Evidence for a *pfcrt*-Associated Chloroquine Efflux System in the Human Malarial Parasite *Plasmodium falciparum*†

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Received January 12, 2005; Revised Manuscript Received May 27, 2005

ABSTRACT: Resistance to the antimalarial drug chloroquine has been linked with polymorphisms within a gene termed *pfcrt* in the human malarial parasite *Plasmodium falciparum*, yet the mechanism by which this gene confers the reduced drug accumulation phenotype associated with resistance is largely unknown. To investigate the role of *pfcrt* in mediating chloroquine resistance, we challenged *P. falciparum* clones differing only in their *pfcrt* allelic form with the "varying-trans" procedure. In this procedure, movement of labeled substrate across a membrane is measured when unlabeled substrate is present on the trans side of the membrane. If a transporter is mediating the substrate flow, a stimulation of cis-to-trans movement may be observed with increasing concentrations of trans substrate. We present evidence for an association of those *pfcrt* alleles found in chloroquine-resistant *P. falciparum* strains with the phenomenon of stimulated chloroquine accumulation under varying-trans conditions. Such an association is not seen with polymorphisms within *pfmdr1*, which encodes a homologue of the human multidrug resistance efflux pump. Our data are interpreted in terms of a model in which *pfcrt* is directly or indirectly involved in carrier-mediated chloroquine efflux from resistant cells.

The spread of chloroquine-resistant (CQR)[1] *Plasmodium falciparum* strains has dashed hopes of global malaria eradication and, due to a paucity of other affordable drugs, has complicated the clinical management of malaria in endemic areas. Chloroquine, which targets the intraerythrocytic stages of *P. falciparum*, accumulates to many millimolars of concentration within the parasite's acidic food vacuole (*1*, *2*). In the food vacuole, hemoglobin taken up from the host cell is digested, and the heme liberated during this process is crystallized to inert hemozoin (*2*, *3*). Chloroquine appears to prevent heme detoxification by complexing with heme (*4*−*7*), resulting in toxic adducts that build up and eventually disrupt the parasite's membranes (*8*, *9*).

Genetic, reverse genetic, and epidemiological studies have linked chloroquine resistance to polymorphisms within a gene termed *pfcrt* residing on *P. falciparum* chromosome 7 (*10*−*13*). *pfcrt* polymorphisms exhibit regional specificity, with a specific CQR allelic form predominant in each of Southeast Asia/Africa, Latin America (*10*), Papua New Guinea (*14*), and possibly the Philippines (*15*). Regardless of their origin, all *pfcrt* alleles from CQR strains encode a conserved $K^{76}T$ amino acid substitution, in addition to other more divergent point mutations, with respect to chloroquine-sensitive (CQS) strains (*10*).

pfcrt encodes a protein of 424 amino acids with 10 predicted transmembrane domains, which is located in the food vacuolar membrane (*10*). How PfCRT mediates chloroquine resistance is largely unclear. Some bioinformatic studies have classified PfCRT into the drug/metabolite transporter superfamily (*16*, *17*), which would be consistent with models of chloroquine resistance postulating that CQR strains lower their intracellular chloroquine concentration by expelling chloroquine via an acquired efflux system (*18*−*20*) or by providing a leak through which protonated chloroquine may diffuse out of the food vacuole driven by its concentration gradient (*21*, *22*). Other reports have proposed a role for PfCRT in transmembrane chloride movement (*23*, *24*). Altered chloride conductance may affect food vacuolar pH and subsequently heme crystallization as well as heme/chloroquine binding kinetics (*25*, *26*) and/or partitioning of the diprotic weak base chloroquine (*1*, *27*).

We have previously investigated the kinetics of chloroquine accumulation under varying-trans conditions and found evidence in favor of a chloroquine efflux system operating in CQR strains (*19*, *20*). The varying-trans procedure assesses solute movement when various concentrations of it are present on both sides of the cell membrane (*28*). When a

† This work was supported by the Landesstiftung Baden-Württemberg and the European Commission.

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[1] Abbreviations: au, arbitrary units; CQ, chloroquine; CQ_{in}, total labeled intracellular chloroquine concentration; CQ_{out}, total labeled extracellular chloroquine concentration; CQ_{pre}, total intracellular preloaded chloroquine concentration; CQS, chloroquine-sensitive; CQR, chloroquine-resistant; LS-Blue, LysoSensor Blue DND-192; *pfcrt*, *Plasmodium falciparum* chloroquine resistance transporter; *pfmdr1*, *P. falciparum* multidrug resistance 1.

10.1021/bi050061f CCC: $30.25
Published on Web 06/28/2005

carrier mediates solute transport across a cell membrane, varying-trans kinetics can exhibit stimulated solute uptake (*28*). Stimulated uptake under varying-trans conditions is not seen in cases of simple passive diffusion or diffusion through channels (*28*). In the case of chloroquine, we observed such stimulated chloroquine accumulation only in CQR strains, not in CQS strains (*19*, *20*). This phenomenon depended on an energy input, could be inhibited by verapamil, and showed a strict substrate specificity for quinoline and methoxyacridine antimalarial drugs (*19*, *20*). On the basis of these data, we proposed that the acquisition of a chloroquine efflux system is a minimal and necessary event in the generation of the chloroquine resistance phenotype (*19*, *20*). Here we assess the contribution of *pfcrt* polymorphisms to the drug efflux phenotype by investigating a series of *P. falciparum* clones differing only in their *pfcrt* allele. In addition, we investigated allelic exchange mutants of *pfmdr1*, encoding a homologue of the human multidrug resistance efflux pump, which some studies have implicated in contributing to chloroquine resistance (*29*−*31*). Our data are interpreted in terms of a model in which *pfcrt* is involved in carrier-mediated chloroquine efflux from CQR cells.

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]Chloroquine (18.8 Ci/mmol), [^{3}H]hypoxanthine (17.9 Ci/mmol), and NCS-II tissue solubilizer were supplied by Amersham. Chloroquine and pyrimethamine were obtained from Sigma, and blasticidin was from ICN. WR99210 was a gift from Jacobus Pharmaceutical Co. (Princeton, NJ).

Parasite Culture and Characterization. P. falciparum strains were cultured as described (*32*). Media in which *pfcrt* allelic exchange mutants were grown was supplemented with 5 nM WR99210 and 5 μM blasticidin (*13*). Media for D10-mdr^{D10}, D10-mdr$^{7G8/1}$, and D10-mdr$^{7G8/3}$ were supplemented with 100 nM pyrimethamine (*31*). Strains 7G8-mdr^{7G8} and 7G8-mdr^{D10} were cultured in media containing 5 nM WR99210. Three days prior to any experiment these drugs were removed from the culture media. The genotype of all strains was confirmed using microsatellite analysis of the PfRRM marker (*33*). The *pfmdr1* alleles and codon 76 of *pfcrt* were determined through PCR−RFLP analysis of isolated genomic DNA (*34*). Other polymorphisms in *pfcrt* were determined by sequencing the relevant region of PCR-amplified, genomic DNA.

Varying-Trans Procedure. This assay has been fully described (*19*). Briefly, *P. falciparum* infected erythrocytes were purified using a strong magnet, as described (*19*). This yielded 98−100% purified trophozoite-infected erythrocytes as directly determined by microscopic examination of Giemsa-stained blood smears. The varying-trans procedure consists of a preloading phase during which cells are incubated for 15 min with different concentrations of cold chloroquine. For CQS strains the following extracellular chloroquine concentrations were used for preloading: 0.0, 0.005, 0.010, 0.025, 0.050, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 μM. For CQR strains, we used 0.0, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 μM. The total amount of chloroquine taken up by the cells during preloading (CQ_{pre}) was determined in independent experiments for each extracellular chloroquine concentration, using [^{3}H]chloroquine. After preloading, cells were washed twice in ice-cold medium and transferred to prewarmed medium (37 °C) containing 43 nM [^{3}H]chloroquine (the probing phase), unless stated otherwise. The amount of label taken up by the cell and that remaining in the extracellular medium were determined after 4 min, as described (*19*). The intracellular chloroquine concentration (CQ_{in}) was calculated from the amount of [^{3}H]chloroquine taken up by the cells and by assuming that the volume of a trophozoite-infected erythrocyte is 75 fL (*35*). Chloroquine accumulation was then expressed as the ratio of the intracellular versus the extracellular chloroquine concentration (CQ_{in}/CQ_{out}). All results are reported as total chloroquine concentrations since the concentration of free drug remains unknown.

Measurement of Intracellular ATP. The concentration of ATP within the parasite was measured using the ATP bioluminescence assay kit CLS II (Roche Applied Science) as described by the manufacturer.

Fluorometric Measurements. P. falciparum infected erythrocytes were washed twice with Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11 mM D-glucose, 10 mM HEPES, 1 mM NaH_2PO_4, pH 7.4) before being allowed to settle onto poly(L-lysine) coated coverslips in a microperfusion chamber, as previously described (*36*). Cells were perfused with Ringer solution containing 1 μM LysoSensor Blue DND-192 (LS-Blue; Molecular Probes). Confocal scanning fluorescence microscopy was performed using a Zeiss LSM510 (Carl Zeiss) equipped with UV laser lines (351 and 364 nm) and an Axiovert 100M microscope. A pinhole of 1 μm was chosen to ensure that fluorescence was from within the food vacuole and not the regions lying above or below. LS-Blue was excited at 364 nm, and the emission was detected using an LP 385 nm filter. In the time series, a frame size of 256 × 256 and a digital zoom of 3 were applied, and measurements were taken every 30 s. This process was optimized to minimize the bleaching of the nonratiometric dye LS-Blue. For quantification of the measurements, a region of interest (ROI) was chosen lying within the food vacuole. This was determined by looking at the DIC image and locating the hemozoin crystal, which coincided with the fluorescence of the LS-Blue fluorochrome. The mean fluorescence of LS-Blue was determined within the regions of interest (ROIs) and plotted as a function of time. Quenching of LS-Blue fluorescence by chloroquine was not observed, as determined by recording the LS-Blue spectrum in the presence of 0, 0.1, and 1.0 mM of chloroquine, using a fluorometer (data not shown).

RESULTS

Defining Conditions for the Varying-Trans Procedure. We first established conditions under which CQS and CQR strains would accumulate comparable concentrations of total chloroquine during the preloading phase. As model parasites we investigated the CQS *P. falciparum* clone HB3 (chloroquine $IC_{50} = 16.0 \pm 1.0$ nM) and the CQR clone Dd2 (chloroquine $IC_{50} = 137.0 \pm 11.0$ nM). To this end, purified erythrocytes infected with either HB3 or Dd2 were incubated for 15 min with different concentrations of labeled chloroquine, ranging from 5 nM to 10 μM, and the amounts of total drug accumulation were determined. We then set the extracellular loading concentrations such that the resulting

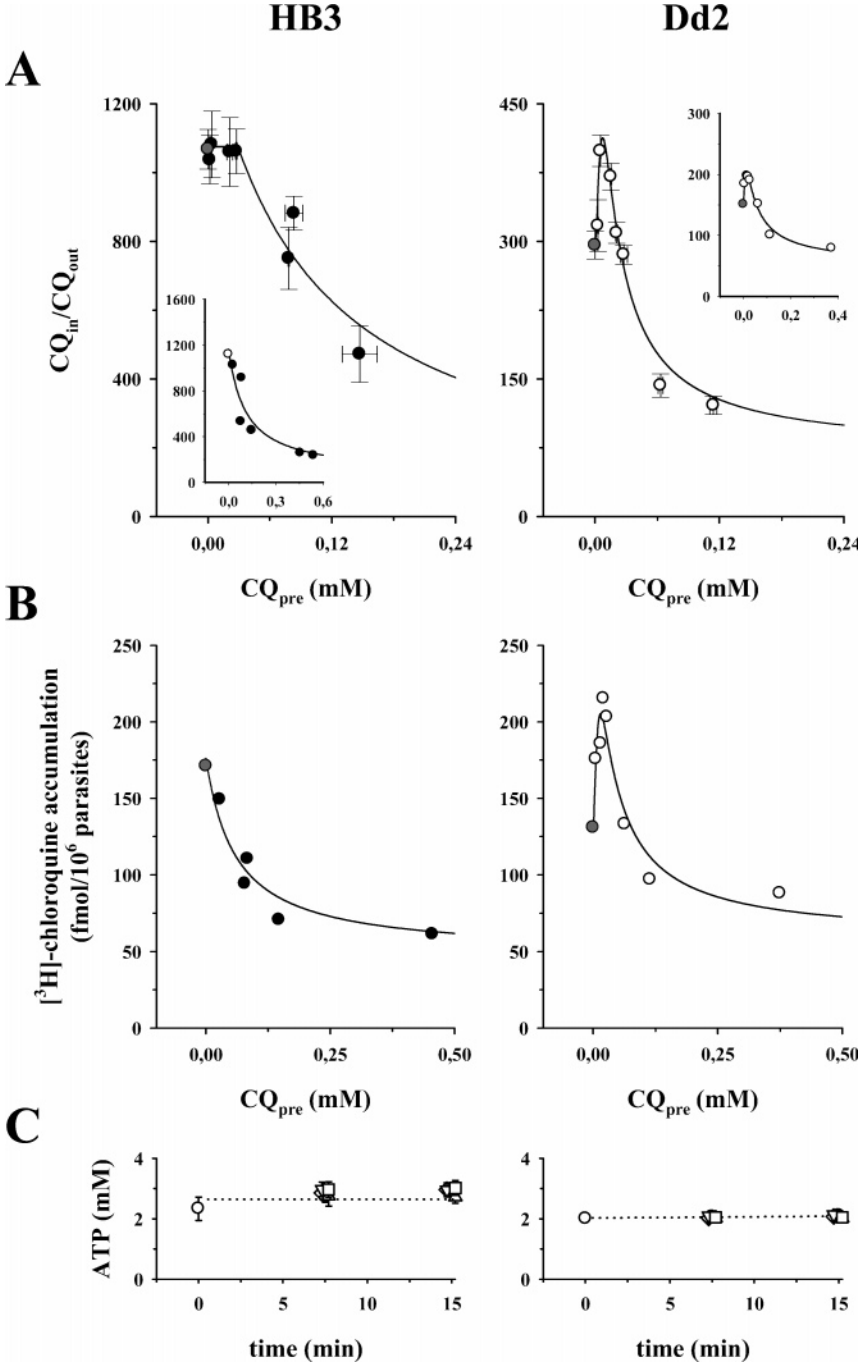

FIGURE 1: Varying-trans kinetics differentiates between CQS and CQR *P. falciparum* strains. (A) Varying-trans kinetics using different probing [^{3}H]chloroquine concentrations. The amount of probing [^{3}H]chloroquine used was 43 nM for the main graphs, 25 nM for the left inset (HB3), and 10 nM for the right inset (Dd2). The level of labeled chloroquine accumulation, given as the ratio of the intracellular versus the extracellular chloroquine concentration (CQ_{in}/CQ_{out}), was analyzed as a function of the total intracellular preloaded chloroquine concentration (CQ_{pre}). The total intracellular preloaded chloroquine concentration was determined in independent experiments. The means ± SEM of at least five independent determinations are shown. (B) Varying-trans kinetics under conditions of equivalent chloroquine accumulation during loading and probing. The amount of probing [^{3}H]chloroquine used was 10 nM for HB3 and 43 nM for Dd2. The amount of labeled chloroquine taken up by the parasite during the probing phase was analyzed as a function of the intracellular preloaded chloroquine concentration. Representative examples of at least three independent determinations are shown. Key: (●) CQS strain HB3; (○) CQR strain Dd2. The control values in the absence of preloaded chloroquine are indicated in gray. For Figures 1−3, the data points were qualitatively fitted using previously described equations (*19*). (C) Temporal changes of ATP concentrations of cells (left, HB3; right, Dd2) incubated with 0 (○), 0.1 μM (◇), 0.5 μM (▽), 1.0 μM (△), and 10.0 μM (□) chloroquine. The means ± SEM of at least three independent determinations are shown.

total intracellular chloroquine concentrations following preloading were equivalent for both types of parasites (Figure 1A). Using these loading conditions, both strains were subjected to the varying-trans procedure, i.e., preincubation with different concentrations of cold chloroquine followed by probing with 43 nM [^{3}H]chloroquine for 4 min. When the results of the varying-trans procedure were analyzed as a function of the total intracellular preloaded chloroquine concentration, the CQR strain revealed stimulated chloroquine accumulation by preloaded chloroquine, while the CQS strain did not (Figure 1A), consistent with our previous data (*19, 20*).

Table 1: Relevant Point Mutations in Pgh-1 (the *pfmdr-1* Gene Product) and PfCRT in Different *P. falciparum* Strains

			Pgh-1					PfCRT								
strain	status	IC_{50} ratio of Dd2	86	184	1034	1042	1246	72	74	75	76	220	271	326	356	371
HB3[a,b]	CQS	0.116	N	F	S	D	D	C	M	N	K	A	Q	N	I	R
D10[b]	CQS	0.301	N	Y	S	N	D	C	M	N	K	A	Q	N	I	R
D10-mdr$^{7G8/1}$ [c]	CQS	0.239	N	Y	S	N	Y	C	M	N	K	A	Q	N	I	R
D10-mdr$^{7G8/3}$ [c]	CQS	0.154	N	Y	C	D	Y	C	M	N	K	A	Q	N	I	R
D10-mdr^{D10} [c]	CQS	0.269	N	Y	S	N	D	C	M	N	K	A	Q	N	I	R
GC03[d]	CQS	0.140	N	F	S	D	D	C	M	N	K	A	Q	N	I	R
C2^{GC03} [d]	CQS	0.117	N	F	S	D	D	C	M	N	K	A	Q	N	I	R
Dd2[b,e]	CQR	1.000	F	Y	C	D	D	C	I	E	T	S	E	S	T	I
C4^{Dd2} [d]	CQR	0.755	N	F	S	D	D	C	I	E	T	S	E	S	T	I
C6^{7G8} [d]	CQR	0.650	N	F	S	D	D	S	M	N	T	S	Q	D	L	R
7G8[b]	CQR	0.736	N	F	C	D	Y	S	M	N	T	S	Q	D	L	R
7G8-mdr^{7G8} [c]	CQR	0.780	N	F	C	D	Y	S	M	N	T	S	Q	D	L	R
7G8-mdr^{D10} [c]	CQR	0.494	N	F	S	N	D	S	M	N	T	S	Q	D	L	R

[a] Reference *37*. [b] Reference *10*. [c] Reference *31*. [d] Reference *13*. [e] Reference *62*.

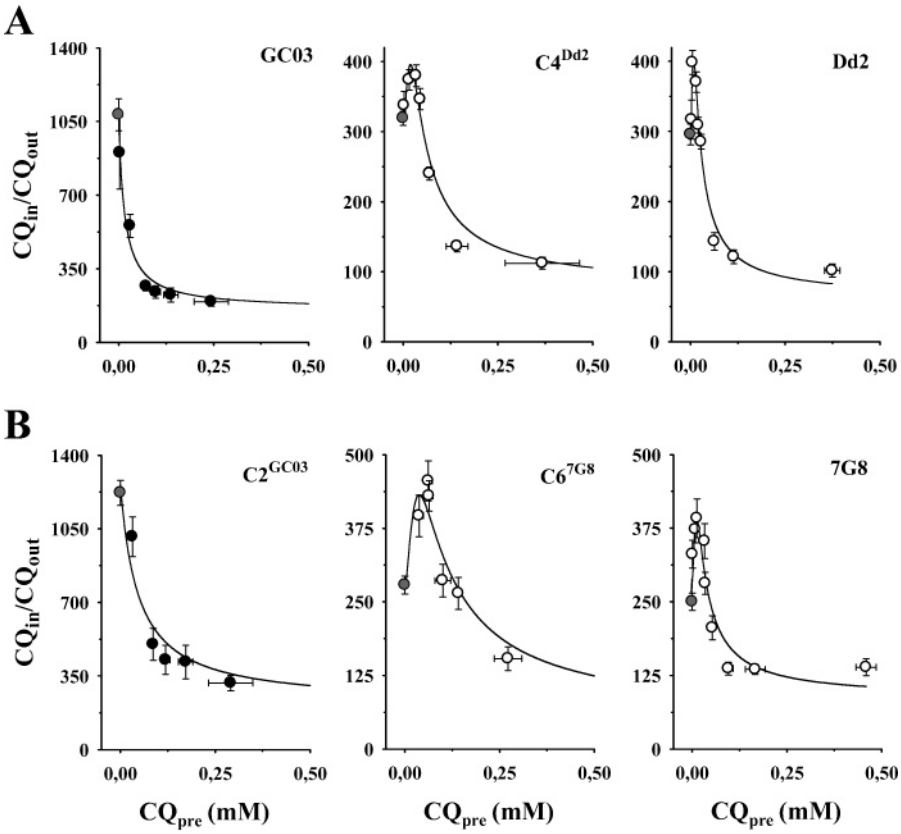

FIGURE 2: Varying-trans kinetics of *pfcrt* allelic exchange mutants. (A) Comparison of the parental strains GC03 and Dd2 with C4^{Dd2} containing the Southeast Asian/African CQR *pfcrt* allele from Dd2. (B) Comparison of the control transfectant C2^{GC03}, the parental strain 7G8, and C6^{7G8} containing the Latin American CQR *pfcrt* allele from 7G8. The level of labeled chloroquine accumulation, given as the ratio of the intracellular versus the extracellular chloroquine concentration (CQ_{in}/CQ_{out}), was analyzed as a function of the total intracellular preloaded chloroquine concentration (CQ_{pre}). The means ± SEM of at least five independent determinations are shown. The control values in the absence of preloaded chloroquine are indicated in gray. Key: (●) CQS; (○) CQR.

We next investigated whether differences in the accumulation of labeled chloroquine during the probing phase affects the outcome of the varying-trans procedure. To address this point, we adjusted the probing, radioactive chloroquine concentration such that comparable amounts of label would accumulate in both HB3 and Dd2 during the probing phase. For HB3 and Dd2, we used 25 and 43 nM labeled chloroquine, respectively. Under conditions of approximately equal amounts of labeled chloroquine accumulation, stimulation was only observed in Dd2 and not in HB3 (Figure 1B). Similarly, labeled probing chloroquine concentrations of 10, 25, 43, and 86 nM had no effect on the outcome of the varying-trans procedure (Figure 1A insets where 25 nM was used for the HB3 cells, and 10 nM for the Dd2 cells). In all cases, Dd2 revealed the stimulation phenomenon while HB3 did not.

In other control experiments we monitored rhodamine 123 uptake by flow cytometry and intracellular ATP levels, as indicators of cell viability, in cells incubated for 15 min in media containing chloroquine concentrations ranging from 0.1 to 10 μM, resembling conditions used for preloading. No changes in rhodamine 123 uptake (data not shown) or in

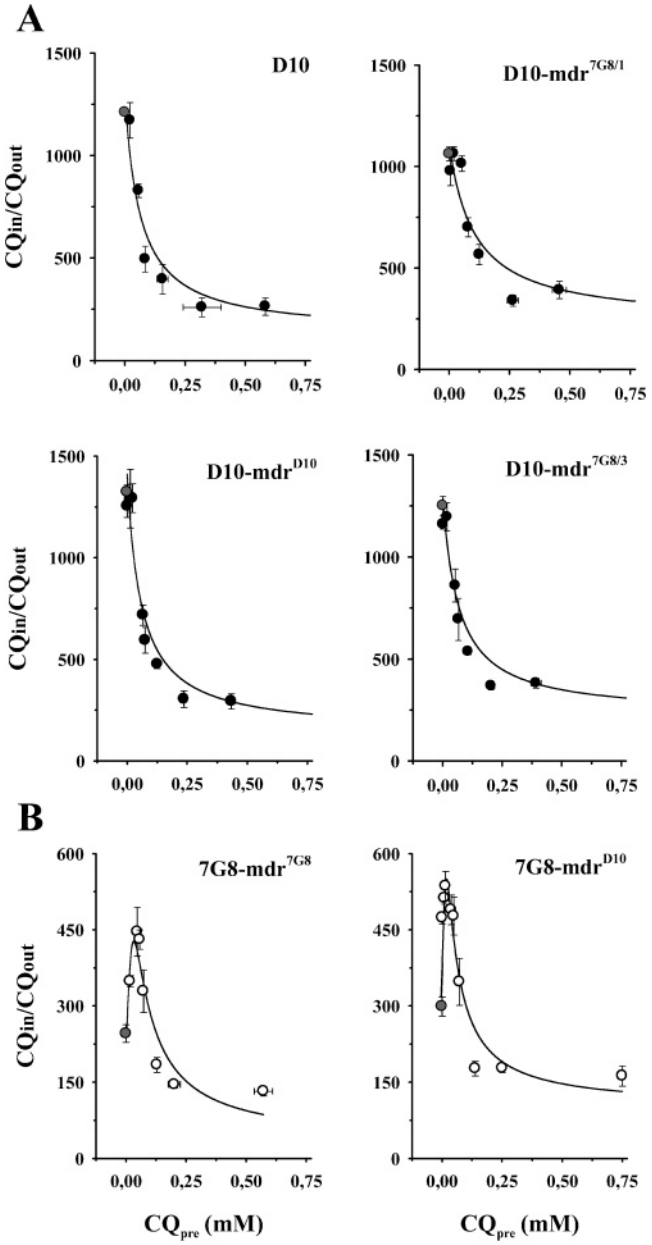

FIGURE 3: Varying-trans kinetics of *pfmdr1* allelic exchange mutants. (A) CQS strain D10 and allelic exchange mutants of *pfmdr1* with a D10 strain background. The superscript indicates the origin of the *pfmdr1* allele (see Table 1). (B) CQR strain 7G8 and allelic exchange mutants of *pfmdr1* with a 7G8 strain background. The level of chloroquine accumulation, given as the ratio of the intracellular versus the extracellular chloroquine concentration (CQ_{in}/CQ_{out}), was analyzed as a function of the total intracellular preloaded chloroquine concentration (CQ_{pre}). The means ± SEM of at least four independent determinations are shown. The control values in the absence of preloaded chloroquine are indicated in gray. Key: (●) CQS; (○) CQR.

intracellular ATP concentrations were observed during the 15 min of incubation, and no differences were observed between control and chloroquine preloaded cells (Figure 1C). Moreover, when cells were returned to the in vitro culture after being preloaded for 15 min with up to 10 μM extracellular chloroquine, they maintained growth rates comparable to those of control cells (data not shown). These data suggest that the cells remain viable during the course of the varying-trans procedure and can tolerate short-term exposure to high concentrations of chloroquine.

Association of Stimulated Chloroquine Accumulation with pfcrt Polymorphisms. We next investigated the kinetics of chloroquine accumulation under varying-trans conditions in a series of previously described *pfcrt* allelic exchange mutants (*13*). The mutants were derived from the CQS clone GC03 by replacing wild-type *pfcrt* with either the Dd2 (Indochina) allele, containing the *pfcrt* polymorphisms found in CQR strains from Asia and Africa, or the 7G8 (Brazil) allele, containing polymorphisms associated with CQR strains from South America and the Oceanic region (Table 1). This yielded the clones $C4^{Dd2}$ and $C6^{7G8}$, respectively, which had both acquired a CQR phenotype (*13*). In addition, we examined GC03, the two CQR strains Dd2 and 7G8, and $C2^{GC03}$, an autologous *pfcrt* replacement mutant retaining the GC03 *pfcrt* allele and the CQS phenotype (Table 1) (*13*).

In GC03 and $C2^{GC03}$, accumulation of [^{3}H]chloroquine declined continuously as the concentration of cold preloaded chloroquine increased (Figure 2), whereas Dd2 and 7G8 exhibit trans-stimulated chloroquine accumulation (Figure 2). Importantly, trans-stimulated chloroquine accumulation was observed in the allelic exchange mutants $C4^{Dd2}$ and $C6^{7G8}$ (Figure 2). Thus, introducing either one of the two predominant CQR *pfcrt* alleles, be it of the Southeast Asian/African or Latin American form, into the genetic background of GC03 bestows upon the mutated strain the phenomenon of trans-stimulated chloroquine accumulation under varying-trans conditions.

Dissociation of Stimulated Chloroquine Accumulation from pfmdr1 Polymorphisms. Some studies have implicated *pfmdr1*, which encodes a homologue of the human multidrug resistance efflux pump, in chloroquine resistance (*29*, *30*, *37*). We therefore investigated the effect of polymorphisms within *pfmdr1* on trans-stimulated chloroquine accumulation using a set of previously described allelic exchange mutants in which *pfmdr1* alleles were exchanged between the CQS strain D10 and the CQR strain 7G8 (Table 1) (*31*). The *pfmdr1* allele of 7G8 encodes four amino acid substitutions with respect to that of D10: $Y^{184}F$, $S^{1034}C$, $N^{1042}D$, and $D^{1246}Y$ (Table 1). The allelic exchange mutants investigated were as follows: $D10\text{-}mdr^{D10}$, which retained the D10 *pfmdr1* sequence; $D10\text{-}mdr^{7G8/3}$ encoding the $S^{1034}C$, $N^{1042}D$, and $D^{1246}Y$ substitutions; $D10\text{-}mdr^{7G8/1}$ encoding only the $D^{1246}Y$ substitution; $7G8\text{-}mdr^{7G8}$, which retained the *pfmdr1* allele of 7G8; and $7G8\text{-}mdr^{D10}$ encoding at positions 1034, 1042, and 1246 wild-type amino acid residues (*31*). All strains with the D10 or 7G8 prefix had the D10 or 7G8 genetic backgrounds, respectively. As seen in Figure 3, replacing the *pfmdr1* allele had no qualitative effect on the outcome of the varying-trans procedure. None of the strains with a D10 genetic background displayed trans-stimulated chloroquine accumulation, whereas all of the strains with a 7G8 genetic background did, irrespective of whether they contained the *pfmdr1* allele from 7G8 or D10.

Effect of Preloaded Chloroquine on Food Vacuolar LysoSensor Blue Fluorescence. In a simple pH-dependent partitioning model, chloroquine may raise the pH of the compartment in which it accumulates depending on the buffering capacity of this compartment (*38*, *39*). We therefore investigated the effect of preloading cells with chloroquine on food vacuolar pH. As a qualitative pH indicator we used LysoSensor Blue DND-192 (LS-Blue). LS-Blue is an acidotropic dye that accumulates in the parasite's food vacuole,

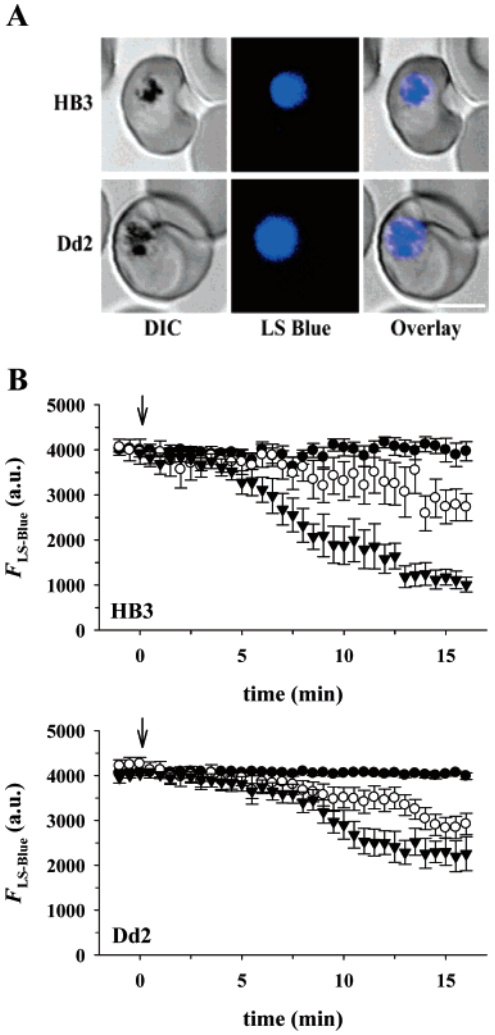

FIGURE 4: Effect of chloroquine on LysoSensor Blue fluorescence. (A) Erythrocytes infected with the CQS strain HB3 or the CQR strain Dd2 were loaded with the qualitative pH indicator LysoSensor Blue DND-192 (LS-Blue). LS-Blue accumulates in the food vacuole, resulting in a bright fluorescence arising from this organelle (here shown in blue). Bar = 5 μm. (B) Temporal changes in LS-Blue fluorescence intensity ($F_{LS-Blue}$) in the food vacuole, provided as arbitrary units (au), were monitored using a confocal laser scanning microscope. At time point zero, indicated by a downward arrow, the perfusion was changed from Ringer solution to a solution containing either 100 nM chloroquine (open circles) or 1 μM bafilomycin A (filled triangles). Control cells were kept continuously in Ringer solution (filled circles). The means ± SEM are shown for at least seven independent determinations.

resulting in a bright fluorescence arising from this organelle (Figure 4A) (*40*). The cytoplasm of the parasite and the host erythrocyte remain nonfluorescent (Figure 4A). Besides its selectivity for the food vacuole, LS-Blue has a high sensitivity in its fluorescence intensity to pH changes, with the fluorescence decreasing with increasing pH (*41*). As LS-Blue lacks a clear isosbestic point, it is precluded from use in ratiometric measurements. Therefore, LS-Blue imaging is better suited for detecting relative changes in pH than for quantification of pH (*41*). Temporal changes in food vacuolar LS-Blue fluorescence intensity were monitored by confocal laser scanning microscopy during perfusion with Ringer's solution containing 100 nM chloroquine. In both HB3 and Dd2, a significant decrease in food vacuolar LS-Blue fluorescence intensity was observed during perfusion for 15 min with medium containing 100 nM chloroquine (Figure 4B, open circles), as compared to Ringer solution (Figure 4B, closed circles), consistent with an alkalinization of the food vacuole. As a control, we investigated the effect of bafilomycin A, an established inhibitor of the food vacuolar H^+-ATPase that maintains the pH in this organelle (*42*, *43*). Inhibition of the food vacuolar ATPase has been shown to result in an alkalinization of this organelle (*42*, *43*). Changing the bath to a medium containing 1 μM bafilomycin A resulted in a rapid loss of LS-Blue fluorescence intensity (Figure 4B, closed triangles) in both HB3 and Dd2, again consistent with an alkalization of the food vacuole.

DISCUSSION

Our data show that stimulated chloroquine accumulation under varying-trans conditions is genetically linked with the *pfcrt* polymorphisms found in CQR strains. This was demonstrated by investigating *pfcrt* allelic exchange mutants in which the wild-type allele was replaced by the mutant Dd2 (Indochina) or 7G8 (Brazil) *pfcrt* alleles (*13*). We have argued that the stimulation phenomenon is a direct consequence of a carrier-mediated process, which extrudes chloroquine from CQR strains (*19*, *20*). At low concentrations, the preloaded chloroquine competes with the labeled chloroquine for transport by the efflux system, thereby blocking efflux of the labeled chloroquine and hence increasing the net entry of label. At high concentrations of preloaded chloroquine, when the efflux system is fully blocked, the preloaded chloroquine competes with the labeled chloroquine for binding to intracellular sites, thus reducing the accumulation of label. In contrast to CQR strains, CQS strains do not show stimulated chloroquine accumulation. Instead, accumulation of labeled chloroquine continuously declines with increasing preloaded chloroquine, which is consistent with the preloaded chloroquine competing with incoming label for binding to some intraparasitic binding site (*19*, *44*, *45*).

The strict linkage between the trans stimulation phenomenon and *pfcrt* polymorphisms suggests that *pfcrt* encodes the chloroquine efflux carrier or a factor regulating its activity; albeit we favor the first model on the basis of the following considerations. Bioinformatic analysis has suggested that PfCRT belongs to the drug/metabolite transporter superfamily, members of which are known to transport both weak bases and divalent organic cations with co- or counterions as a source of secondary energy (*16*, *17*). The PfCRT $K^{76}T$ mutation conserved in all CQR strains investigated thus far is predicted to lie close to the luminal face of the food vacuolar membrane, in a region of the protein proposed to be involved in substrate recognition (*16*). Moreover, PfCRT appears to bind to chloroquine (*21*), suggesting a direct interaction that one would expect if PfCRT transports this drug. Parasites have also acquired discrete point mutations within *pfcrt* when selected for reduced responsiveness to other drugs including quinine, halofantrine, and amantadine (*46*, *47*). This, again, is more consistent with a direct interaction between PfCRT and these drugs and a proposed function of PfCRT in drug transport. The concept of a *pfcrt*-encoded efflux system capable of extruding chloroquine and other drugs is further supported by biochemical studies that have shown that stimulated chloroquine accumulation can be achieved in CQR strains when they are preloaded with

amodiaquine, primaquine, quinacrine, quinine, or quinidine instead of chloroquine (*20*).

Previously, it has been proposed that PfCRT, when mutated, provides a leak through which protonated chloroquine may diffuse out of the food vacuole down its concentration gradient (*21*, *22*). This model, however, is difficult to reconcile with our data as trans stimulation is not seen in cases of simple passive diffusive leaks or diffusion through channels or pores (*28*). Other models suggesting that PfCRT indirectly influences drug partitioning or heme/chloroquine binding kinetics by affecting food vacuolar pH (*24*, *48*, *49*) cannot be ruled out without reference to the transport kinetic data that we marshal in the present paper. On the other hand, the food vacuolar pH changes ascribed to *pfcrt* polymorphisms (*24*, *48*, *49*) may constitute effects associated with possible changes in endogenous transport properties.

In contrast to *pfcrt*, polymorphisms within *pfmdr1* are not linked with trans-stimulated chloroquine accumulation, which we have demonstrated with six *pfmdr1* allelic exchange mutants. These finding suggests that *pfmdr1* is not directly involved in chloroquine efflux. Our data are consistent with genetic and epidemiological data that have found no or only a vague association between chloroquine resistance and the amplification of and/or polymorphisms within *pfmdr1* (*50*−*52*). Regardless, *pfmdr1* may modulate the degree of chloroquine resistance (*31*, *53*, *54*).

In all of our determinations we have measured the total amount of chloroquine in the infected erythrocyte. We have not attempted to discriminate between free chloroquine and that bound to its intracellular binding sites, nor between the different protonated forms of chloroquine. Indeed, in the classic trans stimulation experiments on countertransport in the glucose transporter GLUT1 of the human erythrocyte (*55*), which established the carrier model for membrane transport, it is now known that at least some of the intracellular glucose is bound (*56*), yet this did not significantly affect the result nor the interpretation of these experiments. Similarly, in most subsequent varying-trans procedures, using a large variety of solutes with differing physical properties including charge, hydrophobicity, and hydrophilicity, total intracellularly accumulated solute was considered without differentiating between bound and free forms of the solute (*57*−*60*). In all cases in which trans-stimulated solute accumulation was observed, the phenomenon could be directly related to the presence of a specific solute transporter (*28*, *57*−*60*).

We recognize that chloroquine might have available to itself different binding sites in sensitive or resistant strains. Our argument for the presence of an effective chloroquine transporter in the resistant strains is, however, based only on the results of the varying-trans procedure applied to such resistant strains, not on whether there is a difference in terms of binding sites between the sensitive and the resistant strains. The dependence of chloroquine accumulation on trans chloroquine concentration in CQR cells is not a simple descending hyperbola, but a more complex curve, first rising and then falling as the trans concentration of chloroquine is increased. There is no simple explanation for these complex rising and falling varying-trans kinetics other than the presence of a transporter for the chloroquine. Assuming a simple binding model, it is difficult to see how preloaded chloroquine first gives rise to a steady increasing affinity of labeled chloroquine for binding sites, while the same preloaded chloroquine, at higher concentrations, would give rise to a lower affinity for binding sites. Thus, a change in the binding affinity of sites for chloroquine, induced by increasing intracellular chloroquine concentrations, cannot bring about both a rise and then a fall in labeled chloroquine accumulation. It is of interest that sensitive cells behave according to a simple kinetic scheme, indicating a cis competition for intracellular binding sites, which will also occur at high chloroquine concentrations in the CQR strains.

In the design of the varying-trans procedure, we have chosen conditions under which both CQS and CQR strains accumulate equivalent amounts of total intracellular chloroquine during preloading. Although CQS and CQR strains have the same maximum capacity for chloroquine accumulation (*19*, *45*), CQS strains accumulate substantially more chloroquine than CQR strains do at a given extracellular concentration of chloroquine. Thus, the extracellular chloroquine concentrations used for loading required adjustment to avoid differential accumulation. We also considered whether the labeled probing chloroquine concentration affected the varying-trans procedure, again acknowledging the different drug accumulation ratios between CQS and CQR strains. However, changing the probing chloroquine concentration between 10 and 86 nM did not affect the experimental outcome. There is also no evidence to suggest that the high chloroquine accumulation ratio obscures the stimulation phenomenon in CQS parasites; under conditions in which both CQS and CQR strains accumulate equivalent amounts of total labeled chloroquine during the probing phase (Figure 1B) and unlabeled chloroquine during the preloading phase, stimulated chloroquine accumulation is only observed in CQR stains. Thus, the difference in behavior between CQR and CQS parasites in this stimulation phenomenon cannot be easily explained by differential loading of chloroquine during the preloading phase, differences in the chloroquine accumulation ratios, or differences in labeled chloroquine accumulation during the probing phase.

By virtue of its lipophilic diprotic weak base properties, chloroquine accumulates in acidic compartments, such as the parasite's acidic food vacuole. Protonation of chloroquine may subsequently alkalinize such organelles depending on the buffering capacity of this compartment (*38*, *39*). Indeed, we observed dye fluorescence changes consistent with an alkalinization of the food vacuole in both CQS and CQR strains upon prolonged incubation with chloroquine, in accordance with previous observations (*1*, *27*, *38*, *39*). An alkalinization of the food vacuole by preloaded chloroquine should subsequently diminish the amount of labeled probing chloroquine accumulation, assuming chloroquine diffuses freely across membranes and partitions according to prevailing pH gradients (partitioning model). However, we observe stimulated, not diminished chloroquine accumulation in the CQR strains, in contrast to the predictions made by the partitioning model. Furthermore, CQS parasites also show dye fluorescence changes consistent with alkalinization of their food vacuole upon preloading with chloroquine but do not reveal stimulated chloroquine accumulation. This would suggest that alkalinization of the food vacuole and the trans stimulation effect are unrelated. Thus, relating the trans stimulation effect to chloroquine-induced changes in food

vacuolar pH, which would alter chloroquine partitioning (*27*) or reduce the amount of heme available as target for chloroquine binding (*26*, *48*), would require a convoluted explanation. On the other hand, trans-stimulated chloroquine accumulation is a direct outcome of a carrier-mediated process. In summary, our varying-trans kinetics with differing allelic exchange mutants provides functional in vivo evidence that a *pfcrt*-associated efflux system is the basis of chloroquine resistance in *P. falciparum*.

A recent development in the field further supports our conclusion. *Dictyostelium discoideum* expressing CQR PfCRT in acidic vesicles exhibit reduced chloroquine accumulation in a verapamil-sensitive manner, as compared to untransfected controls or cells expressing the wild-type *pfcrt* allele (*61*). As *D. discoideum* is free of heme, and as only small changes in intravesicular pH were observed with the expression of the different forms of PfCRT (*61*), these findings associate reduced chloroquine accumulation with a PfCRT-mediated drug efflux mechanism and dissociate it from models suggesting that PfCRT affects chloroquine trapping or its binding to heme, thereby supporting major predictions made by our kinetic studies on entire *P. falciparum* infected erythrocytes.

ACKNOWLEDGMENT

We thank E. Wilken and K. Steigleder for technical assistance and A. Cowman for generously providing us with the *pfmdr1* allelic exchange mutants.

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BI050061F