Crystal and Solution Structures of *Plasmodium falciparum* Erythrocyte-binding Antigen 140 Reveal Determinants of Receptor Specificity during Erythrocyte Invasion*

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**Background:** The malaria parasite *Plasmodium falciparum* utilizes PfEBA-140 to invade host erythrocytes.

**Results:** The minimal binding region of PfEBA-140 contains unique structural components compared with other *Plasmodium* invasion ligands.

**Conclusion:** Distinct structural and mechanistic elements of PfEBA-140 binding are likely determinants of receptor specificity.

**Significance:** This study provides the first view into the basis of receptor specificity of *P. falciparum* invasion ligands.

Erythrocyte-binding antigen 140 (PfEBA-140) is a critical *Plasmodium falciparum* erythrocyte invasion ligand that engages glycophorin C on host erythrocytes during malaria infection. The minimal receptor-binding region of PfEBA-140 contains two conserved Duffy-binding-like (DBL) domains, a fold unique to *Plasmodium* species. Here, we present the crystal structure of the receptor-binding region of PfEBA-140 at 2.4 Å resolution. The two-domain binding region is present as a monomer in the asymmetric unit, and the structure reveals novel features in PfEBA-140 that are likely determinants of receptor specificity. Analysis by small-angle x-ray scattering demonstrated that the minimal binding region is monomeric in solution, consistent with the crystal structure. Erythrocyte binding assays showed that the full-length binding region containing the tandem DBL domains is required for erythrocyte engagement, suggesting that both domains contain critical receptor contact sites. The electrostatic surface of PfEBA-140 elucidates a basic patch that constitutes a putative high-affinity binding interface spanning both DBL domains. Mutation of residues within this interface results in severely diminished erythrocyte binding. This study provides insight into the structural basis and mechanism of PfEBA-140 receptor engagement and forms a basis for future studies of this critical interaction. In addition, the solution and crystal structures allow the first identification of likely determinants of erythrocyte receptor specificity for *P. falciparum* invasion ligands. A complete understanding of the PfEBA-140 erythrocyte invasion pathway will aid in the design of invasion inhibitory therapeutics and vaccines.

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Erythrocyte invasion by *Plasmodium* species is mediated by integral membrane proteins of the erythrocyte-binding ligand (EBL) family. During invasion, EBL proteins bind irreversibly and specifically to erythrocyte receptors to create a tight junction between host and parasite membranes. This interaction facilitates merozoite entry into the red blood cell. *Plasmodium falciparum* has a sophisticated invasion machinery with several EBL proteins that each bind a different erythrocyte receptor in a sialic acid-dependent manner (1). Erythrocyte-binding antigen 175 (PfEBA-175), erythrocyte-binding ligand 1 (PfEBL-1), and erythrocyte-binding antigen 140 (PfEBA-140) bind glycophorins A, B, and C, respectively (2–4). A fourth member of this family, erythrocyte-binding antigen 181 (PfEBA-181), binds an unknown receptor (5). The EBL family members are composed of two cysteine-rich regions designated region II (RII) and region VI and contain a type I transmembrane domain and a short cytoplasmic domain (6). Receptor binding has been localized to RII for all members. In the *P. falciparum* EBL family, this region contains two tandem Duffy binding-like (DBL) domains, F1 and F2. The DBL protein fold is unique to *Plasmodium* and is able to recognize and tightly bind a diverse array of host cell receptors. In addition to their critical role during invasion, DBL domains also mediate microvasculature adherence of infected erythrocytes by erythrocyte membrane protein 1 (PfEMP1), a phenomenon directly associated with severe malaria (7). It is unknown how the EBL proteins utilize such a highly conserved domain structure to recognize different erythrocyte receptors and thus provide *P. falciparum* with multiple pathways for invasion. In addition, the role of each individual erythrocyte invasion pathway during natural infection is not fully understood. However, it has been observed that Gerbich negativity is present at high frequency in regions of Papua New Guinea where infection with *P. falciparum* malaria is common (8). Gerbich-negative individuals have a deletion of exon 3 in the glycophorin C (GPC) gene that prevents PfEBA-140 eryth-
rocyte binding and invasion. This observation provides strong evidence that severe malaria has selected for this mutation and illustrates the significance of erythrocyte invasion mediated by PfEBA-140.

As the tandem DBL domains in RII of the four *P. falciparum* EBL proteins can independently bind erythrocytes, they are the focus of combinatorial vaccine efforts. To understand how *P. falciparum* uses the DBL protein fold to recognize different erythrocyte receptors during invasion, we determined the crystal structure and examined the erythrocyte binding profile of RII PfEBA-140. In addition, the solution structure and oligomeric state of this invasion ligand were examined using small-angle x-ray scattering (SAXS). The results presented here elucidate likely determinants of receptor specificity within the EBL family and provide insight into the structural basis of erythrocyte binding by the critical invasion ligand PfEBA-140.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—A codon-optimized construct containing amino acids 143–740 of RII PfEBA-140 with three point mutations (S303A, T469A, and S727A) was cloned for expression. The three mutations were introduced to avoid aberrant glycosylation at putative N-glycosylation sites during expression in mammalian cells that could otherwise impact protein homogeneity. This construct provided high yields of pure protein from *Escherichia coli* and was thus used for crystallization. These amino acid changes did not affect protein function, as demonstrated by erythrocyte binding assays described below. The construct was expressed as inclusion bodies in *E. coli* and recovered using 6 M guanidinium chloride. Following recovery, denatured protein (100 mg/liter) was rapidly diluted in 50 mM Tris (pH 8.0), 10 mM EDTA, 200 mM arginine, 0.1 mM PMSF, 2 mM reduced glutathione, and 0.2 mM oxidized glutathione. After 48 h of refolding at 4 °C, RII PfEBA-140 was concentrated using Amicon centrifugal filters and purified by size exclusion and ion exchange chromatography.

**Crystallization, Data Collection, and Structure Determination**—Crystals were grown using the hanging drop vapor diffusion method by mixing 1 μl of protein at 7.5 mg/ml with 1 μl of
reservoir containing 20% PEG 8000 and 0.1 M HEPES (pH 7.5). Initial crystal hits observed in precipitant screens (Qiagen) were used as seeds to optimize crystal growth. Seeds were generated by transferring the entire crystal drop into 10 μl of 20% PEG 8000 and 0.1 M HEPES (pH 7.5) and vortexing the sample. Crystals were sent for remote data collection at beamline 4.2.2 at the Advanced Light Source, Lawrence Berkeley National Laboratory. Cryoprotectant composed of 30% glycerol, 17.5% PEG 8000, and 0.1 M HEPES (pH 7.5) was introduced gradually into the drop by pipetting. Crystals were isolated with nylon loops and stream-frozen prior to transport.

The RII PfEBA-140 structure was solved by molecular replacement using RII PfEBA-175 as a model in BALBES (9). Automated refinement was performed with PHENIX (10), and manual model building was performed with Coot (11). Refinement was completed once low R-factors and good geometry were obtained (see Table 1). The structure described has been deposited in the Protein Data Bank with accession code 4GF2.

**RESULTS**

**Crystal and Solution Structures of P. falciparum EBA-140**

**Overall Structure of RII PfEBA-140**—To define the structural basis of PfEBA-140 receptor specificity, we solved the crystal structure of RII PfEBA-140 (Fig. 1A and Table 1). RII PfEBA-140 is present as a monomer in the asymmetric unit, and no oligomeric contacts with potential physiological relevance are observed (Fig. 1A). The two DBL domains, F1 (residues 143–422) and F2 (residues 447–740), are connected by a short helical linker. Each DBL domain is composed of three subdomains (Fig. 1B) and contains a unique disulfide bridge pattern relative to other DBL family members (Fig. 1C). The F1 and F2 domains of PfEBA-140 are structurally similar to other DBL domains of *Plasmodium* invasion ligands (Fig. 1D).

**RII PfEBA-140 Erythrocyte Binding Requires Both DBL Domains**—The presence of two structurally conserved domains within the minimal binding region suggests that each domain may be able to independently engage erythrocytes. To assess the functional importance of the putative binding interface, individual amino acid residues within the basic patch were mutated to alanine and tested for deficient erythrocyte binding. Large polar and/or charged residues are often involved in glycoprotein interactions; thus, we focused on these residues for testing (Fig. 2C). Four residues were identified that, when mutated to alanine, resulted in greatly diminished or null erythrocyte binding (Fig. 2D). This result confirms the vital role of individual residues within the putative interface during receptor binding. To test the alternate side of the protein, residues on the face opposite of the basic patch were also mutated to alanine. Mutation of these residues had no effect on binding, suggesting that both domains make essential contacts with GPC during invasion. This result is supported by examination of the electrostatic surface of RII PfEBA-140, which elucidates a putative high-affinity binding interface (Fig. 2C). This region forms an arch of overall positive charge that spans the two DBL domains and would provide an ideal interaction surface for engagement of the highly glycosylated, acidic GPC.

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**Structural Basis of PfEBA-140 Receptor Specificity**—Unique structural elements distinguish RII PfEBA-140 from other DBL domain-containing EBL ligands and are the likely basis of recep-

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**Table 1**

**Data collection and refinement statistics**

Values in parentheses are for highest resolution shell. r.m.s.d., root mean square deviation.

| RII PfEBA-140 |  |
|---------------|---------|
| **Data collection** |  |
| Space group | P2₁ |
| Cell dimensions |  |
| a, b, c (Å) | 65.42, 76.48, 82.34 |
| α, β, γ | 90.00°, 96.80°, 90.00° |
| Resolution (Å) | 20.0±1.0 |
| Rmerge (%) | 5.8 (78.9) |
| Completeness (%) | 99.4 (98.0) |
| Redundancy | 3.72 (3.67) |
| **Refinement** |  |
| Resolution (Å) | 20.0–2.4 |
| No. of reflections | 31,507 |
| Rwork/Rfree (%) | 20.3/23.69 |
| No. of atoms |  |
| Protein | 5339 |
| Ligand/ion | 90 |
| Water | 143 |
| B-factors |  |
| Protein | 67.57 |
| Ligand/ion | 78.16 |
| Water | 53.73 |
| r.m.s.d. |  |
| Bond lengths (Å) | 0.003 |
| Bond angles | 0.607° |
| Ramachandran (%) |  |
| Favored | 97.46 |
| Allowed | 2.54 |
| Disallowed | 0.00 |

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tor specificity. All 26 cysteines in RII PfEBA-140 are involved in disulfide bonds, and two of the disulfide linkages are distinct from other characterized DBL domains (Fig. 1C). The modified disulfide pattern includes a linkage between Cys-7 and Cys-8, as well as a linkage between Cys-9 and Cys-12. In contrast, linkages between Cys-7 and Cys-9 and between Cys-8 and Cys-12 are observed in all other characterized EBL members.

In addition to an altered disulfide pattern, several elements of the RII PfEBA-140 subdomain structure are unique among DBL domains. In RII PfEBA-175, β-fingers present in both DBL domains contain long loops that serve as critical dimeric contacts required for receptor binding (17). In contrast, Plasmodium vivax Duffy-binding protein (PVDBP) and Plasmodium knowlesi Duffy-binding protein (PkDBP), which contain only a single DBL domain in their RII, possess short loops within the β-finger region that do not seem to be important for the function of these ligands (18, 19). The β-finger of F1 PfEBA-140 is similar in length to the β-fingers of the PfEBA-175 F1 and F2 domains. In addition, the F1 β-fingers of PfEBA-140 and PfEBA-175 overlay quite well (Fig. 3A). However, the canonical β-finger motif in F1 PfEBA-140 is replaced with a novel helical segment in the F2 domain (Fig. 3B).

A third critical difference is the orientation of subdomain 3 in F1 PfEBA-140 with respect to the rest of the DBL domain. In most DBL domains, subdomain 3 contains a kink in a long helix facilitated by a glycine residue (Gly-185 in F1 PfEBA-175, Gly-490 in F2 PfEBA-175, Gly-397 in PvDBP, Gly-394 in PkDBP-α, and Gly-627 in F2 PfEBA-140). This glycine is absent in F1 PfEBA-140, and without the inherent flexibility of this residue, the kink in subdomain 3 cannot form. The absence of this kink
leads to a drastic change within the subdomain that propagates through RII PfEBA-140, resulting in a large difference in the hinge angle between the two DBL domains compared with RII PfEBA-175 (Fig. 3, A and C). The splayed-out DBL domains of RII PfEBA-140 would require a large hinge movement to create the mode of dimerization seen in the crystal structure of RII PfEBA-175 (Fig. 3, inset).

Solution Structure of PfEBA-140—The structure and oligomeric state of parasite ligands in solution are important determinants of function. Both the related ligands PvDBP and PfEBA-175 appear to bind receptors as dimers (17, 18). We determined the solution structure and oligomeric state of RII PfEBA-140 by SAXS. The theoretical scatter for the monomeric PfEBA-140 crystal structure resulted in an excellent fit to the experimental SAXS profile, with a $\chi^2$ of 1.46 (Fig. 4). Furthermore, an averaged ab initio reconstruction revealed a molecular envelope that closely resembles the monomeric structure. This result is consistent with the observed crystal contacts and hinge angle between the F1 and F2 domains, suggesting that RII PfEBA-140 is monomeric in solution (Fig. 4). Only monomeric forms of PfEBA-140 have been described here, and there is no evidence for higher order oligomeric states in the absence of GPC.

DISCUSSION

*P. falciparum* field isolates from endemic malaria regions actively utilize multiple erythrocyte invasion ligands, exemplifying the need to characterize each individual pathway (20). Antibodies targeting PfEBA-140 are capable of inhibiting invasion, supporting the critical role of this ligand during invasion (21). Furthermore, PfEBA-140 is naturally immunogenic. Serum isolated from patients infected with *P. falciparum* malaria is reactive to PfEBA-140, and RII was found to be the most immunoreactive element (22). The active immune response to PfEBA-140 provides strong support for its validity as a component of a combinatorial vaccine targeting invasion ligands. The structure described here forms a framework for future studies of the critical interaction between PfEBA-140 and erythrocytes. In addition, identifying the structural basis of receptor specificity within the EBL family is essential to characterizing the full range of invasion pathways utilized by *P. falciparum*. These results will thus aid in the rational development of invasion inhibitory therapeutics and vaccines.

The structural differences identified in RII PfEBA-140 suggest an altered mechanism of binding relative to PfEBA-175 and likely other EBL members. It has been proposed that dimerization is an important mechanistic component of EBL-mediated invasion. Support for this proposed mechanism...
comes from the fact that PvDBP is monomeric in the absence of receptor and dimerizes upon binding its receptor, DARC (18). In addition, PfEBA-175 crystallizes as a dimer and may engage GPA in the dimeric form observed in the crystal structure (17). It is possible that PfEBA-140 follows this EBL pattern and engages GPC as a dimer while existing as a monomer in the absence of receptor. However, to bind GPC using the dimer architecture observed for PfEBA-175, RII PfEBA-140 would need to undergo a large structural hinge movement and display dimeric contacts not observed in PfEBA-175. Specifically, the β-finger motifs in each DBL domain of PfEBA-175 make dimeric contacts. The presence of a unique helical element in place of the canonical β-finger motif in F2 PfEBA-140 may alter the dimeric contacts observed in PfEBA-175, resulting in a novel dimer conformation of PfEBA-140. It is equally possible that PfEBA-140 engages GPC as a monomer and that oligomeric state is an important determinant of receptor specificity.

Receptor recognition by PfEBA-140 is poorly understood; however, it is known that PfEBA-140 erythrocyte engagement is dependent on GPC glycans as well as the protein backbone. The proposed binding interface identified in the crystal structure spans both DBL domains (Fig. 2C). We demonstrated that both domains are required for erythrocyte binding and that mutating individual residues in each domain severely diminishes erythrocyte binding. These results suggest that each DBL domain forms essential contacts with GPC during invasion. In addition, the identification of the positive interface forms a basis for identifying the GPC-binding site. It is probable that vital receptor-binding interactions are located in this putative binding interface due to the concentrated presence of basic residues. The only known essential binding component on GPC is a solitary N-linked glycan at residue 8 (23). Putative O-linked glycans are abundant on GPC, but their role in binding has not been examined in detail. The critical N-linked glycan may make contacts with both DBL domains, explaining the requirement for full-length RII during binding. Alternatively, the N-linked glycan may bind with high specificity to one domain, whereas O-linked glycans and the protein backbone form essential contacts with the other domain. Further studies examining the interaction of RII PfEBA-140 with GPC are required to fully understand the structural and mechanistic basis of receptor recognition during invasion. In conclusion, we have provided insight into the PfEBA-140 binding mechanism, elucidated an important receptor-binding interface, and identified unique structural motifs in RII PfEBA-140 that form the basis of receptor specificity within the EBL family that allows P. falciparum to engage multiple host receptors during invasion.

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