

Identification and Characterization of a Conserved, Stage-Specific Gene Product of *Plasmodium falciparum* Recognized by Parasite Growth Inhibitory Antibodies

Claudia A. Daubenberger,^{1*} Diana Diaz,¹ Marija Curcic,¹ Markus S. Mueller,¹
Tobias Spielmann,¹ Ulrich Certa,² Joachim Lipp,³
and Gerd Pluschke¹

Molecular Immunology, Swiss Tropical Institute, 4002 Basel,¹ and Hoffmann-La Roche Ltd., Roche Genetics, 4070 Basel,² Switzerland, and Vienna International Research Cooperation Center, Department of Vascular Biology and Thrombosis Research, University of Vienna, A1235 Vienna, Austria³

Received 7 October 2002/Returned for modification 25 November 2002/Accepted 23 December 2002

We have identified a novel conserved protein of *Plasmodium falciparum*, designated D13, that is stage-specifically expressed in asexual blood stages of the parasite. The predicted open reading frame (ORF) D13 contains 863 amino acids with a calculated molecular mass of 99.7 kDa and displays a repeat region composed of pentapeptide motives. Northern blot analysis with lysates of synchronized blood stage parasites showed that D13 is highly expressed at the mRNA level during schizogony. The first N'-terminal 138 amino acids of D13 were expressed in *Escherichia coli* and the purified protein was used to generate anti-D13 monoclonal antibodies (MAbs). Using total lysates of blood stage parasites and Western blot analysis, these MAbs stained one single band of ~100 kDa, corresponding to the predicted molecular mass of ORF D13. Western blot analysis demonstrated further that D13 is expressed during schizogony, declines rapidly in early ring stages and is undetectable in trophozoites. D13 protein is localized in individual merozoites in a distinct area, as demonstrated by indirect immunofluorescence analysis. After subcellular fractionation, D13 was confined to the pelleted fraction of the parasite lysate and its extraction by alkaline carbonate buffer treatment indicated that D13 is not a membrane-integral protein. Inclusion of certain anti-D13 MAbs into *in vitro* cultures of blood stage parasites resulted in considerable reduction in parasite growth. The N'-terminal domain encompassing 158 amino acids is 94 and 95%, respectively, identical at the amino acid level between *Plasmodium knowlesi*, *Plasmodium yoelii*, and *P. falciparum*. In summary, we describe a novel stage-specifically expressed, highly conserved gene product of *P. falciparum* that is recognized by parasite growth inhibitory antibodies.

Malaria is a debilitating and frequently fatal disease of the tropics caused by parasites of the genus *Plasmodium*. Four different species cause human malaria, *Plasmodium falciparum* and *Plasmodium vivax* accounting for the majority of the problem. The former causes widespread mortality, and the latter is most prevalent outside Africa. Annually 300 million clinical malaria cases are reported, with over 1 million deaths in sub-Saharan Africa alone (23). Widespread and increasing drug and insecticide resistance has exacerbated the situation, undermining the effectiveness of malaria control methods that depend on chemotherapy and vector control, respectively (21). Novel means to fight the disease are urgently needed, and a vaccine is predicted to have the greatest impact in addition to being the most cost-effective control measure (11, 20).

Access to the sequence of the entire genome of *P. falciparum* has provided the opportunity to deduce the function of many of the predicted proteins through the identification of orthologue genes and motifs in other organism (11). However, as with annotation of the human genome, the annotation of the complete *P. falciparum* genome represents a major challenge. Almost two-thirds of the predicted genes of the published

chromosomes 2 and 3 had no detectable orthologues in other organisms, suggesting that many aspects of parasite biology has still to be discovered (9).

P. falciparum has a complex life cycle involving transmission within and between vertebrate and invertebrate hosts by specialized cell-invasive stages, termed zoots. Sporozoites injected into a human host by the bite of an infective mosquito invade hepatocytes and, after schizogony, release thousands of merozoites capable of invading red blood cells (RBC). All of the clinical symptoms and pathogenic manifestations associated with mammalian malaria infections are caused by the asexual erythrocytic phase of the *Plasmodium* life cycle. After invasion of erythrocytes, merozoites develop into trophozoites, and multiply further within these cells, forming multinucleated blood stage schizonts. These infected RBC rupture, releasing newly formed merozoites into the circulation that can invade new erythrocytes. An intricate series of biological events and developmental processes must occur for this cyclical erythrocytic stage of the infection to continue in a vertebrate host. Thus, the elucidation of molecular mechanisms responsible for recognition and subsequent invasion of erythrocytes by the malaria parasite is of central importance towards the development of new intervention strategies.

In this study properties of a novel protein encoded by an open reading frame (ORF), designated D13, is described. The sequence of D13 is highly conserved in several *P. falciparum*

* Corresponding author. Mailing address: Molecular Immunology, Swiss Tropical Institute, Socinstrasse 57, 4002 Basel, Switzerland. Phone: 41 61 2848217. Fax: 41 61 2718654. E-mail: Claudia.Daubenberger@unibas.ch.

isolates and orthologues of it are identified in the genome of *Plasmodium knowlesi* and *Plasmodium yoelii*. The parasite growth inhibitory activity of anti-D13 monoclonal antibodies (MAbs) is indicative for a functionally essential role of this protein in parasite biology.

MATERIALS AND METHODS

Identification of ORF D13. A cDNA library was constructed from total RNA isolated from *P. falciparum* strain K1 employing the SMART PCR cDNA Library Construction Kit (Clontech) as described (5). Briefly, 2 µg of total RNA was reverse transcribed using a modified oligo(dT) primer, and the SMART oligonucleotide was added to the reaction to serve as a short, extended template at the 5' end of the RNA for reverse transcription. To select for PCR products longer than 0.7 kb, PCR products were run on a 1% agarose gel, selectively excised, and purified. PCR products were ligated into pGem 5 T vector (Promega). DH125 cells (BRL-Life Technologies) were transformed by electroporation with the *P. falciparum* cDNA library. Plasmid DNA of randomly picked clones was digested with enzymes *NotI/NcoI*, and the insert size was analyzed on 1% agarose gels. Clones carrying inserts of more than 1 kb were chosen for further analysis. Linearized DNA was transcribed and translated in vitro as described (6) and positive cDNA clones were characterized further by sequencing employing an ABI 310 automatic sequencer (Perkin-Elmer).

***P. falciparum* culture and Northern blot analysis.** *P. falciparum* strains K1 and FVO were cultured in RPMI 1640 medium (Life Technologies, Inc.) containing gentamicin (50 mg/liter) and 10% A⁺ human serum at a hematocrit of 5%, essentially as described previously (14). In some experiments, the cultures were synchronized by hemolysis of mature trophozoite stage-parasitized erythrocytes in a 5% (wt/vol) sorbitol solution with two sorbitol synchronization steps one cycle before harvesting. Synchronization was confirmed and the level of parasitemia estimated by standard microscopy. Aliquots of cells were taken every 6 h postsynchronization. Cells were washed and total RNA prepared using TRIzol reagent (Gibco-BRL) as described previously (25). Total RNA (25 µg) was separated on 0.8% agarose gel and transferred to Hybond-XL nylon membranes (Amersham Pharmacia Biotech) using a vacuum blotter (Appligene) as described elsewhere (25). Hybridization was performed in an UltraHyb device (Ambion) at 42°C with the [α -³²P]dCTP-labeled D13 probe (5'-terminal 422-bp PCR product) and the pGAPDH probe (complete cDNA; AF03044) (5) generated by random priming using High Prime solution (Roche Biochemicals). High stringency washes were performed at 42°C. Membranes were subjected to autoradiography using X-ray films. Loading of equal amounts of RNA on agarose gels was confirmed by comparison of the intensity of ethidium bromide stained bands of 18S and 28S RNA.

Recombinant expression of N'-terminal fragment of D13. The N'-terminal 139 amino acid residues were recombinant expressed in *Escherichia coli* using the pETBlue2 expression system (Novagen). Briefly, PCR product of D13 was generated from a cDNA library of *P. falciparum* strain K1 employing the following primer combination: 5'-CAACACCATGTTTATGCCACACTTTTGGAGT G-3' and 5'-CGTTTGCTCGAGTGGCAACTTGTAAAGTACCAGGG-3', containing *NcoI* and *XhoI* sites, respectively. The 422-bp amplicon was digested with the restriction enzymes and cloned into the pETBlue2 vector employing its *NcoI* and *XhoI* sites. Recombinant plasmids were sequenced to ensure that the D13 fragment was in the correct reading frame and to exclude PCR errors. Competent *E. coli* Tuner cells (pLys) (Novagen) were transformed with the recombinant plasmid and expression of the fusion protein was induced by the addition of 1 mM isopropyl thiogalactoside (IPTG) (Calbiochem) after the A_{600} reached 0.6. The cells were induced at 37°C for 4 h and were harvested by centrifugation and lysed on ice for 30 min with 8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris/HCl (pH 8.0) and sonicated. After centrifugation at 10,000 × g, the supernatant was loaded onto a nitrilotriacetic acid column (Qiagen) and purified according to manufacturer's instructions. The hexahistidine-tagged recombinant protein was recovered using elution buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris-HCl [pH 4.5], 500 mM imidazole). Purified protein was analyzed by sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis (SDS-15% PAGE), and the protein concentration was determined according to the method of Bradford using bovine serum albumin as the standard. The purified recombinant protein was identified as the expected D13 protein by matrix-assisted laser desorption ionization-time of flight mass spectrometry after tryptic digestion.

Generation of hybridoma cell lines producing anti-D13 MAbs. Hybridoma cell lines were generated from mice immunized essentially as described (18). A group of five mice was immunized subcutaneously three times with 50 µg recombinant D13 protein isolated under denaturing conditions and formulated in

MPL+TDM adjuvant (Sigma Chemicals, St. Louis, Mo.). Three days before cell fusion, the mice received an intravenous booster injection with 20 µg of recombinant D13 in phosphate-buffered saline (PBS). Cells were fused with PAI mouse myeloma cells as a fusion partner (19). Hybrids were selected in hypoxanthine-aminopterin-thymidine (HAT) medium, and cells secreting D13-specific immunoglobulin (IgG) were identified by enzyme-linked immunosorbent assay (ELISA) using Immunolon 4 plates (Dynex Technologies Inc., Chantilly, Va.) coated with D13 protein. Four hybridoma cell lines (named DD1.1, DD1.2, DD1.3, and DD1.4) specific for the D13 N'-terminal fragment could be established and characterized.

Western blot analysis and fractionation of infected erythrocytes. SDS-PAGE was performed essentially as described (5). Briefly, total cell lysates of asynchronous or synchronous cultures of *P. falciparum* (strain FVO) were run on 10% gels. For time course analyses, aliquots of synchronous cultures of malaria-infected erythrocytes were harvested at 6-h intervals and washed with PBS, pH 8.0. The cells were lysed in 10 ml of 0.15% saponin in H₂O, and hemoglobin-depleted infected erythrocytes were collected by centrifugation. Aliquots of the samples were mixed with loading buffer and heated 5 min at 95°C before loading on the gels. As a molecular weight marker, SeeBluePlus (Invitrogen) was used. Separated proteins were transferred electrophoretically to nitrocellulose filter (Protean Nitrocellulose, BA 85; Schleicher & Schuell). Blots were blocked and then incubated with hybridoma supernatant for 1 h. After several washing steps, blots were incubated with goat anti-mouse IgG horseradish peroxidase conjugated Ig (Bio-Rad Laboratories, Hercules, Calif.) for 1 h. Blots were developed using the ECL system according to manufacturer's instructions.

For subcellular fractionation experiments, late stage infected erythrocytes were enriched to 95 to 99% using 60% Percoll gradient essentially as described previously (29). Cells were washed three times in Hanks buffered salt solution and lysed by three cycles of freeze-thaw in 10 volumes of double-distilled water containing protease inhibitors (100 µM phenylmethylsulfonyl fluoride, leupeptin [10 µg/ml], aprotinin [0.4 U/ml], 1 µM pepstatin, 2 µM EDTA [pH 8.0]). A total membrane fraction was collected by ultracentrifugation at 100,000 × g for 1 h at 4°C. For certain experiments, equivalent amounts of the sediments were treated with 100 mM Na₂CO₃, pH 11.3, or with distilled H₂O containing protease inhibitors for 30 min on ice prior to further centrifugation for 1 h at 100,000 × g (6). Samples were solubilized in denaturing SDS sample buffer and equivalent amounts of each sample were subjected to Western blot analysis. Western blot analysis was conducted with anti-D13 MAb DD1.1 and anti-merozoite surface protein 1 (anti-MSP-1) MAb 7/27. MAb 7/27 recognizes the N'-terminal block 1 of MSP-1 (Helg et al., submitted for publication). The blots were developed using the ECL system.

Immunofluorescence analysis of asexual blood stage parasites. Immunofluorescence analysis of blood stage parasites was conducted essentially as described (5). Briefly, multitest immunofluorescence microscopy slides (Flow Labs, Switzerland) were pretreated with 0.01% (wt/vol) poly-L-lysine (Sigma) for 30 min at room temperature and washed. Erythrocytes from in vitro cultivated *P. falciparum* (strain FVO) were washed and mixed with 2 volumes of a solution containing 4% paraformaldehyde and 0.1% Triton X-100. Droplets of 30 µl of cell suspension were added to each well and incubated for 30 min at room temperature. Cells were blocked with blocking solution containing 100-mg/ml fatty acid-free bovine serum albumin in PBS. Cells were incubated with hybridoma supernatants for 1 h. After several washing steps, cells were incubated with secondary antibodies specific for mouse IgG conjugated with Cy3. The immunoreactivity was observed using a Leica TCS NT confocal microscope. Images were acquired with a 63x Plan-Apochromat oil immersion objective (NA 1.32). Pinhole settings were 1 airy unit for all images that were processed with Imapris (Bitplane, Switzerland) and Adobe Photoshop (Mountain View, Calif.).

Sequence analysis of D13 derived from parasite strains. Genomic DNA was prepared from *P. falciparum* strains K1, IFA9, and MAD20 and used for PCR amplification of D13 in order to gain insight into possible nucleotide sequence polymorphism. For sequence analysis, D13 was amplified in two overlapping fragments with the following primer combinations: forward (p17), 5'-CAACAA AATGGTTTATGCCACACTTTTGGAGTGAAG-3', and reverse (p19) 5'-CAG GAATTCACATTTGAACAATTGGATTG-3'; forward (p29), 5'-CCTACTCA GAAGAATAGCATG-3', and reverse (p25), 5'-GTATAGACATGTTTTGTTTC ATATTATTATATAG-3'. Amplifications were performed with the following profile: 5 min 94°C; (25 × 20 s 94°C, 30 s 44°C, 2 min 68°C) 7 min 72°C, soak at 4°C. Amplicons were purified using a PCR product purification kit (Roche Molecular Biochemicals) according to manufacturer's protocol and cloned into the pGEM5 T-vector (Promega, Catalys AG). After isolation of plasmids using the NucleoSpin kit (Macherey-Nagel AG), double-stranded plasmid DNA was sequenced and analyzed employing an ABI PRISM 310 genetic analyzer (Perkin-Elmer). All strains were amplified twice and several independent plasmids were

sequenced on both strands using internal primers designed according to the D13 sequence available from the genome project.

In vitro parasite growth inhibition assays. In vitro growth inhibition assays with *P. falciparum* strain K1 were conducted essentially as described (16). Briefly, synchronous late trophozoites were diluted with fresh RBC to give a parasitemia of 0.5% and were mixed with purified MABs at the indicated concentrations. Parasites were cultivated under an atmosphere of 4% CO₂, 3% O₂, and 93% N₂ at 37°C. Final hematocrit in cultures was adjusted to 0.5%. Each culture was set up in sextuplicate in 96-well flat-bottom culture plates. After 96 h plates were centrifuged at 180 × g for 5 min and culture supernatants were discarded. Pelleted erythrocytes were resuspended in 200 μl of PBS supplemented with hydroethidine fluorescent vital stain (15 μg/ml; Polyscience Inc., Warrington, Pa.) and incubated at room temperature for 45 min. The erythrocytes were washed twice with PBS, resuspended in 400 μl of PBS, and analyzed in a FACScan flow cytometer (Becton-Dickinson, San Jose, Calif.) with CELLQuest program. The hydroethidine emission was detected in the FL2 channel by logarithmic amplification, and the erythrocytes were gated on the basis of their forward and sideward scatters. A total of 30,000 cells per sample were analyzed. Percent inhibition was calculated from the geometric mean parasitemias of sextuplicate test and control wells as 100 × (control – test)/control. Statistical significance was calculated by a two-sided *t* test. Confidence intervals (*P* < 95%) were calculated by antilogging the confidence limits calculated on the log scale.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the GenBank with the accession numbers AF491296 to AF491298.

RESULTS

Identification and sequence analysis of ORF D13 of *P. falciparum*. In order to identify novel secreted and transmembrane gene products of *P. falciparum* involved in host-parasite interactions, a *P. falciparum* cDNA library was screened in an in vitro transcription-translation-translocation assay for genes encoding protein products that are translocated into microsomes and are therefore protected from proteinase K digestion (6). One of the plasmids, designated D13, gave rise to a 37-kDa protein product that resisted proteinase K digestion indicative for membrane association or translocation (data not shown). The 1.2-kb insert of this plasmid was sequenced and results compared with sequence data of the ongoing *P. falciparum* Genome Project available at PlasmoDB database [http://plasmodb.org] (27). One predicted ORF (chr14_1.glm_722) that encompassed the partial sequence of plasmid D13 was identified. The complete predicted ORF D13 is 2,586 bp and codes for 862 amino acid residues, with a calculated molecular mass of 99.7 kDa and an pI of 5.30. The three gene prediction algorithms used by the genome project, Glimmer, Genefinder, and Path, predicted identically D13. Screening of the PlasmoDB database using the Blast program showed that D13 is present on chromosome 14 as a single-copy gene. The identification of the predicted initiation codon is supported by the nucleotide context of the ATG start codon, AAATGG, found in other genes of *P. falciparum* and the lack of alternative start codons anywhere in vicinity (4). D13 is intron-less and the putative protein is rich in asparagine (19.3%), lysine (9.5%), glutamic acid (7.7%), and aspartic acid (7.2%) (Fig. 1). D13 has no predicted N'-terminal signal sequence and other primary structural characteristics of an integral membrane protein are also lacking. Analysis of the predicted secondary structure of D13 with the Predictprotein program package (http://bioc.cubic.colombia.edu/predictprotein) identified several low-complexity, nonglobular regions separating two globular domains located each at the N'- and C'-terminal ends. Search-

ing databases of sequenced genomes of eukaryotes and prokaryotes demonstrated no significant homology to functional domains of other characterized gene products and hence no functional classification can currently be assigned (17).

D13 nucleotide sequences of *P. falciparum* strains K1, MAD20, and IFA9 (13) were compared with the 3D7 sequence available from the genome project. D13 was amplified by PCR from genomic DNA of in vitro-grown blood stage parasites, cloned into pGem5T vector and sequenced as described previously (13). All sequences obtained were identical to the *P. falciparum* 3D7 sequence apart from one nonsynonymous base exchange at nucleotide position 1444 in *P. falciparum* K1. This exchange resulted in a conservative amino acid exchange of D to E and was reconfirmed by a second analysis using an independent PCR product. The nucleotide sequences were deposited in the GenBank with the accession numbers AF491296 to AF491298.

Sequence comparison of D13 of *P. falciparum* using Blast program and the PlasmoDB database [http://plasmodb.org] revealed that full-length putative orthologues of D13 are present in the genome of *P. knowlesi* (chrPkn_008795-6-7284-4963) and *P. yoelii* (chrPyl_cpy805-2-1337-3844) (Fig. 1). When the D13 amino acid sequence of *P. falciparum* was compared using the Blast program to putative orthologues in *P. knowlesi* and *P. yoelii*, sequence identities were 38 and 41%, respectively. Alignment of the deduced amino acid sequences revealed that a repeat region with twelve consecutive repeats of the pentapeptide motif (K/R)(N/S)(D/E)N(I/M/T) is unique for D13 of *P. falciparum* and is absent in the orthologue sequences of the other Plasmodium spp. aligned. This repeat region constituted 8% of the molecule. In contrast to the low overall conservation of the D13 sequence, the N'-terminal domain encompassing the first 158 amino acid residues is highly conserved between the three Plasmodium spp. aligned displaying sequence identities of 94 and 95%, respectively, between *P. knowlesi*, *P. yoelii*, and *P. falciparum* (Fig. 1). Ten strictly conserved cysteine residues with the spacing H₂N-19-C-7-C-6-C-11-C-16-C-14-C-5-C-20-C-7-C-4 are present. Shorter stretches of conserved sequence motifs at the C'-terminal end of the D13 amino acid sequence were also found (Fig. 1).

Stage-specific expression of ORF D13 mRNA in synchronized blood stage parasites. In order to define the transcription pattern of D13, Northern blot analysis was performed using total RNA isolated from asexual blood stage parasites. After hybridization with a radiolabeled PCR product representing the 5'-terminal 422 bp of D13, one specific signal was detected in RNA from unsynchronized blood stage parasites (data not shown). The size of the transcript was about 7.5 kb by comparison with the RNA size marker, indicating the presence of extensive 5'- and 3'-untranslated regions. In parasites from synchronized cultures, this transcript was only detected in RNA of schizonts collected 48 h after synchronization (Fig. 2A, panel I, lane 8). In order to verify the integrity of the RNA preparations isolated at different times after synchronization, the blot was rehybridized with a radiolabeled cDNA of pfGAPDH (5). The pfGAPDH-specific hybridization signal of 3.6 kb was observed in each lane confirming that the RNA was intact and comparable amounts of RNA were present for hybridization (Fig. 2A, panel I and II).

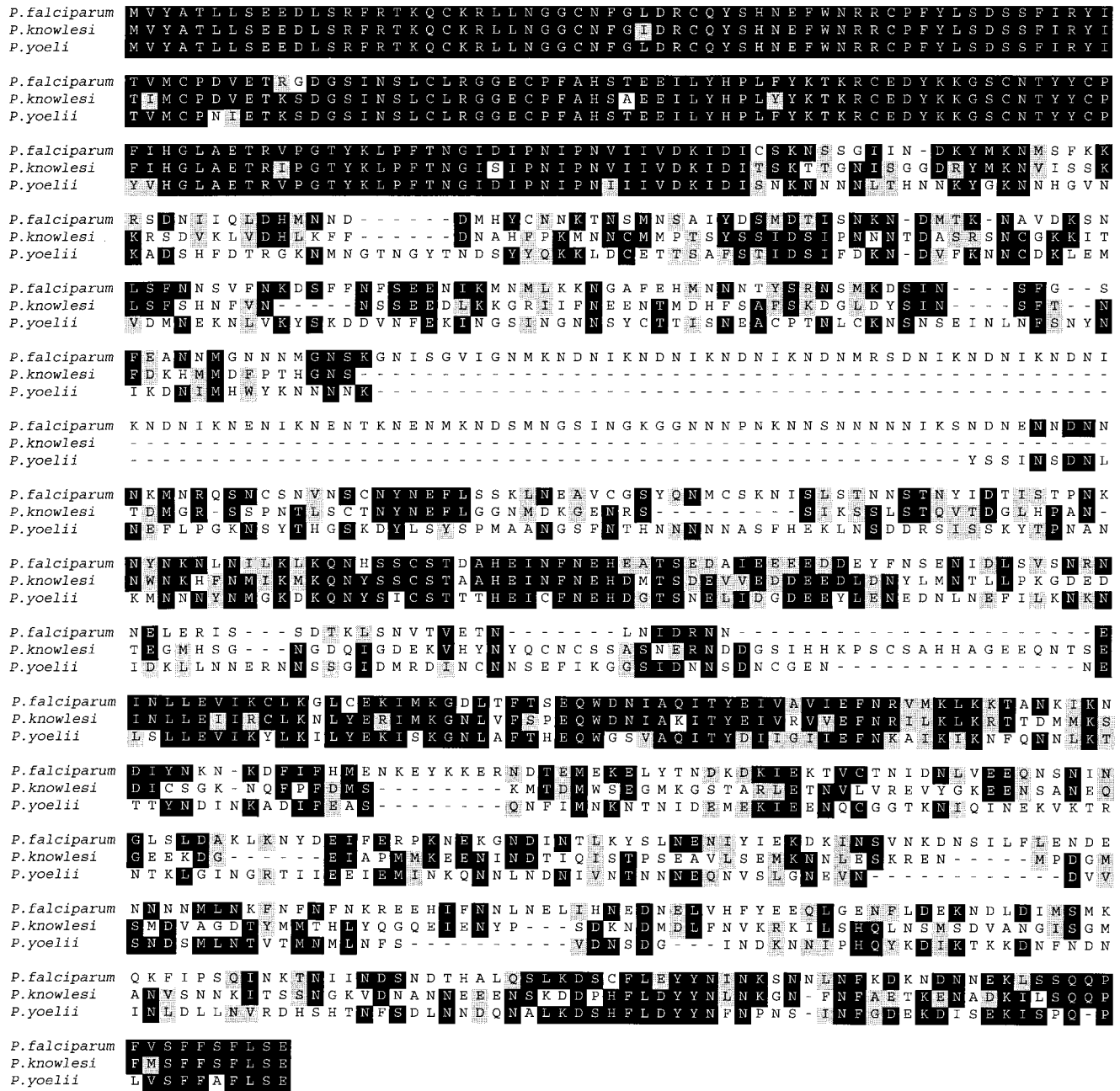


FIG. 1. Alignment of deduced amino acid sequences of D13 orthologues of *P. falciparum*, *P. knowlesi*, and *P. yoelii*. The alignment was done with ClustalW and prepared for display using BOXSHADE (<http://bioweb.pasteur.fr>). Gaps were inserted to give the best fit. (Sequences for *Plasmodium* spp. are available from the PlasmoDB database [<http://www.plasmodb.org>].)

Establishment of D13-specific MABs and analysis of the expression of D13 protein in blood stage parasites. After having established that D13 transcription is largely confined to the schizont stage, we wanted to analyze next whether, when and at what size D13 protein is expressed. The N'-terminal 139 amino acids of D13 were expressed in *E. coli* as hexahistidine tagged fusion protein using the pETBlue2 expression system. Purification by nitrilotriacetic acid-affinity chromatography under denaturing conditions yielded a recombinant protein of the predicted molecular mass of 17 kDa in SDS-PAGE (data not

shown). The purified recombinant protein was identified as the expected D13 protein by matrix-assisted laser desorption ionization-time of flight mass spectrometry. D13-specific MABs were generated from mice carrying human heavy and light immunoglobulin chain replacement mutations (18). After repeated immunization with the recombinant fragment of D13 delivered with MPL+TDM as adjuvant, four anti-D13 MABs were generated that reacted with the recombinant 17 kDa fragment in ELISA (Table 1). DD1.1 and DD1.2, but not DD1.3 and DD1.4, recognized in Western blot analysis of total

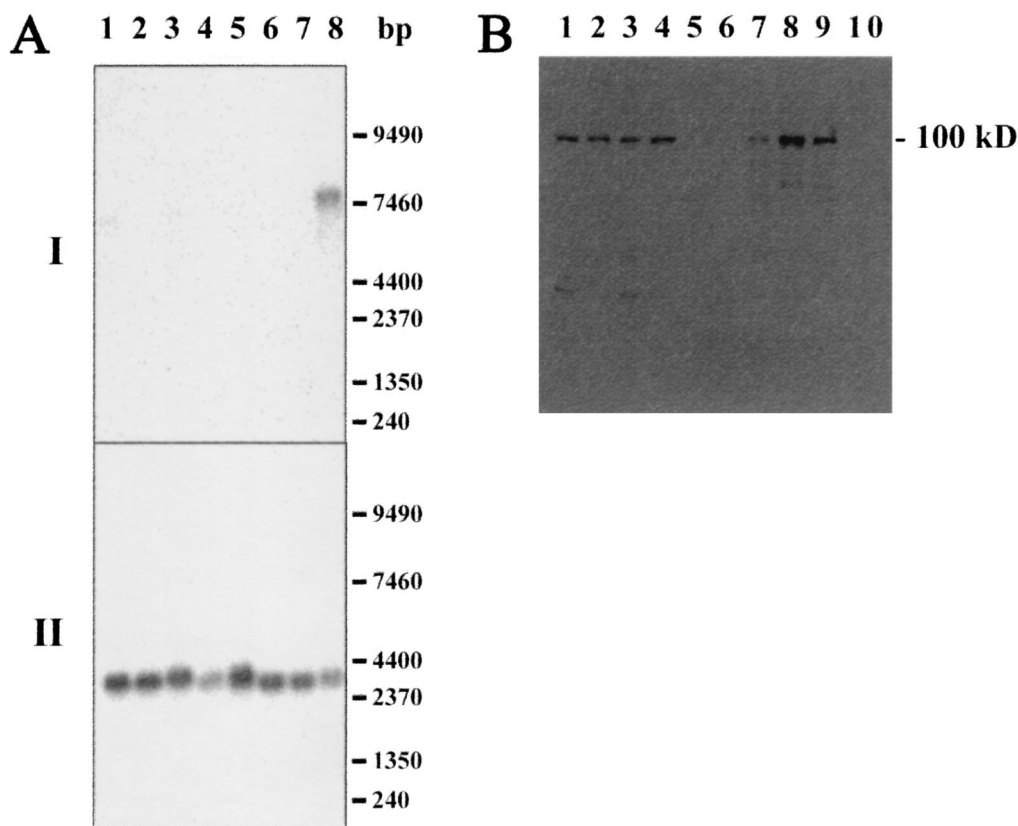


FIG. 2. (A) Northern blot analysis of synchronized asexual blood stage of *P. falciparum*. Panel I shows total RNA (25 µg) from in vitro-grown synchronized *P. falciparum* blood stage parasites that was separated on agarose gels, blotted onto nylon membrane, and hybridized to an [α - 32 P]dCTP-labeled probe corresponding to the 5' terminal 422-bp fragment of D13. Time points analyzed were 0 to 6 h, 6 to 12 h, 12 to 18 h, 18 to 24 h, 24 to 30 h, 30 to 36 h, 36 to 42 h and 42 to 48 h (lanes 1 to 8) after synchronization. Panel II shows the blot rehybridized with an [α - 32 P]dCTP-labeled probe of cDNA of pfGAPDH (5). (B) Western blot analysis of total lysates of blood stage parasites. Total lysates of infected RBC were separated by SDS-10% PAGE under reducing conditions and blotted onto a nitrocellulose membrane. Samples were taken at 6, 12, 18, 24, 30, 36, 42, and 48 h (lanes 1 to 8) after synchronization. Lysates of unsynchronized infected RBC (lane 9) and uninfected RBC (lane 10) were also loaded. The blot was incubated with anti-D13 MAb DD1.1 and developed using the ECL system. MAb DD1.1 recognized a single protein band of about 100 kDa in most lanes containing lysates of infected, but not in uninfected RBC.

lysates of unsynchronized blood stage parasites one distinct band of ~100 kDa (Fig. 2B and data not shown). The size of the identified band corresponded to the predicted molecular weight of 99,7 kDa of ORF D13 (chr14_1.glm_722). As a

representative example, results obtained with MAb DD1.1 are shown in Fig. 2B, lane 9.

In the light of the highly regulated transcription of the D13 gene, we investigated the presence of D13 protein during asexual blood stage development. Aliquots of the lysates from the identical synchronized cultures that had been used for Northern blot analysis were separated by SDS-PAGE and probed with MAb DD1.1. D13 protein was detectable in ring stage parasites (Fig. 2B, lanes 1 to 4), disappeared during the trophozoite stage (Fig. 2B, lanes 5 and 6), and reappeared in the schizont stage (Fig. 2B, lanes 7 and 8). In the late schizont stage at 48 h postsynchronization, the relative abundance was highest (Fig. 2B, lane 8). Uninfected RBC yielded no detectable signal, confirming the specificity of MAbs DD1.1 for a parasite-encoded protein (Fig. 2B, lane 10). Results obtained with MAb DD1.2 were comparable to DD1.1, although considerably weaker signals were obtained (data not shown). Protein staining of SDS-PAGE demonstrated that comparable amounts of total cell lysates from synchronized blood stage parasites were present on nitrocellulose membranes (data not shown).

Immunolocalization of D13 in asexual blood stages of *P.*

TABLE 1. Reactivities of anti-D13 MAbs used in this study

Designation of anti-D13 MAb	Isotype	Reactivity in ELISA to D13 N'-terminal fragment (µg/ml) ^a	Concn (µg/ml) of anti-D13 MAbs yielding a positive result in indirect IFA ^b
DD1.1	IgG1/λ	0.08	0.15
DD1.2	IgG1/λ	0.06	5
DD1.3	IgG2b/λ	0.1	— ^c
DD1.4	IgG2b/λ	0.04	5

^a Purified anti-D13 MAbs were serially diluted and tested for reactivity in ELISA with plates coated with recombinant D13 N'-terminal fragment at a concentration of 5 µg/ml. Given are the concentrations yielding half-maximal binding as measured by optical density at 405 nm.

^b Purified anti-D13 MAbs were serially diluted and used for indirect IFA of unsynchronized parasites fixed onto slides. Antibody binding was assessed by fluorescence microscopy on a Leitz Dialux 20 fluorescence microscope and documented with a Leica DC200 digital camera system. The lowest concentration of anti-D13 MAbs yielding a positive signal in indirect IFA is given.

^c MAb DD1.3 showed no binding in IFA.

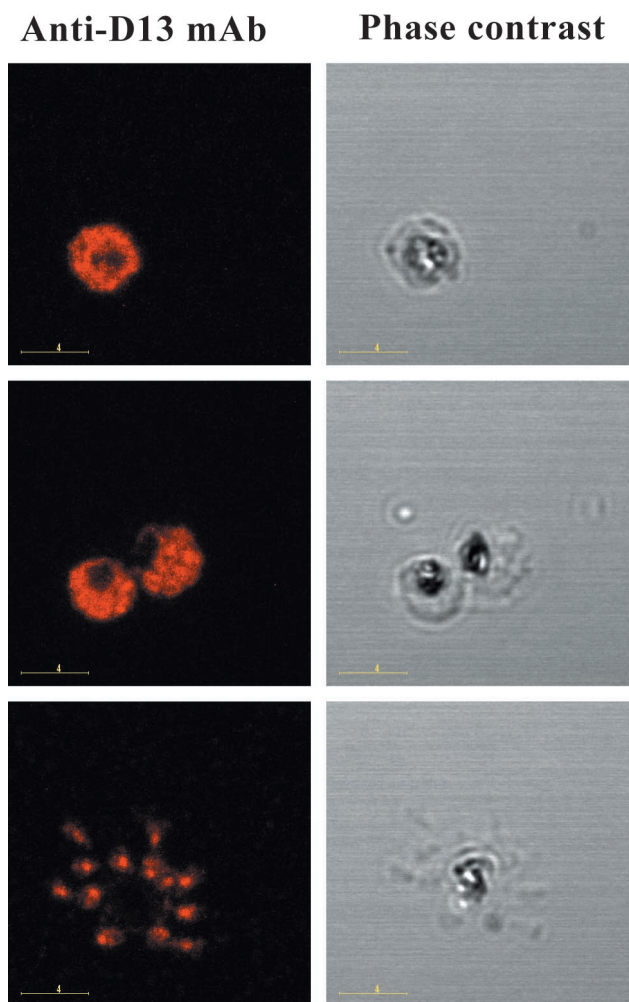


FIG. 3. Immunofluorescence analysis of schizonts, segmenter, and released merozoites of *P. falciparum* using anti-D13 MAb DD1.1 and confocal microscopy. In column I, staining with MAb DD1.1 is shown, while in column II the phase contrast of the corresponding parasites is presented.

falciparum. All anti-D13 MAbs were tested for parasite binding using indirect immunofluorescence assay (IFA). DD1.1 bound strongest to the parasite in IFA, while DD1.2 and DD1.4 yielded only weak signals at high antibody concentrations indicating that their affinity for the native D13 protein was considerably lower compared to DD1.1 (Table 1). Therefore, MAb DD1.1 was used for the characterization of the subcellular localization of D13 protein by IFA and confocal microscopy. In column 1 of Fig. 3, typical results of the indirect IFA are depicted, while in column 2, phase contrast pictures of the corresponding parasites are shown. Specific staining of schizonts (first row), segmenters (second row) and released merozoites (third row) are presented. Interestingly, in the merozoite stage, D13 protein seemed to be concentrated in a distinct area of the cell, while during schizont stages the protein was more evenly distributed in the parasite.

Subcellular fractionation of *P. falciparum* infected erythrocytes. In order to investigate whether D13 protein is associated with parasite membranes, cell fractionation experiments were

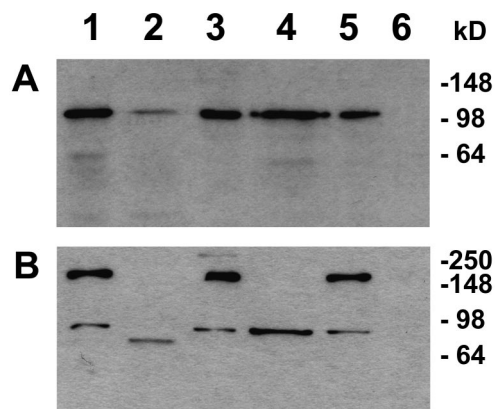


FIG. 4. Association of D13 with the sediment fraction of infected erythrocytes. Schizonts were enriched by Percoll-gradient centrifugation and hypotonically lysed by repeated cycles of freeze-thaw in water. The sediment (lane 1) and supernatant (lane 2) fractions were obtained by centrifugation. Aliquots of membrane fractions were further processed by incubation with alkaline carbonate (lanes 3 and 4) or water (lanes 5 and 6), respectively, and separated by ultracentrifugation in sediment lanes (lanes 3 and 5) and supernatant lanes (lanes 4 and 6) fractions, respectively. The samples were electrophoresed and probed with anti-D13 MAb DD1.1 (Fig. 4A) or anti-MSP-1 MAb 7/27 (Fig. 4B).

performed and the distribution of D13 protein into sediment and supernatant fractions examined using the anti-D13 MAb DD1.1 for Western blot analysis. Infected erythrocytes were Percoll-purified to maximize parasite protein content, washed thoroughly and hypotonically lysed in water. A membrane-containing fraction was pelleted by ultracentrifugation and analyzed. In the sediment fraction derived from infected erythrocytes, a specific band corresponding to the D13 protein could be detected (Fig. 4A, lane 1). This band was considerably weaker in the supernatant fraction of infected erythrocytes (Fig. 4A, lane 2). To analyze the nature of the association of D13 with the pellet fraction, total membrane fractions were stripped of peripherally attached proteins by treatment at high pH with sodium carbonate. After centrifugation, D13 protein could be detected in both the pellet and supernatant fractions, indicating that D13 is not a membrane-integral protein (Fig. 4A, lanes 3 and 4). In pellets incubated with water, D13 protein remained in the sediment and was not detected in the supernatant fraction (Fig. 4A, lanes 5 and 6). Aliquots of the same samples were blotted onto nitrocellulose and probed with MAb specific for the abundant membrane-integral protein MSP-1. MSP-1 is synthesized as a large (~195 kDa) precursor that is held by a glycosyl phosphatidyl inositol anchor on the parasite membrane. It undergoes posttranslational proteolytic processing to produce fragments of 83, 42, 38, and 28 to 30-kDa, which persist as a noncovalently linked complex on the surface of mature merozoites (12). The anti-MSP-1 antibody 7/27 is specific for block 1 of MSP-1 present on both the precursor molecule and the processed 83 kDa fragment. As expected, MAb 7/27 detected a major band of ~195 kDa in the sediment of untreated, alkaline carbonate and water treated pellets but not in the supernatants (Fig. 4B). The smaller band of ~83 kDa represent processing products of MSP-1 (Fig. 4B).

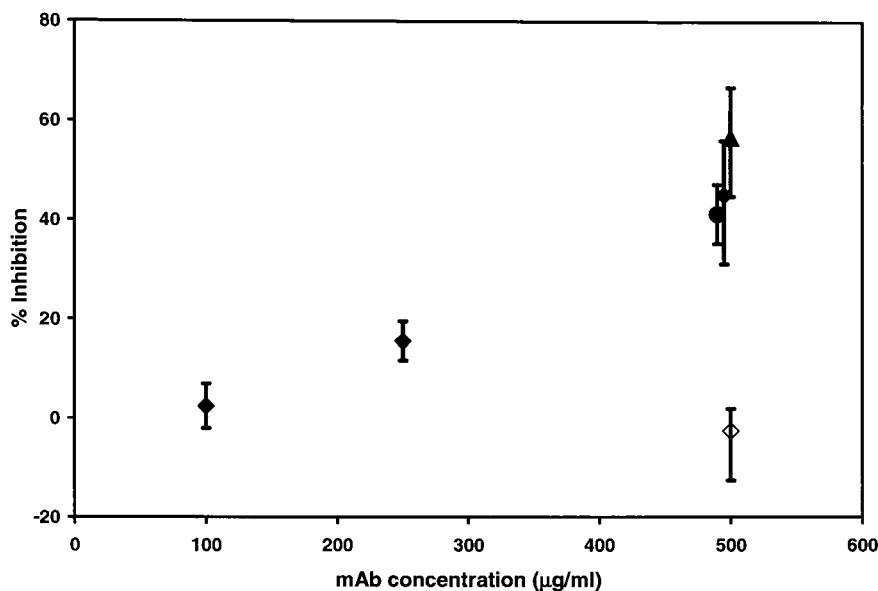


FIG. 5. Parasite growth inhibitory activity of anti-D13 MABs. The vertical bars indicate the 95% confidence intervals. The filled symbols represent results of three separate experiments conducted with MAB DD1.1, while results with the parasite nonbinding MAB DD1.3 are represented by the open symbol (\diamond).

These results indicated that D13 is pelleted with the membrane-fraction but it is not a membrane-integral protein.

***P. falciparum* in vitro growth inhibition assays with anti-D13 MABs.** After having established that MAB DD1.1, DD1.2, and DD1.4 but not DD1.3 bind to native D13 protein expressed in the invasive stage of the parasite blood stage cycle, we conducted in vitro growth inhibition assays for two cycles of merozoite invasion. At 500 $\mu\text{g/ml}$ the anti-D13 MAB DD1.1 showed growth inhibitory effects in three independent experiments (47.6% average growth inhibition) conducted with two different batches of antibody preparations (Fig. 5). This inhibition was statistically significant as judged by a two-sided *t* test. At an antibody concentration of 250 $\mu\text{g/ml}$, the measured inhibition remained statistically significant, while at 100 $\mu\text{g/ml}$ the effect was diminished by dilution. In contrast to the good binding MAB DD1.1, the IFA-negative anti-D13 MAB DD1.3 did not interfere with the growth of the parasite in vitro (Fig. 5).

DISCUSSION

Research conducted in recent years identified the importance of both antibody-dependent and cell-mediated mechanisms of immunity to the erythrocytic stage of *P. falciparum* (15). A protective role for antimalaria antibodies was shown by the pioneering studies of Cohen et al. (3), who showed that protective immunity could be transferred by using the IgG fraction of sera from immune West African adults. Administration of large doses of antimalaria antibodies to children with acute infection resulted in reduction of parasitemia and recovery from clinical illness (3). It was thought that these antibodies interacted with either late-stage schizonts or free merozoites (2). Further studies showed that antimalarial IgG from immune sera of West Africans protected against *P. falciparum*

infection in East Africa (2). Additionally, transfer of purified, pooled hyperimmune IgG from African adults to Thai patients was found to reduce parasite level. These transfer experiments suggest that geographically diverse parasite strains may share antigens important in inducing protection (22). Hence, there is considerable interest in the molecular identification of parasite proteins as potential targets of vaccine-induced antibodies preventing invasion of erythrocytes.

We are currently characterizing secretory and transmembrane gene products of *P. falciparum* stage-specifically expressed during schizogony. Expression screening of a *P. falciparum* cDNA library for in vitro-translated and -translocated products yielded the cDNA clone D13 encompassing the N'-terminal 1.2-kb fragment of a novel predicted ORF (chr14_1.glm_722). The expression of D13 mRNA turned out to be highly regulated and detectable by Northern blot analysis only in schizont development. Therefore, we decided to characterize D13 in more detail. D13 is a single-copy gene localized on chromosome 14 according to the ongoing *P. falciparum* genome project and is predicted to code for a 99.7-kDa protein. D13 contains a region of low-complexity constituted by 12 tandem repeats of a pentapeptide sequence motif that is followed by shorter homopolymer runs of asparagine residues. Many of the malaria antigens that have been characterized in *P. falciparum* contain tandem arrays of relatively short sequences. A number of characteristics allow distinctions to be drawn among such malaria antigens. One group is characterized by one centrally located block of tandem repeats that constitutes a significant proportion of the polypeptide chain. This group includes the S antigens, MSP-2 and the circumsporozoite protein (1). Other antigens contain a single set of repeats comprising a minor segment of the polypeptide chain. This group includes the thrombospondin-related adhesive protein, the exported protein 1, and also D13, since the repeat

region constitutes about 8% of the whole molecule (1). Other characteristics that distinguish between different repeat-containing antigens are diversity of repeat sequences and variation in the number of tandemly repeated sequences. Sequence diversity can be observed both within blocks of repeats and between equivalent repeat segments in allelic gene products (1). In D13, there is variation between the 12 sequence repeats that are based mainly on single base pair exchanges (data not shown). Surprisingly, sequence polymorphism of the low-complexity region of D13 of *P. falciparum* in the laboratory isolates 3D7, K1, and MAD20 and the field isolate IFA9 was not detected although polymorphism of antigens of *P. falciparum* is usually particularly extensive in repeat regions of the molecules (1).

In order to study whether and when D13 protein is expressed during asexual blood stages, the N'-terminal 138 amino acids were expressed in *E. coli* as hexahistidine-tagged protein, purified, and used to raise specific MABs in mice. Immunogenicity of the recombinant protein was low but we were able to establish several anti-D13 MABs. The anti-D13 MABs DD1.1 and DD1.2 recognized a band of 100 kDa in lysates of blood stage parasites in Western blot analysis. This size of the band corresponded to the predicted molecular weight of D13. In IFA, the cross-reactivity of MAB DD1.1, DD1.2, and DD1.4 with the native parasite protein was established. Western blotting and IFA both showed that the abundance of D13 protein was highest in schizonts. It declined during ring stage while D13 was undetectable in trophozoites. D13 protein was detectable for about 30 h during the asexual blood stage cycle, while D13 specific mRNA was present only during the last 6 h of schizont development. Hence, the D13 protein was detectable four to five times longer than the D13-specific mRNA during one asexual developmental cycle.

The strict regulation of D13 mRNA expression together with the highest expression of D13 protein in schizonts suggested an involvement of the protein in the complex biological processes of merozoite development, rupture of mature schizonts, release of merozoites and invasion of fresh erythrocytes. Therefore, we used an in vitro parasite growth inhibition assay to test whether anti-D13 MAB influence the progression of the infective cycle. The results showed that incorporation of the strongest parasite binding anti-D13 MAB DD1.1 inhibited parasite growth by an average of 47.6% in several independent experiments. The non-parasite-binding anti-D13 MAB DD1.3 had no effect on parasite growth. These results indicate that the affinity and/or the epitope recognized by the anti-D13 MABs might be important for the inhibitory function. Currently, it is not possible to assign exactly the subcellular localization of D13 by IFA using confocal microscopy. D13 is not obviously expressed on the surface of merozoites like the MSPs but might be rather concentrated on one distinct pole of the merozoite. The combined results of the in vitro parasite growth inhibition assays with the cell fractionation experiments indicate that D13 is during blood stage development accessible to antibodies in solution and becomes enriched in the pelleted fraction of malaria parasites. However, more detailed analyses, including immuno-electron microscopy, are essential to determine the exact subcellular localization of D13 protein.

Currently, the correlation of in vitro growth inhibitory activities of antibodies with their potential protective capacity in

vivo is incompletely understood. Active immunization studies in animal models are therefore essential to demonstrate unequivocally that the N'-terminal domain of D13 might be a target of protective antibody responses in vivo.

The fact that D13 is conserved in four parasite isolates may suggest that it is not under immune pressure, although sera from donors naturally exposed to malaria contain antibodies reacting with the recombinant N'-terminal domain of D13 in ELISA (unpublished observation). The high level of sequence conservation is in marked contrast to MSPs like MSP-1 and MSP-2 (7, 8, 10). In contrast, the rhoptry-associated protein 2 displays very limited sequence diversity (24). It has been demonstrated that antibodies raised against rhoptry-associated protein 2-derived peptides reduce parasite growth in vitro, indicating that conserved proteins can be targeted by parasite inhibitory antibodies (26).

Besides offering the possibility to improve the annotations of the *P. falciparum* genome through comparative analysis, animal models represent a potent source of information concerning protein function within the context of the infected host or vector (30). Alignment of the deduced amino acid sequences of putative D13 orthologues in *P. falciparum*, *P. knowlesi*, and *P. yoelii* showed that the N'-terminal domain is highly conserved, while the rest of the molecule displayed extensive sequence variation. The high degree of amino acid sequence conservation between rodent, monkey and human malaria species suggests a conserved biological role of the N'-terminal domain in malaria. The prominence of synonymous versus nonsynonymous base exchanges in this domain (data not shown) suggests that apart from differences in the codon usage in different Plasmodium species (28) a negative selection pressure might be operating to preserve a distinct three-dimensional structure. The conserved N'-terminal domain of D13 might bind to and interact tightly with other proteins to conduct its biological function(s). Functional investigations of the biological role(s) of D13 will probably further our understanding of molecular mechanism(s) mediating evasion, recognition, invasion and subsequent establishment of the parasites in host cells.

ACKNOWLEDGMENTS

This work was supported by a grant from the Swiss National Science Foundation to C. A. Daubenberg (3100-061513.00). The Plasmodium Genome Database, a collaborative effort of investigators at the University of Pennsylvania and Monash University Melbourne, Australia, is supported by the Burroughs-Wellcome Fund.

We thank Shinji Okitsu for in vitro cultivation of *P. falciparum* and Bernd Bohrmann for support in confocal microscopy. We thank the scientists and funding agencies comprising the international Malaria Genome Project for making sequence data from the genome of *P. falciparum* (3D7) public prior to publication and the completed sequence.

REFERENCES

- Berzins, K., and R. F. Anders. 1998. The malaria antigens, p. 181-215. In M. Wahlgren and P. Perlman (ed.), *Malaria: molecular and clinical aspects*. Harwood Academic Publishers, London, United Kingdom.
- Cohen, S., and I. A. McGregor. 1963. Gammaglobulin and acquired immunity to malaria, p. 123-159. In P. C. Garnham, A. E. Pierce, and I. Roitt (ed.), *Immunity to protozoa*. Blackwell Scientific Publications, Oxford, United Kingdom.
- Cohen, S., I. A. McGregor, and S. P. Carrington. 1961. Gammaglobulin and acquired immunity to human malaria. *Nature* **192**:733-737.
- Daubenberg, C., V. Heussler, E. Gobright, P. Wijngaard, H. C. Clevers, C. Wells, N. Tsuji, A. Musoke, and D. McKeever. 1997. Molecular characterization of a cognate 70 kDa heat shock protein of the protozoan *Theileria parva*. *Mol. Biochem. Parasitol.* **85**:265-269.

5. Daubenberger, C. A., F. Poltl-Frank, G. Jiang, J. Lipp, U. Certa, and G. Pluschke. Identification and recombinant expression of glyceraldehyde-3-phosphate dehydrogenase of *Plasmodium falciparum*. *Gene* **246**:255–264.
6. Ebel, T., J. F. S. Middleton, A. Frisch, and J. Lipp. 1997. Characterization of a secretory type *Theileria parva* glutaredoxin homologue identified by novel screening procedure. *J. Biol. Chem.* **272**:3042–3048.
7. Ekala, M.-T., H. Jouin, F. Lekoulou, S. Issifou, O. Mercereau-Puijalon, and F. Ntoumi. 2002. *Plasmodium falciparum* merozoite surface protein 1 (MSP1): genotyping and humoral responses to allele-specific variants. *Acta Trop.* **81**:33–46.
8. Fenton, B., J. T. Clark, C. M. A. Khan, J. V. Robinson, D. Walliker, and R. Ridley. 1991. Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen 2 (MSA-2) of the malaria parasite *Plasmodium falciparum*. *Mol. Cell. Biol.* **11**:963–971.
9. Gardner, M. J. 2001. A status report on the sequencing and annotation of the *Plasmodium falciparum* genome. *Mol. Biochem. Parasitol.* **118**:133–138.
10. Gardner, M. J., H. Tettelin, D. J. Carucci, L. M. Cummings, L. Aravind, E. V. Koonin, S. Shallom, T. Mason, K. Yu, C. Fujii, J. Pederson, K. Shen, J. Jing, C. Aston, Z. Lai, D. C. Schwartz, M. Perlea, S. Salzberg, L. Zhou, G. G. Sutton, R. Clayton, O. White, H. O. Smith, C. M. Fraser, and S. L. Hoffman. 1998. Chromosome 2 sequence of the human malaria parasite *Plasmodium falciparum*. *Science* **282**:1126–1132.
11. Hoffman, S. L., G. M. Subramanian, F. Collins, and J. C. Venter. 2002. *Plasmodium*, human and Anopheles genomics and malaria. *Nature* **415**:702–709.
12. Holder, A. A., and R. R. Freeman. 1984. The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. *J. Exp. Med.* **160**:624–629.
13. Jiang, G., C. Daubenberger, W. Huber, H. Matile, M. Tanner, and G. Pluschke. 2000. Sequence diversity of the merozoite surface protein 1 of *Plasmodium falciparum* in clinical isolates from Kilombero District, Tanzania. *Acta Trop.* **51**–61.
14. Matile, H., and R. Pink. 1990. *Plasmodium falciparum* malaria parasite cultures and their use in immunology, p. 221–234. In I. Lefkovits and B. Pernis (ed.), *Immunological methods*, vol. IV. Academic Press, San Diego, Calif.
15. Mohan, K., and M. M. Stevenson. 1998. Acquired immunity to asexual blood stages, p. 467–493. In I. W. Sherman (ed.), *Malaria: parasite biology, pathogenesis, and protection*. ASM Press, Washington, D.C.
16. Moreno, R., F. Poltl-Frank, D. Stuber, H. Matile, M. Mutz, N. A. Weiss, and G. Pluschke. 2001. Rhoptry-associated protein 1-binding monoclonal antibody raised against a heterologous peptide sequence inhibits *Plasmodium falciparum* growth *in vitro*. *Infect. Immun.* **69**:2558–2568.
17. Mulder, N. J., and R. Apwiler. 2001. Tools and resources for identifying protein families, domains and motifs. *Genome Biol.* **3**:2001.1–2001.8.
18. Pluschke, G., A. Joss, J. Marfurt, C. Daubenberger, O. Kashala, M. Zwickl, A. Stief, G. Sansig, B. Schlapfer, S. Linkert, H. van der Putten, N. Hardman, and M. Schroder. 1998. Generation of chimeric monoclonal antibodies from mice that carry human immunoglobulin C γ 1 heavy of C κ light chain gene segments. *J. Immunol. Methods* **215**:27–37.
19. Pörtl-Frank, F., R. Zurbriggen, A. Helg, F. Stuart, J. Robinson, R. Gluck, and G. Pluschke. 1999. Use of reconstituted influenza virus virosomes as an immunopotentiating delivery system for a peptide-based vaccine. *Clin. Exp. Immunol.* **117**:496–503.
20. Richie, R., and A. Saul. 2002. Progress and challenges for malaria vaccines. *Nature* **415**:694–700.
21. Ridley, R. 2002. Medical need, scientific opportunity and the drive for anti-malarial drugs. *Nature* **415**:686–692.
22. Sabchareon, A., T. Burnouf, D. Ouattara, P. Attanah, H. Bouharoun-Tayoun, P. Chantavanich, C. Foucault, T. Chongsuphajasiddhi, and P. Druihle. 1991. Parasitologic and clinical human response to immunoglobulin administration in falciparum malaria. *Am. J. Trop. Med. Hyg.* **45**:297–308.
23. Sachs, J., and P. Malaney. 2002. The economic and social burden of malaria. *Nature* **415**:680–685.
24. Saul, A., J. Cooper, D. Hauquitz, D. Irving, Q. Cheng, A. Stowers, and T. Limpaboon. 1992. The 42-kilodalton rhoptry-associated protein of *Plasmodium falciparum*. *Mol. Biochem. Parasitol.* **50**:139–150.
25. Spielmann, T., and H. Beck. 2000. Analysis of stage-specific transcription in *Plasmodium falciparum* reveals a set of genes exclusively transcribed in ring stage parasites. *Mol. Biochem. Parasitol.* **111**:453–458.
26. Stowers, A., J. A. Cooper, T. Ehrhardt, and A. Saul. 1996. A peptide derived from a B cell epitope of *Plasmodium falciparum* rhoptry associated protein 2 specifically raises antibodies to rhoptry associated protein 1. *Mol. Biochem. Parasitol.* **82**:167–180.
27. The *Plasmodium* Genome Database Collaborative. 2001. PlasmoDB: an integrative database of the *Plasmodium falciparum* genome. Tools for accessing and analyzing finished and unfinished sequence data. *Nucleic Acids Res.* **29**:66–69.
28. van Lin, L. H. M., J. C. Janse, and A. P. Waters. 2000. The conserved genome organization of the non-falciparum malaria species: the need to know more. *Int. J. Parasitol.* **30**:358–370.
29. Wahlgren, M., K. Berzins, P. Perlman, and A. Björkman. 1983. Characterization of the humoral immune response in *Plasmodium falciparum* malaria I. Estimation of antibodies to *P. falciparum* or human erythrocytes by means of microELISA. *Clin. Exp. Immunol.* **54**:127–134.
30. Waters, A. P. 2002. Orthology between the genomes of *Plasmodium falciparum* and rodent malaria parasites: possible practical applications. *Phil. Trans. R. Soc. Lond. Ser. B* **357**:55–63.

Editor: W. A. Petri, Jr.