plasmid DNA was prepared for each with the Qiagen Maxi-prep kit (Qiagen, Inc.) and cotransfected with Bac-N-Blue DNA (Invitrogen) into Escherichia coli (DH5α). The ligation mix was transformed into E. coli competent cells (Gibco-BRL, Gaithersburg, Md.). The fragments were gel purified with the gel extraction kit (Qiagen, Inc.) and ligated into the backbone of glycophorin A. Region II (RII) of EBA-175 has been defined as the receptor-binding domain. RII is divided into regions F1 and F2, which contain duplicated cysteine motifs. We expressed RII in a baculovirus and show that RII binds erythrocytes with a specificity identical to that of the native protein. We found that, consistent with the binding of erythrocytes to COS cells expressing F2, recombinant baculovirus-expressed F2 bound erythrocytes. About 20% of all baculovirus-expressed RII is N-glycosylated, unlike native P. falciparum proteins that remain essentially unglycosylated. However, glycosylation of recombinant RII did not affect its immunogenicity. Antibodies raised against both glycosylated and unglycosylated baculovirus-expressed RII recognized P. falciparum schizonts in immunofluorescence assays and also gave similar enzyme-linked immunosorbent assay titers. Furthermore, these antibodies have similar abilities to block native EBA-175 binding to erythrocytes. These results allow the development of RII as a vaccine candidate for preclinical assessment.

The erythrocytic stage of Plasmodium falciparum kills an estimated 2 million children annually. Efforts to control this global problem have been hampered by the development of drug resistance by the parasite and insecticide resistance by the mosquito host. The development of additional control measures is vitally important, and a malaria vaccine holds great promise for the reduction of morbidity and mortality associated with the disease. An extremely promising vaccine candidate is the 175-kDa P. falciparum erythrocyte-binding protein (EBA-175) (2, 13, 14). EBA-175 is a parasite ligand that binds to its receptor glycoporphin A on erythrocytes during parasite invasion into the erythrocyte (16). The actual receptor-binding domain of EBA-175 is contained within a region of 616 amino acids that is identified as region II (RII) (16). Antibodies against RII block parasite invasion of both sialic acid-dependent and -independent strains of P. falciparum in vitro (12).

We recently described the successful immunization of Aotus monkeys with EBA-175 sequences as a P. falciparum malaria invasion ligand naked DNA vaccine (B. K. L. Sim, D. L. Narum, K. Moch, and S. L. Hoffman, submitted for publication). The DNA vaccine is comprised of sequences encoding a protein-protein vaccine and a DNA prime-protein boost vaccination regimen. Given the cysteine-rich motifs contained within RII, we selected the eukaryotic baculovirus expression system. In this report, we present the production and characterization of recombinant baculovirus RII (rRII) proteins for the human challenge strain 3D7 and the Aotus challenge strain FVO. The rRII proteins have been purified to greater than 95% homogeneity and shown to biologically mimic native EBA-175 binding to human erythrocytes and to induce antibodies that block native EBA-175 binding to human erythrocytes. Finally, given the limited capacity for N-glycosylation of P. falciparum (7), we evaluated the extent of N-glycosylation present within the FVO rRII and the effect that N-glycosylation had on the immunogenicity and induction of EBA-175-blocking antibodies.

MATERIALS AND METHODS

Parasites. P. falciparum strains 3D7 (human strain) and FVO (Aotus adapted) were maintained as previously reported (18). When appropriate, schizonts were purified on Percoll density gradients. P. falciparum strain FVO was metabolically labeled with Tran35S-Label as previously described (16). Cell pellets and supernatant were stored at −70°C.

Construction and expression of recombinant baculovirus 3D7 and FVO EBA-175 RII proteins. The gene fragments encoding 3D7 or FVO RII proteins (amino acids 145 to 760, 1,848 bp) (16) were excised from plasmids VR1020/3D7/RII1 and VR1020/FVORII14, respectively (Sim et al., submitted), with the restriction enzyme BglII (Gibco-BRL, Gaithersburg, Md.). The fragments were gel purified with the gel extraction kit (Qiagen, Inc.) and ligated into the baculovirus transfer vector pMalBacA (Invitrogen, San Diego, Calif.). The ligation mix was transformed into Escherichia coli DH5α competent cells (Gibco-BRL), and transformants were screened by restriction map analysis. Clones pMalBacA/3D7RII6 and pMalBacA/FVORII1/2 were selected and sequence verified. Plasmid DNA was prepared for each with the Qiagen Maxiprep kit (Qiagen, Inc.) and cotransfected with Bac-N-Blue DNA (Invitrogen) into Spodoptera frugiperda (SF21) cells (Invitrogen) following the manufacturer's protocol. Recombinant viral clones, shown as blue plaques, were selected, and the purity of the clones was verified by PCR. SF21 cells were infected with recombinant baculovirus that secreted 3D7 or FVO EBA-175 RII proteins and fermented at a 40-liter scale (Kemp Biotechnologies, Frederick, Md.). rRII protein was probed with anti-RII antibodies generated by immunization with an rRII recombinant EBA-175 RII protein for the purpose of studying a protein-protein vaccine and a DNA prime-protein boost vaccination regimen. Given the cysteine-rich motifs contained within RII, we selected the eukaryotic baculovirus expression system. In this report, we present the production and characterization of recombinant baculovirus RII (rRII) proteins for the human challenge strain 3D7 and the Aotus challenge strain FVO. The rRII proteins have been purified to greater than 95% homogeneity and shown to biologically mimic native EBA-175 binding to human erythrocytes and to induce antibodies that block native EBA-175 binding to human erythrocytes. Finally, given the limited capacity for N-glycosylation of P. falciparum (7), we evaluated the extent of N-glycosylation present within the FVO rRII and the effect that N-glycosylation had on the immunogenicity and induction of EBA-175-blocking antibodies.

A Recombinant Baculovirus-Expressed Plasmodium falciparum Receptor-Binding Domain of Erythrocyte Binding Protein EBA-175 Biologically Mimics Native Protein

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EBA-175 of Plasmodium falciparum is a merozoite ligand that binds its receptor glycoporphin A on erythrocytes during invasion. The ligand-receptor interaction is dependent on sialic acids as well as the protein backbone of glycoporphin A. Region II (RII) of EBA-175 has been defined as the receptor-binding domain. RII is divided into regions F1 and F2, which contain duplicated cysteine motifs. We expressed RII in a baculovirus and show that RII binds erythrocytes with a specificity identical to that of the native protein. We found that, consistent with the binding of erythrocytes to COS cells expressing F2, recombinant baculovirus-expressed F2 bound erythrocytes. About 20% of all baculovirus-expressed RII is N-glycosylated, unlike native P. falciparum proteins that remain essentially unglycosylated. However, glycosylation of recombinant RII did not affect its immunogenicity. Antibodies raised against both glycosylated and unglycosylated baculovirus-expressed RII recognized P. falciparum schizonts in immunofluorescence assays and also gave similar enzyme-linked immunosorbent assay titers. Furthermore, these antibodies have similar abilities to block native EBA-175 binding to erythrocytes. These results allow the development of RII as a vaccine candidate for preclinical assessment.

As a result of this report and our findings, we produced a

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DNA plasmid vaccine (Sim et al., submitted). Culture supernatants were collected and stored frozen until processed as described below.

**rRII purification.** After thawing frozen culture supernatants, phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) was added to make a final concentration of 1 mM. The material was then diluted 1:3 with deionized water; the pH was adjusted to 4.5 with 6 N HCl, and the culture was stirred gently for 15 min at room temperature. Precipitated material was removed by centrifugation at 10,000 × g for 20 min, and the supernatant was passed through a 0.45-μm bottle-top filter (Schleicher & Schuell, Keene, N.H.). The conductivity was determined and adjusted with deionized water if greater than that of the loading buffer. Load material was passed over an SP Sepharose Fast Flow resin (Pharmacia Biotech; Piscataway, N.J.) equilibrated with 25 mM sodium acetate buffer (pH 4.5) containing 100 mM NaCl at 2 to 8°C and washed in the same buffer. Material was eluted by a step elution gradient. The first elution step was performed with 25 mM sodium acetate buffer (pH 4.5) containing 500 mM NaCl, and then rRII was eluted with 25 mM sodium acetate buffer (pH 4.5) containing 1,000 mM NaCl. The eluate pool was dialyzed for 16 to 24 h against 25 mM Tris (pH 8.0) and against 50 volumes of buffer and used immediately or stored frozen (−20 to −80°C). The dialyzed sample was passed over a Q Sepharose Fast Flow resin (Pharmacia Biotech) equilibrated with 25 mM Tris-HCl buffer (pH 8.0) and washed in the same buffer. Bound material was eluted with 25 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl and used immediately or stored frozen. The sample was then passed over another SP Sepharose Fast Flow column equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Bound material was eluted with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl. Fractions were tested for the presence of rRII by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were stained with Coomassie stain or electroblotted for Western blotting. Samples were further enriched for full-length rRII by gel filtration by passing the sample through a HiPrep 16/100 S-100 HR column (Pharmacia Biotech) with 50 mM Tris-HCl buffer (pH 8.0) containing 100 mM NaCl.

The purity of rRII protein was analyzed by reverse-phase column chromatography with a Delta Pak 5 μ, C4 column (Waters, Inc., Milford, Mass.) equilibrated with trisfluoroacetic acid-water and eluted with an acetonitrile gradient with a Waters high-pressure liquid chromatography apparatus. The area under the curve was integrated in order to determine the percentage represented by each molecular species identified. The rRII protein concentration was determined with the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.).

**Recombinant protein characterization.** (i) **N-terminal sequencing.** Purified rRII containing both glycosylated and unglycosylated material was passed over the column twice. Material that did not bind was collected. Material that bound to the column was eluted with 0.5 M methyl-β-o-mannopyranoside (Fluka Chemical, Inc., Ronkonkoma, N.Y.). Bound and unbound samples were analyzed by Coomassie stain, immunostain with anti-RRII antibodies, and lectin blot on samples separated by SDS-PAGE. Lectin blots were performed by incubating with digoxigenin-labeled lectin GNA (Boehringer Mannheim, Inc., Indianapolis, Ind.), washed, and then incubated with an alkaline phosphatase-labeled antidigoxigenin antibody (Boehringer Mannheim, Inc.).

(ii) **Analysis of glycosylation.** Hydrazinolysis and carbohydrate analysis with FVO rRII were performed as previously described (11).

Animals and immunizations. All animal studies were done in compliance with protocols approved by Animal Care and Use committees. Groups of four BALB/c mice were immunized subcutaneously with 16 μg of purified rRII consisting of glycosylated and unglycosylated, unglycosylated, or glycosylated protein. Mice were immunized on days 0, 14, and 28 with Freund's complete and incomplete adjuvants and bled on days 1, 12, 26, and 40. ELISA. For the enzyme-linked immunosorbent assay (ELISA), serum antibodies were assayed essentially as previously described (17). Briefly, high-protein-binding, 96-well, flat-bottomed microtiter ELISA plates (Nunc Microtest Microplates; Fisher Scientific, Pittsburgh, Pa.) were coated with purified rRII protein (1 μg/ml) in phosphate-buffered saline (PBS)-sodium azide buffer and incubated overnight at 4°C. Sera were serially diluted in antibody buffer (0.5% bovine serum albumin–PBS [pH 8.0]) and incubated for 1 h at 37°C. After washing, goat anti-mouse immunoglobulin conjugated with alkaline phosphatase (Promega, Madison, Wis.) was added in a volume of 50 μl per well at a dilution of 1:7,500 and incubated for 1 h at 37°C. Substrate (Pierce, Rockford, Ill.) was added in a volume of 100 μl/well and incubated for 1 h. The optical density at 655 nm (OD₆₅₅) was determined with a Spectra Max 250 (Molecular Devices, Sunnyvale, Calif.). The ELISA results are reported as the reciprocal dilution at which an OD of 0.5 was observed.

Enzyme pretreatment. Human blood was collected in a final 10% citrate-phosphate-dextrose solution for enzymatic treatment of human erythrocytes. Blood was stored at 4°C. Erythrocytes were washed and treated with 0.2 U of *Vibrio cholerae* neuraminidase (Gibco-BRL) per 10⁷ erythrocytes as previously described (6). The enzymatically treated erythrocytes were washed thrice in 100× (vol/vol) packed erythrocytes in RPMI-1640 prior to use in EBA-175 binding studies.

**Erythrocyte binding of rRII protein and blocking of native EBA-175-erythrocyte binding.** Bovine culture supernatant containing rRII protein or an equal volume of culture supernatant was incubated with normal or neuraminidase-treated erythrocytes at room temperature on a rocker for 30 min. Immunoblots were performed on bound and unbound samples (data not shown) obtained as described below. Blocking of binding was performed with 35S-labeled parasite culture supernatant containing labeled FVO EBA-175 as previously described (16). Briefly, 40 μl of labeled EBA-175 was incubated with dilutions of antisera in a final volume of 100 μl with RPMI 1640 containing 10% fetal calf serum (FCS) for 1 h at room temperature. This mixture was then added to 5 × 10⁶ packed erythrocytes and rocked for 30 min at room temperature for binding to occur. SDS-PAGE analysis was performed on eluates of erythrocytes eluted with 9 μl of RPMI 1640–1.5 M NaCl–10% FCS–2 mM phenylmethylsulfonyl fluoride. Gels were dried and quantified with a phosphor-imager (Bio-Rad Molecular Imager FX). The reciprocal dilution resulting in an effective dose that blocked 50% of EBA-175 binding compared with the control was reported. The control was EBA-175 bound to erythrocytes in the presence of preimmune mouse serum.

**RESULTS**

Baculovirus-expressed rRII protein binds human erythrocytes with a specificity similar to that of native EBA-175. The 3D7 and FVO rRII proteins were expressed in the recombinant baculovirus system. The rRII proteins have a molecular mass of 70 kDa, as shown by Coomassie stain and immunoblot (Fig. 1). N-terminal sequencing of the 3D7 rRII protein yielded DRWGS, which was expected based on proper cleavage at the melittin cleavage site (Invitrogen, Inc.). The purity of the rRII protein was greater than 95%, as detected by reverse-phase column chromatography (data not shown). Analysis of the erythrocyte-binding characteristics of the 3D7 and FVO
rRII proteins showed a specificity for binding to human erythrocytes identical to that of EBA-175. rRII binds erythrocytes in a sialic acid-dependent fashion (Fig. 2). Erythrocytes treated with neuraminidase, which are devoid of sialic acids, did not bind rRII proteins (Fig. 2).

rRII protein may be cleaved, and the resultant F2 fragment maintains the capacity to bind human erythrocytes. EBA-175 RII is divided into regions F1 and F2, which contain duplicated cysteine-rich motifs with 13 and 14 cysteines, respectively (Fig. 3A) (1). We observed, during the analysis of purified 3D7 rRII protein, a smaller protein of approximately 40 kDa (Fig. 3B, track 1). The molecular mass of this smaller protein is similar to that of a single F1 or F2 domain. Analysis by immunoblot showed that anti-RII antibodies recognized this 40-kDa protein (Fig. 3B, track 2). N-terminal sequencing of this smaller protein yielded the sequence VLNGNDNTI, which indicates that full-length rRII was cleaved between amino acids S and V at positions 272 and 273, which is located between the cysteine motifs in F1 and F2 (Fig. 3A). The rF2 fragment bound to erythrocytes in an erythrocyte-binding assay (Fig. 3B, track 3), although it appears that the binding of rF2 is less stringent than that of rRII (data not shown).

Glycosylated and nonglycosylated rRII induce antibodies that block EBA-175-erythrocyte binding. Analysis of the deduced amino acid sequence of 3D7 and FVO EBA-175 RII identified five potential sites within RII for N-linked glycosylation (four sites in F1 and one site in F2). Hydrazinolysis studies on the FVO rRII protein determined that FVO rRII contained 20.6% glycosylation. Carbohydrate analysis of the saccharides released by hydrazinolysis showed that the only significant glycan (approximately 90%) was Asn-GlcNAc2-Man3-fucose, which is the dominant saccharide present in baculovirus (4). Given that P. falciparum has a low N-glycosylation capacity (7), we evaluated whether N-glycosylation of FVO rRII protein affected its immunogenicity or altered its capacity to induce antibodies that specifically block the binding of native EBA-175 to human erythrocytes. Three different samples of FVO rRII were prepared. The first sample was the starting material, which contained purified FVO rRII that consisted of both glycosylated and unglycosylated material. The second sample contained only unglycosylated rRII protein, and the third sample contained only glycosylated rRII protein (Fig. 4).

DISCUSSION

Here we report on recombinant baculovirus production of the ligand-receptor binding domain (RII) of EBA-175 for the P. falciparum 3D7 human challenge strain and FVO Aotus challenge strain. A comparison of the deduced FVO and 3D7 RII molecular schematic, showing F1 and F2 cysteine-rich motifs and location of cysteines (Fig. 3A). Panel A shows the cleavage site between the F1 and F2 subdomains (arrow). Panel B shows a Coomassie stain of rRII and cleavage product (40-kDa fragment) (track 1); recognition of rRII and 40-kDa fragment by anti-RII rabbit antibodies by immunoblot (track 2); and binding of rRII and 40-kDa fragment to erythrocytes as detected by immunoblot (track 3).
amino acid sequences of EBA-175 RII shows that only one residue is different at position 286, Glu or Lys, respectively (10). EBA-175 RII may be divided into two regions, F1 and F2, that contain cysteine-rich motifs. The presence of the cysteines suggests that the conformation of this region is significant for the biological function of this ligand-binding region. Therefore, the eukaryotic baculovirus expression system was selected, since this system has been used to successfully express another structurally dependent malaria blood stage protein that was shown to be protective in a primate model (3). The recombinant products were successfully purified using a protocol that is suitable for large-scale purification, which resulted in material which is 95% homogeneous. The level of recombinant protein expression after purification was 1 to 2 mg/liter for each (data not shown).

The interaction of EBA-175 with its receptor glycophorin A is dependent on the ligand-binding sialic acid residues as well as the protein backbone (16). Similarly, the rRII proteins bound to erythrocytes in a sialic acid-dependent manner. Erythrocytes enzymatically treated with neuraminidase, which are devoid of sialic acid residues, did not bind rRII proteins. The fact that the specificity of the ligand-receptor binding interaction is similar for EBA-175 and rRII suggests that the structure of rRII mimics that of native EBA-175 RII.

Previously, F2 was reported to bind erythrocytes, similarly to RII although less stringently, in a transiently transfected COS cell-binding assay (16). In the same study, F1 was not shown to bind erythrocytes. Here we report that rF2 binds to erythrocytes, which is consistent with earlier findings. The biological significance of F2 binding to erythrocytes while F1 apparently does not is unclear.

The role of N-linked glycosylation in *P. falciparum* is still ambiguous. In one report, N-glycosylation was reported to be absent (5); however, in a more recent report, N-glycosylation was reported to be significant for parasite maturation from ring stage parasites to schizonts (8). Hence, low-level N-glycosylation of *P. falciparum* does appear to be possible (7, 8). The presence of 20% N-glycosylation in rRII is therefore nonnative, but this level of N-glycosylation did not interfere with the ability of rRII to bind human erythrocytes or interfere with the induction of blocking antibodies. These results indicate that the baculovirus-expressed N-glycosylated rRII protein is suitable for preclinical studies. The induction of blocking antibodies by rRII is similar to a previous finding, which showed that an RII DNA vaccine plasmid that expressed EBA-175 RII induced EBA-175-blocking antibodies in mice, rabbits, and *Aotus* monkeys (Sim et al., submitted).

In summary, we report on recombinant baculovirus expression of EBA-175 RII, a cysteine-rich ligand-receptor binding domain of *P. falciparum* for both 3D7 and FVO strains. The rRII has been purified to >95% homogeneous using a scalable protocol. Most importantly, rRII protein mimics the sialic acid-dependent binding of native EBA-175 to human erythrocytes. Analysis of N-linked glycosylation has shown that about 20% of rRII is N-glycosylated. Antibodies raised against rRII, which contains glycosylated material, recognize *P. falciparum* schizonts.

### Table 1. Recombinant RII reciprocal antibody titers by ELISA and reciprocal ED$_{50}$ blocking titers for EBA-175 erythrocyte binding

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Reciprocal rRII ELISA Titer (OD 0.5)</th>
<th>Reciprocal ED$_{50}$ Reciprocal EBA-175 Blocking Titer$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RII (+)</td>
<td>RII (-)</td>
</tr>
<tr>
<td>1</td>
<td>110,495</td>
<td>114,838</td>
</tr>
<tr>
<td>2</td>
<td>60,059</td>
<td>(53,407)</td>
</tr>
<tr>
<td>3</td>
<td>187,930</td>
<td>67,331</td>
</tr>
<tr>
<td>4</td>
<td>100,870</td>
<td>—</td>
</tr>
</tbody>
</table>

$^a$ RII (+), glycosylated and unglycosylated FVO rRII; RII (-), unglycosylated FVO rRII; RII (+), glycosylated FVO rRII. Ind, individual.

$^b$ All data are compared to adjuvant control.

$^c$ Mouse died due to unknown cause.
zont-infected erythrocytes by indirect immunofluorescence and block 35S-metabolically labeled EBA-175 binding to human erythrocytes. These results allow the development of rRII as a vaccine candidate for preclinical assessment.

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