Molecular & Biochemical Parasitology 178 (2011) 56–59

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Short technical report

A densely overlapping gene fragmentation approach improves yeast two-hybrid screens for *Plasmodium falciparum* proteins

Hakeenah F. Brown[a], Ling Wang[a], Sudip Khadka[a], Stanley Fields[b,c], Douglas J. LaCount[a,*]

[a] *Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907, USA*
[b] *Howard Hughes Medical Institute*
[c] *Departments of Genome Sciences and Medicine, University of Washington, Box 355065, Seattle, WA 98195, USA*

ARTICLE INFO

Article history:
Received 3 January 2011
Received in revised form 8 April 2011
Accepted 14 April 2011
Available online 20 April 2011

Keywords:
Plasmodium falciparum
Protein expression
Yeast
Saccharomyces cerevisiae
Yeast two-hybrid assay
Split-luciferase assay

ABSTRACT

Use of the yeast two-hybrid assay to study *Plasmodium falciparum* protein–protein interactions is limited by poor expression of *P. falciparum* genes in yeast and lack of easily implemented assays to confirm the results. We report here two methods to create gene fragments – random fragmentation by partial DNAse I digestion and generation of densely overlapping fragments by PCR – that enable most portions of *P. falciparum* genes to be expressed and screened in the yeast two-hybrid assay. The PCR-based method is less technically challenging and facilitates fine-scale mapping of protein interaction domains. Both approaches revealed a putative interaction between PfMyb2 (PF10_0327) and PFC0365w. We developed new plasmids to express the proteins in wheat germ extracts and confirmed the interaction in both the split-luciferase assay and in co-purification experiments with glutathione-S-transferase and HA-tagged proteins. The combination of improved yeast two-hybrid screening approaches and convenient systems to validate interactions enhances the utility of yeast two-hybrid assays for *P. falciparum*.

The yeast two-hybrid assay is a powerful tool for the discovery and characterization of protein–protein interactions. Since it was first reported more than 20 years ago [1], the assay and its many variations have been integral to the analysis of thousands of proteins. However, the yeast two-hybrid assay has not been widely used to study *Plasmodium* protein–protein interactions, due in large part to the poor expression of *P. falciparum* genes in *Saccharomyces cerevisiae* [2]. AT-rich *P. falciparum* sequences resemble *S. cerevisiae* motifs that specify cleavage and polyadenylation of the nascent RNA [2]. These sequences cause *P. falciparum* mRNAs expressed in yeast to be truncated prematurely and result in degradation by the mRNA surveillance pathway [2,3]. To improve expression of *P. falciparum* genes in yeast, we previously reported the identification of yeast strains with mutations in the mRNA processing pathway [3]. Although these strains are useful for expressing *P. falciparum* proteins for functional studies and pair-wise yeast-two-hybrid assays, they grow more slowly and mate less efficiently than parental strains, and are not optimal for library-based yeast two-hybrid screens. Thus, alternative methods are needed to improve the yeast two-hybrid assay for *P. falciparum*. Here we report the development of two approaches to generate fragments of *P. falciparum* genes for use in the yeast two-hybrid assay. These fragmentation approaches enabled identification of interactions that could not be detected with full-length genes and are generally applicable to other systems as well.

Large-scale sequencing of clones from *P. falciparum* yeast two-hybrid libraries revealed gene fragments from a wide range of genes and detected no biases [4]. Since these libraries included only fragments that were expressed in yeast, most *P. falciparum* genes appear to contain sequences that can be expressed even if the full-length gene cannot. To more fully investigate this possibility, we tested two methods to fragment *P. falciparum* genes using *PfMYB2* (*PF10_0327*, 2745 nucleotides, 74% AT) as a test case. *PfMYB2* encodes a 915-amino acid protein with two MYB DNA-binding domains at the N-terminus. Based on the presence of the MYB domains, PfMyb2 has been proposed to function as a helix–turn–helix transcription factor [5].

Random fragments of *PfMYB2* were created using partial DNAse I digestion in the presence of manganese, which promotes the formation of blunt-ended fragments and fragments with one base 5′ or 3′ overhangs. After polishing the ends with T4 DNA polymerase, we ligated double-stranded DNA oligos to the fragments; the oligos were homologous to the sequences flanking the multiple cloning site in the yeast two-hybrid DNA binding domain plasmid pOBD.111 to enable cloning by recombination in yeast. The DNA was then size-fractionated on a Sephacryl S400 column to remove small fragments and unligated oligos. Fragments larger than ~300 base pairs (bp) were PCR-amplified and cloned into pOBD.111 by gap repair in the yeast strain R2HMet [4,6]. Yeast expressing *PfMYB2* fragments were selected on medium lacking tryptophan

* Corresponding author at: Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, RHPH 514, 575 Stadium Mall Drive, West Lafayette, IN 47907, USA. Tel.: +1 765 496 7835; fax: +1 765 494 1414.
E-mail address: dlacount@purdue.edu (D.J. LaCount).

0166-6851/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2011.04.005

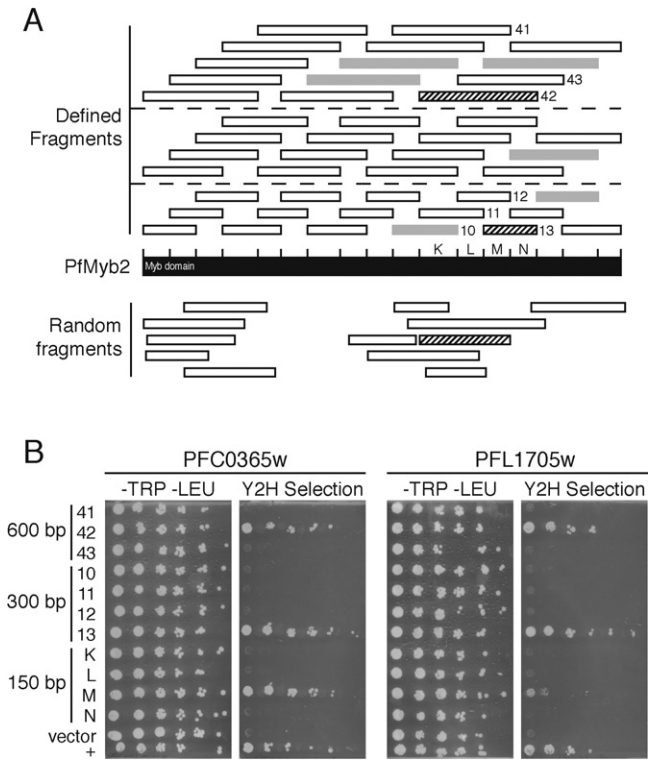

Fig. 1. Gene fragmentation approaches to improve *P. falciparum* yeast two-hybrid screens. (A) Gene fragments generated by partial DNAse I digestion (bottom) and PCR (top). Black bar represents *PfMyb2* (PF10_0327). Bars below *PfMYB2* represent fragments generated by partial DNAse I digestion of *PfMYB2* that were expressed in yeast. Tick marks above the bar indicate the positions of PCR primers, which were spaced at ~150-bp intervals. Short bars above PfMyb2 represent 300-, 450-, and 600-bp fragments; dashed lines separate fragments by size. All fragments were cloned into pOBD.111 by homologous recombination in the yeast strain R2HMet and tested for expression of the fragment by growth on synthetic dropout (SD) medium lacking methionine; the *MET2* gene is fused to the 3′ end of the *PfMYB2* fragment and is expressed only if the *PfMYB2* fragment is also expressed. White bars represent gene fragments that were expressed, whereas gray bars represent those that were not. Numbers and letters indicate fragments that were retested for interaction with *PFC0365w* and *PFL1705w* in part B; letters designate 150-bp fragments. The twelve DNAse I-generated fragments and all 600-bp defined fragments were screened against a *P. falciparum* yeast two-hybrid library. Striped bars indicate fragments that interacted with both *PFC0365w* and *PFL1705w* in the yeast two-hybrid assay. (B) Confirmation of yeast two-hybrid interactions with *PfMYB2* and mapping of the *PfMYB2* protein interaction domain. *PfMYB2* 150-bp fragments K-N, 300-bp fragments #10-13, and 600-bp fragments #41-43 were cloned into pOBD.111 in the yeast strain R2HMet. Similarly, the *PFC0365w* and *PFL1705w* fragments that interacted with *PfMYB2* fragment #42 were PCR-amplified and inserted into pOAD.102 by homologous recombination in the yeast strain BK100. Yeast harboring DNA-binding domain and activation domain plasmids were allowed to mate and then grown on SD medium lacking tryptophan and leucine to select diploid cells containing both plasmids. Yeast were grown to mid log phase, diluted to an OD_{600} of 1.0, serially diluted 5-fold in dH_2O, and plated on SD medium lacking tryptophan and leucine or yeast two-hybrid selection medium (SD medium lacking tryptophan, leucine, methione, uracil, and histidine and containing 1 mM 3 amino-4,5-triazole (3-AT); 3-AT is a competitive inhibitor of the yeast two-hybrid reporter enzyme His3 that is added to growth medium to inhibit background yeast growth).

and methionine. Because the *MET2* gene is fused to the 3′ end of the *PfMYB2* fragment, growth of yeast in the absence of methionine indicates that the *PfMYB2* fragment is expressed. Twelve fragments with diverse start and end points were identified (Fig. 1A, below bar). These fragments included most of the *PfMYB2* gene except for an ~400 bp region near the center. Based on these data, it was not possible to determine if this region could not be expressed in yeast or if too few clones were evaluated.

Because of the incomplete coverage of the random fragments and the technical challenges of the partial DNAse I digestion, we investigated an alternative approach to fragment *PfMYB2*. DNA oligos were designed to hybridize at ~150 bp intervals on *PfMYB2* and used to create a mini-library of densely overlapping gene fragments (Fig. 1A, top, Supplementary Table 1). Every possible 300-, 450-, and 600-bp fragment was PCR amplified and cloned into pOBD.111 by *in vivo* homologous recombination. Fragments expressed in yeast were identified by growth on medium lacking methionine. Of the 45 fragments tested, 39 fragments were expressed at sufficient levels to enable yeast growth. These fragments covered the entire *PfMYB2* gene, indicating that the region for which no random fragments were obtained can be expressed and that the lack of random fragments in this region was due to sampling error.

To identify *P. falciparum* proteins that interacted with *PfMYB2*, all 600-bp defined fragments and the 12 random fragments were screened against a *P. falciparum* activation domain library [4]. Screens were performed manually as described in supplemental methods. Both defined fragment #42 and a random fragment with nearly the same start point interacted with fragments of *PFC0365w* and *PFL1705w*; these genes were the only ones found by both a defined and a random fragment. To confirm these interactions and to further delineate the domains of PfMyb2 implicated in the interactions, we recloned the *PFC0356w* (nucleotides 133–615, amino acids 45–205) and *PFL1705w* (nucleotides 286–639, amino acids 95–213) fragments into the activation domain vector in fresh yeast and tested their ability to interact with a series of *PfMYB2* fragments that overlapped with fragment #42. Both *PFC0365w* and *PFL1705w* interacted with *PfMYB2* fragment 600-bp #42 and the 300-bp fragment #13 (nucleotides 1936–2235, amino acids 645–745). The 150-bp fragment M (nucleotides 1936–2085, amino acids 646–695) interacted with *PFC0365w* to the same extent as larger fragments #42 and #13, suggesting that the entire PFC0365w binding region was encoded by this fragment. In contrast, *PFL1705w* interacted poorly with fragment M and not at all with adjacent fragment N, which was encompassed by fragment #13, suggesting that the binding domain spans the junction of fragments M and N. This experiment delineated a 50 amino acid region of PfMyb2 as the binding domain for PFC0365w and a 100 amino acid region as the binding domain for PFL1705w. Surprisingly, fragment #12, which contained fragment M and fragments #41 and #43, which included the sequences in fragments M and #13, did not interact with either *PFC0365w* and *PFL1705w*.

A second problem with yeast two-hybrid screens of *P. falciparum* genes is the lack of convenient systems to validate interactions. *P. falciparum* proteins express poorly in most heterologous systems, antibodies are not available for the vast majority of *P. falciparum* proteins, and generating transgenic parasites expressing epitope-tagged proteins for colocalization and co-purification experiments is still time-consuming and technically challenging. To facilitate confirmation of yeast two-hybrid interactions, we utilized wheat germ extracts, which have been reported to improve the expression of a wide range of *P. falciparum* proteins [7–9]. Four new plasmids were developed to express PfMyb2, PFC0356w, and PFL1705w in the wheat germ in vitro translation system for use in the split-luciferase assay and co-purifications with glutathione S transferase (GST)- and HA-epitope tagged proteins (Fig. 2A).

In the split-luciferase assay, proteins of interest are fused to N- and C-terminal fragments of luciferase. If the proteins interact, the N- and C-terminal luciferase fragments associate to create a functional luciferase enzyme [10]. Though a number of variants of this system have been described, for these experiments we used fragments of firefly luciferase that were optimized to yield a high signal to background ratio and sufficient enzymatic activity to be easily measured [11]. We first tested the system using the *P. falciparum* interacting proteins PFE1350c (nucleotides 25–405, amino acids 9–135) and PFC0255c (nucleotides 10–411, amino acids 4–137) [4,12], which were fused to the N- and C-FLUC