

A Malaria Invasion Receptor, the 175-Kilodalton Erythrocyte Binding Antigen of *Plasmodium falciparum* Recognizes the Terminal Neu5Ac(α 2-3)Gal- Sequences of Glycophorin A

Palmer A. Orlandi, Francis W. Klotz, and J. David Haynes

Department of Immunology, Walter Reed Army Institute of Research, Washington, D. C. 20307-5100

Abstract. *Plasmodium falciparum* malaria parasites invade human erythrocytes by means of a parasite receptor for erythrocytes, the 175-kD erythrocyte binding antigen (EBA-175). Similar to invasion efficiency, binding requires *N*-acetylneuraminic acid (Neu5Ac) on human erythrocytes, specifically the glycophorins. EBA-175 bound to erythrocytes with receptor-like specificity and was saturable. The specificity of EBA-175 binding was studied to determine if its binding is influenced either by simple electrostatic interaction with the negatively charged Neu5Ac (on the erythrocyte surface); or if Neu5Ac indirectly affected the conformation of an unknown ligand, or if Neu5Ac itself in specific linkage and carbohydrate composition was the primary ligand for EBA-175 as demonstrated for hemagglutinins of influenza viruses. Most Neu5Ac on human erythrocytes is linked to galactose by α 2-3 and α 2-6 linkages on glycophorin A. Soluble Neu5Ac by itself in solution did not competitively inhibit the binding of

EBA-175 to erythrocytes, suggesting that linkage to an underlying sugar is required for binding in contrast to charge alone. Binding was competitively inhibited only by Neu5Ac(α 2-3)Gal-containing oligosaccharides. Similar oligosaccharides containing Neu5Ac(α 2-6)Gal linkages had only slight inhibitory effects. Binding inhibition assays with modified sialic acids and other saccharides confirmed that oligosaccharide composition and linkage were primary factors for efficient binding. EBA-175 bound tightly enough to glycophorin A that the complex could be precipitated with an anti-glycophorin A monoclonal antibody. Selective cleavage of O-linked tetrasaccharides clustered at the NH₂ terminus of glycophorin A markedly reduced binding in inhibition studies. We conclude that the Neu5Ac(α 2,3)-Gal- determinant on O-linked tetrasaccharides of glycophorin A appear to be the preferential erythrocyte ligand for EBA-175.

IF invasion of malaria parasites into human erythrocytes were prevented, the malaria life cycle would be interrupted and disease prevented. Thus, considerable effort has gone into understanding the molecular basis of parasite invasion into erythrocytes. Invasion requires recognition by the parasite of the appropriate host cell during one or more steps that include merozoite attachment, apical reorientation, apical junction formation, release of the contents of apical organelles, and entry into the erythrocyte (16, 21). The glycophorins, major sialoglycoproteins present on the erythrocyte surface, appear to be responsible for the sialic acid-dependent invasion into erythrocytes by *Plasmodium falciparum* malaria merozoites (10, 11, 16, 18, 29, 30, 33-36). Recognition of sialic acid-containing receptors on the erythro-

cyte surface by malaria receptors might be analogous to influenza virus entry into host cells by means of the hemagglutinins which bind with stereospecificity to sialic acid-capped oligosaccharides (46).

Erythrocytes with modifications or deficiencies in the glycophorins show marked reductions in invasion efficiencies by *Plasmodium falciparum* merozoites. Invasion into Ena⁻ erythrocytes (deficient in glycophorin A) (29, 33), M^kM^k erythrocytes (deficient in glycophorins A and B) (18), Tn erythrocytes (missing the terminal sialyl and galactosyl residues of the O-linked oligosaccharide structures on glycophorin [9]) (5, 30), and Cad erythrocytes (with an additional *N*-acetylgalactosamine residue on the O-linked oligosaccharides) (5) showed reduced invasion efficiencies. Similarly, enzymatic modification of erythrocyte surface molecules by either trypsin or neuraminidase, treatments that have a direct effect on the integrity of the glycophorins, render the erythrocyte resistant to invasion (4, 16, 18, 33).

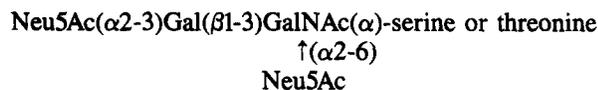
In parallel experiments, erythrocytes with modifications or deficiencies in the glycophorins also had marked reductions in the binding of the 175-kD erythrocyte binding anti-

Palmer A. Orlandi's current address is Membrane Biochemistry Section, Laboratory of Molecular and Cellular Neurobiology, National Institute of Neurological Disorders and Stroke, The National Institutes of Health, Bethesda, Maryland 20982.

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gen of *Plasmodium falciparum* (EBA-175),¹ a receptor apparently needed for efficient invasion (4, 16, 32). EBA-175 is a protein that is released from merozoites into culture supernatants after their release from schizont-infected erythrocytes in the absence of target erythrocytes. Results from previous studies have demonstrated that EBA-175 acts with receptor-like specificity and requires sialic acids on the erythrocyte ligand for binding (4, 32). Similarly, the binding of EBA-175 to invasion-competent erythrocytes correlates closely with merozoite invasion efficiency (4, 32). Further evidence that EBA-175 is a receptor facilitating invasion into erythrocytes was obtained by Sim et al., who reported that antibodies raised against the peptide 4 region of EBA-175 are effective in inhibiting both the binding of EBA-175 to erythrocytes and merozoite invasion *in vitro* (41).

Although a direct interaction between the glycoporphins and EBA-175 has not been demonstrated, the binding characteristics of EBA-175 suggested that EBA-175 binds to the glycoporphins, of which glycoporphin A is the most abundant. Glycoporphin A contains 1 N-linked complex chain and 15 O-linked tetrasaccharides, 6 of which are on immediately adjacent serine and threonine residues and 11 of which are clustered within the first 30 amino acids at the NH₂ terminus. The O-linked tetrasaccharides have the following structure:



These oligosaccharides make up ~60% of the molecular weight of glycoporphin A and are each capped with *N*-acetylneuraminic acid (Neu5Ac), accounting for ~60% of the total sialic acid at the surface of the human erythrocyte (2).

Several characteristics of ligand-receptor interactions remained to be studied: saturability of binding, competitive inhibition of binding with small ligands, ligand stereospecificity, and the demonstration of direct interaction between the ligand and the receptor by immunoprecipitation as a complex. Because sialic acids such as Neu5Ac impart a negative charge and are known to affect binding through electrostatic forces as well as cause conformational changes of adjacent molecules (40), it was of particular interest to show that charge alone did not account for binding and that a small ligand containing Neu5Ac did itself compete for erythrocytes in binding EBA-175. In this study we describe the carbohydrate specificities and structural requirements for binding of EBA-175 as well as the interactions of this malaria lectin with the O-linked tetrasaccharide chains of glycoporphin A.

Materials and Methods

Materials

All materials were obtained as follows: trypsin, PMSF, *N*-glycylneuraminic acid (Neu5Gc), neuramin(α 2-3) lactose (Neu5Ac(α 2-3)Gal(β 1-4)Glc), neuramin (α 2-6)lactose (Neu5Ac(α 2-6)Gal(β 1-4)Glc), and glycoporphin A from Sigma Chemical Co. (St. Louis, MO); *N*-acetylneuraminic (NeuAc), glucosamine, galactosamine, *N*-acetylglucosamine, *N*-acetylgalactosa-

1. **Abbreviations used in this paper:** DST, disialyllacto-*N*-tetraose, Neu5Ac α 2-3Gal β 1-3 (Neu5Ac α 2-6)GlcNAc β 1-3Gal β 1-4Glc. EBA-175, erythrocyte binding antigen-175; Endo F, endo- β -*N*-acetylglucosaminidase F; SRBC, schizont-infected erythrocytes; Lac, lactose, galactose β 1-4glucose; LST a, sialyllacto-*N*-tetraose a, Neu5Ac α 2-3Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc; LST b, sialyllacto-*N*-tetraose b, Gal β 1-3-(Neu5Ac α 2-6)GlcNAc β 1-3Gal β 1-4Glc; NDV, Newcastle disease virus; Neu5Ac, *N*-acetylneuraminic acid; Neu5Ac-Lac, neuramin-lactose; Neu5Gc, *N*-glycylneuraminic acid.

mine, chitobiose, chitotriose, and chitotetraose from Supelco Inc. (Bellefonte, PA); *Vibrio cholerae* neuraminidase (500 U ml⁻¹) and FBS from Gibco Laboratories (Grand Island, NY); endo- β -*N*-acetylglucosaminidase F (Endo F) from Boehringer Mannheim Corp. (Indianapolis, IN); F-50 silicon fluid from General Electric; goat anti-mouse IgG (H+L) Sepharose from Cappel Laboratories (Malvern, PA); protein A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ); sialyllacto-*N*-tetraose a and b (LST a and b) and disialyllacto-*N*-tetraose (DST) from BioCarb chemicals (Lund, Sweden). Immune *Aotus* serum (Ao29) was obtained from monkeys immunized against the Malayan Camp strain of *Plasmodium falciparum* (6, 7). Anti-glycophorin A hybridoma supernatants from monoclonal antibody 453.1.1 (17) were generously supplied by T. J. Hadley (University of Louisville School of Medicine, Louisville, KY) and S. Futrovsky (Walter Reed Army Institute of Research, Washington, DC). Tn and M⁸M⁸ erythrocytes were donated by Dr. L. H. Miller (Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD). Newcastle disease virus (NDV), strain B1, was graciously provided by Dr. T. G. Morrison (University of Massachusetts Medical Center, Worcester, MA).

Culture of Parasites

Cloned Camp strain *Plasmodium falciparum* parasites (Malaysia) were cultured and synchronized as described (27, 44). Schizont-infected erythrocytes (SRBC) were isolated by banding on Percoll gradients (1). SRBC were washed twice in medium containing 10% FBS and cultured for 16 h at 37°C at a cell density of 1.5 × 10⁶ ml⁻¹ with or without [³H]isoleucine in 10% FBS as described previously (4). At the end of the incubation period, during which >95% of schizonts ruptured, the cell pellets and supernatants were collected by centrifugation at 300 g per min per mm⁻¹ (27) and stored at -80°C.

Enzymatic Treatments of Intact Erythrocyte and Glycophorin A Modifications

Treatment of intact human erythrocytes with 50 U ml⁻¹ *Vibrio cholerae* neuraminidase or 1.0 mg ml⁻¹ trypsin was performed as described (4). Erythrocytes were treated with NDV for 60 min at 37°C at a 5% Hct and 10 μg ml⁻¹ of viral protein. Treatment of erythrocytes with Endo F was performed with 3.0 U ml⁻¹ in PBS, pH 7.0, at a 5% Hct overnight at 37°C. Analysis of EBA-175 binding to these enzymatically treated cells was performed as described below. Deglycosylation of the alkali-labile oligosaccharides of glycoporphin A was carried out at 1 mg ml⁻¹ in 0.1 N NaOH at 37°C for 72 h (42). *Vibrio cholerae* neuraminidase treatment of glycoporphin A (1 mg ml⁻¹) was performed with 50 U ml⁻¹ of the enzyme in 0.05 M sodium acetate buffer, pH 5.5, 0.150 M NaCl, and 0.009 mM CaCl₂ overnight at 37°C. It was necessary to add 10% FBS to neuraminidase-treated glycoporphin A samples before assaying for EBA-175 binding to quench any remaining neuraminidase activity. Glycoporphin A was treated with NDV as above in the same buffer containing 5 μg ml⁻¹ of viral protein. Viral particles were pelleted by centrifugation after the incubation period. A control blank containing only NDV and treated similarly was included to control for any residual NDV neuraminidase activity which may affect EBA-175 binding by modification of erythrocyte-bound glycoporphin A. Glycoporphin A at a concentration of 0.3 mg ml⁻¹ was treated with 3.0 U ml⁻¹ Endo F in 0.075 M sodium acetate, pH 7.0, overnight at 37°C. Treated glycoporphin A samples were concentrated and resuspended in PBS twice using Centricon 10 filtration devices (Amicon, Beverly, MA) and adjusted to 1 mg ml⁻¹ before assaying for EBA-175 binding inhibition. Molar concentrations at which binding inhibition is observed are based on the molecular weight of glycoporphin A₂.

Periodate Oxidation/Borohydride Reduction of Intact Erythrocytes

Human erythrocytes were washed several times in serum-free culture medium and resuspended at a 5% Hct in PBS containing the appropriate concentration of IO₄⁻. The suspension was incubated on ice for 15 min (13). Erythrocytes were pelleted and washed twice with 15 ml of 0.1 M glycerol in PBS followed by an additional wash in PBS. Erythrocytes were subsequently treated with NaBH₄ (1 mg ml⁻¹) in PBS at a 5% Hct for 30 min at room temperature. After three washes in PBS, erythrocytes were diluted 50% in serum-free medium and used in binding assays.

Isolation of EBA-175 Affinity-purified from Erythrocytes and Binding Inhibition Assays

Culture supernatants metabolically labeled with [³H]isoleucine were pre-

pared as described above containing 10% FBS. FBS was used in place of human serum to eliminate antibodies that might react with modified erythrocyte surface antigens. No differences were observed in the binding of EBA-175 in the presence of either FBS or human serum. Fresh, human erythrocytes were washed several times in serum-free medium. EBA-175 affinity-purified from erythrocytes was prepared as described previously (19, 32). For binding inhibition assays conducted in the presence of purified saccharides, oligosaccharides, or protein, 0.075 ml of the appropriate substance in PBS, pH 7.0, was combined with 0.025 ml of culture supernatant (3.75×10^5 schizont equivalents). Concentrations were based on the 0.10 ml final volume. This reagent was then added to 0.10 ml of packed erythrocytes (1.0×10^9) and incubated and processed as described (32). After SDS-PAGE and fluorography, EBA-175 binding was quantified by scanning laser densitometry of the fluorographs using an LKB 2022 Ultrascan laser densitometer (LKB Instruments, Inc., Gaithersburg, MD).

SDS-PAGE

Discontinuous SDS-PAGE was performed on 7.5% (1.5 mm) SDS-polyacrylamide gels by the method of Laemmli (25). For fluorography, gels were treated with EN³HANCE (New England Nuclear, Boston, MA) and exposed to Kodak XAR-2 film at -80°C (3, 26). Gels stained for the presence of sialic acid were fixed overnight in 7.5% acetic acid. The gels were then treated with 0.040 M periodic acid for 2 h at 37°C followed by extensive washing for 1 h with distilled water, incubated with ice-cold resorcinol reagent (54.5 mM resorcinol, 0.25 mM CuSO_4 , 5.2 M HCl) for 15 min, heated at 60°C for 10 min, and slowly heated to 80°C until maximum color developed.

Immunoprecipitation

For the immunoprecipitation of the glycoprotein A/EBA-175 complex, culture supernatants were incubated with erythrocytes as described above. The erythrocytes were then pelleted, washed twice in serum-free medium, and lysed with 0.5 ml PBS containing 1% Triton X-100 and 0.002 M PMSF. The solubilized cells were then reacted overnight at 4°C with either 0.5 ml of mAb 453 ascites diluted 1:250 or 0.5 ml of immune *Aotus* serum diluted 1:250 and precipitated as previously described (28). After SDS-PAGE, precipitated antigens were detected by fluorography (3, 26).

Results

Binding of EBA-175

EBA-175 is the major malaria antigen isolated from culture supernatants which binds to and is eluted from normal human erythrocytes with high salt buffer (Fig. 1, lane b) (4, 19, 32, 41). As described here and elsewhere (4, 32), the binding of EBA-175 is a sialic acid-dependent event and is inhibited by pretreatment of erythrocytes with neuraminidase and trypsin (Fig. 1, lanes c and d, respectively). When culture supernatants were incubated with $\text{M}^{\text{h}}\text{M}^{\text{t}}$ or Tn erythrocytes, no EBA-175 binding was detected (Fig. 1, lanes e and f, respectively). In contrast, pretreatment of intact erythrocytes with Endo F had no detectable effect on the binding of EBA-175 when compared with untreated erythrocytes (Fig. 1, lane h).

Immunoprecipitation of the EBA-175/Glycophorin A Complex from Erythrocytes

The direct interaction between erythrocyte-bound glycophorin A and EBA-175 was shown through immunoprecipitation of the receptor-ligand complex (Fig. 2). In the presence of either malaria-immune *Aotus* serum (Fig. 2, lanes a and b) or mAb 453 specific for glycophorin A (17) (Fig. 2, lanes c and d), EBA-175 was immunoprecipitated from lysates of binding-competent erythrocytes (Fig. 2, lanes a and c). Neither antibody precipitated detectable levels of EBA-175 when erythrocytes were pretreated with *Vibrio cholerae* neur-

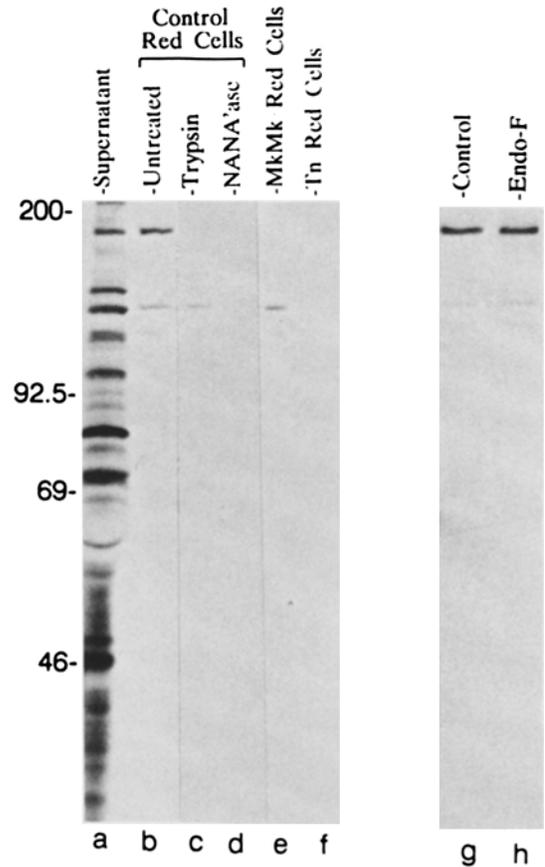


Figure 1. The binding of EBA-175 to human erythrocyte isolates. Culture supernatants metabolically labeled with [^3H]isoleucine were prepared from the Camp strain of *Plasmodium falciparum* as described in Materials and Methods. Erythrocytes were incubated with supernatants, separated by centrifugation through F-50 silicon fluid, and eluted in high salt buffer, and the eluate was subjected to SDS-PAGE as described in Materials and Methods. Shown here are eluates from binding assays using erythrocytes characterized for their modification and expression of glycophorins A and B. Lane a, total culture supernatant; lane b, untreated human erythrocytes; lane c, trypsin-treated human erythrocytes; lane d, neuraminidase-treated erythrocytes; lane e, $\text{M}^{\text{h}}\text{M}^{\text{t}}$ erythrocytes; lane f, Tn erythrocytes; lane g, untreated erythrocytes; lane h, erythrocytes pretreated with Endo F.

aminidase before incubation with culture supernatants (Fig. 2, lanes b and d). The isolation of an EBA-175/glycophorin A complex provides direct evidence that EBA-175 recognizes and binds to sialic acid-containing determinants on glycophorin A and supports previous studies on the identity of the erythrocyte ligand.

Binding of EBA-175 to Erythrocytes and Specific Elution

EBA-175 is released into culture supernatants during schizont rupture (4, 32). The binding of EBA-175 from supernatants to intact human erythrocytes is a saturable event (Fig. 3 A) and is linear at dilute concentrations of these supernatants. The smooth shape of the binding curve is consistent with a single class of high affinity binding sites. The binding of radiolabeled EBA-175 to erythrocytes is also competitively inhibited with increasing concentrations of unlabeled cultured supernatants (data not shown).

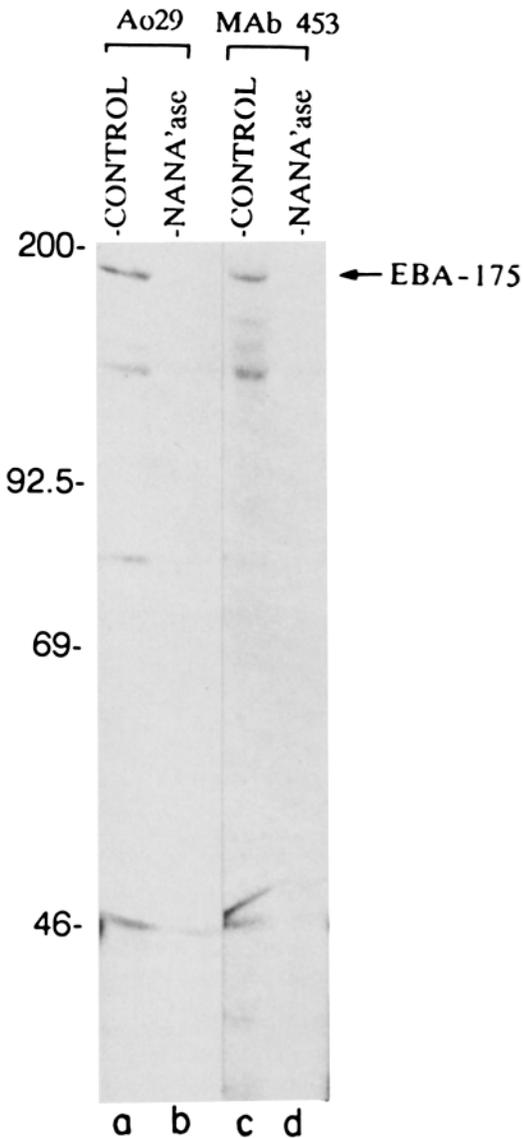


Figure 2. Isolation of the EBA-175/glycophorin A complex from erythrocytes. Normal (lanes *a* and *c*) and neuraminidase-treated (lanes *b* and *d*) human erythrocytes were incubated with [³H]isoleucine-labeled culture supernatants, pelleted, washed, and lysed with 1% Triton X-100 in PBS as described in Materials and Methods. Immunoprecipitations of the erythrocyte lysates with immune *Aotus* serum (Ao29) or mAb 453 were performed as also described in Materials and Methods. Samples were separated by SDS-PAGE and detected by fluorography. Lanes *a* and *b*, erythrocyte lysates immunoprecipitated with immune *Aotus* serum; lanes *c* and *d*, erythrocyte lysates immunoprecipitated with mAb 453.

Erythrocyte-bound EBA-175 can be efficiently eluted with 1 M NaCl (19, 32). Under isotonic conditions, however, only a small percentage of the bound protein freely dissociated (Fig. 3 *B*). Although the binding activity of EBA-175 is dependent on the presence of terminal sialic acid residues, free Neu5Ac was unable to elute EBA-175 (Fig. 3 *B*). In contrast, the effectiveness of Neu5Ac(α 2-3)Lac (Fig. 3 *B*) in eluting bound EBA-175 was similar to high salt and suggested a more defined ligand specificity for binding and elution.

While it has been well established that pretreatment of intact erythrocytes with *Vibrio cholerae* neuraminidase abro-

gates EBA-175 binding, this bacterial enzyme under optimal conditions will cleave all terminal Neu5Ac (8). NDV, however, is specific for (α 2-3)-linked Neu5Ac (8). As shown in Table I, pretreatment of intact erythrocytes with either neuraminidase results in a similar inhibition of EBA-175 binding. These results suggested that terminal Neu5Ac(α 2-3)- residues are required for EBA-175 binding and complement results observed with Neu5Ac(α 2-3)Lac in the elution of erythrocyte-bound EBA-175.

Effects of Soluble Glycophorin A on the Binding of EBA-175 to Erythrocytes

Glycophorin A was treated either with *Vibrio cholerae* neuraminidase or NDV to remove terminal Neu5Ac residues alone; with mild alkali to eliminate the O-linked tetrasaccharides en bloc (42), or with Endo F to enzymatically remove the lone N-linked complex chain (43). SDS-PAGE illustrating the expected electrophoretic patterns of the modified glycophorins is shown in Fig. 4, *A* and *B* (13). These soluble glycophorins were subsequently assayed for their ability to inhibit the binding of EBA-175 to erythrocytes. Untreated glycophorin A was effective in inhibiting the binding of EBA-175 to erythrocytes by 71 and 89% of controls that contained no additions (Fig. 4 *C*) at concentrations of 4.0 and 8.0 μ M of purified protein. Similarly, glycophorin A pretreated with Endo F also retained its inhibitory effects on binding (Fig. 4 *C*). Neither the neuraminidase- nor alkali-treated glycophorin A samples, however, had any significant inhibitory activity at the same concentrations.

Inhibition of EBA-175 Binding to Erythrocytes in the Presence of Soluble Saccharides

The ligand specificity for EBA-175 binding, was defined by the addition of soluble mono- and oligosaccharides to culture supernatants to assess their potential to competitively inhibit the binding of EBA-175 to erythrocytes. As shown in Table II, the most potent inhibitors of EBA-175 binding were Neu5Ac(α 2-3)Lac and Neu5Gc. Binding was slightly affected when assayed in the presence of GlcNAc, with increasing inhibition observed with oligomers of GlcNAc such as chito-tetraose. This suggests a binding specificity comparable to the lectin of *Triticum vulgare* whose binding is specific for NeuAc and GlcNAc and its oligomers (31).

Inhibition by Neu5Ac(α 2-3)Lac was concentration dependent and inhibited binding by 76 and 90% of control binding at concentrations of 25 and 50 mM, respectively (Fig. 5). The degree of binding inhibition observed in the presence of Neu5Ac-Lac was dependent on the sialyl-galactosyl-linkage. The inhibition seen with the α 2-6 isomer, Neu5Ac(α 2-6)Lac, was never greater than 25–35% at 50 mM. Free Neu5Ac and lactose had no significant inhibitory effects on binding at concentrations as high as 50 mM.

The individual contributions of the (α 2-3)- and (α 2-6)-linked Neu5Ac residues of the tetrasaccharides of glycophorin A on the binding of EBA-175 were studied and compared using the purified oligosaccharides LST a, LST b, and DST in binding inhibition assays (Table III). These oligosaccharides reflect the spatial orientation and linkages of the two Neu5Ac residues expressed on the O-linked tetrasaccharides of native glycophorin A. When used in binding inhibition assays the results are consistent with those in the presence of

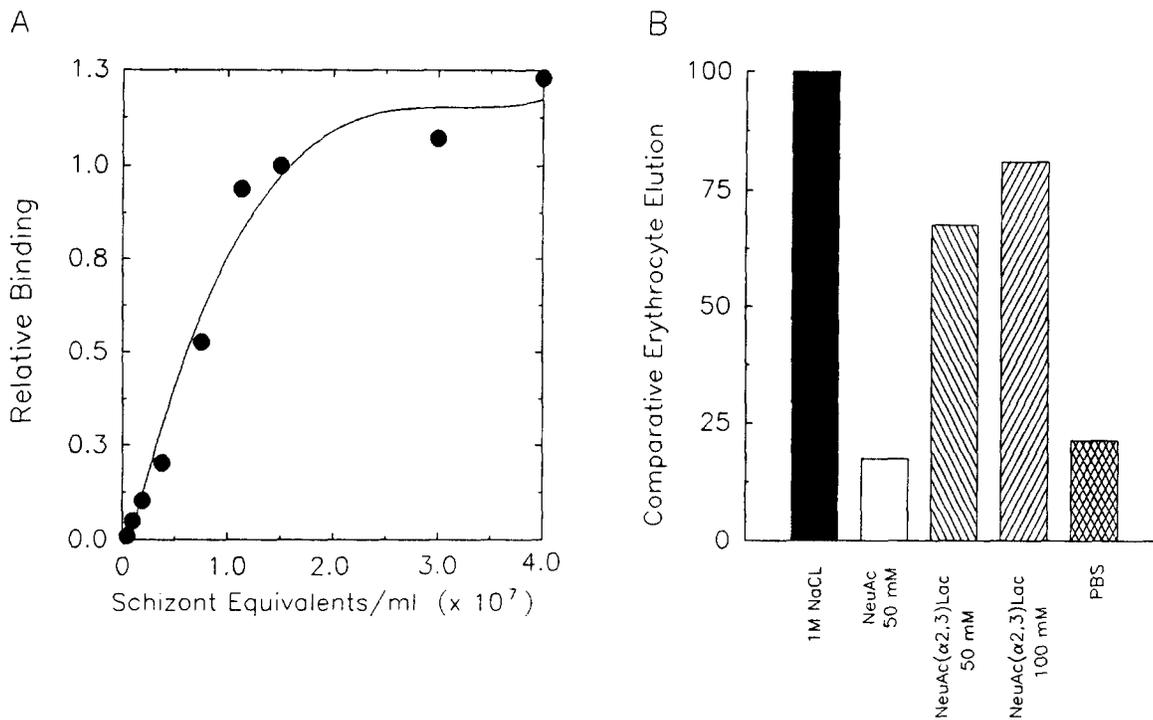


Figure 3. Binding of EBA-175 to erythrocytes. (A) Saturation curve for EBA-175 binding to intact erythrocytes. Culture supernatants metabolically labeled with [3 H]isoleucine prepared at a concentration of 4×10^7 schizont equivalents ml^{-1} , were diluted with complete medium to the indicated concentrations in a final volume of 0.1 ml, incubated with 1.0×10^9 erythrocytes, and assayed for EBA-175 binding as described in Materials and Methods. After separation by SDS-PAGE and detection by fluorography, relative binding was quantified by scanning densitometry of the autoradiographs. (B) Selective elution of erythrocyte-bound EBA-175. Binding assays using 0.1 ml metabolically labeled culture supernatants (1.5×10^7 schizonts ml^{-1}) and 1.0×10^9 erythrocytes were conducted as described above. EBA-175 was then eluted with PBS, or 1 M NaCl, Neu5Ac, or Neu5Ac($\alpha 2,3$)Lac in 0.02 ml in PBS. All elutions were for 20 min at room temperature except for 1 M NaCl, which was for 10 min. After separation by SDS-PAGE and detection by fluorography, relative elution was quantified by scanning densitometry of the autoradiographs.

Neu5Ac-Lac. Oligosaccharides containing the terminal Neu5Ac($\alpha 2,3$)galactosyl- sequence (LST a and DST) were inhibitory (Table II). LST b, containing only the ($\alpha 2,6$)-linked Neu5Ac, was less effective in inhibiting the binding of EBA-175 to erythrocytes, similar to the results seen with Neu5Ac($\alpha 2,6$)Lac.

Effects of Carbohydrate Presentation on the Binding of EBA-175 to Erythrocytes

The relative inhibitory concentrations of Neu5Ac($\alpha 2,3$)Gal-

as it is expressed either on glycoprotein A, fetuin, or freely soluble Neu5Ac($\alpha 2,3$)Lac were compared. Fetuin contains three nonadjacent O-linked tetrasaccharide structures otherwise identical in composition and linkage to the clustered tetrasaccharides of glycoprotein A (42). Whereas the effective concentration of glycoprotein A-bound Neu5Ac($\alpha 2,3$)Gal- associated with 50% inhibition of EBA-175 binding was found at 10^{-5} M, it required two to three orders of magnitude more Neu5Ac($\alpha 2,3$)Gal- on fetuin to achieve the same effect (Fig. 6). Fetuin was only slightly more effective than Neu5Ac($\alpha 2,3$)Lac (Fig. 6). Neuraminidase-treated fetuin with not inhibitory (data not shown).

Table I. Neuraminidase Specificity and EBA-175 Binding

Erythrocyte treatment*	Relative binding [†]	Neuraminidase specificity
None	1.0	
Neuraminidase:		
<i>Vibrio cholerae</i>	0.05	Neu5Ac($\alpha 2,3,6$)-
NDV	0.09	Neu5Ac($\alpha 2,3$)-

* Erythrocytes at a 5% Hct were treated with either 50 U ml^{-1} *Vibrio cholerae* neuraminidase or 10 $\mu\text{g ml}^{-1}$ NDV, strain B1, in serum-free medium for 60 min at 37°C. Erythrocytes were then washed three times in medium containing 10% FBS and assayed for EBA-175 binding as described in Materials and Methods.

[†] Relative binding of EBA-175 was quantified by scanning laser densitometry of fluorographs using an LKB 2022 Ultrascan laser densitometer and are reported as a measure of control binding.

Effects of Periodate Treatment of Intact Erythrocytes

Human erythrocytes were treated with periodate followed by borohydride reduction under conditions previously shown to cleave the exocyclic side chain of sialic acids. These cells were then examined for their potential to bind EBA-175. Erythrocytes treated with periodate concentrations of 0.1–2.0 mM bound EBA-175 as well as untreated erythrocytes (Table IV).

Discussion

The means needed to effectively prevent the invasion of *Plasmodium falciparum* merozoites into erythrocytes requires an understanding of those malarial receptors that participate in

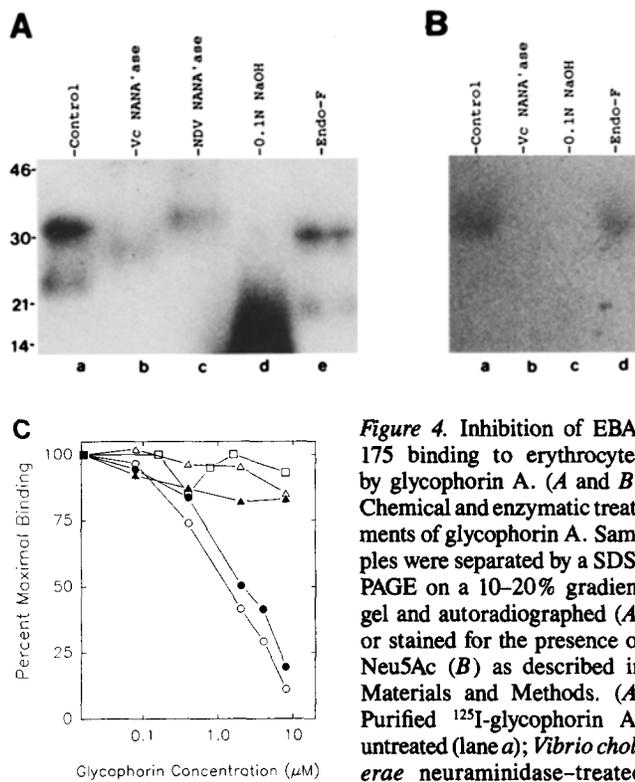


Figure 4. Inhibition of EBA-175 binding to erythrocytes by glycoprotein A. (A and B) Chemical and enzymatic treatments of glycoprotein A. Samples were separated by a SDS-PAGE on a 10–20% gradient gel and autoradiographed (A) or stained for the presence of Neu5Ac (B) as described in Materials and Methods. (A) Purified ^{125}I -glycoprotein A: untreated (lane a); *Vibrio cholerae* neuraminidase-treated (lane b); NDV-treated (lane c); mild alkali-treated (lane d); and Endo F-treated (lane e). (B) Untreated (lane a); *Vibrio cholerae* neuraminidase-treated (lane b); mild alkali-treated (lane c); and Endo F-treated (lane d). (C) Effects of EBA-175 binding to erythrocytes by modified glycoprotein A. Binding assays were conducted with [^3H]isoleucine-labeled culture supernatants as described in Materials and Methods in the presence of increasing concentrations of untreated (○), neuraminidase-treated (*Vibrio cholerae*) (Δ), NDV-treated (□), mild alkali-treated (▲), or Endo F-treated (●). After separation by SDS-PAGE and detection by fluorography, binding was quantified by scanning densitometry of the autoradiographs and expressed as a percentage of control binding as described in Materials and Methods. Values for NDV-treated glycoprotein A were obtained by subtracting densitometry readings of control blanks which contained equivalent volumes of NDV but no glycoprotein A to control for residual NDV neuraminidase activity unquenched by FBS, but which will affect erythrocyte-bound glycoprotein A.

the attachment and invasion process and the ligands on the host cell with which they recognize. In this study we describe the carbohydrate determinants required for efficient binding of EBA-175, a lectin-like malaria receptor. This protein has previously been shown to be an important component in the erythrocyte invasion process by *Plasmodium falciparum* merozoites. The binding of EBA-175 is dependent on the presence of sialic acid-containing determinants on the erythrocyte surface which are sensitive to cleavage by pretreatment with either neuraminidase or trypsin. Such pretreatments abrogate EBA-175 binding (4, 32), prevent merozoite invasion in vitro (4, 16, 30), and can be shown to have a direct effect on the integrity of the NH_2 -terminal domain of glycoproteins A and B in which 11 of 15 O-linked tetrasaccharides and the lone N-linked complex chain are expressed within the first 30 amino acids. Similarly, the binding of EBA-175 reflects the same degree of correlation that exists

Table II. Binding Inhibition of EBA-175 to Erythrocytes

Addition*	Relative binding [‡]
None	1.0
Neu5Ac	0.95
Neu5Gc	0.05
N-Acetylneuramin(α 2-3) lactose	0.15
N-Acetylneuramin(α 2-6) lactose	0.70
Lactose	1.05
Galactose	0.92
Galactosamine	1.09
Glucosamine	0.91
N-Acetylgalactosamine	0.94
N-Acetylglucosamine	0.80
Chitobiose	0.94
Chitotriose	0.86
Chitetraose	0.44
Raffinose	1.05
Stachyose	0.94

* The final concentrations of all oligosaccharides tested were 50 mM and the culture supernatants were adjusted to pH 7.0 before mixing with erythrocytes. [‡] Binding assays were conducted as described in Materials and Methods. Relative binding of EBA-175 was quantified by scanning laser densitometry of fluorographs using an LKB 2022 Ultrascan laser densitometer and are reported as a measure of control binding.

between merozoite invasion and glycoprotein presentation on the erythrocyte surface. Confirmation of the direct interaction of EBA-175 with sialic acid-containing determinants of glycoprotein A on intact erythrocytes was achieved through coprecipitation of the receptor–ligand complex with the anti-glycoprotein mAb 453 (Fig. 2), while the absence of binding to neuraminidase-treated erythrocytes was in agreement with results seen here and elsewhere (Fig. 1, lane d; Fig. 2, and references 4 and 32). Furthermore, the absence of binding to glycoprotein A- and B-deficient M^bM^k erythrocytes (Fig. 1, lane e) suggests that sialic acid elsewhere on the erythrocyte surface is not sufficient for binding EBA-175.

Evidence for the binding of EBA-175 to the alkali-labile tetrasaccharides of glycoprotein A is provided by the results obtained on the effects of soluble, modified glycoprotein A in binding inhibition assays. The inhibition of binding seen in the presence of glycoprotein A is lost if the glycoprotein is pretreated with either *Vibrio cholerae* neuraminidase or NDV to remove terminal Neu5Ac(α 2-3)- residues or with mild alkali to remove the O-linked tetrasaccharide chains before assaying for inhibition.

While electrostatic interactions due to the presence of the negatively charged C-1 carboxyl group of Neu5Ac may contribute to the binding of EBA-175 to some degree, competitive binding inhibition assays with soluble oligosaccharides indicated that saccharide composition and linkage to an underlying sugar are greater factors in binding efficiency. This is in apparent contrast to the reported effects of charge on the invasion of erythrocytes by merozoites (12).

The binding of EBA-175 to erythrocytes is inhibited by the presence of DST, LST a, and Neu5Ac(α 2-3)lactose. These oligosaccharides reflect to a large extent the composition, linkage, and spatial orientation of the NeuAc and Gal-residues of the alkali-labile tetrasaccharides of glycoprotein A. The ability of Neu5Ac(α 2-3)Lac, LST a, and DST to inhibit the binding of EBA-175 to erythrocytes, when contrasted with those seen with Neu5Ac(α 2-6)Lac and LST b, suggested that the terminal carbohydrate sequence Neu5Ac-

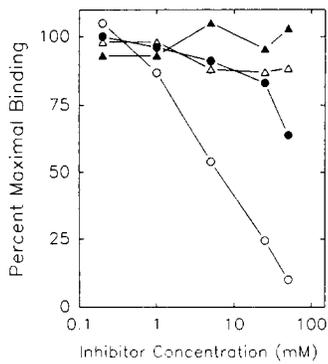


Figure 5. Inhibition of EBA-175 binding to erythrocytes in the presence of Neu5Ac-Lac. Binding assays were conducted with [³H]isoleucine-labeled culture supernatants as described in Materials and Methods with increasing concentrations of the indicated saccharides. After separation by SDS-PAGE and detection by fluorography, binding was quantified by scanning densitometry of the autoradiographs

and expressed as a percentage of control binding as described in Materials and Methods. Neu-5Ac(α2-3) lactose (○), Neu5Ac(α2-6) lactose (●) lactose (Δ), and Neu5Ac (▲).

(α2-3)Gal- was the primary ligand required for efficient binding. The modest effects of the (α2, 6)-linked Neu5Ac residues of LST b may be a consequence of the greater degrees of freedom it possesses in solution. When protein-bound, this ligand may be more restricted and remain relatively inaccessible to the binding domain of EBA-175 as in the native glycoporphin A molecule. This is supported by results obtained with neuraminidase treatment. NDV will not cleave Neu5Ac(α2-6)- residues (8), while its action on intact erythrocytes eliminated EBA-175 binding. Similarly, only the Neu5Ac(α2-3)-linked residues of the O-linked tetrasaccharides of glycoporphin A on intact erythrocytes are susceptible to the action of *Vibrio cholerae* neuraminidase. The inability to liberate the (α2-6)-linked Neu5Ac of these same tetrasaccharides is attributed to steric hindrance (14). This steric hindrance, as well as intrinsic lower affinity, may severely limit the role of α2-6-linked Neu5Ac on glycoporphin for binding EBA-175.

While the data presented here clearly suggest that the Neu5Ac(α2-3)Gal- determinant is necessary for binding, comparison of results obtained from competitive binding inhibition studies with glycoporphin A, fetuin, and Neu5Ac(α2-3)Lac indicate that this determinant alone is not sufficient for the high affinity binding observed with EBA-175 and glycoporphin A. Oligosaccharide density and presentation of the determinants of glycoporphin A may be factors that influence the higher degree of binding. Fetuin, which contains only three nonadjacent O-linked oligosaccharide structures otherwise identical to the 15 clustered on glycoporphin A, and

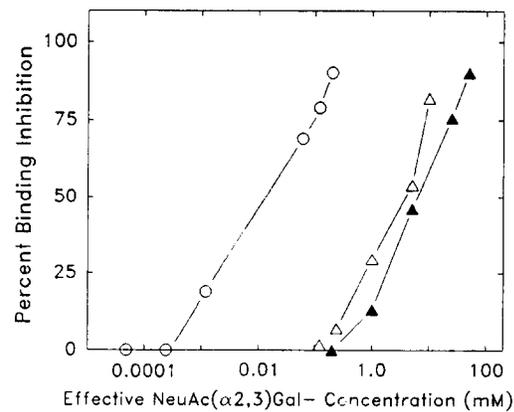


Figure 6. Effects of carbohydrate presentation on the binding of EBA-175 to erythrocytes. Binding assays were conducted with [³H]isoleucine-labeled culture supernatants as described in Materials and Methods in the presence of increasing concentrations of glycoporphin A (○), fetuin (Δ), and Neu5Ac(α2-3) lactose (▲). After separation by SDS-PAGE and detection by fluorography, binding was quantified by scanning densitometry of the autoradiographs and the inhibition was expressed as a percentage of control binding as described in Materials and Methods.

Neu5Ac(α2-3)Lac are inhibitory at similar effective Neu5Ac-(α2-3)Gal- concentrations. This suggests that the tetrasaccharides of fetuin are acting independently of each other due to their distal spatial distribution on the polypeptide backbone and therefore behave similar to free Neu5Ac(α2-3)Lac monomers in solution. Conversely, glycoporphin A contains 11 tetrasaccharide units within the first 30 amino acids. The clustering of these units at the amino terminus of the glycoporphin A dimer may provide for the higher affinity binding of EBA-175 which cannot be mimicked by either Neu5Ac-(α2-3)Lac or the three individual tetrasaccharide units of fetuin. Peptide sequence may also contribute to some degree in binding efficiency and is currently under investigation.

Similar fine specificity has been described for binding of the hemagglutinin glycoprotein of influenza virus used for tissue-specific viral endocytosis into host cells. In comparing the binding activities of these lectin-like pathogen receptors, neither activity is effectively diminished in the presence of free Neu5Ac (15); however, both EBA-175 and influenza A and B viral hemagglutinins recognize a more complex ligand, specific for composition and anomeric linkage. For the much-studied viral hemagglutinin, strains have been iso-

Table III. Inhibition of EBA-175 Binding to Erythrocytes with NeuAc(α2-3)Gal- and NeuAc(α2-6)Gal-containing Oligosaccharides

Oligosaccharide addition*	Structure	Percent inhibition‡
No additions	—	0
LST a	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	85.6
LST b	Galβ1-3GlcNAcβ1-3Galβ1-4Glc ↑(α2-6) Neu5Ac	49.6
DST	Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc ↑(α2-6) Neu5Ac	88.5

* The final concentrations of all oligosaccharides tested were 50 mM and the culture supernatants were adjusted to pH 7.0 before mixing with erythrocytes.
‡ Binding assays were conducted as described in Materials and Methods. EBA-175 binding was quantified by scanning laser densitometry of fluorographs using an LKB 2022 Ultrascan laser densitometer. Relative binding inhibition is expressed as a percentage of control binding.

Table IV. Effects of Periodate Oxidation/Borohydride Reduction of Intact Erythrocytes on the Binding of EBA-175

Erythrocyte treatment*	Relative binding†
None	1.0
NaBH ₄ only	1.27
NaIO ₄ /NaBH ₄ -treated erythrocytes	
0.1 mM	1.27
0.2 mM	1.15
1.0 mM	0.99
2.0 mM	1.08

* Intact erythrocytes were treated with NaIO₄ at the indicated concentrations and reduced with NaBH₄ (1 mg ml⁻¹) as described in Materials and Methods. Control erythrocytes were treated in buffer only or reduced with NaBH₄ only where indicated.

† Binding assays were conducted as described in Materials and Methods using 1 × 10⁹ erythrocytes and 0.1 ml culture supernatant (1.5 × 10⁷ schizont equivalents ml⁻¹). Relative binding of EBA-175 was quantified by scanning laser densitometry of fluorographs using an LKB 2022 Ultrascan laser densitometer and are reported as a measure of control binding.

lated that preferentially bind to oligosaccharides terminating in Neu5Ac(α2-6)Gal- sequences, whereas other isolates require the Neu5Ac(α2-3)Gal- sequences (45, 46). The two- to threefold differences in binding affinities exhibited by these hemagglutinins with respect to the linkage of terminal Neu5Ac have been attributed to a single amino acid change at the binding site (45, 46). X-ray crystallography of the binding site of influenza hemagglutinin (44) also suggests that bulky substitutions at the terminal Neu5Ac(α2-3)-residue may preclude binding. Influenza A and B hemagglutinins do not effectively bind to acetylated derivatives of the exocyclic side chain such as in 9-*O*-Neu5Ac (20). Influenza C hemagglutinins, however, utilize 9-*O*-Neu5Ac acid as an essential component of the cell surface receptor (39). In comparison, EBA-175 cannot bind to this Neu5Ac derivative. Efficiency of binding and merozoite invasion were both found to be inversely related with the levels of the 9-*O*-acetyl derivative on the mouse erythrocyte surface (24). Enzymatic removal of the bulky *O*-acetyl moiety at the C-9 position increased EBA-175 binding with a similar increase in invasion of mouse erythrocytes by *Plasmodium falciparum* merozoites.

In contrast to the sensitivity of binding to the Neu5Ac-Gal linkage and 9-*O*-acetylation, removal of the exocyclic side chain had no appreciable effect on the binding of EBA-175. The binding activity of EBA-175 was effectively unchanged after periodate oxidation and borohydride reduction of intact erythrocytes, conditions specific for external sialic acid-containing glycoproteins (42) (Table IV). This suggested that binding specificity is primarily dependent on linkage and anomeric configuration. Derivatization at sites on the exocyclic side chain may preclude binding due to an imperfect fit in the binding pocket.

During invasion, it has been postulated that there are several parasite-derived proteins that interact with glycoprotein A on the erythrocyte surface during the sequential processes of merozoite invasion. Attachment to and interaction of a lectin-like protein, such as EBA-175, to the oligosaccharides of glycoprotein A, may allow the subsequent exposure of and binding to the polypeptide domain of glycoprotein A near the lipid interface by EBA-175 or by other proteins. Several studies have reported the binding of other malaria antigens to immobilized glycoprotein A and glycoprotein peptides (10, 22, 23, 37). It has also been suggested that Gp195 is a putative

sialic acid-binding protein which interacts weakly with glycoprotein A on erythrocytes (38). The nature of this binding and the ligand specificity have not been thoroughly reported.

As demonstrated in this study, EBA-175 in its interaction with the O-linked tetrasaccharide clusters of glycoprotein A forms a highly specific receptor-ligand complex with the erythrocyte. Further studies may determine whether EBA-175 has multiple binding sites per molecule or whether EBA-175 molecules associate as multimers in their binding to the tetrasaccharide clusters of glycoprotein A. Through self-association or in concert with other malaria proteins, EBA-175 appears to be an essential component in sialic acid-dependent malaria invasion.

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Note Added in Proof. In support of the direct correlation between EBA-175 binding and parasite invasion, we have obtained additional evidence that (α2-3)-linked Neu5Ac is required for optimal invasion by treating intact erythrocytes with NDV (6 μg ml⁻¹ NDV in RPMI medium, pH 6.7, 1 h, 37°C and erythrocytes at 1% Hct). Invasion by Camp strain parasites into washed, treated erythrocytes was significantly decreased (to 10% of that into control, untreated erythrocytes). In another control to show that NDV treatment did not render the erythrocytes refractory to invasion in a non-specific manner, it was found that invasion by 7G8 strain parasites, which additionally possess a sialic acid-independent invasion pathway, into washed, treated erythrocytes was, as expected (20), much less effected (61% of untreated controls).

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