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Molecular basis of binding of the *Plasmodium falciparum* receptor BAEBL to erythrocyte receptor glycophorin C

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Abstract

Plasmodium falciparum invades human erythrocytes by redundant pathways. Unlike *P. vivax* that has one Duffy Binding-Like (DBL) receptor, *P. falciparum* has four members of the DBL receptor family. Furthermore, one of these DBL genes, BAEBL, has polymorphisms at four amino acids in region II; each polymorphism binds to a different erythrocyte receptor. One BAEBL variant (VSTK) binds specifically to erythrocyte glycophorin C and binds poorly to neuraminidase-treated erythrocytes. When the amino acid threonine (T121) in BAEBL (VSTK) is changed to a lysine (VSKK), it no longer requires sialic acid as a receptor. To explore the molecular basis of sialic acid binding, we modeled the structure of region II of BAEBL (VSTK) on the crystal structure of a related DBL receptor, region II of erythrocyte binding antigen-175 (EBA-175). Four charged amino acids, R52, R114, E54 and D125, are predicted to surround T121 in BAEBL (VSTK). They were individually mutated to alanine (R52A, R114A, E54A, and D125A) or lysine (R52K, R114K) and expressed on the surface of Chinese hamster ovary (CHO-K1) cells. BAEBL (VSTK) with mutations in R52 or R114 of BAEBL (VSTK) bound neuraminidase-treated erythrocytes. Unlike the arginine mutations, E54A and D125A still bound poorly to neuraminidase-treated erythrocytes. These findings suggest that the two arginine residues surrounding T121 are critical for the binding specificity of BAEBL (VSTK) to sialic acid and suggest a role for arginine in sialic acid binding independent of its negative charge.

Keywords

Mutations; BAEBL; Glycophorin C; Sialic acid; Erythrocyte receptors

1. Introduction

Receptors on merozoites bind erythrocytes to form a junction at the apical end of merozoites [1]. Invasion of erythrocytes follows junction formation. Region II of the Duffy binding-like (DBL) family of receptors is the domain of the larger protein that binds erythrocytes and forms

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a junction [2,3]. It was first identified through studies on the binding of *Plasmodium vivax* Duffy binding protein to Duffy blood group positive but not to Duffy negative erythrocytes that are resistant to infection by *P. vivax* [4]. There are five proteins in *P. falciparum* that have the structure of the Duffy binding protein of *P. vivax* with the exception that Region II, the Duffy binding domain, is duplicated [5]. Each binds different receptors on erythrocytes by this duplicated domain [3]. In addition to multiple Duffy-like proteins for invasion of erythrocytes in *P. falciparum*, two (BAEBL and JESEBL) have multiple binding receptors as a result of single point mutations [6,7]. For example, BAEBL has four amino acids in the first domain (F1) of the duplicated domains of region II that undergo mutations, each of which has a different erythrocyte receptor [6]. As a result, BAEBL specificity is defined by the sequence of these four mutations. For example, BAEBL (VSTK) fails to bind neuraminidase-treated erythrocytes and Gerbich-negative erythrocytes [6] that lack exon 3 of glycophorin C [8]. Furthermore, glycophorin C inhibits binding only of BAEBL (VSTK) but not of the other BAEBL mutants [9]. N-deglycosylation of normal glycophorin C destroys the ability of glycophorin C to inhibit binding of erythrocytes to BAEBL (VSTK) [9]. However, the N-linked oligosaccharide could not inhibit attachment, suggesting that the receptor on glycophorin C is, in part, an N-linked oligosaccharide, but may also include O-linked oligosaccharides of glycophorin C [9]. The binding definitely requires the sialic acid on glycophorin C [9].

As a result of a single point mutation of BAEBL (VSTK) to BAEBL (VSKK), BAEBL (VSKK) can now bind neuraminidase-treated and Gerbich-negative erythrocytes [6]. The solution of the structure of region II of another member of the *P. falciparum* Duffy binding family, erythrocyte binding antigen-175 (EBA-175) [10], presented us with the opportunity to explore the basis of binding of sialic acid by mutating the sequences predicted to surround threonine (T of VSTK). EBA-175 is closest in sequence to BAEBL compared to the other *P. falciparum* Duffy binding proteins, improving the ability to model BAEBL (VSTK). From the mutational analysis, it appears that the two arginines (R114 and R52) are critical for BAEBL (VSTK) binding, whether they are changed to alanine or lysine.

2. Materials and methods

2.1. Homology Modeling of BAEBL Region II

A structure model for the region II of BAEBL was generated based on the published region II structure of EBA-175 (PDB entry code 1ZRO) using a homology threading modeling software Swiss-Model (<http://swissmodel.expasy.org/SWISS-MODEL.html>). All structural figures were created using PyMol (<http://pymol.sourceforge.net/>).

2.2. Sequence Analysis of erythrocyte-binding regions in DBL family

Protein sequences of Region II of BAEBL (VSTK) (Dd2, GenBank ID: 13926052) and EBA-175 (M. Camp, GenBank ID: 1352334) were aligned using a ClustalW alignment program at EMBL-EBI website (<http://www.ebi.ac.uk/Tools/clustalw2/>).

2.3. Construction of Plasmids for Surface Expression of BAEBL Region II mutants on Chinese hamster ovary (CHO-K1) Cells

Region II of BAEBL from parasite clones Dd2 (VSTK) and HB3 (VSKK) were cloned into the NotI and EcoRI sites of the T8 vector, which encodes a signal peptide and a glycosylphosphatidylinositol anchor to attach the expressed protein to the surface of CHO-K1 cells [11]. Mutations in arginine 52 (to alanine or lysine), arginine 114 (to alanine or lysine), glutamic acid 54 (to alanine) or aspartic acid 125 (to alanine) of BAEBL (VSTK) Region II in recombinant T8 plasmid were made individually. A Duffy-like domain (DBL2) from *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) was cloned into the T8 vector using the same restricted enzyme sites as BAEBL Region II [11]. This domain does not bind erythrocytes

and was used as a negative control in each erythrocyte-binding assay. Another construct containing region II of EBA-175 in T8 vector was also used as control as this insert binds normal erythrocytes and Gerbich-negative erythrocytes but not neuraminidase-treated erythrocytes [11].

2.4. Cell Culture and Transfection

CHO-K1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI medium 1640 with 10% fetal calf serum, 4 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10 mM HEPES, pH 7.55 (Invitrogen, Carlsbad, CA) in a humidified 5% CO₂ incubator at 37° C. Cells in each 10 cm² well of 6-well culture vessel were transfected at 90–95% confluency with 4 µg of plasmid DNA using lipofectamine 2000 (Invitrogen, Carlsbad, CA) as mentioned in the product protocol, and the transfection reaction was gently mixed by bubbling to improve transfection efficiency. Six hours after transfection, transfected CHO-K1 cells were released by 1 ml of 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA), and washed by 2 ml of RPMI medium 1640 with 10% fetal calf serum, 4 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10 mM HEPES, pH 7.55 (Invitrogen, Carlsbad, CA). 1×10^5 transfected cells were transferred onto a 0.7 cm² well of a CC2 treated 8-chamber glass slide (Lab-TekTM II-CC^{2TM}, Thermo Fisher Scientific, Waltham, MA) and cultured in RPMI medium 1640 with 10% fetal calf serum, 4 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10 mM HEPES, pH 7.55 (Invitrogen, Carlsbad, CA) in a humidified 5% CO₂ incubator at 37° C. 20–24 hours after transfection, the transfected cells were used for the erythrocyte binding assays. To evaluate the transfection efficiency of each construct to CHO-K1 cells, expression of recombinant proteins outside of transfected CHO-K1 cells in the same experiment was also determined 20–24 h after transfection using a mouse monoclonal antibody 179 by immunofluorescence assay as described previously [6].

To determine if the number of CHO-K1 cells bound to each well was similar for each transfection of BAEBL variants (VSTK and VSCK) and mutants (VSTK-R52A, VSTK-R52K, VSTK-R114A, and VSTK-R114K), we counted the number of CHO-K1 cells in each well after the binding of normal erythrocytes to CHO-K1 cells. Briefly, after removing unbound erythrocytes, CHO-K1 cells were fixed by 0.16% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 1 ml of RPMI Medium 1640 (Invitrogen, Carlsbad, CA) plus 10 mM HEPES (pH 7.55, Invitrogen, Carlsbad, CA). 1 µl of 0.5% 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen, Carlsbad, CA) in H₂O was added into each well for DNA staining. The amount of DAPI-stained nuclei in each field (0.37 mm²) was counted under ultraviolet light using an inverted microscope (Leica DMIRE 2, Wetzlar, Germany). The average of five fields from each well was multiplied by 190 to give the total number of CHO-K1 cells in 0.7 cm².

2.5. Erythrocyte-Binding Assay

Normal erythrocytes from O⁺ type whole blood were collected and stored at 4° C for up to 2 weeks as described previously [6]. Fresh erythrocytes were treated with 6 mU/ml of neuraminidase (*Vibrio cholerae*; Calbiochem, Gibbstown, NJ) and stored at 4° C in RPMI medium 1640 (Invitrogen, Carlsbad, CA) for up to 1 week as described previously [6]. Gerbich-negative erythrocytes were collected from a donor and stored in liquid nitrogen as described previously [12]. One day before use, frozen Gerbich-negative erythrocytes were thawed and stored at 4° C overnight as described previously [13]. 600 µl of 0.6 % erythrocyte suspension in RPMI medium 1640 with 10% fetal calf serum, 4 mM glutamine, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10 mM HEPES, pH 7.55 (Invitrogen, Carlsbad, CA) was added to transfected CHO-K1 cells and incubated at 37°C for 4 hours to allow erythrocytes to settle down completely. Unbound erythrocytes were removed twice by gravity for 45 min after inverting chamber slide in RPMI Medium 1640 (Invitrogen, Carlsbad, CA) plus 10 mM HEPES

(pH 7.55, Invitrogen, Carlsbad, CA). Transfected CHO-K1 cells with rosettes of at least 5 bound erythrocytes in each well were scored using an inverted microscope (Leica DMIRE 2, Wetzlar, Germany). All rosette numbers from assays with constructs of BAEBL region II were subtracted by the number of rosettes with construct of DBL-2.

2.6. Statistic analysis of different region II of BAEBL binding with normal erythrocytes

The number of rosettes with normal erythrocytes was analyzed using a mixed-effects model [14], with a different fixed effect for BAEBL (yes/no) and mutations (6 mutations), as well as random effects for replicates (4–6 repeats) in a software of R package version 3.1–83 [15].

3. Results

To determine if we could model the structure of BAEBL (VSTK) based on the crystal structure of EBA-175, we compared the primary sequence of F1 of the two genes (Supplemental Fig. 1). The two sequences have 34.6 % homology with the 12 cysteines in BAEBL corresponding in location to the 12 cysteines in EBA-175. EBA-175 has one cysteine that is not found in BAEBL. In addition, the five tryptophanes are located in the identical positions in the two sequences. Besides the homology, there is a high degree of structural similarity between the two sequences (Supplemental Fig. 1). This alignment was possible with only three spaces inserted into BAEBL and one into EBA-175 over a sequence of 315 and 317 amino acids, respectively.

In the model, there were five residues, two arginines (R52 and R114), a tyrosine (Y118), glutamic acid (E54) and aspartic acid (D125), located next to T121 (Fig. 1). In region II of EBA-175, another positively charged amino acid lysine occurred at the corresponding positions of R52 or R114 in region II of BAEBL, while no related amino acid in region II of EBA-175 was found for E54 or D125 in region II of BAEBL (Supplemental Fig. 1). To date, the known receptors for sialic acid include the influenza hemagglutinin, influenza neuraminidase, as well as a family of sialic acid binding immunoglobulin-like lectins (Siglec) [16–18]. Although there is no conserved binding motif among the three sialic acid receptors, both neuraminidase and Siglec receptors relied on charge neutralization between positively charged arginines (R) of the receptor and the negatively charged sialic acid in their ligand recognition. Hemagglutinin, on the other hand, employed a network of hydrogen bonding residues including serine (S), histidine (H), tyrosine (Y), and glutamic acid (E) to coordinate the sialic acid. While the polymorphic T121 in BAEBL is clearly important for sialic acid specificity, the proximity of two arginine residues raises the possibility that they are also involved in the recognition of sialic acid.

To investigate if these charged amino acids are interactive in the binding of T121 to sialic acid on the erythrocyte surface, we individually mutated these amino acids to alanine (A) and cloned them into T8 vector [11]. Recombinant region II proteins of BAEBL (VSTK) mutants (VSTK-R52A, VSTK-R114A, VSTK-E54A, and VSTK-D125A) were expressed on the surface of CHO-K1 cells. In the same experiment, region II of BAEBL (VSKK) and BAEBL (VSTK) were expressed on the surface of CHO-K1 cells as controls. Surface expression was demonstrated by reactivity of a monoclonal antibody against an epitope immediately C-terminal to the parasite protein and just N-terminal to the GPI anchor. All intact cells expressing the recombinant protein reacted with the monoclonal antibody, indicating that the protein was expressed outside of the CHO-K1 cells (Fig. 2A). Transfection efficiency was 60 to 70% in all constructs in CHO-K1 cells. The transfected CHO-K1 cells were evaluated for binding with normal human erythrocytes, neuraminidase-treated erythrocytes and Gerbich-negative erythrocytes. Transfected CHO-K1 cells expressing each recombinant BAEBL region II protein could form rosettes with normal erythrocytes (Fig. 2B). The number of rosettes with neuraminidase-treated and Gerbich-negative erythrocytes was expressed as a percent of the

number of rosettes with normal erythrocytes. All slides were read in a blinded fashion, in that the person reading the slides did not know the recombinant protein transfected or the erythrocytes used for binding. As has been previously described, EBA-175 and BAEBL (VSTK) failed to bind neuraminidase-treated erythrocytes. BAEBL (VSTK) also poorly bound to Gerbich-negative erythrocytes. BAEBL (VSKK) bound well to both of these erythrocytes.

Statistic analysis showed an overall significant effect of BAEBL mutations binding to normal erythrocytes ($p=0.0250$). All of the region II mutants of BAEBL (VSTK) could bind normal erythrocytes, although R52A and R114A bound at a lower rate ($p=0.0308$ and $p=0.0051$, respectively) than the other mutants and the wild type recombinants. R52A and R114A had increased binding to neuraminidase-treated and Gerbich-negative erythrocytes than to normal erythrocytes (Fig. 2C). Like the wild type BAEBL (VSTK), each mutation of E54A and D125A still bound poorly to neuraminidase-treated and Gerbich-negative erythrocytes (Fig. 2C). These studies indicate that R52 and R114 in region II of BAEBL (VSTK) play a critical role in sialic acid-dependant and Gerbich-negative binding of BAEBL (VSTK) to erythrocytes.

However, the results may indicate that sialic acid-dependant binding of BAEBL (VSTK) to erythrocytes requires only positive charge from amino acid positions R52 and R114. To further investigate if the positive charge from R52 or R114 is necessary for BAEBL (VSTK) binding to erythrocytes in a sialic acid-dependent manner, R52 and R114 were individually mutated to lysine to keep the same charge surrounding T121. As shown in Figure 3, each of the lysine mutations markedly increased the binding to neuraminidase-treated erythrocytes, indicating that the positive charge of R52 or R114 was not enough to account for sialic acid-dependent binding of erythrocytes to BAEBL (VSTK). Instead, the arginine itself played a critical role with the threonine (T) in the binding to sialic acids.

4. Discussion

Region II of *P. falciparum* DBL genes is formed by duplicated domains, F1 and F2 [5], both of which are necessary for optimal binding of EBA-175 to erythrocytes [3]. Four amino acid positions with polymorphisms in region II of the *baebl* gene from worldwide *P. falciparum* isolates occurred only in the F1 domain [6]. BAEBL variants that differed in these four amino acids bound to different erythrocyte receptors [6]. Glycophorin C blocks the binding of the parasite protein BAEBL (VSTK) to erythrocytes [9]. Neuraminidase-treated erythrocytes did not bind to BAEBL (VSTK), indicating a critical role of sialic acid, in both N-linked and O-linked oligosaccharides, in its binding to glycophorin C [9]. Gerbich-negative erythrocytes that express a truncated form of glycophorin C with an exon 3 deletion bound poorly to BAEBL (VSTK) expressed on CHO-K1 cells. BAEBL (VSKK) with a single mutation of T121K bound neuraminidase-treated erythrocytes and Gerbich-negative erythrocytes, indicating that the T121 was critical in the binding of sialic acid.

The selective force of BAEBL (VSTK) may have effected the human population in that Gerbich-negative occurs in 50% of the people in the North coastal region of Papua New Guinea where malaria is holoendemic [19,20]. Thus when BAEBL (VSTK) switches to BAEBL (VSKK) by a single point mutation, the specificity to Gerbich-negative and to a new erythrocyte receptor occurs. The new erythrocyte receptor is not present for BAEBL (VSTK). This unusual finding that single point mutations change the erythrocyte specificity were selected to give greater flexibility to *P. falciparum* so that the parasite cannot be eliminated by a deletion of exon 3 of glycophorin C. Unlike *P. falciparum*, *P. vivax* was eliminated from West Africa by a single point mutation in the Duffy blood group promoter [21] and is beginning to be eliminated from Papua New Guinea by the same mutation [22].

The mutations of R52 and R114 to either alanine (A) or lysine (K) resulted in a loss of sialic acid specificity of the parental VSTK strain of BAEBL. This suggests that R52 and R114 in region II of BAEBL, together with T121 contribute to the binding of sialoglycans on the surface of erythrocytes and that the primary interaction is through charge neutralization between the positively charged arginines (R) and the negatively charged sialic acid. Moreover, the failure of the lysine (K) mutations to compensate the wild type arginine (R) phenotype in erythrocyte recognition argues that the presence of charge neutralization is insufficient for the receptor specificity, and that the stereochemistry of the interaction is also important for the receptor specificity. Specifically, the arginine (R) residues could form a bipartite salt bridge with two of the arginine (R) side chain guanidino groups each positioned in hydrogen bonding distance to the two carboxylate groups of the sialic acid, similar to that observed in the Siglec structures [18]. In contrast, mutations of E54A and D125A retained the binding profile of wild type BAEBL (VSTK) to erythrocytes, suggesting these two residues are not involved in defining sialoglycan specificity in the BAEBL (VSTK). The EBA-175 glycan binding site is not in the region of the polymorphic residues of BAEBL (Fig. 4), suggesting a potential distinguished glycan recognition between EBA-175 and BAEBL.

The interpretation of data offered above for sialic acid binding does not explain why T121K, R52A/K or R114A/K confers the ability of BAEBL to bind independent of sialic acid to erythrocytes. Before these single amino acid changes, BAEBL (VSTK) was unable to bind neuraminidase treated erythrocytes. An understanding of this change in specificity to BAEBL (VSKK) will require identification of its erythrocyte receptor and the structure of this erythrocyte receptor with BAEBL (VSKK).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DBL	Duffy Binding-Like
PfEMP1	<i>P. falciparum</i> erythrocyte membrane protein 1
EBA-175	erythrocyte binding antigen-175
CHO-K1 Cells	Chinese hamster ovary cells

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Fig. 1. Two views of the predicted 3-D structure of F1 of Region II of BAEBL (VSTK) modeled from the structure of F1 of EBA-175 [10]. The four polymorphic amino acids can be seen on the left panel. Four charged amino acids surrounding T121 are shown on the right panel, specifically, which are two positively charged arginines (R52 and R114) in blue, and two negatively charged amino acids (E54 and D125) in red.

Fig. 2.

The effect of alanine mutations of amino acids R52, R114, E54 and D125 in region II of *P. falciparum* receptor BAEBL (VSTK) on its binding specificity to erythrocytes. Transfected CHO-K1 cells expressing mutated region II of *P. falciparum* erythrocyte-binding receptor BAEBL were assayed for binding with normal, neuraminidase-treated and Gerbich-negative erythrocytes. Positions of the mutations are shown in Figure 1 and Supplemental Figure 1. (A) Expression of each region II of BAEBL on the surface of CHO-K1 cells was tested by immunofluorescence assay. Transfection efficiencies of all constructs to CHO-K1 cells were in the range of 60–70%. (B) The recombinant region II proteins bound normal erythrocytes and formed rosettes. (C) Transfected cells with five or more bound erythrocytes were counted as rosettes. Total rosette numbers of binding with normal erythrocytes are mean ± SE (standard error). The number of independent experiments is in parentheses. The amounts of CHO-K1 cells expressing different BAEBL recombinant proteins (VSTK, VSKK, VSTK-R52A, and VSTK-R114A) bound to each well are between $1.4\text{--}1.5 \times 10^5$. Data from neuraminidase-treated and Gerbich-negative erythrocytes are expressed as the percentage of rosettes compared to rosettes for normal erythrocytes. Bars represent 10 μm in panel (A) and (B). RBCs, red blood cells.

Fig. 3.

Lysine mutations of amino acids R52 and R114 in region II of *P. falciparum* receptor BAEBL (VSTK) alter its erythrocyte receptor specificity. As described in the legend of Figure 2, surface expression of each recombinant BAEBL region II protein was shown in panel (A) and typical rosettes with normal erythrocytes were shown in panel (B). (C) Data from neuraminidase-treated and Gerbich-negative erythrocytes are expressed as the percentage of rosettes compared to rosettes for normal erythrocytes. The control data for BAEBL (VSKK) and BAEBL (VSTK) are the same as those shown in Figure 2. Transfected cells with five or more bound erythrocytes were counted as rosettes. Total rosette numbers of binding with normal erythrocytes are mean $\pm$ SE (standard error). The number of independent experiments is in parentheses. The amount of CHO-K1 cells bound to each well is between $1.4\text{--}1.5 \times 10^5$ in each case. Transfection efficiencies of all constructs to CHO-K1 cells were in the range of 60–70%. Bars represent 10 μ m in panel (A) and (B). RBCs, red blood cells.

Fig. 4. The dimeric structure of EBA-175 [10] is shown as ribbons with the two monomers colored in cyan and green. The known glycan binding sites of EBA-175 are labeled in yellow. The polymorphic residues (VSTK) are labeled in red and the mutational sites, R52, E54, R114 and D125 are labeled in white.

Table 1

Position of amino acid polymorphisms in F1 domain of BAEBL

Variants	Binding [*] to			Glycophorin C blocking ^{†b}
	Normal erythrocytes	Neuraminidase-treated erythrocytes	Gerbich-negative erythrocytes	
E12 (ISKK) ^a	++	–	++	–
M. Camp (INRE) ^a	++	++	++	–
HB3 (VSKK)	++	++	++	NT
Dd2 (VSTK)	++	–	–	+
Dd2E54A	++	–	–	NT
Dd2D125A	++	–	–	NT
Dd2R52A	+	++	+	NT
Dd2R52K	++	++	++	NT
Dd2R114A	+	++	++	NT
Dd2R114K	++	++	++	NT

^{*} Binding of erythrocytes to CHO-K1 cells expressing the recombinant BAEBL region II protein.

++ strong binding; +, weak binding; –, no binding.

^a Refers to data from a previous publication [6].

[†] Blocking of binding of BAEBL variants from *P. falciparum* culture supernatants to normal erythrocytes by glycophorin C. +, blocking; –, no blocking; NT, not tested.

^b Refers to data from a previous publication [9].