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Analysis of Chloroquine Resistance Transporter (CRT) Isoforms and Orthologues in *S. cerevisiae* Yeast

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Abstract

Previous work from our laboratory optimized MeOH – inducible expression of the *P. falciparum* malarial parasite transporter PfCRT in *P. pastoris* yeast. These strains are useful for many experiments, but do not allow for inducible protein expression under ambient growth conditions. We have therefore optimized galactose – inducible expression of PfCRT in *S. cerevisiae* yeast. We find that expression of PfCRT confers CQ hypersensitivity to growing yeast and that this is due to plasma membrane localization of the transporter. We use quantitative analyses of growth rates to compare hypersensitivity for yeast expressing various PfCRT isoforms. We also report successful high level inducible expression of the *P. vivax* orthologue, PvCRT, and compare CQ hypersensitivity for PvCRT vs PfCRT expressing yeast. We test the hypothesis that hypersensitivity is due to increased transport of CQ into yeast expressing the transporters via direct ³H – CQ transport experiments, and analyze the effect that membrane potential has on transport. The data suggest important new tools for rapid functional screening of PfCRT and PvCRT isoforms and provide further evidence for a model wherein membrane potential promotes charged CQ transport by PfCRT. Data also support our previous conclusion that wild type PfCRT is capable of CQ transport, and provide a basis for understanding the lack of correspondence between PvCRT mutations and resistance to CQ in the important malarial parasite *P. vivax*.

Keywords

malaria; drug resistance; chloroquine; PvCRT; PfCRT

Since the isolation of the *pfcr* gene 10 years ago [1], much has been learned regarding the encoded PfCRT protein and its role in conferring chloroquine resistance (CQR) in *P. falciparum* malarial parasites [2, 3]. Geographically distinct patterns of amino acid substitutions in PfCRT are associated with a variety of different CQR phenotypes in *P. falciparum* isolates from around the globe. The different phenotypes are further characterized by cross resistance patterns to other drugs and variable contributions from additional allele inheritances, gene mutations or gene over-expression events that work in concert with PfCRT mutations to cause antimalarial multidrug resistance [4-6]. One additional gene that appears to be involved in multidrug resistance is *pfmdr1*, which encodes a human P- glycoprotein orthologue that is mutated and / or alternately expressed in many CQR isolates [7-9].

Further elucidation of the PfCRT molecular mechanism is essential for controlling current and newly evolving drug resistance phenomena, and for developing second tier drug

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therapies. As one example of the former, CQR has been found in the related malarial parasite *P. vivax*, similar to *P. falciparum* [10,11], albeit in a different geographically disposed pattern. However, unlike the case for PfCRT and *P. falciparum*, amino acid substitutions in the PvCRT orthologue are apparently not required for *P. vivax* CQR [10]. It would be helpful to translate concepts elucidated for *P. falciparum* CQR towards progress in understanding *P. vivax* CQR. Unfortunately, many approaches for studying CQR in *P. vivax* are inaccessible since there are no convenient laboratory – based methods available for culturing *P. vivax*. Heterologous expression of key *P. vivax* proteins, compared side – by – side with their *P. falciparum* orthologues, might provide one avenue for elucidating some aspects of *P. vivax* biology. For example, a model for why PvCRT mutations are apparently not related to *P. vivax* CQR could conceivably be generated from such experiments.

Previously, we have shown that both wild type (“HB3” isoform) and mutant CQR associated (“Dd2” [S.E. Asian] and “7G8” [S. American]) isoforms bind CQ [12,13]. Surprisingly, affinity constants for equilibrium binding of ^3H -CQ were found to be similar for HB3 and Dd2 PfCRT [12]. Subsequent use of a CQ photoaffinity analogue and mass spectrometry allowed us to map a single specific quinoline binding site for HB3 PfCRT [13]. The predicted site lies proximal to PfCRT residues that have been found to be mutated in CQR conferring isoforms.

At low external doses of CQ, infected red blood cells harboring CQR parasites accumulate less CQ relative to QCS [14-16]. Also, CQ is known to concentrate within the parasite digestive vacuole (DV) [16,17], and subcellular PfCRT protein localization includes high levels within the DV membrane [1,18]. It has thus been proposed that PfCRT mediates CQ transport from the DV in some fashion, in order to lower CQ accumulation within the DV. As summarized elsewhere [2], there is considerable support for this overall model, yet a number of issues remain to be resolved. Two recent studies have provided direct evidence for PfCRT mediated CQ transport. One uses oocytes injected with *pfprt* cDNA to measure slow, but heightened accumulation of ^3H -CQ into the oocytes vs time [19] and the other uses purified PfCRT protein reconstituted into proteoliposomes (PLs) and a novel fluorescent CQ reporter to measure pH gradient (ΔpH) and electrical potential ($\Delta\Psi$) stimulated efflux from the PLs [20]. Some differences in the two studies are found, in particular, estimates of turnover (drug transported per PfCRT per second) are much slower for the oocyte experiments relative to PL, and the PL study reports transport by both HB3 and Dd2 PfCRT isoforms, whereas the oocyte study suggests that only Dd2 PfCRT is capable of drug transport (see [2] for a more detailed discussion).

For these reasons and others, we have endeavored to devise additional convenient model systems for the study of PfCRT isoforms as well as PvCRT and other malarial transport proteins. Metabolic induction of transporters in *S. cerevisiae* growing under ambient conditions is in theory another such system, but high AT bias and unusual gene structure for some *P. falciparum* genes, as well as cellular targeting issues, often complicates application of these techniques. In this study, we have perfected pYES2 based galactose-inducible expression of PfCRT and PvCRT. We use this tool to further analyze questions related to PfCRT-mediated CQ transport, and to also test for putative CQ transport by the important PfCRT orthologue PvCRT.

Materials and Methods

Materials

Yeast DOB media and DOB with galactose and raffinose were obtained in powder form from MP Biomedicals, Solon, OH. Cell culture plastics were from BD Falcon. Glass beads for yeast cell lysis were from B. Braun Biotech, Allentown, PA. Anti-HexaHis-HRP and

anti-V5-HRP antibodies were from Qiagen (Valencia, CA) and Invitrogen (Carlsbad CA), respectively. ^3H – CQ was from American Radiolabelled Chemicals Inc (St. Louis MO). All other chemicals were reagent grade or better, were purchased from Sigma (St. Louis MO) and used without additional purification.

Yeast Strains and Methods

CH1305 (MATa *ade2 ade3 ura3-52 leu2 lys2-801*) was supplied by J.F. Cannon [21]. Solid and liquid media were prepared as described in Sherman *et al.* [22], and included synthetic complete (SC) media lacking one or more specified amino acids, as well as rich medium (YPAD or YPD). Induction of CRT protein expression was achieved by adding 2% galactose and 1% raffinose to SC media (then called SGR media) lacking uracil. Standard procedures were used for yeast manipulations [22]. Parental host strains were maintained in YPAD or YPD media and grown at 30°C. Yeast transformations were done by the lithium acetate procedure [23] with 1 µg of pYES2 backbone containing specified construct (into the same host strain for each set of plasmids described.) Transformants were plated onto SD media lacking uracil. All subsequent cultures originated from frozen stock glycerol suspensions of individual clones.

Plasmids

A codon-optimized version [24] of the wild type *P. falciparum* chloroquine resistance transporter (*pfCRT*) cDNA sequence, designed with a 5'-Kozak sequence and both V5 and polyhistidine C-terminal epitope tags, was synthesized by Genescript (Piscataway, NJ) and cloned into vector pUC57 to create pUC57/PfHB3vh. The PfHB3vh fragment was restricted from pUC57/PfHB3vh with Kpn1 and EcoRI and subcloned behind the GAL1 promoter of Kpn1/EcoRI-digested plasmid pYES2 (kindly provided by Dr. William Fonzi, Georgetown University) to create pYES2/PfHB3vh. To generate PfHB3_{PMA}, encoding HB3 PfCRT fused in frame to the N-terminal domain of the yeast plasma membrane ATPase (PMA), the 333-bp fragment encoding the first 111 amino acids of PMA was synthesized by Genescript and inserted into pUC57/PfHB3vh to create pUC57/PfHB3_{PMA}vh. In this construct, the 50 N-terminal amino acids of wild type (isoform HB3) PfCRT were replaced by the N terminal 111 amino acids of yeast plasma membrane ATPase (PMA1). The PfHB3_{PMA}vh fragment was restricted from pUC57/PfHB3_{PMA}vh with Kpn1 and EcoRI and subcloned behind the GAL1 promoter of Kpn1/EcoRI-digested plasmid pYES2. pYES2/PfDd2vh and pYES2/Pf7G8vh, encoding epitope tagged CQR – associated Dd2 and 7G8 isoforms of PfCRT respectively, were created by ligating fragments of pPIC3.5/PfDd2h and pPIC3.5/Pf7G8h (see [12,13]), digested with SexAI and AarI, into SexAI/AarI-digested pYES2/PfHB3vh (See Table 1).

To express *P. vivax* chloroquine resistance transporter (PvCRT), pYES2/PfHB3vh was linearized with BstEII and pPICZ-A/PvCRTmh containing the native *pvcrt* cDNA was linearized with SacII. After filling in, fragments were further restricted with KpnI and ligated to plasmid pYES2 to create pYES2/PvCRTvh. Site-directed mutagenesis via the Stratagene QUICKChange method was used to set V5 and hexaHis epitope tags back in frame. pYES2/PvCRTvh was used to create a “Dd2-like” isoform of PvCRT. Primers PfQ271^E, PfN326^S, PfI356^T, PfM371^I, PfMIK74-76^{IET} (see Table 2) were used to create pYES2/PvCRT^{Dd2}vh. All final plasmid constructs were confirmed by direct DNA sequencing.

Isolation of yeast membrane fractions

Overnight cultures of yeast cells grown in inducing SGR-ura medium to midlog phase were harvested at 2000g for 5 min at 25 °C. Cells were washed in harvest buffer (100 mM glucose/50 mM imidazole/5 mM DTT, pH 7.5) and lysed by rapid mixing in the presence of

glass beads (1.00–1.05 mm diameter). Suspensions (10 g wet weight of cells, 20 mL of breaking buffer [0.25 M sucrose/0.1 M glucose/50 mM imidazole/1.0 mM MgCl₂, pH 7.50], equal volume of glass beads) were homogenized using a BioSpec BeadBeater (Bartlesville, OK) at 3000 rpm for 20 min at 4 °C (for large scale preparations) or, for 30 seconds followed by 30 seconds on ice, 8 times, for small scale preparations. Suspensions were decanted away from the glass beads and centrifuged twice at 1000g and once at 3000g for 5 min each, to remove unlysed cells and cellular debris. The supernatant was collected and spun again at 100,000g to collect the crude membrane (CM) fragment. To prepare pure plasma membrane (PM) fractions, these fragments were acid-precipitated via the procedure of Goffeau and Dufour [25]. CM or purified PM fraction was stored in suspension medium (10 mM imidazole/1 mM MgCl₂) and analyzed by Western blot and amido black assays.

Western Blotting

Strains expressing HB3 *Pf*CRT, Dd2 *Pf*CRT, 7G8 *Pf*CRT, *Pv*CRT, LacZ or empty vector were grown at 30°C to OD⁶⁰⁰ ~ 1.0 in either inducing SGR or non inducing SD media lacking uracil. Cellular membrane fractions were prepared as described above, protein content was determined via amido black, and aliquots stored at –80 °C. For western blot analysis, gel loading dye was added, extracts denatured at 65°C for 5 min, and proteins first resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) 12% at 110 V for ~ 2 h. Proteins were then transferred to polyvinylidene difluoride membranes in transfer buffer (20% methanol/150 mM glycine/20 mM Tris base/0.1% SDS) at 40 mA for 16 hours. Membranes were then washed with phosphate buffered saline-Tween (PBS-T) solution twice (10 min each), blocked with 5% milk in PBS-T for 1 h, incubated with either anti-HexaHis-HRP or anti-V5-HRP for 1 h, washed three times with PBS-T (5 min each), and detected using Amersham Hyperfilm ECL (Piscataway, NJ).

Colony Formation Assays

To test the effects of CQ on growing yeast cells expressing CRTs, strain CH1305 harboring CRT plasmids (see above), pYES2/LacZ, or empty pYES2 vector were initially assessed by colony spot assays (see Results). Cell suspensions used to inoculate agar plates were grown at 30°C to mid-exponential phase in non inducing SD media lacking uracil. Suspensions were measured, equilibrated to an OD = 0.1, and transferred to wells of a 96 well plate. Three 10-fold dilutions were made for each cell suspension with a multi-channel pipette, and 5 µL from each well were then replica plated side by side on either non inducing SD or inducing SGR media lacking uracil, and vs specified concentrations of CQ (see Results).

Quantitative Growth Rate Analysis

Susceptibilities of yeast strains to CQ were quantitatively assessed by automated growth curve analysis in liquid media. Cell suspensions were grown at 30°C to mid-exponential phase in non inducing SD media lacking uracil. Cells density was adjusted to OD = 0.1 and cells transferred to 96-well plates containing 200 µL SD or SGR media lacking uracil, plus various concentrations of drug. Plates were wrapped in parafilm and placed in a Tecan GENious plate reader (Durham, NC) and growth curve data collected using the following parameters: measurement wavelength, 595 nm; number of flashes, 3; number of kinetic cycles, 125; kinetic interval, 30 minutes; valid temperature range, 28 - 32 °C; orbital shake duration, 30 seconds. For ΔpH and Δψ dependent growth curve analysis yeast harboring isoforms of CRT were assayed in synthetic complete media containing additional 100 mM KCl and buffered with 100 mM MES – Tris (see Results). Growth under each condition was measured in triplicate via back dilution of the strain grown under normal non inducing conditions (SD media lacking uracil). These experimental conditions are designed to indirectly probe sensitivity of yeast strains in response to decreases in ΔpH. Under normal conditions, medium external pH (pH_{ex}) ~ 5.0 and the yeast PM maintains a high ΔpH but

low $\Delta\psi$ [26,27]. Alkalinization of the external medium lowers ΔpH and cells then use PM K^+ channels to increase $\Delta\psi$ such that substantial $\Delta\psi$ compensates for loss of pH [26,27].

[^3H] CQ Accumulation Assay

Transformed CH1305 derived strains were grown to OD^{600} 0.5-1.0 in 300 mls of SGR medium. Cells were counted with a hemacytometer, centrifuged $1000 \times g$ for 5 minutes, washed twice in transport buffer (2.0% glucose/10 mM MES, pH 6.1), and resuspended in approximately 1/100th of the original culture volume in assay buffer (2×10^9 cells mL^{-1}). For uptake experiments, 50 μL of cell suspension was added to 150 μL of transport buffer containing ^3H -CQ at a final concentration of 1 μM (0.01 mCi/mM). At specific times, reactions were stopped by addition of 3 mL of ice-cold quench buffer and cells were rapidly filtered through Whatman GF/C filters (Piscataway, NJ). Filters were immediately washed twice with 3 mls of ice cold-quenching buffer (50 mM CQ / 20 mM $\text{NaC}_6\text{H}_7\text{O}_7$ / 5 mM MgSO_4 / 1 mM EDTA / 1 mM K_2HPO_4 / 1 mM CaCl_2 / 1 mM NaCl) transferred to scintillation vials containing 14 mls of Ecoscint A scintillation cocktail (National Diagnostics; Atlanta GE) and radioactivity measured using a Beckman LS6500 scintillation counter (Brea, CA). Uptake at 0 time was determined by simultaneously mixing cells in ice-cold transport buffer media and ice-cold wash buffer. For kinetic analyses, uptake rates were obtained in triplicate for each strain as described in Results.

Results

Previous results from our group showed that codon optimization of the 72 % A-T *pfCRT* gene was required for efficient heterologous expression of PfCRT protein in either *P. pastoris* or *S. cerevisiae* yeast [24]. The former expression system provides tightly controlled, AOX1 promoter mediated induction by MeOH and acceptable yields of protein for purification and proteoliposome reconstitution [13,20]. However, the system does not allow for analysis of PfCRT effects on growing yeast. The latter used a constitutively active, non-inducible Ste6 promoter. Using the pYES system and codon optimized *pfCRT* we are now able to strongly induce PfCRT expression in tightly controlled fashion for growing yeast using galactose (Fig. 1A). Expression is similar for multiple isoforms of PfCRT (Fig. 1A,B). Also, using slightly modified *pvCRT* cDNA (see Methods), we are able to use this system for similar inducible heterologous expression of the PfCRT orthologue PvCRT (Fig. 1B, lanes 4,5 vs lanes 1,2,3). Fig. 1C shows that 50 – 60 % of the PfCRT protein expressed in these yeast is PM localized (see caption), and that the protein can be exclusively localized to the PM via attachment of a N- terminal leader sequence from the yeast H^+ ATPase (see [28]).

Unlike previous heterologous expression methods, these galactose-inducible strains allow us to carefully quantify CRT – dependent phenotypes for actively growing yeast via careful side – by – side comparison of transformed strains grown in glucose (SD medium) vs. galactose (SGR medium). Since PfCRT amino acid substitutions confer reduced susceptibility to CQ growth inhibitory effects in *P. falciparum*, we hypothesized that expression of PfCRT might also alter the sensitivity of growing yeast to the drug. Indeed, Fig. 2 shows that inducing PfCRT or PvCRT using SGR medium (+galactose / raffinose, - glucose; right hand side Fig. 2) significantly reduces growth in a CQ concentration - dependent fashion. That is, yeast can tolerate CQ in the growth medium up to about 150 mM without appreciable effects on growth (Fig. 2, left hand side), and expression of PfCRT or PvCRT isoforms in and of themselves similarly does not affect growth (compare top panels “no drug” for the left hand [non-inducing] and right hand [inducing conditions] sides, Fig. 2). However, when PfCRT or PvCRT proteins are expressed, increasing the concentration of external CQ progressively inhibits colony formation in a CRT specific fashion (Fig 2, right hand side, compare two left most rows for “EV” [empty vector] and “LacZ” [gal inducible β galactosidase expression], vs. other rows for CRT expressors).

Taking the results shown in Fig. 2 and Fig. 1C together, one obvious hypothesis is that similar to plasma membrane expression of PfCRT in oocytes [19], PfCRT at the yeast plasma membrane facilitates influx of toxic CQ, promoting increased sensitivity to the drug, in a [CQ] dependent fashion. The similar CQ dependent growth inhibited phenotype of yeast expressing the PMA – PfCRT fusion protein (row 8, right hand side Fig. 2) supports this idea, since expression of the fusion protein is PM specific (Fig. 1C). We expect PM topology of the expressed CRT protein to conserve cytosolic disposition of cytosolic domains, and indeed, such a topology is strongly supported by earlier work with inside – out vesicles made from yeast constitutively expressing PfCRT [24]. Since PfCRT protein expressed in the DV is believed to facilitate transport of CQ from inside the DV to the cytosol of the malarial parasite [2], then this yeast PM topology would be predicted to facilitate influx of CQ from growth medium to the yeast cytosol.

Interestingly then, in these colony formation assays (Fig. 2), the most pronounced CQ dependent growth inhibition is seen for PvCRT expressing yeast (row 5, right hand side Fig 2), suggesting that even though PvCRT mutations have not yet been associated with *P. vivax* CQR, this orthologue also interacts with CQ. As shown in Fig. 2 (5th, 6th rows, right hand side), mutating PvCRT to a “Dd2 – like” PvCRT (see Methods) did not significantly change the hypersensitivity phenotype. To test interaction between CQ and PvCRT further, we partially purified wild type PvCRT protein from these yeast, reconstituted the protein into proteoliposomes, and examined AzBCQ photolabelling as previously described in depth for PfCRT protein [13]. As shown in a representative blot (Fig. 3), 50 % competition of photolabelling with the high affinity CQ analogue is achieved at approximately 8 - fold molar excess of underivitized CQ (Fig. 1C). Previously, we found that 50 % competition of photolabelling occurred at 22, 25 and 6 fold excess underivitized CQ for HB3, Dd2, and 7G8 PfCRT isoforms, respectively [13]. Thus, under de-energized conditions CQ probe apparent affinity for PvCRT is the second highest we have yet measured for any CRT protein.

Colony formation assays are a convenient phenotypic screen, but to more rigorously quantify effects of PfCRT and PvCRT expression on yeast CQ hypersensitivity, we performed quantitative growth analyses (Fig. 4). In these experiments, seeding yeast density was normalized and strains aliquoted in triplicate into wells containing inducing (SGR, “B” right hand side Fig 4) or non inducing (SD, “A” left hand side, Fig 4) media. In each case, solid lines in each panel in Fig. 4 are growth in the absence of CQ, whereas dotted lines are growth in the same medium containing 100 mM CQ. Again, quantitative growth analysis shows that at this concentration CQ alone has no effect on growth of any of the strains, assuming CRT protein is not being induced (left hand side, Fig. 4, compare solid vs dotted lines). Similarly, when strains harboring either empty vector (top panel, right hand side Fig. 4) or vector expressing β galactosidase (data not shown) are grown in inducing medium, no significant effect is seen +/- CQ (e.g. compare solid vs dotted line, top right panel Fig. 4). However, when CRT is induced (panels 2 – 5 from top, right hand side Fig. 4) the yeast clearly grow much slower in the presence of 100 mM CQ, consistent with the colony formation data shown in Fig. 2. Notably, yeast expressing wild type PfCRT (“HB3” isoform, 2nd panel from top, Fig. 4) grow as slowly as do yeast expressing the mutant PfCRT isoforms found in CQR *P. falciparum* (“Dd2” and “7G8” isoforms, 3rd and 4th panels from top, left hand side Fig. 4). Similar to earlier work with purified protein reconstituted into proteoliposomes [20], this suggests that wild type PfCRT is also capable of transporting CQ under some conditions. Interestingly, yeast expressing wild type PvCRT grow the slowest (bottom panel, right hand side).

After performing these growth curve analyses multiple times, doubling times for each well (δ) were then determined by converting growth vs. time to semilog plots and calculating

slope. Fig. 5 summarizes these averaged data for strains growing in the presence of 100 mM CQ under either non inducing conditions (black bars) or inducing conditions (gray bars) and at either low external pH ($= 5.0$, panel A) or high external pH ($pH = 7.5$, panel B, see Methods). We wished to inspect growth effects at a range of pH because as external pH is raised for growing yeast, plasma membrane ΔpH is reduced, but $\Delta \Psi$ is increased to preserve electrochemical driving force for essential membrane transport (see Methods and [26,27]).

At low external pH (high ΔpH , $\Delta \Psi$ near zero), the growth inhibition due presumably to CRT mediated influx of CQ is again most pronounced for yeast expressing PvCRT (Fig 5A, far right hand side). Also, growth inhibition for strains expressing HB3 wild type (CQS associated) PfCRT is similar to that for strains expressing mutant (CQR associated) Dd2 PfCRT, and at this pH / $\Delta \Psi$, CQR associated mutant 7G8 PfCRT confers less dramatic growth inhibition relative to HB3 wild type.

However, as external pH is raised to 7.5, such that ΔpH is near zero (internal pH for yeast is approximately 8.0) but $\Delta \Psi$ is near maximum [26,27], the growth inhibition effect promoted by 7G8 PfCRT and Dd2 PfCRT is noticeably more severe than that promoted by HB3 PfCRT (Fig. 5 panel B). That is, elevating $\Delta \Psi$ has a more significant effect on growth inhibition caused by mutant (CQR associated) PfCRT isoforms (from 100 – 200 minutes to 300 – 500 minutes for 7G8 and Dd2 PfCRTs) than by wild type (CQS associated) PfCRT (from ~ 200 minutes to ~250 minutes) or than the PvCRT orthologue (~ 400 minutes in both cases, compare Fig 5A vs 5B). To examine this more closely, Fig. 5C shows a plot of the difference in strain doubling times (growth in the presence of CQ under CRT inducing conditions minus growth plus CQ under non inducing conditions; [y-axis]) vs pH (x-axis) for strains expressing no PfCRT (◆), or HB3 (■) vs Dd2 (▲) PfCRT. Control doubling rate (◆) is mildly dependent on pH but is not slowed by CQ, instead, a very mild stimulation by glucose is seen at higher pH (yielding points just below the x axis). Strains expressing PfCRT are slowed in their growth upon addition of CQ in a pH / $\Delta \Psi$ dependent manner. The effect is exacerbated quite significantly as pH / $\Delta \Psi$ are increased, with the change for Dd2 PfCRT expressing yeast greater than for yeast expressing wild type PfCRT. A similarly heightened effect is seen for 7G8 PfCRT expressing yeast (not shown).

As suggested above, one obvious hypothesis for these effects on growth is that PfCRT at the PM of the yeast is facilitating heightened transport of CQ into the yeast. To test this more directly, we performed 3H – CQ uptake experiments. Figure 6 shows representative 3H – CQ accumulation vs time for induced Dd2 PfCRT yeast (open circles) and induced yeast harboring empty vector (closed circles) at pH 5.5 (panel A) and 7.5 (panel B). Clearly, expression of PfCRT in the induced yeast leads to increased accumulation of 3H -CQ. Fig. 7 summarizes 30 minute accumulation data for various strains assayed at low (black bars) vs high (gray bars) pH. Consistent with the PfCRT isoform dependent growth effects discussed above, yeast strains expressing mutant (CQR associated) Dd2 and 7G8 PfCRT accumulate more drug at high pH (low ΔpH , high $\Delta \Psi$) vs low pH (high ΔpH , low $\Delta \Psi$; see caption), whereas yeast expressing HB3 wild type PfCRT or PvCRT accumulate similar levels at both pH / $\Delta \Psi$. Fig 8 and Table 3 summarize initial rate analysis of these uptake data. Importantly, neglecting slower background CQ accumulation (yeast grown under inducing conditions but harboring empty vector), the rank order of initial rate of drug transport is PvCRT > Dd2 PfCRT > 7G8 PfCRT > HB3 PfCRT, at high ΔpH / low $\Delta \Psi$. Consistent with plateau data (Fig. 7), rate does not increase for HB3 PfCRT or PvCRT expressing yeast at lower ΔpH / higher $\Delta \Psi$, but does increase for 7G8 and Dd2 PfCRT expressing yeast, such that at high $\Delta \Psi$ the rank order of initial rate of drug transport is PvCRT ~ Dd2 PfCRT ~ 7G8 PfCRT >> HB3 PfCRT (Table 3).

Discussion

Mutated PfCRT is believed to cause resistance to the antiparasitic effects of CQ via mediating increased flux of CQ from the malarial parasite DV [2,19,20]. Data in this paper help to further elucidate key features of that transport. Previous yeast heterologous expression of PfCRT via MeOH induction, or via constitutively active Ste6 promoter [24] did not allow for convenient analysis of PfCRT function in actively growing yeast under ambient growth conditions. However, the high fidelity of the glu / gal induction system now provides new convenient growth assays (Fig. 2, 4) that can be used to characterize the function of various PfCRT and PvCRT isoforms. The simple growth assays that are now possible should be useful for screening putative PfCRT and PvCRT inhibitors, and / or drug resistance reversal agents that act via interaction with PfCRT. Although often overlooked, PfCRT has no strong homologues other than additional *Plasmodium* CRT proteins, and appears to be an essential protein [29]. Although not yet proven for *P. vivax*, it is likely that PvCRT is also essential. These proteins therefore represent important, unique targets for development of new antimalarial drugs. Rapid screening of candidate CRT inhibitors vs. CQ hypersensitivity might provide one inexpensive way to prioritize lead compounds.

We find a reasonably good correspondence between the degree of CQ – induced growth inhibition vs. heightened rate of CQ accumulation promoted by expression of CRT protein (Fig. 4,5 vs Table 3). Since yeast intracellular compartmentalization of the diprotic weak base CQ is complex, involving the yeast vacuole and other compartments, we suggest altered initial rates of CQ transport are a less ambiguous indicator of CRT function than net accumulation at 30 minutes. That is, net accumulation at ≥ 30 min is due to a variety of overlapping complex effects, many of which do not involve PfCRT or PvCRT. We also note that the protein – polysaccharide outer membrane of yeast changes cellular permeability of CQ relative to what would be seen for intact parasites (14 – 16). Nonetheless, relative differences in accumulation across the yeast strains qualitatively parallel the effects seen for initial rates, and are again consistent with trends in altered growth phenotypes due to expression of CRTs. Our model to explain these data is simple: expression of the known CQ transporter PfCRT at the PM of yeast promotes heightened accumulation of toxic CQ, leading to significant growth inhibition of these yeast.

In contrast to expression of PfCRT in yeast, PvCRT expression did not require extensive codon optimization [24]. The *P. vivax* genome is ~ 58% A+T content whereas *P. falciparum* is ~ 81 % A+T [30]. Correspondingly, *pvcrt* cDNA is 54 % A+T whereas the *pfert* is 72 %. Interestingly, we find that the PvCRT orthologue promotes more significant growth inhibition and appears to transport CQ *better* than do the mutant (CQR associated) Dd2 and 7G8 PfCRT isoforms. This provides a surprising, but simple hypothesis for why PvCRT mutations have not been associated with CQR in *P. vivax*, even though CQR clearly exists for this species and is on the rise [11]. Namely, since the wild type PvCRT transporter is already as capable of CQ transport as are the mutant PfCRT isoforms, there is perhaps little to “gain” (in terms of further increased drug transport ability) by acquisition of PvCRT mutations. These data and arguments then suggest that CQ IC₅₀ should be intrinsically higher for *P. vivax* relative to *P. falciparum*. This is predicted because CQ IC₅₀ is related to the relative intra – DV accumulation of CQ [15,16]. IC₅₀ assays for *P. vivax* are difficult since currently the parasite cannot be conveniently cultured *in vitro*. However, available data that compare *P. falciparum* and *P. vivax* patient isolates side – by – side with the same assay [31] show that CQ IC₅₀ is indeed intrinsically higher for *P. vivax* relative to *P. falciparum*. That is, at least with respect to growth inhibition by CQ, *P. vivax* is intrinsically more resistant to CQ because of a higher endogenous (wild type PvCRT mediated) ability to allow diffusion of accumulated CQ²⁺ back out of the DV. We note that this does not necessarily

mean that *P. vivax* are more resistant to cytotoxic effects of CQ relative to *P. falciparum*, since DV accumulation of CQ and CQ LD₅₀ do not appear to be related [16].

Growth inhibition and direct ³H-CQ transport data in this paper strongly support the notion that, similar to wild type PvCRT, wild type CQS – associated isoform HB3 PfCRT is able to transport CQ in some fashion. Currently, there are differing conclusions on this point [2]. Martin *et al.* [19] do not measure appreciable CQ uptake for oocytes expressing HB3 PfCRT, but do measure uptake for oocytes expressing Dd2 PfCRT. In contrast, Paguio *et al.* [20] reported that both HB3 and Dd2 isoforms of PfCRT were capable of mediating electrochemically downhill, $\Delta\Psi$ – dependent, transport of a fluorescent CQ analogue. Some possible explanations for the discrepancy have been discussed previously [2] and include the important point that membrane potential in the two experimental systems (live oocytes vs reconstituted proteoliposomes) are very different.

Also, data in this paper further support the notion that CQ transport by PfCRT is stimulated by $\Delta\Psi$ (see also [20]). This is not unexpected since the physiologic environment for PfCRT and PvCRT is the DV membrane, across which a high electrochemical potential (positive inside) is expected. PfCRT topology in the parasite DV membrane places cytosolic domains at the negative side with respect to $\Delta\Psi$ (and at alkaline pH), and intra DV domains of PfCRT facing positive (and acid pH). For growing yeast, external pH is acid and $\Delta\Psi$ across the plasma membrane is positive outside, negative inside, thus PfCRT topology in yeast plasma membrane preserves the physiologic orientation with respect to electrochemical driving force. Because yeast are capable of generating high $\Delta\Psi$ when external pH is artificially raised [26,27], membrane potential stimulatory effects for CQ transport by PfCRT can be observed, similar to how generation of high artificial $\Delta\Psi$ for proteoliposomes promotes increased transport of a fluorescent CQ analogue [20].

Interestingly, an increase in CQ transport upon elevating $\Delta\Psi$ is only found for mutant (CQR associated) Dd2 and 7G8 PfCRT isoforms. Neither wild type HB3 PfCRT or wild type PvCRT appeared to be stimulated by higher $\Delta\Psi$ with respect to growth inhibitory phenotypes, or with respect to whole cell ³H – CQ accumulation. Consistent with previous work with purified PfCRT and a fluorescent CQ probe [20], we suggest that electrochemical potential differences across the parasite DV membrane are important for heightened electrochemically passive CQ²⁺ facilitated diffusion by mutant PfCRT in CQR parasites. In support of this concept, some studies have published data that show mutant PfCRT promotes lower intra-DV pH (0.2 – 0.4 pH unit higher DV membrane Δ pH; [32-34]), however, other studies have not measured these small DV Δ pH changes [35-37]. If cytosolic pH remains constant for CQR parasites (or is perhaps even elevated for some particularly drug resistant strains, see [38]), the increased driving force presumably provided by this increased Δ pH may promote reduced DV [CQ²⁺] for CQR parasites. Increases in $\Delta\Psi$ across the DV membrane for CQR parasites may indeed exist, but this awaits additional experimental measurement. Currently, reliable quantification of $\Delta\Psi$ for an organellar membrane of a living intracellular parasite is technically challenging, but further work may be able to test these predictions. In any case, clear differences in DV volume for CQS vs CQR malarial parasites strongly suggest altered osmolyte traffic exists for the CQR DV [39]. If these osmolytes include ions or charged solutes for which the DV membrane normally has high permeability, perturbation of $\Delta\Psi$ is expected.

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Bibliography

1. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naudé B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Wellems TE. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell*. 2000; 6:861–871. [PubMed: 11090624]
2. Roepe PD. PfCRT – Mediated Drug Transport in Malarial Parasites. *Biochemistry*. 2011; 50:163–171.
3. Valderramos SG, Fidock DA. Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci*. 2006; 11:594–601. [PubMed: 16996622]
4. Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE. Dissecting the loci of low-level quinine resistance in malaria parasites. *Mol Microbiol*. 2004; 52:985–997. [PubMed: 15130119]
5. Patel JJ, Thacker D, Tan JC, Pleeter P, Checkley L, Gonzales JM, Deng B, Roepe PD, Cooper RA, Ferdig MT. Chloroquine susceptibility and reversibility in a *Plasmodium falciparum* genetic cross. *Mol Microbiol*. 2010; 78:770–787. [PubMed: 20807203]
6. Sá JM, Twu O, Hayton K, Reyes S, Fay MP, Ringwald P, Wellems TE. Geographic patterns of *Plasmodium falciparum* drug resistance distinguished by differential responses to amodiaquine and chloroquine. *Proc Natl Acad Sci U S A*. 2009; 106:18883–18889. [PubMed: 19884511]
7. Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF. Amplification of a gene related to mammalian *mdr* genes in drug-resistant *Plasmodium falciparum*. *Science*. 1989; 244:1184–1186. [PubMed: 2658061]
8. Foote SJ, Kyle DE, Martin RK, Oduola AM, Forsyth K, Kemp DJ, Cowman AF. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *P. falciparum*. *Nature*. 1990; 345:255–258. [PubMed: 2185424]
9. Pleeter P, Lekostaj JK, Roepe PD. Purified *Plasmodium falciparum* multi-drug resistance protein (PfMDR 1) binds a high affinity chloroquine analogue. *Mol Biochem Parasitol*. 2010; 173:158–161. [PubMed: 20546803]
10. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, Wootton JC, Wellems TE. Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. *J Infect Dis*. 2001; 183:1653–1661. [PubMed: 11343215]
11. Baird KJ. Resistance to therapies for infection by *Plasmodium vivax*. *Clin. Microbiol. Rev*. 2009; 22:508–534. [PubMed: 19597012]
12. Zhang H, Paguio M, Roepe PD. The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. *Biochemistry*. 2004; 43:8290–8296. [PubMed: 15222741]
13. Lekostaj JK, Natarajan JK, Paguio MF, Wolf C, Roepe PD. Photoaffinity labeling of the *Plasmodium falciparum* chloroquine resistance transporter with a novel perfluorophenylazido chloroquine. *Biochemistry*. 2008; 47:10394–10406. [PubMed: 18767816]
14. Geary TG, Jensen JB, Ginsburg H. Uptake of [3H]chloroquine by drug-sensitive and -resistant strains of the human malaria parasite *Plasmodium falciparum*. *Biochem Pharmacol*. 1986; 35:3805–3812. [PubMed: 3535803]
15. Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM, Ward SA. Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum* *in vitro*. *Antimicrob Agents Chemother*. 1998; 42:682–686. [PubMed: 9517951]
16. Cabrera M, Paguio MF, Xie C, Roepe PD. Reduced digestive vacuolar accumulation of chloroquine is not linked to resistance to chloroquine toxicity. *Biochemistry*. 2009; 48:11152–11154. [PubMed: 19883122]
17. Sullivan DJ Jr, Gluzman IY, Russell DG, Goldberg DE. On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci U S A*. 1996; 93:11865–11870. [PubMed: 8876229]

18. Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. *Mol Pharmacol*. 2002; 61:35–42. [PubMed: 11752204]
19. Martin RE, Marchetti RV, Cowan AI, Howitt SM, Bröer S, Kirk K. Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science*. 2009; 325:1680–1682. [PubMed: 19779197]
20. Paguio MF, Cabrera M, Roepe PD. Chloroquine transport in *Plasmodium falciparum*. 2. Analysis of PfCRT-mediated drug transport using proteoliposomes and a fluorescent chloroquine probe. *Biochemistry*. 2009; 48:9482–9491. [PubMed: 19725576]
21. Nigavekar, Shraddha S.; Cannon, John. F. Characterization of genes that are synthetically lethal with *ade3* or *leu2* in *Saccharomyces cerevisiae*. *Yeast*. 2002; 19:115–122. [PubMed: 11788966]
22. Sherman, F.; Baim, SB.; Hampsey, DM.; Goodhue, CT.; Friedman, LR.; Stiles, JI. Translational Control, Current Communications in Molecular Biology. Matthews, MB., editor. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY: 1986.
23. Ito H, Fukuda Y, Murata K, Kimura A. Transformation of intact yeast cells treated with alkali cations. *Bacteriology*. 1983; 153:163–168.
24. Zhang H, Howard EM, Roepe PD. Analysis of the antimalarial drug resistance protein PfCRT expressed in yeast. *J. Biol. Chem*. 2002; 277:49767–49775. [PubMed: 12351620]
25. Goffeau A, Dufour JP. Plasma membrane ATPase from the yeast *Saccharomyces cerevisiae*. *Methods Enzymol*. 1988; 157:528–533. [PubMed: 2906716]
26. Fritz F, Howard EM, Hoffman MM, Roepe PD. Evidence for Altered Ion Transport in *S. cerevisiae* Overexpressing Human MDR 1 Protein. *Biochemistry*. 1999; 38:4214–4226. [PubMed: 10194338]
27. Orij R, Postmus J, Ter Beek A, Brul S, Smits GJ. In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth. *Microbiology*. 2009; 155:268–278. [PubMed: 19118367]
28. Downie MJ, El Bissati K, Bobenchik AM, Nic Lochlainn L, Amerik A, Zufferey R, Kirk K, Ben Mamoun C. PfNT2, a permease of the equilibrative nucleoside transporter family in the endoplasmic reticulum of *Plasmodium falciparum*. *J Biol Chem*. 2010; 285:20827–20833. [PubMed: 20439460]
29. Waller KL, Muhle RA, Ursos LMB, Verdier-Pinard D, Fujioka H, Roepe PD, Fidock DA. Chloroquine resistance modulated in vitro by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter (PfCRT). *J. Biol. Chem*. 2003; 278:33593–33601. [PubMed: 12813054]
30. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, Cheng Q, Coulson RM, Crabb BS, Del Portillo HA, Essien K, Feldblyum TV, Fernandez-Becerra C, Gilson PR, Gueye AH, Guo X, Kang'a S, Kooij TW, Korsinczky M, Meyer EV, Nene V, Paulsen I, White O, Ralph SA, Ren Q, Sargeant TJ, Salzberg SL, Stoeckert CJ, Sullivan SA, Yamamoto MM, Hoffman SL, Wortman JR, Gardner MJ, Galinski MR, Barnwell JW, Fraser-Liggett CM. Comparative Genomics of the Neglected Human Malarial Parasite *Plasmodium vivax*. *Nature*. 2008; 455:757–763. [PubMed: 18843361]
31. Russell B, Chalfein F, Prasetyorini B, Kenangalem E, Piera K, Suwanarusk R, Brockman A, Prayoga P, Sugiarto P, Cheng Q, Tjitra E, Anstey NM, Price RN. Determinants of in vitro drug susceptibility testing of *Plasmodium vivax*. *Antimicrob Agents Chemother*. 2008; 52:1040–1045. [PubMed: 18180357]
32. Bennett TN, Kosar AD, Ursos LMB, Dzekunov S, Sidhu ABS, Fidock DA, Roepe PD. Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. *Mol. & Biochem. Parasitol*. 2004; 133:99–114. [PubMed: 14668017]
33. Reeves DC, Liebelt DA, Lakshmanan V, Roepe PD, Fidock DA, Akabas MH. Chloroquine-resistant isoforms of the *Plasmodium falciparum* chloroquine resistance transporter acidify lysosomal pH in HEK293 cells more than chloroquine-sensitive isoforms. *Mol Biochem Parasitol*. 2006; 150:288–299. [PubMed: 17014918]

34. Naude B, Brzostowski JA, Kimmel AR, Wellem TE. *Dictyostelium discoideum* expresses a malaria chloroquine resistance mechanism upon transfection with mutant, but not wild-type, *Plasmodium falciparum* transporter PfCRT. J. of Biol. Chem. 2005; 280:25596–25603. [PubMed: 15883156]
35. Hayward R, Saliba KJ, Kirk K. The pH of the digestive vacuole of *Plasmodium falciparum* is not associated with chloroquine resistance. J Cell Sci. 2006; 119:1016–1025. [PubMed: 16492710]
36. Klonis N, Tan O, Jackson K, Goldberg D, Klemba M, Tilley L. Evaluation of pH during cytosomal endocytosis and vacuolar catabolism of haemoglobin in *Plasmodium falciparum*. Biochem J. 2007; 407:343–354. [PubMed: 17696875]
37. Kuhn Y, Rohrbach P, Lanzer M. Quantitative pH measurements in *Plasmodium falciparum*-infected erythrocytes using pHluorin. Cell Microbiol. 2007; 9:1004–1013. [PubMed: 17381432]
38. Bennett TN, Patel J, Ferdig MT, Roepe PD. Altered *Plasmodium falciparum* Na^+ / H^+ Exchange Activity is Correlated with Quinine Resistance. Mol. Biochem. Parasitol. 2007; 153:48–58. [PubMed: 17353059]
39. Glikoreijevic B, McAllister R, Urbach J, Roepe PD. Spinning Disk Confocal Microscopy of Intraerythrocytic Malarial Parasites II: Quantification of Digestive Vacuolar Volume. Biochemistry. 2006; 45:12411–12423. [PubMed: 17029397]

FIG. 1. Western Blot analysis of gal – inducible CRT protein expression

A: Crude membrane preparations of *S. cerevisiae* transformed with different isoforms of PfCRT grown under non-inducing glucose conditions (lanes 1, 3, 5, 7) vs inducing galactose / raffinose (lanes 2, 4, 6, 8). Each lane contains 10 µg of protein. Each blot uses crude yeast membranes (total membrane fraction) prepared from CH1305 strains transformed with pYES2/PfHB3vh (Lanes 1 and 2), pYES2/PfDd2vh (Lane 3 and 4), pYES2/Pf7G8vh (Lane 5 and 6) or pYES2/PfHB3_{PMA}vh (Lanes 7 and 8). The V5-tagged proteins were detected with anti-V5-HRP. Top arrow (dashed) points to PfHB3_{PMA}vh protein, bottom (solid) arrow points to normal length PfCRT proteins. **B:** Crude membranes were prepared from CH1305 strains transformed with pYES2/PfHB3vh (Lane 1), pYES2/PfDd2vh (Lane 2), pYES2/Pf7G8vh (Lane 3), pYES2/PvCRTvh (Lane 4) or pYES2/PvCRT_{Dd2}vh (Lane 5) and equivalent membrane protein (quantified by amido black assay) run side – by – side. Top panel shows anti-pentaHis staining, bottom shows anti-V5 staining, both of which indicate that approximately equivalent levels of the isoforms and orthologues are expressed in the different strains. **C:** Comparison of relative amounts of PfHB3vh and pYES2/PfHB3_{PMA}vh in plasma membrane fractions. Lanes 1 and 5 are 5 µg of crude membranes prepared from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/

PfHB3_{PMA}vh. Lanes 2 and 6 are 5 μ g of pellet harvested at $75,000 \times g$ (intermediate step in pure plasma membrane preparation, harboring non – plasma membrane cellular membranes) from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/PfHB3_{PMA}vh. Lanes 3, 4 and 7, 8 contain 5 and 10 μ g, respectively, of pure plasma membranes harvested from CH1305 strains transformed with pYES2/PfHB3vh and pYES2/ PfHB3_{PMA}vh. Top arrow indicates PMA1-fusion PfHB3 isoform and bottom arrow indicates PfHB3 isoform. High mass bands near 100 kDa in lanes 7,8 are likely PMA1 – HB3CRT aggregates, and band near 15 kDa is an apparent protease degradation product. Densitometry of bands indicated approximately 50 – 60 percent of CRT is within the plasma membrane fraction without N terminal modification, but for pYES2/ PfHB3_{PMA}vh, > 10 times is found in the plasma membrane relative to all other cell membranes.

FIG. 2. Plate Assays for CQ and CRT – dependent growth inhibition

Susceptibilities of CH1305 yeast harboring pYES2 (“empty vector” or “EV”), pYES2/LacZ, pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh, pYES2/PvCRTvh, pYES2/PvDd2vh or pYES2/PfHB3_{PMA}vh. Suspensions for inoculating plates were grown at 30°C to mid-exponential phase in SD media - uracil. Suspensions equilibrated to OD = 0.1 were transferred to 96 well plates, 10-fold serial dilutions were made, and 5 uls of each (10^4 , 10^3 , 10^2 cells; top, middle and bottom row, respectively, each “box” defined by white dashed lines) was then replica plated on SD or SGR media – uracil, +/- concentrations CQ. Plates were incubated side-by-side for approximately 3 days at 30°C.

FIG. 3. AzBCQ Photolabelling of Partially Purified PvCRT

A: Representative streptavidin-HRP detection of AzBCQ photolabelled PvCRT vs. competition with underivitized CQ (see [9] and [13] for detailed description of methods). Lane 1: no CQ competitor, Lane 2-6: 5-, 10-, 20-, 40-, 80-fold molar excess of CQ relative to AzBCQ, respectively. **B:** PentaHis-HRP detection of PvCRT in the same samples as in **A**, showing equal amounts of protein were used in each photolabelling reaction (see [13]). **C:** Plot of densitometry of bands as in **A**, vs. [CQ] competitor. As described [13], results from 3 different experiments (three photolabelling reactions, three separate sets of gels) were averaged, data represent mean $\pm$ S.E.M. Fitting this curve shows that 50 % maximal inhibition of PvCRT CQ analogue photolabelling occurs at 8.5 ($\pm$ 1.3) – fold molar excess of underivitized CQ.

FIG. 4. Quantitative growth curve analysis

Susceptibilities of yeast strain CH1305 harboring pYES2 (Empty vector, top), pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh or pYES2/PvCRTvh were assessed by quantitative growth curve analysis in liquid medium, pH = 5.0. Cultures were inoculated with 10^5 cells/ml synthetic complete medium with (A, left side panels) 3% glucose (SD-ura) alone (solid lines) or with 100 mM CQ (dashed), or (B, right side panels) synthetic complete medium with 2% galactose and 1% raffinose (SGR-ura) alone (solid), or SGR-ura with 100 mM CQ (dashed). Average growth curves from three replicates are shown, see Fig. 5 for curve fit analysis.

FIG. 5. Summary of Quantitative Growth Analysis

Doubling rates for data obtained as in Fig. 4 were computed as described. **A:** Inducing SGR medium, pH = 5.0 with (gray bars) or without (black bars) 100 mM CQ, **B:** Inducing SGR medium, buffered to pH = 7, with (gray bars) or without (black bars) 75 mM CQ. **C:** Induction and pH / $\Delta\Psi$ -dependent effects on growth rate. Growth rates obtained for non-inducing medium + 75 mM CQ were subtracted from rates obtained for inducing medium + 75 mM CQ. The growth rate differences for yeast expressing Dd2 PfCRT (top, triangles) were significantly higher relative to yeast expressing HB3 PfCRT (middle, squares) for all pH / $\Delta\Psi$ ($P < .05$, unpaired t tests in each comparison). As expected, growth under the two conditions did not differ significantly for yeast harboring empty vector (bottom, diamonds). The data represent the means (+ S.E.M) of 4 independent experiments.

FIG. 6. ^3H -CQ accumulation in yeast +/- CRT expression

CH1305 harboring pYES2 (closed circles) or pYES2/PfDd2vh (open circles) were grown to exponential phase in SGR/-ura, harvested and assayed for [^3H]-CQ uptake at pH 5.5 (A) or 7.5 (B). The data represent the means ($\pm$ S.D.) for 3 - 5 separate experiments.

FIG. 7. Summary of ^3H – CQ accumulation data for different yeast strains
 CH1305 harboring pYES2 (“EV”), pYES2/PfHB3vh, pYES2/PfDd2vh, pYES2/Pf7G8vh or pYES2/PvCRT were grown to exponential phase in SGR/-ura, harvested and assayed for [^3H] CQ uptake at 30 min (near plateau, see Fig. 6) at either pH 5.5 (black bars) or pH 7.5 (gray bars). Similar results are obtained at 60 min time (not shown, see Fig. 6). Each bar represents the average of 3 separate experiments. * $P > .2$, ** $P = .08$, *** $P < .01$. Differences in uptake at pH 7.5 vs. 5.5 are significant for 7G8 ($P \leq .05$, unpaired t tests) and suggestive for Dd2 ($P = .08$, unpaired t tests), but not significant for any of the other strains.

FIG. 8. Initial rate analysis of ³H – CQ accumulation

Representative initial rate analysis of ³H – CQ accumulation (see also Table 3 for a summary). Gray squares are CH1305 expressing pYES2/PfDd2vh, black diamonds CH1305 expressing pYES2. Data were obtained at pH 5.5 and are the results of three experiments, +/– S.D. See Table 3 for a summary of initial rate data.

Table 1

Plasmids used in the present study.

Plasmid	Feature	Source
pYES2	GAL1 PROM_MCS_V5_HIS, URA3 2 μ	Commercial vector
pPIC3.5/HB3hb	PfHB3hisbad, HIS	Roepe 2004
pYES2.1/LacZ	LacZ, URA3, 2 μ	Commercial vector
pUC57/PfHB3vh	pBR322, PfHB3vh	Present Study
pUC57/PfHB3 _{PMA} vh	pBR322, PfHB3PMAvh	Present Study
pYES2/PfHB3vh	PfHB3vh, URA3, 2 μ	Present Study
pYES2/PfHB3 _{PMA} vh	PfHB3PMAvh, URA3, 2 μ	Present Study
pYES2/PfDd2vh	PfDd2vh, URA3, 2 μ	Present Study
pYES2/Pf7G8vh	Pf7G8vh, URA3, 2 μ	Present Study
pYES2/PvCRTvh	PvCRTvh, URA3, 2 μ	Present Study
pYES2/PvCRTDd2vh	PvCRT“Dd2”vh, URA3, 2 μ	Present Study

Table 2

Oligonucleotides used in the present study.

Name	Sequence
PfDd2 ^R 371 ^I	5'-CTTAGCAGGTGATGTCGTAATAGAACC ACGTTTCTT ^I -3
PVCRTinsrG	5'-CACCCCGGGCCGGGTCACCCATTTCG-3'
PVCRTinsGR	5'-CGAATGGGTGACCCGGCCCGGGTG-3'
PfQ271 ^E	5'-CACCTCCCATTTTGAAGGAGATCAATTGCCCTTCTCGG-3'
PfN326 ^S	5'GAAAACCTTCATAGCCTATTCCTTTTTCAGCATTTGCGACAATTAATC-3'

Table 3

Initial Rates of ^3H – CQ accumulation (fMoles CQ / min / 10^6 cells) for yeast expressing various CRT isoforms / orthologues (see Figure 8 for representative raw data). Data shown are average of 3 determinations.

^3H – CQ accumulation initial rates (fmoles CQ per min)		
pH	5.5	7.5
EV (none)	47.9	50.5
HB3 Pf	120.2	120.5
Dd2 Pf	160.0	181.7
7G8 Pf	150.5	176.9
PvCRT	188.6	183.0