**Dictyostelium discoideum** Expresses a Malaria Chloroquine Resistance Mechanism upon Transfection with Mutant, but Not Wild-type, *Plasmodium falciparum* Transporter PfCRT*

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Chloroquine resistance in *Plasmodium falciparum* malaria results from mutations in PfCRT, a member of a unique family of transporters present in apicomplexan parasites and *Dictyostelium discoideum*. Mechanisms that have been proposed to explain chloroquine resistance are difficult to evaluate within malaria parasites. Here we report on the targeted expression of wild-type and mutant forms of PfCRT to acidic vesicles in *D. discoideum*. We show that wild-type PfCRT has minimal effect on the accumulation of chloroquine by *D. discoideum*, whereas forms of PfCRT carrying a key charge-loss mutation of lysine 76 (e.g. K76T) enable *D. discoideum* to expel chloroquine. As in *P. falciparum*, the chloroquine resistance phenotype conferred on transformed *D. discoideum* can be reversed by the channel-blocking agent verapamil. Although intravesicular pH levels in *D. discoideum* show small acidic changes with the expression of different forms of PfCRT, these changes would tend to promote intravesicular trapping of chloroquine (a weak base) and do not account for reduced drug accumulation in transformed *D. discoideum*. Our results instead support outward-directed chloroquine efflux for the mechanism of chloroquine resistance by mutant PfCRT. This mechanism shows structural specificity as *D. discoideum* transformants that expel chloroquine do not expel piperaquine, a bisquinoline analog of chloroquine used frequently against chloroquine-resistant parasites in Southeast Asia. PfCRT, nevertheless, may have some ability to act on quinine and quinidine. Transformed *D. discoideum* will be useful for further studies of the chloroquine resistance mechanism and may assist in the development and evaluation of new antimalarial drugs.

Mortality because of malaria in African children has increased dramatically with the spread of *Plasmodium falciparum* parasites resistant to chloroquine (1). Yet there are no replacement drugs available with the efficacy, safety, ease of use, and low cost that once made chloroquine the antimalarial drug of choice (2). New drug developments will be supported by a detailed understanding of the chloroquine resistance mechanism and of antimalarial compounds that can reverse or circumvent it.

Chloroquine-resistant (CQR) parasites accumulate less chloroquine in their digestive vacuoles than do chloroquine-sensitive (CQS) parasites (3–5). This reduces toxicity to the parasite that otherwise results from the interaction of chloroquine with hematin molecules released by digestion of host erythrocyte hemoglobin (6). Mutations in PfCRT, a transporter of the *P. falciparum* digestive vacuole membrane, are linked to chloroquine resistance (7–10), but the mechanism by which these mutations reduce the accumulation of chloroquine remains unclear. Leading proposals for the resistance mechanism include: 1) energy-dependent efflux of chloroquine from CQR but not CQS parasites (4, 11–13); 2) reduced import of chloroquine into CQR relative to CQS parasites (14, 15); and 3) decreased efficiency of the chloroquine-hematin interaction resulting from increased acidification of the digestive vacuole (16). The correct resistance mechanism will need to explain the critical role of charge loss at position 76 in PfCRT by mutation from lysine to neutral amino acids (7–9), to account for the ability of verapamil to increase chloroquine accumulation in CQR but not CQS *P. falciparum* (4, 17), and to explain why certain antimalarial drugs that are structurally related to chloroquine remain effective against CQR parasites (18–20).

PfCRT belongs to a newly described family of transporters in two deeply divided lineages of eukaryotes. In the crown group, three members of this family (paralogs DdCRTp1, DdCRTp2, and DdCRTp3) occur in the soil-living amoeba *Dictyostelium discoideum*; in the alveolate clade, single copies of transporter homologs have been identified in apicomplexan parasites including *Cryptosporidium parvum*, *Toxoplasma gondii*, *Eimeria tenella*, *Theileria annulata*, and several *Plasmodium* species (21) (Fig. 1, A and B). Transporters of this family are characterized by 10 predicted transmembrane domains and by a number of conserved amino acids in and between these domains, including T/S/T/G and G/C/G/S/T/G/C/D/N/C sequences in the 3rd and 7th inter-transmembrane regions, respectively. These and certain other features place this family within a larger superfamily of drug/metabolite transporters (22, 23). Whereas native functions for PfCRT and its homologs have not yet been established, evidence indicates that some of these transporters reside on the acidic vesicle membranes of apicomplexans and *D. discoideum*. In addition to the localization of PfCRT to the digestive vacuoles of *P. falciparum*, we have confirmed that the *D. discoideum*
transporter DdCRTp1 (also termed SSA662) (21) is present on acidic endosomes (“Results”).

Studying chloroquine resistance within *Plasmodium falciparum*-infected erythrocytes is complicated by the effects of hematin inside of the parasite digestive vacuole. Furthermore, the haploid nature of intraerythrocytic parasites and an apparently essential native function of PfCRT have so far limited its genetic manipulation in *P. falciparum* to complementation and allelic exchange experiments (24). Heterologous systems capable of expressing PfCRT on hematopoietic cells and vesicles prepared from them have not simulated the reduced chloroquine accumulation phenotype of *P. falciparum*. *D. discoideum* is an attractive alternative for the heterologous expression of PfCRT because of its abundant and characterized system of acidic vesicles, its high AT base pair content compared with that of *P. falciparum*, and the relative facility with which its cells can be grown and genetically manipulated in the laboratory. Here we describe successful transfer of the *P. falciparum* chloroquine resistance phenotype to *D. discoideum* cells and use results from these transformants to evaluate different hypotheses for the mechanism of resistance. We show that expression of mutant but not wild-type forms of PfCRT can reduce accumulation of chloroquine in *D. discoideum*, and that this reduced-accumulation phenotype is reversed by the channel blocker verapamil. PfCRT transfection appears to produce small changes in the intravesicular pH of *D. discoideum*, but these do not explain reduced drug accumulation and instead point to active drug efflux as the mechanism of chloroquine resistance. In contrast to chloroquine, quinine and quinidine show complex patterns of accumulation in *D. discoideum* expressing different forms of PfCRT. Finally, we demonstrate that the chloroquine efflux mechanism in *D. discoideum* transformants does not reduce accumulation of an important chloroquine analog (piperaquine) used against CQR parasites in Southeast Asia.

**EXPERIMENTAL PROCEDURES**

Phylogenetic Analysis—Predicted protein sequences of transporters in the PfCRT family were obtained from the following GenBank™ deposits and database web sites: *Plasmodium berghei* PfCRT, accession number AF314645; *P. falciparum* (D2) PfCRT, accession number AF030684; *Plasmodium knowlesi* PfCRT, accession number AF314646; *T. gondii* TcpCRT, toxodb.org; *E. tenella* EtCRT, www.sanger.ac.uk/Projects/T._tenella/; *C. parvum* CpCRT, accession number EAL34645; *T. annulata* TaCRT, www.sanger.ac.uk/Projects/T._annulata/; *D. discoideum* DdCRTp1 (SSA662), accession number AF217500 (DictyBase ID number DDD9217505); DdCRTp3, accession number EAL72462 (DictyBase ID number DDB01970876). Prefixes, e.g., “Dd,” were included in each sequence name to distinguish the species of its origin. An “h” or “p” was appended at the end of each name to designate the sequence as a homolog or to indicate the likelihood that the *D. discoideum* sequences are homologous or paralogs of one another. Computer predicted protein alignment was by TCoffee version 1.76 (29) (available at igb-server.crs-nrs.fr/TCoffee/teoffee/cgi/index.cgi). THMMMM and TMpred programs were used to predict transmembrane segments (30, 31). Maximum likelihood phylogenetic reconstructions from predicted protein sequences were computed using TREE PUZZLE version 5.2 (www.tree-puzzle.de) (32).

The construction and *D. discoideum* Transformation—Plasmid pEXP4 (33) was used to express full-length PfCRT or fusion proteins of the PCRT sequence together with the N-terminal portion of the *D. discoideum* transporter DdCRTp1. Plasmid pDEXH-GFP (34) was used to express the DdCRTp1 protein with a green fluorescent protein (GFP) tag at its C-terminal end. Transformants of *D. discoideum* were generated using exponential growth-phase cells (1–3 × 10⁶ cells/ml) of the AX2 line (35). The cells were washed twice in cold H₂O transfection buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM MgSO₄, 5 mM NaHCO₃, 1 mM NaH₂PO₄, pH 7.0) and resuspended at 5 × 10⁷ cells/ml. One-hundred μl of cell suspension were mixed with 10 μg of plasmid in 10 μl Tris, 1 mM EDTA, pH 7.4 (TE), and electroporated twice using a Bio-Rad Gene Pulser (settings: 0.8 kV/25 μF, with a 5-s break between shocks). The electrooporated cells were incubated on ice for 5 min and transferred to a tissue culture plate containing 10 ml of D3-T Basic Media (KD Medical) at 20 °C, the cultivation temperature for *D. discoideum*. G418 (20 μg/ml, Invitrogen) was added after 24 h for transformant selection, and clones were picked by pipette 5–7 days later. Cells were cultivated as shaking suspensions in D3-T Basic Media supplemented with ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), and tetracycline (25 μg/ml) (Sigma) at 20 °C. Expression of plasmid-encoded proteins was assessed by SDS-PAGE as described below.

**Immunofluorescence and Immunoblotting Analysis**—For immunofluorescence studies, cells in exponential growth phase (1–3 × 10⁶ cells/ml) were allowed to adhere for 15 min to glass coverslips coated with 0.01% poly-l-lysine (Sigma). The cells were fixed for 15 min in 100% cold methanol and probed for 3 h either with an anti-PfCRT polyclonal rabbit antibody (7) or monoclonal antibody 221–35 against the A-subunit of *D. discoideum* vacuolar H⁺-ATPase (kindly provided by Markus Maniak, Max-Planck-Institut für Biochemie, Martinsried, Germany) (36). After washing with 0.2% Tween 20 (Bio-Rad) in phosphate-buffered saline (10 mM NaPO₄, pH 7.4), the fixed cells were incubated for a further 3 h with either FITC-conjugated donkey anti-rabbit or TRITC-conjugated donkey ant-mouse immunoglobulin G (IgG) (Jackson Immunoresearch). Images were captured using a Leica TCS-SP2 AOS confocal microscope (Leica Microsystems GmbH). For immunoblot analysis, proteins from whole cells were separated by SDS-PAGE and blotted to nitrocellulose membranes. The blots were probed with anti-PfCRT rabbit antibody (7) or developed using horseradish peroxidase conjugated to donkey anti-rabbit IgG (Jackson Immunoresearch).

Isolation of TRITC-loaded Endosomes—*D. discoideum* cells were resuspended at a count of 1–3 × 10⁶ cells/ml in 10 ml of D3-T Basic Media containing 4 mg/ml TRITC-dextran (70 kDa; Sigma) and incubated on a rotary shaker at 20 °C for 2 h. The cells were harvested by centrifugation, washed with cold phosphate buffer (PB; 10 mM NaPO₄, pH 7.4) and washed with homogenization buffer (25 mM KCl, 5 mM MgCl₂, 100 mM sucrose, 50 mM Tris-HCl pH 7.6) containing the Complete™ protease inhibitor mixture (Roche Diagnostics). Purification of the endosomes was performed by the protocol of Clarke et al. (37). The cells were resuspended in 2 ml of homogenization buffer, placed in a syringe, and forced twice through a stack of two polycarbonate filters (Nuclepore, 5 μm pore size). After the cell lysate was centrifuged at 500 × g at 4 °C to remove unbroken cells, 1.5 ml of the supernatant was brought to 4 ml with homogenization buffer, layered onto a Percoll gradient (10 ml each of 70, 65, and 60% Percoll in homogenization buffer) and spun for 1 h at 80,000 × g at 4 °C. The TRITC-loaded endosomes were recovered in a faint red band near the top of the gradient. Intact TRITC-loaded vesicles were resuspended in homogenization buffer (with or without verapamil) and PfCRT protein was detected by immunoblotting as described above.

**Determination of ³H-Drug Accumulation in *D. discoideum*—**

[^3H]Chloroquine (25.7 Ci/mmol) was from Amersham Biosciences; [¹H]Quinidine and [¹H]quinidine (both 20 Ci/mmol) were from American Radiolabeled Chemicals, Inc.; [¹H]piperaquine was custom prepared by tritium exchange (15 Ci/mmol, American Radiolabeled Chemicals, Inc.). *D. discoideum* cells during exponential phase growth were pelleted and resuspended to 1.1 × 10⁷ cells/ml in PB. Cell numbers were determined from a curve of optical density at 590 nm versus cell counts by hemocytometer. Eighty μl of the cell suspension were added to 40 μl of 3-fold concentrated [³H]-drug in PB with or without verapamil and incubated at 20 °C. Each experiment was repeated in parallel on ice (0–4 °C) to determine the extent of nonspecific interaction of [³H]-drug with the cells. The cells were kept in suspension by vortexing every 10 min. After 1 h, the cells were separated from the radioactive incubation medium by layering the suspension on top of 250 μl of silicon oil (Wacker Silicones, 10:1 mixture of PDM20 and PDM200) in a microcentrifuge tube and centrifuging at 13,000 rpm for 30 s in a fixed angle rotor (Sorvall Biofuge Pico). The tubes were placed on dry ice to freeze their contents, inverted, and sliced so that the bottoms containing complete pellets could be transferred into scintillation vials containing 100 μl of NCS II tissue solubilizer (Amersham Biosciences). The vials were vortexed vigorously to dissolve the cell pellets and 20 μl of glacial acetic acid (Sigma) were added to neutralize the pH. After 5 min, 3 ml of Ultima Gold liquid scintillation mixture (PerkinElmer Life Sciences) were added and the vials were allowed to stand overnight in the dark at room temperature. Samples were counted in a Wallac Trilux 1650 Beta

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*Expression of Chloroquine Resistance in Dictyostelium*
scintillation counter (PerkinElmer Life Sciences). Values for nonspecific drug interaction at 0–4 °C (for chloroquine, typically <5% of the counts from cells at 20 °C that do not express the CQR phenotype) were subtracted from those obtained at 20 °C and drug uptake (femtomoles/10^6 cells/ml) was calculated as described (15). Intracellular space of the pellets was determined using [14C]inulin carboxylic acid (Amersham Biosciences) (38).

Measurement of Endosomal pH—D. discoideum cells during exponential growth phase (1–3 × 10^6 cells/ml) were harvested and resuspended to 1 × 10^6 cells/ml in D3-T Basic Media in a shaking flask at 225 rpm at 20 °C. TRITC-dextran (70 kDa; Molecular Probes) and FITC-dextran (77 kDa, Sigma) were added to final concentrations of 4 and 0.4 mg/ml, respectively. After 15 min, the cells were centrifuged at 750 × g for 5 min, washed once with D3-T Basic Media, and resuspended to 1 × 10^6 cells/ml. One-ml aliquots were removed at appropriate time points and added to 100 μl of 2 mg/ml trypan blue (Sigma) in 20 mg sodium citrate, 150 mM NaCl, pH 4.5, solution to quench extracellular fluorescence. The cells were pelleted at 500 × g for 3 min and resuspended in 1 ml of PB. Fluorescence was measured using excitation and emission wavelengths of 480 and 520 nm for FITC-dextran, and 544 and 574 nm for TRITC-dextran. The endosomal pH was estimated from a standard curve of FITC/TRITC fluorescence ratios in 0.1 M potassium phosphate buffer at pre-determined pH values.

RESULTS

Wild-type and Mutant Forms of PfCRT Can Be Targeted to Acidic Vesicles of D. discoideum—Our initial experiments to express full-length PfCRT in D. discoideum yielded high amounts of protein that was mislocalized throughout the cell and not targeted to acidic vesicles (data not shown). We therefore designed a modified expression construct in which the N-terminal coding region of the D. discoideum transporter DdCRTp1 replaced the corresponding N-terminal coding region of PfCRT ahead of the first transmembrane segment (Fig. 1, A and C). Immunofluorescence microscopy of cells probed with α-PfCRT antibody (7) and with an antibody to vacuolar H^+ATPase of D. discoideum (36) showed that chimeric protein from this modified construct was efficiently targeted to the acidic vesicles (Fig. 2A).

We performed two additional experiments to further confirm targeting of the DdCRTp1-directed chimera to acidic endosomes. In the first experiment, we transformed D. discoideum cells with a modified pDEXH-GFP plasmid (34) to express DdCRTp1 tagged with GFP at its C-terminal end. After these transformants were exposed to 4 mg/ml TRITC-dextran for 2 h to load the vesicles of the endosomal pathway, TRITC fluorescence from the endosomes was found to be circumscribed by GFP signal at the vesicle membranes (Fig. 2B). In the second experiment, D. discoideum cells expressing the chimeric DdCRTp1/PfCRT protein were exposed to TRITC-dextran, and the loaded endosomes were isolated by cell fractionation and Percoll gradient separation (37). Immunoblot analysis with anti-PfCRT antibody verified concentrated localization of the chimeric protein to the isolated TRITC-dextran-loaded endosomes (Fig. 2C).

Expression of Mutant PfCRT with a Key Charge-loss Mutation Enables D. discoideum to Expel Chloroquine—To evaluate the effects of the PfCRT sequence on chloroquine accumulation by D. discoideum, we generated transfectants expressing chimeric proteins containing either the wild-type PfCRT (WT-CRT) sequence from CQS P. falciparum or a mutant transporter sequence from a representative CQR P. falciparum line (Dd2) from Southeast Asia (SEA-CRT, carrying PfCRT mutations M74I/N75E/K76T/A220S/Q271E/N326S/I356T/R371I) (7). After confirming expression of these chimeric proteins in transformed cells, we compared the intracellular accumulations of [3H]chloroquine at 4 °C and 20 °C, the cultivation temperature for D. discoideum. Accumulation of [3H]chloroquine at 4 °C was comparable for all D. discoideum lines tested and provided a measure of nonspecific interaction, in agreement with the findings of Wünsch et al. (39) for P. falciparum. However, at 20 °C, D. discoideum expressing SEA-CRT accumulated significantly lower levels of [3H]chloroquine than untransformed cells or cells expressing WT-CRT. After subtracting nonspecific 4 °C uptake of [3H]chloroquine, more than 3-fold differences in drug accumulation were typically detected between SEA-CRT- and WT-CRT-transformed D. discoideum; no significant differences were observed between WT-CRT-transformed and untransformed cells (Fig. 3A). These results were confirmed in multiple experiments using two independent sets of transformed cell lines (data not shown).

Naturally CQR P. falciparum parasites carry multiple mutations in PfCRT that always include a key mutation from lysine to threonine at PfCRT position 76 (K76T) (7, 9). The K76T mutation causes loss of a positive charge that is thought to facilitate the transport of chloroquine in its diprotic form. Various mutations elsewhere in PfCRT (e.g. M74I/N75E/A220S/Q271E/N326S/I356T/R371I) regularly accompany K76T and may help preserve a critical function of the transporter in malaria strains (9, 23). To explore the effect of position 76 mutation in our D. discoideum transformants, we modified the SEA-CRT construct to encode lysine instead of threonine at position 76 (SEA-CRT<sup>K76L</sup>). This change ablated the ability of the expressed chimeric transporter to reduce the accumulation of [3H]chloroquine in transformed cells (Fig. 3A). We also modified the WT-CRT construct to encode threonine instead of lysine at position 76 (WT-CRT<sup>K76T</sup>). This single amino acid change in the wild-type protein reduced [3H]chloroquine accumulation by 20–40% but did not lower it to the levels observed in SEA-CRT transformants (Fig. 3A). A complete reduction of [3H]chloroquine accumulation to the level of SEA-CRT-transformed cells presumably requires the additional mutations in PfCRT that accompany K76T in naturally resistant P. falciparum.

Verapamil Reversal Is a Feature of the Chloroquine Resistance Phenotype in Both P. falciparum Parasites and D. discoideum Transformants—Channel blocking agents such as verapamil and amantadine can partially chemosensitize CQR P. falciparum but do not significantly affect the chloroquine response of CQS parasites (17, 40). Several lines of experimental evidence suggest that this chemosensitization involves the interaction of these blocking agents with mutant forms of PfCRT in CQR parasites (10, 40, 41). We compared the effect of verapamil on [3H]chloroquine accumulation by our transformed lines of D. discoideum. At 20 μM, verapamil partially reversed the reduction of [3H]chloroquine accumulation in the SEA-CRT and WT-CRT<sup>K76T</sup>-transformed lines, such that it increased from 30 to 60% and from 50 to 75% the level in control untransformed cells, respectively (Fig. 3B). At 40 μM, verapamil completely reversed the SEA-CRT phenotype in transformed cells such that the accumulation of [3H]chloroquine was the same as in untransformed D. discoideum (Fig. 3B). The relative accumulations of [3H]chloroquine in cells transformed with WT-CRT or SEA-CRT<sup>K76L</sup> did not differ significantly from those in untransformed cells at any verapamil concentration, consistent with the observation that verapamil does not enhance the drug response of CQS P. falciparum (17). We note that the chloroquine resistance phenotype in transformed D. discoideum could be completely reversed by verapamil because this organism tolerates verapamil concentrations well above those that are lethal to P. falciparum (8–16 μM) (17).

Verapamil also produced general increases of [3H]chloroquine accumulation in all transformed and untransformed lines of D. discoideum (Fig. 3B). Untransformed cells exposed to 80 μM verapamil accumulated 30% more [3H]chloroquine than cells not exposed to any verapamil. The origin of this effect resides with D. discoideum itself and remains to be explained.
It is possible that one or more of the three *D. discoideum* homologs of PfCRT provide a background component of verapamil-reversible chloroquine transport by the cells.

**Intravesicular pH Levels in *D. discoideum* Show Small Acidic Changes with the Expression of Wild-type or Mutant PfCRT Sequences**—We estimated the intravesicular pH of endosomes in our *D. discoideum* lines after a 15-min pulse uptake of TRITC- and FITC-dextrans (36). Intra-endosomal fluorescence ratios were measured and converted to pH using a standard curve of FITC/TRITC fluorescences in the pH range of 4.5 to 6.5 (Fig. 4, A and B). Results indicated that endosomal pH values rose from ~4.9–5.1 to 5.4–5.7 over the course of 1 h, consistent with movement of both dextrans from acidic prelysosomal vesicles to more neutral postlysosomal vesicles (36, 42). These increases appeared to be slightly less for the WT-CRT- and SEA-CRT-transformed lines than for untransformed *D. discoideum* (Fig. 4A). The fluorescence ratios also suggested slightly greater acidity for the vesicles of SEA-PfCRT relative to WT-CRT.

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**Fig. 1.** Phylogenetic analysis of the PICRT transporter family and a map of the expression cassette used for *D. discoideum* transformation. A, the alignment for phylogenetic analysis was determined from predicted protein sequences of the *P. falciparum* Dd2 line (PICRT; SEA sequence), the three paralogs of *D. discoideum* (DdCRTp1, DdCRTp2, and DdCRTp3), and the PICRT homologs of *C. parvum* (CpCRTp1), *T. gondii* (TgCRTp1), *E. tenella* (EtCRTp1), and *T. annulata* (TaCRTp1). Approximate locations of the 10 predicted transmembrane segments are overlined (poor prediction scores were obtained for the 10th transmembrane segments of *E. tenella* and *T. gondii*). The key Thr76 change involved in chloroquine resistance is circled in the first transmembrane segment of PICRT. An arrowhead marks the position at which the encoded N-terminal sequence of PICRT was replaced by the N-terminal sequence of DdCRTp1 to improve targeting to *D. discoideum* vesicles. The N-terminal portion of the *E. tenella* sequence is incomplete because of uncertainty in the first exon and no availability of the corresponding cDNA sequence.

B, phylogenetic relationships of protein sequences in the PICRT family are displayed in a maximum likelihood tree. Branch lengths, drawn to scale, represent distances computed from the Müller-Vingron substitution model (55). The deep branches of *P. parvum* and *T. annulata* at the base of the apicomplexan group are consistent with previous phylogenetic analyses (56, 57). Support for the internal branches of the unrooted tree is indicated in percent. Alignments of the *P. berghei* and *P. knowlesi* homologs relative to PICRT are provided by Nomura et al. (21). C, a readily modifiable plasmid was used to express chimeric PICRT proteins in *D. discoideum*. Grey shading indicates the portion of the *D. discoideum* DdCRTp1 gene that replaces the N-terminal open reading frame (ORF) of pfcr (codons 1M to 49T); black fill indicates the ensuing portion of the pfcr ORF (codons 50N to 424). Transcription is under control of the actin 15 (*act15*) promoter and the 2H3 termination sequence (33). *ampr* and *neor* indicate approximate positions of the ampicillin and G418 resistance cassettes used for selection in *Escherichia coli* and *D. discoideum*, respectively. Restriction enzyme sites: *E*, EcoRI; *N*, NotI; *S*, SpeI; *Xb*, XbaI; *X*, XhoI.
Expression of Chloroquine Resistance in Dictyostelium

Quinine and Quinidine Accumulation Are Traits of Greater Complexity than Chloroquine Accumulation in D. discoideum Transformants—Mutations in PfCRT can influence P. falciparum responses to certain quinoline drugs other than chloroquine. Some of these responses, for example, to quinine and its stereoisomer quinidine, involve complex interactions among multiple genes and differ from chloroquine response in that PfCRT is not the primary determinant (41). How these quinoline lines might interact with PfCRT remains unclear. We examined the accumulation of [3H]quinine and [3H]quinidine in our D. discoideum transformants. Transformed D. discoideum expressing the PfCRT sequence of the CQR SEA (Dd2) parasite line differed little from untransformed control cells in [3H]quinine or [3H]quinidine accumulation (Fig. 5, A and B). In contrast, WT-CRT transformants demonstrated 2–3-fold greater accumulation of both of these drugs. We investigated the accumulation of [3H]quinine and [3H]quinidine in D. discoideum lines transformed with SEA-CRTT76K, WT-CRT*, and SEA-CRT*T76I (encoding an isoleucine instead of threonine at PfCRT position 76). [3H]Quinidine accumulation by these lines was not significantly different from that of D. discoideum transformed with WT-CRT (Fig. 5B), whereas their [3H]quinine accumulation was midway between that of WT-CRT-transformed cells and untransformed controls (Fig. 5A).

We also examined the effect of verapamil on accumulation of [3H]quinine and [3H]quinidine by our D. discoideum transformants. Verapamil produced little or no increase in the accumulation of [3H]quinine by any of the transformants used in these experiments (Fig. 5B). Verapamil did appear to slightly increase the [3H]quinine accumulation of untransformed as well as SEA-CRT and SEA-CRTT76K-transformed cells, but not that of WT-CRT and WT-CRT*PfCRT-transformed cells (Fig. 5A).

Verapamil had a greater effect on [3H]quinine accumulation by D. discoideum expressing SEA-CRT*T76I, approximately doubling the accumulation (Fig. 5A). This effect is opposite to the antagonism of quinine accumulation that verapamil produces in P. falciparum expressing isoleucine at position 76 (9) and is further evidence for complex effects of multiple transporters on quinine response. D. discoideum Transformants Expressing the Chloroquine Resistance Phenotype Are Not Able to Expel Piperaquine, a Bisquinoline Analog of Chloroquine—Certain structural analogs of chloroquine can act with comparable efficacy against CQR as well as CQS P. falciparum (43). These analogs may be poorly recognized by existing forms of PfCRT and therefore
may not be expelled by the resistance mechanism. Piperaquine, a bisquinoline that has two aromatic ring moieties of chloroquine connected by a symmetric alky-l-amine bridge (Fig. 6a), is an important example of an analog that has been consistently successful against CQR strains since the 1970s (46, 47). Piperaquine is also a leading candidate for partnership with dihydroartemisinin in artemisinin-class combination therapy against malaria (46, 47). We performed experiments to test if PICRT can act on piperaquine. The accumulation of [3H]piperaquine was not significantly different between untransformed D. discoideum and the four transformed lines expressing various forms of PICRT (Fig. 6B). Furthermore, verapamil did not affect relative piperaquine accumulations in these lines, even at concentrations that completely reverse the chloroquine resistance phenotype in cells expressing the SEA-CRT sequence (data not shown).

**DISCUSSION**

The results of this study show that mutant forms of PICRT properly targeted to acidic vesicles are sufficient to confer the main features of chloroquine resistance to D. discoideum. These features include reduced accumulation of chloroquine in whole cells and reversal of this reduction by verapamil. Because D. discoideum cells are relatively insensitive to chloroquine as well as to verapamil, the effects of these agents on the chloroquine resistance mechanism can be studied without the toxicity that complicates studies of P. falciparum parasites. Also, D. discoideum vesicles have the advantage of being free of hematin, thus enabling a separation of the mechanisms of chloroquine action and chloroquine resistance that is not available in studies of P. falciparum parasites.

**Acidic Vesicle pH Changes Do Not Account for the Chloroquine-resistance Mechanism in D. discoideum Transformants**

One of the outcomes of our study is evidence against hypotheses ascribing the P. falciparum chloroquine resistance phenotype to changes in vacuolar pH. Yayon et al. (48, 49) proposed that an elevated vacuolar pH in CQR relative to CQS parasites could account for reduced drug accumulation because chloroquine carries two basic amine groups and is concentrated by the square of the proton gradient in acid compartments (50). Our results, however, show trends of acidic vesicle pH toward lower pH values in D. discoideum lines transformed with the PICRT sequences of either CQS or CQR P. falciparum. Photometry of individual intraerythrocytic parasites under continuous perfusion has been reported from other studies to show significantly more acidic digestive vacuole pH values in CQR relative to CQS P. falciparum (16, 51). However, the reports of such pH differences have been controversial and are paradoxical, in part because they predict steeper protein gradients that should drive increased accumulation of chloroquine in CQR P. falciparum (52, 53). One explanation for this paradox is that a more acidic vacuolar pH in the CQR parasite vacuole would enhance the aggregation of heme and reduce its capacity to bind chloroquine in the digestive vacuole, thereby accounting for a reduced-accumulation phenotype (54). Our D. discoideum transformants, however, do not support large pH shifts and altered heme binding capacity as an explanation for the chloroquine resistance phenotype. The pH values of TRITC/FITC-loaded vesicles in D. discoideum transformed by the PICRT sequences of CQS or CQR parasites differed by 0.1–0.2 unit on average and did not show the difference of 0.4 unit reported for CQR relative to CQS P. falciparum (16). More importantly, the absence of hematin pigment from D. discoideum cells discounts the possibility that an acidic pH shift in their vesicles could paradoxically reduce chloroquine accumulation by an effect of hematin on drug binding.

**Mutations Expand the Native Substrate Repertoire of PICRT to Include Chloroquine**

Because the expression of PICRT from CQR malaria parasites reduces chloroquine accumulation in D. discoideum to levels below that of control or WT-CRT-transformed cells, we favor a mechanism by which PICRT expels chloroquine from the acidic vacuole, the compartment of accumulation in P. falciparum and, presumably, in D. discoideum as well. Evidence for an energy-dependent outward-directed chloroquine efflux mechanism (4, 11–13, 40) outweighs that for...
earlier proposals of reduced drug import into the acidic vacuole (14, 15). Genetic studies that link mutations in PCRT to chloroquine resistance as well as its specific reversal by channel blocking agents such as verapamil and amantadine (7, 10, 41) also favor active drug export over reduced uptake, because reversers are more likely to act by blocking the PCRT-mediated efflux than by enhancing PCRT-mediated influx of the drug (40).

In accordance with these observations, our transformed D. discoideum lines acquired the verapamil-reversible chloroquine efflux phenotype upon a single amino acid change from lysine to threonine in wild-type PCRT. Conversely, this feature of reversibility was fully ablated by a complementary change from threonine to lysine in mutant forms of PCRT. Such specific effects suggest that the charged lysine in position 76 inhibits the binding interaction of chloroquine with PCRT in CQS parasites, whereas neutral threonine at this position in CQR parasites enables PCRT to expel the drug in its diprotic form. Indeed, Zhang et al. (25) recently reported that purified membranes and inside-out plasma membranes from P. pastoris expressing PCRT specifically bound chloroquine at physiologically relevant concentrations. Specific binding of chloroquine to PCRT might also explain why certain chemical analogs of chloroquine that differ only in alkyl side chain length are equally effective against CQR and CQS forms of P. falciparum (13, 18, 19).

Wild-type PCRT is thought to serve an essential transport function at the P. falciparum digestive vacuolar membrane because extensive genetic experiments to knockout or otherwise interrupt its gene have not been successful (10). The ability of charge-loss mutations at position 76 to provide the gained function of chloroquine transport represents an expansion of the native substrate repertoire of PCRT. Although this native repertoire has not been established, it may include short peptide and amino acids from hemoglobin digestion in the P. falciparum digestive vacuole. Experiments to evaluate this possibility may now be feasible with vesicles from PCRT-transformed D. discoideum.

Quinoline and Quinidine Accumulation May Be Affected by Multiple Transporters, Including PCRT—In contrast to chloroquine, quinoline and quinidine exhibit patterns of accumulation and verapamil reversal that suggest PCRT acts as only one influence among many on these two drugs in transformed D. discoideum. Various models that invoke the direct or indirect interplay of PCRT with endogenous D. discoideum transporters can be postulated to account for these findings. These would be consistent with our knowledge of genetics of quinine resistance in P. falciparum, which is more complex than the genetics of chloroquine resistance and has been shown to involve five or more quantitative trait loci, including the genes for PCRT, a P-glycoprotein homolog, and, possibly, a Na\(^+\)-H\(^+\) exchanger (41). Interactions of PCRT with endogenous transporters in heterologous systems have also been suggested from studies of transformed X. laevis oocytes (27).

CQR P. falciparum Remains Sensitive to Piperazine and Will Require a New Mechanism of Resistance to Escape It—We have shown in these studies that piperazine accumulation by D. discoideum is not affected by expression of PCRT or its mutant forms. Furthermore, verapamil does not distinguish the accumulation of piperazine in the untransformed and transformed lines of D. discoideum. The mutant forms of PCRT we have studied therefore do not act to transport piperazine. These findings help explain why piperazine has remained highly successful against CQR strains in Southeast Asia since its introduction in the 1970s. Extensive use of this drug in China and its recent incorporation in combination therapy with artemisinin derivatives have not yet led to confirmed reports of piperazine-resistant P. falciparum strains (44–47). Taken together, these observations suggest that piperazine has potential for further extensive use against CQR malaria and that a unique mechanism will be required for the evolution of piperazine resistance in P. falciparum.

D. discoideum Transformants Offer New Approaches in Antimalarial Drug Research—D. discoideum cells transformed with PCRT and its mutant forms will expand our knowledge of structure-activity-relationships in chloroquine resistance and in its reversal by channel blocking agents. Their investigation will help explain why PCRT acts on chloroquine yet remains remarkably unable to recognize and transport certain analogs of this drug, including piperazine. Because D. discoideum is hematin-free and can provide acute vacuoles and membranes for biophysical studies, its transformants will promote deeper examination of the PCRT mechanism independent of the complexities of P. falciparum-infected erythrocytes. Native substrates of the transporter and cofactors involved in energy-dependent transport can now be explored. D. discoideum transformants may also facilitate the testing of leads in antimalarial drug development programs and perhaps suggest new ways to attack Plasmodium parasites in malaria.

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Dictyostelium discoideum Expresses a Malaria Chloroquine Resistance Mechanism upon Transfection with Mutant, but Not Wild-type, Plasmodium falciparum Transporter PfCRT

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