Malaria Journal

BioMed Central

Methodology

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Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions

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Published: 17 December 2003

Malaria Journal 2003, **2**:47

Received: 24 November 2003
Accepted: 17 December 2003

This article is available from: http://www.malariajournal.com/content/2/1/47

Abstract

Background: The development of *Plasmodium falciparum* within human erythrocytes induces a wide array of changes in the ultrastructure, function and antigenic properties of the host cell. Numerous proteins encoded by the parasite have been shown to interact with the erythrocyte membrane. The identification of new interactions between human erythrocyte and *P. falciparum* proteins has formed a key area of malaria research. To circumvent the difficulties provided by conventional protein techniques, a novel application of the phage display technology was utilised.

Methods: *P. falciparum* phage display libraries were created and biopanned against purified erythrocyte membrane proteins. The identification of interacting and in-frame amino acid sequences was achieved by sequencing parasite cDNA inserts and performing bioinformatic analyses in the PlasmoDB database.

Results: Following four rounds of biopanning, sequencing and bioinformatic investigations, seven *P. falciparum* proteins with significant binding specificity toward human erythrocyte spectrin and protein 4.1 were identified. The specificity of these *P. falciparum* proteins were demonstrated by the marked enrichment of the respective in-frame binding sequences from a fourth round phage display library.

Conclusion: The construction and biopanning of *P. falciparum* phage display expression libraries provide a novel approach for the identification of new interactions between the parasite and the erythrocyte membrane.

Background

Malaria remains one of the most serious and widespread causes of pathogen-specific mortality in humans. Between 300 and 500 million people are affected globally with the mortality rate estimated to be 2.5 million deaths each year http://www.malaria.org/. Control of malaria is becoming increasingly difficult. The spread of resistance of both the parasite and the mosquito vector to anti-malaria drugs and insecticides, ensures that malaria remains an expanding health risk. Therefore, new approaches are needed to combat the disease. These include the identification of novel therapeutic agents and the development of vaccines targeted to various stages of the parasite life cycle [1].

The virulence and widespread prevalence of *Plasmodium falciparum* has made this parasite the best-studied species

of malaria protozoa that infect humans. The invasion of erythrocytes by *P. falciparum* alters the host cells morphologically and functionally. *P. falciparum* has been shown to extensively modify the erythrocyte membrane in order to facilitate merozoite entry, intra-erythrocytic parasite development and exit from the erythrocytes. Only a handful of parasite proteins have been shown to interact with erythrocyte cytoskeletal proteins, including key structural modulators such as spectrin and protein 4.1 [2]. Despite our increasing knowledge, many questions remain unanswered concerning protein-protein interactions between host and parasite.

The isolation of proteins from *P. falciparum* using conventional protein techniques has limited the discovery of new protein interactions. The inability to maintain a very high parasitaemia of *P. falciparum* in continuous culture has hindered means to purify parasite-derived proteins in large quantities. Furthermore, the presence of contaminating erythrocyte membrane proteins in *P. falciparum* protein extracts makes it extremely difficult to obtain preparations of sufficient purity. To circumvent these limitations, phage display technology was used for characterizing biomolecular interactions.

Phage display involves the construction and assembly of peptide libraries, which comprise millions of short, variable amino acid sequences that are displayed on the surface of bacteriophage virions [3]. To date, research into *P. falciparum* phage display has focused only on two applications: the expression of random peptide fragments derived from a selected malaria protein [4] and production of antibody libraries for biopanning against a malaria protein [5]. In this study, we demonstrate a novel approach using *P. falciparum* phage display libraries for mapping unidentified protein interactions between human erythrocyte membrane proteins and the malaria parasite.

Methods

Construction of P. falciparum *phage display libraries*

The *P. falciparum* FCR-3 strain was cultured *in vitro* according to the method of Trager and Jensen [6]. Parasites were extracted from infected erythrocytes and total RNA isolated using guanidinium isothiocyanate [7]. Messenger RNA was extracted using the Dynal® (Oslo, Norway) mRNA direct kit. Two-base anchored (oligodT_{12}VN) primers (2 μg) were used for reverse transcribing 4 μg of *P. falciparum* mRNA. cDNA was synthesized and end modified according to Novagen's (Wisconsin, USA) T7Select10-3 Orient Express ™ cDNA cloning (random primer) system. Excess linkers and cDNA smaller than 300 base pairs (bp) were removed by gel filtration. *P. falciparum* phage display libraries were created by cloning the modified cDNA into the T7Select10-3b *EcoRI*/*HindIII* vector arms. Recombinant vectors were subsequently packaged in T7 bacteriophage packaging extracts and propagated in *Escherichia coli* (strain BLT5403) during which the fusion proteins were expressed and displayed on the phage surface.

Biopanning

P. falciparum phage display cDNA libraries were screened by biopanning against immobilized human erythrocyte spectrin and protein 4.1. These proteins were isolated from erythrocyte membranes by using a glycine-NaOH (pH 9.8) buffer containing Tween 20 [8]. Spectrin and protein 4.1 were subsequently concentrated using Slide-A-lyzer cassettes (Pierce, USA) and polyethylene glycol 20,000 (Sigma-Aldrich, USA) and biotinylated with D-Biotin-N-hydroxysuccinimide ester (Roche, Germany). Thirty to 120 μg biotinylated protein (0.6 μg/μl-2.0 μg/μl) were immobilized on streptavidin-coated magnetic beads (Roche, Germany) at room temperature for 2 hours. Unbound protein was removed by washing with Tris-buffered saline (TBS), pH 7.5. Libraries were reacted overnight at 4°C with streptavidin-coated beads to pre-select background binding. The remaining phage were then incubated overnight against spectrin and protein 4.1 coated streptavidin beads at 4°C. Unbound phage were removed by washing with TBS plus 0.1 % (v/v) Tween 20. Bound phage were eluted from beads for 10–20 minutes at room temperature by adding 200 μl 1% SDS, and then amplified overnight at 37°C in 50 ml early log-phase BLT5403 *E. coli* cultures. Following amplification, sodium chloride was added to lysed cultures to a final concentration of 0.5 M and cellular debris cleared by centrifugation at 8000 × g for 10 minutes. The supernatant was subjected to another round of biopanning. After fourth round biopanning, the final lysate was titred and DNA was extracted from individual plaques by incubation in 50 μl 10 mM EDTA (pH 8.0) at 65°C for 10 minutes. *P. falciparum* insert sizes were determined by PCR using 1 μl phage lysate, 12.5 μl PCR Master Mix (Roche, Germany) and 0.25 μl T7Select specific primers. The DNA sequence and correct reading frames of insert sequences were determined using the PCR product pre-sequencing and Sequenase Version 2.0 DNA sequencing kits (Amersham Biosciences, UK). Sequences were verified for correct reading frame and matched to annotated *P. falciparum* genes by performing bioinformatic analyses in the PlasmoDB database http://www.plasmodb.org/.

Results and Discussion

The average size of *P. falciparum* cDNA synthesized was greater than 722 bp (Fig. 1). The recommended molar ratio of insert to vector (1:1 to 3:1 insert: vector for an average cDNA size of 1.5 kbp) was, therefore, used for cloning *P. falciparum* cDNA into the T7Select vector. However, the majority of *P. falciparum* cDNA inserts after

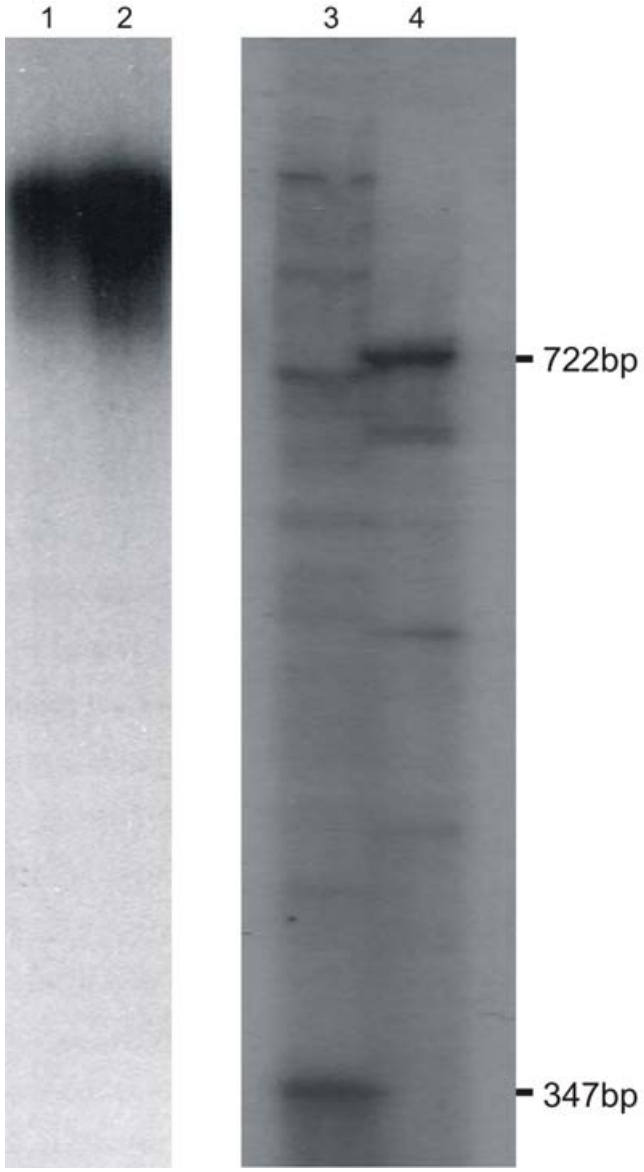

Figure 1
Gel filtration of *P. falciparum* cDNA. Lanes 1 and 2 represent end-modified cDNA, labeled with [α-^{32}P] dATP (Amersham, Biosciences, UK), after gel filtration. To determine the average size of *P. falciparum* cDNA, size markers were synthesized by PCR utilizing primers specific for human erythrocyte α-spectrin (exon 2) and band 3 (exon 18 and 19) sequences (lanes 3 and 4). *P. falciparum* cDNA appears as a smear of an average size greater than 722 bp in length. cDNA and PCR products were evaluated using an 8 % denaturing polyacrylamide gel and autoradiography.

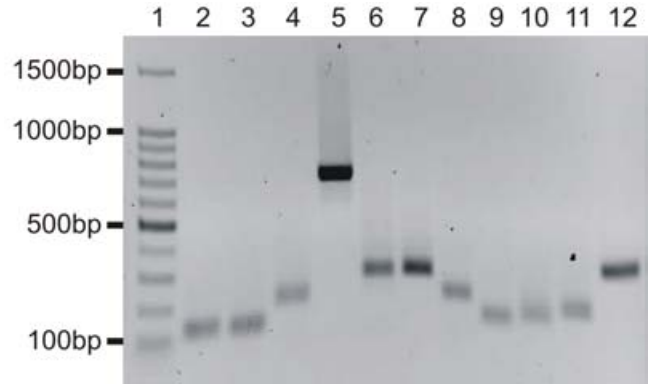

Figure 2
PCR amplification of *P. falciparum* cDNA inserts cloned into the T7Select10-3b vector. All fragments include 107 bp of vector sequence. Lanes 2–12 depict amplified DNA from selected phage recombinants. Lane 1 is a 100 bp DNA ladder (Promega, USA). PCR products were evaluated using 1% agarose gel electrophoresis and ethidium bromide staining.

fourth round biopanning were 100–200 bp in length, and the largest insert was 600 bp (Fig. 2). The presence of small inserts was unexpected, since gel filtration of the modified cDNA should have excluded fragments smaller than 300 bp, and electrophoretic analysis (Fig. 1) did not reveal any smaller fragments. However, smaller cDNA fragments would have incorporated a very low amount of radiolabel, resulting in their apparent absence from the gel (Fig. 1). Inefficient size exclusion during column chromatography presumably resulted in a higher molar ratio of smaller to larger cDNA fragments, which would therefore have facilitated a favourable cloning efficiency for the smaller fragments. The efficiency of *in vitro* packaging and/ or ligation between *P. falciparum* cDNA inserts and T7Select10-3b vector was significantly lower (10^4–10^6 plaque forming units/μg vector), when compared to a positive control (10^9 plaque forming units/μg vector). This may be accounted for by having selected an insert: vector ratio favourable for cloning fragments of ± 1.5 kbp.

Sequencing analyses of fourth round virions and subsequent blast searches in PlasmoDB, revealed in-frame insert sequences which were mapped to predicted *P. falciparum* genes. Seventeen parasite sequences were in-frame, representing approximately one third of the inserts sequenced. However, several of these inserts were found more than once and only seven represented unique open reading frames. This was attributed to the significant degree of enrichment of each of these clones from the initial library. This demonstrates that the in-frame *P. falciparum* binding sequences identified by biopanning are highly specific for human erythrocyte spectrin and protein 4.1. The seven annotated proteins are shown in Table 1. Ebl-1 like protein, a Duffy binding-like protein is

Table 1: *P. falciparum* proteins containing binding sequences specific for human erythrocyte membrane proteins spectrin and protein 4.1 *P. falciparum* phage display libraries were biopanned against spectrin and protein 4.1. This allowed for the identification of seven in-frame parasite sequences that were subsequently mapped to annotated proteins in PlasmoDB.

PlasmoDB V 4.1 identification	Predicted function	Amino acid position of spectrin/ protein 4.1 binding sequences
MAL13P1.278	putative serine/ threonine kinase	786–815
MAL6P1.145	putative phosophotransferase	28–77
PFI1570c	putative aminopeptidase	216–245
PFA0125c	putative Ebl-1 like protein	971–1001
PFL1130c	hypothetical protein	3713–3742
PF13_0071	hypothetical protein	209–273
PF14_0201	hypothetical protein	801–860

synthesized in the merozoite micronemes and may be involved in erythrocyte entry [9]. The biochemical modification of spectrin and protein 4.1 by the putative kinase, aminopeptidase and phosphotransferase may result in the structural re-organisation of the erythrocyte skeleton, thus facilitating the survival of *P. falciparum*.

P. falciparum phage display libraries were successfully used to identify malaria protein sequences involved in binding important structural components of the erythrocyte membrane, namely spectrin and protein 4.1. The expression and analyses of the identified *P. falciparum* proteins/binding domains will aid in unravelling the binding kinetics of these recombinant proteins with spectrin and protein 4.1.

Conclusions

P. falciparum phage display libraries provide a powerful tool and novel approach for identifying new protein interactions between the parasite and the human erythrocyte membrane. These libraries were used to identify seven parasite proteins that bind human erythrocyte spectrin and/or protein 4.1. The function of these *P. falciparum* proteins remains to be determined, however, they are potentially involved in the entry and/or exit of merozoites from the human erythrocyte. Furthermore, these proteins may also be involved in parasite growth and survival during intra-erythrocytic development.

Authors' contribution

The construction of *P. falciparum* phage display libraries was optimised by SBL. Biopanning of phage display libraries against spectrin and protein 4.1, with subsequent sequencing and bioinformatic analyses, was carried out by SBL and RL respectively. TLC, the principal investigator, was responsible for the study design and co-ordination. All authors read and approved the final manuscript.

Acknowledgements

We thank the Department of Pharmacy and Pharmacology, University of the Witwatersrand, for supplying stock cultures of *P. falciparum* (strain FCR-3). This material is based upon work supported by the National Research Foundation under grant number 2053200, as well as research grants from the Medical Research Council of South Africa, National Health Laboratory Services and University of the Witwatersrand.

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