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Recommended Citation

Salinas, Nichole D.; Paing, May M.; and Tolia, Niraj H., "Critical glycosylated residues in exon three of erythrocyte Glycophorin A engage Plasmodium falciparum EBA-175 and define receptor specificity." *mBio*. 5, 5. e01606-14. (2014).

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2014. Critical Glycosylated Residues in Exon Three of Erythrocyte Glycophorin A Engage *Plasmodium falciparum* EBA-175 and Define Receptor Specificity. mBio 5(5): .
doi:10.1128/mBio.01606-14.

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Critical Glycosylated Residues in Exon Three of Erythrocyte Glycophorin A Engage *Plasmodium falciparum* EBA-175 and Define Receptor Specificity

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N.D.S. and M.M.P. contributed equally to this work.

ABSTRACT Erythrocyte invasion is an essential step in the pathogenesis of malaria. The erythrocyte binding-like (EBL) family of *Plasmodium falciparum* proteins recognizes glycophorins (Gp) on erythrocytes and plays a critical role in attachment during invasion. However, the molecular basis for specific receptor recognition by each parasite ligand has remained elusive, as is the case with the ligand/receptor pair *P. falciparum* EBA-175 (PfEBA-175)/GpA. This is due largely to difficulties in producing properly glycosylated and functional receptors. Here, we developed an expression system to produce recombinant glycosylated and functional GpA, as well as mutations and truncations. We identified the essential binding region and determinants for PfEBA-175 engagement, demonstrated that these determinants are required for the inhibition of parasite growth, and identified the glycans important in mediating the PfEBA-175–GpA interaction. The results suggest that PfEBA-175 engages multiple glycans of GpA encoded by exon 3 and that the presentation of glycans is likely required for high-avidity binding. The absence of exon 3 in GpB and GpE due to a splice site mutation confers specific recognition of GpA by PfEBA-175. We speculate that GpB and GpE may have arisen due to selective pressure to lose the PfEBA-175 binding site in GpA. The expression system described here has wider application for examining other EBL members important in parasite invasion, as well as additional pathogens that recognize glycophorins. The ability to define critical binding determinants in receptor–ligand interactions, as well as a system to genetically manipulate glycosylated receptors, opens new avenues for the design of interventions that disrupt parasite invasion.

IMPORTANCE *Plasmodium falciparum* uses distinct ligands that bind host cell receptors for invasion of red blood cells (RBCs) during malaria infection. A key entry pathway involves *P. falciparum* EBA-175 (PfEBA-175) recognizing glycophorin A (GpA) on RBCs. Despite knowledge of this protein–protein interaction, the complete mechanism for specific receptor engagement is not known. PfEBA-175 recognizes GpA but is unable to engage the related RBC receptor GpB or GpE. Understanding the necessary elements that enable PfEBA-175 to specifically recognize GpA is critical in developing specific and potent inhibitors of PfEBA-175 that disrupt host cell invasion and aid in malaria control. Here, we describe a novel system to produce and manipulate the host receptor GpA. Using this system, we probed the elements in GpA necessary for engagement and thus for host cell invasion. These studies have important implications for understanding how ligands and receptors interact and for the future development of malaria interventions.

Received 9 July 2014 Accepted 4 August 2014 Published 9 September 2014

Citation Salinas ND, Paing MM, Tolia NH. 2014. Critical glycosylated residues in exon three of erythrocyte glycophorin A engage *Plasmodium falciparum* EBA-175 and define receptor specificity. mBio 5(5):01606–14. doi:10.1128/mBio.01606-14.

Editor Louis H. Miller, National Institute of Allergy and Infectious Diseases

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Forty percent of the world's population is at risk of malaria infection, and more than 800,000 lives, mostly children, are lost annually (1). Of the six *Plasmodium* species that cause malaria in humans, infection with *Plasmodium falciparum* results in the majority of disease and death across sub-Saharan Africa. The asexual parasite stage within the bloodstream is responsible for all disease pathology, and proteins critical for invasion of erythrocytes are attractive vaccine targets for malaria control.

The parasite ligands and host receptors involved in invasion include the erythrocyte binding-like (EBL) family of *P. falciparum* proteins (2, 3) that bind to glycophorins (Gp) on erythrocytes:

P. falciparum EBA-175 (PfEBA-175) binds to GpA (4–8), PfEBA-140 binds to GpC (9–16), and PfEBL-1 binds to GpB (17, 18). A fourth EBL member, PfEBA-181, plays a role in invasion, but the erythrocyte receptor is unknown (14, 19, 20). The *P. falciparum* EBL proteins are membrane embedded and are characterized (2) by the presence of two structurally and functionally conserved Duffy-binding-like (DBL) domains, designated region II (RII) domains (6, 7, 9, 10, 15, 18, 21), and a conserved cysteine-rich domain, labeled region VI (22).

GpA is the major sialylated protein on the surface of erythrocytes (23, 24). GpA exists as a cell surface dimer, with interactions

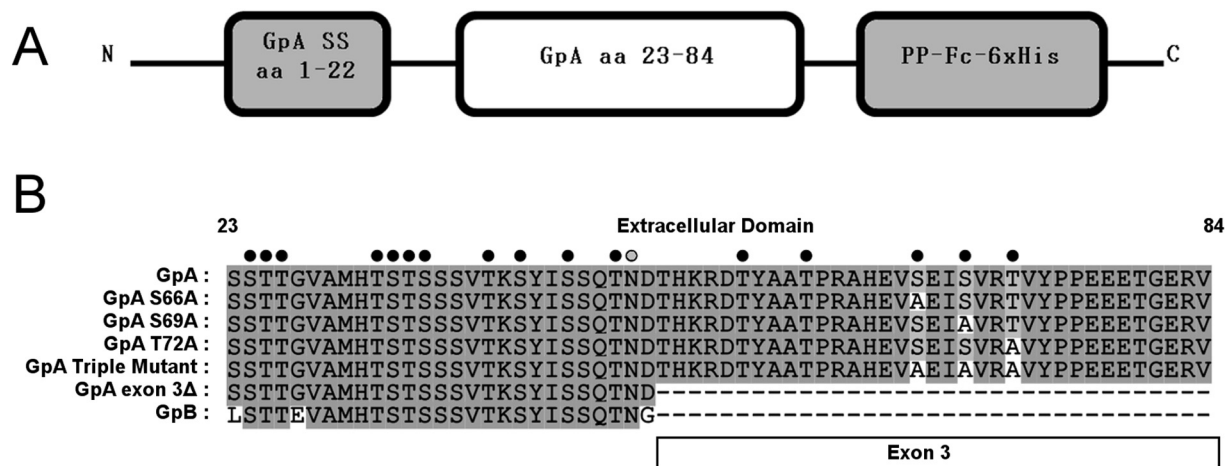


FIG 1 Schematic of constructs for glycoprotein A (GpA) expression. (A) Domain structure of the GpA constructs, including the signal sequence (GpA SS), the extracellular domain (GpA amino acids 23 to 84), and the Fc fusion protein with the PreScission protease (PP) and 6×His tag. (B) Amino acid sequence alignment of GpA constructs used and GpB for comparison. O-glycans are indicated by black circles, and the single N-glycan is indicated by a grey circle. Sequence similarity is indicated by grey shading.

mediated through the transmembrane helices (25–28). The GpA gene contains seven exons, three of which encode the signal sequence and extracellular domain (23, 24, 29). The extracellular domain contains 16 O-glycans and one N-glycan, with the glycans comprising ~50% of the total molecular weight for this glycoprotein receptor (23, 24, 29, 30). Sialic acid moieties on GpA are a critical determinant for PfEBA-175 engagement of erythrocytes, since neuraminidase treatment to remove α2-3-linked sialic acid from the erythrocyte surface abolishes PfEBA-175 binding to erythrocytes (5, 6). Furthermore, mutant Tn and Cad erythrocytes have aberrant O-glycosylation and are resistant to invasion by *P. falciparum* (31).

The crystal structure of PfEBA-175 RII in complex with sialyllactose identified a total of six glycan binding pockets per PfEBA-175 RII dimer (7). The presence of multiple pockets for sialic acid binding highlights that multiple glycans in GpA are engaged by PfEBA-175. EBL ligands have been shown to engage receptors through receptor-mediated ligand dimerization, facilitating multimeric assembly to form stable and strong receptor-ligand complexes (21, 32–34). Dimeric GpA likely induces dimerization of PfEBA-175, leading to multimeric assembly of the complex and high-avidity binding (7, 35). This binding requires both DBL domains in RII (8) and is enhanced by additional regions in PfEBA-175 (35). Receptor binding and multimeric assembly are critical for parasite invasion, since an antibody (36) that targets the GpA-binding pockets and the dimer interface of PfEBA-175 RII potentially neutralizes *P. falciparum* invasion (37, 38). Together, these results demonstrate that direct receptor blockade of the PfEBA-175–GpA interaction is a viable method for parasite neutralization.

Although the ligands and their respective receptors have been known for years, the molecular basis for specific receptor recognition by parasite ligands is still unclear, in spite of available crystal structures (7, 15, 16). PfEBA-175 selectively binds to GpA but not to GpB (6), but the determinants for receptor specificity have remained elusive due to difficulties in expressing properly glycosylated receptors or glycan mutants for studies. GpA shares ~95% sequence homology with members within the same class, GpB and

GpE (23, 29). However, only GpA expresses exon 3 due to a splice site mutation in GpB and GpE that eliminates the exon, and the inclusion of exon 3 results in multiple O-glycans that are unique to GpA. Glycophorins are under positive selection, and 43 known naturally occurring variants of GpA have been identified (23, 29, 39). Many of these variants map to a zone of recombination in exon 3 and are likely to result in functional consequences.

Here we describe molecular determinants for specific engagement of GpA by PfEBA-175 during host cell invasion. We demonstrate that properly glycosylated and functional recombinant GpA can be produced and purified in large quantities using a mammalian expression system. We show that the critical binding region of GpA for PfEBA-175 maps to distinct glycosylation residues in exon 3. The absence of exon 3 glycans in GpA prevents binding to PfEBA-175, and a GpA construct similar to GpB is unable to engage PfEBA-175. We further show that recombinant GpA disrupts parasite growth of three *P. falciparum* strains and that the glycans in exon 3 are essential for parasite neutralization by recombinant GpA.

RESULTS

Recombinant GpA, GpA exon 3Δ, and GpA glycosylation mutants are expressed and purified to high yields from a mammalian cell culture system. We previously reported preliminary expression and purification conditions for the recombinant GpA extracellular domain (rGpA) (37). We optimized expression and purification conditions to improve the yield of rGpA, necessary for further study. All recombinant GpA constructs were produced by a fusion of the extracellular domain of GpA to an antibody Fc domain with a C-terminal His tag (Fig. 1). The fusion constructs were transiently transfected into 293F cells, and the optimal DNA/polyethyleneimine (PEI) ratio and time of expression were determined for each construct (Fig. 2A; see also Fig. S1 in the supplemental material). A DNA/PEI ratio of 1:2 produced the highest yields and was used for all subsequent transfections of recombinant glycoproteins. Expression for all constructs was observed 24 h posttransfection and continued to increase over time. Large-scale expression was conducted at the optimal DNA/PEI ratio for

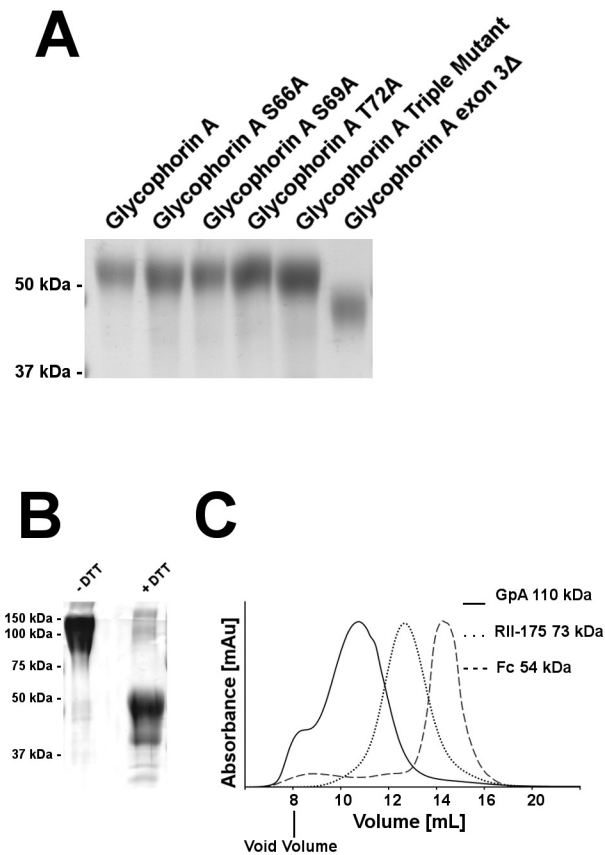


FIG 2 Expression, purification, and analysis of recombinant GpA and variants expressed as fusions to an Fc domain and 6-His tag. (A) Reduced SDS-PAGE gel of recombinant GpA, recombinant GpA glycan mutants, and recombinant GpA exon 3 Δ . Apparent molecular mass estimates by SDS-PAGE are consistent with heavily glycosylated samples. (B) SDS-PAGE gel of rGpA under oxidizing (–DTT) and reducing (+DTT) conditions reveal that rGpA is a disulfide linked dimer. (C) Size exclusion chromatography of recombinant GpA, RII-175 (73 kDa), and the Fc-His domain (54 kDa) alone show that rGpA elutes before RII-175 or Fc, consistent with a molecular mass of 110 kDa.

4 days, and the proteins were purified as described in Materials and Methods. All constructs yielded 5 to 10 mg of protein per liter of harvested medium.

All protein samples were glycosylated as evidenced by significantly higher apparent molecular masses in SDS-PAGE (Fig. 2A and B; see also Table S1 in the supplemental material). The theoretical molecular masses for rGpA-Fc-His and single mutants are 34.3 kDa, that for the rGpA-Fc-His triple mutant is 34.2 kDa, and that for rGpA exon 3 Δ -Fc-His is 30.3 kDa (see Table S1). All constructs migrated ~20 kDa larger than the predicted molecular mass on reduced SDS-PAGE gels, consistent with the presence of numerous glycans (Fig. 2A). The rGpA triple mutant migrated at a smaller apparent molecular mass than wild-type rGpA, consistent with the removal of three O-glycans (Fig. 2A). rGpA exon 3 Δ also migrates at a smaller apparent molecular mass than wild-type rGpA, consistent with the removal of exon 3 (Fig. 2A).

The antibody Fc domain forms a disulfide-linked dimer. Consistent with disulfide-linked dimerization, rGpA-Fc-His fusions have an apparent molecular mass of ~110 kDa in SDS-PAGE in the absence of reducing conditions and an apparent molecular mass of ~55 kDa when reduced (Fig. 2B). In addition, rGpA-Fc-

His eluted earlier than region II of PfeBA-175 (RII-175) in size exclusion chromatography, consistent with a molecular mass larger than those of the 73-kDa PfeBA-175 RII and the 54-kDa Fc domain alone (Fig. 2C). Finally, the molecular mass of rGpA in solution measured by sedimentation equilibrium analytical ultracentrifugation was 107.6 ± 3.5 (see Fig. S2 in the supplemental material). These results demonstrate that rGpA is dimeric in solution.

Recombinant GpA is correctly glycosylated and recognized by both an anti-GpA antibody and lectins. To assess the glycosylation state and functionality of the recombinant proteins, purified samples were examined by antibody and lectin recognition. An anti-GpA antibody recognizes endogenous GpA (40) and inhibits PfeBA-175 RII binding to erythrocytes (8). This anti-GpA antibody bound untreated (UT), neuraminidase (NA)-treated, or PNGase F-treated rGpA but did not bind to the Fc control that lacks the GpA extracellular domain (Fig. 3A). This demonstrates that the recombinant protein is GpA and that binding of the antibody is specific for the GpA extracellular domain of the fusion protein. The anti-GpA antibody failed to recognize rGpA exon 3 Δ , while an anti-6 \times His tag antibody recognizes both GpA and GpA exon 3 Δ (Fig. 3B), further emphasizing its specificity for GpA and suggesting that the antibody epitope lies in residues 50 to 84 of the GpA extracellular domain.

Lectin soybean agglutinin (SBA) and peanut agglutinin (PNA) are known to bind different sugar moieties on glycosylated proteins. SBA from *Glycine max* binds to α - and β -N-acetyl-galactosamine (GalNAc), which are found predominantly on O-linked glycans. The GalNAc in O-linked glycans is made accessible by removal of the terminal sialic acid by NA treatment. Sialic acid removal results in a large increase in binding observed when SBA binds to NA-treated rGpA and rGpA exon 3 Δ (Fig. 3C and D). SBA's specificity for O-linked glycans demonstrates that rGpA and rGpA exon 3 Δ contain correctly glycosylated O-linked residues. PNA from *Arachis hypogaea* is specific for β -galactose (β -Gal), which can be found in both O-linked glycans and N-linked glycans. Again, β -Gal is made accessible for binding by removal of the terminal sialic acid by NA treatment. Similar to SBA, PNA shows an increase in binding to NA-treated rGpA and rGpA exon 3 Δ due to increased accessibility upon sialic acid removal (Fig. 3E and F). Together, these results demonstrate that rGpA and rGpA exon 3 Δ are properly glycosylated.

Glycans encoded by exon 3 are critical for PfeBA-175 RII binding to GpA. To further determine the role of the glycans in PfeBA-175 RII binding to GpA, individual glycan mutants, as well as a triple glycan mutant in exon 3 of rGpA, were expressed and purified (Fig. 1B and 2A). A direct protein-protein interaction assay by affinity pulldown with PfeBA-175 RII was conducted using rGpA, glycosylation mutants, and rGpA exon 3 Δ (Fig. 4; see also Fig. S3 in the supplemental material). PfeBA-175 RII bound rGpA but not NA-treated rGpA, as expected ($P \leq 0.001$) (Fig. 4). rGpA exon 3 Δ contains a sequence and glycosylation pattern similar to those of GpB, and, consistent with PfeBA-175 specificity for GpA over GpB, rGpA exon 3 Δ was incapable of supporting binding to PfeBA-175 RII (Fig. 4) and the reduction in binding was significant ($P \leq 0.01$) (Fig. 4B). The individual glycosylation mutants had various effects on binding (Fig. 4). rGpA S66A had a minimal effect on binding (Fig. 4), implying that it is not involved or was minimally involved in PfeBA-175 RII binding and the reduction observed was not significant (Fig. 4B). On the other hand,

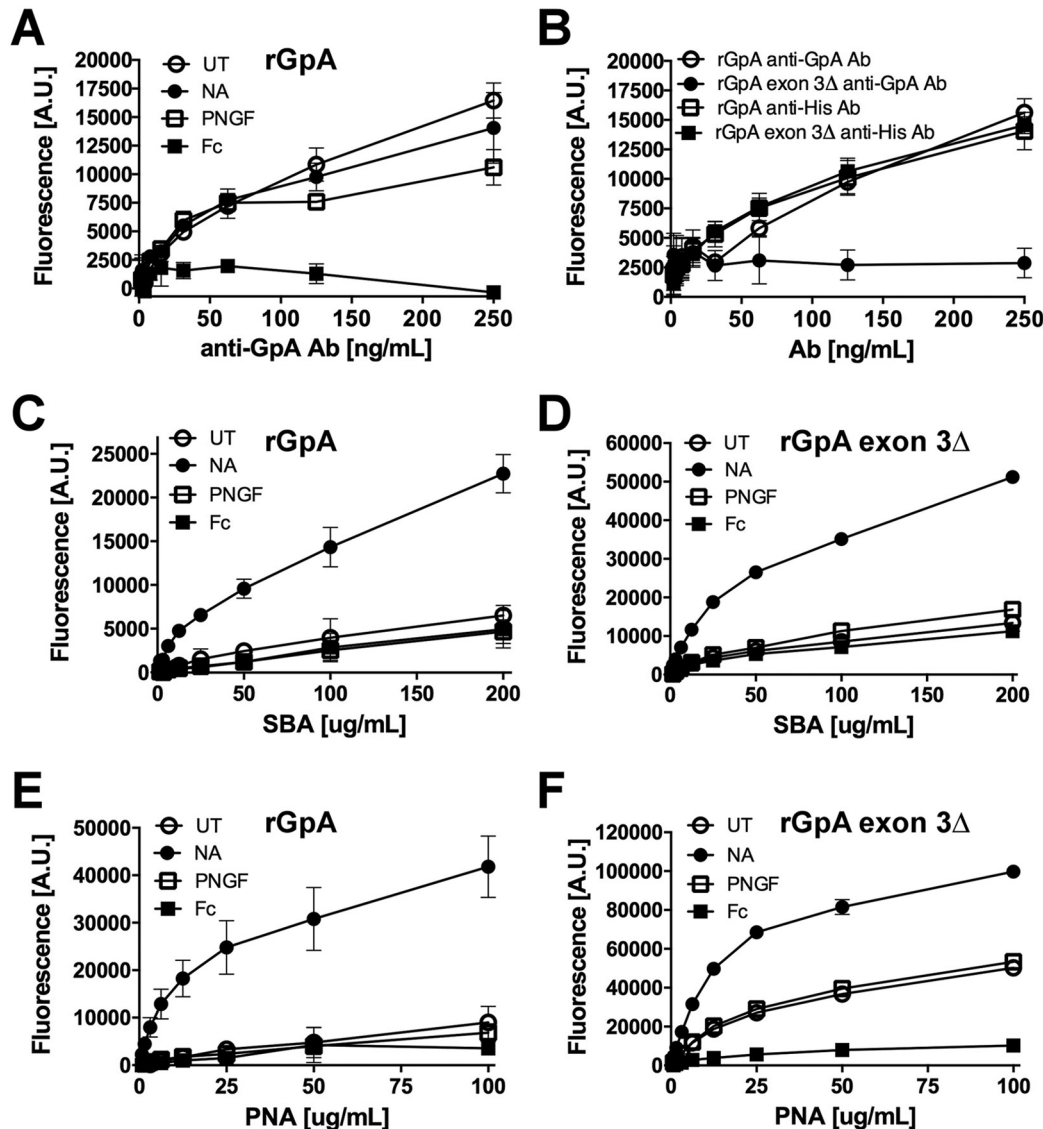


FIG 3 Recognition of untreated and enzyme-treated rGpA and rGpA exon 3Δ by antibodies and lectins. UT, untreated; NA, neuraminidase treated; PNGF, PNGase F treated; Fc, Fc domain alone. (A) Anti-GpA monoclonal antibody (Ab) binding to rGpA. The antibody binds to UT rGpA but not Fc. NA and PNGF treatment of rGpA did not affect binding to the antibody. (B) Anti-GpA monoclonal antibody binding to rGpA exon 3Δ. The antibody is unable to bind rGpA exon 3Δ, while it retains the ability to bind rGpA. A positive control for binding using an anti-His antibody binds to both rGpA and rGpA exon 3Δ, demonstrating that the inability of the anti-GpA antibody is not due to a lack of protein. (C) SBA from *Glycine max* binding to rGpA. Binding is observed upon NA treatment, since sialic acid removal exposes GalNAc residues predominantly found in *O*-glycans. (D) SBA from *Glycine max* binding to rGpA exon 3Δ. Again, binding is observed upon NA treatment. (E) Lectin PNA from *Arachis hypogaea* binding to rGpA. PNA binding is observed only on NA treatment, since removal of the sialic acid exposes the correct sugar residue for binding. PNA does not bind the Fc domain. (F) PNA from *Arachis hypogaea* binding to rGpA exon 3Δ. Again, binding is observed upon NA treatment. A.U. = absorbance units.

both rGpA S69A and rGpA T72A significantly reduced binding ($P \leq 0.05$) (Fig. 4B). This implies that multiple glycans are involved in binding of PfEBA-175 RII to GpA, although neither mutation alone resulted in a complete elimination of binding. In contrast to the individual mutants, an rGpA triple glycan mutant containing all three individual glycan mutants (S66A/S69A/T72A) is able to completely eliminate binding of PfEBA-175 RII ($P \leq 0.01$) (Fig. 4). This demonstrates that multiple glycans encoded by exon 3 are necessary for PfEBA-175 RII binding.

Recombinant GpA competes with endogenous GpA on red blood cells for binding to PfEBA-175 and inhibits parasite inva-

sion. We assessed the functionality of purified, fully glycosylated rGpA by analyzing its ability to compete with endogenous GpA on erythrocytes by quantifying inhibition of parasite invasion of erythrocytes. We assayed for changes in parasite growth by adding increasing concentrations (0.3 to 300 μ M) of purified rGpA to synchronized schizont-stage parasite culture and determined the parasitemia 48 h later for four different laboratory strains of *P. falciparum*. Recombinant GpA inhibited parasite growth in three strains with 50% inhibitory concentrations (IC_{50} s) of $43 \pm 19 \mu$ M, $25 \pm 17 \mu$ M, and $52 \pm 12 \mu$ M for 3D7, Dd2, and FVO/FCR1, respectively (Fig. 5; see also Fig. S4 and Table S2 in the

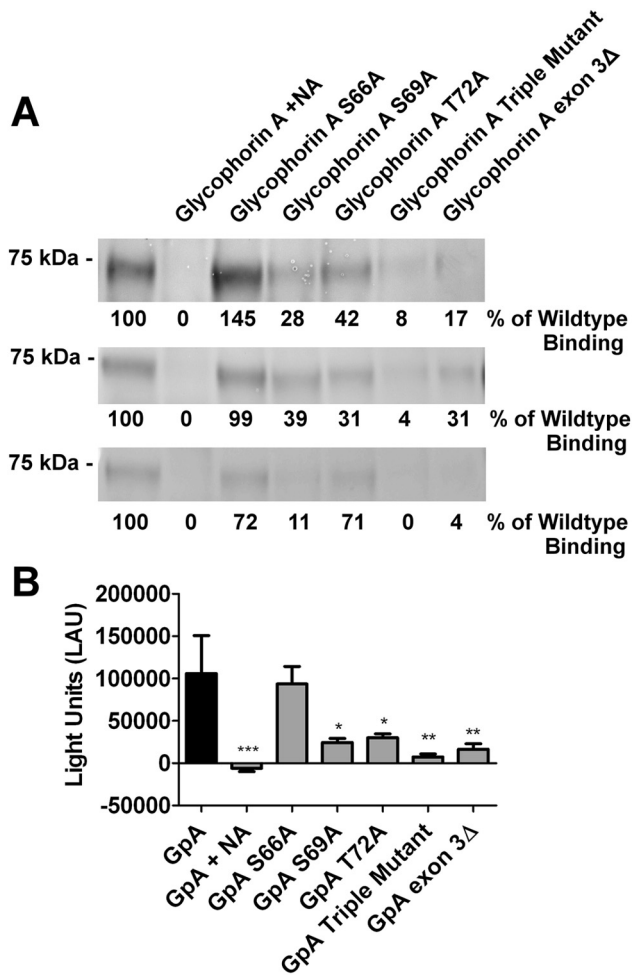


FIG 4 PfEBA-175 RII binding depends on glycans in exon 3. (A) Anti-PfEBA-175 RII Western blots after pulldown with rGpA wild type, mutant rGpAs, and rGpA exon 3Δ. The panels are three independent pulldowns, demonstrating experimental reproducibility. PfEBA-175 RII binds to rGpA (lane 1), and neuraminidase (NA) treatment of rGpA abolishes binding (lane 2). The single mutations S66A (lane 3), S69A (lane 4), and T72A (lane 5) have various effects on binding. The triple mutation S66A/S69A/T72A (lane 6) completely abolishes binding. rGpA exon 3Δ (lane 7) is unable to significantly bind PfEBA-175 RII, indicating that residues 23 to 49 of GpA are unable to support efficient binding. (B) Band intensities were quantified by densitometry and plotted as means \pm SEM. Significance compared to result for GpA was determined by a one-way ANOVA. Asterisks denote $P < 0.001$ (***), $P < 0.01$ (**), and $P < 0.05$ (*).

supplemental material). These IC_{50} s are reported as the means and standard deviations of data from three independent experiments. The Fc domain alone did not significantly inhibit parasite growth (see Table S2), demonstrating a specific dependence on the rGpA for inhibition. These data suggest that rGpA is fully functional and can compete with endogenous GpA for binding to the known ligand PfEBA-175. rGpA did not inhibit parasite growth of a fourth *P. falciparum* strain HB3 (see Fig. S5), since this strain invades erythrocytes through sialic acid-independent pathways and does not appear to use PfEBA-175 (14, 41). Together these results suggest that rGpA inhibits parasite invasion by targeting PfEBA-175 specifically.

Binding of recombinant GpA to PfEBA-175 and inhibition of

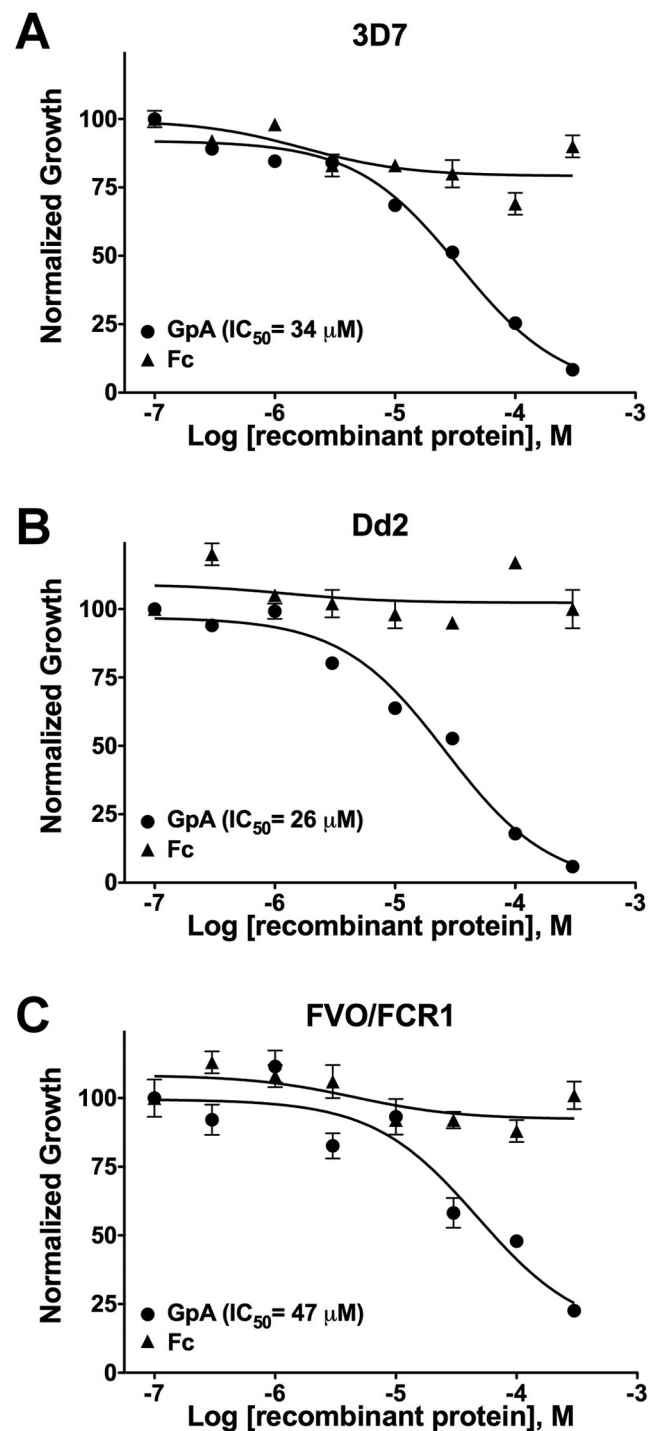


FIG 5 Recombinant GpA is fully functional and can compete with endogenous GpA on erythrocytes to inhibit parasite invasion. Purified rGpA or Fc was serially diluted and tested for its ability to inhibit parasite growth. Representative inhibition curves are shown for the 3D7 (A), Dd2 (B), or FVO/FCR1 (C) strain. Growth inhibition by rGpA was determined by microscopy analysis of parasitemia, and the results are expressed as growth normalized to that of untreated control wells ($0 \mu M$).

parasite growth are mediated through the sialic acid moieties on exon 3. The ability to express and purify not only wild-type rGpA

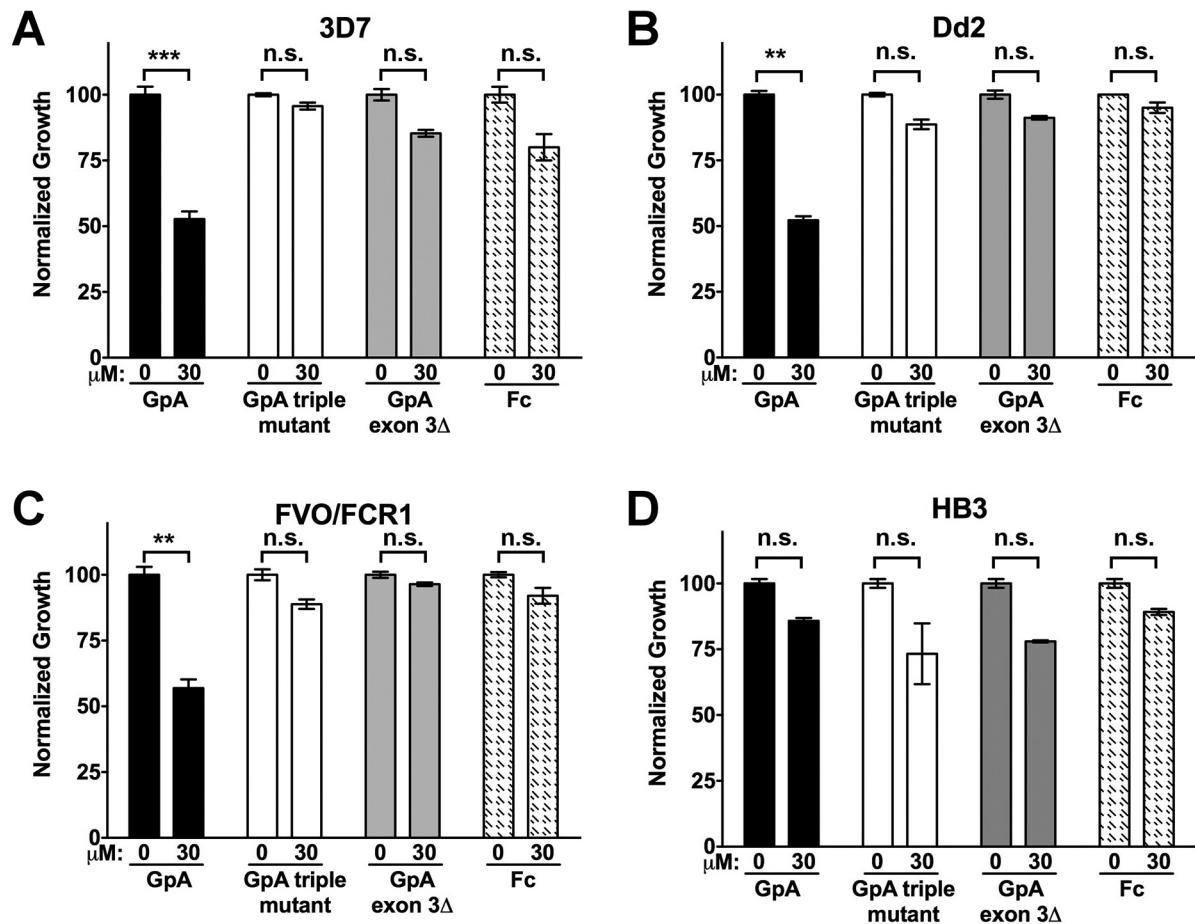


FIG 6 rGpA triple mutant and rGpA exon 3Δ fail to inhibit parasite invasion of 3D7, Dd2, or FVO/FCR1. rGpA, rGpA triple mutant, or rGpA exon 3Δ was purified and used at 30 μ M in a growth inhibition assay with the 3D7 (A), Dd2 (B), FVO/FCR1 (C), or HB3 (D) strain. Fc was included in the experiment as a control. HB3 showed no inhibition by rGpA, since this strain invades through sialic-acid-independent pathways that do not rely on PfEBA-175. Parasitemia at the end was analyzed by microscopy, and growth was normalized to that of untreated control wells (0 μ M). Data shown are means \pm SEM from three independent experiments, each done in triplicate. Asterisks denote $P < 0.001$ (***) and $P < 0.01$ (**), and “n.s.” denotes not significant.

but also the rGpA triple glycan mutant and rGpA exon 3Δ enabled us to map the binding region on GpA that is critical for engagement with PfEBA-175 during invasion of erythrocytes by *P. falciparum*. We next examined whether rGpA lacking the critical O-glycosylation residues in exon 3 can block parasite growth. A near-IC₅₀ concentration of rGpA (30 μ M) was utilized in analyzing the effects of the rGpA triple mutant and rGpA exon 3Δ on parasite growth. While wild-type rGpA at a 30 μ M concentration could inhibit parasite growth to ~50% in all three sialic acid-dependent strains, neither the rGpA triple mutant nor rGpA exon 3Δ inhibited parasite growth significantly (Fig. 6A to C), providing further support that sialic acid moieties on exon 3 of GpA are necessary for binding to PfEBA-175. This also demonstrates that the O-glycans in residues 23 to 49 of the GpA extracellular domain that are conserved in GpB are dispensable for binding to PfEBA-175 and inhibition of parasite growth in sialic acid-dependent strains. In addition, we tested the growth of the sialic acid-independent HB3 strain (14, 41) in the presence of rGpA and found that it was not significantly affected by GpA, the triple glycan mutant, or rGpA exon 3Δ (Fig. 6D; see also Fig. S5 in the supplemental material).

DISCUSSION

The EBL family of ligands on the parasite surface serve as attractive targets for vaccine development, since antibodies against these ligands have been shown to be inhibitory and PfEBA-175 is recognized by antibodies in individuals with naturally acquired immunity (36, 37, 42, 43). Although EBL ligands are functionally redundant, PfEBA-175 is an excellent vaccine candidate (44, 45) because it is a major invasion pathway (46) and antibodies that target PfEBA-175 inhibit parasite growth of multiple strains of *P. falciparum*, including those that use PfEBA-175-independent invasion pathways (47). Toward development of more efficacious vaccines, greater understanding of interactions between surface receptors and ligands is needed to better focus vaccine responses. Here we describe a necessary step in facilitating a complete understanding of the PfEBA-175–glycophorin A interaction.

Region II of PfEBA-175 is responsible for binding to GpA on the surface of erythrocytes (6–8), and structural studies suggest that PfEBA-175 RII dimerizes around a dimer of GpA engaging three distinct glycans from each GpA monomer (7). The identification of six glycan binding sites per PfEBA-175 RII dimer implies

that multiple glycans are engaged to facilitate high-avidity binding of PfEBA-175 to GpA.

Prior studies have relied on extraction of GpA from erythrocytes due to the lack of expression systems for protein production and genetic manipulation. Toward understanding the mechanism of PfEBA-175 binding to GpA, we developed a novel glycoprotein expression system to efficiently produce large quantities of correctly glycosylated recombinant GpA. This system also allows the generation of glycan mutants by single amino acid mutation and by truncation. We described a protocol to express large quantities of soluble recombinant GpA by transient transfection of HEK293F cells and reported that recombinant GpA and glycan mutants can be produced with yields of 5 to 10 mg/liter of harvested medium. The success in producing glycosylated protein was essential in mapping the interface between PfEBA-175 and GpA, since glycosylation is required for binding. The glycosylated protein products were assessed for adequate glycosylation and functionality through antibody and lectin binding.

The data presented here from direct protein-protein interaction, parasite growth inhibition assays, and direct manipulation of GpA and its glycosylation reveal that the critical binding domain corresponds to multiple glycans encoded by exon 3 of GpA. This finding provides insight into how PfEBA-175 discriminates between GpA and GpB, the latter of which lacks exon 3. Exon 3 of GpA has five *O*-glycans (T55, T59, S66, S69, and T72) that are unique to GpA (30). To determine the role of glycans in this region, single glycan mutants of three (S66, S69, and T72) of the five glycosylated residues and a triple glycan mutant were tested for binding to PfEBA-175 RII in a direct protein-protein interaction assay. Individual glycan showed mutants reduced binding, but only the triple mutant showed a complete loss of binding, demonstrating that multiple glycans of exon 3 are necessary for engagement. The other two single mutants (mutated at T55 and T59) could not be assayed due to difficulties in protein expression, and it is plausible that these two *O*-glycans may also be involved in binding. Furthermore, rGpA exon 3Δ and the triple mutant both retain at least 11 *O*-glycans and are still unable to engage PfEBA-175 RII, suggesting that general sialic acid binding is insufficient for PfEBA-175 engagement of GpA. Together these results suggest that the identity, location, and presentation of the three glycans in exon 3 are critical for high-avidity binding of PfEBA-175 to GpA on erythrocytes. These results are consistent with findings of prior studies that suggested the region encoded by exon 3 is important for engagement, since red blood cell (RBC)-extracted GpA but not GpB disrupted binding of erythrocytes to PfEBA-175 expressed on the surface of tissue culture cells (rosetting assays) (6). Further, separation of GpA into two pieces by protease digestion within the protein sequence encoded by exon 3 abolished inhibition by GpA in *in vitro* rosetting assays, and neither each piece alone nor a combination of both fragments was able to inhibit binding (6).

The growth inhibition assay (GIA) results demonstrated the role of glycans in the parasite invasion process. Recombinant GpA inhibited parasite growth in culture, likely as a result of direct competition with endogenous GpA on erythrocytes. In contrast, the recombinant GpA triple glycan mutant could not bind to PfEBA-175, was unlikely to impede endogenous GpA binding to PfEBA-175, and did not inhibit parasite growth. Likewise, rGpA exon 3Δ is similar to GpB and retains 11 of the 16 *O*-glycans of GpA but could not bind PfEBA-175 and did not affect parasite growth. These results indicate that residues 23 to 49 and the gly-

cans in this region of GpA/B are dispensable for engagement with PfEBA-175. This is consistent with *P. falciparum* invading Mg erythrocytes at the same rate as normal erythrocytes even though Mg erythrocytes lack glycosylation of residues 24, 25, and 26 (29, 31). Taken together, these results demonstrate the functional importance of exon 3 *O*-glycans in the PfEBA-175 invasion pathway.

An intriguing observation is that recombinant GpA inhibited three distinct *P. falciparum* strains with differing dependence on sialic acid for invasion. W2mef (the parental line of Dd2) has been reported to be primarily sialic acid dependent compared to the 3D7 and FVO/FCR1 strains (42, 46). Consistent with this observation, we found that rGpA inhibited Dd2 slightly better than 3D7 or FVO/FCR1. However, inhibition of parasite growth by rGpA was observed in all three strains, demonstrating that these strains depend on PfEBA-175 for invasion. This is supported by the observation that anti-PfEBA-175 antibodies inhibit *P. falciparum* strains independent of their sialic dependence for invasion and identification of the PfEBA-175–GpA pathway as a dominant invasion pathway (44, 46, 47). To ensure that inhibition by rGpA was not due to off-target effects independent of PfEBA-175, we examined inhibition of HB3, a strain that invades predominantly through pathways not involving PfEBA-175 (14, 41). HB3 was unaffected by rGpA, suggesting that the inhibitory effects target PfEBA-175 specifically. Minor reductions in growth were observed with nonspecific proteins (Fc), although these were not significant in any case. The studies described here enable mapping of the determinants for GpA engagement by PfEBA-175 by multiple *P. falciparum* strains.

The production of recombinant GpA and multiple mutants is an important innovation for the field, since it now allows for the future study of known GpA variants, as well as other ligand-receptor systems. Three particular GpA variants of interest are Vr, Hop, and Nob, which map to exon 3, and all may affect the number of *O*-glycans present in this exon (29). The Vr variant corresponds to a Ser-to-Tyr change at position 66, and it is one of the glycosylated residues investigated in this study. Mutating Ser to Ala (S66A) resulted in a partial but not significant reduction of PfEBA-175 RII binding to GpA in our pulldown assay. It is conceivable that the S66Y variant has a diminished capacity to engage PfEBA-175, although further studies are needed. The other variants, Hop and Nob, potentially result in the addition of glycans to exon 3. Both Hop and Nob have the Arg68Thr mutation, while Nob carries a second mutation, Tyr71Ser (29). The potential addition of *O*-glycans immediately adjacent to existing *O*-glycans could influence binding. The ability to express multiple mutants in large quantities opens new areas of research not possible previously due to limitations in reagent availability.

Malaria is thought to have shaped human evolution by promoting the evolution of surface receptors on erythrocytes to prevent infection. The archetypal change is the loss of the Duffy antigen/receptor for chemokines on red blood cells, which reduces infection by *P. vivax* (48, 49). In addition, individuals in areas of Papua New Guinea to which malaria is endemic have selected for loss of GpC, which is thought to prevent *P. falciparum* infections that initiate via PfEBA-140 (11). The mapping of the PfEBA-175 binding site in GpA and the lack of this binding site in GpB and GpE are supportive of selection to evade infection. The results raise the possibility that GpB and GpE arose due to selective pressure to evade malaria infection by eliminating the PfEBA-175 binding site and a predominant invasion route for *P. falciparum*.

The mapping of the critical binding domain to exon 3 has implications for understanding species tropism. In nonhuman primates, GpA contains exon 3, with the major zone of variation being present in this exon (50, 51). Determining the influence of polymorphisms unique to humans versus other primates requires further experimentation that is now feasible with the novel expression system described here. Glycophorins function as receptors for other critical human pathogens, and this study opens up new avenues of research to identify receptor-binding determinants in these systems (52–54). In conclusion, the results presented here have a wide application for mapping receptor-ligand interactions and have important implications for the design and development of peptidoglycan mimetics that aim to disrupt the essential process of parasite invasion.

MATERIALS AND METHODS

Glycoprotein expression and purification. rGpA (amino acids 23 to 84 of the extracellular domain), rGpA glycosylation mutants, and rGpA exon 3Δ (amino acids 23 to 49 of the extracellular domain) (Fig. 1A) were cloned into a pHLFchis vector containing the GpA signal sequence and a PreScission protease site inserted between the glycophorin domain and the Fc domain. The constructs were transiently transfected into HEK293F cells using a DNA/PEI ratio of 1:2 with the concentration of the DNA at 1 μg/ml of culture as described elsewhere (37). For the DNA/PEI ratio experiment, 4 days posttransfection, the medium was harvested and diluted 3-fold with binding buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 10 mM imidazole [pH 8]) and incubated with Ni²⁺-agarose beads. Recombinant proteins were eluted from the beads with elution buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 500 mM imidazole [pH 8]). For all other experiments, 4 days posttransfection, the medium was harvested and diluted 3-fold with binding buffer (50 mM Tris [pH 8] and 50 mM NaCl). The protein was then eluted with a 5× column volume of elution buffer (50 mM Tris [pH 8] and 500 mM NaCl). The eluted protein was then diluted 3-fold with binding buffer (50 mM Tris [pH 8] and 10 mM imidazole [pH 8]) and incubated with Ni²⁺-agarose beads, which were then washed with a 3× column volume with wash buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 10 mM imidazole [pH 8]). Recombinant proteins were eluted from the beads with 5 column volumes of elution buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 500 mM imidazole [pH 8]). Recombinant proteins were concentrated and buffer exchanged into phosphate-buffered saline (PBS) using a 10-kDa-molecular-mass-cutoff Amicon Ultra centrifugal filter (Millipore). Proteins required for growth inhibition assays were purified under sterile conditions.

Analytical ultracentrifugation. Sedimentation equilibrium experiments were carried out in a Beckman/Coulter XL-A analytical ultracentrifuge (Beckman/Coulter, Palo Alto, CA) using an An60Ti rotor at 10°C. Absorbance measurements at a wavelength of 266 nm for three independent replicates at three concentrations of 5 μM, 7 μM, and 8 μM were obtained at speeds of 6,000 rpm (6K), 7K, and 8K. A partial specific volume for fully glycosylated rGpA of 0.693633 was calculated by using the software program Sednterp (55), and a global fit analysis was performed in the program Ultrascan II, version 9.9 (56). The molecular mass reported is the mean ± standard deviation for the three independent replicates at all speeds analyzed.

Enzyme treatments. Glycoproteins were treated with 400 mU of neuraminidase (NA) from *Clostridium perfringens* (Sigma) or 154 mU of PNGase F (NEB) overnight at 4°C in PBS. rGpA-Fc-His or rGpA exon 3Δ-Fc-His was treated with PreScission protease for 1 h at room temperature in PBS. The Fc-His portion (Fc) was then purified using Ni²⁺-agarose beads by binding Fc to the beads with binding buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 10 mM imidazole [pH 8]). Fc was eluted

from the beads with elution buffer (50 mM Tris [pH 8], 100 mM sodium chloride, and 500 mM imidazole [pH 8]).

Lectin and antibody binding. Nunc 96-well Maxisorb plates were coated by incubation overnight at 4°C with 10 μg of each protein sample and were blocked with 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Plates were subsequently incubated with either anti-GpA antibody (Santa Cruz Biotech), anti-6×His antibody (Invitrogen), *Glycine max* soybean agglutinin (SBA)-Alexa Fluor 488 (Invitrogen), or *Arachis hypogaea* peanut agglutinin (PNA)-Alexa Fluor 488 (Invitrogen) for 1 h at room temperature in the dark. Plates incubated with anti-GpA or anti-6×His antibodies received an additional incubation with anti-mouse IgG-Alexa Fluor 488 antibody (Invitrogen) for 30 min at room temperature in the dark. The plates were washed with PBS-Tween 20 (0.1%) after every step and were read with either a FLUOstar Omega (BMG Labtech) or POLARstar Omega (BMG Labtech) plate reader.

Direct protein-protein interaction assay for GpA engagement of PFEBA-175. Direct interaction studies were performed as previously described (37). PFEBA-175 RII was obtained as reported previously (8, 37). Eight micromolar rGpA, rGpA mutants, or rGpA exon 3Δ, either untreated or treated with NA as described above, was incubated with 3 μM PFEBA-175 RII and 20 μl of Ni²⁺-agarose beads in a total of 100 μl in pull-down buffer (50 mM Tris [pH 8], 50 mM imidazole, 100 mM sodium chloride, and 0.1% Triton X-100) for 1 h at room temperature. The beads were then washed 3 times with pull-down buffer, resuspended in 50 μl (for analysis by using a Fujifilm FLA-5000 system) or 100 μl (for analysis by autoradiography) of 4× protein sample dye, separated by SDS-PAGE, and immunoblotted with an anti-PFEBA-175 antibody (36, 37) to detect PFEBA-175 RII bound to GpA. The immunoblot was imaged using either film or a Fujifilm FLA-5000 phosphorimager. Three independent pull-downs with two technical replicates utilizing different antibody concentrations for the technical replicates were quantified using the Fuji phosphorimager and Image Gauge V4.23 software. Band intensities were then analyzed in a one-way analysis of variance (ANOVA) of all six blots in the software program Prism 5, and the raw data were plotted as means ± standard errors of means (SEM) with significance values.

Parasite growth inhibition assay. The *P. falciparum* strains 3D7, Dd2, FVO/FCR1, and HB3 were cultured in fresh human O⁺ red blood cells in RPMI 1640 medium with 0.5% Albumax and synchronized with successive rounds of 5% sorbitol treatment as described previously (57). Growth inhibition assays (58) were performed in a 96-well format with a starting parasitemia of 0.1 to 0.5%. Briefly, synchronized cultures with 5 to 10% parasitemia were used to isolate erythrocytes infected with late stages of the parasite using a magnetic bead column (MACSQuant Columns; Miltenyi Biotec). Giemsa-stained smears of the culture and flow cytometry by acridine orange staining of the parasites were performed as needed to evaluate parasitemia of 0.1 to 0.5% at the start of invasion assay. The cultures were then incubated in the absence or presence of various concentrations of rGpA, the rGpA triple mutant, rGpA exon 3Δ, or Fc control for 48 h at 2% hematocrit in triplicate wells. Final parasitemia was assessed by light microscopy analysis. Giemsa-stained thin smears were prepared from each well, and 10 random field images per smear (30 total images per data point) were acquired using a Zeiss Axioskop microscope equipped with a 100× oil immersion lens (1.3 numerical aperture [NA]) and an AxioCam MRm camera with Axiovision v. 3.1 software (Carl Zeiss). The number of parasite-infected erythrocytes in each image was visually counted, while the total number of erythrocytes in each image was analyzed using the Volocity 6.3 Cellular Imaging software program (PerkinElmer). Parasitemia was calculated as the number of parasite-infected erythrocytes divided by the total number of erythrocytes. The microscopist was blinded to the experimental group designation of each smear. Each growth inhibition assay was performed three independent times, with each concentration in triplicate. For the rGpA IC₅₀ analysis, nonlinear dose-inhibition curve fitting and individual IC₅₀s for each growth inhibition assay were derived using Prism 5.0 software. The mean

IC₅₀ and standard deviation of results from the three independent experiments was reported.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01606-14/-/DCSupplemental>.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.1 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 0.1 MB.
Table S1, DOC file, 0.1 MB.
Table S2, DOC file, 0.1 MB.

ACKNOWLEDGMENTS

We thank B. M. Malpede and J. Park for insightful discussions and for reviewing the manuscript. We also thank R. Galletto for assistance with the analytical ultracentrifugation, L. D. Sibley and A. Alaganan for assistance with densitometry, and A. Odom and L. Imlay for use of the BMG Labtech plate readers.

This work was supported by NIH grant R01AI080792, the Edward Mallinckrodt, Jr. Foundation grant, and Burroughs Wellcome Fund to N.H.T. Experimental support was also provided by the Facility of the Rheumatic Diseases Core Center under award number P30AR048335.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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