A P-glycoprotein Homologue of *Plasmodium falciparum* Is Localized on the Digestive Vacuole

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Abstract. Resistance to chloroquine in *Plasmodium falciparum* bears a striking similarity to the multi-drug resistance (MDR) phenotype of mammalian tumor cells which is mediated by overexpression of P-glycoprotein. We show here that the *P. falciparum* homologue of the P-glycoprotein (Pgh1) is a 160,000-D protein that is expressed throughout the asexual erythrocytic life cycle of the parasite. Quantitative immunoblotting analysis has shown that the protein is expressed at approximately equal levels in chloroquine resistant and sensitive isolates suggesting that overexpression of Pgh1 is not essential for chloroquine resistance. The chloroquine-resistant cloned line FAC8 however, does express approximately threefold more Pgh1 protein than other isolates which is most likely because of the increased *pfmdr1* gene copy number present in this isolate. Immunofluorescence and immunoelectron microscopy has demonstrated that Pgh1 is localized on the membrane of the digestive vacuole of mature parasites. This subcellular localization suggests that Pgh1 may modulate intracellular chloroquine concentrations and has important implications for the normal physiological function of this protein.

PLASMODIUM falciparum causes the most lethal form of human malaria and is responsible for much morbidity and a large number of deaths throughout the tropical world. One of the most important forms of control of this parasite has involved the use of antimalarial drugs, in particular, chloroquine. However, the emergence of resistance to chloroquine in the late 1950s, followed by the spread of resistance to virtually all parts of the world where malaria is endemic has severely compromised the effectiveness of this drug. The development of *P. falciparum* parasites that are resistant to several other drugs as well as chloroquine has created a dilemma in the choice of therapeutic and prophylactic regimes to control this disease.

Chloroquine resistance in *P. falciparum* has striking similarities to the multi-drug resistance (MDR)[1] phenotype that has been described in mammalian tumor cells (Riordan et al., 1985; Gros et al., 1986, 1988). Multi-drug-resistant tumor cells are able to expel a large array of chemically distinct antitumor drugs thus preventing accumulation of lethal concentrations of the drug within the cell (Skovsgaard, 1978; Horio et al., 1988). This is characterized by amplification of multi-drug-resistance genes and the overexpression of a 170-kD protein termed P-glycoprotein which is an ATP-binding molecule located on the plasma membrane of the cell (see Endicott and Ling, 1989). The MDR phenotype of tumor cells can be inhibited by several drugs including calcium channel blockers. Chloroquine resistance in *P. falciparum* can similarly be inhibited by many of the drugs that reverse the MDR phenotype in tumor cell lines (Martin et al., 1987; Bitonti et al., 1988). In addition, efflux of chloroquine from resistant *P. falciparum* parasites is 40–50-fold increased compared to chloroquine-sensitive parasites (Krogstad et al., 1987).

P. falciparum has two genes (*pfmdr1* and *pfmdr2*) that are homologous to the *MDR* genes in mammals, and they belong to a large gene family that includes molecules such as bacterial transport proteins and the product of the recently isolated cystic fibrosis gene (CFTR) (Riordan et al., 1989). The *pfmdr1* gene has been found to be amplified in some chloroquine-resistant lines of *P. falciparum* and this can involve large segments of DNA (Foote et al., 1989), an observation that is similar to that seen in mammalian tumor cells (Endicott and Ling, 1989).

The sequences of a large number of *pfmdr1* genes from both resistant and sensitive parasites have suggested that several alleles of *pfmdr1* are closely linked to chloroquine resistance (Foote et al., 1990). Using 1–4 key nucleotide differences it was possible to correctly predict the chloroquine resistance or sensitivity status of 34 of 36 isolates examined. These results support the hypothesis that chloroquine resistance is multigenic, as one of the alleles linked to chloroquine resistance has a single amino acid change. If this was the only change necessary to obtain chloroquine resistance it would be expected to occur very frequently and in a multifocal pattern as is the case with pyrimethamine resistance (Cowman et al., 1988; Peterson et al., 1988). In contrast to pyrimethamine resistance, chloroquine resistance arose in

1. *Abbreviations used in this paper*: GST, glutathione-S-transferase; MDR, multi-drug resistant; PCR, polymerase chain reaction.

only two parts of the world and from these two foci, in South East Asia and South America, it has spread throughout the regions where malaria is endemic.

This data is in contrast to a study that analyzed the progeny of a genetic cross between a chloroquine-resistant and -sensitive cloned line of *P. falciparum* which showed that chloroquine resistance segregated independently of the *pfmdr1* and *pfmdr2* genes (Wellems et al., 1990). Sequencing of the *pfmdr1* gene of the chloroquine sensitive line used in the genetic cross showed that it had one of the amino acid changes linked to chloroquine resistance (Foote et al., 1990). This suggests that the chloroquine-sensitive cloned line (HB3) may have a *pfmdr1* allele that is competent for chloroquine resistance but lacks the second gene(s) that appears to be necessary for the phenotypic expression of resistance.

The protein product of the *pfmdr1* gene has not as yet been characterized and in this study we have analyzed the level of expression of the protein and its subcellular localization and show that it is present on the digestive vacuole of trophozoites. This localization has important implications for the normal function of the protein and its possible role in the mechanism of chloroquine resistance.

Materials and Methods

Parasites

The *P. falciparum* isolate FC27 was obtained through a collaboration with the Papua-New Guinea Institute of Medical Research (Madang, Papua-New Guinea). The cloned isolates HB3 (Honduras), 3D7 (a cloned line from the isolate NF5 was obtained from an airport worker in Amsterdam), and K1 (Thailand) was obtained from D. Walliker (Edinburgh University, Edinburgh, Scotland). FAC8 is a cloned line derived from ITG2F6 a Brazilian cloned line which was obtained from the laboratory of L. Miller, National Institutes of Health, Bethesda, Maryland. CSL2, originating from Thailand, was obtained from M. B. Moloney at the Commonwealth Serum Laboratories (Melbourne, Australia). 7G8 (Brazil) and V1 (Vietnam) were obtained from R. Howard (DNAX, San Francisco, CA). KF1776 (Papua New Guinea) gametocytes were kindly provided by K. Forsyth of this institute. Parasites were grown in RPMI-Hepes, 5.8% $NaHCO_3$, with 10% human serum as described (Trager and Jensen, 1976).

Preparation of Affinity-purified Antibodies to Pgh1

The region of the *pfmdr1* sequence between 4,372 to 4,842 bp (Foote et al., 1989), which encoded the COOH-terminal 168 amino acids of the protein, was amplified using the polymerase chain reaction (PCR) and the fragment was subcloned into pGex-3X (Smith and Johnson, 1988) to produce an abundant Pgh1 fusion protein with glutathione-S-transferase (GST). The fusion protein was separated by SDS-PAGE, excised from the gel and electroeluted. The protein was methanol precipitated, redissolved in 0.1% SDS, and antisera raised by immunizing rabbits together with Freund's complete adjuvant for the priming immunization and incomplete Freund's adjuvant for subsequent immunizations. Antibodies to the Pgh1 portion of the fusion protein were obtained by depleting antibodies to the GST part of the fusion protein by absorbing the sera with GST immobilized on CNBr-activated Sepharose. The flow through from the column was then loaded onto a second Sepharose affinity column to which the GST-Pgh1 fusion protein had been conjugated. The antibodies were eluted from this column using 0.1 M glycine, pH 2.6, 0.15 M NaCl, and these antibodies were used in all of the experiments described.

Electrophoresis and Immunoblotting

All protein samples were analyzed by SDS-PAGE (Laemmli, 1970) and electrophoretically transferred to nitrocellulose as described (Towbin et al., 1979). Nitrocellulose blots were processed in TBS (50 mM Tris, pH 7.5, 150 mM NaCl) using BSA as a blocking agent. Affinity-purified antibodies to Pgh1 were used at a dilution of 1:20 to 1:50 while antibodies against the *P. falciparum* homologue of hsp70 (Pfhsp70) (Bianco et al., 1986), were used at a 1:1000 dilution. Bound antibody was detected using ^{125}I-protein A followed by autoradiography with an intensifying screen. In some experiments, bound antibody was detected colorimetrically with alkaline phosphatase–conjugated anti-rabbit IgG and the bromochloroindolyl phosphate/nitro-blue tetrazolium substrate system.

Preparation of Parasites from the Asexual Life Cycle

Six 30-ml dishes of the parasite isolate K1 were mixed and synchronized twice at a 4-h interval with 5% sorbitol and the parasites replaced in six 30-ml dishes at 10% parasitemia and 2% hematocrit in fresh erythrocytes. One 30-ml dish was taken at 8-h intervals over 48 h and the parasites purified by lysis of the uninfected erythrocytes in 0.15% saponin. Equal amounts of parasite material from each time point were analyzed by SDS-PAGE and both Pgh1 and Pfhsp70 were detected by immunoblotting.

Preparation of Purified Trophozoites

Trophozoites from each isolate were obtained by synchronization with 5% sorbitol and the parasites were plated at 10% parasitemia and 2% hematocrit. The mature trophozoites were harvested 24 h later and the trophozoite-infected erythrocytes further purified by separation over a Percoll step gradient with concentration steps of 90, 80, 60, and 40% made in RPMI-Hepes. Mature trophozoites separating between the 60–80% step were harvested and washed in serum-free RPMI-Hepes. The concentration of the total number of trophozoites was calculated by counting on a hemocytometer and the parasites were frozen at −70°C in aliquots of 10^8 parasites. Erythrocytes containing more than one trophozoite were determined by visualizing the number of food vacuoles present. The food vacuole was easily distinguished by the presence of hemozoin granules.

Quantitation of Pgh1 in Mature Trophozoites

The level of Pgh1 in mature trophozoites was quantitated by determining the range of pure trophozoites for five different isolates that gave a linear signal in immunoblotting experiments with affinity-purified antibodies to Pgh1. Aliquots of 2.5×10^5, 5×10^5, 10^6, 5×10^6, and 10^7 were separated by SDS-PAGE and immunoblots probed with the affinity-purified anti-Pgh1 or anti-Pfhsp70 antibodies (Bianco et al., 1986) followed by ^{125}I-protein A. The intensity of the signal was determined by exposure of the filters to a storage phosphor screen for 24 h and quantitation using a Phosphorimager (model 400A; Molecular Dynamics, Sunnyvale, CA) and Image Quant software. This showed that the signal for anti-Pgh1 and Pfhsp70 was linear in the range 2.5×10^5 to 5×10^6 trophozoites. 5×10^5 parasites were used in all subsequent quantitation experiments for all isolates. 5×10^5 trophozoites of each of the isolates purified on Percoll gradients were separated by SDS-PAGE and immunoblots probed with the same affinity-purified anti-Pgh1 and Pfhsp70 antibodies used above. Hybridization on the filters was quantitated as above using the phosphorimager.

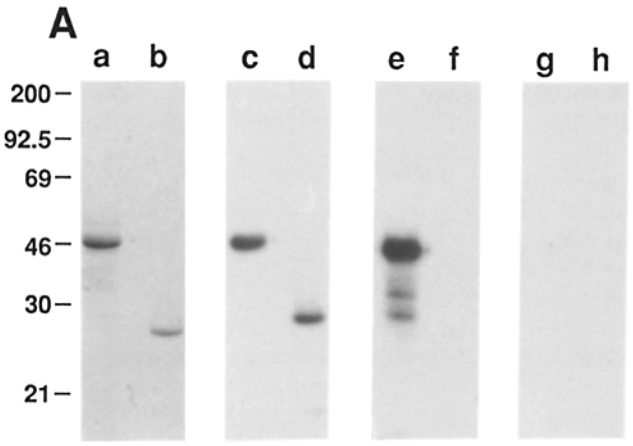
Immunofluorescence

Parasitized erythrocytes were washed twice with human tonicity PBS, pH 7.3 and used for preparation of thin blood films. After air drying, the slides were fixed in acetone at −20°C for 20 min and stored desiccated at −70°C until use. Smears of parasitized erythrocytes were reacted with affinity-purified anti-Pgh1 antibodies or normal rabbit serum for 60 min at room temperature, washed, and incubated with FITC-conjugated anti-rabbit IgG antibodies. The slides were washed in human tonicity PBS and mounted in 90% glycerol, 10% human tonicity PBS containing *p*-phenylenediamine. Viewing and photography were performed under UV or transmitted illumination at ×1000 magnification. Indirect immunofluorescence was repeated a number of times using different preparations of anti-Pgh1 affinity-purified antibodies on a number of different isolates (3D7, K1, and 7G8).

Indirect immunofluorescence of purified digestive vacuoles was done on a small aliquot of vacuoles air dried onto slides, fixed in acetone, and reacted with affinity-purified anti-Pgh1 antibodies or normal rabbit serum as described above.

Immunoelectron Microscopy

The method for postembedding immunolabeling was similar to that described previously (Culvenor and Crewther, 1990). Cultures were fixed in 0.25% glutaraldehyde, 0.1 M phosphate buffer, pH 7.4, for 10 min at room temperature followed by addition of 0.05 M NH_4Cl and washing in 0.1 M



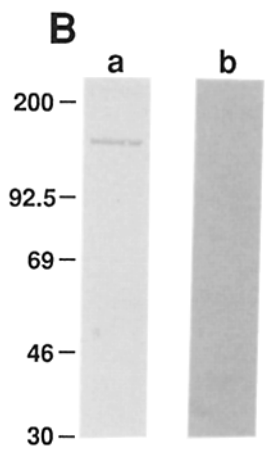


Figure 1. Affinity-purified anti-Pghl antibodies are specific for a 160,000-D protein. (*A*) Purified Pghl-GST fusion protein and GST were separated by SDS-PAGE and the proteins transferred to nitrocellulose and treated in the following way: (lanes *a* and *b*) the proteins were visualized by staining with amido black; (lanes *c* and *d*) probed with antibodies against purified GST protein and ^{125}I-protein A as described in Materials and Methods; (lanes *e* and *f*) probed with affinity-purified anti-Pghl antibodies and ^{125}I-protein A; (lanes *g* and *h*) the same amount of affinity-purified anti-Pghl antibodies were preincubated with 250 μg of Pghl-GST fusion protein in 200 μl of PBS. (*B*) Purified trophozoites of FAC8 were separated by SDS-PAGE and transferred to nitrocellulose. (Lane *a*) Probed with affinity-purified anti-Pghl antibodies and 1:7500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG. Protein bands were visualized using bromochloroindolyl phosphate/nitro-blue tetrazolium substrate system. (Lane *b*) The affinity-purified anti-Pghl antibodies were preincubated with 250 μg of Pghl-GST fusion protein in 200 μl of PBS.

phosphate buffer. Samples were dehydrated in 70% ethanol and embedded in LR White resin which was then polymerized at 50°C for 4 h or 37°C for 5 d. Thin sections were incubated with affinity-purified anti-Pghl antibodies diluted in 0.05 M phosphate, pH 7.4, 0.1% Tween 20, 1% BSA (phos-Tween-BSA) for 1 h at room temperature and after washing in phos-Tween, were incubated for 1 h in protein A-gold (Janssens) diluted in phos-Tween-BSA. Sections were then washed and stained with uranyl acetate.

Purification of Digestive Vacuoles from Trophozoites

Digestive vacuoles from mature trophozoites were obtained essentially by the method previously published (Goldberg et al., 1990) with a few minor alterations. Briefly, the method involved harvesting of 10 × 30-ml dishes of synchronized trophozoites at 10% parasitemia and 2% hematocrit and washing three times in 100 ml of PBS containing 1.5 mM $MgCl_2$. The mature parasites were lysed with 5 vol of 5% sorbitol at room temperature for 15 min followed by centrifugation at 650 *g*. 0.05 vol of 1% saponin in PBS and 0.01 volumes of 50% streptomycin sulphate were added to the supernatant and incubated at room temperature for 10 min. The crude preparation of vacuoles was pelleted at 1,500 *g*. The pellet was washed with 10 ml of 1.5 mM $MgCl_2$, 0.5% streptomycin sulphate in human tonicity PBS and resuspended in 1 ml of 0.25 M sucrose, 10 mM sodium phosphate, and 0.5% streptomycin sulphate, pH 7.1. The resuspension was triturated through a 27-gauge needle 10 times. 13 ml of 42% Percoll, 0.25 M sucrose, 1.5 mM $MgCl_2$ pH 7.0, was added and the gradient centrifuged to equilibrium at 16,000 *g* in a centrifuge (Sorvall Instruments Div., Newton, CT) with an SS34 rotor. The black band at the bottom containing the purified digestive vacuoles was harvested and washed in 0.25 M sucrose, 10 mM sodium phosphate, 1.5 mM $MgCl_2$, pH 7.1. The yield of vacuoles was calculated by counting the vesicles using a hemocytometer.

Results

Identification of the pfmdr1 Protein Product

The *pfmdr1* protein product of *P. falciparum* (Pghl) was identified with antibodies raised to a fragment of the *pfmdr1* gene product expressed in *Escherichi coli* (Foote et al., 1989). A DNA fragment, that encoded the COOH-terminal 168 amino acids of the protein, was cloned into the expression vector pGex-3X (Smith and Johnson, 1988) to produce a fusion protein which was purified and used to immunize rabbits. Antibodies to the *pfmdr1* portion of the fusion protein were first depleted of specificity to the GST part of the fusion protein and affinity-purified antibodies (see Materials and Methods) were used in all of the experiments described.

To test the specificity of the affinity-purified antibodies produced against the Pghl part of the fusion protein they were used to probe immunoblots of purified Pghl-GST fusion protein and the GST protein (Fig. 1 *A*). Antibodies to GST recognize both the Pghl-GST fusion and as expected the GST protein however, the affinity-purified antibodies to Pghl

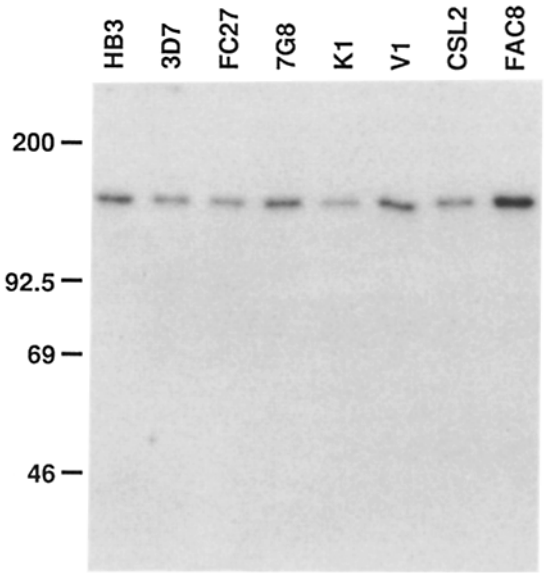


Figure 2. Identification of Pghl in trophozoites of *P. falciparum*. Synchronized trophozoites were purified on Percoll gradients and 10^6 separated by SDS-PAGE. The proteins were transferred by electroelution to nitrocellulose and probed with the affinity-purified anti-Pghl antibodies and ^{125}I-protein A as described in Materials and Methods. The filter was exposed to Agfa curix X-ray film with an intensifying screen for 16 h. The lanes contain the chloroquine-sensitive isolates HB3, 3D7, and FC27 and the chloroquine-resistant isolates; 7G8, K1, V1, CSL2, and FAC8.

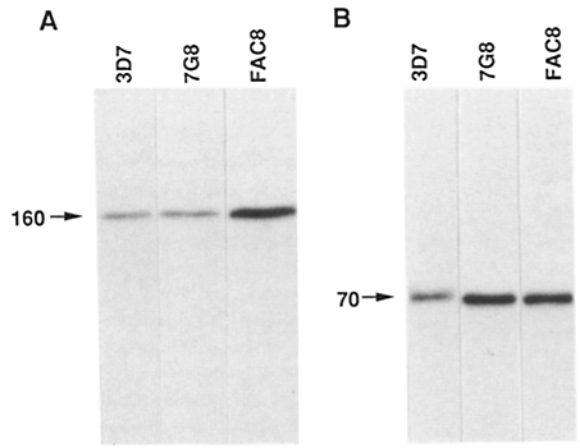


Figure 3. Expression of Phgl in chloroquine-resistant and -sensitive *P. falciparum.* Trophozoites (5×10^5) that had been synchronized and purified on Percoll gradients were separated by SDS-PAGE and the proteins electroblotted onto nitrocellulose. The filters were probed with either (*A*) the affinity-purified anti-Pgh1 antiserum or (*B*) anti-Pfhsp70 and developed using ^{125}I-protein A as described in Materials and Methods. The filter was exposed to X-ray film for 16 h. The order of loading is 3D7, 7G8, and FAC8 for both panels. The signal obtained for both Pgh1 and Pfhsp70 was quantitated using a phosphorimager (see Materials and Methods).

react only with the Pgh1-GST fusion protein indicating that specificities to the GST portion of the protein have been removed. To show that the antibodies reacting with the Pgh1 portion of the fusion protein were specific we preincubated the antibodies with excess purified Pgh1-GST fusion protein before probing the immunoblot. The Pgh1-GST fusion protein competed out the reactivity to the fusion protein indicating that the interaction of the affinity-purified anti–Pgh1 antibodies was specific. The affinity-purified anti–Pgh1 antibodies were then used to probe immunoblots of purified trophozoites from the cloned isolate FAC8 and a protein of 160,000 D was detected (Fig. 1 *B*). To show that the interaction of the antibodies to this protein was specific, we preadsorbed the anti-Pgh1 antibodies with excess Pgh1-GST fusion protein followed by probing an immunoblot containing purified FAC8 trophozoites. The fusion protein competed out the reactivity to the 160,000-D protein indicating that the antibodies are specific for this protein. We have also made antibodies to a region of the NH_2 terminus of Pgh1 and these react with the same 160,000-D protein (data not shown) as the COOH-terminal affinity–purified antibodies described here.

When the affinity-purified antibodies were used to probe immunoblots of purified trophozoites, from both chloroquine-resistant and -sensitive isolates, a protein of 160,000 D was recognized in all cases (Fig. 2). This is in agreement with the molecular weight that was predicted from the nucleotide sequence of *pfmdr1* (Foote et al., 1990). Equal numbers of trophozoites were loaded in all lanes and the protein band detected for both chloroquine-resistant and -sensitive isolates was approximately the same intensity except in the case of FAC8. The protein band in FAC8 was reproducibly much more intense than that seen in the other parasite isolates suggesting that the protein was expressed at a higher level in this chloroquine-resistant cloned line.

A Chloroquine-resistant Isolate Expresses Increased Levels of Pgh1

The MDR phenotype in mammalian tumor cells is characterized by overexpression of P-glycoprotein as a result of amplification of the *MDR* genes (Endicott and Ling, 1989). Since the *pfmdr1* gene is also amplified in some chloroquine-resistant isolates of *P. falciparum* (Foote et al., 1989) it seemed likely that these may also overexpress the Pgh1 protein. To test if chloroquine resistant lines of *P. falciparum* express increased amounts of Pgh1 compared to chloroquine-sensitive isolates we determined the level of Pgh1 in different isolates by quantitative immunoblotting. Several dilutions of trophozoites isolated from synchronized cultures of 3D7, 7G8, and FAC8 were separated by SDS-PAGE and an immunoblot probed with the affinity-purified antibodies to Pgh1 to determine the linear range of detection of Pgh1 (see Materials and Methods). The filter was exposed to a storage phosphor screen and the intensity of each protein band was determined by scanning using a phosphorimager. Detection of the Pgh1 protein band was linear from 2.5×10^5 to 5×10^6. Consequently, 5×10^5 purified trophozoites from a number of chloroquine-resistant and -sensitive parasites were separated by SDS-PAGE and immunoblots probed with the same affinity-purified antiserum (Fig. 3 *A*). The 160,000-D band was present in all parasites tested including both chloroquine-resistant (7G8 and FAC8) and -sensitive parasites (3D7). Equal amounts of the trophozoite preparations were probed with antibodies to the Pfhsp70 protein (Fig. 3 *B*) a molecule that is expressed throughout the asexual erythrocytic life cycle (Fig. 3 *B*) (Bianco et al., 1986).

The ratio of Pgh1 to Pfhsp70 was determined from at least three different experiments by measuring the density of each of the signals using the phosphorimager (Table I). The level of expression of the Pgh1 protein was similar in the chloroquine-sensitive clone 3D7 and the chloroquine-resistant clone 7G8, but the level of expression in FAC8 was consistently approximately threefold greater. This is in agreement with previous results that showed increased *pfmdr1* RNA expression in FAC8 compared to 3D7 (Foote et al., 1989).

The P-glycoprotein Homologue 1 Is Expressed throughout the Erythrocytic Asexual Life Cycle

Chloroquine has its antimalarial effect on the mature stages of the parasite asexual cycle (Ward, 1988) and therefore, if Pgh1 was involved in chloroquine resistance, it would be expected to be expressed in these stages. To determine the stage specificity of Pgh1 expression we synchronized *P. falciparum* parasites in the ring stage of the life cycle and sampled ali-

Table I. Quantitation of Pgh1 in Chloroquine Resistant and Sensitive P. falciparum

Isolate	Ratio*
	n‡
3D7	0.35 ± 0.03 (3)
7G8	0.41 ± 0.03 (4)
FAC8	1.1 ± 0.1 (3)

* The protein bands were quantitated using the phosphorimager and expressed as the Pgh1:Pfhsp70 ratio.
‡ n is the number of experiments performed.

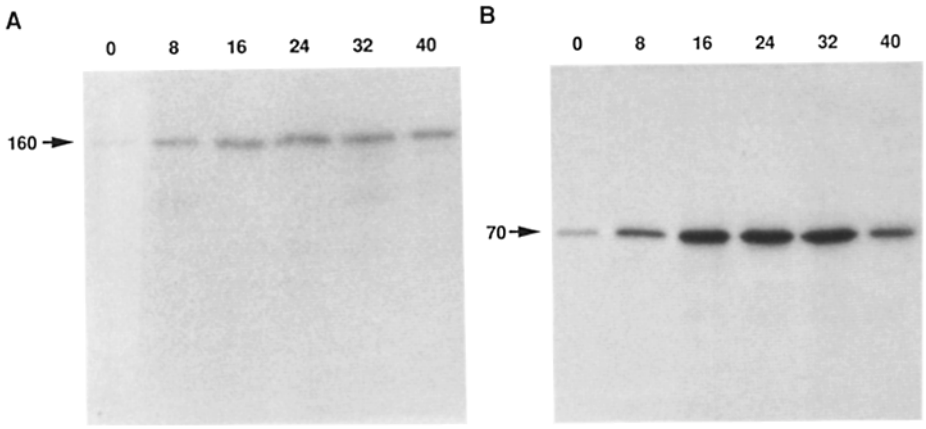


Figure 4. Analysis of Pgh1 throughout the erythrocytic asexual life cycle. Parasites from the cloned line K1 were synchronized twice with 5% sorbitol at 4-h intervals replated at 10% parasitemia and 2% hematocrit and samples taken from 0 to 48 h at 8-h time points. Parasites were purified by saponin lysis of the uninfected erythrocytes and 10^6 parasites separated by SDS-PAGE and transferred by electroelution to nitrocellulose. The filters were probed with (*A*) the affinity-purified anti-Pgh1 antiserum and (*B*) anti-Pfhsp70 and developed using ^{125}I-protein A as described in Materials and Methods. The time points indicated correspond to the following stages as shown by microscopy viewing of smears stained with Giemsa: 0 h, rings; 8 h, rings; 16 h, trophozoites; 24 h, trophozoites; 32 h, schizonts; 40 h, schizonts.

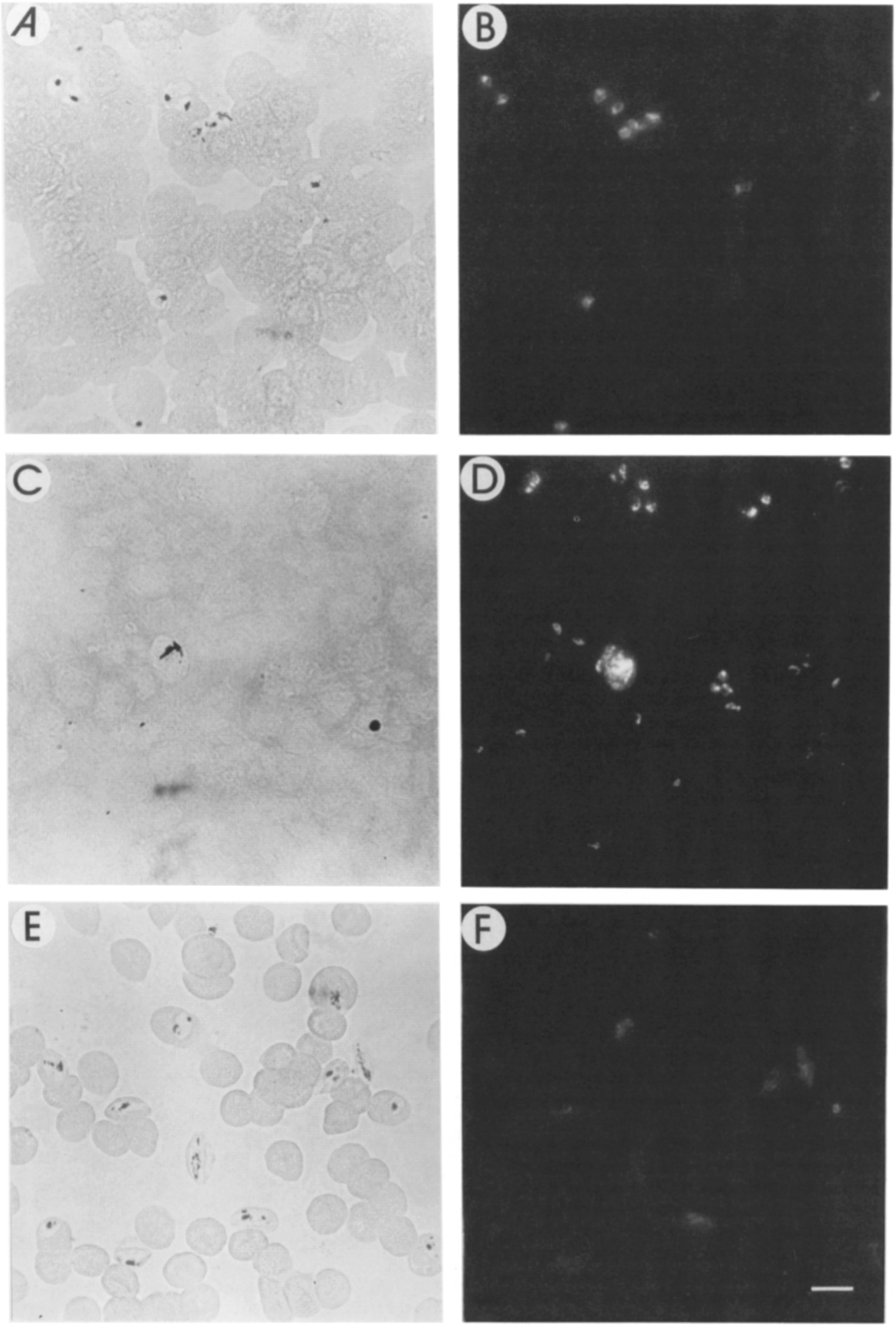
quots at 8-h intervals throughout the 48-h asexual cycle. Equal numbers of parasites were separated by SDS-PAGE and an immunoblot of the transferred proteins was probed with the anti-Pgh1 antibodies (Fig. 4 *A*). A single band of 160,000 D was observed throughout the asexual life cycle but with increasing intensity in mature stages. An immunoblot of the same samples when probed with antibodies to Pfhsp70 (Bianco et al., 1986), as expected, showed a band of 70,000 D that increased with intensity as the parasite matured (Fig. 4 *B*). This increase in abundance of both Pfhsp70 and Pgh1 presumably occurs as the parasite matures and increases in size (Bianco et al., 1986). From this data it is clear that Pgh1 is expressed throughout the asexual life cycle, and importantly is present in the mature stages when chloroquine has its effect on the parasite.

Detection of Pgh1 by Immunofluorescence Microscopy

The affinity-purified antibodies raised against Pgh1 were used to label acetone-fixed smears of parasitized erythrocytes from all asexual stages as well as gametocytes to define the subcellular localization of the protein. As expected the antibodies reacted with all stages of the parasite including gametocytes and did not show any reactivity with uninfected erythrocytes (Fig. 5). Trophozoites had a very distinctive pattern with virtually all reactivity surrounding the digestive vacuole (Fig. 5 *B*). This was seen from the light microscopy which shows the hemozoin granule surrounded by intense fluorescence (Fig. 5 *A*). This suggested that Pgh1 was present on the surface of the digestive vacuole. Much weaker fluorescence was seen over the entire parasite suggesting that there may be a very low amount of the protein present elsewhere within the cell, possibly the parasite plasma membrane. Ring stages showed clear staining with the antibodies including regions of more intense staining against a background of fluorescence over the parasite (Fig. 5 *C*). The schizont stage showed much more scattered fluorescence. However, the most intense staining surrounded the residual body of the parasite. This organelle is the remnant of the digestive vacuole of the parasite as can be seen by the presence of the hemozoin granules (Fig. 5 *C*). There was also staining over regions that represent the developing merozoites as well as small dots of fluorescence which may be the precursor to the digestive vacuole. All stages of gametocytes show generally weak fluorescence (Fig. 5, *E* and *F*) however, there is more intense staining surrounding the hemozoin as is seen in trophozoites and schizonts. Affinity-purified antibodies to a NH_2-terminal portion of Pgh1 have been made and these give the same pattern of immunofluorescence on *P. falciparum* erythrocytic asexual stages (data not shown) as the anti-Pgh1 antibodies described here.

Pgh1 Is Localized on the Digestive Vacuole of Trophozoites

The subcellular localization of Pgh1 was investigated using immunoelectron microscopy by labeling sections of asynchronous cultures using affinity-purified antibodies and protein A-10-nm gold (Fig. 6). The *P. falciparum*-cloned isolate ITG2F6 (the parent of FAC8) showed no label above background over the erythrocyte but gold particles were clearly present over the membrane of the digestive vacuole of trophozoites. Labeling was also present over the membranes of free digestive vacuoles which remain after cell disruption. These vacuoles contain characteristic crystals of hemozoin. No labeling over background could be discerned in ring stages but in schizont stages there was weak but significant labeling over the residual body membrane indicating that the protein is most concentrated around the digestive vacuole of the trophozoite. This pattern of labeling has not previously been seen for any antigens that have been localized by immunoelectron microscopy in *P. falciparum* (Culvenor et al. In press; Culvenor and Crewther, 1990). The *P. falciparum* isolates FC27, V1, D10, HB3, IC1, and FCR3 were also analyzed by immunogold EM and gave the same pattern as that shown for ITG2F6. ITG2F6 (the parent of FAC8), IC1, and FCR3 showed consistently more labeling over the food vacu-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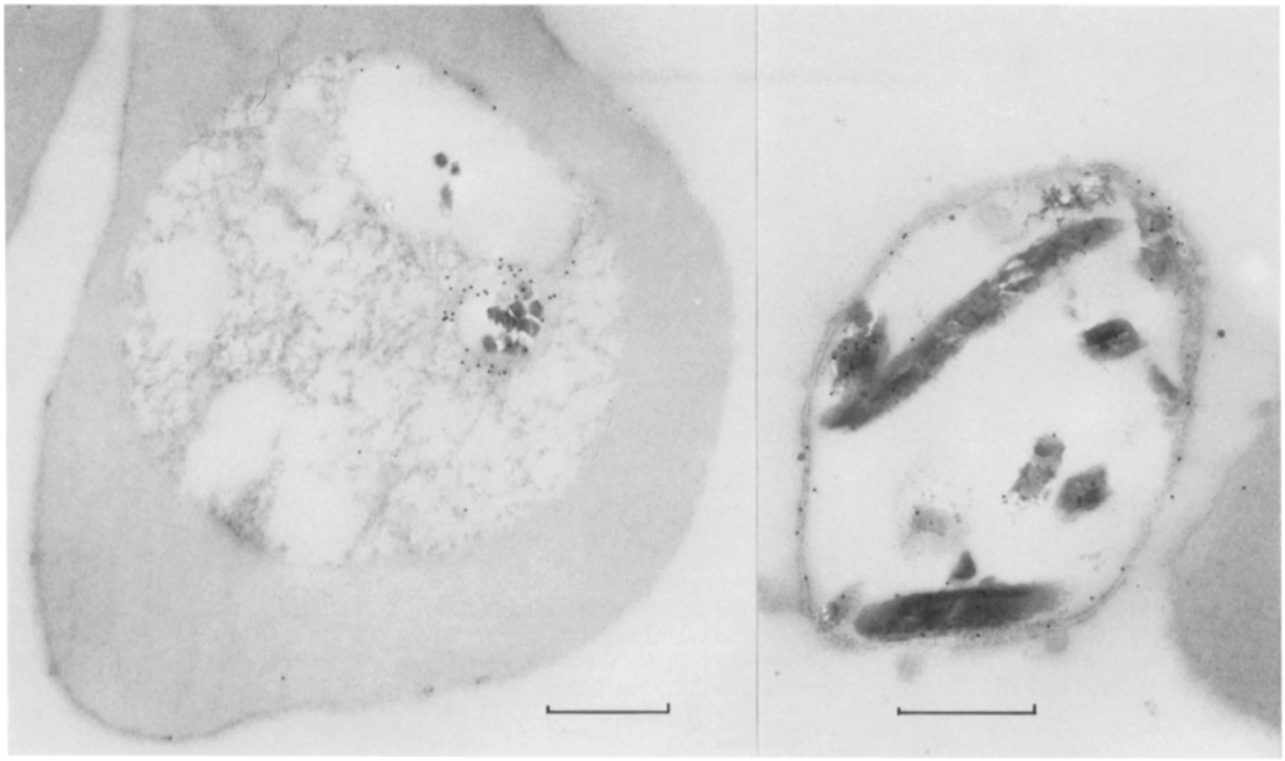

Figure 6. Immunogold EM localization of Pgh1. Asynchronous cultures of the cloned line ITG2F6 were analyzed by postembedding immunolabeling as described in Materials and Methods. The antibody used was the affinity-purified anti-Pgh1 followed by protein A conjugated to 10-nm gold particles. A trophozoite is shown on the left and a free digestive vacuole on the right. Bars, 0.5 μm.

ole membrane than any of the other isolates. Interestingly these isolates have more than one *pfmdr1* gene as does FAC8.

Pgh1 from Purified Digestive Vacuoles

We purified the digestive vacuole from both chloroquine-resistant and chloroquine-sensitive isolates of *P. falciparum* (Goldberg et al., 1990) to provide additional evidence that the Pgh1 molecule was present in this organelle. Synchronized trophozoites were disrupted by sorbitol lysis and a crude preparation of digestive vacuoles was obtained by centrifugation. The pellet was fractionated on a Percoll gradient and a preparation of pure digestive vacuoles was harvested from the bottom of the gradient. The vacuoles consisted of vesicles of $\sim$2 μm in diameter and all contained hemozoin crystals, a characteristic of digestive vacuoles (Fig. 7 *A*). The vacuoles were dried onto a slide, fixed in acetone, and examined by indirect immunofluorescence microscopy using the affinity-purified antibodies to the COOH terminus of Pgh1 (Fig. 7 *B*). All of the digestive vacuoles were fluorescent over the entire organelle consistent with the protein being in the membrane of these vesicles. Serum obtained from prebleeds of the same rabbits used to produce the anti-Pgh1 antibodies before injection of the fusion protein showed no reactivity to the food vacuoles as expected.

Equal numbers of trophozoites and digestive vacuoles were separated by SDS-PAGE and an immunoblot probed with affinity-purified antibodies to Pgh1 (Fig. 8). A single band was present in both trophozoites and purified digestive vacuoles from chloroquine-resistant and -sensitive isolates. The intensity of the band in the trophozoites was approximately the same as that seen in the corresponding purified digestive vacuoles suggesting that most of the protein expressed in trophozoites is present in this organelle. There is a consistently higher level of reactivity to Pgh1 in both FAC8 trophozoites and purified digestive vacuoles suggesting that this cloned line expresses a higher level of the protein.

Discussion

Is Pgh1 Involved in Chloroquine Resistance?

The P-glycoproteins of mammalian cells are present on the plasma membrane and transport cytotoxic drugs out of the cell and thereby prevent their accumulation in toxic concen-

Figure 5. Indirect immunofluorescence using affinity-purified anti-Pgh1 antibody. Smears were made from synchronized K1 cultures as described in Materials and Methods. (*A* and *B*) Light microscopy and UV illumination of trophozoite-infected erythrocytes. Parasites contain hemozoin pigment (dark crystals) within the digestive vacuole. (*C* and *D*) Schizont and ring stages of the parasite. The schizont contains hemozoin pigment which is visible by transmission microscopy (*C*). Ring stages are not easily visible under transmission microscopy. (*E* and *F*) Mixed gametocyte stages from the parasite isolate KF1776. Bar, 10 μm.

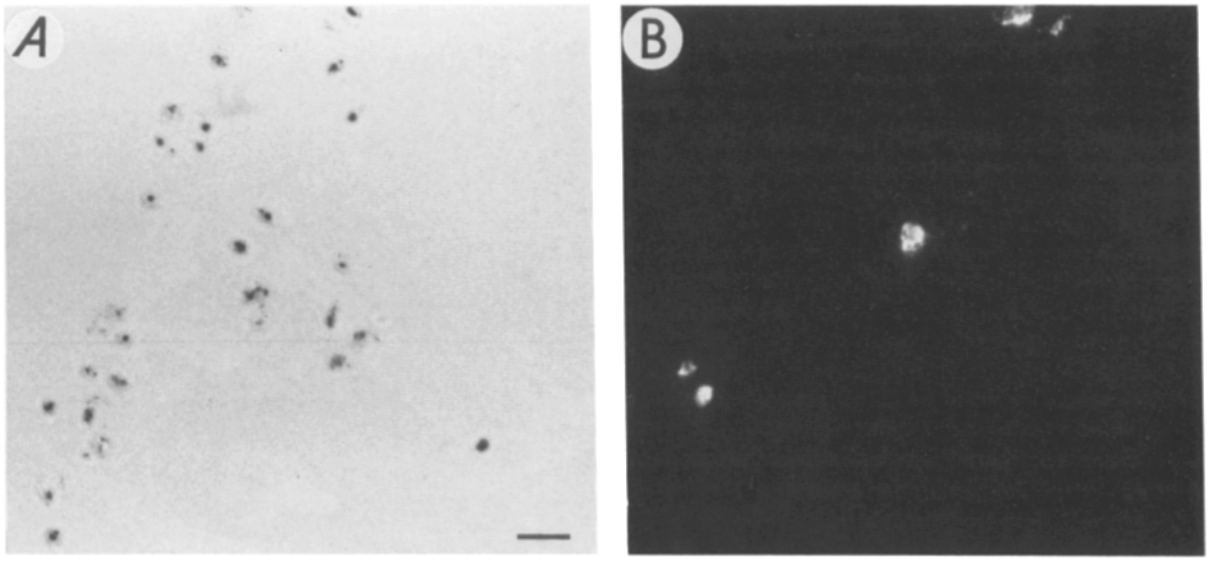

Figure 7. Indirect immunofluorescence of purified digestive vacuoles. Vacuoles were purified as described previously (Goldberg et al., 1990; and Materials and Methods) by a Percoll gradient and the vesicles resuspended in 0.25 M sucrose, 10 mM sodium phosphate, 1.5 mM $MgCl_2$, pH 7.1. (*A*) A wet mount of the purified vacuoles was photographed at ×1000. All vacuoles contained hemozoin granules typical of the digestive vacuole. (*B*) Vacuoles were dried onto a slide and fixed with acetone and reacted with the affinity-purified anti-Pgh1 antibodies followed by FITC-conjugated goat anti-rabbit IgG. Bar, 10 μm.

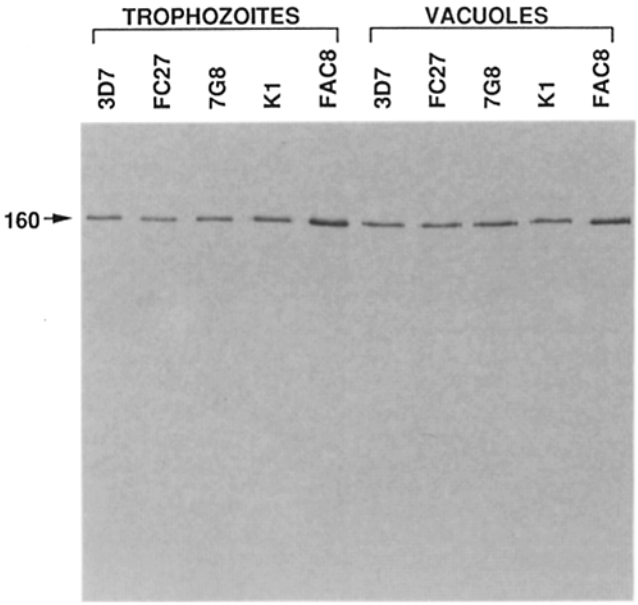

Figure 8. Protein blot analysis of Pgh1 in purified vacuoles. Trophozoites are in the order as labeled: 3D7, FC27, 7G8, K1, and FAC8. Digestive vacuoles of 3D7, FC27, 7G8, K1, and FAC8 are in the same order. Vacuoles were purified as described (Goldberg et al., 1990; Materials and Methods) and the yield counted using a hemocytometer so that an equal number could be loaded onto each track. 10^6 each of purified vacuoles and synchronized trophozoites were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The filter was reacted with the rabbit affinity-purified anti-Pgh1 antibodies followed by 1:7500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG. Protein bands were then visualized using the bromochloroindolyl phosphate/nitro-blue tetrazolium substrate system.

trations (Endicott and Ling, 1989). It has been proposed that the *pfmdr1* gene of *P. falciparum* is linked to chloroquine resistance (Foote et al., 1989, 1990) however, there has been no analysis of the protein product of this gene or functional linkage of it to the chloroquine resistance phenotype. Chloroquine has its antimalarial effect on mature stages of *P. falciparum* and it is concentrated in the digestive vacuole (Krogstad et al., 1985; Krogstad and Schlesinger, 1987; Yayon et al., 1985), which is the most likely site of action of this and related drugs (Ward, 1988).

In this paper we have shown that the *pfmdr1* gene encodes a 160,000-D protein that is localized at the surface of the digestive vacuole of trophozoites. Pgh1 is expressed throughout the erythrocytic asexual life cycle consequently, its stage specificity and subcellular localization support the contention that it is involved in efflux of chloroquine from the digestive vacuole. A transport molecule such as Pgh1, present on the surface of the digestive vacuole, would be able to remove chloroquine from its site of action. This mechanism by itself however, would not remove chloroquine from the parasitized cell.

It has been suggested that chloroquine accumulates in the digestive vacuole as a result of its weak base properties (Homewood et al., 1972) however, there may be a requirement for a chloroquine transporter into the parasite cytoplasm (Warhurst, 1988). In the medium (pH 7.4) the drug would be neutral or singly protonated and would be able to move through membranes into the erythrocyte. It could then move down a pH gradient into the parasite cytoplasm (pH 6.8) and then into the digestive vacuole of the parasite (pH 5). There is however, disagreement on the pH of the parasite cytoplasm and it may be as high as pH 7.6 which would invalidate the weak base effect and suggests that a transport

molecule is necessary for chloroquine uptake (Warhurst, 1988; Ginsberg, 1988). Irrespective of the exact mode of chloroquine uptake it is clear that the drug is concentrated in the digestive vacuole. Within this organelle it would be doubly protonated and transport back into the parasite cytoplasm would require an active transport mechanism. There would be a tendency for the neutral or singly protonated form of the drug to move back into the digestive vacuole down the pH gradient but a concentration gradient would also move chloroquine out of the cell. Therefore an efficient transport molecule that removes chloroquine from the digestive vacuole would allow the drug to be removed from the cell. This is consistent with the efflux of the drug from the parasite (Krogstad, 1987) and the ultrastructural observation in *P. berghei* that a chloroquine analogue was localized in the digestive vacuole of a chloroquine-sensitive parasite but was detected only in the cytoplasm of a chloroquine-resistant isolate (Moreau et al., 1986).

What Is the Normal Function of Pgh1?

As Pgh1 is present on the digestive vacuole membrane of mature stages of *P. falciparum* its function may involve transport into or out of this organelle. Most proteins belonging to the *MDR*-like ATP-binding super family are involved in transport of molecules, such as amino acids and proteins, across membranes. The *HylB* gene (Mackman and Holland, 1984) of *E. coli* is required for transport of hemolysin, a 110,000-D protein, and the *cyaB* gene (Glaser et al., 1988) of *Bordetella pertussis* is responsible for secretion of a calmodulin-dependent adenylate cyclase. It has been suggested that the peroxisomal membrane protein PMP70 which is a member of the MDR family is part of the import machinery for peroxisomal proteins (Kamijo et al., 1990). If the direction of transport of Pgh1 is into the digestive vacuole it may be required for the transport of proteins required for the function of this organelle. The bacterial protein *hisP* of *Salmonella typhimurium* is a peripheral membrane component of periplasmic active transport system of histidine, lysine, arginine, and ornithine. Also *malK* from *E. coli* is involved in the transport of maltose and maltodextrins and both of these proteins share homology with the *MDR* gene family and are members of the *mdr*-like ATP-binding superfamily. *P. falciparum* obtains most of its energy requirements from the digestion, within the digestive vacuole, of globin and it is possible that Pgh1 is necessary for transport of breakdown products of globin from the digestive vacuole into the cytoplasm of the parasite so that they can be used.

Overexpression of Pgh1 Is not Necessary for Chloroquine Resistance

The MDR phenotype in mammalian tumor cells is associated with overexpression of P-glycoprotein and the level of resistance of these cells to the cytotoxic drugs usually reflects the amount of expression of this transport protein (Endicott and Ling, 1989). The Pgh1 protein of *P. falciparum* is expressed at approximately equivalent levels in the chloroquine-sensitive and most of the chloroquine-resistant isolates. Therefore overexpression of this protein is not necessary for chloroquine resistance. Previous studies have identified a number of amino acid differences within the pfmdr1 gene, between chloroquine-sensitive and -resistant isolates (Foote et al., 1990). On the basis of these chloroquine-resistance-linked nucleotide changes in the *pfmdr* gene it was possible to predict the chloroquine resistance status of 34 of 36 isolates of *P. falciparum* and it was suggested that these amino acid changes may be important in the mechanism of resistance. As there is no overexpression of the Pgh1 protein in most chloroquine-resistant isolates the amino acid changes in the Pgh1 protein must be important if this protein plays a role in resistance to this drug.

The chloroquine-resistant cloned line FAC8 expresses more of the Pgh1 protein than any other isolate tested and interestingly it has a threefold increased copy number of the pfmdr1 gene compared to the other isolates (Foote et al., 1989). FAC8 pfmdr1 gene is located on a 100-kb amplicon that is present in three copies along chromosome 5 and the increased expression of Pgh1 is probably a result of this increase in pfmdr1 gene copy number. If Pgh1 is involved as an efflux pump in chloroquine resistance, an increase in expression of this protein may be expected to show a concomitant increase in the level of chloroquine resistance. The level of resistance of FAC8 to chloroquine is not greater than most of the chloroquine-resistant isolates that show no increase in expression of Pgh1 (Foote et al., 1989). Therefore there is no direct correlation between overexpression of the Pgh1 protein and the level of chloroquine resistance. However, the localization of the Pgh1 protein to the surface of the digestive vacuole of mature asexual stage parasites and the availability of these purified organelles in large numbers (Goldberg et al., 1990) make it possible to initiate biochemical analysis on the structure and function of this protein and its potential role in chloroquine resistance.

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