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Molecular & Biochemical Parasitology 139 (2005) 133–139
MOLECULAR & BIOCHEMICAL PARASITOLOGY

Identification of putative *Plasmodium falciparum* mefloquine resistance genes

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Received 21 May 2004; received in revised form 12 October 2004; accepted 15 October 2004
Available online 26 November 2004

Abstract

Mefloquine is an effective antimalarial drug; however, resistant strains of the human malarial pathogen, *Plasmodium falciparum*, are beginning to arise. The yeast *Saccharomyces cerevisiae* is sensitive to mefloquine, enabling a screen for *P. falciparum* genes involved in resistance. Yeast were transformed with a *P. falciparum* expression library, followed by selection on mefloquine plates and sequencing of plasmids that conferred resistance. We characterized the four genes that conferred the strongest mefloquine-resistant phenotype in yeast. All four (PFD0090c, PFI0195c, PF10_0372 and PF14_0649) are uncharacterized *P. falciparum* genes from distinct chromosomes (4, 9, 10 and 14, respectively). The mefloquine-resistant phenotype was dependent on induction of the *P. falciparum* gene and independent of vector context. PFI0195c, which likely encodes a small GTPase activator (GAP), also conferred resistance to cycloheximide and halofantrine in yeast. Immunolocalization of the encoded protein to the Golgi complex in yeast is consistent with potential GAP function. The other three candidate proteins localized to the cytoplasm and plasma membrane (PF14_0649), nuclear envelope/ER (PF10_0372) and Golgi (PFD0090c) of yeast. Analysis of mefloquine-resistant *P. falciparum* strains and the mefloquine-sensitive strain, W2, by sequencing and semi-quantitative RT-PCR identified no relevant mutations in the resistant strains but showed that PFI0195c was upregulated in two out of three resistant strains and PF14_0649 was upregulated in all resistant strains tested.
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Keywords: *Plasmodium falciparum*; Mefloquine; Drug resistance; *Saccharomyces cerevisiae*

1. Introduction

Malaria affects over 200 million people in the tropical and subtropical regions of the world. There are four species of *Plasmodium* that cause human malaria. The most dangerous of these is *P. falciparum*, which kills 2 million annually, mostly children under 5 years of age [1]. Malaria management has relied on vector control of the *Anopheles* mosquito and on chemotherapy and chemoprophylaxis. The antimalarial drug chloroquine was heavily used until the late 1950s when chloroquine-resistant strains emerged at several locations and rapidly spread throughout the world [1]. Many countries now use other drugs, such as Fansidar (a combination of the two antifolates pyrimethamine and sulfadoxin) and mefloquine (MFQ). Antifolate resistance is well documented worldwide and mefloquine resistance, already a problem in southeast Asia, is threatening to spread to other regions [2].

Little is known about either mefloquine's mode of action and targets in *P. falciparum* or the resistance that develops to it. The *P. falciparum* multi-drug resistant gene (pfmdr1) encodes an ATP-binding cassette (ABC) transporter (Pgh1), which is often upregulated and/or mutated in mefloquine-resistant strains [3,4]. However, mefloquine-resistant strains have been reported that contain no pfmdr1 alterations [5–7], suggesting that other genes are also involved in mefloquine resistance. We sought to identify additional mefloquine re-

Abbreviations: MFQ, mefloquine; GAP, GTPase activating protein
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doi:10.1016/j.molbiopara.2004.10.005

sistance genes by a genome-wide screen in the yeast *Saccharomyces cerevisiae*. Transformation of *P. falciparum* is not yet efficient enough for the large-scale library transformation possible in model organisms, and the screening of thousands of transformants in individual drug assays is similarly not yet feasible. *S. cerevisiae* can be transformed efficiently and screened with comparative ease in a short time period, is sensitive to mefloquine [8], and has been used successfully to characterize *Plasmodium* resistance to antifolate drugs [9]. We transformed yeast with a *P. falciparum* expression library under the control of a yeast inducible promoter and screened for putative MFQ resistance genes. We characterized four genes, PFD0090c, PFI0195c, PF10_0372 and PF14_0649, which were identified in this screen.

2. Materials and methods

2.1. A yeast screen for P. falciparum resistance genes

The *S. cerevisiae* W303-1B (*MATα ade*2-1 *trp*1-1 *can*1-100 *leu*2-3,112 *his*3-11,15 *ura*3) strain was grown in 5 mL YPD [10] at 30 °C overnight. This culture was added to 45 mL YPD and grown at 30 °C until OD 660 = 1.0 (approx. 4 h), then transformed with a *P. falciparum* cDNA library (a gift of G. Subramanian and Louis Miller, NIH) in the pB42AD vector (Clontech, Palo Alto, CA) using the TRAFCO high efficiency transformation method (http://www.mcb.ucdavis.edu/faculty-labs/powers/Protocols/Pages/TRAFOTransformaion.htm). The library contains cDNA from all intraerythrocytic stages of the *P. falciparum* reference strain, 3D7. The transformation mix was plated on 2% galactose/raffinose agar plates lacking tryptophan to induce *P. falciparum* gene expression and to select for the library, and containing 400 μM MFQ. The plates were incubated for 3 days at 30 °C. The 364 colonies selected at this concentration of MFQ were patched on 2% galactose/raffinose minus tryptophan plates and replica-plated onto plates containing either 400, 500, 600 or 800 μM MFQ. All 364 colonies grew on the 400 μM MFQ plates and the four colonies that survived > 400 μM MFQ plus others totaling 80 were picked for analysis. Each was grown in 2 mL of 2% glucose minus tryptophan media at 30 °C overnight. Plasmids were recovered using a Qiagen miniprep (Qiagen, Valencia, CA) kit, with the addition of glass beads and 10 min of vortexing, prior to the lysis step. The resulting DNA was then transformed into *E. coli* (DH5α) by electroporation, and individual transformants were grown in 2 mL Luria Broth with 100 μg/mL ampicillin at 37 °C overnight. Plasmids were harvested using the standard Qiagen miniprep protocol. Each of the 78 resulting plasmids was sequenced and compared by BLAST against the *P. falciparum* genome database at http://www.plasmodb.org. Plasmids were retested by transformation back into *S. cerevisiae* and patched onto plates containing increasing concentrations of MFQ.

2.2. Serial dilution spot assays

Yeast were grown in 2% galactose/raffinose minus tryptophan media at 30 °C overnight, then diluted to either 10^6 cells/mL or 10^4 cells/mL. Ten μl were spotted, in duplicate, onto 2% galactose/raffinose minus tryptophan media, MFQ plates containing either no drug, 200, 300, 400, 500 or 600 μM MFQ. Plates were then placed at 30 °C and photographed daily for 7–8 days.

2.3. Liquid culture drug assays

Yeast were grown in 2% galactose/raffinose minus tryptophan media at 30° to mid-log phase (OD 660 = 0.5–0.8), then diluted to 5×10^6 cells/mL and added to a 96-well plate containing either no drug or 0.5, 1, or 2 mg/mL G418; 10, 50, or 100 ng/mL cycloheximide; 5, 10 or 25 μM halofantrine; 1, 2 or 3 mM quinine; 25, 50, 100, or 500 μM chloroquine; 25, 50 or 100 μM mefloquine. Growth was measured after 24 h at 30 °C by absorbance at $\lambda = 660$. Each strain was tested in triplicate and at least three experiments were performed.

2.4. Cloning into pYES vector

Isolated pB42AD plasmids were used to subclone the *P. falciparum* cDNA sequence into the pYES2.1/V5-His-TOPO (Invitrogen, Carlsbad, CA) vector using the primers 5′-ATGCCTTATGATGTGCCAGATTATGCCTCT and 3′-CCAAACCTCTGGCGAAGAAGTCCAAAGT. Full-length genes were cloned from *P. falciparum* 3D7 cDNA into pYES by TA-TOPO cloning (Invitrogen's protocol) in yeast using the following primers: PF14_0649: 5′-ATGAAACAGGCAAGTAAAAACTTCAGCC, 3′-ATGGTATCTTCCTCCTCGGCATC; PF10_0372: 5′-ATGGGGTCACAAATTCAACCATCAAG, 3′-CTTGTCACGATTTAATCTATTTGGATCTTC; PFD0090c: 5′-ATGTGTAATAAATTGTCAAGGGGTAG, 3′-CATCAAGTGTAGGGTATCATTATAGTCC; PFI0195c: 5′-ATGATAGTAGGTAACAAATTTTGGG, 3′-ATATTCTTCCATTTCTCTTTTTTCATTAAT. These primers were also used for sequencing of these genes from MFQ-resistant and MFQ-sensitive *P. falciparum* strains.

2.5. Immunolocalization in yeast

The three full-length genes (PF10_0372, PFD0090c and PFI0195c) and PF14_0649 fragment in the vector pYES2.1 were transformed into the yeast W303-1B strain, and transformants were grown overnight in 5 mL of 2% glucose minus uracil media at 30 °C. Each sample was washed with ddH20 and added to 50 mL of 2% raffinose/galactose minus uracil media and incubated at 30 °C for an additional 4 h (final cell density of $\sim 1 \times 10^7$) to induce expression. Cells were fixed by addition of 6.75 mL 37% formaldehyde followed by incubation at room temperature for 30 min, pelleting and resuspension in 5 mL phosphate-buffered formaldehyde (100 mM K_2HPO_4/KH_2PO_4, 0.5 mM $MgCl_2$, 5% formaldehyde, pH

6.5) for 2 h at room temperature. Cells were then washed two times in solution A (40 mM K_2HPO_4/KH_2PO_4, 0.5 mM $MgCl_2$, 1.2 M sorbitol, pH 6.5) prior to cell wall digestion for 30 min at 30 °C in 1 mL solution A with glusalase and zymolyase. Following digestion, cells were washed again with solution A and stored overnight at 4 °C. The next day, a 10 μL cell suspension was applied to each slide well, aspirated and allowed to dry before the slide was washed in methanol at −20 °C for 6 min and then acetone at −20 °C for 30 s. Slides were then blocked with 1% milk PBS for 30 min, incubated with anti-V5 antibody (1:200) (Invitrogen, Carlsbad, CA) in 1% milk PBS for 1.5 h, washed 10 times with PBS, incubated with anti-mouse IgG-FITC antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1.5 h in the dark and washed 10 times with PBS. One microliter mounting media, containing DAPI, was added and slides were sealed. Slides were viewed on a Zeiss Axiovert Delta Vision microscope at 100× magnification supported by SoftWorx software.

2.6. Sequencing of P. falciparum genes from MFQ-resistant and MFQ-sensitive strains

Sequences were obtained using a Big Dye Terminator kit, protocol and ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). Genomic DNA and/or cDNA, made from mRNA using Superscript II reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) and protocol, from the following lines: W2, Dd2, HB3, 3D7 (RNAs were a gift of C. Ben Mamoun, University of Connecticut), C10 (RNA was a gift of Jean Feagin, SBRI) and W2Mef (parasites were a gift of D. Kyle, WRAIR). Each was sequenced at least three times to rule out RT and PCR errors.

2.7. Quantitative RT-PCR from MFQ-resistant and MFQ-sensitive P. falciparum strains

Reverse transcription reactions were performed on *P. falciparum* mRNA from the W2, Dd2, 3D7 and W2Mef lines using Superscript II RT (Invitrogen, Carlsbad, CA) and protocol. Each reaction was performed with and without RT enzyme prior to amplification of cDNAs by specific primers to rule out genomic DNA contamination. Each of the following primer pairs was used in multiplex PCR reactions (http://uni-ulm.de/∼dkaufman/rt-pcr.html) with actin primer pairs and with varied RT product input (0.5, 1 and 2 μL) and 22–25 cycles of 94 °C for 20 s, 55 °C for 10 s, 50 °C for 10 s and 65 °C for 4.5 min. Primers are as follows: PfActin: 5′-ATGGGAGAAGAAGATGTTCAAGCTTTAG, 3′-GGATACTTTAATGTTAATATACCTCTCTTGG; PFD0090c: 5′-ATGTGTAATAAATTGTCAAGGGGTAGTAATATG, 3′-CTTTTTTTAGTTTCGGTATTTGTGTTCCTC; PF10_0372: 5′-ATGGGGTCACAAATTCAACCATCAAG, 3′-CTTGTCACGATTTAATCTATTTGGATCTTC; PF14_0649: 5′-TTAAATGACGACAATAATAATAAACAAGTGTCC, 3′-GTACTATTGTAATATTGATATTGTTCATTTTCC; PFI0195c5: 5′-ATGATAGTAGGTAACAAATTTTGGGACG, 3′-GATATACCCTGATGATAATCATTTGTTATTGG; pfmdr1: 5′-ATGGGTAAAGAGCAGAAAGAGAAAAAAG, 3′-GGATTAATATCATCACCTAAATTCATGTTC.

The resulting DNA was fractionated on a 2% agarose gel in TBE, stained with ethidium bromide and analyzed on a Macintosh computer using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/) to determine relative band intensities. Each sample was normalized to its internal actin control and relative transcript intensities for each experiment were compared to levels present in the W2 (MFQ-sensitive) sample, which was normalized to 1. Multiple experiments were then averaged.

3. Results and discussion

3.1. Screen for P. falciparum genes that confer mefloquine resistance in S. cerevisiae

The yeast strain W303-1B is incapable of growth on 200 μM MFQ plates (data not shown). Yeast commonly require drug concentrations well above that needed for tissue culture cells [11], especially on plates where only a fraction of the total surface area of each cell contacts the drug-containing agar. The thick cell wall that surrounds the yeast is a formidable barrier and endogenous drug pumps, such as Pdr5, are able to exclude many compounds [12] such that intracellular concentrations are likely to be much lower. We transformed W303-1B with a library of plasmids containing *P. falciparum* cDNA fused to the B42 activation domain, under the control of a galactose-inducible promoter. Mefloquine-resistant colonies were selected on plates containing 400 μM MFQ and were patched to plates of increasing MFQ concentration to determine the degree of resistance (Fig. 1). To determine if the resistance phenotype is plasmid dependent, we recovered the plasmids from 80 colonies, retransformed them into yeast, and retested on MFQ-containing plates (Fig. 1). Seventy-eight of the eighty grew on these plates (data not shown), indicating that MFQ resistance is conferred by the library plasmid. We replica-plated these 78 MFQ-resistant colonies to plates of varying MFQ concentration and identified four (Table 1) that exhibited resistance above 400 μM MFQ, with PFI0195c showing the strongest resistance phenotype. These genes were further characterized.

Table 1
Putative mefloquine resistance genes isolated

Identity	Function	Size (bp)	Isolated (bp)	MFQR (μM)[a]
PFI0195c	Putative GAP	1281	586–1020	600
PF14_0649	Unknown	7677	4815–5703	500
PF10_0372	Unknown	363	1–363	500
PFD0090c	Unknown	1287	1–488	500

[a] Degree of mefloquine resistance tabulated from multiple patching experiments.

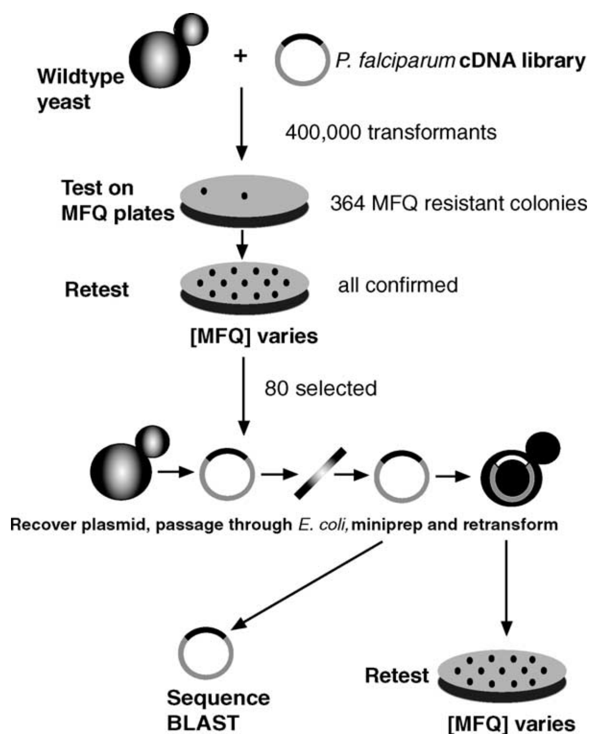

Fig. 1. Protocol for identifying putative *P. falciparum* MFQ resistance genes in yeast.

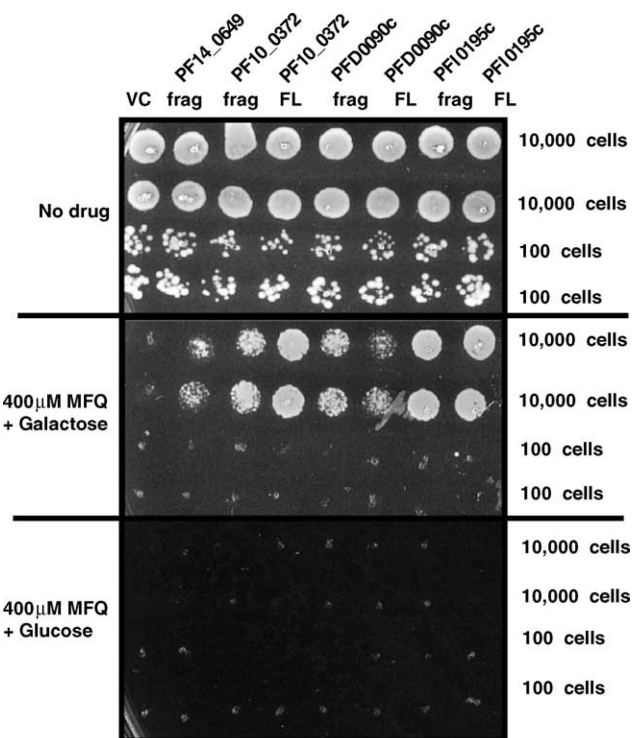

Fig. 2. MFQ resistance phenotype is inducible and independent of vector context. Serial dilutions of yeast bearing either a pYES vector control (VC), fragment from a putative MFQ resistance gene isolated in the original screen and subcloned into pYES, or the corresponding full length (FL) gene cloned into pYES were tested on plates with and without MFQ under promoter inducing (galactose/raffinose) or repressing (glucose) conditions.

3.2. Analysis of the MFQ resistance phenotype

In order to quantify MFQ resistance, we employed a serial dilution spotting assay (Fig. 2). The spotting assay was performed under both promoter-inducing (galactose) and non-inducing (glucose) conditions in order to assess whether expression of the *P. falciparum* DNA was required for the MFQ resistance phenotype. Because the library was generated using oligo (dT) priming and the *P. falciparum* genome is 80% AT rich, cDNAs cloned into the library often contain only a C-terminal or internal fragment of a gene. Three of the four library inserts were fragments, whereas PF10_0372, due to its small size, was recovered at full length (Table 1). The corresponding full-length open reading frames (predicted by PlasmoDB) were cloned from *P. falciparum* cDNA, except PF14_0649, which was too large to be cloned in its entirety. The vector used in the original screen fuses a two-hybrid activation domain and a nuclear localization signal to the *P. falciparum* inserts. To confirm that the activation domain did not influence the phenotype, we cloned both the gene fragments and their corresponding full-length open reading frames into the pYES vector which contains no domain fusion. Fig. 2 shows that these genes expressed in the pYES vector also conferred MFQ resistance, and that both the isolated fragments and full-length genes conferred varying degrees of resistance. In addition, it was necessary for each putative MFQ resistance gene to be induced in order for it to confer resistance.

We also quantified resistance by liquid culture drug assays. A comparison of the strains on the middle plate in Fig. 2 shows that the PF10_0372 fragment conferred less MFQ resistance ($55 \pm 3.2\%$ growth in liquid culture at 200 µM MFQ compared with growth at no MFQ) than the full length gene, which conferred $78 \pm 1.1\%$ relative growth at the same concentration of MFQ. By contrast, the PFD0090c fragment conferred more resistance ($76 \pm 1.2\%$ relative growth) than the corresponding full length gene ($69 \pm 1.1\%$ relative growth). The PFI0195c gene and its fragment, with relative growth rates of $81 \pm 1.3\%$ and $80 \pm 1.2\%$, respectively, conferred similar MFQ resistance. Fig. 3 (top panel) shows that, as in the replica-plating and serial dilution spotting assays, PFI1095c conferred the greatest resistance.

3.3. PFI0195c confers multi-drug resistance

Some drug resistance proteins, particularly those involved in drug efflux like the ATP-binding cassette drug transporters, confer resistance to multiple unrelated drugs [12]. In order to determine if any of the putative MFQ resistance genes isolated in our screen confer resistance to multiple unrelated drugs, we repeated the liquid drug assay with chloroquine, halofantrine, quinine, cycloheximide and G418. Both PFI0195c and PF14_0649 conferred cross-resistance to halofantrine (Fig. 3, middle panel). Only one gene, PFI0195c,

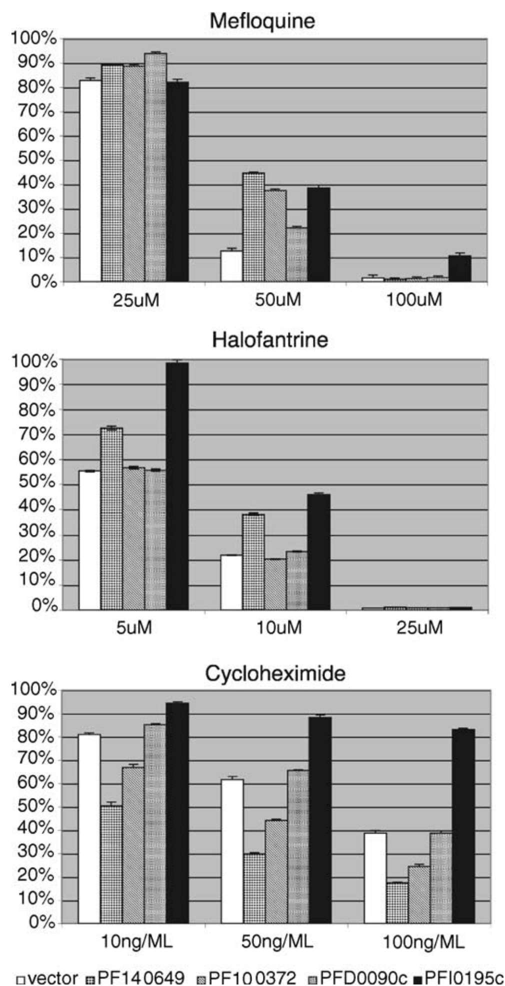

Fig. 3. Comparison of resistance to MFQ, cycloheximide and halofantrine. (Top) Similar patterns of MFQ resistance were conferred by putative MFQ resistance genes when yeast were grown in liquid culture. Yeast carrying each gene was tested at varying MFQ concentration, as shown, and compared to the rate of growth of the no drug control. (Middle) Yeast carrying each gene was tested at varying halofantrine concentration, as shown, and compared to the rate of growth of the no drug control. (Bottom) Yeast carrying each gene was tested at varying cycloheximide concentration, as shown, and compared to the rate of growth of the no drug control.

conferred additional resistance to cycloheximide (Fig. 3, lower panel). PFI0195c is a putative GTPase activating protein (Table 1) with homology to yeast GTPase activating proteins involved in vesicular transport of proteins and regulation of endocytosis and exocytosis [13]. Therefore, it is possible that changes in protein transport overcome the deleterious effects on protein production in cells treated with cycloheximide. Interestingly, yeast expressing PF14_0649 and, to a lesser extent, PF10_0372, were cycloheximide-sensitive. Possibly the exogenous expression of these *P. falciparum* proteins in yeast sensitizes the protein synthesis machinery to drugs like cycloheximide. None of the MFQ resistance genes conferred resistance or sensitivity to the antimalarial chloroquine or to G418 (data not shown). Additionally, as reported in Delling et al. [8], we were unable to test the effects of quinine on yeast because they were quinine-insensitive, even at a concentration of 2 mM (data not shown).

3.4. Localization of the P. falciparum proteins in yeast

To learn more about the potential function of these genes, we localized the resulting proteins in yeast by tagging each with a V5 epitope and labeling the resulting fusion protein with a FITC-conjugated secondary antibody. The protein encoded by the 296 C-terminal residues of PF14_0649 localized in a diffuse staining pattern punctuated by discrete spots at the cell surface (Fig. 4), suggesting that it localized to the cytoplasm and specialized pockets on the plasma membrane. Such localization suggests that the C-terminal tail of this predicted multi-trans-membrane spanning protein partially localizes to the plasma membrane despite lacking its predicted trans-membrane domains. We hypothesize that it could potentially signal from there to downstream components of a multi-drug resistance response. However, since we observed localization to both cytoplasm and plasma membrane, we do not know whether plasma membrane localization is necessary for function. The protein encoded by PF10_0372 localized to the nuclear envelope/ER (Fig. 4). The proteins encoded by PFD0090c and PFI0195c showed a punctate staining pattern (Fig. 4) suggestive of ER/Golgi localization in yeast. This localization matches that of a Rab GTPase activating protein [14], which PFI0195c resembles. PFI0195c also co-localized with the yeast Rab GTPase, Ypt6 (see inset panel in Fig. 4), which is involved in vesicular trafficking and located in the ER/Golgi [13,15]. If PFI0195c activates Ypt6, the increased vesicular transport could affect drug efflux. Alternatively, if the target of mefloquine is within the vesicular transport pathway, increased activation might confer resistance.

3.5. PF14_0649 and PFI0195c are upregulated in MFQ-resistant P. falciparum

We examined whether any of these four genes were mutated or upregulated in MFQ-resistant *P. falciparum* lines as compared to MFQ-sensitive strains. The coding sequences of all four open reading frames (except the large ORF of PF14_0649, for which only the sequence corresponding to the fragment recovered from the screen was obtained) were sequenced in six lines (MFQ-resistant: 3D7, Dd2, W2Mef; MFQ-sensitive: HB3, W2; MFQ status unknown: C10). We found only one synonymous change in PFI0195c which did not correlate with resistance status. We performed semi-quantitative RT-PCR on the RNA from four asynchronous lines (3D7, Dd2, W2 and W2Mef), assessing the relative levels of the transcripts of the four resistance genes as well as that of the pfmdr1 gene, which is known to be upregulated in the resistant lines Dd2 and W2Mef [3,4,16]. Transcript levels were statistically equivalent to those seen in the W2 line except for pfmdr1, which was 4–6.5-fold higher in W2Mef, Dd2 and 3D7. Increased pfmdr1 expression in the 3D7 line correlates with this line having an MFQ IC50

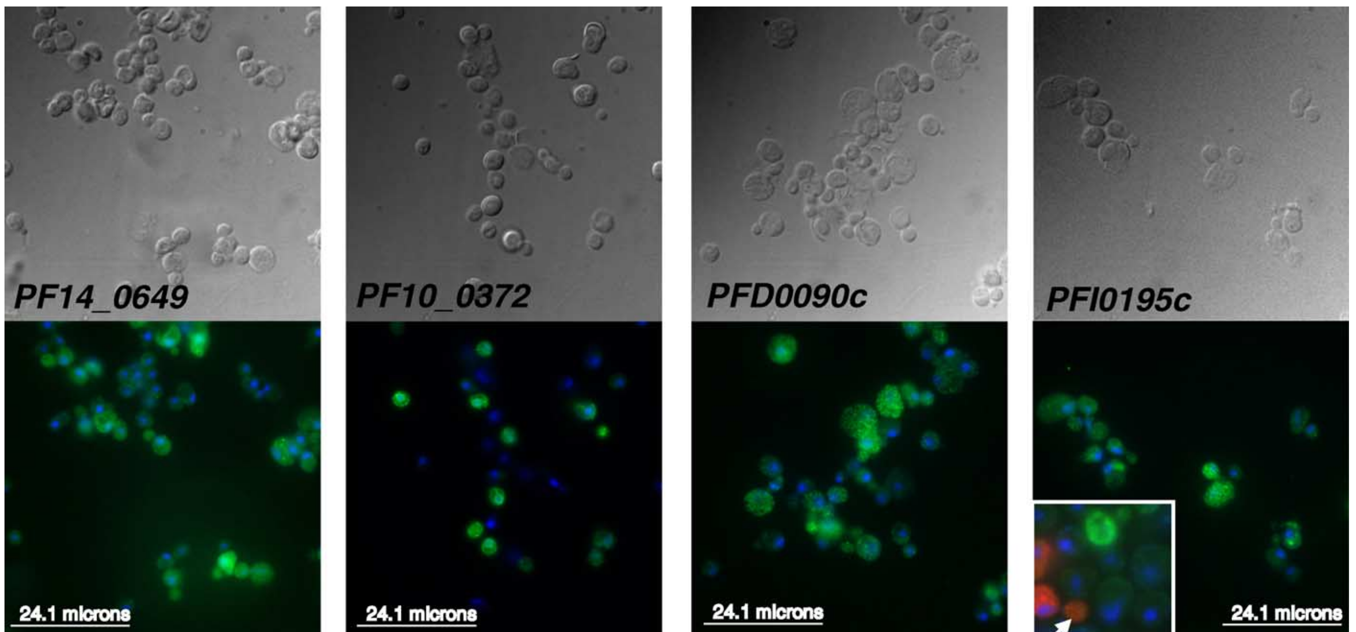

Fig. 4. Immunolocalization of *P. falciparum* proteins in yeast. Proteins were tagged with a V5 epitope and labeled with a V5 antibody followed by a FITC-conjugated secondary antibody, shown in green. Nuclei were stained with DAPI, shown in blue. Inset in right-most panel shows co-localization of Ypt6 (labeled with Cy5 in red) and PFI0195c (labeled with FITC in green).

of 20 nM (C. Ben Mamoun, personal communication), similar to that reported for the MFQ-resistant line Dd2. The PF14_0649 transcript was 2.5–5 fold higher in all MFQ-resistant lines tested compared to W2. PFI0195c transcript levels were 1.75 fold higher in Dd2 and 5-fold higher in 3D7 but equivalent in W2Mef to W2 (Fig. 5). Although the RNA was harvested from asynchronous lines grown in several different laboratories, each sample was compared to an internal actin control. Additionally, pfmdr1 levels acted as a positive control, because they are known to be increased in the Dd2 and W2Mef lines. A comparison of the transcripts of the actin, pfmdr1 and putative mefloquine resistance genes to expression profiles from the large data sets of DeRisi and coworkers [17] and Winzeler and coworkers [18] also indicated that the RNAs reflect a mixed parasite population.

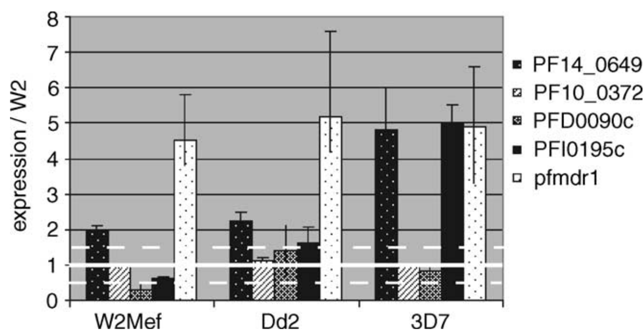

Fig. 5. Transcript levels in MFQ-resistant *P. falciparum* lines. Quantitative RT-PCR was performed on four strains of *P. falciparum* with primer probes to the four putative MFQ resistance genes and to pfmdr1 (positive control). Shown are the results of three or more experiments as means ± 1 S.D. Solid white line indicates no difference relative to W2 and dashed lines indicate 0.5 and 1.5 the W2 level.

4. Conclusions

Successful yeast models of resistance to antimalarial antifolates have been generated [9] that have furthered our understanding and prediction of resistance mutations. This knowledge has contributed to the development of better antifolates [19]. In these models, the target of the drug is known: dihydrofolate reductase. However, as is the case for many drugs, the target of mefloquine is not known. Here, we have demonstrated the feasibility of using yeast to screen for genes involved in mefloquine resistance. A similar experiment in *P. falciparum* is currently not feasible, and our screen was simple and inexpensive to perform. However, biological differences between any model organism and the system it is meant to reflect lead to results that must be interpreted with caution. We examined sequence and expression level differences in several *P. falciparum* lines and found that one of the genes identified in our screen (PF14_0649) was upregulated in all of the resistant lines tested. The PF14_0649 protein was partially membrane-localized in yeast and contains predicted trans-membrane spanning domains, although it does not resemble most transporters or pumps [20,21]. However, it could act as a modulator of a drug transporter such as the *Plasmodium* pfmdr1-encoded protein, Pgh1, which is similarly localized and whose transcript is upregulated in all of the MFQ-resistant strains we tested. Interestingly, PFI0195c was also upregulated in two of the three resistant strains tested.

PFI0195c is responsible for the strongest resistance we identified, contains a TBC domain (indicative of Rab GAP function), has homology to the yeast Rab GAPs, Mdr1 and Gyp6 (activators of Ypt6), and co-localizes to the Golgi of yeast with Ypt6, suggesting that it may mediate resistance in yeast, and possibly in *P. falciparum*, through its effects on vesicular transport pathways. This evidence, coupled with data suggesting that mefloquine inhibits parasite endocytosis [22], leads us to propose that one of the components of the endocytosis pathway (possibly a Rab-GTPase) may be a target of mefloquine.

Interestingly, in addition to being upregulated in MFQ-resistant parasites, both PF14_0649 and PFI0195c conferred mefloquine and halofantrine cross-resistance to yeast, a resistance pattern commonly seen in malaria parasites [16,23]. Therefore, we conclude that both PF14_0649 and PFI0195c are good candidates for MFQ resistance genes. Further analysis of these genes will be necessary to determine if they play a role in *P. falciparum* MFQ resistance.

Acknowledgements

We thank G.M. Subramanian and Louis Miller for providing the pB42AD-*P. falciparum* cDNA library; C. Ben Mamoun for advice and the gift of 3D7, Dd2, W2 and HB3 mRNAs; Jean Feagin for advice, the gift of C10 mRNA and 3D7 genomic DNA and the use of her lab to grow *P. falciparum*; Dennis Kyle for the gift of W2 and W2Mef parasites; Bethany Fox and Trisha Davis for use of the DeltaVision microscope and aid in image generation; those who maintain the Plasmodium Genome Database; WRAIR for mefloquine; and Sanjay Desai for critical reading of the manuscript.

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