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Precise Timing of Expression of a *Plasmodium falciparum*-derived Transgene in *Plasmodium berghei* Is a Critical Determinant of Subsequent Subcellular Localization*

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The development of transfection technology for malaria parasites holds significant promise for a more detailed characterization of molecules targeted by vaccines or drugs. One asexual blood stage vaccine candidate, apical membrane antigen-1 (AMA-1) of merozoite rhoptries has been shown to be the target of inhibitory, protective antibodies in both *in vitro* and *in vivo* studies. We have investigated heterologous (trans-species) expression of the human malaria *Plasmodium falciparum* AMA-1 (PF83/AMA-1) in the rodent parasite *Plasmodium berghei*. Transfected *P. berghei* expressed correctly folded and processed PF83/AMA-1 under control of both *pb66/ama-1* and *dhfr-ts* promoters. Timing of expression was highly promoter-dependent and was critical for subsequent subcellular localization. Under control of *pb66/ama-1*, PF83/AMA-1 expression and localization in *P. berghei* was limited to the rhoptries of mature schizonts, similar to that observed for PF83/AMA-1 in *P. falciparum*. In contrast the *dhfr-ts* promoter permitted PF83/AMA-1 expression throughout schizogony as well as in gametocytes and gametes. Localization was aberrant and included direct expression at the merozoite and gamete surface. Processing from the full-length 83-kDa protein to a 66-kDa protein was observed not only in schizonts but also in gametocytes, indicating that processing could be mediated outside of rhoptries by a common protease. Trans-species expressed PF83/AMA-1 was highly immunogenic in mice, resulting in a response against a functionally critical domain of the molecule.

The protozoan parasite *Plasmodium falciparum* is a causative agent of malaria, one of the major human infectious diseases. In the search for new methods to combat the disease, the advent of transfection technology for *Plasmodium* species is critical, because it offers the opportunity to relate genotype to phenotype, and this will permit a more rational design of vaccines and drugs. To date, stable episomal maintenance of plasmid DNA introduced into *Plasmodium* has been reported (1–3) as well as site-directed integration of DNA into the parasite genome (4–8). This technology also offers the possibility to dissect the thus far poorly characterized *Plasmodium* promoter function (9–11) and study the relation between the tightly controlled timing of expression and the subcellular trafficking and localization of stage-specific proteins. Trans-species expression of malarial antigens will allow targeted development of attenuated parasite vaccines and opens possibilities for complementation of otherwise detrimental integration into essential genes. Apical membrane antigen-1 (AMA-1)[1] is an attractive candidate for such studies, because it appears to be intimately involved in red cell invasion (12). Expression and post-translational N-terminal proteolytic cleavage of AMA-1 are restricted to the final stages of schizogony (13), during which the protein is localized within the neck of the rhoptry, an apical secretory organelle of the merozoite involved in red cell invasion (14). AMA-1 is a major candidate for inclusion in a malaria blood stage vaccine following *in vivo* experiments in nonhuman primates and rodents showing that AMA-1 can induce protective immune responses (15–17).

Here we report for the first time in a malaria parasite the development of drug-selectable trans-species expression of a second gene (in addition to the selectable marker) and its use to investigate the role of the promoter on subcellular localization of the trans-species expressed protein. *P. falciparum* AMA-1 (PF83/AMA-1) expression in the rodent malaria *Plasmodium berghei* was driven by the stage-specific *P. berghei* AMA-1 promoter (*pb66/ama-1*) or the more constitutive *P. berghei* dihydrofolate reductase-thymidylate synthase *(dhfr-ts)* promoter. The type of promoter control determined the timing and subsequent subcellular localization of PF83/AMA-1, which markedly differed between the two promoters. In addition, the trans-species expressed protein proved to be highly immunogenic in mice, resulting in antibodies to a critical functional determinant of PF83/AMA-1.

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[1] The abbreviations used are: AMA-1, apical membrane antigen-1; DHFR-TS, dihydrofolate reductase-thymidylate synthase; IFA, immunofluorescent assay; ORF, open reading frame; kb, kilobase pair(s); mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

DNA Constructs

The selection cassette controlling *Toxoplasma gondii* DHFR-TS expression and the pUC19 backbone (element $pD_B.D_{Tm}.D_B.$, nomenclature following Ref. 18) were common to all constructs used for transfection and were as described previously for transfection of *Plasmodium knowlesi* (3) except that *Tg dhfr-ts* (GenBank™ accession number L08489) was conservatively mutagenized (pAlter kit, Promega, Madison, WI) to remove *Eco*RI and *Kpn*I sites by use of oligonucleotides ToxM1 CCATGAAGAGTTCCAGTAC (base pairs 3722–3741) and ToxM2 CAACGGGGTTCCCTACGAC (base pairs 3064–3083), the altered residue being underlined. Through a series of cloning steps plasmids $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$ and $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$ and two plasmids that were identical but for reversed orientation of *pf83/ama-1* open reading frame (ORF) ($pD_B.D_{Tm}.D_B./D_B.\alpha\ A_F.D_B.$ and $pD_B.D_{Tm}.D_B./A_B.\alpha\ A_F.D_B.$) were derived (see Fig. 1). *Pf83/ama-1* was base pairs 1–1869 (complete ORF) of *P. falciparum* 7G8 strain (19). 5′ *pb66* was a 1.5-kb polymerase chain reaction fragment lying immediately 5′ to the *pb66/ama-1* ORF that had been amplified using sequence derived from a λZAP *P. berghei* ANKA genomic library probed with *pf83/ama-1*. Recombinant DNA manipulations and analyses were performed according to standard procedures (20).

Derivation and Maintenance of Transfected Parasites

P. berghei ANKA clone 15cy1 schizonts derived from infected Wistar rats were electroporated with constructs $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$, $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$, or a mixture of $pD_B.D_{Tm}.D_B./D_B.\alpha\ A_F.D_B.$ and $pD_B.D_{Tm}.D_B./A_B.\alpha\ A_F.D_B.$ as previously reported (1) except that cytomix was used (2). Electroporated parasites (2×10^8) were injected intravenously into phenylhydrazine-treated naive Wistar rats, and pyrimethamine treatment was begun (1). 9–10 days later when parasitemias had reached ≥0.5%, 100 μl of blood was transferred intraperitoneally to six Swiss mice, and pyrimethamine pressure maintained was. 4–6 days after infection, when parasitemias had reached 5%, mice were bled by cardiac puncture to provide parasites for DNA analysis and cryopreserved stocks. Parasites for analyses detailed below were derived from Swiss mice that had been infected with cryopreserved parasites and maintained under pyrimethamine pressure.

Analysis of Transfected Parasites

Leukocytes were removed from infected blood (Plasmodipur, Eurodiagnostica, Apeldoorn, The Netherlands) that was then either used directly or cultured for a further 12–24 h in an atmosphere of 5% O_2, 5% CO_2, 90% N_2 (21). Parasite DNA and RNA was isolated (Gentra Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Western blots following reduced SDS-polyacrylamide gel electrophoresis (Phast-system, 10–15% gradient gel, Amersham Pharmacia Biotech, Uppsala, Sweden) used parasite stages that had been enriched by Nycodenz centrifugation (21) and stored at −80 °C.

For purification of gametocytes, rats were infected with 10^8 *in vitro* matured, Nycodenz-purified schizont-infected red cells isolated from an infected rat at 1% parasitemia. 27 h after infection rats were bled, and gametocytes were Nycodenz purified (21).

Rat mAb Development and in Vitro Inhibition of Invasion Assay

The pan-specific AMA-1 rat mAb 28G2dc1 recognizes a linear determinant at the highly conserved C terminus and immunoprecipitates both the full-length 83-kDa and processed 66-kDa forms of PF83/AMA-1; rat mAb 58F8dc1 recognizes a linear determinant in the N-terminal region and only immunoprecipitates the 83-kDa form (13). Additional mAbs were developed from rats that had been immunized with recombinant PF83/AMA-1 (22) essentially as described (13).

For *in vitro* inhibition of invasion assays, purified IgG was incubated in triplicate with schizont-infected red blood cells at a parasitemia of 0.04% to 0.1% in 96-well flat-bottomed plates (Costar, Cambridge, MA) in a total volume of 100 μl (1.0–1.5% hematocrit) (T_{0h}). After two cycles of invasion (T_{65h}), 25 μl of RPMI 1640 containing 10% human serum, and [^{3}H]hypoxanthine (Amersham International, 's-Hertogenbosch, The Netherlands) was added to each well to yield a final concentration of 20 μCi ml^{-1}. Parasites were harvested (T_{87h}) onto glass fiber filters using a Skatron cell harvester (Suffolk, UK), and [^{3}H]hypoxanthine incorporation was determined by liquid scintillation spectrometry. Parasite growth inhibition, reported as a percentage, was determined as follows: ((mean $cpm_{control}$ − mean $cpm_{experimental}$)/mean $cpm_{control}$) × 100. The cpm for red blood cells alone was subtracted from all averages prior to determining the percentage of inhibition. All analyses of statistical significance were performed by Student's *t* test.

IFA and Immunoelectron Microscopy

Methanol-fixed thin films were prepared and used for IFA as described previously (13), and in some experiments mAbs that had been directly succinamide-conjugated with fluorophores (Molecular Probes, Leiden, The Netherlands) were used according to the manufacturer's instructions. Slides were mounted in anti-fade (5% (w/v) 1,4-diazobicyclo[2.2.2 octane], 10 mM Tris-HCl, pH 8, 90% glycerol) to which 4,6-diamidino-2-phenylindole (23) at a final concentration of 1.9 μM was added. Photographs were taken with a Photometrics CH250 cooled CCD camera mounted on a Leica DMRXA microscope. Digital images that were generated were all treated identically. IFA on fresh unfixed gametes was performed with a mixture of 28G2dc1, 58F8dc1, and 4G2dc1 mAb culture supernatants on homogenized mid-guts of *Anopheles stephensi* at various times after they had fed on infected Swiss mice (24). Immunoelectron microscopy was performed on gametocytes and schizonts transfected with PF83/AMA-1 expressed under the *P. berghei dhfr-ts* promoter. Cells harvested as for IFA were fixed (20 min on ice in 0.1% (v/v) double-distilled glutaraldehyde in RPMI), washed four times in fresh ice-cold RPMI, then dehydrated in a series of ethanols cooled progressively from 0 to −20 °C, infiltrated with LR White Resin overnight, and polymerized at room temperature under indirect ultraviolet light for 48 h (25). Sectioned material was stained using mAb 58F8dc1 at 25 μg ml^{-1} and secondary goat anti-rat antibodies labeled with 10-nm gold. Grids were post-stained for 2 min in 2% aqueous uranyl acetate. Controls were parasites transfected with *pf83/ama-1* ORF in the reverse orientation.

ELISA

Total IgG—ELISA plates (Greiner, Labortechnik, Solingen, Germany) were coated overnight at +4 °C with 100 ng ml^{-1} PF83-7G8-1 (22) in phosphate-buffered saline/0.02% NaN_3, pH 7.4, and blocked with 3% bovine serum albumin/phosphate-buffered saline. Dilutions of mouse sera in antibody buffer (0.5% bovine serum albumin/phosphate-buffered saline, pH 8.0) were tested in triplicate, and bound IgG was detected by goat anti-mouse IgG coupled to alkaline phosphatase as described previously (26).

Competition ELISA—ELISA plates were prepared as above. Rat mAb 4G2dc1 was coupled to alkaline phosphatase (Sigma-Aldrich N.V./S.A., Bornem, Belgium) (27). Duplicate mouse sera (diluted 1:100) and mAb 4G2dc1 (30 μg ml^{-1}) in an optimized concentration of alkaline phosphatase coupled 4G2dc1 in antibody buffer were incubated in wells coated with PF83-7G8-1 (90 min, 37 °C). The plates were washed, substrate was added (2 h), and the A_{405} was determined. Inhibition of 4G2dc1 binding was calculated using the average OD reading for each group of mice relative to the average OD reading for a pool of normal Swiss mouse serum.

Immunization of Mice with Transfected P. berghei

To assess specific antibody production in animals infected with transfected parasites, infected mice received regular pyrimethamine treatment to maintain plasmids in the parasite population. Parasitemia was controlled when it reached 1% by sulfadiazine treatment (10 mg/liter drinking water) for 2–4 days (28) until parasites were barely detectable by Giemsa thin film. Mice were chloroquine-treated after 5 weeks to kill all remaining parasites and allow recovery and were reinfected with the same transfected parasites 3 weeks later (2×10^7 schizonts/mouse intraperitoneally). To counteract bone marrow suppression induced by pyrimethamine, these mice received folinic acid intraperitoneally once per week (400 μg/kg).

RESULTS

Transfected P. berghei Parasites Express PF83/AMA-1—The rodent malaria *P. berghei* was transfected with constructs that were designed to express PF83/AMA-1 controlled by two different promoters. The selection cassette was based on the ability to confer resistance to pyrimethamine, and for this an engineered *T. gondii dhfr-ts* gene was chosen because of its high resistance levels and the reduced likelihood of unwanted homologous integration (3). To allow easier manipulation of the selection cassette *T. gondii dhfr-ts* was mutagenized to remove *Eco*RI and *Kpn*I sites. This mutant *T. gondii dhfr-ts* was flanked by *P. berghei dhfr-ts* control regions. In addition to the selectable marker cassette, vectors $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$ (Fig. 1*A*) and $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$ (Fig. 1*B*) respectively employed the *pb66/ama-1* promoter or the *P. berghei dhfr-ts*

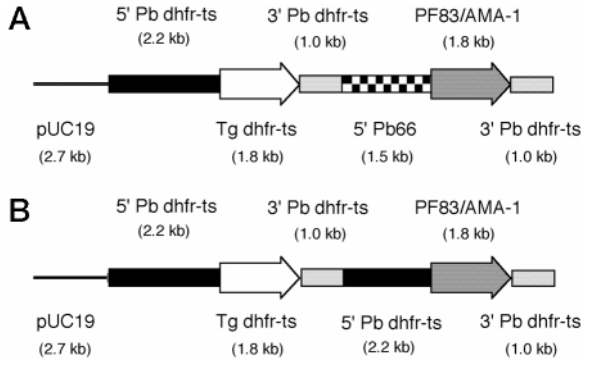

FIG. 1. **Plasmid constructs used for the trans-species expression of PF83/AMA-1 in *P. berghei*.** Plasmids contained *Tg dhfr-ts* controlled by *P. berghei dhfr-ts* 5′ and 3′ regions to enable selection of transfected parasites on the basis of pyrimethamine resistance. PF83/AMA-1 expression was under control of a 1.5-kb 5′ region of *pb66/ama-1* ($pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$) (*A*) or a 2.2-kb 5′ region of *P. berghei dhfr-ts* ($pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$) (*B*) and 3′ *P. berghei dhfr-ts* regions. Constructs $pD_B.D_{Tm}.D_B./A_B.\alpha\ A_F.D_B.$ and $pD_B.D_{Tm}.D_B./D_B.\alpha\ A_F.D_B.$ are identical except that the *pf83/ama-1* ORF is in reversed orientation.

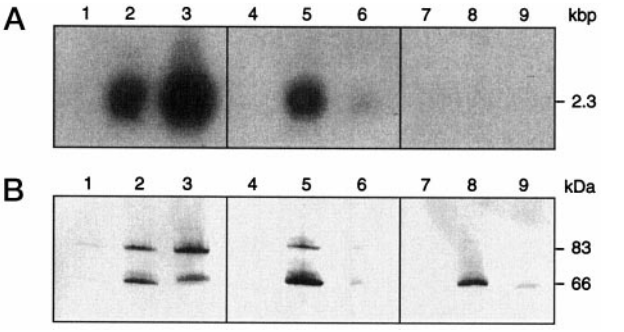

FIG. 2. **Northern blot (*A*) and Western blot (*B*) analyses of transfected *P. berghei* parasites.** Parasites were transfected with vector $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$ (*lanes 1–3*), vector $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$ (*lanes 4–6*), or a mixture of vectors $pD_B.D_{Tm}.D_B./A_B.\alpha\ A_F.D_B.$ and $pD_B.D_{Tm}.D_B./D_B.\alpha\ A_F.D_B.$ (*lanes 7–9*). Parasites were purified at ring stage (*lanes 1*, *4*, and *7*), schizont stage (*lanes 2*, *5*, and *8*), and gametocyte stage of development (*lanes 3*, *6*, and *9*). *A*, total RNA was isolated from purified parasite populations and fractionated on a 1% agarose-formaldehyde gel (RNA from 10^7 parasites/lane), blotted onto a nylon membrane, and probed with a ^{32}P-labeled *pf83/ama-1* ectodomain probe under stringent conditions. The 2.3-kb mRNA (*lanes 2*, *3*, and *5*) mirrors PF83/AMA-1 protein expression. *B*, extracts were fractionated by SDS-polyacrylamide gel electrophoresis under reducing conditions (5×10^5 parasite equivalents/lane) and blotted onto a nitrocellulose membrane. The blot was reacted with the panspecific mAb 28G2dc1. 83- and 66-kDa forms of PF83/AMA-1 are evident in transfected schizonts (*lanes 2* and *5*) and in gametocytes when under *dhfr-ts*-promoter control (*lane 3*). Control transfected schizonts only express the 66-kDa Pb66/AMA-1 (*lane 8*).

promoter to control PF83/AMA-1 expression. Transfection of *P. berghei* schizont-infected red blood cells with these constructs yielded pyrimethamine-resistant parasites. Southern blot, polymerase chain reaction, and plasmid rescue analyses showed that these parasites contained the *T. gondii dhfr-ts* gene, indicating that the *T. gondii* DHFR-TS is active in *P. berghei* and that the mutagenesis of *T. gondii dhfr-ts* had no detrimental effects on DHFR-TS expression (data not shown).

Pyrimethamine-resistant transgenic *P. berghei* parasite populations obtained from mice were matured *in vitro* and analyzed for the presence of the AMA-1 protein of the human malaria *P. falciparum*. IFA with mAb 58F8dc1 specific for the N-terminal region of PF83/AMA-1 reacted strongly with recombinant *P. berghei* but not with control *P. berghei* parasites, indicating that PF83/AMA-1 was expressed.

Developmental Stage-specific Trans-species Expression of PF83/AMA-1 Is Promoter-mediated—Northern blots of total RNA isolated from 1×10^7 rings, schizonts, or gametocytes and probed with *pf83/ama-1* showed high levels of a 2.3-kb mRNA in schizonts and gametocytes of parasites transfected with the construct containing the *P. berghei dhfr-ts* promoter, whereas under control of the *pb66/ama-1* promoter, transcription was only observed in schizonts (Fig. 2*A*, *lanes 2*, *3*, and *5*). The weak hybridization signal in *lane 9* (gametocytes transfected with the *pb66* promoter construct) can be accounted for by slight contamination of this preparation with mature schizonts (verified in Giemsa-stained thin films). No transcripts were detected in ring stage parasites nor in parasites transfected with *pf83/ama-1* in the reverse orientation. Hybridization of these Northerns with a *T. gondii dhfr-ts* probe showed transcription of the selectable marker gene in all schizont and gametocyte lanes but not in the lanes from ring stage parasites.

Western blot analysis using a pan-specific mAb (28G2dc1) that reacts with a linear determinant present in all *Plasmodium* AMA-1 molecules identified to date revealed expression of PF83/AMA-1 as a full-length 83-kDa protein in mature schizonts. No expression was detected in ring stage parasites, irrespective of the promoter used to drive expression (Fig. 2*B*, *lanes 2* and *5*). In addition, expression controlled by the *dhfr-ts* promoter was detected in gametocytes (Fig. 2*B*, *lane 3*). Analogous to the situation in *P. falciparum* (13), N-terminal proteolytic processing to a form of approximately 66 kDa was observed (Fig. 2*B*, *lanes 2* and *5*). This processed form migrated as the slightly larger molecule in a 66-kDa doublet, the other component of which was authentic *P. berghei* AMA-1, which was also reactive with mAb 28G2dc1 (Fig. 2*B*, *lanes 2*, *5*, and *8*). As expected authentic P. berghei AMA-1 expression was restricted to schizonts. Confirmation of this expression profile for full-length PF83/AMA-1 expression was obtained through reactivity of the 83-kDa AMA-1 with the N-terminal mAb 58F8dc1, which does not react with P. berghei AMA-1 (data not shown). Interestingly, *P. berghei* gametocytes that express PF83/AMA-1 under control of *P. berghei dhfr-ts* also showed processing to the 66-kDa form (Fig. 2*B*, *lane 3*). No PF83/AMA-1 expression was evident when *P. berghei* was transfected with constructs containing *pf83/ama-1* in the reverse orientation (Fig. 2*B*, *lanes 7*, *8*, and *9*). The weak 66-kDa signal in the gametocyte *lanes 6* and 9 can be accounted for by minor contamination of the gametocyte preparations with mature schizonts.

Trans-species Expressed PF83/AMA-1 Attains a Functional Conformation—AMA-1 contains multiple disulfide links (29) that generate species-specific epitopes that are critical to vaccine efficacy (11, 17) and protein function (30). Rat mAbs capable of blocking *P. falciparum* AMA-1 function were selected from a panel of mAbs characterized by recognition of reduction-sensitive, native, parasite determinants present on both full-length and processed PF83/AMA-1. Thus all mAbs in this panel immunoprecipitated both full-length (83-kDa) and processed (66-kDa) forms of PF83/AMA-1 from Triton X-100 extracts of metabolically radiolabeled *P. falciparum* schizonts but were not reactive with Western blots of *P. falciparum* schizonts. One of these mAbs, 4G2dc1 (IgG2a) consistently inhibited asexual *P. falciparum* multiplication *in vitro* (by 60–70% compared with control mAbs of the same isotype). This mAb reacted by IFA with all ten *P. falciparum* strains of diverse geographical origin analyzed to date. Critically, 4G2dc1 also recognized transfected *P. berghei* parasites by IFA when PF83/AMA-1 was expressed under both *pb66* and *pb-dhfr* promoter control.

Temporal Regulation of Trans-species PF83/AMA-1 Expression—A more detailed analysis of PF83/AMA-1 protein expression in transgenic *P. berghei* was performed by IFA with mAb 4G2dc1. Parasites transfected with $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$,

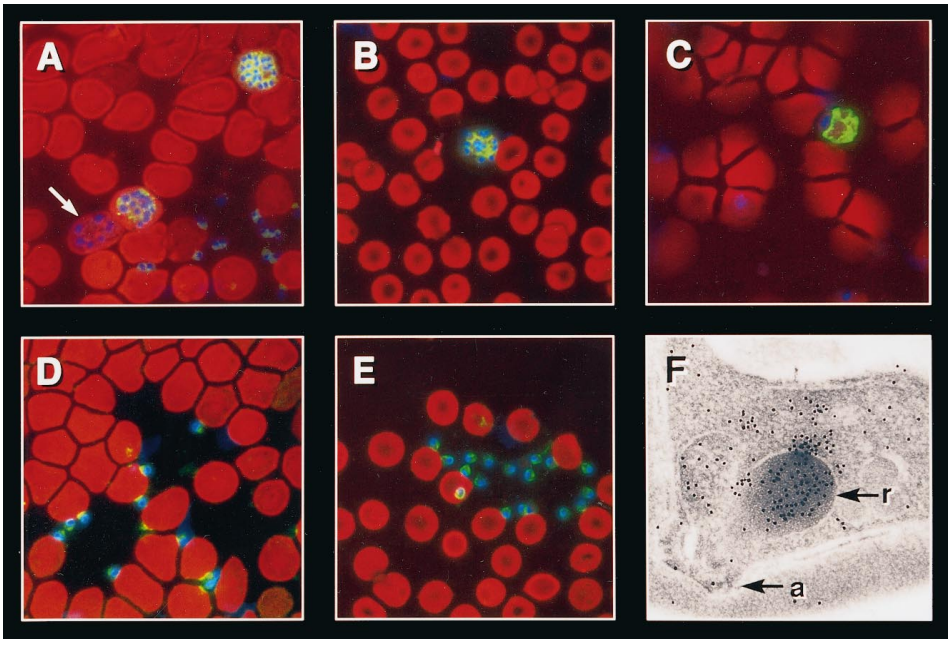

FIG. 3. **Immunofluorescent and immunoelectron microscopic analyses of transfected *P. berghei* parasites expressing PF83/AMA-1.** For IFA, methanol-fixed thin films were reacted with the PF83/AMA-1-specific mAb 4G2dc1, and parasite nuclei were stained with 4,6-diamidino-2-phenylindole. *A*, *P. berghei* schizont-infected red cells at various stages of development (six and twelve nuclei, and segmented schizont) expressing PF83/AMA-1 under control of the *pb66/ama-1* promoter. PF83/AMA-1 expression is barely visible at the six-nucleus stage (*arrow*), but prominent at the twelve-nucleus stage (*middle parasite*) and in segmented schizonts (*top parasite*). *B*, *P. berghei* mature schizont-infected red cell expressing PF83/AMA-1 under control of the *dhfr-ts* promoter, showing high level expression. Similar expression was observed in mature trophozoites (not shown). *C*, *P. berghei* gametocyte expressing PF83/AMA-1 under control of the *dhfr-ts* promoter. *D* and *E*, free merozoites expressing PF83/AMA-1 under control of the *pb66/ama*-1 promoter showing apically restricted fluorescence (*D*) and under control of the *dhfr-ts* promoter showing predominantly circumferential and cytoplasmic fluorescence (*E*). *F*, electron microscopic section through a developing merozoite within a schizont of *P. berghei* expressing PF83/AMA-1 under control of the *dhfr-ts* promoter. Note the rhoptry body staining along with adjacent regions of cytoplasm. The section was immunostained with 58F8dc1 (specific for the N terminus of PF83/AMA-1), and secondary antibodies were labeled with 10-nm gold (magnification, ×64,000).

using the *pb66/ama-1* promoter to control PF83/AMA-1 expression, expressed PF83/AMA-1 only in maturing schizonts with six or more nuclei (Fig. 3*A*). In contrast, in parasites obtained after transfection with $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$, using the *dhfr-ts* promoter to control expression, PF83/AMA-1 was initially observed in maturing trophozoites and during onset of schizogony and could be detected throughout schizont development (Fig. 3*B*). PF83/AMA-1 expression in native conformation was also evident in gametocytes of both sexes when under control of the *dhfr-ts* promoter (Fig. 3*C*). IFA analysis of activated gametes in homogenized mosquito mid-gut revealed PF83/AMA-1 expression 1 h after a blood meal but not 24 h after feeding of the mosquitoes (data not shown). Parasites obtained after transfection with the construct containing the *pb66/ama-1* promoter consistently failed to show expression of PF83/AMA-1 in gametocytes and gametes (data not shown).

Subcellular Localization of Trans-species Expressed PF83/AMA-1 Is Promoter-dependent—Quantitation of PF83/AMA-1 expression under control of the *pb66/ama-1* promoter by counting fluorescence patterns in 1000 free merozoites yielded PF83/AMA-1 localization entirely to the apex of 95% of the merozoites (Fig. 3*D*). In contrast, expression under control of the *dhfr-ts* promoter yielded in 85% of the merozoites a strong circumferential and cytoplasmic staining in addition to occasional weak apical staining (Fig. 3*E*). This difference in localization is already evident in maturing schizonts (Fig. 3, *A* and *B*), where the protein when expressed under *ama-1* promoter control is apparently associated with developing organelles, whereas under *dhfr* promoter control, a much more diffuse localization is evident.

Immunoelectron microscopy analysis of expression under the *dhfr-ts* promoter shows that PF83/AMA-1 is distributed patchily in the cytoplasm of merozoites, and in some rhoptries, it localizes to the rhoptry body but is not found in micronemes or dense granules (Fig. 3*F*). In maturing gametocytes the protein is associated with the endoplasmic reticulum network (not shown). IFA analyses of gametes performed on unfixed material 1 h after mosquito feeding revealed PF83/AMA-1 expression at the gamete surface.

Immunization with Transfected P. berghei Parasites Yields High Titer Antibodies to PF83/AMA-1—To determine whether *P. berghei* parasites that expressed PF83/AMA-1 could induce an immune response against PF83/AMA-1, two groups of Swiss mice were infected with *P. berghei* transfected either with mixed $pD_B.D_{Tm}.D_B./D_B.A_F.D_B.$ and $pD_B.D_{Tm}.D_B./A_B.A_F.D_B.$ (forward *pf83/ama-1*) or with mixed $pD_B.D_{Tm}.D_B./D_B.\alpha\ A_F.D_B.$ and $pD_B.D_{Tm}.D_B./A_B.\alpha\ A_F.D_B.$ (reverse *pf83/ama-1*). Sulfadiazine modulation of parasite growth was used to permit two sequential peaks of parasitemia of approximately 1% (28). Parasitemia peaks were observed at the end of the first week after infection and at the beginning of the third week after infection. A second infection was given in week 9. Expression of PF83/AMA-1 was monitored by IFA on parasites that were obtained from infected mice and matured (18 h) *in vitro* and was evident throughout the experiment. Antibodies reactive with PF83/AMA-1 were already detected at week 4 as determined by ELISA on pooled sera (end point titer 1:20,000; data not shown), and by week 11 antibody titers were >1:100,000 (Fig. 4), whereas in the control group titers of approximately 1:10,000 were observed. This control group reactivity can be explained by cross-reactive antibodies induced by *P. berghei* AMA-1. To unequivocally demonstrate the appearance of PF83/AMA-1 specific reactivity and determine whether the functional epitope defined by mAb 4G2dc1 was recognized, a competition ELISA was performed. Only antibodies of mice

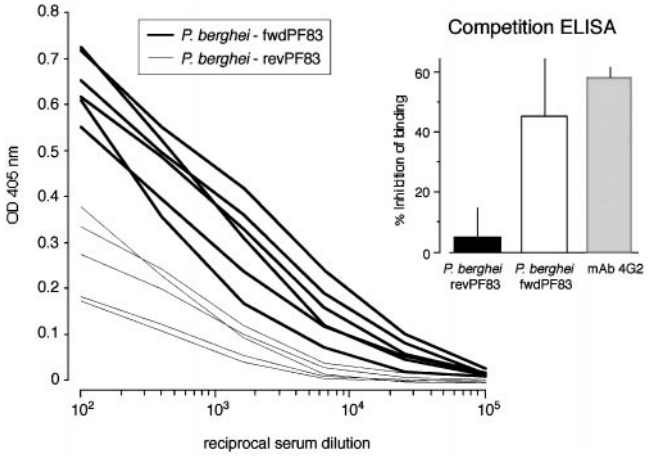

FIG. 4. **ELISA titration of individual mouse sera 11 weeks after infection with transfected *P. berghei*.** Mice were infected with *P. berghei* transfected with *pf83/ama-1* (*thick lines*) or with reverse ORF *pf83/ama-1* (*thin lines*), allowed to experience two waves of parasitemia over a 4-week period, and were finally boosted with 2×10^7 schizonts at week 8. Titration was performed using recombinant full-length PF83/AMA-1 as solid phase antigen. The *inset* shows results of a competition ELISA demonstrating that only IgG from the experimental group of mice competes with mAb 4G2dc1 for binding to PF83/AMA-1 (>45% inhibition of binding). *Lines* over the *bars* indicate standard deviations.

immunized with transfected *P. berghei* parasites that were expressing PF83/AMA-1 effectively competed with mAb 4G2dc1 (Fig. 4), demonstrating that components of the immune response were directed against a critical functional epitope of PF83/AMA-1.

DISCUSSION

The development of transgene expression systems for malaria will allow detailed study of parasite cell biology. Mechanisms underlying drug resistance, protein trafficking, and molecular function can be explored in greater depth than previously possible, and manipulation of parasite phenotype for evaluation of attenuated vaccines, for example toward higher immunogenicity and lower pathogenicity, becomes feasible. Here we report on the first studies of trans-species expression of a human malaria vaccine candidate antigen and show (i) that the protein is expressed in a conformationally and functionally relevant form, (ii) that depending on the time of expression it is differentially routed within the developing parasite, and (iii) that it is highly immunogenic within the context of a rodent malaria parasite. These studies employed an asexual blood stage vaccine candidate, AMA-1, a single copy gene that has a well defined, tightly controlled, stage-specific expression pattern (13, 14, 31) and contains targeting signals for the rhoptries, organelles involved in red cell invasion. Vaccine experiments to date have shown that deletion mutants of AMA-1 are not induced under immune pressure (15, 16), indicating it is an essential molecule. As a prelude to AMA-1 knockout experiments, which we expect to be lethal because the parasite genome during asexual phase development is haploid, we have developed trans-species complementation capabilities for this molecule. In this study two promoters controlled PF83/AMA-1 expression, the authentic *P. berghei ama-1* promoter, and the *P. berghei dhfr-ts* promoter. Both promoters induced expression of conformationally intact, full-length PF83/AMA-1. However, the time of appearance and the subcellular distribution of the protein were substantially different. PF83/AMA-1 conformation was assessed by reactivity with a mAb (4G2dc1) that inhibited *P. falciparum* invasion of erythrocytes *in vitro*. This mAb is reactive with an invariant, reduction-sensitive conformational and functional epitope and provides a new tool to assess the correct conformation of *P. falciparum* AMA-1 molecules.

Trans-species PF83/AMA-1 expression under control of the *pb66/ama-1* promoter was analogous to authentic PF83/AMA-1 expression in *P. falciparum* (13), restricted to schizonts with six or more nuclei. This demonstrates that the 1.5 kb of the *pb66/ama-1* upstream region present in the expression construct contains sufficient information to drive stage-specific expression of PF83/AMA-1. Under *P. berghei dhfr-ts* promoter control, PF83/AMA-1 was expressed throughout schizogony as well as in gametocytes, coordinated with DHFR-TS expression from the genomic copy (32) in developmental stages where DNA synthesis is ongoing. Although, because eight male gametes are produced from each microgametocyte, it was not surprising that under *dhfr-ts* promoter control PF83/AMA-1 was expressed in male gametocytes, approximately 30% of PF83/AMA-1-expressing gametocytes were female. It remains to be determined whether these results indicate that DHFR-TS is also normally synthesized in female gametocytes (perhaps in preparation for post-fertilization DNA synthesis) or whether the *dhfr-ts* driven expression observed is a consequence of loss of transcriptional control because of incomplete 5′ elements or loss of chromosomal positioning. Despite prolonged exposure we found no evidence for *dhfr-ts* mRNA transcripts in ring stages, in contrast to a previous report (32).

The timing of expression markedly influenced the subcellular localization of PF83/AMA-1. When expressed under the *ama-1* promoter, the protein is routed to the rhoptries, as is authentic AMA-1 (13, 14, 33). In contrast, expression controlled by the *dhfr-ts* promoter during the onset of schizogony, when rhoptries are absent, as well as in gametes that do not contain rhoptries, results in targeting to the parasite surface as well as in a cytoplasmic localization, as is evident from both IFA and immunoelectron microscopy data. When rhoptries are being formed, *dhfr-ts*-controlled PF83/AMA-1 is also routed to the rhoptries but is notably localized to the rhoptry body (Fig. 3*F*) rather than authentically localized in the rhoptry neck (14). At this stage cytoplasmic localization is also detected, but the fate of this cytoplasmically localized protein is not yet clear. Additional immunoelectron microscopy studies are currently being performed to more closely define the localization of authentic and trans-species expressed AMA-1 in relation to the promoter used to drive expression. Exchanging putative signal and targeting sequences in future transfection experiments will start to unravel the trafficking of rhoptry and other organellar proteins to their final destination.

Proteolytic processing of PF83/AMA-1 from an 83- to a 66-kDa form occurred both in schizonts and in gametocytes, suggesting that the processing is mediated by a common parasite protease that is not restricted to the rhoptry. This processing event does not occur in *P. berghei* AMA-1 because it is synthesized *de novo* as a 66-kDa molecule, as are all other AMA-1 forms reported to date. This extra N-terminal region, present only in *P. falciparum,* is of unknown function, but its cleavage seems to be associated with capacity for merozoite invasion of red cells (13).

Although there are significant obstacles to the development of vaccines based on the malaria parasite itself, it is possible that effective vaccines may ultimately be based upon attenuated parasites or upon nonpathogenic species that are genetically modified to carry heterologous target proteins. To evaluate the immunogenicity of PF83/AMA-1 expressed in the context of a *P. berghei* infection, mice were chronically infected with transgenic *P. berghei* parasites. The observed responses and the fine specificity thereof demonstrate that trans-species expression of the human *P. falciparum* antigen PF83/AMA-1 in

P. berghei can elicit a strong immune response directed against a functionally important region of this molecule. Given the protection induced in primate models after AMA-1 vaccination (15, 16), it will be interesting to evaluate whether trans-species expression can provide protection against a challenge with a heterologous parasite species. This is not possible in the system described here because rodents are not susceptible to infection with *P. falciparum*. However, as a prelude to such studies in other systems we have recently shown that expression of AMA-1 from other primate malaria species is feasible in *P. knowlesi*,[2] a parasite of relatively broad host specificity.

In summary, we have demonstrated drug-selectable trans-species expression of a second gene (in addition to the selectable marker) in a malaria parasite. *P. falciparum* AMA-1 expression in *P. berghei* under control of the stage-specific *P. berghei pb66/ama-1* promoter or the more constitutive *P. berghei dhfr-ts* promoter resulted in a timing and subsequent subcellular localization of PF83/AMA-1, which markedly differed between the two promoters. In addition, antibodies to a critical functional determinant of PF83/AMA-1 were elicited in mice, emphasizing strong immunogenicity of the trans-species expressed protein.

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