Use of split-dihydrofolate reductase for the detection of protein-protein interactions and simultaneous selection of multiple plasmids in *Plasmodium falciparum*

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Highlights

- *In vivo* detection of protein-protein interactions in *Plasmodium* using split-DHFR
- Simultaneous selection of two plasmids using a single antifolate drug

Abstract

Defining protein-protein interactions is fundamental to the understanding of gene function. Protein-fragment complementation assays have been used for the analysis of protein-protein interactions in various organisms. The split-dihydrofolate reductase (DHFR) protein-fragment complementation assay utilises two complementary fragments of the enzyme fused to a pair of potentially interacting proteins. If these proteins interact, the DHFR fragments associate, fold into their native structure, reconstitute their function and confer resistance to antifolate drugs. We show that murine DHFR fragments fused to interacting proteins reconstitute a functional enzyme and confer resistance to the antifolate drug WR99210 in *Plasmodium falciparum*. These data demonstrate that the split-DHFR method can be used to detect *in vivo* protein-protein interactions in the parasite. Additionally, we show that split-DHFR fragments can be used as selection markers, permitting simultaneous selection of two plasmids in the presence of a single antifolate drug. Taken together, these experiments show that split-DHFR represents a valuable tool for the characterisation of *Plasmodium* protein function and genetic manipulation of the parasite.

Keywords

Malaria, split-dihydrofolate reductase, protein-fragment complementation assay, *Plasmodium* transfection, co-transfection, selection marker

1. Introduction

Understanding the cellular roles of the large proportion of uncharacterised malaria parasite proteins remains a significant challenge, with the functions of many essential proteins currently undefined [1]. The identification of binding partners represents a key approach to elucidating protein function and several methods have been developed to characterise protein-protein interactions. Some of these approaches have been applied to malaria parasite proteins including affinity purification, blue-native gel analysis, two-hybrid analysis, *in vitro* split-luciferase, and the AVEXIS assay [2-6]. *In vivo* protein-fragment complementation assays are used in various organisms for the detection of protein interactions [7], but have not been used in *Plasmodium*.

Protein-fragment complementation strategies use colorimetric, fluorescent or luminescent signals, or resistance to drugs to detect interaction between proteins [8-11]. One widely used approach utilises the enzyme dihydrofolate reductase (DHFR) as a protein interaction reporter [12]. Two inactive fragments of DHFR, referred to as DHFR1,2 and DHFR3, are fused to two proteins of interest and expressed in a cell. *In vivo* interaction between the two proteins fused to the DHFR fragments brings the two halves of the enzyme into close proximity. When fused to interacting proteins the DHFR fragments co-assemble reconstituting an active, functional enzyme that confers resistance to antifolate drugs [11, 13]. In the absence of interacting fusion partners the DHFR fragments do not co-assemble. Thus, resistance to an antifolate drug is used as an indicator of protein interaction. Binary or close to binary interactions are typically detected as reconstitution of the complementary fragments requires that the termini of the interacting partners are within close proximity.

The split-DHFR assay has been used in bacterial, yeast, plant and mammalian cells, utilising methotrexate or trimethoprim resistance as indicators of protein-protein interactions [11, 14-17]. The assay captures *in vivo* interactions of both soluble and membrane proteins in the cytoplasm and has been used to analyse spatial proximity of protein subunits within larger protein complexes [18], to elucidate whole-organelle interactomes [19] and genome-wide interactomes [13]. Additionally, the approach has been used to monitor changes in protein-protein interactions in response to DNA damage [20], and to detect drug perturbations of cellular pathways by proteome-wide monitoring the propensity of proteins to homodimerise [21].

Here we show that split-DHFR fragments, expressed from two separate plasmids in *Plasmodium falciparum*, reconstitute a functional DHFR enzyme. The activity requires that the fragments are fused to interacting proteins, indicating that the method can be used to detect protein interactions in the parasite.

2. Methods

2.1. Plasmids

Plasmids for expression of DHFR fragments were based on the vector pEFGFP [22]. pEFGFP was modified such that the first expression cassette utilises the HSP86 promoter, and the second expression cassette uses the calmodulin promoter. Plasmid names comprise an abbreviation of the protein expressed from the first cassette separated by a / symbol from an abbreviation of the protein expressed in the second cassette. In plasmid $ACP_{GFP}/LZ_{DHFR1,2}$ the sequence encoding residues 1-60 of acyl carrier protein (ACP) fused to GFP, was inserted next to the HSP86 promoter. A sequence encoding the GCN4 leucine zipper fused to DHFR1,2 was amplified from p41-ZL-DHFR1,2 [23] and inserted next to the calmodulin promoter.

In the plasmid $mCherry/LZ_{DHFR3}$, sequences encoding mCherry and the GCN4 leucine zipper fused to DHFR3 were inserted adjacent to the HSP86 and calmodulin promoter, respectively. The leucine zipper-DHFR3 sequence was amplified from p41-ZL-DHFR3 [23]. For the $PF3D7\_0402000_{mCherry}/LZ_{DHFR3}$ construct, PF3D7_0402000 fused to mCherry was inserted in the first expression cassette, and the sequence encoding the leucine zipper-DHFR3 fusion in the second cassette. Plasmid $mCherry/_{DHFR3}$ was assembled as for $mCherry/LZ_{DHFR3}$ except that the DHFR3 fragment was amplified without the sequence encoding the leucine zipper. Sequences of all plasmids are provided in the supplementary methods.

2.2. In vitro parasite transfection and culture

Parasites were transfected using a previously described method [24]. Briefly, DNA for transfection was precipitated in 300 mM sodium acetate and 66% ethanol, washed in 70% ethanol, dried and resuspended in sterile water. After resuspension, the DNA concentration was determined such that 100 µg of each plasmid was used for transfection. Red blood cells were washed once with cytomix and 400 µl of packed cells were electroporated with 100 µg of each plasmid using a Bio-Rad Xcell^{TM} electroporator (310 V, 950 µF) [24]. Asynchronous parasites were added to electroporated blood cells, washed with RPMI and resuspended in medium containing Albumax II. WR99210 was added to a final concentration of 2.5 nM one day after electroporation, and medium containing drug was replaced every two days throughout the selection. Cultures were grown *in vitro* in medium containing Albumax II and maintained in 5% CO_2, 5% O_2 and

balance N_2. Giemsa stained blood smears were used to determine parasitemia by counting numbers of infected and uninfected red blood cells; at parasitemias below 0.2% numbers of infected cells within each field of view were counted and number of uninfected cells were estimated.

2.3. Fluorescence microscopy

Parasites were imaged by placing a drop of culture between a microscope slide and coverslip. Images of fluorescent parasites were acquired with a Zeiss Observer Z1 microscope, using a 100x phase contrast lens, an AxioCam MRm camera and ZEN Pro 2012 software. Images were processed using Image J. Images of mCherry-tagged PF3D7_0402000 were deconvolved using Zen Pro 2012 software using the 'excellent slow (constrained iterative)' method; other images were not deconvolved.

2.4. Western blotting

Expression of GFP and mCherry-tagged proteins was analysed by SDS-PAGE using 12% gels and western blotting. Schizonts were purified by Percoll gradient [25]; approximately 1 x 10^6 parasites were loaded per lane. Western blots were probed with rabbit anti-GFP antibody (Torrey Pines: TP401) diluted 1:2000, or rabbit anti-RFP antibody (MBL International: PM005) diluted 1:2000. The secondary antibody goat anti-rabbit (Thermo Scientific: 35568) was diluted 1:4000. Nitrocellulose membranes were scanned with a LI-COR Odyssey CLx imager.

3. Results

To determine whether the split-DHFR method can be used in *Plasmodium falciparum*, plasmids were constructed for expression of the complementary DHFR1,2 and DHFR3 fragments, derived from the murine DHFR enzyme [11]. A short sequence encoding the leucine zipper domain from the *Saccharomyces cerevisiae* GCN4 protein was fused to the N-terminus of each DHFR fragment [11]. The leucine zipper sequence is known to homodimerise and when fused to DHFR1,2 and DHFR3, it mediates association of these fragments to reconstitute a functional DHFR enzyme (Fig.1A). In the following sections, fusions of the GCN4 leucine zipper to DHFR1,2 and DHFR3 are referred to as $LZ_{DHFR1,2}$ and LZ_{DHFR3}, respectively.

For expression of $LZ_{DHFR1,2}$, a plasmid referred to as $ACP_{GFP}/LZ_{DHFR1,2}$ was assembled (Fig.1B). In addition to expressing $LZ_{DHFR1,2}$, the plasmid also contained a cassette for expression of the acyl carrier protein (ACP) fragment fused to GFP, which localises to the parasite apicoplast, a visually distinct compartment [26]. This expression cassette was included only to confirm by fluorescence microscopy that parasites were successfully transfected with this plasmid. Similarly, two plasmids expressing LZ_{DHFR3} were generated (mCherry/LZ_{DHFR3} and PF3D7_0402000$_{mCherry}$/LZ_{DHFR3}, Fig.1B); these also contained an expression cassette expressing either mCherry targeted to the parasite cytoplasm, or mCherry fused to the *Plasmodium* exported protein PF3D7_0402000, which is targeted to the red blood cell cytoskeleton, respectively [27-29]. The mCherry and PF3D7_0402000 expression cassettes were incorporated only to allow identification by microscopy of parasites containing the plasmids.

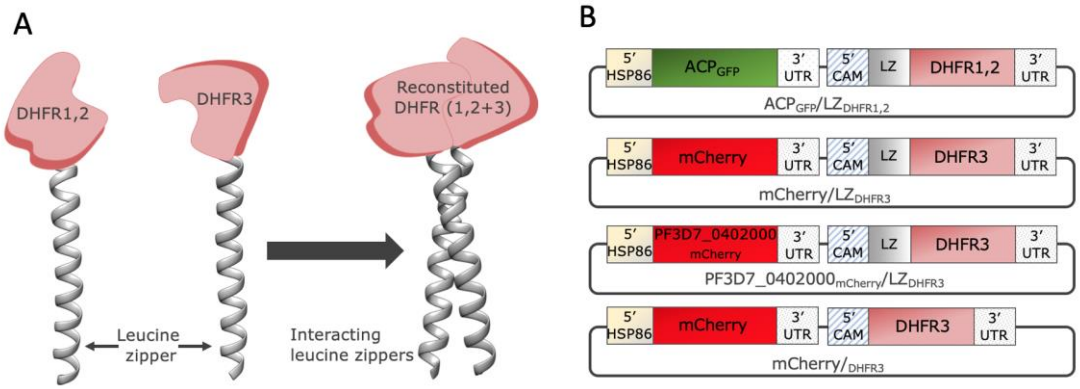

Figure 1. (A) Interacting protein domains fused to DHFR1,2 and DHFR3 fragments mediate assembly into an active DHFR enzyme. (B) Schematic of plasmids used to express DHFR fragments.

Expression of the LZ_{DHFR} fragments and reconstitution of the split-DHFR enzyme in *Plasmodium falciparum* was tested by transfecting parasites with individual or combinations of split-DHFR plasmids in the presence of WR99210, an antifolate drug that is known to inhibit the natively expressed parasite DHFR enzyme [30]. Human DHFR is not inhibited by WR99210; consequently, it can be used to select for parasites transfected with plasmids expressing human DHFR. As the sequence of human DHFR is 91% identical to that of murine DHFR, it was anticipated that murine DHFR reconstituted from the two enzyme fragments might also confer resistance to WR99210.

Plasmids $ACP_{GFP}/LZ_{DHFR1,2}$ and mCherry/LZ_{DHFR3} were co-transfected into parasites and selected using WR99210. Resistant parasites were obtained and parasitemia exceeded 1% after approximately four weeks (Fig.2A). Expression of both GFP-tagged ACP and cytoplasmic mCherry with the expected localisation was confirmed by fluorescence microscopy, indicating that the parasites contained both plasmids (Fig.2B, top row); ~90% of parasites expressed both proteins. Expression of both proteins was also confirmed by western blotting using anti-GFP and anti-mCherry antibodies (Fig.2C, lanes 2 and 5, respectively). Proteins were of the expected size; as ACP is proteolytically processed during transport to the apicoplast, several bands are present as seen previously [26]. Similarly, co-transfection of plasmids $ACP_{GFP}/LZ_{DHFR1,2}$ and PF3D7_0402000$_{mCherry}$/LZ_{DHFR3} yielded resistant parasites (Fig.2A, B, C); ~85% of which expressed both fluorescent proteins.

Significantly, transfection with individual plasmids encoding either DHFR1,2 or DHFR3 alone did not yield resistant parasites after approximately 8 weeks of selection (Fig.2A). Taken together, these data show that the leucine zipper-split-DHFR fragments, when co-expressed in *Plasmodium falciparum* can reconstitute a functional DHFR enzyme and that co-expression of both fragments is essential for this activity.

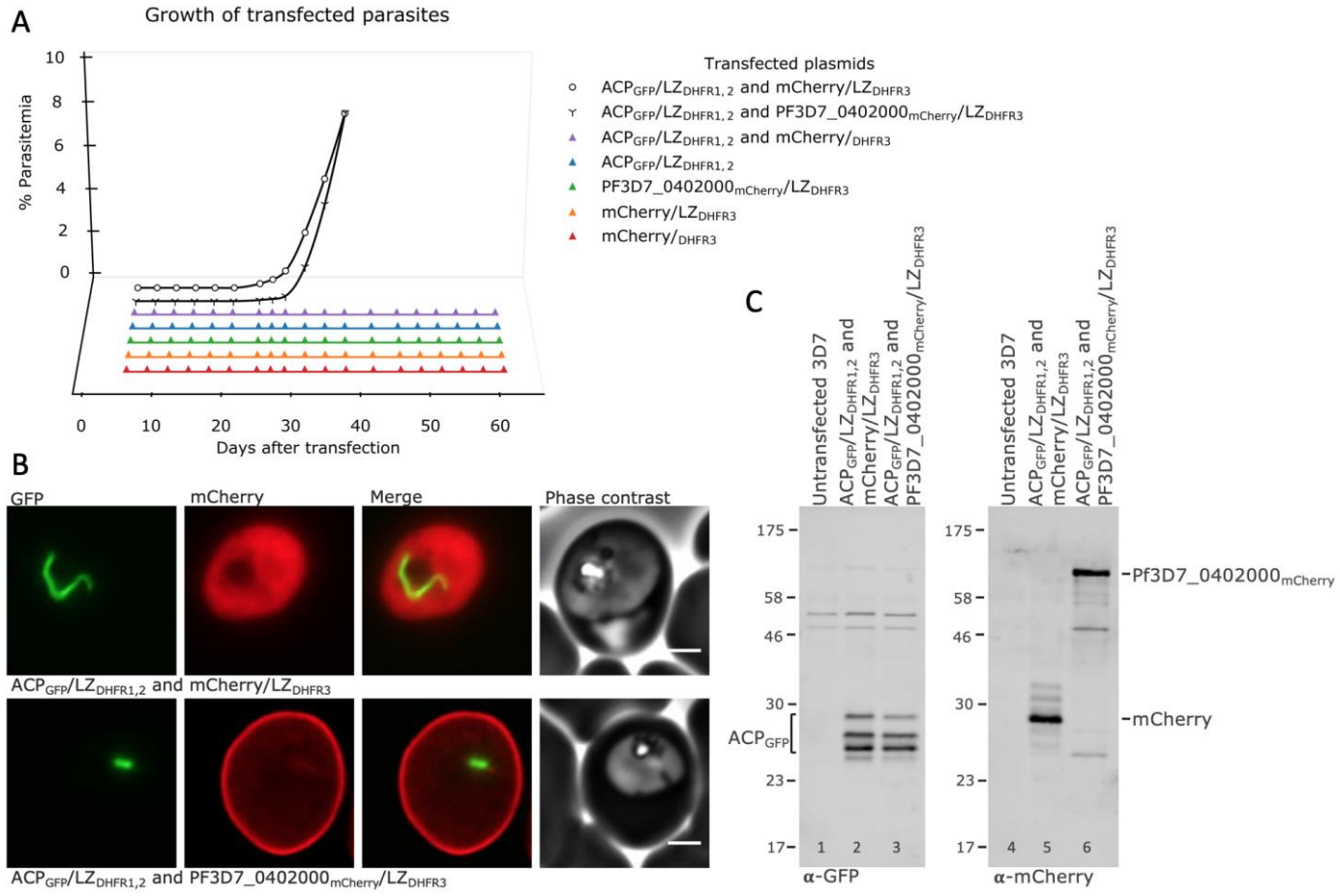

Figure 2. (A) Parasitemia of transfected cultures determined using Giemsa stained slides. The result shown is representative of three independent experiments. (B) Fluorescence microscopy images of WR-resistant parasites transfected with indicated plasmids; scale bar, 2μm. (C) Western blots of transfected parasites probed with anti-GFP

and anti-mCherry antibodies. Transfected plasmids are indicated above each lane.

To confirm that reconstitution of DHFR activity requires the DHFR fragments to be fused to interacting proteins, a plasmid expressing DHFR3 without a leucine zipper sequence was generated, mCherry-DHFR3 (Fig.1B). This plasmid was co-transfected with $ACP_{GFP}/LZ_{DHFR1,2}$. No resistant parasites were obtained after 8 weeks of selection (Fig.2A). This indicates that reconstitution of a functional DHFR complex requires the DHFR1,2 and DHFR3 fragments to be fused to interacting proteins.

4. Discussion

Taken together these data show that split-DHFR can be used to detect protein interactions *in vivo* in *P. falciparum*. Additionally, as genetic manipulation of *Plasmodium* parasites becomes more sophisticated, the need for introduction of multiple plasmids into parasites is likely to increase. Although multiple selection markers are available for use in *P. falciparum* [31], we show that the split-DHFR approach can be used to simultaneously select for two plasmids using a single drug selection marker, thus leaving a large range of selection markers for subsequent manipulations. Additionally, genetic screens in model organisms have in many cases utilised selection markers that are only active in a particular cellular compartment [32-34], thus allowing identification of genes required for protein localisation or trafficking. As reconstitution of split-DHFR requires that both fragments be localised in the same cellular compartment, it may be adapted for use in similar screening strategies in the malaria parasite.

For the widely used mouse malaria parasite *Plasmodium berghei*, selection markers are more limited and typically a sequential strategy is used to transfect two plasmids [31, 35]. The dihydrofolate reductase thymidylate synthase gene from either *Toxoplasma gondii* or *P. berghei*, that confers resistance to pyrimethamine but not WR99210, are used for selection of the first plasmid. Human DHFR, which confers resistance to both pyrimethamine and WR99210 is then used to select for a second plasmid. In principle, split-DHFR could be used to simultaneously select for two plasmids in *P. berghei*, thus reducing the time and number of animals required. This may represent a method to complement other emerging strategies to overcome the dearth of selection markers that can be used in this parasite species and others [36-39].

The split-DHFR approach represents a valuable method for the *in vivo* characterisation of protein interactions in *Plasmodium*. Whilst the split-DHFR approach may be unsuitable for characterising briefly expressed proteins with short half-lives, it represents an invaluable tool for studying a significant proportion of parasite proteins. Indeed, systematic genome-wide analysis of protein interactions has successfully been performed in *S. cerevisiae* using libraries encoding split-DHFR-tagged proteins [13]. While similar analysis of *Plasmodium* proteins would currently be technically challenging, screening a single parasite protein against a library of plasmids representing either the entire parasite proteome or a subset thereof, represents a feasible strategy for the characterisation of protein-protein interactions. As our ability to genetically manipulate the parasite improves this may allow pairwise, genome-wide analysis of protein interactions. Identification of protein interacting partners is a fundamental step towards revealing the function of *Plasmodium* proteins, facilitating our understanding of cell physiology, drug resistance and development of novel therapies and vaccines.

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Declaration of interest

None.

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Supplementary information:

Sequences of plasmids used are shown below.

Plasmid sequence: $ACP_{GFP}/LZ_{DHFR1,2}$

GCGGCCGCTAAGCGGGGGAAAGGGGCCATTGGATATATATTTAGTATTCCATAGTATATAGTTAAATATAATAATATTATAGTAAATTTTTGCATG
AATTTAAAGAAAAAAAAAAAAAATAACAAAAAGGAGTATTATATAAATATAACAAAATAATAAATACTATACCTATATATATATATATATATA
TACATATATATAAATATATAAATATATTTATATAAAAATAATATTTTATATATAATATTATATTTTTATAATATCTTTTTTTTTTTTAAATATCATCTT
TCTTTCTTTCTTTCTTTTTTTTTTTTTTGATTTATATATTATTTTATTTTGTATTTACAAAATATAATAAAATAATTTCATGTTTAGCAAAAATTTTAAA
TAGAAAATAATAATATAAAATAAAAAGCATCATTTAATATAAAATAATAAAATTAAAATAAAATAAGATAATTACCAAAAATAATAAAAAAAAT
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Plasmid sequence: mCherry/LZ$_{DHFR3}$

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