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 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) 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 containing Toxoplasma gondii DHFR-TS expression and the pUC19 backbone (element pDp66DpAMA-1, 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 mutated (pAlter kit, Promega, Madison, WI) to remove EcoRI and KpnI sites by use of oligonucleotides ToxM1 CCATGAGAAGTTCCAGTAC (base pairs 3722–3741) and ToxM2 CAACGGGGTTGCCCCAGCAC (base pairs 3064–3083), the altered residue being underlined. Through a series of cloning steps plasmids pDp83DpAMA-1, pDpAMA-1, and pDpAMA-2 were produced that were identical but for reversed orientation of p83/ama-1 open reading frame (ORF) (pDpAMA-1). Phenotypically DHFR-TS positive Wistar rats were treated with pyrimethamine, and pyrimethamine treatment was begun (1). 9–10 days later when parasitemias had reached 0.1% (v/v) double-distilled glutaraldehyde in RPMI, washed four times in fresh ice-cold RPMI, then dehydrated in a series of ethanol 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 p83/ama-1 ORF in the reverse orientation.

ELISA

Total IgG—ELISA plates (Greiner, Labotechnik, Solingen, Germany) were coated overnight at +4 °C with 100 ng ml−1 PF83-TGS-1 (22) in phosphate-buffered saline/0.02% NaN3, 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-TGS-1 (90 min, 37 °C). The plates were washed, substrate was added (2 h), and the A405 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 × 107 schizonts/mouse intraperitoneally). To counteract bone marrow suppression induced by pyrimethamine, these mice received folic 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 (5). To allow easier manipulation of the selection cassette T. gondii dhfr-ts was mutagenized to remove EcoRI and KpnI sites. This mutant T. gondii dhfr-ts was flanked by P. berghei dhfr-ts control regions. In addition to the selectable marker cassette, vectors pDpAMA-1, pAMA-1, and pAMA-2 were employed the pb66/ama-1 promoter or the P. berghei dhfr-ts...
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, polyacrylamide 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 5F8dc1 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 1×10⁷ 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. 2A, 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 Northern blots 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. 2B, lanes 2 and 5). In addition, expression controlled by the dhfr-ts promoter was detected in gametocytes (Fig. 2B, lane 3). Analogous to the situation in *P. falciparum* (13), N-terminal proteolytic processing to a form of approximately 66 kDa was observed (Fig. 2B, 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. 2B, 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 5F8dc1, 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. 2B, 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. 2B, 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 showed species-specific epitopes that are critical to vac

**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<sub>B</sub>DT<sub>bm</sub>D<sub>N</sub>/A<sub>B</sub>A<sub>F</sub>D<sub>N</sub>,
Trans-species Expression in Plasmodium berghei

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. 3A). In contrast, in parasites obtained after transfection with pDB.DTm.DB./DB.AF.DB., using the dhfr-ts promoter to control expression, PF83/AMA-1 was initially observed in mature merozoites and during onset of schizogony and could be detected throughout schizont development (Fig. 3B). PF83/AMA-1 expression in native conformation was also evident in gametocytes of both sexes when under control of the dhfr-ts promoter (Fig. 3C). IFA analysis of activated gametocytes 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 cytofluorescence patterns in 1000 free merozoites yielded PF83/AMA-1 localization entirely to the apex of 95% of the merozoites (Fig. 3D). 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. 3E). This difference in localization is already evident in maturing schizonts (Fig. 3A 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. 3F). In maturing gametocytes the protein is associated with the endoplasmic reticulum network (not shown). IFA analyses of gametocytes 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 pDB.DTm.DB./DB.AP.DB. and pDB.DTm.DB./AP.DB. (forward pf83/ama-1) or with mixed pDB.DTm.DB./DB.AP.AP.DB. (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
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 proteins to their final destination.

**Trans-species Expression in *Plasmodium berghei***

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 gametocytes 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 not normally localized to the rhoptry body (Fig. 3F) 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 rhoptries and other organelar 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
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*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 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 *P. falciparum* in *P. berghei* can elicit a strong immune response directed against other primate malaria species that is feasible in *P. knowlesi*, 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* pb66 promoters 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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\(^{2}\) C. H. M. Kocken, A. M. van der Wel, and A. W. Thomas, unpublished observation.