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Plasmodium yoelii: Combinatorial expression of variants of the 235 kDa rhoptry antigen during infection

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Abstract

The 235 kDa rhoptry protein Py235 of *Plasmodium yoelii*, has been implicated in erythrocyte invasion by the merozoite forms of the parasite. Py235 is encoded by a large, highly polymorphic gene family, members of which appear to be differentially transcribed. However, it is not clear how many variants are expressed at the protein level during an infection cycle and whether or not these variants are expressed selectively or combinatorially. Certain monoclonal antibodies to Py235 have been shown to attenuate parasite virulence upon passive transfer into mice, suggesting that this antigen or its derivatives may be useful vaccine candidates. To provide a basis for this, we sought to identify those variants that are recognised by the host immune system, and to establish the pattern of expression of the antigen in mice during infection. Using Py235 monoclonal antibodies as probes, we isolated distinct antigenic variants from an expression library, suggesting that the antigen repertoire is potentially large and that different Py235 variants may be produced during infection. The implications of these observations are discussed with respect to the ability of a cloned parasite line to express distinct antigenic variants *in vivo*.

Index Descriptors and Abbreviations: Plasmodium; *Plasmodium yoelii*; Rhoptry organelle protein; Expression cloning; Antigenic variation; Py235, *Plasmodium yoelii* 235 kDa protein; Malaria; Apicomplexa; Virulence; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; Monoclonal antibodies; Gene copy number; Merozoite; Polymorphic gene; Erythrocyte-binding protein; Infection; Red cell invasion; Normocyte; Reticulocyte

1. Introduction

The malaria parasite *Plasmodium* spp. employs a number of proteins to facilitate cell invasion (Robson et al., 1988; Schrevel et al., 1993; Sim et al., 1994). Among these is Py235, which belongs to a family of high molecular mass (~235 kDa) proteins first identified in *P. yoelii*. Py235 has segmental homology with the reticulocyte-binding protein of *P. vivax* (Galinski et al., 1992; Keen et al., 1994). Orthologues of this family have also been identified in *P. falciparum* and shown to be required for invasion and phenotypic variation (Rayner et al., 2000; Duraisingh et al., 2003). Ultrathin cryomicrotomy and immunoelectron microscopy showed that Py235 and its isoforms are ensconced within the rhoptry organelles of the merozoites from where they are extruded onto the cell surface immediately prior to erythrocyte invasion, presumably to facilitate cell entry (Aikawa et al., 1978; Oka et al., 1984). Thus passive transfer of monoclonal antibodies to these proteins has been found to protect mice against the lethal *P. yoelii* YM strain. These antibodies were specific to the merozoites and attenuated parasite virulence by inducing a switch in target cell-type preference from mature red blood cells to reticulocytes for invasion (Freeman et al., 1980; Holder and Freeman, 1981). This resulted in the conversion of a fulminating and potentially lethal infection, to a self-limiting one typical of infection by the non-lethal *P. yoelii* 17X strain. This suggests that Py235 may subserve a vital function in merozoite infectivity although it remains unclear how it carries out such a function.

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Some of the genes encoding Py235 have been cloned and are present in multiple copies within the parasite genome (Keen et al., 1990,1994; Borre et al., 1995; Khan et al., 2001; Owen et al., 1999); this has now been confirmed in the sequencing of the genome (Carlton et al., 2002). Those gene copies that have been characterized so far differ in their restriction profiles and in the length of a repetitive sequence at the C-terminus of Py235 (Khan et al., 2001). We do not know how many Py235 variants are expressed by the parasite during infection or indeed which variants are critical for inducing host immune responses. By a combination of expression cloning and transcription analyses we found that distinct antigenic variants may be expressed simultaneously during infection. The teleology for such a multiplicity of antigen expression and/or presentation from an apparent parasite clone is discussed against the background of *Plasmodium* immunobiology.

2. Materials and methods

2.1. Infection, parasite isolation and purification of genomic DNA

CBA mice were infected with *P. yoelii* YM; at a parasitaemia of 40–55%, the mice were exsanguinated into heparin by cardiac puncture. Red cells were separated from (most) white cells by sequentially passing the blood through two cellulose columns (Homewood, 1976). The red cells were lysed with 10% saponin to release the parasites, which were subsequently washed with several volumes of ice-cold PBS. High molecular weight parasite genomic DNA was isolated by standard techniques (Berger and Kimmel, 1987).

2.2. Construction and screening of cDNA library

Polyadenylated (A^+) mRNA was purified from the parasites using the QuickPrep *Micro* mRNA Purification kit (Amersham Pharmacia Biotech, UK). To avoid cloning poly A-rich parasite genomic DNA, residual contaminating DNA was eliminated by treatment with FPLC*pure* RNase-free DNase I (Amersham Pharmacia Biotech) for 15 min at 37 °C; mRNA was precipitated with glycogen carrier, 100% ethanol and 2.5 M potassium acetate pH 5.0 for 2 h at −20 °C. Five micrograms of mRNA was reverse transcribed with the TimeSaver cDNA synthesis kit (Amersham Pharmacia Biotech) using random primers for first strand cDNA synthesis. After second strand synthesis, the cDNA was purified on a Sepharose CL-4B spin column (Amersham) and ligated to *Eco*RI/*Not*I adaptors for 2 h at 16 °C. The ligation mixture was phenol:chloroform-extracted and co-precipitated with 2 βg of λZAPII (Stratagene, Belgium) pre-digested with *Eco*RI and treated with calf intestinal alkaline phosphatase. The precipitated DNA was washed with 70% ethanol and air-dried. It was then ligated with T4 DNA ligase at 16 °C overnight in a total volume of 20 μl; 5 μl of this ligation mixture was used for packaging using the Gigapack II XL Packaging extract (Stratagene) as instructed by the manufacturer. To determine library titre and score recombinant phage, serial dilutions of the library were used to inoculate XL1-Blue MRF′ cells which had been cultured overnight in LB broth/0.2% maltose/10 mM $MgSO_4$; the cells were plated on LB agar containing isopropyl-β-D-thiogalactopyranoside (IPTG)/5-bromo-4-chloro-3-indolyl-D-galacto-pyranoside (Xgal).

Based on the library titre, XL1-Blue MRF′ cells were transduced with the phage library and grown on 24 × 24 cm agar plates (Nunc) to form a confluent lawn of plaques. Plaques were transferred onto Hybond N^+ membranes (Amersham Pharmacia Biotech) and screened according to the Gigapack II protocol. The membrane was pre-hybridized and then hybridized with *E3*, a full-length (6.7 kb), intronless, single exon genomic clone (Keen et al., 1994), labelled with [α-^{32}P]dCTP (Amersham Pharmacia Biotech). Labelling reactions were performed using the T^{7}Quickprime kit (Amersham Pharmacia Biotech). Hybridizations were performed at 65 °C for 18 h in 5× SSPE, 0.5% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA. Post-hybridization treatment included a final wash of the filter in 1x SSC, 0.05% SDS at 65 °C for 1 h. The membrane was exposed to X-ray film (Fujifilm, UK) overnight at −80 °C.

2.3. Phage and phagemid rescue

Positive plaques identified in Section 2.2 were isolated and purified by two more cycles of plating and screening with radiolabelled-*E3* at decreasing plaque densities. Phage were eluted from agar plugs of positive plaques according to Gigapack II protocol; 200 μl of the eluted phage was used for *in vivo* excision or phagemid rescue with the Rapid Excision kit (Stratagene) according to the manufacturer's instructions. Single colonies were picked and grown overnight in LB broth supplemented with 100 mg/ml ampicillin. Miniprep plasmid DNA was isolated, restricted with *Eco*RI to determine insert sizes, and Southern blotted using radiolabelled-*E3* to verify their authenticity.

2.4. Expression screening and sequencing of recombinant phagemids

Bacterial cultures of positive clones obtained from *in vivo* excision were streaked on an LB agar plate containing 10 mM IPTG and 100 μg/ml ampicillin. After ~6–8 h growth at 37 °C, an Immobilon-P membrane (Millipore, UK) pre-soaked in 10 mM IPTG was laid over the colonies; growth was continued overnight at 37 °C. The membrane was lifted and the colonies were lysed *in situ* in an atmosphere of chloroform (Harlow and Lane, 1988). The membrane blot was incubated in blocking buffer (5% non-fat dry milk in Tris-buffered saline, 0.05% Tween, TBST) for 1 h at room temperature. A cocktail of the monoclonal antibodies 25.37, 25.77 and 25.86 (Freeman et al., 1980; Holder and Freeman, 1981) was added and