sorting and trafficking of type 1 membrane proteins to the micronemes and rhoptries (Di Cristina et al., 2000; Hoppe et al., 2000; Reiss et al., 2001). This is in contrast to EBA-175 in *P. falciparum*, a protein that does not require tyrosine-based motifs or indeed the cytoplasmic tail for correct sorting to the micronemes, although this region is essential for the function of this protein in invasion.

The rhoptry protein RAP1 acts as an escorter for RAP2 in *P. falciparum*, suggesting that complex formation may be important in sorting and subcellular targeting in Apicomplexan parasites (Baldi et al., 2000). Similarly, in *T. gondii*, the type 1 transmembrane protein MIC6 and MIC8 act as escorters for the soluble proteins MIC1, MIC4, and MIC3 for trafficking to the micronemes, and these complexes appear to play an important role in the invasion of tachyzoites into mammalian cells (Reiss et al., 2001). The cytoplasmic domains of MIC6 and MIC8 appear to contain the information required for correct microneme localization of the complexes, as has been shown for MIC2 (Di Cristina et al., 2000). Deletion of the transmembrane and cytoplasmic domain of MIC8 led to incorrect localization of the protein (Cerede et al., 2002), and this is similar to the results observed with deletion of the 3' cysteine-rich region, transmembrane, and cytoplasmic domain of EBA-175 (Fig. 3). However, it was unexpected that the cytoplasmic domain of EBA-175 was not required for protein sorting to the micronemes. Correct sorting of truncated EBA-175 lacking the cytoplasmic tail to the micronemes suggests another protein with the necessary localization signals may be involved in directing this protein and its paralogues to their subcellular localization.

The results presented here suggest a model for trafficking of EBA-175 that involves an escorter protein specifying the targeting information required for microneme localization (Fig. 6, A and B). We presume that EBA-175 and other microneme proteins are processed through the classical secretory pathway involving the ER and Golgi followed by unknown sorting machinery in the TGN (Fig. 6 A). The trafficking of EBA-175 to the micronemes is independent of the cytoplasmic domain, and we propose that an escorter protein forming a complex is required for this specific sorting event (Fig. 6 B). The inability of EBA-175 Δ 230 protein to be trafficked to the micronemes suggests that the cysteine-rich region and/or the transmembrane domain are involved in complex formation. It is likely that the 3' cysteine-rich region is important in protein sorting, as it is conserved in other paralogues that function in merozoite invasion, and it is likely that these proteins are sorted and localized to the micronemes by identical mechanisms.

Furthermore, functional EBA-175 requires linkage of the cytoplasmic domain to the invasion machinery either directly or via an adaptor protein (Fig. 6 C). This interaction can be substituted by the cytoplasmic tail of TRAP, suggesting that this family of proteins have the information required for function in the invasion process encrypted in their cytoplasmic tail. An increased understanding of the

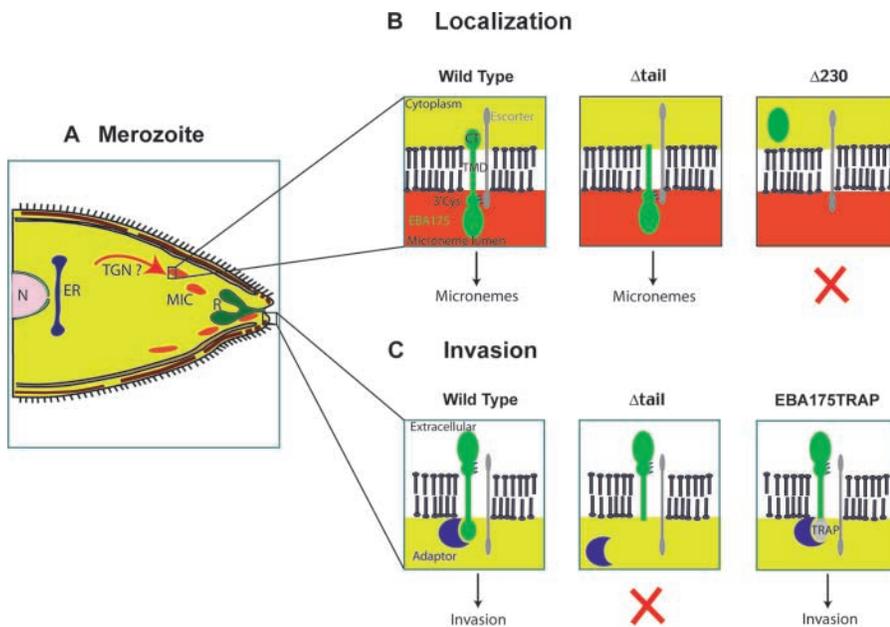


Figure 6. **A proposed model of trafficking and function for EBA-175.** (A) Schematic representation of a merozoite.

Secretory proteins enter the lumen of the endoplasmic reticulum (ER) with their NH₂-terminal signal peptide. The molecular mechanism of pre- and post-Golgi trafficking (TGN) and the differential sorting of microneme (MIC) and rhoptry (R) proteins to their final destination is unclear.

(B) In a post-Golgi sorting event, EBA-175 is trafficked to the micronemes by an escorter protein. The protein-protein interaction may take place via the conserved 3' cysteine-rich region. The information of neither microneme localization nor interaction with the escorter protein is encrypted in the cytoplasmic domain of EBA-175. Deletion of the cytoplasmic domain does not affect sorting to the micronemes. Sorting was disrupted by deletion of the transmembrane domain and the 3' cysteine rich region.

(C) The intracellular function of EBA-175 is dependent on the interaction of an adaptor protein with the cytoplasmic domain. The deletion of this region abolishes the binding of the adaptor protein and leads to disruption of its function. The cytoplasmic domain can be substituted by the cytoplasmic domain of the sporozoite protein TRAP, suggesting that EBA-175 and TRAP have functionally equivalent roles in the active invasion process.

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role of the cytoplasmic tail of these proteins in invasion will help identify potential new drug targets that block the infectivity of this parasite at the liver and erythrocyte stage of the life cycle.

Materials and methods

Parasite strains and transfection

P. falciparum asexual stages were cultured in human O⁺ erythrocytes according to standard procedures (Trager and Jensen, 1976). Human O⁺ were provided by the Blood Bank, Melbourne (Melbourne, Australia). W2mef is derived from the Indochina III/CDC strain. W2mef and 3D7 parasites were transfected as described previously (Crabb and Cowman, 1996; Wu et al., 1996) with 80 μ g purified plasmid DNA (QIAGEN). Positive selection for transfectants was achieved using 10 nM WR99210, an antifolate that selects for the presence of the human *dhfr* gene (Fidock and Wellems, 1997).

Nucleic acid and sequence analysis

Chromosome 7 of *P. falciparum* was sequenced by the Sanger Center (<http://www.sanger.ac.uk>; Gardner et al., 2002). Preparation of genomic DNA was performed as described previously (Triglia et al., 1998). DNA sequencing was performed using BigDye[®] Terminator Cycle sequencing (PerkinElmer). Southern blotting was performed using standard procedures. In vitro mutagenesis of the 3' region of *EBA-175* was achieved using cDNA in either a one- or two-step primer-directed PCR mutagenesis method with proof reading Vent polymerase (Stratagene). An ~1-kb fragment of the 3' end of the *EBA-175* gene was amplified with the following primers and sequenced in order to detect unwanted mutations: 175-S, 5'-TGAGGATCCAGGAAATGATACATCT-GAAATGTCGCA-3'; 175 3'R-AS, 5'-AGTCTCGAGTCATATCTTAAATTTAATATCATT-3'; 175Y₁₄₆₄-AS, 5'-GCGCCTCGAGTCATATCTTAAATTTAATATCATTATATCCTCATGCCTTCAG-3'; 175EDD₁₄₇₀-AS, 5'-AGTCTCGAGTCATATCTTAAATTTAATACCATTATACCCCATGGTA-3'; 175 Δ 15-AS, 5'-GCGCCTCGAGTCAATCGTTGATATTAGTTT-3'; 175 Δ tail-AS, 5'-AGCCTCGAGTCAACTTTGATATTTGGCTTGTGA-3'; 175Y₁₄₁₉-AS, 5'-CTTTGACCTTTGGCTTGTGAAGC-3'; 175Y₁₄₁₉-S, 5'-GCTTCACAAGCCAAAGGCAAAG-3'; 175TRAP-AS, 5'-GGCAAAGGGTGTTCACCTTTGATATTTGGCTTGTGAAGC-3'; TRAP-S, 5'-GCAACACCTTTGCCGGA-3'; TRAP-AS, 5'-AGTCTCGAGTCAATTCACCTCGTTTCTTC-3'. The bases in italics refer to restriction enzyme sites used for cloning.

All PCR products were subcloned into BamHI/XhoI pBluescript[®], amplified, and digested with BamHI and XhoI. Using the compatibility of the 5' overhangs of BglIII and BamHI, the fragments were subsequently cloned

into a BglIII/XhoI precut pHH1 transfection vector (Reed et al., 2000b). To construct pHH1-YY and pHH1Y Δ 15, the pBluescript[®] construct pBSEBA175Y₁₄₁₉ was used as a template for PCR mutagenesis.

Antisera and immunoblots

Rabbit and mouse antisera were raised against the cytoplasmic domain of EBA175 and P_{TRAP} using the pGEX system (Amersham Biosciences). Other antibodies used in immunodetection were rabbit anti-EBA175 (Reed et al., 2000b) and anti-HSP70 antibodies. For immunoblots, parasite proteins from a synchronized culture were separated on 6% SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher & Schuell). EBA175-CT, TRAP-CT, and EBA-175 rabbit antisera were diluted 1:250, and HSP70 antisera was diluted 1:5,000 in PBS with 1% wt/vol skim milk. The secondary antibody was sheep anti-rabbit IgG HRP (Silenus Laboratories). The immunoblots were developed by chemiluminescence using ECL (Amersham Biosciences).

Real-time RT-PCR

Synchronized parasites were harvested in the late schizont stage and total RNA was isolated using TRIzol[®] (Invitrogen). RNA was further purified by DNase digestion on an RNeasy[®] column (QIAGEN). 2 μ g of total RNA were reverse transcribed either with or without SuperScript[™] II using Anchor T and random nonamers (Invitrogen). The LightCycler[™] (Roche) was used to quantitate cDNA using QuantiTect SYBR[®] Green PCR Kit (QIAGEN) and gene specific primers. Reaction conditions were 94°C for 15 min, 45°C for 20 min, and 65°C for 30 min. EBA175F, 5'-AATTTCTGTAAATATTGTGACCATAT-3'; EBA175R, 5'-GATACTGCACAACACAGATTCTT-3'; ACTINF, 5'-TGCACCACAGAGAAAAT-3'; ACTINR, 5'-ACTTGGTCTGATTCATCGT-3'; H2BF, 5'-TGGTCCAGATGGAAA-GAAA-3'; and H2BR, 5'-TCGAAAGTATCAACAAGGAATG-3'.

Serial dilutions of 3D7 genomic DNA were used as a standard reference control. The relative expression ratios of EBA175 compared with the two reference genes, actin and histone 2B, were calculated for each strain after subtracting the signal generated by samples with no reverse transcriptase.

Immunofluorescence

Immunofluorescence assays were performed with synchronized parasites. Late schizonts were smeared, air dried, and fixed for 1 min with 100% methanol at -20°C. Slides were incubated for 1 h with a mixture of rabbit anti-EBA175 (1:1,000) or anti-TRAP-CT (1:1,000) and mouse anti-EBA181 (1:1,250), washed three times for 10 min with 0.05% Tween 20-PBS, and then incubated for 1 h with fluorescein isothiocyanate-labeled sheep anti-mouse IgG antibodies (Silenus Laboratories), rhodamine-labeled goat anti-rabbit IgG antibodies (CHEMICON International) and DAPI (1:1,250;

Boehringer). Dual-color fluorescence images were captured using a microscope (Axioskop 2; Carl Zeiss MicroImaging, Inc.) and a digital camera (PCO sensicam).

Erythrocyte enzyme treatments and invasion assay

Parasite erythrocyte invasion assays were performed to characterize the invasion phenotype of the W2mef-EBA175 transgenic lines (Reed et al., 2000a). Surface modification of the erythrocytes was achieved by similar treatment as described elsewhere (Dolan et al., 1994): 500 μ l 1 μ g/ml chymotrypsin of packed erythrocytes were incubated with either 50 mU neuraminidase or 1.5 mg/ml trypsin. Tightly synchronized parasite cultures were treated with neuraminidase and trypsin to prevent reinvasion. Ring stage cultures were adjusted to 2% parasitemia and treated with neuraminidase for 1 h at 37°C (50 mU for 200 μ l packed, infected erythrocytes in 1 ml RPMI 1640/Hepes/0.2% NaHCO₃). After washing twice with RPMI 1640/Hepes/0.2%NaHCO₃, the infected cells were incubated with trypsin (1 mg/ml in RPMI 1640/Hepes/0.2% NaHCO₃) for 1 h at 37°C followed by incubation with trypsin inhibitor (1 mg/ml in RPMI 1640/Hepes/0.2% NaHCO₃; Sigma-Aldrich) for 10 min at RT. The cultures were washed, resuspended in complete medium, and incubated overnight under standard conditions. The invasion assays were set up the next day by mixing equal amounts of infected double-enzyme treated cells (schizont stage) with uninfected normal or enzyme-modified erythrocytes to a final hematocrit of 3%. 100- μ l aliquots of each culture in triplicate were put into a 96-well microtiter plate. The parasitemia in each well was counted by FACScan™ using Retic-COUNT™ (Becton Dickinson). After 36 h of incubation, the parasitemia of each well was counted again by FACScan™. Parasitemias were confirmed by microscopic counting of Giemsa-stained thin blood smears and subsequently compared with the starting parasitemia. Control wells with unstained culture and stained uninfected erythrocytes were included in FACScan™ and in the microscopic counting to enable background correction.

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