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Immunogenicity of the *Plasmodium falciparum* Glutamate-Rich Protein Expressed by Vaccinia Virus

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The *glurp* gene of *Plasmodium falciparum* F32 has been inserted into a vaccinia virus, and the recombinant virus was designated VVG4. Expression of *glurp* in VVG4-infected Vero cells was analyzed by immunoprecipitation and revealed a primary GLURP product of approximately 220,000 Da; GLURP was detected both intracellularly and in culture supernatants. To study the immunogenicity of vaccinia virus-expressed GLURP, mice were immunized with VVG4 and serum samples were analyzed for antibody reactivity with three polypeptides, covering almost the entire GLURP molecule; these three polypeptides were produced in recombinant form in *Escherichia coli*. The immune response was primarily directed against a carboxy-terminal repeat region. The mouse anti-GLURP serum recognized authentic GLURP by immunoprecipitation analysis from *P. falciparum* grown in vitro. These results demonstrate that vaccinia virus-expressed *glurp* product can induce a humoral immune response against GLURP derived from blood-stage parasites.

The glutamate-rich protein (GLURP) of *Plasmodium falciparum* is an exoantigen which is considered a possible malaria vaccine candidate, in part because it is highly antigenic and synthesized during all stages of the parasite in the vertebrate host (2) and also because the level of antibodies to GLURP in serum is positively correlated to immunity against clinical malaria. Thus, seroepidemiological studies performed in high-transmission areas in Liberia (5, 6), in Burkina Faso (3), and in The Gambia (7) have demonstrated a high prevalence in adults of antibodies against a recombinant GLURP fragment consisting of the carboxy-terminal 783 amino acid residues ($GLURP_{489–1271}$). Høgh et al. (9) found that high levels of antibodies to the same GLURP fragment were associated with protection against high parasite densities in Liberian children aged 5 to 8 years but not in younger children (2 to 4 years old). Although this correlation was not observed for Gambian children, immunoglobulin G antibodies to GLURP were associated with reduced morbidity 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, the recombinant GLURP fragment $GLURP_{816–1134}$ was found to specifically activate lymphocytes from malaria-exposed individuals (2). Thus, GLURP may participate in eliciting protective immunity against malaria.

Vaccinia virus (VAC) has recently been used as an expression system for a number of plasmodial antigen genes (11, 13–17, 20) because of its efficiency as a live delivery system and because recombinant proteins produced by VAC are post-translationally modified and hence may resemble the native proteins more closely than recombinant proteins produced in a prokaryotic expression system.

As part of a continuing effort to evaluate the vaccine potential of GLURP, we have constructed a VAC recombinant producing GLURP and characterized the VAC-expressed GLURP product. The immunogenicity of VAC-expressed GLURP was investigated in mice, and an immunodominant domain was localized.

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MATERIALS AND METHODS

Cells, viruses, and parasites. VAC (WR strain) and recombinant VAC were propagated in BSC-1 or Vero cells. Human thymidine kinase-negative (TK^-) 143, BSC-1, and Vero cells were grown as monolayers in Eagle's minimal essential medium (GIBCO), supplemented with 10% fetal bovine serum (GIBCO). *P. falciparum* F32 was kept in continuous culture in a modified Trager and Jensen system (21) as described by Jepsen and Andersen (10). The parasites were grown in 10% (vol/vol) human (group A^+) erythrocytes.

Plasmid constructions. Plasmid pGLURP, containing the entire GLURP-coding sequence on a 3,832-bp *Bam*HI-*Hin*dIII fragment, was made as follows. PCR was carried out as previously described (2) with total chromosomal DNA extracted from *P. falciparum* F32 as the template. The primers were 5′-CGTCCTGGATCCATCGAGGGTAGGATGAGA AACCTTTTCCATATTACCATTTG-3′, corresponding to nucleotides 1 to 29 of the GLURP-coding sequence with a 5′ extension containing a *Bam*HI recognition site, and 5′-CGA CCGAAGCTTAAATGTTTTGGAAAAATATTAC-3′, complementary to nucleotides 3793 to 3813 and providing a 3′ extension with a *Hin*dIII site. The amplified fragment was cloned into *Bam*HI-*Hin*dIII-digested pUC19 from which the *Eco*RI and *Kpn*I sites had been removed; this modification of pUC19 had been carried out by digesting with *Eco*RI, filling in protruding ends with the Klenow fragment of *Escherichia coli* DNA polymerase I, digesting with *Sma*I, and closing with DNA ligase. The internal *Eco*RI-*Kpn*I fragment of *glurp* was excised and replaced with the corresponding fragment from pRD15 (2), and the remaining parts of the cloned fragment were sequenced to ensure that no errors had been introduced by the PCR. The plasmid insertion vector pMST1, used for introducing *glurp* into the VAC genome, was made by digesting pGLURP with *Bam*HI-*Hin*dIII, isolating the 3.8-kbp fragment containing *glurp*, making the ends blunt with the Klenow

fragment of DNA polymerase I, and cloning the fragment into the *Sma*I site of the VAC insertion vector pVVSLI (22).

Transfection and isolation of recombinant viruses. BSC-1 cells (10^6) were infected with wild-type VAC (WR strain) at a multiplicity of infection of 0.03 PFU per cell. At 4 h postinfection, 10 ng of linearized plasmid DNA (pMST1) was transformed into the cells by electroporation (Gene Pulser; Bio-Rad, Richmond, Calif.; with settings at 200 V and 500 μF). After 2 days of incubation at 37°C, viruses were harvested from cell pellets by several cycles of freezing and thawing. Serial dilutions of viruses were plated on TK^- 143 cells in six-well multidishes (Nunc, Roskilde, Denmark) and then overlaid with 0.8% agarose (GIBCO) in growth medium containing 5-bromo-2′-deoxyuridine (25 μg/ml). Individual TK^- plaques were picked, plaque purified three times on TK^- 143 cells, and then tested for *glurp* expression by indirect immunofluorescence using polyclonal rabbit antiserum raised against recombinant GLURP produced in *E. coli* (2).

Indirect immunofluorescence. The production of GLURP in VAC-infected BSC-1 or Vero cells was examined by indirect immunofluorescence. Briefly, cells were infected with recombinant or wild-type VAC at a multiplicity of infection of 3 PFU per cell, and 10 h postinfection cells were fixed in methanol at −20°C for 10 min and examined for *glurp* expression with rabbit anti-GLURP serum (2) and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (Dakopatts, Glostrup, Denmark) as described elsewhere (8).

Polyacrylamide gel electrophoresis (PAGE). Sodium dodecyl sulfate (SDS)-PAGE of VAC-encoded polypeptides and *P. falciparum* proteins was performed according to the method of Laemmli (12) on 7.5% polyacrylamide gels.

Metabolic labelling and immunoprecipitation of VAC-encoded polypeptides. BSC-1 or Vero cells were infected with wild-type or recombinant VAC at a multiplicity of infection of 10, and 10 h postinfection metabolic labelling was performed with L-[4,5-^{3}H]leucine (Amersham; 100 μCi/ml of medium; 144 Ci/mmol) added to monolayer in leucine-free Eagle's minimal essential medium (GIBCO). After 4 h, the cells were harvested and resuspended in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS), and the culture supernatants were adjusted to 50 mM Tris-HCl (pH 8.0)–0.1% SDS. Whole-cell lysates were obtained by subjecting cell suspensions to several cycles of freezing and thawing followed by shearing in an Ultra-Turrax T25 mincer (Janke and Kunkel, Staufen, Germany) and centrifugation at 10,000 × *g* for 10 min to remove cell debris. Aliquots of whole-cell lysate and culture supernatants were stored at −20°C until use.

Immunoprecipitations were performed as follows. Rabbit anti-GLURP serum (2) was added to 0.5 ml of whole-cell lysate or to 250 μl of culture supernatant plus 250 μl of 2× RIPA buffer, and the mixtures were incubated overnight at 4°C with rotation. Seventy-five microliters of a 50% suspension of protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) bound to mouse anti-rabbit immunoglobulin G in RIPA buffer was added to each immunoprecipitation solution, and the mixture was incubated with rotation for 4 h at 4°C. The beads were washed several times with RIPA buffer and resuspended in 50 μl of Laemmli sample buffer. The immunoprecipitates were separated by SDS-PAGE on 7.5% polyacrylamide gels and subsequently treated with Amplify (Amersham) prior to exposure to X-ray film.

Immunization of mice. Ten female BALB/c mice (6 to 8 weeks old) were randomly assigned to two groups, immunized subcutaneously at the tail root on days 0 and 14, and bled on days 0, 14, and 28. One group received 10^8 PFU of VVG4, and the other group received 10^8 PFU of wild-type VAC in 0.2 ml of phosphate-buffered saline (pH 7.4).

Reactivity of mouse anti-VVG4 sera with authentic GLURP. Metabolic labelling of *P. falciparum* polypeptides was performed with L-[4,5-^{3}H]leucine (Amersham; 100 μCi/ml of medium; 144 Ci/mmol) added to an asynchronous culture of *P. falciparum* F32 (parasitemia, 25%) in leucine-free medium. Parasites were allowed to develop for 18 h and were then harvested by centrifugation at 3,000 × *g* for 10 min. After labelling, the cells were washed twice with serum-free RPMI medium and resuspended at a hematocrit of 2% in RIPA buffer containing 0.5 mM phenylmethylsulfonyl fluoride. The culture supernatant was adjusted to 50 mM Tris-HCl (pH 8.0)–0.1% SDS–0.5 mM phenylmethylsulfonyl fluoride. Aliquots of cell suspensions and culture supernatants were stored at −20°C until use. Prior to immunoprecipitation, the parasite pellet was sonicated four times for 20 s each time and cell debris was removed by centrifugation at 10,000 × *g* for 10 min, giving rise to a whole-cell lysate. Labelled polypeptides were subjected to immunoprecipitation and SDS-PAGE as described above, except that mouse anti-VVG4 serum and goat anti-mouse immunoglobulin (Dakopatts) were used as the first and second antibodies, respectively.

Enzyme-linked immunosorbent assay (ELISA). GLURP is composed of a nonrepeat region and two repeat regions, referred to as R0 ($GLURP_{94-489}$) and as R1 ($GLURP_{489-705}$) and R2 ($GLURP_{705-1178}$) (numbers correspond to amino acid residue endpoints), respectively. In order to localize immunogenic domains on VAC-expressed GLURP, three gene fragments containing each of the three regions were produced in *E. coli* as polypeptides fused to a stretch of six His residues and a factor Xa cleavage site (19). The products were purified by metal chelate affinity chromatography on a Ni^{2+}-iminodiacetic acid column as described previously (23). ELISAs were performed as previously described in detail (5) with each of the three recombinant polypeptides R0, R1, and R2 as coating antigens in concentrations of 0.5, 0.5, and 0.1 μg/ml, respectively.

RESULTS AND DISCUSSION

Plasmid pMST1, containing the *glurp* gene under the control of a synthetic VAC consensus late promoter (22) and interrupting the VAC thymidine kinase gene, was introduced into BSC-1 cells previously infected with VAC wild-type strain WR. Several VAC recombinants were isolated as plaques from TK^- 143 cells and tested by indirect immunofluorescence for their ability to produce GLURP after infection of BSC-1 cells. GLURP-specific rabbit serum raised against purified recombinant $GLURP_{705-1178}$ was used as the first antibody in the immunofluorescence study, and the results obtained with the VAC recombinant, VVG4, are shown in Fig. 1A. A strong fluorescence was observed in the cytoplasm of VVG4-infected cells, while there was no discernible fluorescence in wild-type VAC-infected cells (Fig. 1B).

The expression of GLURP was further analyzed in VVG4-infected Vero cells by labelling with ^{3}H-leucine, followed by immunoprecipitation with the GLURP-specific rabbit antiserum and subsequent SDS-PAGE. From the data presented in Fig. 2 it appears that in addition to wild-type VAC-encoded polypeptides (lanes 1 and 2), VVG4 encodes three polypeptides with molecular weights of 180,000, 200,000, and 220,000, respectively (lane 4), that all react with GLURP antiserum. A polypeptide migrating as a single band at 220 kDa is observed in the culture medium of VVG4-infected cells (lane 3), indicating that the GLURP signal peptide is functional in mediat-

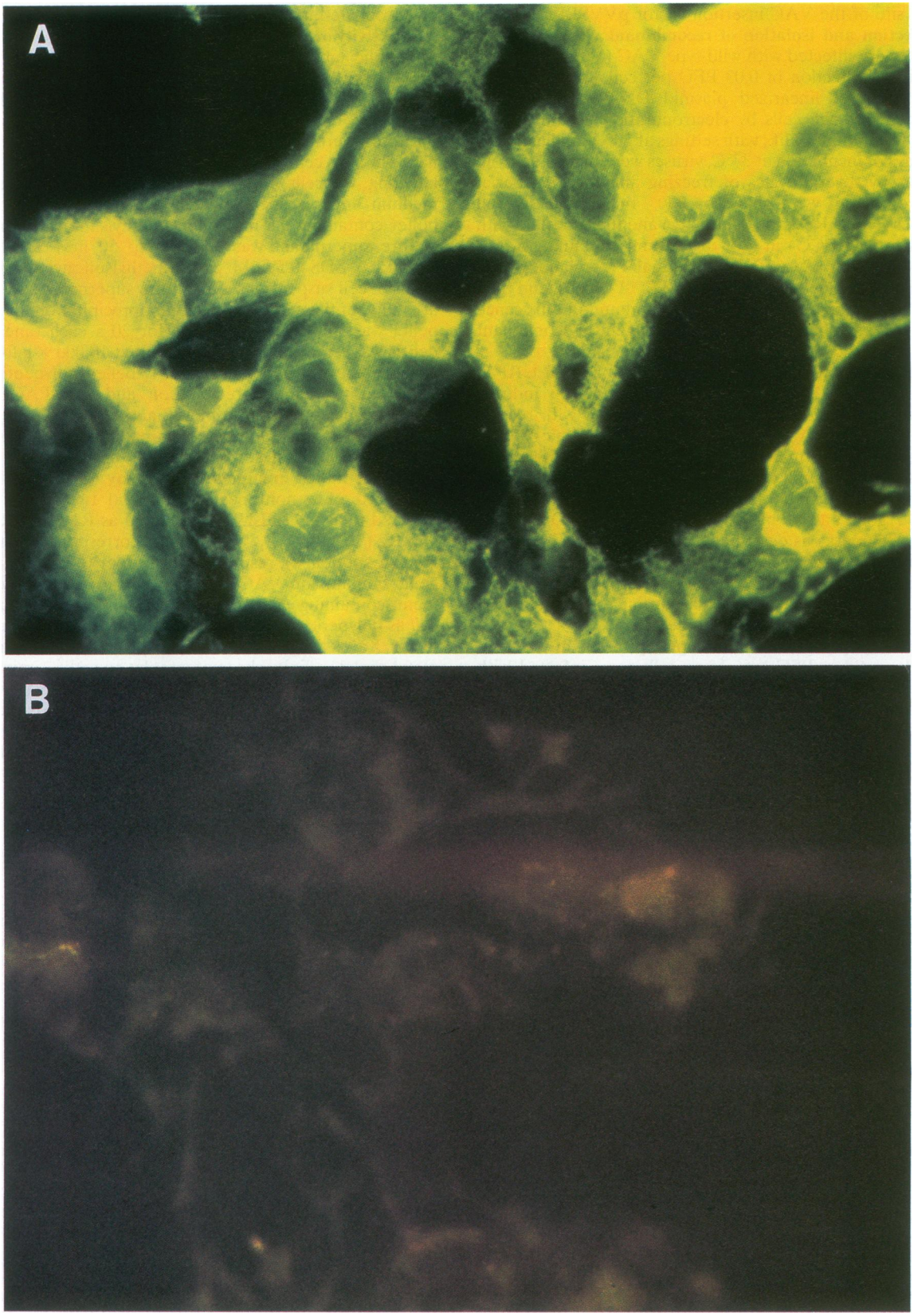

FIG. 1. Intracellular expression of *glurp* in VAC-infected BSC-1 cells. Indirect immunofluorescence of recombinant VVG4 (A) or wild-type (B) VAC-infected BSC-1 cells.

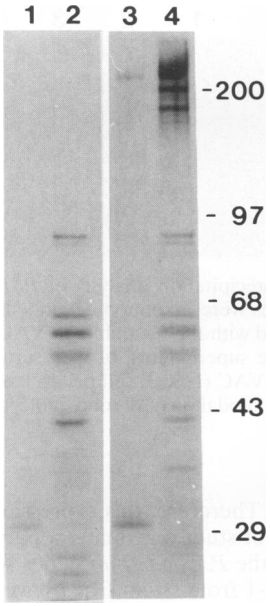

FIG. 2. Characterization of GLURP polypeptides made by VVG4. Vero cells were infected with VVG4 or wild-type VAC and at 10 h postinfection were labelled with L-[4,5-^{3}H]leucine for 4 h. Subsequently, cells and culture supernatants were harvested separately. Polypeptides were immunoprecipitated with GLURP-specific polyclonal rabbit antiserum, and the precipitated polypeptides were analyzed by SDS-PAGE (7.5% polyacrylamide). Gels were treated with Amplify (Amersham), dried, and exposed to X-ray film. Lane 1, wild-type VAC-infected culture supernatant; lane 2, wild-type VAC-infected cell pellet; lane 3, VVG4-infected culture supernatant; lane 4, VVG4-infected cell pellet. The sizes (in kilodaltons) of molecular mass markers (Bethesda Research Laboratories) are indicated.

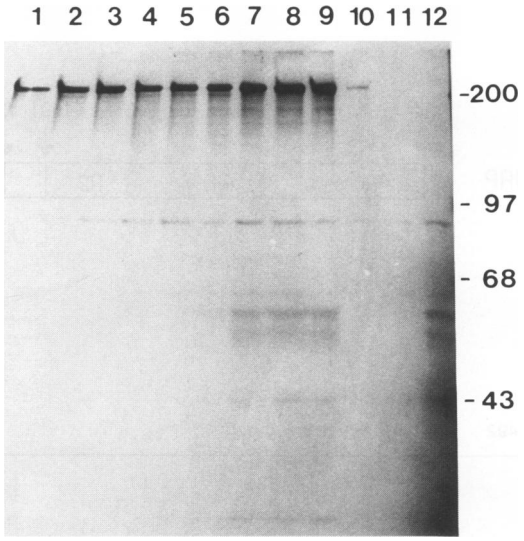

FIG. 3. Pulse-chase analysis of GLURP expression by VVG4. Vero cells infected with VVG4 were at 10 h postinfection pulse-labelled with L-[4,5-^{3}H]leucine for 10 min and chased with Eagle's minimal essential medium containing 10× leucine (GIBCO) at 0 h (lane 10), 15 min (lane 1), 30 min (lane 2), 45 min (lane 3), 60 min (lane 4), 75 min (lane 5), 90 min (lane 6), 120 min (lane 7), 150 min (lane 8), and 180 min (lane 9). As a control, Vero cells were infected accordingly with wild-type VAC and chased at 0 min (lane 11) and 180 min (lane 12). Cell pellets were subjected to immunoprecipitation, and precipitates were analyzed by SDS-PAGE and autoradiography as described in the legend to Fig. 2. GLURP polypeptides are indicated. The sizes (in kilodaltons) of molecular mass markers (Bethesda Research Laboratories) are indicated.

ing the transport of GLURP into the endoplasmic reticulum, from where the protein is further transported outside the cell. This is consistent with the presence of GLURP extracellularly (2). Equivalent results were obtained in VVG4-infected BSC-1 cells (data not shown).

A pulse-chase experiment was performed with VVG4-infected and wild-type VAC-infected Vero cells to study the possible biosynthetic relationship among the three intracellular GLURP polypeptides (Fig. 3). After a 10-min pulse, only the large 220,000-Da polypeptide was labelled, in addition to wild-type VAC-encoded polypeptides. During the chase, smaller polypeptides, of which two corresponded to the 180,000- and 200,000-molecular-weight polypeptides, were labelled. Thus, we conclude that VAC recombinant GLURP is primarily produced as a 220,000-Da polypeptide which is secreted into the culture supernatant, and the smaller intracellular GLURP polypeptides are presumably results of intracellular degradation of the 220,000-Da polypeptide.

The discrepancy of 70,000 between the observed and the calculated molecular weights of GLURP is probably not related to posttranslational modifications. First, we were unable to demonstrate incorporation of [^{14}C]glucosamine into polypeptides immunoprecipitated with the anti-GLURP rabbit serum, indicating that VAC-expressed GLURP was not glycosylated in Vero or BSC-1 cells (data not shown). Second, the observed molecular weight of GLURP, or fragments thereof, produced in *E. coli* (2) was also larger than the calculated molecular weight, suggesting that anomalous migration of GLURP was an artifact of the SDS-PAGE system, presumably because this large polypeptide contains many repeating negative charges.

To investigate the immunogenicity of VAC-expressed GLURP, we immunized a group of five mice with VVG4 and analyzed the serum samples taken on days 0, 14, and 28 for antibody reactivity against three purified GLURP polypeptides produced in *E. coli* (19). The three polypeptides represent the amino-terminal nonrepeat region R0 ($GLURP_{94-489}$), the central repeat region R1 ($GLURP_{489-705}$), and the carboxy-terminal repeat region R2 ($GLURP_{705-1178}$) (Fig. 4A). Following the second immunization, mice developed high titers of serum antibodies primarily to $GLURP_{705-1178}$ (Fig. 4B). Following a third immunization, there was no further increase in the level of anti-$GLURP_{705-1178}$ antibodies, while increasing titers against $GLURP_{94-489}$ and $GLURP_{489-705}$ were observed (data not shown). A group of control mice immunized with the wild-type VAC did not develop anti-GLURP antibodies. The immunodominance of the R2 region may be explained by the clustering of putative antigenic sites in this portion of GLURP (1). As determined by the computer program Antigenic of the Genetics Computer Group Sequence Analysis Software Package, version 7 (4), the antigenic sequence HEIVEVEEI (single-letter amino acid code) was found eight times in the region between the amino acid residues 888 and 1051 and may thus contribute significantly to the immunodominance of the R2 region.

The immunogenicity of VAC-expressed GLURP was also analyzed by immunoprecipitation of parasite-derived polypeptides with sera from mice immunized with VVG4. As demonstrated in Fig. 5 (lane 1), such sera immunoprecipitated a single polypeptide of approximately 220,000 Da, the molecular

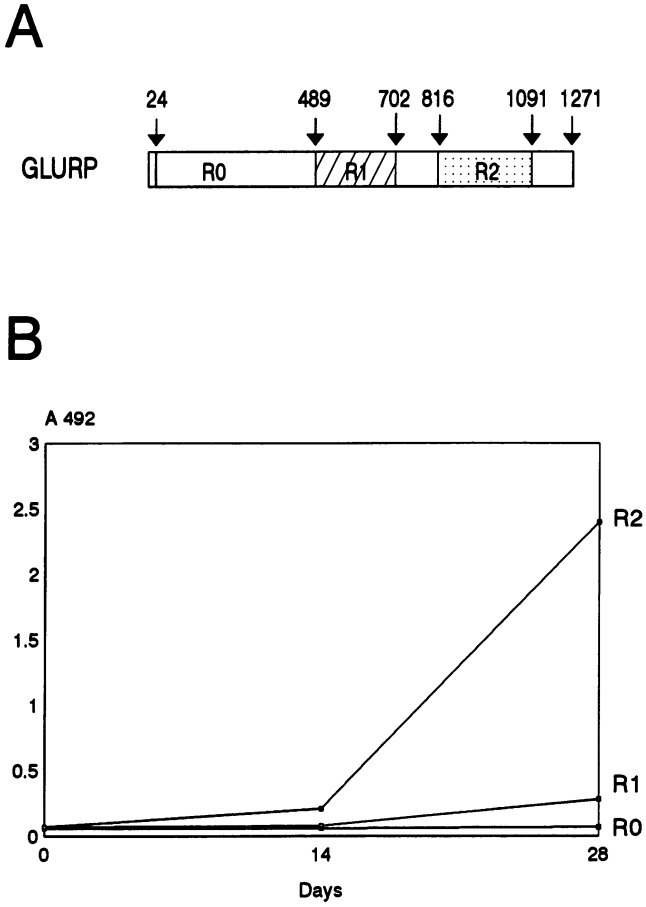

FIG. 4. (A) Schematic representation of GLURP. The open box indicates the amino acid sequence of GLURP. R0 indicates the nonrepeat region, R1 is the central repeat region, and R2 is the carboxy-terminal repeat region. Numbers above the box indicate amino acid residues. (B) The mean ELISA titers of five mice immunized with VVG4 on days 0 and 14. The microdilution plates were coated with R0 ($GLURP_{94-489}$ [0.5 μg/ml]), R1 ($GLURP_{489-705}$ [0.5 μg/ml]), or R2 ($GLURP_{705-1178}$ [0.1 μg/ml]), and the serum samples were analyzed in a 100-fold dilution. The measurements represent the means for triplicate assays from five individual mouse serum samples.

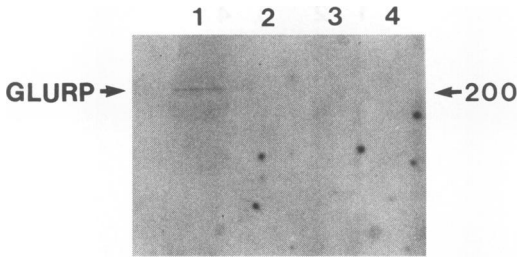

FIG. 5. Immunoprecipitation analysis of *P. falciparum* F32 cells. Labelled polypeptides were immunoprecipitated with serum samples from mice immunized with the recombinant VAC, VVG4 (lane 1, cell pellet; lane 2, culture supernatant), or with serum from mice immunized with wild-type VAC (lane 3, cell pellet; lane 4, culture supernatant). The size (in kilodaltons) of one molecular mass marker is indicated.

mass previously found for GLURP in SDS-PAGE. The 220,000-Da polypeptide could not be detected in the culture supernatant (lane 2), indicating that GLURP is present in small amounts outside the parasitized erythrocyte. Also, the 220,000-Da polypeptide could not be detected in the control experiment in which parasite polypeptides were immunoprecipitated with sera from mice immunized with wild-type VAC (lanes 3 and 4). Thus, GLURP produced by the recombinant VAC VVG4 elicits a humoral immune response in mice that is reactive with parasite-derived GLURP.

Expression in VAC has the theoretical advantage that eukaryotic proteins such as *P. falciparum* gene products are made in a more authentic form than they would be in a prokaryotic host such as *E. coli*. Moreover, VAC is supposedly an efficient delivery system for recombinant antigens, since these should be synthesized continuously after vaccination. Thus, the antigen will be presented to the vaccinated organism for an extended time period and presumably in a native configuration, which theoretically should result in optimum immune response. Therefore, this expression system has been used in a number of studies of malaria parasite antigens. For instance, recently the *P. falciparum* genes for RESA, MSA-1, MSA-2, and AMA-1 from strain FC27 have been expressed in VAC and the resulting VAC recombinants have been used for immunization of *Saimiri* monkeys in a challenge study (16). These studies failed to demonstrate protection against a subsequent parasite challenge; however, as discussed by Tine et al. (20), the lack of protection may be due to an insufficient immune response against protective epitopes. Another challenge experiment in which mice were first immunized with a recombinant influenza virus expressing the gene for the *Plasmodium yoelii* circumsporozoite protein and then a VAC recombinant expressing the same gene did result in induction of protective immunity against malaria (18).

In this study, we have described the construction and characterization of the VAC recombinant VVG4, expressing the *P. falciparum* antigen gene *glurp*. Because of the broad host range of VAC, *glurp* expression could be analyzed both in tissue culture and in animals. We found that GLURP encoded by VVG4, as authentic GLURP, is produced as a 220,000-Da polypeptide which is secreted into the culture supernatant. Also, VAC-expressed *glurp* is strongly immunogenic in mice, and it was demonstrated that the immune response is primarily directed against the carboxy-terminal repeat region R2. Moreover, antibodies raised against VAC-expressed recombinant GLURP recognize authentic GLURP derived from blood-stage parasites, showing that the two molecules are immunologically homologous. Thus, further analysis of VAC-expressed GLURP in immunization and challenge studies may contribute to our understanding of protective immunity against malaria.

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