Published in final edited form as:

Mol Biochem Parasitol. 2012 May ; 183(1): 23–31. doi:10.1016/j.molbiopara.2012.01.002.

Identification of a specific region of *Plasmodium falciparum* EBL-1 that binds to host receptor glycophorin B and inhibits merozoite invasion in human red blood cells

Xuerong Li^{1,2,‡}, Marina Marinkovic^{1,‡}, Crystal Russo¹, C. James McKnight³, Theresa L. Coetzer⁴, and Athar H. Chishti^{1,2,*}

¹Department of Molecular Physiology and Pharmacology, Tufts University School of Medicine, Boston, MA 02111, USA, ¹Sackler School Programs in Physiology, Pharmacology, and Microbiology

²Department of Pharmacology, University of Illinois College of Medicine, Chicago, IL 60612, USA

³Department of Physiology and Biophysics, Boston University School of Medicine Boston, MA 02118

⁴Department of Molecular Medicine and Haematology, University of the Witwatersrand, National Health Laboratory Service, Johannesburg, South Africa

Abstract

The malaria parasite *Plasmodium falciparum* invades human erythrocytes through multiple pathways utilizing several ligand-receptor interactions. These interactions are broadly classified in two groups according to their dependency on sialic acid residues. Here, we focus on the sialic acid-dependent pathway by using purified glycophorins and red blood cells (RBCs) to screen a cDNA phage display library derived from *Plasmodium falciparum* FCR3 strain, a sialic acid-dependent strain. This screen identified several parasite proteins including the erythrocyte-binding ligand-1, EBL-1. The phage cDNA insert encoded the 69-amino acid peptide, termed F2i, which is located within the F2 region of the DBL domain, designated here as D2, of EBL-1. Recombinant D2 and F2i polypeptides bound to purified glycophorins and RBCs, and the F2i peptide was found to interfere with binding of D2 domain to its receptor. Both D2 and F2i polypeptides bound to trypsin-treated but not neuraminidase or chymotrypsin-treated erythrocytes, consistent with known glycophorin B resistance to trypsin, and neither the D2 nor F2i polypeptide bound to glycophorin B-deficient erythrocytes. Importantly, purified D2 and F2i polypeptides partially inhibited merozoite reinvasion in human erythrocytes. Our results show that the host erythrocyte receptor glycophorin B directly interacts with the DBL domain of parasite EBL-1, and the core binding site is contained within the 69 amino acid F2i region (residues 601–669) of the DBL domain. Together, these findings suggest that a recombinant F2i peptide with stabilized structure could provide a protective function at blood stage infection and represents a valuable addition to a multi-subunit vaccine against malaria.

© 2012 Elsevier B.V. All rights reserved.

*Corresponding author at: TUSM, 150 Harrison Avenue, Jaharis Building, Room 714, Boston, MA 02111, U.S.A., Tel +1-617-636-3457, Fax +1-617-636-3568, athar.chishti@tufts.edu.

‡Authors contributed equally to this work.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Malaria; Phage display; *Plasmodium falciparum*; Erythrocytes; Glycophorin B; EBL-1

1. Introduction

Plasmodium falciparum merozoites are known to use multiple pathways to invade human red blood cells (RBCs) [1, 2]. These pathways are grouped into two classes depending upon the presence of sialic acid on host receptors. Some parasite strains use ligands that preferentially bind to host receptors lacking sialic acid, whereas others, such as FCR3, are restricted to the sialic acid-dependent pathways [3]. Neuraminidase treated RBCs lack sialic acid residues, and are known to be highly resistant to certain strains of *P. falciparum* including FCR3, Camp, Dd2, and FVO [3]. The *P. falciparum* FCR3 strain is completely dependent upon the presence of sialic acid residues for invasion, since it is unable to propagate in neuraminidase-treated RBCs [3]. The major sialoglycoproteins in the RBC membrane are glycophorins and thus are implicated in merozoite invasion [4–6]. Soluble glycophorins as well as antibodies against glycophorins block parasite invasion *in vitro* [7, 8]. Moreover, RBCs genetically deficient in specific glycophorins are known to confer partial resistance to invasion by *P. falciparum* [9, 10]. The four glycophorins in human erythrocytes, glycophorin-A (GPA), glycophorin-B (GPB), glycophorin-C (GPC), and glycophorin-D (GPD) constitute ~2% of the total membrane protein mass. GPA and GPB are encoded by two distinct genes, with GPA constituting the dominant glycophorin in human erythrocytes. In contrast, GPC and GPD are encoded by a single gene. GPD contains a truncated amino terminal cytoplasmic domain, so that the carboxyl-terminal amino acids (residues 21–128) are identical between GPD and GPC [11]. Thus, human erythrocytes carry three glycophorins with distinct extracellular domains, and these proteins serve as the main receptors involved in the sialic acid-dependent pathways.

The first *P. falciparum* ligand identified that mediates RBC invasion through a sialic acid-dependent pathway was the erythrocyte-binding antigen-175 (EBA-175). EBL-175 binds to GPA, the most abundant glycophorin in human RBCs, and this biochemical interaction has been extensively characterized [12–15]. EBA-175 belongs to the Duffy binding-like (DBL) protein family, which includes EBA-140 (BAEBL), EBA-165 (PEBL), EBA-181 (JESEBL), and EBL-1 [16]. EBA-165 is unlikely to play any direct role in invasion since it does not express a functional protein due to a frame shift mutation in the coding region [17]. EBA-140 specifically binds to GPC, binding is sialic acid-dependent, and it is unable to bind to Gerbich negative human erythrocytes lacking exon 3 in the glycophorin C gene [18, 19]. Similarly, the Leach phenotype erythrocytes, lacking GPC, are partially resistant to invasion by *P. falciparum* [20]. The molecular identity of the erythrocyte receptor for EBA-181 is unknown at present. EBA-181 binds to RBCs in a sialic acid-dependent manner. The receptor is trypsin-resistant and chymotrypsin-sensitive, but the receptor is not GPB. Moreover, the EBA-181 knockout parasites showed no change in invasion efficiency, implying possible redundancy of this interaction [21, 22]. A phage display screen identified the 10 kDa domain of erythrocyte membrane protein 4.1R as a binding motif for parasite EBA-181, suggesting a cytoskeletal function of EBA-181 under certain conditions [23]. Recently, Mayer and colleagues reported the identification of GPB as a host receptor for parasite EBL-1 [24]. Their finding originated from the highly polymorphic nature of the GPB gene, particularly the unusually high occurrence of the GPB-null phenotype in the Efe pygmies of the Democratic Republic of Congo [25].

In this study, we utilized purified glycophorins and human RBCs to screen a phage display cDNA library from FCR3, a sialic acid-dependent *Plasmodium falciparum* strain. Several

parasite proteins, including EBL-1, were identified from the library screen as they specifically bind to both glycoporphins and RBCs. Here, we provide evidence that the major binding site of EBL-1 to GPB is located within the 69-amino acid segment, termed F2i, of the D2 domain of EBL-1. Importantly, both recombinant F2i peptide and D2 domain recognizing the GPB partially inhibited the merozoite invasion of human erythrocytes.

2. Materials and Methods

2.1. Parasite culture

P. falciparum (FCR3 strain) was maintained continuously in a culture using standard conditions [26]. The parasite culture was maintained in 75 cm² flasks at 37°C under 5% CO₂, 5% O₂, and 90% N₂ at 5% hematocrit of fresh type O⁺ human erythrocytes in a complete medium containing RPMI-1640 medium with L-glutamine and 25 mM HEPES (Invitrogen) supplemented with 10 mg/ml gentamycin, 200 μM Hypoxanthine, 0.2% NaHCO₃, and 0.5% Albumax II (Invitrogen), and adjusted to a pH of 7.4. Ring-stage parasites were synchronized using 5% sorbitol and the late stage parasites were enriched to greater than 95% parasitemia by centrifugation through a gradient of 65% (v/v) Percoll (GE Healthcare).

2.2. *P. falciparum* phage display library preparation

P. falciparum (FCR3 strain, asynchronous) T7 phage display cDNA library was generated and amplified by the liquid lysate amplification method as described before [27]. The typical size of the *P. falciparum* cDNA inserts was more than 722 bp [27]. The parasite phage library was propagated using a log-phase culture of *E. coli* BLT5403 and amplified by shaking for 2–3 hours at 37°C in LB medium containing carbenicillin (100 μg/ml) until cell lysis occurred. The phage particles were clarified by centrifuging the cell lysate at 12,000 × g for 20 minutes. The amplified-library lysates were stored at 4°C for short term or at –70°C with 15% glycerol for long term storage. The library titer was determined by the plaque assay.

2.3. Phage display library screen using purified human glycoporphins or intact RBCs

The biopanning technique was used to screen the *P. falciparum* phage library against purified human MN-glycoporphins (Sigma, #G5017) or freshly isolated RBCs [28]. Four rounds of biopanning were performed on immobilized glycoporphins on the ELISA plate or on RBCs. The final phage solution was titrated, and the plaques were grown in LB medium containing carbenicillin (100 μg/ml) for 3h at 37°C. The phage DNA was then extracted from individual amplified phage plaques using the QIAprep Spin M13 kit (Qiagen). Partial sequencing of the phage DNA was carried out with T7 select down primer 5'-AACCCCTCAAGACCCGTTTA-3' using the BigDye Terminator version 3.0 Cycle Sequencing Kit (Applied Biosystems).

2.4. Recombinant protein expression and purification in *E. coli*

The Duffy-binding-like (DBL) domain (D2) of EBL-1 gene (GenBank accession number: AY769519) was amplified by PCR from *P. falciparum* (FCR3) genomic DNA and cloned into pET32a (Novagen) to generate recombinant plasmid pET32a-D2. The PCR primers used were 5'-GCCGGATCCTGTGGGAAGAAAATAAAGG-3' (sense, BamHI) and 5'-GCCGTCGACACATACCATACAAGCCTCT-3' (antisense, SalI). Recombinant D2 of EBL-1 was expressed in *E. coli* BL21(DE3) as a fusion to the thioredoxin, Trx, and His₆ tag. The thioredoxin tag was chosen to enhance solubility of the recombinant protein. The soluble Trx-D2 fusion protein was affinity-purified using Ni-NTA beads. A 207-bp region of F2 in EBL-1 domain 2 (named here as F2i), which corresponded to the phage insert

cDNA sequence of EBL-1 recognized in the screen using RBCs and glycophorins, was PCR amplified from pET32a-D2 and cloned into pGEX-6P-2 (GE Healthcare) using the forward primer 5'-GCCGGATCCTGCAATGCTATATTGGGAAG-3' (sense, BamHI) and reverse primer 5'-GCCGTCGACTACCATACTAGACCATATTA-3' (antisense, SalI). The recombinant F2i was expressed in *E. coli* DH5 α as a fusion to GST. The soluble GST-F2i fusion protein was affinity-purified using GSH beads. Expression of recombinant proteins was induced by 0.4 mM IPTG at 25°C for 3 hours, cells were pelleted at 3,750 rpm for 15 minutes in a Sorvall 5C RC Plus at 4°C, and cell lysis was carried out in the presence of lysozyme (1.0 mg/ml), DNase II (5.0 ug/ml), and RNase (10.0 ug/ml) with protease inhibitors for 30 minutes on ice, followed by sonication and centrifugation at 3,750 rpm for 20 minutes to recover the soluble fractions for affinity purification. The identity of each fusion protein was confirmed by Western blotting using HRP-conjugated anti-His₆ monoclonal antibodies (Santa Cruz Biotechnology; Invitrogen) and anti-GST polyclonal antibody (Santa Cruz Biotechnology). It is to be noted that due to the instability and storage conditions of various fusion proteins, purification of recombinant proteins was repeated several times with consistent results across different protein preparations. For example, data shown in Figures 3 A–C and Figure 4 A–B were confirmed with multiple preparations of TRX-D2.

For the circular dichroism (CD) measurements, the F2i fragment was subcloned into pET32a using BamHI and SalI sites and expressed in *E. coli* BL21(DE3). The expression of Trx-F2i was induced by 0.4 mM IPTG at 25°C for 4h. The cell lysis was carried out in the presence of 20 mM sodium phosphate, pH 7.8, 500 mM NaCl, 1.0 mg/ml lysozyme, 10% glycerol, and multiple protease inhibitors. After incubation for 30 min on ice, the cell pellet was sonicated, treated with 5.0 ug/ml DNAase II and 10 ug/ml RNase A, and centrifuged to recover the soluble fraction. The soluble Trx-F2i protein was affinity-purified using Ni-NTA beads in the presence of 10% glycerol, and dialyzed to a final buffer concentration of 4.0 mM sodium phosphate, pH 7.8, and 5% glycerol for CD analysis.

2.5. Circular dichroism spectroscopy

The CD spectra were collected using an Aviv 62DS spectrometer. Both Trx-F2i and Trx proteins were measured in buffer consisting of 4.0 mM sodium phosphate, 5% glycerol, pH 7.8, and the CD spectra were generated using a 2 mm path length cuvette containing 200 μ L of 5.0 mM sample at 20°C. The scans were performed with five repeats at an average of 1.0 nm increment recordings from 190 to 250 nm. The sample concentrations were determined using absorbance at 280 nm.

2.6. ELISA

Purified glycophorins (1–2 μ g) in 100 μ l of 100 mM NaHCO₃ buffer (pH 9.5) were added to the wells of a 96-well ELISA plate (Corning). The plate was incubated at 4°C overnight. The wells were blocked for at least 2 h at room temperature with 300 μ l of the blocking buffer PBST (3% BSA in 0.1% Tween-20, 3.2 mM Na₂HPO₄, 0.5 mM KH₂PO₄, 1.3 mM KCl, 135 mM NaCl, pH 7.4). Trx-D2 and GST-F2i protein were added to the coated wells in varying concentrations (0–10 μ M) and incubated at room temperature for 3 h. The wells were washed with PBST, and each sample was incubated with HRP-conjugated anti-His₆ and anti-GST antibodies, respectively, for 2 h at room temperature. The wells were washed with PBST extensively, and bound proteins were detected using a solution of TMB substrate (Pierce). The reaction was stopped after 15 min by adding 100 μ l of 2.0 M H₂SO₄, and the color was measured at 450 nm. Trx and GST tag proteins were used as negative controls and subtracted for each Trx-D2 and GST-F2i reading, respectively. The dissociation constants (means $\pm$ S.E.) were determined from duplicate experiments.

2.7. Binding of recombinant D2 domain and F2i peptide of EBL-1 to RBCs

For the binding assays, the Trx-D2 or GST-F2i fusion proteins were incubated with 50 μ l of packed RBCs in RPMI-1640 medium to a final volume of 800 μ l for 2h at room temperature with gentle rotation. The mixture was then placed on top of 500 μ l of silicon oil and centrifuged at 14,000 g for 1 min. The supernatant and silicon oil were removed and bound proteins were eluted by incubating with 20 μ l of 1.5 M NaCl for 10 min at room temperature, followed by centrifugation at 12,000 g for 1 min. The eluted proteins were separated by SDS-PAGE and identified by immunoblotting.

The RBCs were treated with enzymes as described before [4]. Briefly, RBCs were incubated with neuraminidase (66 mU/ml), trypsin (1.0 mg/ml), and chymotrypsin (1.0 mg/ml) at 37°C for 1h, treated with soybean trypsin/chymotrypsin inhibitor, and extensively washed prior to binding assays. The efficiency of enzyme treatment was confirmed by Western blotting using anti-GPA, anti-GPC, and anti-Band 3 antibodies as previously described [29]. Glycophorin B-deficient (S-s-U) RBCs were obtained from a donor from the Virginia Blood Services (Richmond, VA) where the cells were tested to confirm the absence of cell surface GPB. The Trx-D2 fusion protein was incubated with 50 μ l of packed GPB-deficient cells in RPMI-1640 medium and treated as described before. Similarly, for the binding inhibition assays, the GST-F2i fusion protein (1 μ M or 10 μ M) was incubated with RBCs in RPMI-1640 medium for 1 h at room temperature, then, the Trx-D2 protein (1 μ M) was added and further incubated for 2 h at room temperature. The erythrocytes were sedimented and the bound proteins were eluted as above and analyzed by Western blotting. Equal amount of Trx (1 μ M) was used as a negative control.

2.8. Erythrocyte invasion assay

Trx-D2 and GST-F2i proteins were tested for their capacity to inhibit invasion of *P. falciparum* FCR3 merozoites to RBCs. Parasite cultures were synchronized using 5% sorbitol treatment, and schizont-stage infected-RBCs were purified by centrifugation on a 63% Percoll gradient. Parasites were washed with RPMI-1640 and mixed with uninfected RBCs in malaria culture medium. The mixture of infected and uninfected RBCs was seeded into a 96-well plate containing varying concentrations of Trx-D2 or GST-F2i protein (10 μ M, 5 μ M, 2.5 μ M, 1.2 μ M, 0.6 μ M, 0.3 μ M) to a final volume of 200 μ l, 2% hematocrit, and 2% parasitemia. Purified GST and Trx protein tags served as negative controls. Parasites were incubated for 18 to 20 h at 37°C to allow parasite reinvasion. Following the incubation period, thin smears of each well were made, and stained with Giemsa. Approximately 1,000 RBCs were counted for each sample to quantify parasitemia, and the percent inhibition of invasion was calculated relative to the control sample as follows: (parasitemia for GST or Trx control – parasitemia for sample containing GST-F2i or Trx-D2) / parasitemia for GST or Trx control. Experiments were carried out in triplicate, and each condition was performed at least twice. Data are presented as mean $\pm$ S.D.

3. Results

3.1. Plasmodium falciparum phage display cDNA library screen

To identify *P. falciparum* proteins interacting directly with glycophorins, purified glycophorins were used to screen a sialic acid-dependent strain of *P. falciparum* (FCR3) phage display cDNA library. After 4 rounds of biopanning against immobilized glycophorins, the phage DNA was extracted and sequenced from individual positive plaques. Sequence analysis of the plaques and subsequent BLAST searches using the databases at NCBI and PlasmoDB revealed several *P. falciparum* genes including EBL-1, EBA-175, and hypothetical proteins PFA0420w and MAL13P1.249. When RBCs were used as bait, nucleotide sequence analysis of the positive phage clones revealed that the cDNA

inserts encoded proteins EBL-1, EBA-175, MSP1, hypothetical proteins Pf07_0024, PFL1130c, PF13_0167, and PFF0220w. An identical 207-bp nucleotide sequence of EBL-1 was found in multiple phage clones using glycophorins and RBCs as bait. This cDNA insert segment, designated here as F2i, was in the correct open reading frame encoding 69 amino acids. The 69 amino acid insert spans from residues 601 to 669 of the coding sequence of EBL-1. Subsequent sequence alignment analysis placed the 69 amino acid insert, F2i, within the F2 segment of the DBL domain, D2, of EBL-1 (Fig.1).

3.2. Binding specificity of EBL-1 domains to glycophorins

Based on the results of phage display cDNA library screen, we tested direct binding of recombinant EBL-1 domains D2 and F2i to glycophorins using an ELISA. The D2 and F2i segments were expressed in *E. coli* and purified as Trx-D2 and GST-F2i fusion proteins, respectively. Both fusion constructs expressed as soluble proteins in bacteria (Fig. 2). Purified Trx-D2 protein expressed with the correct predicted molecular mass of 92 kDa (Fig. 2, lanes 1–3). Purified GST-F2i protein expressed as a band with the predicted molecular mass of 34 kDa, with additional truncated products including a dominant band running at the GST tag alone (Fig. 2, lanes 4–6). The expression of Trx-D2 and GST-F2i was confirmed by Western blotting using antibodies against His₆ and GST, respectively (Fig. 2).

To quantify direct interaction between EBL-1 domains and glycophorins, a serial dilution ELISA was performed using recombinant Trx-D2 and GST-F2i. Glycophorins were coated on the ELISA plate, followed by the incubation of serially diluted Trx-D2 or GST-F2i, establishing a concentration-dependent interaction between EBL-1 fusion proteins with the immobilized glycophorins (Fig. 3A). As negative controls, Trx and GST proteins showed little interaction with the immobilized glycophorins (Fig. 3A). Similarly, no signal was observed without any coating of glycophorins. Dissociation constants for direct binding of soluble Trx-D2 and GST-F2i to immobilized glycophorins were calculated to be 692 ± 41 nM and 836 ± 57 nM, respectively. Notwithstanding the potential modulatory effects of other glycophorins present in the mixture of glycophorins used in the ELISA, these results suggest that the F2i peptide in the context of the full length D2 domain of EBL-1 showed similar binding affinity for the immobilized glycophorin-B.

3.3. F2i segment mediates EBL-1 domain 2 interaction with human RBCs

To determine direct binding of EBL-1 domains with intact erythrocytes, the Trx-D2 and GST-F2i fusion proteins were individually incubated with freshly isolated erythrocytes. Both Trx-D2 and GST-F2i fusion proteins bound specifically to erythrocytes, whereas Trx and GST proteins in an equivalent concentration did not interact with erythrocytes (Fig. 3B, 3C). To investigate if the F2i segment interferes with the binding of the D2 domain, RBCs were pre-treated with GST-F2i fusion protein and then incubated with Trx-D2 protein. Binding of Trx-D2 protein to the RBCs was reduced with an increasing concentration of GST-F2i protein (Fig. 3B). The GST protein alone did not affect Trx-D2 binding to erythrocytes. Although a low-affinity interaction of erythrocytes with other regions of the D2 domain of EBL-1 cannot be ruled out at this stage, our data suggest that the D2 domain of EBL-1 interacts directly with the surface of intact erythrocytes, and the major binding site of the D2 domain is likely to be contained within the F2i segment.

3.4. Sialic acid-dependent interaction of EBL-1 D2 domain with erythrocytes

To further characterize the binding properties of Trx-D2 domain with intact erythrocytes, binding assays were performed using erythrocytes treated individually with neuraminidase, trypsin, and chymotrypsin. Bound proteins were eluted and analyzed by SDS-PAGE and Western blotting. The Trx-D2 domain fusion protein did not bind to either neuraminidase or chymotrypsin-treated erythrocytes indicating that the D2 domain of EBL-1 specifically

interacts with erythrocytes in a sialic acid-dependent manner (Fig. 4A). In contrast, the Trx-D2 domain bound to trypsin-treated erythrocytes with the same efficiency as normal untreated erythrocytes (Fig. 4A). These results indicate that the D2 domain of EBL-1 binds to human erythrocytes via a receptor that is trypsin-resistant but chymotrypsin-sensitive. Prior studies have established that glycoporphins A, C, and D are sensitive to trypsin treatment of intact erythrocytes [12, 18, 30, 31]. These observations, combined with direct interaction of D2 and F2i fusion proteins with purified glycoporphins (Fig. 3A), suggest that glycoporphin B is the receptor for EBL-1. This conclusion is consistent with the known characteristics of erythrocyte glycoporphin B that is sensitive to neuraminidase and chymotrypsin and resistant to trypsin. Our findings are also in agreement with the recently published evidence that glycoporphin B is the receptor for EBL-1 [24]

3.5. The D2 domain of EBL-1 does not bind to glycoporphin B null human erythrocytes

To further confirm the identity of glycoporphin B as a receptor for the D2 domain of EBL-1, we utilized human erythrocytes with complete deficiency of glycoporphin B (S-s-U-). Glycoporphin B deficient (S-s-U-) erythrocytes were obtained from the Virginia Blood Services where they had been tested to confirm the absence of cell surface glycoporphin B [24]. The hematological properties of glycoporphin B deficient (S-s-U-) erythrocytes are nearly identical to normal erythrocytes except for the lack of glycoporphin B [32]. This phenotype occurs with very low frequency, and previous studies have suggested that glycoporphin B plays a role in merozoite invasion as individuals lacking glycoporphin B are relatively less susceptible to malaria parasite infection [33, 34]. We performed binding assays using recombinant Trx-D2 domain of EBL-1 and glycoporphin B deficient human erythrocytes. Our data demonstrate that the Trx-D2 domain does not bind to erythrocytes lacking glycoporphin B (S-s-U-) as compared to normal glycoporphin B positive (+) erythrocytes (Fig. 4A, lane 7).

3.6. Inhibition of merozoite invasion by recombinant D2 and F2i fusions of EBL-1

To investigate the biological significance of molecular interactions between parasite EBL1 and erythrocyte glycoporphin B, erythrocyte reinvasion inhibition assays were performed using the sialic-acid dependent FCR3 strain of *P. falciparum*. Purified schizont stage parasite-infected erythrocytes were incubated with an increasing concentration of soluble Trx-D2 and GST-F2i fusion proteins. *In vitro* erythrocyte invasion assays were performed, and the results show a dose-dependent inhibition of parasite invasion in erythrocytes by both Trx-D2 and GST-F2i (Fig. 4B). No trophozoite or schizont accumulation was observed during the invasion inhibition assay, indicating that the Trx-D2 and GST-F2i fusions added to the culture medium did not affect the intracellular maturation and release of the merozoites from erythrocytes. The Trx-D2 and GST-F2i fusions at a concentration of 10 μ M inhibited merozoite invasion in human erythrocytes by ~31% and ~27%, respectively. Due to the limitations of invasion assays, specifically volume restrictions and relatively low molar fusion protein concentrations, higher concentrations of fusions could not be tested in the invasion assays under these conditions. All invasion inhibition experiments were performed in triplicate and minimum variation was observed within each experiment. The Trx and GST proteins were used as negative controls under the same conditions, resulting in baseline inhibition of erythrocyte invasion (Fig. 4B). These data suggest that the D2 and F2i segments of EBL-1 inhibit merozoite invasion presumably by competing with merozoites for specific binding to glycoporphin B receptor on the erythrocyte cell surface. These findings provide additional evidence for a functional role of EBL-1 in the parasite invasion process. Our results suggest that a direct interaction between F2i segment of parasite EBL-1 and host glycoporphin B plays an important functional role in the malaria parasite invasion process in human erythrocytes

3.7. Circular dichroism (CD) analysis of F2i segment of EBL-1

Because of the relatively small size of the F2i segment, consisting of 69 amino acids, it was necessary to assess the secondary structural integrity of F2i by circular dichroism spectroscopy. The F2i fragment was subcloned into pET32a vector and expressed as Trx-F2i fusion protein (Fig. 2, lanes 7–9). This cloning and expression step was necessary because of the presence of multiple bands in the GST-F2i fusion (Fig. 2, lanes 4–6) that complicated the interpretation of CD data. Although the purified Trx-F2i protein showed minimal degradation, the fusion protein was expressed predominantly in the inclusion bodies of transformed bacteria. Soluble Trx-F2i fusion protein was recovered by extracting the bacterial pellet with high salts in the presence of glycerol. Affinity-purified Trx-F2i required the presence of 5–10% glycerol for solubility. Using qualitative sedimentation assays, we confirmed the binding of Trx-F2i fusion protein to RBCs and trypsin-treated RBCs, but not to neuraminidase or chymotrypsin-treated RBCs (data not shown). It is noteworthy that the presence of glycerol at higher concentrations of Trx-F2i interfered with RBCs thus precluding the quantification of binding parameters and invasion assays.

Affinity-purified Trx and Trx-F2i proteins were subjected to CD analysis under identical conditions. The CD spectra and spectral difference are shown in Fig. 5. Based on the sequence and structural homology of known EBL-175 domain, the expected secondary structure of F2i peptide was predicted as three helices [35]. The spectral difference showed the largest negative intensity at 222 nm and 208 nm, which is characteristic of helices. While a small contribution from beta sheet structure (intensity at ~217 nm) cannot be ruled out, it is clear that there is little random coil structure (intensity at 190–200 nm). These data suggest that the F2i segment is likely to form a helical folded conformation in native EBL-1 protein.

4. Discussion

Plasmodium falciparum utilizes a repertoire of ligand-receptor interactions to invade human erythrocytes. These interactions are broadly grouped into two invasion pathways. The first group engages multiple parasite proteins to erythrocyte surface receptors, and these interactions are dependent upon the sialic acid content of host receptors. The second group of invasion pathways follows a similar strategy except that the parasite protein interactions with host receptors are sialic acid-independent [36]. The erythrocyte glycophorins are considered as the principal mediators of the sialic acid-dependent pathways (Fig. 6), whereas the host anion transporter band 3 and complement receptor 1 have recently been shown to mediate components of the sialic acid-independent pathways [29, 37–39]. These two seemingly distinct yet inter-dependent invasion routes ensure the malaria parasite to enter human erythrocytes efficiently and swiftly.

Intense interest in elucidating the components of the ligand-receptor interactions of two invasion pathways is driven by the need to identify novel parasite antigenic epitopes required for the development of malaria vaccine. Our previous studies have identified components of the sialic acid-independent invasion pathway. We identified parasite MSP1-MSP9 complex binding to host band 3 as one potential ligand-receptor interaction for the sialic acid-independent pathway [37]. Moreover, our earlier observations with band 3 null mouse erythrocytes [40], and by others on human erythrocytes [41], have shown a close association of host band 3 with glycophorin A in the erythrocyte membrane. Since the retention of glycophorin A on the erythrocyte surface is not feasible without band 3 [42], we proposed a model whereby the band 3-glycophorin A complex serves as a major receptor for *P. falciparum* invasion in human erythrocytes [37]. This model provides a molecular framework for integrating both sialic acid-dependent and independent pathways of merozoite invasion in human erythrocytes.

To test this model, we screened a phage display cDNA library to identify parasite proteins that bind to erythrocyte glycoporphins. Phage display technique has been successfully used in the malaria field for identifying new epitopes of peptides and antibodies using parasite antigens such as AMA-1, MSP1, CSP, and Pfs48/25 [28, 43–46]. Our strategy was to screen a phage display cDNA library made from the sialic acid-dependent strain of *P. falciparum* (FCR3) using a mixture of purified glycoporphins as bait. In addition, we performed a similar phage display screen using intact erythrocytes as bait. The details of the phage display library used in the present study have been published before [27]. Since glycoporphin A is the major glycoporphin present in human erythrocytes, purified preparations of glycoporphins isolated from human RBCs generally consists of 85% glycoporphin A, 10% glycoporphin B, 4% glycoporphin C, and 1% glycoporphin D [15]. Indeed, both phage display screens identified EBA-175 as a parasite ligand binding to purified glycoporphins as well as intact erythrocytes. Since EBA-175 is a well-known parasite ligand for glycoporphin A, its identification further supports the feasibility of our experimental strategy for identifying novel parasite ligands for other glycoporphins (Fig. 6).

Both screens identified multiple phage clones encoding a 69 amino acid insert of EBL-1 (Fig. 1). It is to be noted that the two screens pulled out dozens of specific phage clones; however, we amplified and sequenced only about 25 phage clones for further analysis. Among the successfully sequenced clones, five encoded EBA-175, three encoded EBL-1, and several clones encoded hypothetical proteins. We did not find any phage clone encoding the EBA-140 in our limited number of sequenced clones. Like EBA-175, the EBL-1 belongs to a family of Duffy-binding-like proteins. EBL-1 is the largest member of the ebl family of genes; the single copy gene is located on chromosome 13, and encodes a polypeptide of 2,647 amino acids with a predicted mass of 304.5 kDa. The full length EBL-1 protein contains two Cys-rich domains within an amino-terminal DBL domain (D2), which includes F1 and F2 sub-domains, and a c-Cys domain adjacent to the C-terminal transmembrane domain [47]. The c-Cys domain is relatively better conserved than the DBL domains in the EBL family of proteins. While other EBL family members contain eight conserved cysteine residues, the EBL-1 contains four cysteine residues [47]. The precise function of the c-Cys domains has not been determined. In the EBL family of *P. falciparum*, the DBL domains generally mediate the erythrocyte binding function. Interestingly, a recent study examined the binding of several EBL-1-derived peptides with human erythrocytes, and found that several binding peptides were located within the F1 and F2 sub-domains of EBL-1 [48]. These observations are consistent with our findings that the phage-encoded 69 amino acid peptide termed F2i (residues 601–669), bearing the binding site for glycoporphin B, is located within the F2 sub-domain of DBL domain in EBL-1.

The interactions between parasite ligands and erythrocyte surface receptors are often characterized by their enzyme sensitivity to neuraminidase, trypsin, and chymotrypsin [3, 12, 18, 21, 24]. Using a similar approach, our data indicate that both D2 and F2i segments of EBL-1 bind to human RBCs in a trypsin-resistant manner, and these interactions are sensitive to neuraminidase and chymotrypsin (Fig. 4A). Among the four glycoporphins, only glycoporphin B is resistant to trypsin but sensitive to chymotrypsin, and the sialic acid residues of all glycoporphins can be removed by neuraminidase. These observations suggested that glycoporphin B is the likely receptor for EBL-1. This prediction was further confirmed by the lack of EBL-1 D2 domain binding to glycoporphin B-null human erythrocytes (S-s-U-) (Fig. 4A).

A recent study has identified glycoporphin B as a receptor for EBL-1 [24]. In this study, the region 2 (DBL domain) of EBL-1 was expressed in CHO-K1 cells, and its binding to glycoporphin B positive RBCs, but not with glycoporphin B-null RBCs, was demonstrated [24]. Individual F1 and F2 sub-domains of EBL-1 were also expressed in CHO-K1 cells, but

these constructs did not support binding to human RBCs [24]. Our phage display screens revealed that the glycophorin B binding peptide is located within the F2 subdomain of EBL-1 (Fig. 1A). In addition, our study provides the first direct evidence that both D2 and F2i segments of EBL-1 inhibit merozoite invasion in human erythrocytes (Fig. 4B). This result is consistent with previous findings showing reduced merozoite invasion in glycophorin B-deficient erythrocytes by several parasite strains [49]. Similarly, a previous study showed that the 7G8 strain of *P. falciparum* cannot invade glycophorin B-deficient erythrocytes, whereas other strains could invade these cells at 72–79% efficiency as compared to normal erythrocytes [50]. In contrast, a subsequent study showed that *P. falciparum* 7G8 strain can invade glycophorin B-deficient erythrocytes with ~50% efficiency [49]. It is also noteworthy that several *Plasmodium falciparum* strains do not express apparently functional forms of EBL-1, and may be under selective genetic pressure that is unique to this gene, particularly within the F2 segment of the DBL/D2 domain [51, 52]. Irrespective of the extent of receptor utilization by the invading merozoites, our findings identify a relatively short F2i segment of EBL-1 as the cognate parasite ligand for glycophorin B on human erythrocytes. We suggest that an optimized 69-amino acid synthetic peptide of EBL-1 could be considered for inclusion in the multi-subunit malaria vaccine.

Acknowledgments

We are very grateful to Dr. Ghislaine Mayer of the Virginia Commonwealth University, Richmond, VA, for kindly providing us glycophorin B-null human erythrocytes. We are also indebted to Dr. Louis Miller of the NIH for advice and guidance in obtaining the glycophorin B-null erythrocytes. We are grateful to Dr. Brian Kay of the University of Illinois at Chicago for his invaluable advice about the phage display library screens, and thank Rolf Fendel, Ravi Ranjan, Changling Ma, Danielle Donzal, and Donna-Marie Mironchuk for suggestions, proofreading, and graphic arts of the manuscript. This work was supported by the NIH Grants HL60961 and HL89517 (A.C.), and the National Research Foundation of South Africa GUN 2069449 and the Medical Research Council of South Africa (T.C.). XL designed and performed majority of the experiments, and participated in manuscript writing. MM and CR designed and performed the recombinant protein expression experiments. MM managed the resubmission of the original manuscript. JM performed the circular dichroism experiments. TC made the phage display library and participated in manuscript editing. AC designed and supervised the research project, and participated in manuscript writing. Dr. Li's current address is Department of Parasitology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, China.

References

1. Cowman AF, Crabb BS. Invasion of red blood cells by malaria parasites. *Cell*. 2006; 124:755–766. [PubMed: 16497586]
2. Gaur D, Mayer DC, Miller LH. Parasite ligand-host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. *Int J Parasitol*. 2004; 34:1413–1429. [PubMed: 15582519]
3. Dolan SA, Miller LH, Welles TE. Evidence for a switching mechanism in the invasion of erythrocytes by *Plasmodium falciparum*. *J Clin Invest*. 1990; 86:618–624. [PubMed: 2200806]
4. Facer CA. Erythrocyte sialoglycoproteins and *Plasmodium falciparum* invasion. *Trans R Soc Trop Med Hyg*. 1983; 77:524–530. [PubMed: 6356506]
5. Howard RJ, Haynes JD, McGinniss MH, Miller LH. Studies on the role of red blood cell glycoproteins as receptors for invasion by *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol*. 1982; 6:303–315. [PubMed: 6757749]
6. Pasvol G, Jungery M, Weatherall DJ, Parsons SF, Anstee DJ, Tanner MJ. Glycophorin as a possible receptor for *Plasmodium falciparum*. *Lancet*. 1982; 2:947–950. [PubMed: 6127459]
7. Perkins ME. Binding of glycophorins to *Plasmodium falciparum* merozoites. *Mol Biochem Parasitol*. 1984; 10:67–78. [PubMed: 6363923]
8. Perkins M. Inhibitory effects of erythrocyte membrane proteins on the in vitro invasion of the human malarial parasite (*Plasmodium falciparum*) into its host cell. *J Cell Biol*. 1981; 90:563–567. [PubMed: 7026577]

9. Miller LH, Haynes JD, McAuliffe FM, Shiroishi T, Durocher JR, McGinniss MH. Evidence for differences in erythrocyte surface receptors for the malarial parasites, *Plasmodium falciparum* and *Plasmodium knowlesi*. *J Exp Med*. 1977; 146:277–281. [PubMed: 327014]
10. Pasvol G, Wainscoat JS, Weatherall DJ. Erythrocytes deficiency in glycophorin resist invasion by the malarial parasite *Plasmodium falciparum*. *Nature*. 1982; 297:64–66. [PubMed: 7040988]
11. Cartron JP, Rahuel C. Human erythrocyte glycophorins: protein and gene structure analyses. *Transfus Med Rev*. 1992; 6:63–92. [PubMed: 1591491]
12. Sim BK, Chitnis CE, Wasniowska K, Hadley TJ, Miller LH. Receptor and ligand domains for invasion of erythrocytes by *Plasmodium falciparum*. *Science*. 1994; 264:1941–1944. [PubMed: 8009226]
13. Ockenhouse CF, Barbosa A, Blackall DP, Murphy CI, Kashala O, Dutta S, et al. Sialic acid-dependent binding of baculovirus-expressed recombinant antigens from *Plasmodium falciparum* EBA-175 to Glycophorin A. *Mol Biochem Parasitol*. 2001; 113:9–21. [PubMed: 11254950]
14. Tolia NH, Enemark EJ, Sim BK, Joshua-Tor L. Structural basis for the EBA-175 erythrocyte invasion pathway of the malaria parasite *Plasmodium falciparum*. *Cell*. 2005; 122:183–193. [PubMed: 16051144]
15. Bharara R, Singh S, Pattnaik P, Chitnis CE, Sharma A. Structural analogs of sialic acid interfere with the binding of erythrocyte binding antigen-175 to glycophorin A, an interaction crucial for erythrocyte invasion by *Plasmodium falciparum*. *Mol Biochem Parasitol*. 2004; 138:123–129. [PubMed: 15500923]
16. Adams JH, Blair PL, Kaneko O, Peterson DS. An expanding ebl family of *Plasmodium falciparum*. *Trends Parasitol*. 2001; 17:297–299. [PubMed: 11378038]
17. Triglia T, Thompson JK, Cowman AF. An EBA175 homologue which is transcribed but not translated in erythrocytic stages of *Plasmodium falciparum*. *Mol Biochem Parasitol*. 2001; 116:55–63. [PubMed: 11463466]
18. Maier AG, Duraisingh MT, Reeder JC, Patel SS, Kazura JW, Zimmerman PA, et al. *Plasmodium falciparum* erythrocyte invasion through glycophorin C and selection for Gerbich negativity in human populations. *Nat Med*. 2003; 9:87–92. [PubMed: 12469115]
19. Patel SS, Mehlotra RK, Kastens W, Mgone CS, Kazura JW, Zimmerman PA. The association of the glycophorin C exon 3 deletion with ovalocytosis and malaria susceptibility in the Wosera, Papua New Guinea. *Blood*. 2001; 98:3489–3491. [PubMed: 11719395]
20. Chishti AH, Palek J, Fisher D, Maalouf GJ, Liu SC. Reduced invasion and growth of *Plasmodium falciparum* into elliptocytic red blood cells with a combined deficiency of protein 4.1, glycophorin C, and p55. *Blood*. 1996; 87:3462–3469. [PubMed: 8605365]
21. Gilberger TW, Thompson JK, Triglia T, Good RT, Duraisingh MT, Cowman AF. A novel erythrocyte binding antigen-175 paralogue from *Plasmodium falciparum* defines a new trypsin-resistant receptor on human erythrocytes. *J Biol Chem*. 2003; 278:14480–14486. [PubMed: 12556470]
22. Mayer DC, Mu JB, Kaneko O, Duan J, Su XZ, Miller LH. Polymorphism in the *Plasmodium falciparum* erythrocyte-binding ligand JESEBL/EBA-181 alters its receptor specificity. *Proc Natl Acad Sci U S A*. 2004; 101:2518–2523. [PubMed: 14983041]
23. Lanzillotti R, Coetzer TL. The 10 kDa domain of human erythrocyte protein 4.1 binds the *Plasmodium falciparum* EBA-181 protein. *Malar J*. 2006; 5:100. [PubMed: 17087826]
24. Mayer DC, Cofie J, Jiang L, Hartl DL, Tracy E, Kabat J, et al. Glycophorin B is the erythrocyte receptor of *Plasmodium falciparum* erythrocyte-binding ligand, EBL-1. *Proc Natl Acad Sci U S A*. 2009; 106:5348–5352. [PubMed: 19279206]
25. Fraser GR, Giblett ER, Motulsky AG. Population genetic studies in the Congo. 3. Blood groups (ABO, MNSS, Rh, Jsa). *Am J Hum Genet*. 1966; 18:546–552. [PubMed: 4959344]
26. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science*. 1976; 193:673–675. [PubMed: 781840]
27. Lauterbach SB, Lanzillotti R, Coetzer TL. Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions. *Malar J*. 2003; 2:47. [PubMed: 14678570]
28. Lanzillotti R, Coetzer TL. Phage display: a useful tool for malaria research? *Trends Parasitol*. 2008; 24:18–23. [PubMed: 18037345]

29. Goel VK, Li X, Chen H, Liu SC, Chishti AH, Oh SS. Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes. *Proc Natl Acad Sci U S A*. 2003; 100:5164–5169. [PubMed: 12692305]
30. Lobo CA, Rodriguez M, Reid M, Lustigman S. Glycophorin C is the receptor for the *Plasmodium falciparum* erythrocyte binding ligand PfEBP-2 (baeb1). *Blood*. 2003; 101:4628–4631. [PubMed: 12576308]
31. Duraisingh MT, Maier AG, Triglia T, Cowman AF. Erythrocyte-binding antigen 175 mediates invasion in *Plasmodium falciparum* utilizing sialic acid-dependent and -independent pathways. *Proc Natl Acad Sci U S A*. 2003; 100:4796–4801. [PubMed: 12672957]
32. Huang CH, Johe K, Moulds JJ, Siebert PD, Fukuda M, Blumenfeld OO. Delta glycophorin (glycophorin B) gene deletion in two individuals homozygous for the S--s--U-- blood group phenotype. *Blood*. 1987; 70:1830–1835. [PubMed: 2823938]
33. Garratty G. Blood group antigens as tumor markers, parasitic/bacterial/viral receptors, and their association with immunologically important proteins. *Immunol Invest*. 1995; 24:213–232. [PubMed: 7713584]
34. Dolan SA, Proctor JL, Alling DW, Okubo Y, Wellems TE, Miller LH. Glycophorin B as an EBA-175 independent *Plasmodium falciparum* receptor of human erythrocytes. *Mol Biochem Parasitol*. 1994; 64:55–63. [PubMed: 8078523]
35. Cole C, Barber JD, Barton GJ. The Jpred 3 secondary structure prediction server. *Nucleic Acids Res*. 2008; 36:W197–W201. [PubMed: 18463136]
36. Stubbs J, Simpson KM, Triglia T, Plouffe D, Tonkin CJ, Duraisingh MT, et al. Molecular mechanism for switching of *P. falciparum* invasion pathways into human erythrocytes. *Science*. 2005; 309:1384–1387. [PubMed: 16123303]
37. Li X, Chen H, Oo TH, Daly TM, Bergman LW, Liu SC, et al. A co-ligand complex anchors *Plasmodium falciparum* merozoites to the erythrocyte invasion receptor band 3. *J Biol Chem*. 2004; 279:5765–5771. [PubMed: 14630931]
38. Kushwaha A, Perween A, Mukund S, Majumdar S, Bhardwaj D, Chowdhury NR, et al. Amino terminus of *Plasmodium falciparum* acidic basic repeat antigen interacts with the erythrocyte membrane through band 3 protein. *Mol Biochem Parasitol*. 2002; 122:45–54. [PubMed: 12076769]
39. Spadafora C, Awandare GA, Kopydlowski KM, Czege J, Moch JK, Finberg RW, et al. Complement Receptor 1 Is a Sialic Acid-Independent Erythrocyte Receptor of *Plasmodium falciparum*. *PLoS Pathog*. 2010; 6:e1000968. [PubMed: 20585558]
40. Hassoun H, Hanada T, Lutchman M, Sahr KE, Palek J, Hanspal M, et al. Complete deficiency of glycophorin A in red blood cells from mice with targeted inactivation of the band 3 (AE1) gene. *Blood*. 1998; 91:2146–2151. [PubMed: 9490702]
41. Ribeiro ML, Alloisio N, Almeida H, Gomes C, Texier P, Lemos C, et al. Severe hereditary spherocytosis and distal renal tubular acidosis associated with the total absence of band 3. *Blood*. 2000; 96:1602–1604. [PubMed: 10942416]
42. Poole J. Red cell antigens on band 3 and glycophorin A. *Blood Rev*. 2000; 14:31–43. [PubMed: 10805259]
43. Chappel JA, Rogers WO, Hoffman SL, Kang AS. Molecular dissection of the human antibody response to the structural repeat epitope of *Plasmodium falciparum* sporozoite from a protected donor. *Malar J*. 2004; 3:28. [PubMed: 15283866]
44. Keizer DW, Miles LA, Li F, Nair M, Anders RF, Coley AM, et al. Structures of phage-display peptides that bind to the malarial surface protein, apical membrane antigen 1, and block erythrocyte invasion. *Biochemistry*. 2003; 42:9915–9923. [PubMed: 12924940]
45. Sowa KM, Cavanagh DR, Creasey AM, Raats J, McBride J, Sauerwein R, et al. Isolation of a monoclonal antibody from a malaria patient-derived phage display library recognising the Block 2 region of *Plasmodium falciparum* merozoite surface protein-1. *Mol Biochem Parasitol*. 2001; 112:143–147. [PubMed: 11166396]
46. Roeffen WF, Raats JM, Teelen K, Hoet RM, Eling WM, van Venrooij WJ, et al. Recombinant human antibodies specific for the Pfs48/45 protein of the malaria parasite *Plasmodium falciparum*. *J Biol Chem*. 2001; 276:19807–19811. [PubMed: 11279092]

47. Peterson DS, Wellems TE. EBL-1, a putative erythrocyte binding protein of *Plasmodium falciparum*, maps within a favored linkage group in two genetic crosses. *Mol Biochem Parasitol.* 2000; 105:105–113. [PubMed: 10613703]
48. Curtidor H, Rodriguez LE, Ocampo M, Lopez R, Garcia JE, Valbuena J, et al. Specific erythrocyte binding capacity and biological activity of *Plasmodium falciparum* erythrocyte binding ligand 1 (EBL-1)-derived peptides. *Protein Sci.* 2005; 14:464–473. [PubMed: 15659376]
49. Gaur D, Storry JR, Reid ME, Barnwell JW, Miller LH. *Plasmodium falciparum* is able to invade erythrocytes through a trypsin-resistant pathway independent of glycophorin B. *Infect Immun.* 2003; 71:6742–6746. [PubMed: 14638759]
50. Rayner JC, Vargas-Serrato E, Huber CS, Galinski MR, Barnwell JW. A *Plasmodium falciparum* homologue of *Plasmodium vivax* reticulocyte binding protein (PvRBP1) defines a trypsin-resistant erythrocyte invasion pathway. *J Exp Med.* 2001; 194:1571–1581. [PubMed: 11733572]
51. Drummond PB, Peterson DS. An analysis of genetic diversity within the ligand domains of the *Plasmodium falciparum* ebl-1 gene. *Mol Biochem Parasitol.* 2005; 140:241–245. [PubMed: 15760663]
52. Githui EK, Peterson DS, Aman RA, Abdi AI. Prevalence of 5' insertion mutants and analysis of single nucleotide polymorphism in the erythrocyte binding-like 1 (ebl-1) gene in Kenyan *Plasmodium falciparum* field isolates. *Infect Genet Evol.* 2010; 10:834–839. [PubMed: 19879379]

Figure 1.

Schematic representation of the primary structure of *Plasmodium falciparum* EBL-1. (A) EBL-1 consists of a putative signal peptide (SP) sequence, Duffy-binding-like (DBL) ligand domain consisting of two cysteine-rich regions F1 and F2, transmembrane (TM) domain, and putative cytoplasmic domain (CD). The location of EBL-1 constructs D2 (DBL) and F2i are indicated and their respective amino acid positions are also marked. Plasmids encoding D2 and F2i were used to express the recombinant proteins. (B) Amino acid sequence of DBL (D2) domain of EBL-1. The F1 and F2 sub-domains are boxed with dash and solid line, respectively. The F2i sequence is underlined and highlighted.

Figure 2.

Expression and purification of recombinant *Plasmodium falciparum* EBL-1 segments in *E. coli*. Lane 1, Coomassie blue stained SDS-PAGE of purified Trx-D2; lane 2, Western blot using anti-His₆ mAb of purified Trx-D2 and Trx (lane 3). Coomassie blue stained gel of purified GST-F2i (lane 4), and Western blot using anti-GST of purified GST-F2i (lane 5) and GST (lane 6). Coomassie blue stained gel of purified Trx-F2i (lane 7) and Western blot using anti-His₆ of purified Trx-F2i (lane 8) and Trx (lane 9). Arrowheads show the location of polypeptides.

Figure 3.

Plasmodium falciparum EBL-1 binds to purified glycoproteins and human RBCs. (A) A concentration-dependent binding of Trx-D2 and GST-F2i to purified glycoproteins. Binding of Trx-D2 (filled circles), GST-F2i (filled squares), Trx (empty circles), and GST (empty squares) to immobilized glycoproteins as detected by ELISA using anti-His₆ mAb and anti-GST antibody. Values are means $\pm$ S.E. (B) Western blot using anti-His₆ mAb showing binding of Trx-D2 to untreated RBCs and GST-F2i to pre-treated RBCs. Lane 1, RBCs + Trx (1 μ M) (control); lane 2, RBCs + Trx-D2 (1 μ M); lane 3, RBCs + Trx-D2 (1 μ M) + GST (1 μ M); lane 4, RBCs + Trx-D2 (1 μ M) + GST-F2i (1 μ M); lane 5, RBCs + Trx-D2 (1

μM) + GST-F2i (10 μM). (C) Western blot using anti-GST antibody shows the binding of GST-F2i to RBCs. Lane 1, RBCs + GST-F2i (2 μM); lane 2, RBCs + GST (2 μM) control.

Figure 4.

(A) Interaction of malaria parasite EBL-1 D2 domain to human erythrocytes. The Trx-D2 fusion protein binds to trypsin-treated RBCs but not to neuraminidase, chymotrypsin, and glycophorin B-null RBCs. Western blots using anti-His₆ mAb to detect Trx-D2: Lane 1, Trx-D2 + normal RBCs; lane 2, Trx-D2 + neuraminidase(Nm)-treated RBCs; lane 3, Trx-D2 + trypsin (T) treated RBCs; lane 4, Trx-D2 + chymotrypsin (Ch) treated RBCs; lane 5, purified Trx-D2 as control; lane 6, Trx-D2 + glycophorin B positive (GPB⁺) RBCs; lane 7, Trx-D2 + glycophorin B-null (GPB⁻) RBCs. (B) Recombinant EBL-1 fragments inhibit merozoite invasion in human RBCs. Synchronized *P. falciparum* cultures were incubated with Trx-D2 fusion (filled circles), GST-F2i (filled squares), Trx (empty circles), and GST

(empty squares) at increasing concentrations, and invasion was assessed by counting the newly formed rings. The data are presented as % of invasion inhibition with respect of untreated cultures, with values as means $\pm$ S.E.

Figure 5. Circular dichroism (CD) analysis. The difference between the CD spectra of Trx-F2i and Trx in millidegrees as a function of the wavelength in nm. Insert: CD signal of Trx-F2i (filled circles) and Trx (empty circles), and the difference (triangles).

Figure 6.

A sialic acid-dependent parasite invasion pathway model. Cartoon shows the erythrocyte glycoprotein receptors and malaria parasite EBL ligand interactions during parasite invasion in human red blood cells. EBL-1 is involved in erythrocyte receptor GPB recognition, and plays a functional role in the merozoite invasion process. Human erythrocyte GPA and GPC/D have been previously identified as receptors for *P. falciparum* ligands EBA-175 and EBA-140 (BAEBL), respectively.