Expression of full-length *Plasmodium falciparum* P48/45 in *P. berghei* blood stages: A method to express and evaluate vaccine antigens

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**ABSTRACT**

The transmission-blocking vaccine candidate Pf64/45 from the human malaria parasite *Plasmodium falciparum* is known to be difficult to express in heterologous systems, either as full-length protein or as correctly folded protein fragments that retain conformational epitopes. In this study we express full-length Pf64/45 in the rodent parasite *P. berghei*. Pf64/45 is expressed as a transgene under control of the strong *P. berghei* schizont-specific *mmsp1* gene promoter (Pfs48/45@PbMSP1). Pfs48/45@PbMSP1 schizont-infected red blood cells produced full-length Pf64/45 and the structural integrity of Pfs48/45 was confirmed using a panel of conformation-specific monoclonal antibodies that bind to different Pfs48/45 epitopes. Sera from mice immunized with transgenic Pfs48/45@PbMSP1 schizonts showed strong transmission-reducing activity in mosquitoes infected with *P. falciparum* using standard membrane feeding. These results demonstrate that transgenic rodent malaria parasites expressing human malaria antigens may be used as means to evaluate immunogenicity and functionality of difficult to express malaria vaccine candidate antigens.

1. Introduction

Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed [1,2]. This hampers the screening of *Plasmodium* antigens in immunization studies for their suitability as vaccine candidate antigens. Preclinical evaluation of *Plasmodium* antigens often involves immunizing rodents with recombinant *Plasmodium* proteins followed by an examination of induced immune responses, either in vivo using rodent models of malaria or in vitro by performing functional assays with human malaria parasites incubated with immune sera [3]. Multiple factors contribute to inefficient expression of *Plasmodium* proteins, such as the high AT content of *Plasmodium* genes, large size and often unique protein structure (i.e. encoding repeated stretches of amino acids) and unique post-translational modifications [1,4]. This is particularly evident for cysteine-rich proteins where correct folding depends on accurate formation of disulfide bridges to form domains specific for *Plasmodium* proteins [5–7]. Transgenic rodent malaria parasites (RMP) expressing human malaria parasite (HMP) proteins are increasingly used to evaluate and rank order candidate malaria vaccines before investing in scalable manufacture to support advancement to clinical testing [3]. Such transgenic RMP have been used in preclinical assays to evaluate vaccine potential of HMP proteins, both in vivo where mice are immunized with HMP antigens and subsequently challenged with transgenic RMP expressing the cognate HMP or in *in vitro* assays where immune sera or antibodies are evaluated for inhibition of parasite growth or invasion. Both the functional complementation of RMP genes by the HMP orthologs [3] and analysis of HMP expression using antisera, provide evidence for correct expression...
of functional HMP proteins in transgenic RMP [8]. Based on these studies, we reasoned that transgenic RMP can be used as expression systems to more efficiently express, screen, validate and down-select HMP antigens as potential novel malaria vaccine candidates [2,9]. Further, the expression of conformationally-accurate Plasmodium proteins could be used to generate epitope-specific monoclonal antibodies, which in turn can be used to better characterize the vaccine antigen.

The use of RMP would circumvent many of the above-mentioned problems associated with expression in heterologous expression systems including, but not limited to, peculiarities of post-translational modifications and Plasmodium-specific domains involved in protein trafficking and cellular location. As a proof of concept, we generated transgenic P. berghei (Pb) parasites that express full length Pfs48/45 from P. falciparum (Pf). The Pfs48/45 protein is expressed in
Fig. 1. Generation, genotype and phenotype analyses of Pf48/45@PbMSP1, a transgenic P. berghei parasite expressing P. falciparum Pf48/45 in schizonts. (A) Schematic representation of the introduction of the Pf48/45 expression cassette into the GIMO<sub>PBANKA</sub> parasite (line 1596c11). Construct pL1707 contains the Pf48/45 gene flanked by the map1 promoter region and the 3′ pbdhfr UTR. This construct is integrated into the modified P. berghei 230p locus of GIMO<sub>PBANKA</sub>, which contains the hdrofr:yfcu selectable marker (SM) cassette by double cross-over homologous recombination at the homology regions (230p; grey boxes). Negative selection with 5-FC selects for parasites that have the SM cassette replaced by the Pf48/45 expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (upper panel) and Southern analysis of PFG-separated chromosomes (lower panel) confirm correct integration of construct pL1707 in line 1807c2 parasites. PCR shows the absence of the hdrofr:yfcu marker and the presence of the Pf48/45. 5′ integration PCR (5′ int; primers p5/p6), 3′ integration PCR (3′ int; primers p7/p8), hdrofr:yfcu (primers p1/p2). Pf48/45 (primers p3/p4). Primer locations and product sizes are shown in A and primer sequences in Table S1. Hybridization of PFG-separated chromosomes with a mixture of two probes (the hdrofr probe and a control probe recognizing p25 gene on chromosome 3) shows the removal of the SM cassette marker in the 230p locus on chromosome 3 in 1807c2 parasites. (C) Western analysis of Pf48/45 expression in protein extracts of purified gametocytes of P. falciparum (PF Gam), purified schizonts of wild type P. berghei (1596c11) and purified schizonts of Pf48/45@PbMSP1 (1807c2l). As a positive control, recombinant P. falciparum Pf48/45 fragment fused to GLURP R0 domain (R0.10c) was included (expected molecular size is 150 kDa). Blots were stained with 4 different anti-Pf48/45 antibodies (45.1-3, 45.5) that recognize different epitopes. Anti-PyMSP1 antibody staining was used as a loading control. (D) Immuno-fluorescence analyses of Pf48/45 expression in purified schizonts of Pf48/45@PbMSP1 (1807c2l), and the reference parent P. berghei GIMO line (i.e. WT; 1596c11). Fixed parasites were stained with four different rat anti-Pf48/45 mAbs (45.1-3, 45.5) and rabbit anti-PyMSP1 antibody followed by secondary conjugated antibodies anti-rabbit IgG Alexa Fluor *488 (green) or anti-rat IgG Alexa Fluor *594 (red). Nuclei stained with the DNA-specific dye Hoechst 33,342 (H). All pictures were recorded with the same exposure/gain times; anti-rabbit IgG Alexa Fluor *488 (green) 0.7 s; anti-rat IgG Alexa Fluor *594 (red) 0.6 s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). BF: bright field; M: merged. Scale bar: 2 μm.

Plasmodium gametocytes and gametes [10,11] and contains multiple cysteine-rich domains with multiple disulfide bonds [12–14]. These constitute distinct conformational B cell epitopes that can be recognized by several monoclonal antibodies some of which have transmission-blocking (TB) activity [15]. Pf48/45 becomes exposed on the surface of gametes once the parasite is taken up in blood meal by a mosquito and here the antigen can be targeted by antibodies and other components of the blood meal [16]. Expression of Pf48/45 for TB immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein [17,18]. The limited reactivity of recombinant Pf48/45 with monoclonal antibodies against conformational epitopes of Pf48/45 has indicated this misfolding [19,20].

2. Results and discussion

In this study the coding sequence of the gene encoding Pf48/45 (PF3D7_1346700) was introduced into the redundant P. berghei p230p gene locus (PBANKA_0306000) [3,21,22]. The Pf48/45 gene was placed under control of 1.3 kb of the promoter region of the schizont-specific Pb map1 gene (PBANKA_0831000). This promoter was chosen since map1 is one of the highest transcribed genes in developing Pb schizonts [23] and the Pb schizont stage can be easily produced and purified in large quantities [24]. The transgenic parasite (Pf48/45@PbMSP1) was generated by the method of GIMO transfection and selection [21]. Using this method transgenes can be rapidly introduced into the p230p gene locus in a GIMO<sub>PBANKA</sub> parent line by replacing the positive-negative selectable marker expression cassette by the transgene expression cassette (Supplementary M&M and Fig. 1A). Correct replacement of the selectable marker cassette and insertion of the Pf48/45 expression cassette in a cloned line of Pf48/45@PbMSP1 (1807c2l) was confirmed by diagnostic PCR and Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (Fig. 1B). Analysis of the growth rate of transgenic Pf48/45@PbMSP1 parasites during the cloning period demonstrated normal growth of blood stages, comparable to wild type (WT) PBANKA parasites (i.e. all mice (n = 3) achieved a 0.5–2% parasitemia on day 8, after inoculation with a single infected red blood cell). To obtain transgenic schizonts, parasites were cultured overnight using standard methods to produce and purify Pf schizonts (Supplementary M&M).

We confirmed expression of Pf48/45 in the transgenic schizonts by Western and immuno-fluorescence analysis using four anti-Pf48/45 monoclonal antibodies 8SRF45.1 (45.1), 8SRF45.2b (45.2b), 8SRF45.3 (45.3), and 8SRF45.5 (45.5). Three of these (45.1, 45.2b and 45.3) recognize conformational epitopes (epitopes I, IIb and III respectively) in the C terminal region of Pf48/45 [14].

In Western analysis all the antibodies recognize a protein of the expected size (48 kDa) in protein extracts from Pf48/45@PbMSP1 schizonts and WT Pf gametocytes but not in extracts from a Pb line that does not express Pf48/45 (i.e. GIMO<sub>PBANKA</sub> line 1596c11). As a positive control, recombinant protein that contains a fragment of P. falciparum Pf48/45 fused to the GLURP R0 domain (R0.10c) was included and as expected a 150 kDa band was present after probing with monoclonal antibodies 45.1, 45.2b and 45.3 but was not present after probing with 45.5 (Fig. 1C) [25]. We next examined the presence of Pf48/45 epitopes using the anti-Pf48/45 antibodies by immuno-fluorescence assay (IFA) (Fig. 1D). All antibodies recognized Pf48/45 produced in the Pf48/45@PbMSP1 schizonts and did not react with proteins of WT Pf schizonts (Fig. 1C and 1D). These results demonstrate that transgenic Pf schizonts can effectively express full length Pf48/45, which retains a number of conformational epitopes. Pf48/45, like MSP1, contains a GPI anchor and is present at the plasma membrane of Pf gametocytes/gametes [26]. The immuno-fluorescence analyses indicate that Pf48/45 was located in the cytoplasm of the transgenic merozoites as the fluorescence signals did not completely overlap with fluorescence signals obtained with anti-MSP1 antibodies, which stain MSP1 at the merozoite plasma membrane in mature schizonts (Fig. 1D), Possible reasons for this observation is that GPI attachment may be different between rodent and human Plasmodium parasites, or that the attachment of Pf48/45 onto P. berghei merozoites would require the presence of other Plasmodium proteins normally present in gametocytes/gametes.

In order to estimate the proportion of Pf48/45 present in the protein lysates of Pf48/45@PbMSP1 schizonts we performed a quantitative Western Blot analysis. Densitometry analysis of signals obtained after probing known amounts of Pf48/45@PbMSP1 protein schizont lysates and a dilution series of recombinant Pf48/45 with anti-Pf48/45 monoclonal 45.1, revealed that the intensity of the schizont lysate signals corresponds to less than 1 ng of recombinant Pf48/45 (Supplementary Fig. S1), indicating that is between 0.25 - 0.12% of the total schizont lysate is Pf48/45 (Fig. 2A).

Next, we examined if Pf48/45@PbMSP1 transgenic schizont lysate could be used to raise sera that could block Pf transmission in mosquitoes, presumably by Pf48/45 specific antibodies. Two groups of 10 C57BL/6 mice were immunized 4 times (2 week interval) with lysates of 1 × 10<sup>6</sup> schizonts of either Pf48/45@PbMSP1 or WT (c15cy1) parasites (Fig. 2B). Purified schizonts were inactivated by three rounds of freezing on dry ice followed by thawing at room temperature before immunization and schizont lysates were injected intravenously. Two
weeks after the final immunization, serum was collected from all animals and a serum pool made for each group. Total IgG was isolated from the pooled sera and tested for transmission-reducing activity (TR activity) in standard membrane feeding assays (SMFA) using Pf gametocytes (Supplementary M&M). Pf gametocytes were fed to A. stephensi mosquitoes in the presence of IgG obtained from mice immunized with schizonts of either Pfs48/45@PbMSP1 or WT. In the first experiment, IgG (1500 μg/ml) from Pfs48/45@PbMSP1-immunized mice showed 99.8% inhibition in oocyst density (p = 0.001) compared to the IgG obtained from WT immunized (Fig. 2C). Next, TR activity was determined in SMFA using a dilution series of the IgG obtained from Pfs48/45@PbMSP1-immunized mice. Significant TR activity with IgG from Pfs48/45@PbMSP1-immunized mice was still observed at a concentration of 187 μg/ml (p = 0.014) compared to the control IgG (Fig. 2C). The quantitative Western blot analysis (Fig. 2A) indicated that is between 0.25-0.12% of the total Pfs48/45@PbMSP1 schizont lysate was Pfs48/45 and therefore it is likely that the majority of the IgG from the immunized mice is not directed against Pfs48/45.
failure to induce TR activity of IgG of mice immunized with WT schizont lysate indicates that the small proportion of anti-Pfs48/45-Ab mediates the TR activity after Pfs48/45@PbMSP1 schizont lysate immunization. The strong TR activity mediated by the total IgG isolated from Pfs48/45@PbMSP1 immunized mice (Fig. 2C), indicates that Pfs48/45 expressed in P. berghei can induce antibodies with potent TR activity.

Combined, our proof-of-concept studies demonstrate that transgenic Pb schizonts can be used as a system to produce a difficult to express HMP protein that is correctly folded and retains conformational epitopes of the native protein. This opens possibilities to use this expression system to evaluate the immunogenicity of other difficult to express antigens or specific domains of these parasites. Studies using sera obtained from mice immunized with Pb proteins expressed by transgenic Pb parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molbiopara.2018.07.009.

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