The 3' portion of the gene for a *Plasmodium yoelii* merozoite surface antigen encodes the epitope recognized by a protective monoclonal antibody

(malaria/λgt11 expression library/nucleic acid sequence/vaccine development)

JAMES M. BURNS, JR., THOMAS M. DALY, AKHIL B. VAIDYA, AND CAROLE A. LONG

Malaria Research Group, Department of Microbiology and Immunology, Hahnemann University, Philadelphia, PA 19102

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ABSTRACT   The 230-kDa merozoite antigen of the murine malarial parasite *Plasmodium yoelii* provides a potential model system for the development of a protective erythrocytic stage vaccine. To characterize this antigen at the molecular level, isolated *P. yoelii* 17XL DNA was used to construct a genomic library in the expression vector λgt11. A monoclonal antibody, mAb 302, which passively protected mice against *P. yoelii* challenge infection, was used to identify a λgt11 recombinant clone encoding a portion of the 230-kDa antigen of this parasite. Using this clone as a probe, we identified an mRNA of 7.6 kilobases by RNA blot analysis. Nucleic acid sequence analysis of the clone showed that the epitope recognized by the protective mAb 302 is encoded by the 3' portion of the gene for the 230-kDa antigen. The deduced amino acid sequence revealed that this antigen also contains the tandemly repeated tetrapeptide Gly-Ala-Val-Pro, a series of 10 cysteine residues located within the terminal 110 amino acids, and a potential membrane anchor of 18 hydrophobic residues. Comparison of this C-terminal sequence with the carboxyl segment of the 195-kDa merozoite antigen of *Plasmodium falciparum* revealed nucleic acid and amino acid sequence similarities ranging from 40% to 70%. The localization of a B-cell epitope recognized by the protective mAb 302 to this carboxyl region of the *P. yoelii* antigen, combined with the limited strain variability in this region of the homologous 195-kDa antigen of *P. falciparum*, has implications for the development of an effective erythrocytic stage malarial vaccine.

The development of an anti-malarial vaccine has focused on the identification and characterization of plasmodial antigens inducing protective immune responses. During infection, the parasites progress through various developmental stages, presenting the host with a complex array of antigens. The elicited immune responses are equally complex, stage specific, and involve both humoral and cell-mediated mechanisms (1, 2). Despite this diversity, sporozoite, erythrocytic, and gametocyte stage-specific antigens have emerged as potential vaccine candidates (3).

During the erythrocytic phases of development, a class of high molecular weight merozoite surface antigens has been identified in rodent (4, 5), simian (6), and human (7) plasmodial species. These surface proteins may be involved in merozoite invasion of erythrocytes (8), and host immune responses directed toward them have provided significant levels of protection against challenge infection (9–12). Consequently, evaluation of these antigens as vaccine components has led to an increasing need for the development of experimental model systems.

Previously, this laboratory reported the production of a monoclonal antibody, mAb 302, which protected mice pas-

sively against challenge infection with *Plasmodium yoelii* (13). This antibody recognized specifically a 230-kDa merozoite antigen, PY230. Prior evidence indicated that this antigen is homologous to a 195-kDa merozoite antigen of the human malarial parasite *Plasmodium falciparum*, PF195 (5). We now report the cloning and sequencing of a 2-kilobase (kb) portion of the PY230 gene encoding the B-cell epitope recognized by the protective mAb 302. The epitope is located in the carboxyl-terminal portion of the molecule, which contains regions of both nucleic acid and protein sequence similarity with the 195-kDa *P. falciparum* antigen.*

MATERIALS AND METHODS

Experimental Infections. Male BALB/c or C57BL/6 mice, 6–10 weeks old, were purchased from The Jackson Laboratory. Infections were initiated by injection of parasitized erythrocytes of the lethal 17XL or nonlethal 17X strains of *P. yoelii* and were monitored as described (13).

Antibodies. Hyperimmune antisera against *P. yoelii* (HIS) were obtained from animals infected repeatedly with the nonlethal *P. yoelii* 17X (14). mAb 302 was obtained from either tissue culture supernatants or from ascites fluids of mice carrying the mAb 302 hybridoma tumor. Polyclonal antisera against the PY230 antigen were generated by immunization of BALB/c mice with parasite antigens complexed with mAb 302 in Freund's complete adjuvant. These immune complexes were prepared by immunoprecipitation of PY230 from a solubilized preparation of *P. yoelii* 17XL-infected erythrocytes. Antisera were collected following subcutaneous immunization at four monthly intervals.

Library Construction. Parasitized blood from 50 C57BL/6 mice was collected on day 6 postinfection with *P. yoelii* 17XL, when parasitemias averaged 40%. Leukocytes were removed from the pooled blood by passage over columns of microcrystalline cellulose (15). Parasite DNA was extracted as described (16) and digested by using the "star" activity of *Eco*RI (20 mM Tris·HCl, pH 8.5/2 mM MnCl$_2$/10 mM dithiothreitol/25 units of *Eco*RI per μg of DNA). A genomic expression library in λgt11 (Stratagene, San Diego, CA) was constructed according to the methods described by Huynh *et al.* (17).

Library Screening. Screening of the λgt11 expression library with antibody probes was carried out as described (17) using *Escherichia coli* strain Y1090 (American Type Culture Collection no. 37197). Lysates of *E. coli* Y1089 (American Type Culture Collection no. 37196) lysogenized with λgt11 were coupled to Affi-Gel 10 (Bio-Rad) and used as

Abbreviations: mAb, monoclonal antibody(ies); HIS, *Plasmodium yoelii* hyperimmune antiserum.
*This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03612).

an immunoadsorbent to remove reactivity against λgt11 and *E. coli* proteins from all antibody preparations. Antibody bound to duplicate nitrocellulose lifts was detected with ^{125}I-labeled protein A (Amersham) followed by autoradiography.

Recombinant λgt11 lysogens were generated by infecting the *E. coli* strain Y1089 and β-galactosidase fusion protein expression was induced as described (17). Bacteria pelleted from 1-ml culture samples were resuspended in NaDodSO₄/polyacrylamide gel sample buffer (0.125 M Tris·HCl, pH 6.8/2.5% NaDodSO₄/5.0% 2-mercaptoethanol), boiled, and electrophoresed on 7.5% NaDodSO₄/polyacrylamide gels according to Laemmli (18). β-Galactosidase fusion proteins were identified by immunoblot analysis (19, 20) using either HIS or mAb 302. Bound antibody was detected by using ^{125}I-labeled protein A followed by autoradiography.

RNA Blot Analysis. Parasitized erythrocytes were isolated from the blood of *P. yoelii* 17XL-infected mice as described above, and lysed in phosphate-buffered saline (10 mM sodium phosphate/150 mM sodium chloride, pH 7.4) containing 0.1% glucose and 0.01% saponin. RNA was extracted from the pelleted parasites by the guanidinium isothiocyanate/cesium chloride method, and separated into poly(A)⁺ and poly(A)⁻ fractions by oligo(dT)-cellulose column chromatography (21). This RNA was electrophoresed through agarose gels containing formaldehyde and transferred to nitrocellulose (21). The 1.85-kb *Eco*RI/*Eco*RV restriction fragment of the recombinant clone used as a probe (see below) was radiolabeled by nick-translation in the presence of [³²P]dCTP (Amersham) to a specific activity of 4 × 10⁷ cpm/μg. Blots were prehybridized, hybridized with denatured probe, and washed under conditions previously reported (16). Bound probe was then visualized by autoradiography.

DNA Sequencing. Restriction fragments of the 4.3-kb insert (*Eco*RI/*Pvu* II, *Pvu* II/*Pst* I, *Pst* I/*Eco*RV, *Eco*RV/*Xba* I) were ligated into the polylinker region of both M13mp18 and M13mp19 (New England Biolabs) (22). A series of clones containing overlapping deletions of each of these subcloned fragments was generated by either controlled exonuclease III digestion of isolated replicating form DNA (23) or by the rapid deletion method of Dale *et al.* (24) using single-stranded phage DNA. Template DNA from JM101-infected cells was isolated (22) and sequenced by the Sanger dideoxy chain-termination method (25) using dATP [³⁵S] (Amersham). Computer-assisted sequence analysis and comparisons were facilitated by using the ALIGN programs of the Bionet Resource available through the Intelligenetics Division of IntelliCorp (Palo Alto, CA).

RESULTS

Construction of *P. yoelii* 17XL λgt11 Library. *P. yoelii* 17XL DNA was extracted from parasitized blood cells after removal of mouse leukocytes. After digestion of the parasite DNA using the star activity of *Eco*RI, fragments in the 1- to 8-kb range were ligated into the *Eco*RI site of the β-galactosidase gene of λgt11. The resulting genomic expression library contained 7 × 10⁶ phage, 86% being recombinants, as assayed by the loss of β-galactosidase activity. To assess the production of parasite antigens as β-galactosidase fusion proteins, 10,000 recombinant phage were initially screened with HIS. Approximately 60 to 70 plaques gave strongly positive signals. No positive signals were observed with normal mouse serum.

Isolation of λgt11 Clones Encoding the Epitope Recognized by mAb 302. To identify recombinants containing segments of the PY230 gene, 50,000 phage were screened with the protective mAb 302. Five positive clones were detected. After plaque purification procedures and two additional
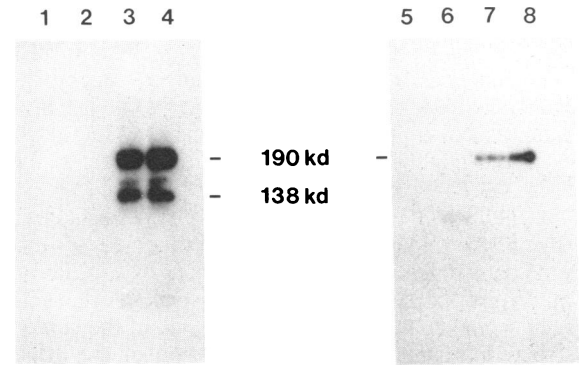

FIG. 1. Immunoblot of the β-galactosidase fusion proteins of the PY230 λgt11 clones, probed with HIS (lanes 1–4) to *P. yoelii* or with mAb 302 (lanes 5–8). Lanes: 1 and 5, bacterial extracts of Y1089 cells alone; 2 and 6, lysates of the same host lysogenized with wild-type λgt11; 3 and 7, a Y1089 lysogen of recombinant clone λ1.4. A similar lysogen of recombinant clone λ6.3 is shown in lanes 4 and 8. kd, kDa.

rounds of screening, two recombinants, λ1.4 and λ6.3, remained positive. That these clones contained PY230 coding sequences was confirmed by using a polyclonal antiserum raised against the PY230 antigen.

Recombinant phage λ1.4 and λ6.3 were lysogenized in the *E. coli* strain Y1089, grown up, and induced; bacterial extracts were separated on 7.5% NaDodSO₄/polyacrylamide gels. Immunoblots of these gels identified in both recombinants a β-galactosidase fusion protein of 190 kDa when probed with either HIS (Fig. 1, lanes 3 and 4) or mAb 302 (lanes 7 and 8). An additional recombinant protein of 138 kDa, presumably a degradation product, was detected only with HIS. These recombinant proteins were not detected in extracts of Y1089 cells alone (lanes 1 and 5) or Y1089 cells lysogenized with wild-type λgt11 (lanes 2 and 6). Since the β-galactosidase protein sequence accounts for 114 kDa of the fusion protein, these clones encode ≈76 kDa of the PY230 antigen, including the determinant recognized by mAb 302.

Identification of PY230 mRNA. Isolation and digestion of phage DNA from λ1.4 and λ6.3 showed both to contain an *Eco*RI insert of 4.3 kb. Since the isolated clones appeared to be identical, only the insert of λ6.3 was subcloned into the *Eco*RI site of the plasmid pUC9. Subsequently, a 1.85-kb *Eco*RI/*Eco*RV fragment of this insert was radiolabeled by nick-translation and hybridized to an RNA blot of *P. yoelii* 17XL RNA, separated into poly(A)⁺ and poly(A)⁻ fractions. This probe detected a 7.6-kb mRNA present in both the total RNA preparation (Fig. 2, lane 1) as well as the
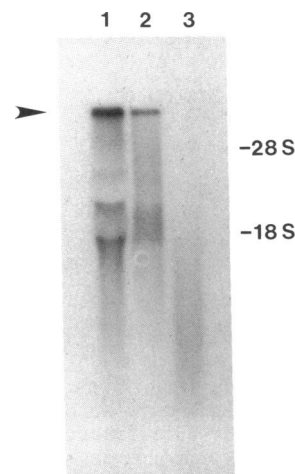

FIG. 2. RNA blot analysis of *P. yoelii* 17XL RNA. Fractionated parasite RNA was probed with a radiolabeled 1.85-kb *Eco*RI/*Eco*RV fragment of the λ6.3 recombinant clone. Lanes: 1, 20 μg of total *P. yoelii* 17XL RNA; 2 and 3, 1 μg of poly(A)⁺ and 20 μg of poly(A)⁻ parasite RNA, respectively. The positions of mouse 28S and 18S ribosomal RNA, used as markers, are indicated.

poly(A)$^+$ fraction (lane 2). A transcript of approximately this size would be expected to encode a large peptide of 230 kDa. Previous findings indicated that the mRNA encoding the PY230 antigen was operationally poly(A)$^-$ (14). The present identification of a poly(A)$^+$ message may be due to differences in the methods of RNA extraction and fractionation used.

C Terminus of PY230 Contains the Epitope Recognized by the Protective mAb 302. Based on the size of the β-galactosidase fusion protein, we estimated that ≈2 kb of the 4.3-kb insert would be sufficient to encode the 76-kDa malarial segment. A partial restriction map of the clone was constructed (Fig. 3A) and the orientation of the insert in λgt11 was determined relative to the *Pst* I site (data not shown). Restriction fragments of the 2 kb of sequence

proximal to the β-galactosidase gene were subcloned into both M13mp18 and M13mp19. A series of overlapping clones were generated through controlled exonuclease digestion of either replicating form or single-stranded phage DNA (Fig. 3A). The complete sequence of both the coding and noncoding strands of this portion of the clone was determined by the Sanger dideoxy chain-termination method. Fig. 3B shows the compiled nucleic acid sequence data with the deduced amino acid sequence, covering 2430 base pairs. Examination of the sequence reveals a single open reading frame encoding a polypeptide of 680 amino acids, having a calculated molecular mass of 77,077 Da. Furthermore, the 5' end of this clone is joined, in-frame, with the λgt11 β-galactosidase sequence at the *Eco*RI site. Prior to termination, the sequence encodes a stretch of 18 hydrophobic amino acids
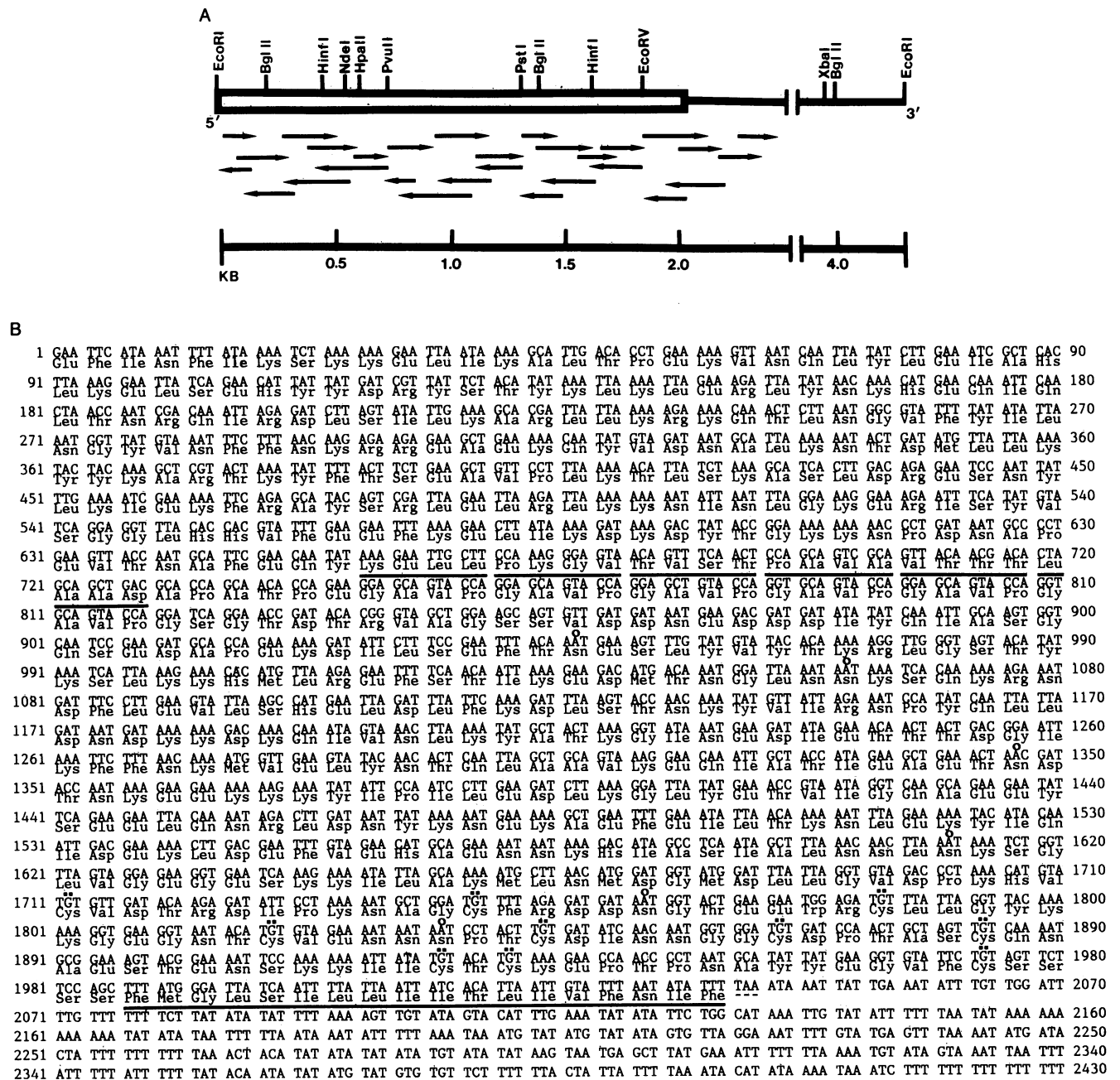

FIG. 3. (A) Restriction map and DNA sequencing scheme for the λ6.3 PY230 clone. Arrows directed left to right represent overlapping M13 clones used to determine the coding-strand sequence, while arrows in the opposite orientation represent M13 clones used to obtain the sequence of the noncoding strand. (B) Nucleic acid and deduced amino acid sequence of the coding region of the λ6.3 PY230 clone. Overlined amino acids designate the repeated tetrapeptide, while underlined residues indicate a potential membrane anchor. Solid squares mark each of the carboxyl-terminal cysteine residues. Potential N-linked glycosylation sites are shown by an open circle.

presumably functioning as a membrane anchor sequence (26). Following the first in-frame termination codon, the sequence contains multiple termination codons in all three reading frames. The A+T content is similar to that previously found in other plasmodial genes (27), 71% in the coding region and 88% in the 3' untranslated segment. Also noteworthy is a series of 10 cysteine residues located in the final 110 amino acids of the sequence. Further upstream, the sequence contains the tetrapeptide Gly-Ala-Val-Pro repeated tandemly six times. The deduced amino acid sequence also shows six potential sites for N-linked glycosylation, although it has proven difficult to demonstrate carbohydrate on this molecule (28). Most significantly, however, these data show that the epitope recognized by the protective mAb 302 is located in the carboxyl-terminal 76 kDa of the PY230 antigen.

Comparison Between the C-Terminal Portion of PY230 and the 195-kDa Antigen of *P. falciparum*. Computer-assisted analysis aligned this C-terminal sequence of PY230 with the carboxyl segment of the homologous 195-kDa antigen of the Wellcome strain of *P. falciparum*. Nucleic acid sequence similarity ranged from ≈40% to ≈70% (Fig. 4A) with similar levels in the protein sequence, considering conservative amino acid substitutions (Fig. 4B). Interestingly, a terminal series of cysteine residues was conserved in both antigens; specifically, 10 of the final 110 amino acids in PY230, and 11 of the final 100 amino acids in PF195. Furthermore, alignment of the protein sequences of these antigens showed that 9 of the 10 PY230 cysteine residues were located in a similar position relative to those of PF195.

DISCUSSION

Through the construction of a *P. yoelii* 17XL λgt11 expression library, we have cloned and sequenced the portion of

the PY230 gene encoding the epitope recognized by the protective mAb 302. Our data show that this epitope is located in the carboxyl-terminal 76 kDa of the PY230 antigen. The relationship between this PY230 antigen and the 195-kDa antigen of *P. falciparum* has been strengthened by the demonstration of nucleic acid and protein sequence homology in the C-terminal sequences.

Previous studies in animal models have implicated this high molecular weight class of blood stage antigens in the induction of protective immune responses. Specifically, the passive administration of mAb 302 provided dramatic *in vivo* protection against *P. yoelii* infection (13). In addition, purified preparations of PY230 have been used successfully to immunize mice against lethal challenge (9, 10). mAb against the analogous 250-kDa antigen of *Plasmodium knowlesi* blocked merozoite invasion of rhesus monkey erythrocytes (6). Consequently, the homologous 195-kDa merozoite antigen of *P. falciparum* has drawn considerable attention as a vaccine candidate. In a recent report, *Aotus* monkeys immunized with this antigen emulsified in Freund's complete adjuvant were totally protected from lethal challenge infection with the homologous parasite (12).

Immunologic and molecular biologic analyses have revealed that the PF195 polypeptide contains regions that show considerable polymorphism between strains, whereas other areas of the molecule are more conserved. Using a panel of mAb, McBride *et al.* (29) demonstrated both strain-common and strain-restricted epitopes on this PF195 antigen. Sequence data available on several PF195 variants defined a region in the N-terminal portion of the molecule encoding an immunogenic, polymorphic, repeated determinant (30–32). This variable epitope is present on a 76- to 83-kDa processed fragment of PF195 and appears to be shed from the merozoite surface at the time of erythrocyte invasion (33–35). Although considerable research efforts have
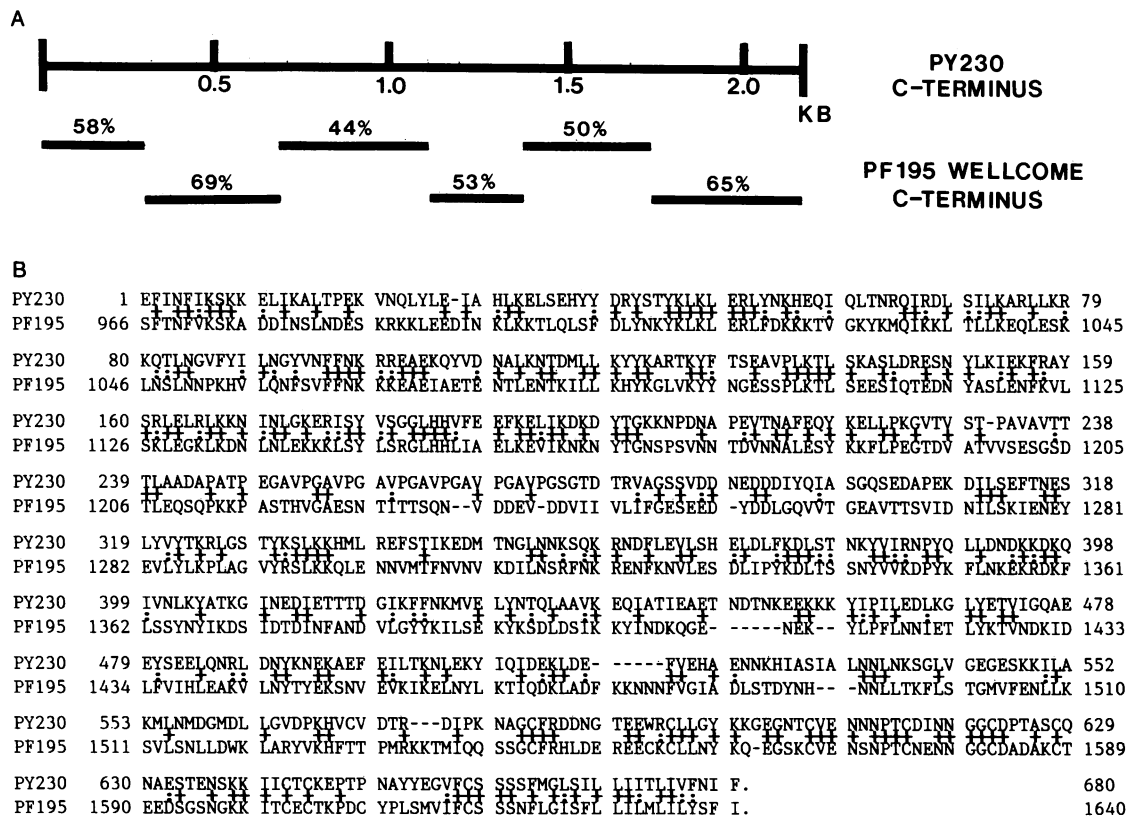
A

B

FIG. 4. Similarity between the C-terminal portions of the PY230 and the PF195 sequences. (A) Comparisons of nucleic acid sequences. (B) Protein sequence similarity with + representing identity and : representing conservative amino acid substitutions. Amino acids are identified by the single-letter code.

focused on this region of the molecule, its role in the induction of a protective immune response remains questionable. Successful vaccination of *Saimiri* monkeys has been accomplished with purified preparations of PF195, although the protective effect did not appear to be strain specific (11). These data suggest that conserved regions of PF195, located outside the N-terminal repeats, may elicit protective immune responses *in vivo*.

Several PF195 epitopes common to different strains of this parasite have been defined by various mAb (29), with three of these mapping to the carboxyl terminus of the molecule (36). In addition, one mAb recognizing a determinant in this region has been shown to block merozoite invasion *in vitro* (37). These epitopes are detected on a glycosylated 40- to 50-kDa processed fragment of PF195, shown to be expressed on the merozoite surface (36–38). Available data also suggest that a portion of this peptide is retained by the parasite following schizont rupture and subsequent invasion of erythrocytes (34, 38). Furthermore, studies at the nucleic acid level indicate that portions of the region encoding the C terminus appear conserved between various *P. falciparum* isolates (39, 40).

The role of the C-terminal portion of these merozoite antigens in the erythrocytic stage of parasite development is unclear. Recently, McBride and Heidrich (38) reported that the carboxyl-terminal PF195 fragment noncovalently associates with other parasite surface antigens, forming an antigenic complex. Since only a limited number of different antigens are expressed on the merozoite surface (33), this complex may be important in facilitating parasite invasion of erythrocytes.

The data presented here support the contention that C-terminal epitopes of these blood stage antigens may be important in the induction of protective immune responses. Cloned sequences of this region of the PY230 gene encode the B-cell epitope recognized by a protective mAb. The relatedness of the carboxyl segments of the PY230 antigen of the rodent parasite and the PF195 antigen of the human plasmodial parasite point to a need for the conservation of these sequences between species. Particularly, the preservation of nine C-terminal cysteine residues in homologous positions in both antigens suggests that this region of the molecule may provide some structural domain necessary for parasite development. This, combined with the lack of variability in this region between *P. falciparum* isolates, has significant implications for vaccine development. Using the *P. yoelii* murine model, we will be able to test, *in vivo*, the potential of this region to induce protective responses as well as the factors regulating these responses.

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