**Glycophorin B is the Erythrocyte Receptor of Plasmodium Falciparum Erythrocyte-Binding Ligand, EBL-1**

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In the war against Plasmodium, humans have evolved to eliminate or modify proteins on the erythrocyte surface that serve as receptors for parasite invasion, such as the Duffy blood group, a receptor for Plasmodium vivax, and the Gerbich-negative modification of glycophorin C for Plasmodium falciparum. In turn, the parasite counters with expansion and diversification of ligand families. The high degree of polymorphism in glycophorin B found in malaria-endemic regions suggests that it also may be a receptor for Plasmodium, but, to date, none has been identified. We provide evidence from erythrocyte-binding that glycophorin B is a receptor for the P. falciparum protein EBL-1, a member of the Duffy-binding-like erythrocyte-binding protein (DBL-EBP) receptor family. The erythrocyte-binding domain, region 2 of EBL-1, expressed on CHO-K1 cells, bound glycophorin B but not glycophorin B-null erythrocytes. In addition, glycophorin B* but not glycophorin B-null erythrocytes adsorbed native EBL-1 from the P. falciparum culture supernatant. Interestingly, the Efe pygmies of the Ituri forest in the Democratic Republic of the Congo have the highest gene frequency of glycophorin B-null in the world, raising the possibility that the DBL-EBP family may have expanded in response to the high frequency of glycophorin B-null in the population.

Duffy-binding-like erythrocyte-binding protein | invasion | malaria | red blood cells

The asexual erythrocytic phase of the life cycle of Plasmodium falciparum produces all of the clinical symptoms, disease, and pathology associated with malaria. During this phase, merozoites released from schizont-infected erythrocytes invade uninfected erythrocytes. Invasion depends on distinct molecular interactions between ligands on the merozoite, the invasive form of the parasite, and host receptors on the erythrocyte membrane. To avoid infection, humans have evolved to eliminate or modify erythrocyte surface proteins that serve as receptors for parasite invasion. Perhaps one of the best examples of this evolutionary process is the loss of the Duffy blood group in Africa. Plasmodium vivax depends on two ligands for erythrocyte invasion: the Duffy-binding protein (DBP) that binds the Duffy blood group antigen (1, 2) and the reticulocyte homology protein that binds to an unknown receptor on reticulocytes (3). The Duffy blood group-null went almost to fixation in West Africa and is being selected in Papua New Guinea as a result of the inability of P. vivax to invade Duffy-negative erythrocytes (4).

Unlike P. vivax, P. falciparum has highly redundant, alternate invasion pathways that use several different receptor families. For example, P. vivax has only one gene, DBP, in the Duffy-binding-like erythrocyte-binding protein (DBL-EBP) family, whereas P. falciparum has four DBL-EBP genes: erythrocyte-binding antigen 175 (EBA-175), erythrocyte-binding antigen 140 (BAEEL/ EBA-140), erythrocyte-binding antigen 181 (JEB/ EBA-181), and erythrocyte-binding ligand-1 (EBL-1) (5-10). Consequently, no erythrocyte has been identified that is refractory to P. falciparum invasion. The erythrocyte-binding domains for P. vivax DBL-EBP and for three of the P. falciparum DBL-EBPs reside in the N-terminal cysteine-rich region, region 2 (8, 11, 12). In P. falciparum, the region 2 domain is duplicated; both domains are required for optimum binding to erythrocytes. The erythrocyte receptors for the P. falciparum DBL-EBPs include glycophorin A for EBA-175 and glycophorin C for one of the BAEBL/ EBA-140 proteins (6, 12). The receptor for JESEBL/EBA-181 is not known. No P. falciparum ligand has been found to bind glycophorin B, even though glycophorin B is highly polymorphic, suggesting that it is under a strong selective pressure. The polymorphisms are especially high in Africans and in African–Americans where multiple mutations exist, including the S-s-U-blood group (glycophorin B-null) that has a gene frequency of 59% in the Ituri forest pygmies (13, 14).

Here, we provide evidence that the fourth DBL-EBP family member, EBL-1, binds to glycophorin B. Region 2 of EBL-1 expressed on CHO-K1 cells bound to normal erythrocytes but not to glycophorin B-null erythrocytes. Interestingly, the Efe pygmies of the Ituri forest in the Democratic Republic of the Congo have the highest gene frequency of glycophorin B-null in the world, raising the possibility that the DBL-EBP family may have expanded in response to the high frequency of glycophorin B-null in the population.

**Results**

**Localization of EBL-1.** The spatial and temporal expression of EBL-1 has not been reported. To verify that the location of EBL-1 is similar to that of other members of the DBL-EBP family, we localized EBL-1 by fluorescent confocal microscopy. Antibodies specific for region 2 of EBL-1 of the P. falciparum clone Du2/Nm colocalized with RAPI, a rhoptry marker, and BAEBL, a microneme marker (Fig. 1). Despite the apparent complete colocalization of EBL-1-specific antibodies with RAPI and partial colocalization with BAEBL antibodies, the localization of EBL-1 indicates that it may be involved in invasion.
specific for region 2 of EBL-1 contained a protein of 11 kDa, the predicted size of EBL-1 (Fig. 2A). To characterize further the binding specificity of EBL-1, we studied binding to neuraminidase-, chymotrypsin-, and trypsin-treated human erythrocytes and human erythrocytes with genetically modified blood groups. Treatment with neuraminidase or chymotrypsin eliminated the ability of EBL-1 to bind human erythrocytes (Fig. 3A). However, EBL-1 bound to trypsin-treated erythrocytes in the same manner as untreated erythrocytes, indicating that the erythrocyte receptor required sialic acid attached to a peptide backbone that was susceptible to chymotrypsin but not to trypsin (Fig. 3B). One such protein on the erythrocyte with these characteristics is glycophorin B.

To determine further the binding specificity of EBL-1, we used S-s-U- (glycophorin B-null) human erythrocytes from two different donors. S-s-U- erythrocytes from both donors did not bind EBL-1 from the lysates of the Dd2/Nm clone of P. falciparum (Fig. 4A). In contrast, EBA-175, the ligand for glycophorin A, bound to S-s-U- erythrocytes (Fig. 4B).

Expression of EBL-1. To determine the temporal expression profile of EBL-1 during the intraerythrocytic stage, protein extracts from rings, trophozoites, and schizonts of P. falciparum clone Dd2/Nm were used for immunoblotting with antibodies specific for region 2 of EBL-1. We found that EBL-1 is expressed only in the schizont stage of the intraerythrocytic life cycle. The expression profile is similar to that of other DBL-EBPs. In control experiments, EBL-1 was not observed in protein extracts from P. falciparum clone HB3 that lacks the ebl-1 gene (9), nor was it observed with the preimmune sera (Fig. S1).

Binding Characteristics of EBL-1. To measure the binding of EBL-1 to human erythrocytes, we performed erythrocyte-binding assays by using soluble, metabolically labeled P. falciparum proteins. Schizont-infected erythrocytes were cultured without uninfected erythrocytes so that released merozoites would remain in the supernatant. Under such conditions, erythrocyte-binding proteins of the DBL-EBP family have been identified in the culture supernatants. Immunoprecipitation of culture supernatant of Dd2/Nm merozoites with antibodies specific for region 2 of EBL-1 contained a protein of ~300 kDa, the predicted size of EBL-1 (Fig. 2A). Similarly, immunoprecipitation of the Dd2/Nm supernatant with antibodies specific for region 2 of EBA-175 contained a protein of ~200 kDa. The antibodies specific for EBL-1 failed to immunoprecipitate a 300-kDa protein from the EBL-1-null clone HB3 (Fig. 2B) whereas an antibody to EBA-175 immunoprecipitated a protein of the correct size. To determine whether EBL-1 bound to erythrocytes, culture supernatant was incubated with ~50 µL of packed erythrocytes. The supernatant after absorption with human erythrocytes no longer contained EBL-1 and had a reduced amount of EBA-175, respectively. Treatment of the adsorbed erythrocytes with 1.5 M NaCl released both EBL-1 and EBA-175 from the erythrocytes, respectively (Fig. 2A).

Glycophorin B is the Erythrocyte Receptor of EBL-1. To characterize further the binding specificity of EBL-1, we studied binding to neuraminidase-, chymotrypsin-, and trypsin-treated human erythrocytes and human erythrocytes with genetically modified blood groups. Treatment with neuraminidase or chymotrypsin eliminated the ability of EBL-1 to bind human erythrocytes (Fig. 3A). However, EBL-1 bound to trypsin-treated erythrocytes in the same manner as untreated erythrocytes, indicating that the erythrocyte receptor required sialic acid attached to a peptide backbone that was susceptible to chymotrypsin but not to trypsin (Fig. 3B). One such protein on the erythrocyte with these characteristics is glycophorin B.

To define further the binding specificity of EBL-1, we used S-s-U- (glycophorin B-null) human erythrocytes from two different donors. S-s-U- erythrocytes from both donors did not bind EBL-1 from the lysates of the Dd2/Nm clone of P. falciparum (Fig. 4A). In contrast, EBA-175, the ligand for glycophorin A, bound to S-s-U- erythrocytes (Fig. 4B).
Fig. 4. EBL-1 does not bind to S-s-U- erythrocytes that lack glycophorin B. Proteins in culture supernatant were adsorbed to S-s-U- erythrocytes. The supernatant and the eluate were immunoprecipitated with antibodies to EBL-1 (A) or to EBA-175 (B). The same pattern of rosetting was observed when region 2 of EBL-1 was expressed on COS-7 cells. We found that the rosettes formed by region 2 of EBL-1 contained fewer erythrocytes than the rosettes formed by region 2 of EBA-175 (Fig. 5A). These rosettes were also less dense than those formed by region 2 of BAEBL/BA-140 and region 2 of JESEBL/BA-181. The region near the transmembrane sequence of EBL-1 did not bind human erythrocytes as would be expected. Region 2 of DBL-EBP in the P. falciparum genes is duplicated (F1 and F2 domains) compared with the P. vivax region 2 where only one copy is observed. The F1 and F2 domains of EBL-1 expressed alone on CHO-K1 cells did not bind to human erythrocytes, indicating that both domains are required for erythrocyte recognition.

**Discussion**

Here, we have provided evidence that the receptor for EBL-1, a member of the DBL-EBP family of receptors, is glycophorin B. First, EBL-1 from P. falciparum culture supernatant binds to glycophorin B and not to glycophorin B-null erythrocytes as indicated by immunoprecipitation with antibodies specific for region 2 of EBL-1. The immunoprecipitated protein was in the range of the predicted size of EBL-1. The antibodies specific for EBL-1 did not precipitate a protein from the supernatant of P. falciparum clone HB3 in which the ebl-1 gene is deleted in the genome (9). Second, region 2 of EBL-1 expressed on the surface of CHO-K1 cells bound glycophorin B erythrocytes but not glycophorin B-null erythrocytes in two genetically unrelated individuals.

Glycophorin B has long been suspected to be a receptor for P. falciparum because of the high level of polymorphism in individuals living in malaria-endemic regions. However, because P. falciparum has multiple redundant pathways of invasion, no difference has been observed between the invasion of glycophorin B-null and glycophorin B-positive erythrocytes. For example, invasion of erythrocytes by the P. falciparum Dd2/Nm clone that expresses EBL-1 and the HB3 clone that lacks EBL-1 is similar compared with invasion of glycophorin B-positive erythrocytes (Dd2, 79%; HB3, 72%) (16). EBL-1 and the other members of the DBL-EBP family share a high degree of sequence homology in region 2 and contain three exons at the 3' end that encode the transmembrane region and the cytoplasmic domain. EBL-1, however, differs in the 3' cysteine-rich region, the most conserved region of the DBL-EBP genes is duplicated (F1 and F2 domains) compared with the P. vivax region 2 where only one copy is observed. The F1 and F2 domains of EBL-1 expressed alone on CHO-K1 cells did not bind to human erythrocytes, indicating that both domains are required for erythrocyte recognition.

Glycophorin B shows a high degree of polymorphism in malaria-endemic areas, including S-s-U-, Henshaw with variable sequence of the first 5 aa, and Miltenberger mutations that have recombination between glycophorin A and B (17). The Miltenberger mutations in malaria-endemic areas always lead to loss of glycophorin B. The most extreme example is the 59% gene frequency on S-s-U- erythrocytes in the Efe pygmies of the Ituri...
Forest in the Democratic Republic of Congo (13). This region also has chimpanzees and gorillas that are infected with *Plasmodium reichenowi*, a *P. falciparum*-like *Plasmodium*. Evidence indicates that *P. falciparum* was spread through the world ~100,000 years ago (18), with a more recent expansion of infection with the introduction of agriculture in Africa and the diversification of the *Anopheles gambiae* mosquito complex ~10,000 years ago (19). We can only speculate as to when *P. falciparum* spread to the human population. It may have originated from *P. reichenowi* of the higher apes. It is possible that the pygmies lost glycophhorin B to resist *P. falciparum* after it first entered humans, and then the parasite may have undergone diversification of its invasion pathways and mutations to silence EBL-1. We speculate that the *P. reichenowi* genome may have a smaller number of DBL-EBP family members, one being syntenic with EBL-1.

Theoretical studies published as SI Text indicate that a null allele of glycophrorin B would need to afford only a modest level of protection against malaria in heterozygous to increase in frequency from a single mutant to an allele frequency of 0.59 in the relevant time frame. Assuming a constant population size 1,000–10,000 individuals, one need invoke a selective advantage of only 1% in homozygous-null genotypes to have a single copy of the null mutant allele increase to a frequency of 59% across an interval of 100,000 years (5,000 generations). A shorter time entails stronger selection, but even for 10,000 years (500 generations), a selective advantage of only 10% in homozygous-null genotypes is required. Both cases require partial dominance corresponding to 10–20% as much protection in heterozygous genotypes as in the homozygous-null. Table S1 also includes models of heterozygote superiority, where again the levels of selection that need be invoked are quite modest.

**Materials and Methods**

**Antisera.** Antisera specific for EBL-1 region 2 of Dd2/Nm were generated by immunization of rabbits with a DNA vaccine created with the vector VR1050 (kindly supplied by S. Hoffman, Naval Medical Research Center, Silver Spring, MD). This vector contains the T cell epitopes P2P30 from tetanus toxoid. Region 2 gene fragments of EBL-1 were amplified from *P. falciparum* clone Dd2/Nm and cloned into the VR1050 vector, described as VR1012tPap2p30 in refs. 19 and 20 but now renamed VR1050 (W. O. Rogers, personal communication). This vector contains the T cell epitopes P2P30 from tetanus toxoid. The inserts for regions 2 of Dd2/Nm spanned from amino acid D200 to refs. 19 and 20 but now renamed VR1050 (W. O. Rogers, personal communication). This vector contains the T cell epitopes P2P30 from tetanus toxoid. The inserts for regions 2 of Dd2/Nm spanned from amino acid D200 to

**Expression of Region 2 of EBL-1 on CHO-K1 Cells and COS-7 Cells.** Expression of region 2 of EBL-1 in CHO-K1 (American Type Culture Collection) were cultured in RPMI medium 1640 as described in ref. 12. Transfected cells were used for immunofluorescence or erythrocyte-binding assays 48 h after transfection.

**Immunolocalization of EBL-1.** The methods for immunolocalization of EBL-1 by confocal microscopy were performed as described in ref. 7. The secondary antisera consisted of Alexa Fluor 488-conjugated goat anti-rat IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) diluted 1:200 blocking buffer and 10% goat serum in PBS (Invitrogen). The membranes were blocked with 5% nonfat dry milk in PBS and 1% Tween 20 for 2 h at 4 °C. The membranes were stained with mouse monoclonal antibodies and Alexa Fluor 594-conjugated goat anti-mouse IgG (Invitrogen). The secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) diluted 1:200 in blocking buffer. The nuclei were stained with DAPI (Invitrogen). The cells were mounted labeled parasites in Prolong Antifade (Invitrogen).

**Immunoblot Analysis.** Equal concentrations of ring, trophozoite, and schizont stage protein samples from the clones Dd2/Nm were extracted in Laemmli sample buffer and separated on 8% SDS/polyacrylamide gels. The proteins were transferred to PVDF membranes. The membranes were blocked in 10% nonfat dry milk in PBS and 1% Tween 20 overnight at 4 °C. The membranes were incubated with primary antibodies for 1 h at room temperature. After washes, the membranes were incubated with 1:10,000 dilution of goat anti-rabbit, horseradish peroxidase-conjugated antibodies. The membranes were washed, and antibody complexes were detected with enhanced chemiluminescence (GE Healthcare).

**Enzyme Treatment of Erythrocytes.** Erythrocytes were collected in 10% citrate phosphate dextrose and stored at 4 °C. Erythrocyte samples were obtained from culture supernatant of schizont-infected erythrocytes that released merozoites in the absence of uninfected erythrocytes. The parasites were left to lyse and release proteins into the culture supernatant. The Dd2/Nm and HB3 clones of *P. falciparum* were cultured as described in ref. 7 with the following exceptions. Schizont-infected erythrocytes (5 × 10³ per mL of culture medium) were used during the metabolic labeling with 200 μCi/mL [³⁵S]Cys-Met. The culture supernatant was ultracentrifuged in a Beckman Optima TLX ultracentrifuge (Beckman–Coulter) at 40,000 rpm (88,600 × g) for 10 min at 4 °C before storage of the supernatant at 80 °C.

**Immunoprecipitation.** Proteins in the supernatant and in the diluted eluate were immunoprecipitated as described in ref. 7 with the following exceptions. The supernatant (50 μL) was diluted into 250 μL of NETT (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) supplemented with 0.5% BSA (ICN) to decrease background. Radiolabeled supernatant (50 μL) was preabsorbed with protein A-Sepharose as described elsewhere (7). The supernatant was split into 2 equal volumes and immunoprecipitated with 5 μL of anti-EBL-1 region 2 and 1 μL of anti-EBA-175 region 2, as described above. Protein G-Sepharose (40 μL, 50% (vol/vol)); GE Healthcare) was added to remove the immune complexes.

**Metabolic Labeling of Parasite Proteins.** Soluble, metabolically labeled parasite proteins were obtained from culture supernatant of schizont-infected erythrocytes that released merozoites in the absence of uninfected erythrocytes. The parasites were left to lyse and release proteins into the culture supernatant. The Dd2/Nm and HB3 clones of *P. falciparum* were cultured as described in ref. 7 with the following exceptions. Schizont-infected erythrocytes (5 × 10³ per mL of culture medium) were used during the metabolic labeling with 200 μCi/mL [³⁵S]Cys-Met. The culture supernatant was ultracentrifuged in a Beckman Optima TLX ultracentrifuge (Beckman–Coulter) at 40,000 rpm (88,600 × g) for 10 min at 4 °C before storage of the supernatant at 80 °C.
DL6 and ID3 directed against the herpes simplex glycoprotein D sequences as described (11, 15, and gifts from R. Eisenberg and G. Cohen, University of Pennsylvania, Philadelphia), and rabbit polyclonal antisera directed against region II of EBL-1, respectively.

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