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A Second Merozoite Surface Protein (MSP-4) of *Plasmodium falciparum* That Contains an Epidermal Growth Factor-Like Domain

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Merozoite surface proteins of *Plasmodium falciparum* play a critical role in the invasion of human erythrocytes by the malaria parasite. Here we describe the identification of a novel protein with a molecular mass of 40 kDa that is found on the merozoite surface of *P. falciparum*. We call this protein merozoite surface protein 4 (MSP-4). Evidence for the surface location of MSP-4 includes (i) a staining pattern that is consistent with merozoite surface location in indirect immunofluorescent studies of cultured parasites, (ii) localization of MSP-4 in the detergent phase in Triton X-114 partitioning studies, and (iii) nucleotide sequencing studies which predict the presence of an N-terminal signal sequence and a hydrophobic C-terminal sequence in the protein. Immunoprecipitation studies of biosynthetically labelled parasites with [^{3}H]myristic acid indicated that MSP-4 is anchored on the merozoite surface by a glycosylphosphatidylinositol moiety. Of considerable interest is the presence of a single epidermal growth factor-like domain at the C terminus of the MSP-4 protein, making it the second protein with such a structure to be found on the merozoite surface.

Malaria is one of the most important causes of human morbidity and mortality worldwide, with approximately 200 million cases of infection yearly resulting in approximately 2 million deaths. Clinical symptoms occur during the phase of infection in which human erythrocytes are invaded by the asexual form of the parasite, the merozoite. Erythrocyte invasion is a multistep process that commences when the merozoite makes contact with the erythrocyte and then reorients so that its apex is brought into apposition with the erythrocyte membrane. Specialized organelles in the merozoite apex release their contents, resulting in perturbation of the erythrocyte membrane and a localized loss of the underlying membrane skeleton (42). The erythrocyte forms a tight junction around the circumference of the merozoite, and the merozoite moves into the erythrocyte, where it is surrounded by a vacuolar membrane (36).

The molecular players in this process are gradually being identified. There are a number of integral membrane proteins on the merozoite surface that in turn anchor peripheral membrane proteins that make up the gauzy coat of the merozoite (12, 24). The coat is the first part of the merozoite to contact the erythrocyte during the invasion process (36). The apical organelles of the merozoite release a number of proteins, both integral membrane proteins and soluble proteins. The integral membrane proteins on the merozoite surface identified to date include merozoite surface protein 1 (MSP-1) (25) and MSP-2 (44). MSP-1 is a 185- to 200-kDa protein that is anchored via a glycosylphosphatidylinositol (GPI) moiety to the merozoite surface. At about the time of merozoite release, MSP-1 undergoes a series of proteolytic processing events to generate four polypeptides that form a noncovalent complex on the merozoite surface. The entire complex is anchored by the 42-kDa C-terminal fragment which contains the GPI moiety. A second processing event cleaves this fragment into a 19-kDa fragment that remains anchored on the parasite surface and a 33-kDa fragment that is shed together with the other polypeptides of the complex (4). The 19-kDa GPI-anchored fragment consists of two epidermal growth factor (EGF)-like domains. MSP-2 is a 45- to 56-kDa protein that is also anchored to the merozoite surface by GPI. MSP-2 varies greatly in both molecular mass and antigenicity among parasite strains. Most of this variation is due to the presence of a large block of tandem repeats in the central region of the protein. These repeats vary in number and sequence in different forms of MSP-2 (44, 45).

The precise functions of these two proteins in the invasion process have not yet been elucidated. It is clear that both play important roles, as antibodies directed against both MSP-1 and MSP-2 prevent the invasion of erythrocytes by merozoites (8, 34, 41). In particular, it has previously been shown that the EGF-like domain of MSP-1 is an important antibody target and that antibodies to this region inhibit both the secondary processing step and the invasion of erythrocytes (3). Previous studies have shown that the immunization of mice with the 19-kDa fragment of MSP-1 from the murine malaria parasite *P. yoelii* can induce a strong effect in mice that protects against subsequent live-parasite challenge (17, 32). Such studies have important implications for the development of a vaccine to protect humans against malaria infection.

The identification of further proteins on the surface of the merozoite is clearly an important step both in the development of a vaccine against malaria and in increasing our understanding of the invasion process. We report here the identification of a third integral membrane protein of merozoites, MSP-4. Al-

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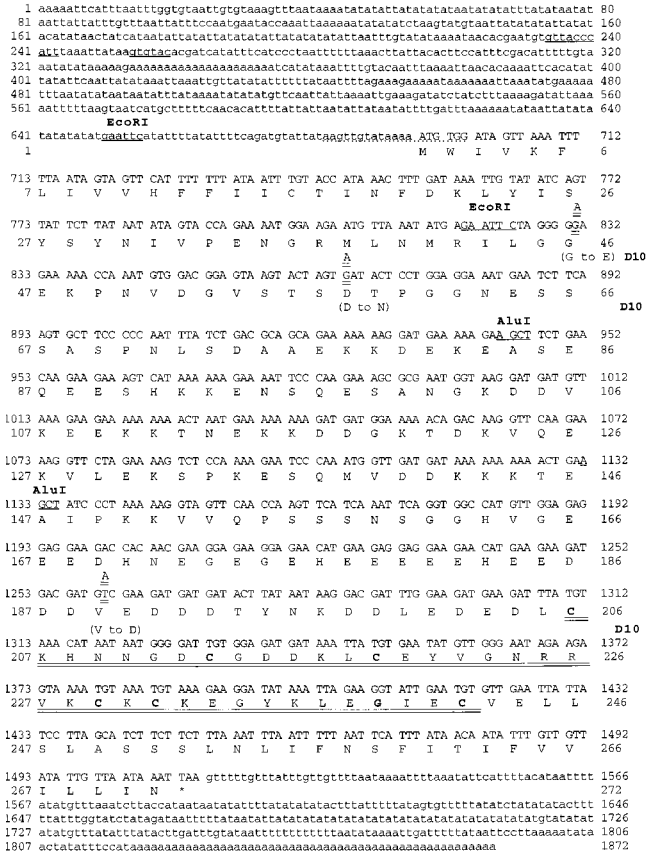


FIG. 1. Nucleotide and predicted amino acid sequences of a full-length cDNA encoding MSP-4 from *P. falciparum* w2mef. Nucleotide and amino acid numbers are shown. Noncoding DNA sequences are shown in lowercase. The restriction sites important in this study are underlined and labelled. The putative EGF-like domain is indicated by double underlining; the cysteine residues and single glycine residue of the EGF-like domain are shown in bold. Nucleotides underlined in the 5′ noncoding region represent GC-rich consensus sequence elements. The nucleotides indicated by dotted underlining around the ATG start codon are consistent with a consensus sequence for translational initiation sites in *Protozoa* spp. Nucleotides and amino acids in the *P. falciparum* D10 sequence that differ from those in the w2mef sequence are indicated by double underlines (nucleotides) and parentheses (amino acid residues).

though this is the third protein of this group, we are obliged to use the nomenclature MSP-4, as other workers have previously described a peripheral membrane protein of *P. falciparum* that lacks any membrane anchoring sequences and named it MSP-3 (35, 38, 39). The molecule we describe here is a 40-kDa protein that is first synthesized at the late ring stage and transported to the parasite surface, where it is anchored to the merozoite membrane by a GPI moiety. MSP-4 contains a single EGF-like domain at the C terminus of the protein.

MATERIALS AND METHODS

Construction and screening of *P. falciparum* cDNA library. A cDNA clone, Ag960.1, was isolated by screening a pGex2T cDNA expression library, prepared from *P. falciparum* D10 parasites, with a pool of hyperimmune sera collected from malaria-infected individuals of Papua New Guinea as previously described (35). Clone Ag960.1 is 200 bp long and corresponds to nucleotides 818 to 1014 of the sequence shown in Fig. 1. Clone Ag960.1 was used to screen a genomic library that had been prepared by shearing genomic DNA from *P. falciparum* (strain D10) after passing it 200 times through a 30-gauge needle and size selecting fragments of between 2 and 5 kb after agarose gel electrophoresis. Purified genomic DNA was then incubated in Klenow buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$) in the presence of DNA polymerase I (Klenow fragment) and 100 μM deoxynucleotide triphosphates at 37°C for 1 h. *Eco*RI adapters were ligated to the genomic DNA, which was subsequently cloned into λgt10, packaged into phage heads (GigaPack Gold; Stratagene, La Jolla, Calif.), and grown on *Escherichia coli* C600*hfl*. A 2-kb genomic clone (Ag960.2) was isolated and sequenced. A 125-bp *Alu*I-*Eco*RI subfragment of clone Ag960.2 (Ag960.2-Alu) was used to screen a second cDNA library made from mRNA of *P. falciparum* w2mef (a gift of S. Karcz). To construct the w2mef cDNA library, mRNA collected from *P. falciparum* w2mef (37) was reverse transcribed and ligated into λgt10 after the addition of *Eco*RI adapters as previously described (48). Several positive clones were isolated and excised from λgt10 by restriction digestion with *Eco*RI. These fragments ranged in size from 950 to 1,100 bp, and the longest was designated w2960.3′. All w2mef cDNA fragments were subcloned into M13 and pBluescriptII SKM13− plasmid vectors (Stratagene) and sequenced on both strands. All clones contained the same *Eco*RI site observed in clones Ag960.1 and Ag960.2, and all extended past the stop codon and into the 3′ noncoding region of the *MSP-4* gene. To obtain sequence 5′ to this internal *Eco*RI site, a genomic library was constructed from DNA of *P. falciparum* D10 after restriction endonuclease digestion with *Alu*I, ligation of *Eco*RI adapters, and ligation to *Eco*RI-digested λgt10 vector. Ligated DNA was packaged with GigaPack Gold (Stratagene), and the resulting phage particles were grown on *E. coli* C600*hfl*. This *Alu*I genomic library was screened with the 125-bp subfragment Ag960.2-Alu. Several clones containing inserts of approximately 1.6 kb (designated Ag960.5′Alu) and one of approximately 300 bp were excised from λgt10 with *Eco*RI, subcloned into M13 and pBluescriptII SKM13− vectors (Stratagene), and sequenced. To obtain the cDNA sequence from the 5′ end of the *MSP-4* gene, the w2mef cDNA library was rescreened with the genomic fragment Ag960.5′ Alu. Positive λgt10 clones (designated w2960.5′) were isolated and purified, and the inserts were subcloned into M13 vectors after excision with *Eco*RI. To obtain contiguous sequence across internal *Eco*RI sites, DNA purified from some λgt10 w2960.5′ clones was amplified by PCR with MSP-4-specific antisense primers and either λgt10 forward or reverse primers. All M13 and plasmid clones and PCR-amplified DNA were sequenced on both strands.

DNA sequencing of MSP-4. Cycle sequencing was performed with either universal −21M13 forward dye-labelled primers (Applied Biosystems [ABI], Foster City, Calif.) or synthetic oligonucleotides (Joint Protein Structure Laboratory, Ludwig Institute, Melbourne, Australia) with a dye-terminator sequencing kit (ABI). Sequence reaction products were fractionated on a model 373A automated DNA sequencer, and sequences were analyzed either by SeqEd analysis or with AutoAssembler software (ABI).

Northern blot analysis. mRNA was purified from late-trophozoite- and early-schizont-stage *P. falciparum* D10 parasites as previously described (35). Northern blot analysis was carried out by agarose gel electrophoresis of 2.5 μg of poly(A)$^+$ mRNA followed by the transfer of RNA to a nylon membrane by standard procedures (35). Size standards were provided by an RNA ladder ranging from 0.24 to 9.5 kb (Gibco BRL, Bethesda, Md.). The Northern blot was probed with the 125-bp *Alu*I-*Eco*RI subfragment of Ag960.2 (Ag960.2-Alu).

Generation of antibody reagents against MSP-4. Both human and rabbit anti-MSP-4 antibodies were prepared. For human antibodies, a glutathione *S*-transferase (GST)–MSP-4 polypeptide fusion product was generated from the original expression clone Ag960.1. The MSP-4 sequence expressed as a fusion originated from *P. falciparum* D10 and corresponds to amino acids 43 to 107 of the predicted polypeptide sequence (Fig. 1). This fusion protein was coupled to CNBr-activated Sepharose 4B (AMRAD Pharmacia Biotech, Melbourne, Australia) according to the manufacturer's instructions. A pool of antisera collected from a number of malaria-infected individuals of Papua New Guinea was affinity purified on this column and eluted as previously described (14).

To prepare rabbit anti-MSP-4 antibodies, a second fusion protein was generated by expressing a 62-residue N-terminal fragment of MSP-4 from strain w2mef (corresponding to amino acids 84 to 146 of the predicted polypeptide sequence [Fig. 1]) as a fusion with GST. Briefly, a 189-bp *Alu*I subfragment of clone w2960.3′ (Ag960.4) was ligated into the expression vector pGex1 and transfected into *E. coli* by standard procedures. Positive colonies were grown overnight in Luria broth at 37°C, *E. coli* cells were harvested, and the fusion protein (VM912C) was purified by affinity chromatography on glutathione agarose as described elsewhere (43). The purified fusion protein was dialyzed extensively against phosphate-buffered saline. Antibodies were generated in two rabbits after primary immunization by intramuscular injection at two sites with a total of 50 μg of recombinant fusion protein emulsified in TiterMax adjuvant. All subsequent inoculations were by subcutaneous injection at three sites with a total of 50 μg of recombinant fusion protein VM912C emulsified in TiterMax adjuvant. Rabbits were inoculated a total of five times at 1, 2, 4, and 10 months after primary immunization. The rabbit antibody used in these experiments was obtained from rabbit R1806 after the third immunization.

Anti-MSP-4 antibodies raised in rabbits to MSP-4–GST fusion product VM912C were depleted of anti-GST reactivity by dilution of rabbit antisera in 5% nonfat milk powder in TBS/T (0.05 M Tris-HCl [pH 7.4], 0.15 M NaCl, 0.05% Tween 20) and incubation in the presence of 100 μg of purified GST per ml at 4°C overnight.

To test for anti-GST antibody depletion, *P. falciparum* FAF6 parasites were harvested from asynchronous in vitro cultures and solubilized in sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl [pH 6.8], 4% SDS). Parasite lysates were fractionated on an SDS–12% polyacrylamide gel and transferred electrophoretically to nitrocellulose filters. Filters were incubated in 5% nonfat

milk powder in TBS/T overnight at 4°C prior to the addition of either anti-GST-depleted anti-MSP-4 rabbit antiserum or rabbit anti-GST antibodies for 1 h at room temperature. Filters were then washed three times for 10 min each in TBS/T. Antibody reactivities to immunoblotted proteins were detected with anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Silenus Laboratories) and subsequently with ECL immunodetection reagents (Amersham International, Buckinghamshire, United Kingdom) according to the manufacturer's instructions.

Immunoblots. *P. falciparum* extracts were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5 or 10% polyacrylamide gels and immunoblotted as described previously (14). Immunoblots were incubated with various antibody reagents for 2 h at room temperature, and reactive proteins were detected with ^{125}I-labelled protein A, followed by autoradiography.

Biosynthetic labelling and immunoprecipitation. *P. falciparum* (strain w2mef) parasites were cultured in vitro to a parasitemia of 10% at 2% hematocrit. A 5-ml sample of parasite culture was grown at 37°C in 100 μCi of [9,10(*n*)-^{3}H]myristic acid per ml for 4 h. Labelled parasite materials were isolated by saponin lysis by the method of Rosenthal (40). Parasite pellets were snap frozen at −70°C until required. For immunoprecipitation, frozen cell pellets were extracted for 1 h at 4°C in 10 volumes of T-NET (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.02% NaN_3, 0.5% Triton X-100 [pH 8.0]) containing a cocktail of protease inhibitors (35). Insoluble materials were removed by centrifugation at 15,000 × *g* for 10 min at 4°C, and supernatants were preabsorbed on protein A-Sepharose to remove nonspecific binding prior to immunoprecipitation with specific antisera as previously described (15).

Triton X-114 partitioning experiments. Triton X-114 partitioning experiments were performed as previously described (46). Briefly, *P. falciparum* D10 parasites were lysed in the presence of 0.5% Triton X-114 and incubated at 4°C for 2 h. The parasite lysate was centrifuged at 11,000 × *g* for 15 min at 4°C, and the pelleted material was washed three times in 0.5% Triton X-114 to minimize contamination with aqueous-phase proteins. The supernatant was loaded onto a cushion of 6% sucrose in 0.06% Triton X-114. Phase separation was conducted by incubation at 37°C for 5 min, followed by centrifugation at 5,000 × *g* at 37°C. The detergent-depleted layer was washed three times in 200 μl of 11.4% Triton X-114 to remove any contaminating hydrophobic material. The Triton X-114-enriched layer was resuspended in cold phosphate-buffered saline. All samples were subjected to SDS-PAGE, proteins were transferred electrophoretically to nylon membranes, and filters were probed with affinity-purified human anti-MSP-4 antibodies. Reactivity was detected by autoradiography with ^{125}I-labelled protein A.

Indirect immunofluorescence assays. Indirect immunofluorescence assays were performed essentially as previously described (2), except that parasites were purified on Percoll gradients prior to the preparation of parasite smears. The primary antibody was rabbit antiserum to MSP-4–GST fusion protein VM912C, and the secondary antibody was fluorescein-conjugated anti-rabbit immunoglobulin (Sigma, St. Louis, Mo.).

Nucleotide sequence accession number. The GenBank accession number for the *MSP-4* cDNA sequence from *P. falciparum* w2mef is U85260.

RESULTS

Isolation of a cDNA clone expressing a novel protein. We have previously described the construction of a cDNA library in expression vector pGex2T with cDNA synthesized from mRNA of *P. falciparum* D10, a clone of the Papua New Guinean isolate FCQ27 (35). The library was screened with a pool of human antisera collected from villagers living in an area of Papua New Guinea where malaria is endemic. Numerous reactive clones were isolated, and one of these, Ag960.1, appeared to express a novel polypeptide, based on the absence of reactivity with antisera to known antigens of *P. falciparum* and the absence of hybridization of the insert DNA with known antigen gene probes (data not shown). Ag960.1 contained a single 200-bp insert that encoded an open reading frame, resulting in the expression of an in-frame fusion protein with GST (data not shown).

A *P. falciparum* (strain D10) genomic library was screened with the 200-bp insert, and the hybridizing clones identified were used to screen a λgt10 cDNA library from strain w2mef. Numerous overlapping w2mef cDNA clones were identified and used to create a composite 1,872-bp region of the *MSP-4* gene, encompassing a full-length open reading frame and 5′ and 3′ untranslated sequences (Fig. 1). Sequence analysis revealed a long 5′ untranslated region (694 bp), a feature previously observed in other malarial antigens, such as the knob-associated histidine-rich protein, actin II, and MSP-1 (31). The 5′ untranslated region has an AT content of 88%, a consistent feature of such regions from *P. falciparum*. It also contains the sequence element GTGTAC (Fig. 1), previously reported for the 5′ untranslated regions of other plasmodial genes, such as that encoding glycophorin-binding protein 130 (30). A single long open reading frame of 816 bp commences at nucleotide 695 and terminates at nucleotide position 1510. The 3′ untranslated sequence is AT rich (91%), another feature typical of plasmodial sequences (13). Northern blot analysis of *P. falciparum* mRNA identified a transcript of 1.7 kb (Fig. 2). This suggests that the cDNA sequence presented in Fig. 1 represents a full-length or nearly full-length transcript. Hybridization of insert Ag960.2-Alu to *P. falciparum* chromosomes separated by pulsed-field gel electrophoresis showed that the gene encoding MSP-4 lies on chromosome 2 (data not shown).

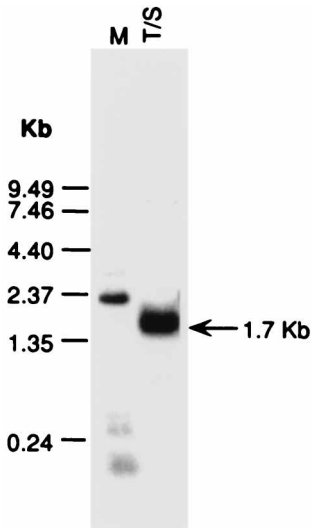
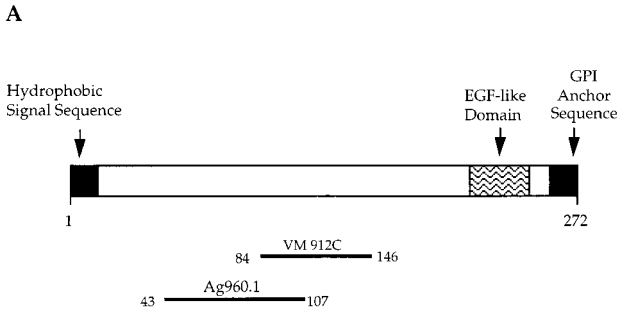


FIG. 2. Northern blot analysis of poly(A)$^+$ mRNA from *P. falciparum* (D10) probed with a 125-bp *Eco*RI-*Alu*I subfragment of the gene encoding MSP-4 (Ag960.2-Alu). Lanes: M, RNA ladder ranging from 0.24 to 9.5 kb; T/S, mRNA purified from trophozoite- and schizont-stage parasites.

MSP-4 contains a hydrophobic sequence at each end. The *MSP-4* gene encodes a 272-amino-acid protein with a calculated molecular mass of 30.4 kDa and an observed molecular mass of approximately 40 kDa. The mature protein is relatively hydrophilic, consisting of 14.3% glutamic acid, 9.6% aspartic acid, 11.4% lysine, and 8.8% serine. An analysis of the MSP-4 sequence identified two hydrophobic regions, one each at the extreme N- and C-terminal ends (Fig. 3). The N-terminal hydrophobic stretch consists of 15 predominantly hydrophobic amino acids, which is characteristic of a secretory signal sequence. The C terminus of MSP-4 consists of 19 hydrophobic residues preceded by 3 consecutive serine residues. The length and position of this hydrophobic region, together with the presence of serine residues, are strongly reminiscent of the arrangement found in several surface proteins of *P. falciparum* and *Trypanosoma brucei* (Fig. 3C) that are anchored to the cell membrane by GPI moieties (18, 23, 26, 44). This arrangement of residues is believed to provide the signal for cleavage of the polypeptide chain and attachment of a GPI moiety.

A striking structural feature of the predicted polypeptide sequence of MSP-4 is present just N-terminal to the putative GPI attachment site. Six cysteine residues are clustered in a pattern that is consistent with a consensus sequence for EGF-like domains (**C**xn **C**xn **C**xn **C**x **G** x2 **C**, where x denotes any



B

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h-EGF:          C-X7-C-X4-C-X10-CNC-X5 -GERC
MSP-1 EGF 1:    C-X4-C-X5-C-X9 -CKC-X7 -GDKC
MSP-1 EGF 2:    C-X6-C-X5-C-X13-CEC-X10-GIFC
MSP-4 EGF:      C-X6-C-X5-C-X9 -CKC-X7 -GIEC
consensus       C-Xn-C-Xn-C-Xn -CXC-Xn -GX2C
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C

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P.falciparum        CSP   KMEKC  SSVFNVVNSSIGLIMVLSFLFLN
                    MSP-2 SLLSN  SSNIASINKFVVLISATLVLSFAIFI
                    MSP-1 VIFCS  SSNFLGISFLLILMLILYSFI
                    MSP-4 LSLAS  SSLNLIFNSFITIFVVILLIN
T.brucei     MITat 1.4a  ENNACKD SSILVTKKFALTVVSAAFVALLF
VSG's         ILTat 1.3  EGETCKD SSFILNKQFALSVVSAAFAALLF
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FIG. 3. Analysis of the predicted structural features of the MSP-4 protein. (A) Schematic representation of the predicted polypeptide structure of MSP-4. The position of the EGF-like domain is indicated by a box with wavy lines. Black boxes on the left and right represent signals for secretion and attachment of a GPI anchor, respectively. Horizontal bars below the schematic indicate regions of the MSP-4 polypeptide that were expressed as fusion proteins with GST. VM912C is the 62-residue expression clone from the N terminus of MSP-4 that was used to generate rabbit anti-MSP-4 antibodies. Ag960.1 is the 65-residue GST–MSP-4 fusion product of clone Ag960.1 used to affinity purify human anti-MSP-4 antibodies. Residue numbers are indicated. (B) Sequence alignments comparing the spacing of cysteine residues (in bold) in the EGF-like domain of MSP-4 with the spacing in the two EGF-like domains of MSP-1 and the cysteine-rich domain of human EGF (h-EGF) (1). Conserved glycine residues of the EGF-like domains and h-EGF are underlined. (C) Comparison of the C-terminal hydrophobic sequence of MSP-4 with those of five protozoal proteins (three from *P. falciparum* and two from *T. brucei*) known to be anchored to a membrane via the attachment of a GPI moiety. The two serine residues that may act as signals for cleavage are aligned, and the putative cleavage site in each *P. falciparum* protein is denoted by an arrow. The exact cleavage site in each *T. brucei* protein is known and indicated by an arrow. CSP, circumsporozoite surface antigen; VSG, variant surface glycoprotein.

amino acid and n denotes any number) (1, 9, 10). The EGF-like domain of MSP-4 is also consistent with the more stringent consensus sequence proposed by Campbell and Bork [$Cx_{2-7}Cx_{1-4}$(G/A)xCx_{1-13}ttaxCxCccGax_{1-6}GxxC, where a denotes an aromatic amino acid, t denotes a nonhydrophobic amino acid, and x denotes any amino acid, respectively (6)]. The positioning of the glycine and tyrosine residues (amino acids 234 and 235 [Fig. 1]) between the fifth and sixth cysteine

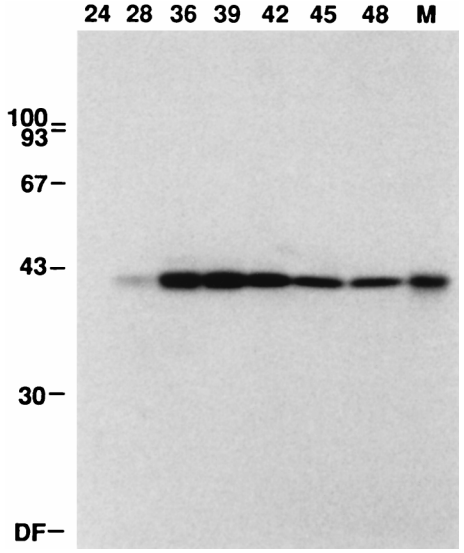

FIG. 4. Immunoblot analysis of D10 parasites sampled at 24, 28, 36, 39, 42, 45, and 48 h postsynchronization to ring-stage parasites. Proteins were separated on a 10% polyacrylamide gel, electroblotted, probed with affinity-purified human anti-MSP-4 antibodies, and detected with ^{125}I-labelled protein A. M, merozoites grown in vitro and harvested at the end of one complete asexual cycle; DF, dye front. Molecular mass standards (in kilodaltons) are given on the left.

residues of the MSP-4 putative EGF-like domain is highly conserved and provides further evidence for the existence of this structural feature in MSP-4. Other *P. falciparum* proteins which contain EGF-like domains are known, and their EGF-like domains are compared to that of MSP-4 in Fig. 3B.

MSP-4 is most abundant in late stages of the parasite and is present in merozoites. Northern blot analysis showed that the MSP-4 transcript is present in mature stages of the parasite. To further study the expression of MSP-4, a pool of sera collected from patients living in areas where malaria is endemic was incubated with a fusion protein expressed by clone Ag960.1 (strain D10) and reactive antibodies were eluted. These affinity-purified human antibodies were used to probe an immunoblot of *P. falciparum* D10 parasite lysates. Asynchronous parasites were cultured in vitro, harvested, and treated three times with 5% D-sorbitol to enrich for only ring forms of the parasite (29). Synchronized parasites were grown for 24 h in vitro and then sampled at 24, 28, 36, 39, 42, 45, and 48 h postsynchronization, representing various stages of the asexual-parasite life cycle. Parasite lysates were subjected to SDS-PAGE and electrotransferred onto nitrocellulose membranes. Affinity-purified human antibodies reacted with a single band with a molecular mass of 40 kDa (Fig. 4). Expression of the MSP-4 protein varied over the course of the life cycle, being first observed in parasite lysates taken at 28 h postsynchronization and reaching the highest level of expression at between 36 and 39 h postsynchronization. This corresponds to the time of the appearance of trophozoite- and schizont-stage parasites in *P. falciparum* cultures. MSP-4 was also found in isolated merozoites. For a panel of different parasite strains, MSP-4 was present in all isolates and did not appear to vary in size (data not shown).

To generate antibody reagents for further characterization of MSP-4, an N-terminal fragment of MSP-4 (strain w2mef) was expressed as a fusion protein (VM912C) with GST and used to immunize rabbits. To test the specificity and activity of the rabbit antibody, lysates of schizont-infected erythrocytes of

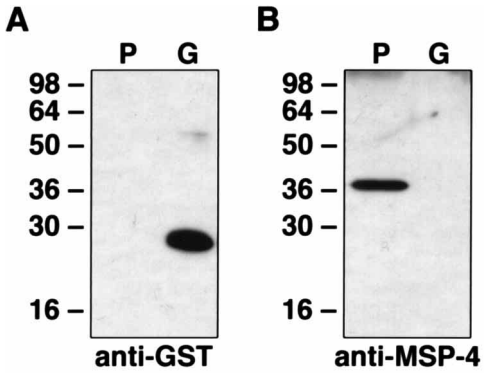

FIG. 5. Immunoblot analysis of *P. falciparum* FAF6 parasite lysates (lanes P) and purified GST protein (lanes G) fractionated on SDS–12% polyacrylamide gels and electrophoretically transferred to produce duplicate nitrocellulose filters. Filters were probed with rabbit anti-GST antibodies (A) or rabbit anti-MSP-4 antibodies after depletion of anti-GST antibody activity (B). Molecular mass standards (in kilodaltons) are shown on the left in each panel.

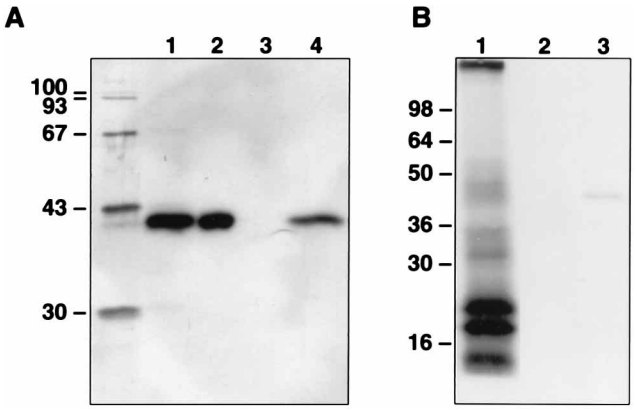

FIG. 6. (A) Phase separation studies of *P. falciparum* D10. Parasite proteins were lysed in the presence of Triton X-114, and after phase separation at 37°C, aliquots of various samples were subjected to SDS-PAGE and electroblotted. Immunoblots were probed with affinity-purified human anti-MSP-4 antibodies. The fractions shown are a Triton X-114 lysate of infected erythrocytes (lane 1), the Triton X-114-insoluble pellet (lane 2), the Triton X-114-depleted aqueous phase (lane 3), and the Triton X-114 detergent phase (lane 4). (B) Immunoprecipitation by rabbit anti-MSP-4 antibodies of w2mef parasites biosynthetically labelled with [9,10(n)-^{3}H]myristic acid. Lane 1, total labelled material; lane 2, immunoprecipitate with preimmune rabbit antibodies; lane 3, immunoprecipitate with rabbit antibodies raised against a 62-residue N-terminal fragment of MSP-4 from strain w2mef. Molecular mass standards (in kilodaltons) are given on the left in each panel.

P. falciparum D10 were immunoblotted and probed with rabbit anti-MSP-4 antibodies collected 10 weeks after primary immunization. The same blot was probed with affinity-purified human anti-MSP-4 antibodies. Proteins were detected with ^{125}I-labelled protein A. Identical 40-kDa bands were visualized with both antisera (data not shown). These rabbit antibodies were used in subsequent experiments.

As rabbit antisera were raised against GST fusion proteins, it was essential to demonstrate that reactivities with parasite lysates were due to antibodies directed to MSP-4. To do this, rabbit antisera were reacted with duplicate immunoblots of fractionated *P. falciparum* FAF6 parasite lysates and GST. One immunoblot was reacted with rabbit anti-GST antibodies (Fig. 5A), and the other was reacted with rabbit antisera to GST–MSP-4 fusion protein VM912C after depletion of anti-GST antibody activity (Fig. 5B). Anti-GST antibodies reacted only with GST and failed to recognize any parasite protein, whereas anti-GST-depleted antisera to MSP-4 reacted only with parasite materials and failed to recognize the GST fusion protein.

Evidence for the presence of a GPI moiety in MSP-4. Infected parasites were harvested from in vitro cultures and subjected to Triton X-114 partitioning to separate proteins into detergent-soluble and aqueous-phase proteins (46). Previous studies have shown that integral membrane proteins such as MSP-2 partition in the Triton X-114 detergent-phase fraction because of the presence of a hydrophobic membrane-anchoring GPI moiety (MSP-2) (44) or a hydrophobic transmembrane domain (apical membrane antigen 1 [AMA-1]) (15). Phase-separated proteins were electroblotted to nitrocellulose and probed with affinity-purified human anti-MSP-4 antibodies (Fig. 6A). MSP-4 was highly enriched in the detergent phase, suggesting a hydrophobic region which would associate with the cell membrane.

The C-terminal sequence of MSP-4 is similar to that found in a number of GPI-anchored proteins. We examined whether MSP-4 contains a GPI moiety by performing a biosynthetic labelling experiment in which D10 parasites were cultivated in the presence of [9,10(n)-^{3}H]myristic acid. Parasite cultures were labelled for 4 h at the trophozoite stage, harvested, solubilized in Triton X-100, and immunoprecipitated with rabbit anti-MSP-4 antiserum. Autoradiography revealed a labelled band with a molecular mass of 40 kDa, consistent with the proposition that MSP-4 is a GPI-anchored surface protein. This protein was not immunoprecipitated by preimmune rabbit serum (Fig. 6B).

MSP-4 is located on the merozoite surface. Indirect immunofluorescence studies were carried out with acetone-fixed cultures of *P. falciparum* and rabbit antibodies raised to GST–MSP-4 fusion protein VM912C. The pattern of fluorescence on schizonts included the typical bunch-of-grapes appearance, with accentuated staining at the rim of the merozoite (Fig. 7). Such staining is characteristic of merozoite surface proteins. An examination of early-ring-stage parasites failed to detect fluorescence, suggesting that the region of MSP-4 recognized by these antibodies is not present or expressed in these early-

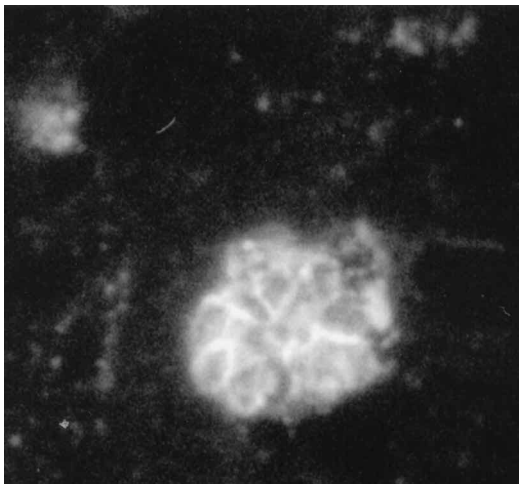

FIG. 7. Indirect immunofluorescence studies with rabbit anti-MSP-4 antibodies. *P. falciparum*-infected erythrocytes were stained with antisera from rabbits immunized with an N-terminal fragment of MSP-4 (from strain w2mef) expressed as a fusion with GST (VM912C). Staining of schizonts shows a typical bunch-of-grapes appearance, which is characteristic of merozoite surface proteins.

stage parasites (data not shown). Parasitized cells examined with rabbit anti-GST antibodies revealed no detectable fluorescence (data not shown).

Sequence differences between strains of *P. falciparum* in the gene encoding MSP-4. To generate the complete sequence of MSP-4 from strain w2mef, various genomic libraries prepared from *P. falciparum* D10 were screened and isolated clones were sequenced. A comparison of the *MSP-4* gene sequences from w2mef and D10 revealed three nucleotide differences (Fig. 1). All the nucleotide substitutions result in amino acid changes. Compared to the w2mef sequence, the D10 sequence has a glutamic acid substitution for glycine at amino acid position 46, an asparagine substitution for aspartic acid at position 58, and an aspartic acid substitution for valine at position 189. In addition, the predicted polypeptide sequence of MSP-4 from strain D10 contains an insertion of one glycine residue between amino acid positions 87 and 88 relative to the w2mef MSP-4 sequence (Fig. 1).

DISCUSSION

Four *P. falciparum* proteins have been unequivocally identified as integral membrane proteins of merozoites. Two, MSP-1 and MSP-2, are found on the merozoite surface at the time of schizont rupture, and two, EBA-175 and AMA-1, are released from apical organelles (24). Our results suggest that we have found another member of the group of resident merozoite surface proteins, MSP-4. The evidence for this is based on the observations that MSP-4 is found in isolated merozoites and can be biosynthetically labelled with [9,10(*n*)-^{3}H]myristic acid. Gene sequencing studies revealed that it has a hydrophobic signal sequence and a typical GPI attachment motif at the C terminus. MSP-4 partitioned into the Triton X-114-soluble phase, in which membrane-associated proteins are concentrated, and indirect immunofluorescence assays demonstrated staining that is consistent with merozoite surface location. We performed some preliminary immunoelectron microscopy studies that demonstrated labelling of the circumference of free merozoites with 5 nM gold particles, a distribution that is characteristic of merozoite surface localization (16), providing further confirmation of the merozoite surface location of MSP-4.

Previous biosynthetic labelling studies of *P. falciparum* with [^{3}H]glucosamine have identified a total of four proteins that incorporate this moiety (22). Under the conditions used, glucosamine was incorporated into the GPI anchor found on several malaria surface antigens. Two of these labelled proteins were shown to be MSP-1 and MSP-2 (22). Of the remaining two unidentified proteins, one was shown to have a molecular mass of 40 kDa and to be invariant in size in different isolates. We suggest that this labelled protein, called GP4, corresponds to the MSP-4 protein described here. The identity of the 51,200-Da protein reported in that study remains unknown.

The discovery of an EGF-like domain in MSP-4 is particularly interesting, as this structural feature has been found in three other malaria surface proteins. One of these is MSP-1, and the others are surface antigens of the zygote and ookinete stages, Pfs25 and Pgs28 (20, 28). In all three antigens, the EGF-like domain is an important functional element, with antibodies directed against it interfering with the parasite life cycle. Several studies have previously shown that antibodies recognizing the EGF-like domains of MSP-1 are capable of blocking parasite growth in vitro (3, 7, 8, 11, 34). Other studies have previously shown that active immunization with the EGF-like domain blocks subsequent parasite growth or protects animals from infection (7, 17). Recombinant Pfs25 expressed in vaccinia virus on the surfaces of mammalian cells has previously been shown to bind transmission-blocking antibodies (27). Similarly, antiserum raised against Pgs28 can block parasite infectivity to mosquitoes (20). Since the four EGF-like domains of Pfs25 and Pgs28 represent the body of the protein flanked only by hydrophobic signal and anchor sequences that are cleaved to produce the mature protein, one can presume that the transmission-blocking antibodies are directed to the EGF-like domains. These parallels make it tempting to speculate that the EGF-like domain of MSP-4 is also capable of inducing antibodies that block in vitro invasion, but this remains to be investigated.

Although EGF-like domains are found in many proteins with diverse functions, it has previously been observed that all proteins containing EGF-like domains are extracellular and that the function of many of them clearly is to facilitate interactions between two proteins (19). It may be that the EGF-like domains of MSP-1 and MSP-4 are involved in protein-protein interactions during merozoite invasion, perhaps in stabilizing some of the peripheral membrane proteins in the merozoite coat. Alternatively, the recent identification of EGF binding sites on human erythrocytes (21) raises the possibility that these proteins are involved in directly binding erythrocytes during the invasion process. Certainly the observation that the EGF-like domain of MSP-1 is carried into the erythrocyte during invasion is consistent with such a proposal (3).

A recent comparison of the DNA sequence presented here with DNA sequences in databases showed a very high match to a 249-bp genomic DNA sequence reported to encode a part of SALSA, a 70-kDa sporozoite- and liver-stage antigen of *P. falciparum* (5). Our work clearly demonstrates the existence of a 40-kDa merozoite surface protein that is encoded by the *MSP-4* gene. We determined this sequence from a full-length cDNA clone corresponding to the transcript size seen on Northern blots of asexual-stage parasites. It seems unlikely that the sequence reported here could encode a polypeptide with a molecular mass of 70 kDa. Sporozoite materials are extremely difficult to obtain so we cannot determine whether the gene encoding MSP-4 is transcribed in this stage. Southern blot analysis of digested genomic DNA with subfragment Ag960.2 revealed bands that are consistent with the presence of a single *MSP-4* gene in the *P. falciparum* genome (data not shown). Thus, the relationship between SALSA and MSP-4 remains to be determined, but it seems reasonable to suggest that they share cross-reactive epitopes.

A striking feature of other merozoite surface proteins is the occurrence of a number of distinct alleles within populations of the parasite. These differing alleles within circulating parasites are thought to result from selection by the host immune system. Variation is particularly marked for MSP-2 but is also a feature of MSP-1 sequences. Alleles encode proteins that differ in molecular mass and antigenicity. We have been unable to detect variation in the size of MSP-4 in a number of different parasite isolates. However, a comparison of the *MSP-4* cDNA sequence (w2mef) and the genomic sequence from D10 revealed a limited amount of variation between strains at the DNA level. Since the w2mef sequence is generated from cDNA and the D10 sequence is genomic, it is possible that these amino acid differences are due to errors generated during reverse transcription of mRNA. However, it is more likely that such mutations are real and that the MSP-4 molecule varies between strains by a number of point mutations in a fashion similar to that of AMA-1 (33, 47). Further sequencing studies are required to confirm and extend the data.

Certain features of MSP-4 make it a particularly interesting

subject for further studies to determine its efficacy as a vaccine component. Most notably, it contains an EGF-like domain, which in other malaria antigens is the target of parasite-inhibitory antibodies. It is relatively small (272 residues), making it more likely to be capable of being expressed as a full-length product in recombinant expression systems. The use of affinity-purified antibodies to identify the corresponding parasite protein clearly demonstrates that this protein is immunogenic during the course of infection. We have affinity purified anti-MSP-4 antibodies from patient sera collected in Papua New Guinea, Vietnam, and Kenya, demonstrating that the protein is widely distributed in different parasite populations and that it is immunogenic for a number of different patient groups (data not shown). Studies to assess the immunogenicity and antigenicity of MSP-4 and its potential as a component of an antimalaria vaccine are planned.

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