

# Antigenicity and Immunogenicity of Recombinant Glutamate-Rich Protein of *Plasmodium falciparum* Expressed in *Escherichia coli*

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**A recombinant *Plasmodium falciparum* glutamate-rich protein (GLURP) was produced in *Escherichia coli* as a nearly full-length protein. In order to map immunodominant regions on GLURP, the nonrepetitive amino-terminal region (R0) as well as the central repeat region (R1) and the carboxy-terminal repeat region (R2) were also produced as separate products. All four purified gene products reacted specifically with serum samples from adults living in an area of Liberia where malaria is holoendemic. It appears that the human immune response against GLURP is primarily directed against the R2 region because 94% of the serum samples reacted with this region in an immunoassay. Antibody reactivity against the R0 region was also observed in 75% of the serum samples, while the R1 region showed only weak antibody-binding activity. When the nearly full-length GLURP molecule was adsorbed to Al(OH)<sub>3</sub> it was found to be immunogenic in mice. In these experiments, the antibody response was almost exclusively directed against the R2 region. When anti-GLURP sera were obtained from rabbits immunized with the three regions, R0, R1, and R2, respectively, they recognized in immunoprecipitation experiments authentic GLURP from *P. falciparum* grown in vitro. These results demonstrate that GLURP produced in *E. coli* can induce a humoral immune response against GLURP derived from blood-stage parasites.**

Repeatedly infected individuals living in areas where malaria is endemic gradually acquire partial protective immunity against the malaria parasite *Plasmodium falciparum* (16, 17). With the aim of identifying parasite antigens which may evoke protective immunity, a number of antigen genes have been cloned and characterized (see references 15 and 16 and references therein). One of these is the *glurp* gene coding for an immunodominant glutamate-rich protein (GLURP) (2).

Nucleotide sequencing of *glurp* revealed an open reading frame of 1,271 amino acids including an amino-terminal non-repetitive region (R0) and two blocks of repetitive sequences (R1 and R2) (2). The predicted polypeptide contains hydrophobic amino- and carboxy-terminal regions characteristic of secreted proteins, consistent with the presence of GLURP in *P. falciparum* culture supernatants (2). Antibodies against GLURP were found to react with the asexual, hepatic, and gametocyte stages of the parasite (2), suggesting that GLURP is synthesized throughout the entire life cycle of *P. falciparum* in the vertebrate host. Moreover, Southern blotting and PCR analysis of the R2 repeat region performed on geographically different *P. falciparum* isolates revealed little polymorphism (2).

Seroepidemiological studies performed in areas with high levels of transmission have demonstrated a high degree of prevalence of antibodies reacting with a recombinant GLURP fragment containing the carboxy-terminal 783 amino acid residues (GLURP<sub>489–1271</sub>) in adults from Liberia (5, 6) and Gambia (7). A negative correlation between the immunoglobulin G (IgG) response against GLURP<sub>489–1271</sub> and parasite density was found in 5- to 9-year-old children but not in younger children (2 to 4 years old) (3, 9). Although this correlation was not observed for Gambian children, IgG

antibodies to GLURP were associated with reduced morbidity owing to *P. falciparum* infections in the next rainy season in children 5 to 8 years old but not in children 2 to 4 years old (7). Moreover, a shorter recombinant GLURP fragment, GLURP<sub>816–1134</sub>, was found to specifically activate lymphocytes from malaria-exposed individuals (2). Thus, GLURP may participate in eliciting protective immunity against malaria.

As part of a continuing effort to evaluate the vaccine potential of GLURP, the nearly full-length protein and sub-fragments were expressed in *Escherichia coli* and were purified to homogeneity. The antigenicities and immunogenicities of the recombinant proteins were analyzed in detail.

## MATERIALS AND METHODS

**Bacterial strains, parasites, and growth conditions.** *E. coli* JM105 (*endA1 thi rpsL sbcB15 hsdR4 Δlac-proAB*) [*F'* *traD36 proAB<sup>+</sup> lacI<sup>q</sup>ZΔM15*] (Bethesda Research Laboratories, Gaithersburg, Md.) containing the indicated plasmids was grown in Luria broth (14) supplemented with ampicillin (100 μg/ml). *P. falciparum* F32 was kept in continuous culture in a modified Trager and Jensen system (20) as described by Jepsen and Andersen (11). The parasites were grown in 10% (vol/vol) human group A<sup>+</sup> erythrocytes.

**DNA techniques.** Restriction enzymes and T4 DNA ligase were used as recommended by the supplier (Bethesda Research Laboratories). DNA sequencing was accomplished by the chain termination method essentially as described by Messing (13).

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of *E. coli* and *P. falciparum* proteins was performed as described by Laemmli (12) on 7.5% polyacrylamide gels.

**Construction of plasmids.** The structures of the various plasmids used in the study are shown in Fig. 1. All plasmid constructions were verified by DNA sequencing.

(i) **pMST24.** Two complementary synthetic oligonucleotides (5'-GAT CTC ATC ACC ATC ACC ATC ACA TCG AGG GCC GCA and 5'-GAT CTG CGG CCC TCG ATG TGA TGG TGA TGG TGA TGA) coding for a stretch of six His residues followed by a factor Xa cleavage site were synthesized, annealed, and cloned into the unique *Bgl*II site of the expression plasmid pGH433 (19), generating plasmid pMST24. The mRNA for the corresponding peptide is translated from a plasmid-encoded translational start site and is controlled by a *tac* promoter. The plasmid also encodes the *lac* repressor to ensure tight control of gene expression.

(ii) **pMST25.** Plasmid pGLURP (18) was digested with *Eco*RI and *Sau*96I, and the protruding 5' ends were filled with the Klenow fragment of *E. coli* DNA

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polymerase I. The resulting 651-bp DNA fragment was ligated to *Bam*HI-digested pMST24 after the protruding 5' ends had been blunt ended with the Klenow fragment of DNA polymerase I.

(iii) **pMST27.** Plasmid pGLURP was digested with *Sau*96I, and the resulting 1,416-bp DNA fragment was cloned into *Bam*HI-digested pMST24 after filling in all sticky ends with the Klenow fragment of DNA polymerase I.

(iv) **pMST31.** Plasmid pGLURP was digested with *Eco*RV and *Hind*III, the protruding 5' ends were made blunt ended, and the resulting 3,533-bp DNA fragment was cloned into *Sma*I-digested pMST24.

(v) **pMST32.** Plasmid pGLURP was digested with *Eco*RV and *Eco*R1, the 5' ends were filled in, and the resulting 1,183-bp DNA fragment was cloned into *Sma*I-digested pMST24.

**Purification of recombinant proteins.** A stretch of six His residues selectively bind the metal ion  $Ni^{2+}$ , allowing purification of the fusion proteins by metal chelate affinity chromatography (8). Fusion proteins were purified on an  $Ni^{2+}$ -IDA column (iminodiacetic acid-epoxy activated Sepharose 6B fast flow column [Sigma Chemical Co., St. Louis, Mo.]) as described previously in detail (21). Protein samples were desalted on an Econo-Pac 10DG column (Bio-Rad, Richmond, Calif.) and were concentrated by freeze-drying to a final concentration of approximately 1 mg/ml. The His tag was removed by cleavage with factor Xa as recommended by the manufacturer (Boehringer, Mannheim, Germany), and the protein samples were subsequently dialyzed against the column buffer (20 mM L-histidine [pH 6.0]) and applied to the fast-performance liquid chromatography (Pharmacia, Uppsala, Sweden) Mono Q (HR 5/5) column. The protein was eluted from the Mono Q column by applying a gradient of 0 to 0.5 M NaCl in 20 mM L-histidine (pH 6.0) at a flow rate of 1 ml/min. Fractions (1 ml) containing the GLURP fusion protein were rechromatographed under the same conditions. Analysis of all fractions was performed by SDS-PAGE. The protein concentration was measured by the method of Bradford (4).

**ELISA and serum samples.** Enzyme-linked immunosorbent assays (ELISAs) were performed as previously described in detail (5). The coating concentrations of GLURP<sub>94-1271</sub>, GLURP<sub>94-489</sub>, GLURP<sub>489-705</sub>, and GLURP<sub>705-1178</sub> were 0.25, 0.5, 0.5, and 0.1  $\mu$ g/ml, respectively. The antisera used were either polyclonal rabbit sera against recombinant GLURP fragments, serum samples from adults (clinically healthy male blood donors) living in an area in Liberia where malaria is holoendemic, or serum samples from Danish donors never exposed to malaria.

**Immunization.** Twenty-five female BALB/c mice (age, 6 to 8 weeks) were randomly assigned to five groups. Mice were immunized three times intraperitoneally at 2-week intervals and were bled on days 0, 14, 28, and 42. Each immunization dose contained 5  $\mu$ g of either GLURP<sub>94-1271</sub>, GLURP<sub>94-489</sub>, GLURP<sub>489-705</sub>, or GLURP<sub>705-1178</sub> antigen, each in a purified form, dissolved in 0.5 ml of 10 mM phosphate-buffered saline and supplemented with 1 mg of Al(OH)<sub>3</sub> (Superfos Biosector, Vedbæk, Denmark). Mice in the control group were injected accordingly with a GLURP-free preparation. The serum samples were diluted 200-fold, and the titers in the sera of mice before and after immunization were assessed by ELISA, with each of the recombinant GLURP fragments used as the coating antigens.

Rabbit sera were obtained from rabbits immunized subcutaneously five times with 50  $\mu$ g of purified GLURP fragments emulsified with Freund's adjuvant. First, three immunizations were carried out at 1-week intervals, and then immunizations were performed at three 3-week intervals. The first detectable antibody response to R0 was observed 90 days after the first immunization and peaked 60 days later.

**Metabolic labelling and immunoprecipitation of *P. falciparum* polypeptides.** Metabolic labelling of *P. falciparum* polypeptides and immunoprecipitation analysis were performed as described previously (18).

**Statistical analysis.** A two-way analysis of variance, with the factors immunization and test antigen, was carried out on the log-transformed ELISA titer. The variations between mice were thus included in the residual variations. The antilog of the least significant differences between means in two immunization groups was estimated.

## RESULTS

**Expression of *P. falciparum* glurp fragments.** GLURP of *P. falciparum* is composed of a nonrepeat region and two repeat regions, referred to as R0 and R1 and R2, respectively (Fig. 1). In order to locate the immunodominant regions on GLURP, three gene fragments coding for each of the three regions and one gene fragment encoding R0, R1, and R2 were cloned into the expression vector pMST24 (Fig. 1; the cloned regions are designated according to their amino acid residue endpoints), producing in-frame fusion proteins to a stretch of six His residues and a factor Xa cleavage site. The products were purified by metal chelate affinity chromatography on an  $Ni^{2+}$ -IDA column and were subsequently analyzed by SDS-PAGE. The plasmids pMST32 (lane 1), pMST25 (lane 2), pMST27

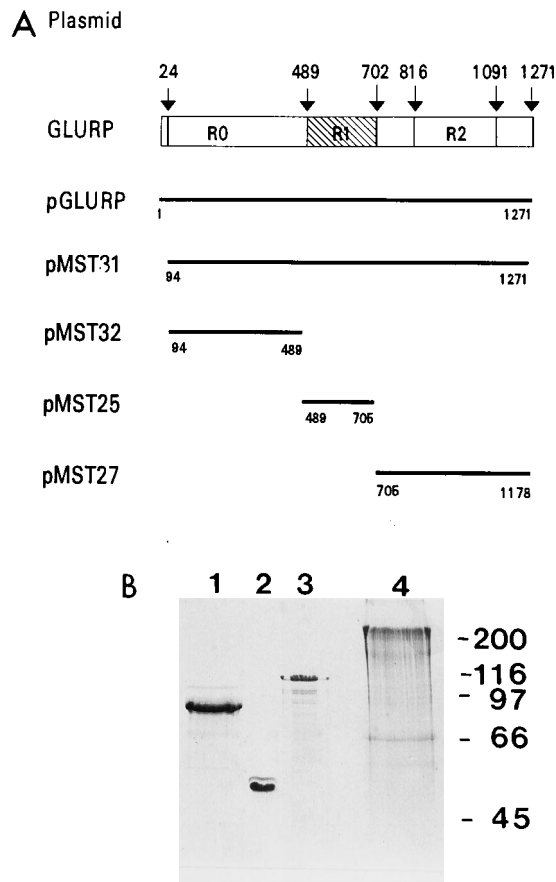


FIG. 1. (A) Structure and properties of specific plasmids. The open box at the top indicates the amino acid sequence of GLURP. R0 indicates the nonrepeat region, R1 indicates the central repeat region, and R2 indicates the carboxy-terminal repeat region. Numbers above the box indicate amino acid residues. The lines below represent *P. falciparum* DNA cloned into plasmid pMST24. (B) Coomassie blue-stained SDS-7.5% polyacrylamide gel of purified GLURP fusion proteins produced in JM105. Lane 1, pMST32; lane 2, pMST25; lane 3, pMST27; lane 4, pMST31. Numbers to the right indicate the size and position of the molecular weight markers (in thousands).

(lane 3), and pMST31 (lane 4) produced major products of 92, 59, 105, and 220 kDa, respectively. Additional lower-molecular-mass bands were observed in the purified products from cells containing pMST27 (encoding R2 [lane 3]) and pMST31 (encoding the nearly full-length GLURP [lane 4]). When they were analyzed by immunoblotting, these smaller products were specifically recognized, as were the full-length products, by immune sera from individuals living in areas where malaria is endemic (data not shown), indicating that the smaller polypeptides may result from incomplete translation of the mRNA and/or from protease cleavage of the primary GLURP product. In accordance with previous observations (5), the four major GLURP fragments migrated with relative molecular masses of approximately twice the calculated molecular masses. The removal of the His tag could be monitored by SDS-PAGE since the mobilities of all GLURP fragments were visibly affected by the presence of the six His residues (data not shown).

**Antigenicities of GLURP fragments.** The antigenicity of each GLURP fragment was evaluated by ELISA. An optimal coating concentration for each antigen was determined (GLURP<sub>94-1271</sub>, R0, R1, and R2 at 0.25, 0.5, 0.5, and 0.1

TABLE 1. IgG antibody reactivity of sera from humans clinically immune to malaria with recombinant GLURP polypeptides

Recombinant protein	No. (%) of reactive serum specimens <sup>a</sup>
GLURP <sub>94-1271</sub> .....	92 (80.7)
GLURP <sub>94-489</sub> (R0).....	85 (74.6)
GLURP <sub>489-705</sub> (R1).....	11 (9.6)
GLURP <sub>705-1178</sub> (R2).....	107 (93.9)

<sup>a</sup> Specimens with a response greater than the 95% fractile for the 165 nonimmune Danish serum samples. A total of 114 serum specimens were tested.

μg/ml, respectively), and each antigen was evaluated against serum samples from adults living in an area of Liberia where malaria is holoendemic. Serum samples from Danish donors never exposed to malaria were used as controls. For each of the four recombinant GLURP products, Table 1 shows the number of serum samples with a response which was greater than the 95% fractile for Danish donors. It appears that GLURP<sub>94-1271</sub> reacted in the IgG ELISA with 80.7% of the 114 serum samples from humans clinically immune to malaria. The amino-terminal protein R0 was reactive with 74.6% of the serum samples, while 93.9% of the serum specimens from humans immune to malaria reacted when the carboxy-terminal protein R2 was used as the antigen. Only 9.6% of the serum samples showed reactivity with R1.

The association between the positive and negative IgG ELISA reactivities of the 114 malaria-positive serum specimens with the four recombinant GLURP fragments is given in Table 2. Eleven serum specimens were reactive with all of the GLURP regions, whereas the other sera failed to react with one or more of the regions. Only two of the serum samples were unreactive with all of the GLURP fragments. However, these two serum samples were from individuals of immigrant families who were generally less exposed to malaria during childhood and adolescence. Of the 92 serum specimens which reacted with the nearly full-length GLURP protein, 11 serum specimens reacted with all of the GLURP regions, 65 serum specimens reacted with R0 and R2 but not R1, 15 serum specimens reacted with R2 only, and 1 serum specimen was unreactive with any of the three GLURP regions. Twenty of the serum specimens that were unreactive with the nearly full-length GLURP were reactive with regions R1 and R2, or one of these, possibly because of the serologically favorable conformations of these proteins compared with

TABLE 2. Association between positive and negative antibody reactions against the four GLURP fragments in malaria-positive serum samples<sup>a</sup>

R0	R1	R2	No. of serum specimens GLURP <sub>94-1271</sub>	
			+	-
-	-	-	1	2
-	-	+	15	11
+	-	-	0	4
+	-	+	65	5
+	+	+	11	0
All			92	22

<sup>a</sup> Specimens with a response greater than the 95% fractile for Danish donors were considered positive (+), and specimens with a lower response were considered negative (-).

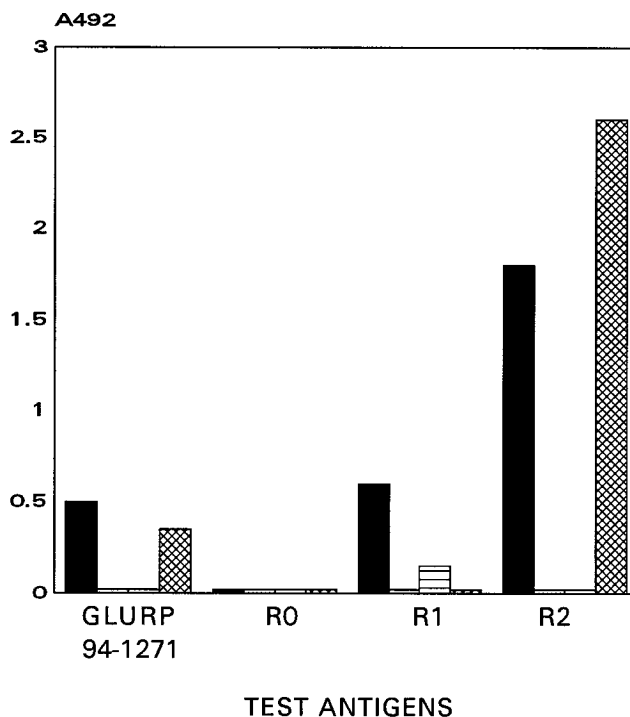


FIG. 2. Mean ELISA titer of sera from five mice immunized three times with GLURP<sub>94-1271</sub> (solid bars), R0 (open bars), R1 (bars with horizontal lines), or R2 (bars with crosshatched lines) and tested against GLURP<sub>94-1271</sub>, R0, R1, and R2. Bars represent the geometric mean of triplicate assays resulting from individual mouse sera corrected for the background [determined with a group of mice receiving only Al(OH)<sub>3</sub>]. Means which differed by more than a factor of 2.4 were statistically significant at the 5% level. The y axis shows the A<sub>492</sub>.

that of the nearly full-length protein. Alternatively, this observation may be explained by a higher concentration of specific epitopes when R0 or R2 is used as a coating antigen compared with that when the nearly full-length GLURP is used as the coating antigen.

**Immunogenicities of the recombinant GLURP fragments.** The humoral response against B-cell epitopes on recombinant GLURP was determined in mice. Individual groups of mice were each immunized intraperitoneally three times at 2-week intervals with one of the GLURP proteins: GLURP<sub>94-1271</sub>, R0, R1, and R2. Serum samples taken on day 42 were then tested by ELISA for IgG antibody activity. Figure 2 shows the data obtained when GLURP<sub>94-1271</sub>, R0, R1, and R2 were used as the coating antigens, and it appears that only GLURP fragments containing the R2 repeat region, i.e., GLURP<sub>94-1271</sub> and R2, are immunogenic with Al(OH)<sub>3</sub> as the adjuvant. Thus, the immunodominant part of GLURP is located between amino acid residues 705 and 1178.

**Reactivities of rabbit anti-GLURP sera with authentic GLURP.** The immunogenicity of recombinant GLURP was also analyzed by immunoprecipitation of parasite-derived polypeptides with sera from rabbits immunized with each of the three recombinant polypeptides, R0, R1, and R2, respectively. As demonstrated in Fig. 3 (lanes 2, 4, and 6), all serum specimens from rabbits immunized with the three polypeptides, respectively, immunoprecipitated a polypeptide of approximately 220,000 Da, the apparent molecular mass previously found for GLURP by SDS-PAGE. The 220,000-Da polypeptide could not be detected in the culture supernatant (lanes 1, 3, and 5), indicating that GLURP is present in relatively low



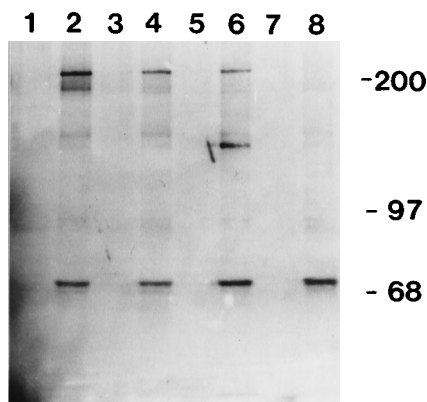


FIG. 3. Immunoprecipitation analysis of *P. falciparum* F32 cells. Cells were grown and labelled as previously described (18). Labelled polypeptides were subjected to immunoprecipitation with serum samples from rabbits immunized with the recombinant R0 (lane 1, culture supernatant; lane 2, cell pellet), R1 (lane 3, culture supernatant; lane 4, cell pellet), R2 (lane 5, culture supernatant; lane 6, cell pellet), or preimmune serum (lane 7, culture supernatant; lane 8, cell pellet). The sizes (in kilodaltons) of the molecular mass markers are indicated.

concentrations outside of parasitized erythrocytes. Additional polypeptides of 190,000 and 140,000 Da were also precipitated by the anti-R0 and anti-R2 sera. Neither the 220,000-Da, the 190,000-Da, nor the 140,000-Da polypeptides could be detected in the control experiment in which parasite polypeptides were immunoprecipitated with sera from preimmunized rabbits (lanes 7 and 8).

## DISCUSSION

In the present study, we analyzed in detail the human antibody response to the *P. falciparum* GLURP in individuals from an area in Liberia where malaria is holoendemic as well as the immunogenicity of recombinant GLURP in mice. In order to identify the main antigenic determinants of GLURP, four GLURP fragments were produced in *E. coli* and purified. One fragment represented the nearly full-length protein, and the other three fragments covered either the nonrepeat region R0, the R1 repeat region, or the R2 repeat region, respectively (Fig. 1). All vector-encoded amino acids were removed by cleavage with factor Xa at a genetically engineered recognition site to ensure that only GLURP-specific antibodies were detected.

We found that the human immune response against GLURP is primarily directed against the carboxy-terminal portion of the molecule containing the R2 repeat region, because 94% of the serum samples had high IgG titers against R2 (Table 1). This is in accordance with the findings of 98 and 95% IgG seropositivity rates against GLURP<sub>489-1271</sub> in adult Liberians and adult Gambians, respectively (5, 7). The amino-terminal nonrepeat region R0 reacted with 75% of the serum samples, while the central repeat region R1 reacted with only about 10% of the tested sera from humans clinically immune to malaria.

The immunodominance of the R2 region is further illustrated by focusing on the 92 serum samples which tested positive against GLURP<sub>94-1271</sub>. Of these, 76 were positive against both R0 and R2, while 15 of the samples tested positive against R2 only (Table 2).

The immunogenicity of GLURP was studied in mice with Al(OH)<sub>3</sub> used as the adjuvant since Al(OH)<sub>3</sub> can also be used in humans. We find that the R2 repeat region gave rise to a stronger immune response than the other regions of GLURP,

both when it was injected by itself and in the context of the nearly full-length protein (Fig. 2). Thus, the high seropositivity rate in the serum samples from Liberians against R2 is likely because the R2 region is a main antigenic determinant relative to R0 and R1. This immunodominance may be related to the highly repetitive nature of the R2 region (1), which consists of 14 well-conserved repeat units, each of which is composed of 19 or 20 residues. Moreover, as described previously the repeat unit contains the sequence HEIVEVEEI, which has a high antigenic index (18). The R1 region, which gave rise to a weaker immune response, consists of only six repeat units of 15 poorly conserved amino acid residues.

Another important antigenic determinant of GLURP, the nonrepeat region R0, is not immunogenic when Al(OH)<sub>3</sub> is used as the adjuvant. To obtain a significant antibody response toward R0, rabbits had to be immunized with R0 in complete Freund's adjuvant for 2 months. Thus, the high seropositivity rate against R0 in adult Liberians may reflect the need for a long period of exposure to *P. falciparum* before a humoral response is raised against this part of GLURP. Alternatively, GLURP may be posttranslationally modified by the parasite in such a way that the authentic R0 region yields a stronger antibody response than the recombinant R0. For example, glycosylation is known to be of importance for the immunogenicity of other *P. falciparum* proteins (10). It is not known how the immune response against R0 is correlated with the age of the infected individual because this is the first time that this portion of the molecule has been expressed and purified. However, it is tempting to speculate that because of the lack of immunogenicity of the R0 region, individuals would have to be infected with the parasite for a prolonged period of time before a humoral response is achieved. Thus, nonimmune young children might be seronegative, while sera from partially protected older children and adults might contain antibodies against the R0 region on GLURP.

Antibodies raised against each of the three regions, R0, R1, and R2, recognized authentic GLURP by immunoprecipitation analysis (Fig. 3). The anti-R0 serum and anti-R2 serum also recognized polypeptides of 190,000 and 140,000 Da, respectively. The natures of these polypeptides are unknown.

In conclusion, GLURP produced in *E. coli* is recognized by sera from humans clinically immune to malaria and is capable of eliciting in animals a humoral immune response that is reactive with parasite-derived GLURP.

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