Expression of the P-glycoprotein Homologue1 on Food Vacuoles isolated from Chloroquine-Sensitive and Resistant *Plasmodium Falciparum* Strains

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Abstract

Worldwide occurrence of chloroquine resistance is an expanding problem in prophylaxis and treatment of malaria. Similarities between the drug resistance phenotype in certain cancers and in malaria suggest that homologue multidrug resistance proteins might be involved in the mechanism of resistance.
In this thesis, the expression of a putative multidrug resistance protein of the malaria parasite *Plasmodium falciparum*, the P-glycoprotein homologue1 (Pgh1), was quantified on food vacuoles, the site of action of chloroquine.

Chloroquine susceptibility was determined in 8 different *P. falciparum* strains. Food vacuoles were isolated from trophozoites of two chloroquine-sensitive (3D7 and D10) and three chloroquine-resistant (FAC8, K1 and RSA11) strains.
Antibodies against an 18 amino acid long peptide of Pgh1 were raised, as well as two other antibodies against the N-terminal ATP-binding site and the C-terminus of Pgh1. With these antibodies, Pgh1 was detected on isolated food vacuoles and on trophozoites by immunoblotting. The exact Pgh1 expression levels on food vacuoles were measured with digital image analysis.
The chloroquine-sensitive strains 3D7 and D10 and the chloroquine-resistant strains K1 and RSA11 expressed equal amounts of Pgh1. The chloroquine-resistant FAC8 strain expressed at least three times more vacuolar Pgh1. No correlation was found between chloroquine IC_{50} and vacuolar Pgh1 expression levels.
Phosphorylation studies on intact food vacuoles indicated that Pgh1 is not a major kinase substrate.

List of Abbreviations

Å	Ångstrom
ABC	ATP-binding cassette
ADP	Adenosine 5'-diphosphate
Am. persulfate	Ammonium persulfate
APAD	3-Acetyl pyridine adenine dinucleotide
ATP	Adenosine 5'-triphosphate
BC	Before christ
bp	Base pair
BSA	Bovine serum albumin
^0C	Degrees celcius
CFA	Complete Freund's adjuvant
Ci	Curie
CQ	Chloroquine
Da	Dalton
DDT	1,1 Bis (p-Chlorophenyl)-2,2,2-trichloroethane
DMF	Dimethylformaimide
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
ETH	Eidgenössische Technische Hochschule
FPIX	Ferriprotoporphyrin IX
G	Gauge
g	Grams
GSH	Glutathione
GST	Glutathione S-transferase
GTP	Guanosine 5'-triphosphate
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HRP	Horseradish peroxidase
i.e.	Id est

IC_{50}	Drug concentration required to inhibit parasite growth by 50%
IPTG	Isopropyl ß-D-thiogalactopyranoside
kD	Kilodalton
l	Liter
LDH	Lactate dehyrogenase
M	Molar
µ	Micro
mA	Milliampere
MBS	Maleimidobenzyl-N-hydroxysuccinimide ester
MDR	Multidrug resistance/resistant
min	Minute/s
ml	Milliliter
MP/T/P	5% milkpowder, 0.1% Twee-20 in PBS
n	Nano
NBT	Nitro blue tetrazolium
nm	Nanometer
OD	Optical density
P.falciparum	Plasmodium falciparum
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PES	Phenazine ethosulfate
Pfmdr	Plasmodium falciparum multidrug resistance gene/protein
Pgh1	P-glycoprotein homologue 1
P-gp	P-glycoprotein
pH	Negative logarithm of the hydrogen ion concentration
Pipes	(Piperazine-N,N' bis[2-ethane-sulfonic acid];1,4-piperazine diethanesulfonic acid)
pLDH	Parasite lactate dehydrogenase
PMSF	Phenylmethylsulfonyl Fluoride
pRBC	Parasitised red blood cell
RBC	Red blood cell

rpm	Revolutions per minute
RSA	Republic of South Africa
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate gel electrophoresis
TE	Tris EDTA
TIFF	Tag image file formate
V	Volt/s
v/v	Volume/volume
w/v	Weight/volume
WHO	World Health Organization

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1. Introduction

1.1. Introduction to Malaria

Malaria as a disease was already known in Chinese medicine before 2500 BC. The same illness was described in ancient Rome and in the Middle Ages. A hundred years ago malaria was common in most parts of the world, including Europe, where it disappeared in the mid-20th century [Wernsdorfer and McGregor, 1988]. At present malaria is still the most important parasitic disease in Africa, South East Asia and Latin America. Of the four species of human malaria parasites *Plasmodium falciparum* occurs most frequently in the tropics. The wide-spread occurrence of *P. falciparum* and the fact that it is the cause of the most severe form of human malaria has resulted in malaria research being focused mainly on *P. falciparum*.

After success in the early 1950s with the insecticide DDT, there was hope that the malaria vector and therefore the disease could be eradicated. There is now consensus that this is not possible and that sustainable combat and control of malaria must include both vector control as well as interruption of transmission and treatment and healing of patients.

A major group of antimalarial drugs is derived from quinine and includes mefloquine, halofantrine and chloroquine [Cowman, 1995]. Chloroquine, a drug for prophylaxis, treatment and healing of patients was first synthesised in Germany in 1934. It's antimalarial activity and pharmacology was evaluated more than 10 years later and it 'became recognised as the antimalarial drug of choice throughout the world' [Wernsdorfer and McGregor, 1988].

1.2. Mode of Action of Chloroquine

The intraerythrocytic malaria parasite mostly gets nourishment from haemoglobin that is taken up from the host cytoplasm [Coombs and North, 1991; Goldberg and Slater, 1992; Dorn et al., 1995]. It is degraded in the food vacuole, a lysosome-like organelle. Hydrolytic enzymes are known to assist digestion, cleave haemoglobin to single amino acids and heme derivative, ferriprotoporphyrin IX (FPIX). FPIX as a monomer is toxic to the parasite. It is polymerized to a crystalline structure called haemozoin (also called ß-haematin or malaria pigment). This insoluble polymer can easily be identified by light microscopy as a dense dark brown structure and is non-toxic to the parasite.

The effectiveness of quinine-like compounds such as chloroquine is limited to the asexual intraerythrocytic stages, which all produce haemozoin. It was shown that chloroquine and other drugs such as quinine and mefloquine inhibit the sequestration of FPIX to haemozoin [Egan et al., 1994; Dorn et al., 1995]. The accumulation of undigested haemoglobin leads to a swelling of the food vacuole and death of the parasite [Sinden, 1982].

It was initially proposed that chloroquine binds to FPIX preventing polymerization [Wellems, 1992]. Yet affinities of other quinolone ring drugs such as amodiaquine and halofantrine to FPIX did not correlate with antimalarial activity [Warhurst, 1987; Wellems, 1992]. In 1992 Slater and Cerami isolated a haem polymerase from *P. falciparum* trophozoite extracts [Slater and Cerami, 1992]. This haem polymerase could promote polymerisation of haem to haemozoin. Addition of chloroquine or quinine to the haem and enzyme solution, inhibited the polymerisation of haem to haemozoin; strong evidence that chloroquine can directly inhibit the haem polymerase.

Subsequently other groups have shown that spontaneous formation of haemozoin occurs under conditions similar to those in food vacuoles and can be blocked by chloroquine, quinine and amodiaquin [Egan et al., 1994; Dorn et al., 1995], all of which are known to bind haem. Recently an expanded model for the antimalarial action of chloroquine was proposed (Ginsburg et al., 1998). This research group showed that only 30% of heam is polymerised to haemozoin within the food vacuole. Nonpolymerized haem would exit the food vacuole where it would be degraded by glutathione. Chloroquine as well as amodiaquin would inhibit this degradation. Haem would therefore accumulate in membranes and disturb homeostasis in the parasite.

1.3. Chloroquine Resistance

Resistance to chloroquine was first reported in South America and South East Asia after approximately 15 years of wide-spread use of chloroquine. [Wernsdorfer and McGregor, 1988; figure 1.1].

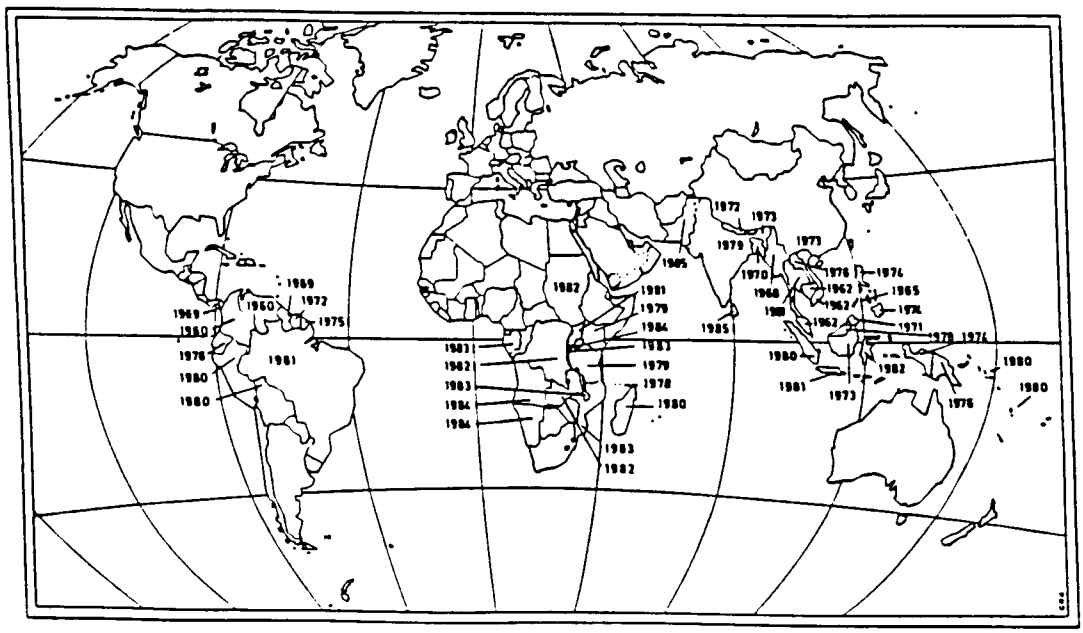


Figure 1.1. Chronology of occurance and spread of chloroquine-resistant *P.falciparum* malaria, 1960-1984. From Wernsdorfer and McGregor, 1988.

In 1999 chloroquine resistance is widely distributed in all geographical areas where *P. falciparum* is endemic. In these regions, mefloquine or a combination of chloroquine with proguanil, is recommended by the WHO as prophylaxis [Prophylaxis of Malaria, 1996].

1.4. Mechanism of Resistance

Chloroquine-resistant *P. falciparum* parasites incorporate less drug than sensitive parasites [Yayon et al., 1985; Krogstad et al., 1987; Saliba, 1997; Saliba et al., 1998]. This suggests that the mechanism of resistance is not directly coupled to the mode of action of the drug. It has also been shown that, in a cell free environment, digestive vacuoles of chloroquine-resistant strains accumulate less drug than vacuoles from drug sensitive strains of *P. falciparum* [Saliba, 1997; Saliba et al., 1998]. These findings suggest that in resistant parasites chloroquine cannot get to its site of action.

In red blood cells infected with parasites, it is thought that chloroquine as an uncharged molecule, passes through the membranes of the erythrocyte and the parasite. Within the parasite it would pass through the membrane of the food vacuole. As chloroquine is a weak base, it would become diprotonated in the acid environment of the food vacuole. Once charged it would not easily penetrate the vacuolar membrane, and thus accumulates in the vacuole.

Uninfected red blood cells accumulate much less chloroquine than infected red blood cells [Fitch, 1970; Sanchez et al., 1997]. There are two possible explanations for the fact that less chloroquine accumulates in resistant trophozoites and food vacuoles isolated from resistant parasites. Either a decreased uptake of chloroquine occurs or there is an enhanced efflux system. A combination of these mechanisms may also be possible.

The food vacuole of the malaria parasite has a pH of 5.2 [Yayon et al., 1984; Krogstad et al., 1985]. In mammalian lysosomes this acidity is due to a membrane H-ATPase which pumps H^+ into the lumen of the organelle [Alberts et al., 1994]. Digestive vacuoles of *P. falciparum* also have a membrane ATPase [Choi and Mego, 1988].

In 1991 Ginsburg and Stein demonstrated that drug resistance 'is compatible with the existence of a weakened proton pump in resistant parasite strains' [Ginsburg and Stein, 1991]. This model was strongly supported by experiments in which the uptake of chloroquine was lowered by bafilomycin A_1 , a specific inhibitor of vacuolar ATPases [Bray et al., 1992].

On the other hand, an enhanced, energy-dependent efflux of chloroquine in resistant strains was reported. [Krogstad et al., 1987]. Verapamil, an agent that reverses multidrug resistance in tumour cells, reverses chloroquine resistance in malaria parasites [Martin et al., 1987].

1.5. ABC Transporters and Multidrug Resistant Proteins

ABC transporters are proteins that have 2 characteristic ATP-binding subunits [Higgins et al., 1990; Hyde et al., 1990; Higgins, 1992]. The protein has 2 large hydrophobic segments anchored in the membrane which act as transport systems or pumps. A wide variety of substrates is transported, including sugars, inorganic ions, hydrophobic drugs, amino acids, peptides and even proteins. Some ABC transporters are uptake systems that accumulate substrate in the cell; others export substrate from the cell [Higgins et al., 1990].

Fig. 1.2. shows a schematic structural organisation of an ABC transporter.

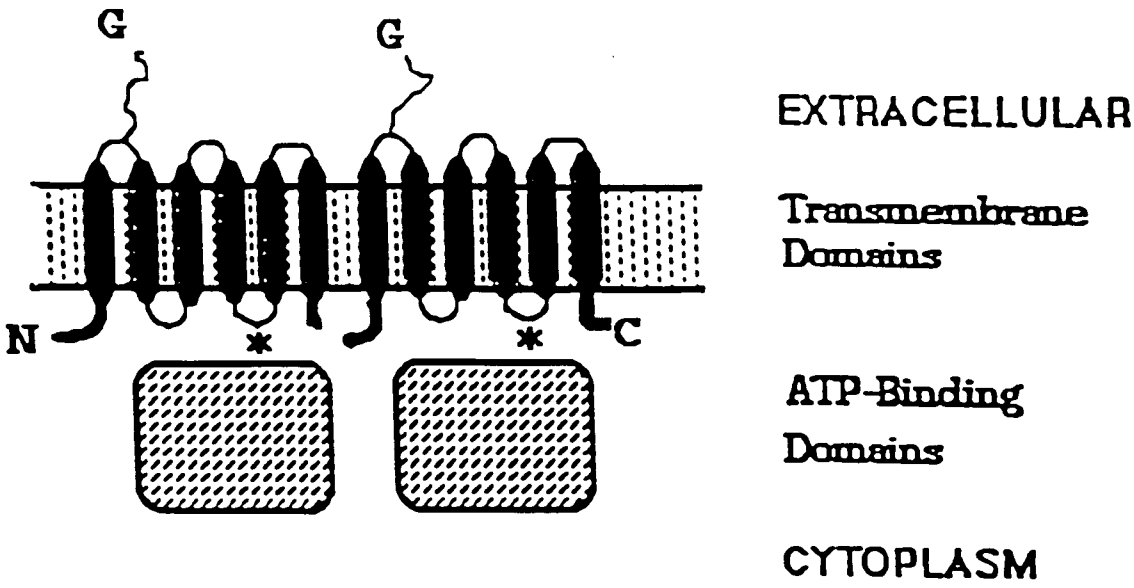


Fig. 1.2. Schematic structural organsation of an ABC transporter.

From Higgins CF (1992), ABC transporter: microorganisms to man. Annu. Rev. Cell. Biol., 8: 67-113.

The typical ABC transporter consists of four membrane-associated subunits. Two are highly hydrophobic subunits, each with six membrane-spanning segments that are thought to form a translocation pathway for substrates. Two are ATP-binding domains that are on the cytoplasmic face of the membrane. Some ABC tansporters are glycosylated at the extracellular face of the membrane. The N- and C-termini face the cytosol.

Most members of the ABC family have been described in prokaryotes but many ABC transporters are also being discovered in eurkaryotes. New members of the ABC family are continually being identified; for example in 1990, 3 ABC transporters were known in yeast. In 1996, when the complete genome of yeast

was sequenced, 16 different ABC transporters had been described [Goffeau A, personal communication, 1997].

The two ATP-binding domains of the ABC transporters are about 200 amino acids long. Each of these highly conserved sequences includes two short motifs: the Walker motifs [Walker et al., 1982]. These motifs are associated with nucleotide-binding properties. Walker motif A is a glycine rich loop and seems to be involved in phosphoryl transfer. In MDR (multidrug resistant proteins), Walker motif B is a leucine-rich region.

Both of the two ATP-binding domains of each ABC transporter are required for function. A mutation in either ATP-binding domain of P-glycoprotein (a subfamily of ABC transporters) dramatically reduces transport activity. It is estimated that two ATP molecules are hydrolysed for each molecule of transported substrate [Mimmack et al., 1989].

It is assumed that ATP binding and hydrolysis induce a conformational change in the ATP-binding domain. This alteration is transmitted to the transmembrane segments that mediate translocation of substrate across the membrane.

An ABC transporter family of eukaryotic cells with great clinical importance is the multidrug resistant (MDR) proteins [Gros et al., 1986; Gros et al., 1988 and Hsu et al., 1989]. In human cancer cells, MDR proteins are responsible for resistance against various therapeutic agents. In cells expressing MDR proteins, cytotoxic drugs are efficiently pumped out and are therefore unable to mediate their therapeutic effect.

P-glycoprotein (P-gp) is a phosphorylated and glycosylated ABC transporter that pumps hydrophilic drugs out of cells in an ATP-dependent fashion. P-gps are encoded by mdr genes. An overexpression of this 170 kd protein leads to multidrug

resistance as it reduces the cytoplasmic concentration of drugs [Gottesman and Pastan, 1988; Endicott, 1989].

P-glycoprotein shows a broad specificity. It transports a wide range of chemically dissimilar drugs. A mechanism to explain transport was proposed by Higgins and Gottesman in 1992. The substrate recognition is a two-step process. First the drug is intercalated in the lipid bilayer where it can be recognised by the transporter, which pumps it out of the bilayer [Higgins and Gottesman, 1992]. In view of this, it is easily seen that the number and the density of expressed pgp transporters could correlate with the degree of resistance of the cell.

P-glycoprotein can be labeled with lipophilic agents such as forskolin [Morris et al., 1991]. MDR drugs compete with forskolin for labeling. This implies that the substrate binding-site is accessible from the lipid phase i.e. the transmembrane segment.

1.6. Pfmdr and Pfef3-rl Genes

Similarities between multidrug resistance in cancer and chloroquine resistance in *P. falciparum* suggest that homologous proteins maybe involved. Three genes with homology to ABC transporters have been identified in *P. falciparum*, namely pfmdr1, pfmdr2 and pfef3-rl. [Foote et al., 1989; Zalis et al., 1993 and Rubio and Cowman, 1996].

The pfmdr1 gene encodes a 162 kd protein which has been termed p-glycoprotein homologue 1. Similar to the p-glycoproteins, Pgh1 consists of 2 ATP-binding cassettes and 12 transmembrane segments. It has been shown that Pgh1 is localized on the surface of digestive vacuoles of *P. falciparum* [Cowman et al., 1991]. There is evidence that Pgh1 is also expressed on the cell surface of *P.falciparum* [Cowman et al., 1991; Saliba, 1997].

The corresponding protein of the pfmdr2 gene has 10 predicted transmembrane segments: four at the N-terminus and 6 at the C-terminus. The two halves of the protein are linked by a small hydrophilic linker. There is probably only one nucleotide-binding site which is situated at the carboxy terminus. [Zalis et al., 1993]. The pfmdr2 protein has not been linked to chloroquine resistance.

The protein product of the pfef3-rl gene has two putative nuleotide-binding sites. It is a hydrophylic protein and contains no transmembrane segments. At present there is also no evidence that this protein is involved in chloroquine resistance [Rubio and Cowman, 1996].

1.7. Pgh1

Cowman et al. [1991] raised antibodies against an N-terminal peptide, a 164 amino acid long fragment that included the N-terminal ATP-binding site of Pgh1 and against a 168 amino acid long C-terminal fragment of Pgh1 [Cowman et al., 1991; Karcz et al., 1993]. Antibodies directed against the C-terminus identified the pfmdr1 protein product in trophozoites of 8 different *P. falciparum* strains in immunoblotting. Three strains (HB3, 3D7 and FC27) were chloroquine-sensitive; the others (7G8, K1, V1, CSL2 and FAC8) were resistant strains. Pgh1 was also identified on food vacuoles by Western blotting as well as by immuno-electron microscopy [Cowman et al., 1991 and Karcz et al., 1993]. As Pgh1 is expressed in chloroquine-sensitive and resistant strains it has been implied that an overexpression of Pgh1 is not essential for chloroquine resistance [Cowman et al., 1991]. The fact that in the malaria parasite the ATP-binding sites of Pgh1 face the cytosol leads to the conclusion that this protein transports substrates either out of the cell or into the vacuole [Karcz et al., 1993].

1.8. Mutations on the Pfmdr1-Gene and Chloroquine Resistance

Wellems et al. [1990] performed a genetic cross on a chloroquine-sensitive (HB3) and a resistant (Dd2) clone. 8 recombinants were chloroquine-sensitive, similar to the parental clone HB3. 8 other progeny of these clones were chloroquine-resistant, identical to the Dd2 clone. Hybridization experiments with DNA fragments of these 16 clones and the 2 parental clones with a pfmdr1 probe showed that the phenotype of these clones could not be predicted by the hybridization pattern. This implied that chloroquine resistance would not be linked to mutations in the pfmdr1 gene. On the other hand Foote et al. [1990] proposed that 2 different mutations on pfmdr1, the K1 allele type and the 7G8 allele type would be linked to chloroquine resistance. These mutations generated 2 new restriction sites for NspI and EcoRV [Frean et al., 1992]. Yet digestion of PCR amplified pfmdr1 DNA from 17 different strains with these enzymes did not show a correlation between the genotype and the chloroquine susceptibility [Haruki et al., 1994]. This was again evidence that chloroquine resistance is not regulated by mutations in the pfmdr1 gene.

In order to analyze the pfmdr1 gene and its protein product Volkman et al. [1995] developed a system in yeast where they complemented the mutated ste6 gene with pfmdr1. Ste6 is also a member of the ABC transporter family and the protein product exports the a-factor mating pheromone in yeast. The pfmdr1 product was expressed in yeast and it could be shown that yeast cells with Pgh1 were able to transport the mating pheromone a-factor. In this system Pgh1 clearly acted as a transporter. If the mutated ste6 gene was complemented with the above mentioned 7G8 mutant variant of Pgh1 ability to transport the a-factor mating pheromone as well as the mating frequency was reduced.

1.9. Aim of the Study

The transport protein Pgh1 is expressed on membranes of trophozoites and food vacuoles from chloroquine-sensitive and resistant strains. Although it is suggested that there is no difference in the expression of Pgh1 on trophozoites from strains with different chloroquine susceptibility the level of expression on the vacuole, the putative place of action of chloroquine, was not quantified in these experiments [Cowman et al., 1991].

In this project the level of Pgh1 expression on functional food vacuoles is examined. In order to achieve this, antibodies to Pgh1 will be raised. These antibodies will be used to identify Pgh1 in extracts of pure, intact and functional food vacuoles isolated from different strains of *P. falciparum*. The exact level of Pgh1 expression at the vacuolar level will be compared with the chloroquine susceptibility of these strains.

Whether Pgh1 phosphorylation can be linked to chloroquine resistance will also be tested.

2. Materials and Methods

2.1. Parasite Cultures

Plasmodium falciparum parasites were cultured as described [Trager and Jensen, 1976] using washed 0^+ erythrocytes at a haematocrit of 5% and A^+ serum (final concentration 10%) obtained from the Western Province Blood Bank, South Africa. Parasites were grown in RPMI 1640 with glutamine (Biowhittaker), supplemented with hypoxanthine (44mg/L, Sigma), Hepes-buffer (6g/L, Sigma), glucose (4g/L, Sigma) and $NaHCO_3$(2.1g/L, Sigma). As antibiotic, gentamycin (50mg/L, Pharmacare Limited, RSA) was used.

Cultures were maintained at 37^0C in sterile Petri dishes in desiccator cabinets or in sealed flasks under an atmosphere of 93% N_2, 4% CO_2 and 3% O_2. Synchrony was maintained by Sorbitol treatment [Lambers and Vanderberg, 1979].

The chloroquine-sensitve clone D10 was derived from FCQ27, an isolate from Papua New Guinea [Ekong et al., 1993]. RSA3 is a culture adapted isolate from KwaZulu, South Africa [Freese et al., 1991]. 3D7, a clone from the NF54 strain which was isolated from an airport worker in Amsterdam, was donated by C. Weiss, London School of Hygiene and Tropical Medicine.

The chloroquine-resistant strains from South Africa, RSA11 and RSA15 are culture adapted isolates from KwaZulu and Transvaal, respectively [Freese et al., 1991]. FAC8 is a clone from ITG2FG which is a Brazilian cloned cell-line [Biggs et al., 1989]. The FCR3 strain originates from Gambia [Nguyen-Dinh and Trager, 1978] and the K1 strain is from Thailand [Thaithong and Beale, 1981]. W2 is a cloned parasite line which was derived from the Indochina III strain [Oduola et al., 1988].

2. 2. Determination of the IC_{50} Values for Chloroquine

The viability of parasites under chloroquine pressure was determined by measuring the activity of the parasite lactate dehydrogenase (pLDH), [Makler and Hinrichs, 1993]. The pLDH which converts lactate to pyruvate uses the coenzyme 3-acetyl pyridine adenine dinucleotide (APAD). In this reaction APAD is reduced to APADH. Reduced APAD can in turn reduce nitro blue tetrazolium (NBT). The formation of this purple dye can be monitored in a photospectrometer. The LDH from the red blood cells does not interfere with this enzymatic assay as it converts APAD to APADH at a negligible rate [Makler and Hinrichs, 1993].

Parasitised red blood cells (pRBC), synchronized in the trophozoite stage with a parasitemia of 2 % and a haematocrit of 2% were incubated with complete culture medium at different chloroquine concentrations. The concentrations ranged from 2μM – 3.9nM or from 1μM – 1.95nM , respectively. RBC were used as a blank and as a positive control the pLDH activity of pRBC cultured in the absence of chloroquine was measured. All chloroquine dose response experiments were done in triplicate. The incubation was performed in microtiter plates in sealed desiccator cabinets (as described under 2.1.) for 48 hours. After this incubation 10μl of the resuspended culture was mixed with 100μl of the Malstat™ reagent and 25μl of the 1.96mM nitroblue tetrazolium (NBT)/0.24M phenazine ethosulfate (PES) solution. The formation of a purple colour indicated the parasite survival and was read at 620nm using a 7520 Microplate Reader, Cambridge Technology.

Chloroquine IC_{50} values were calculated using the GraphPad Prism Software.

2. 3. Trophozoite Isolation

Erythrocytes infected with parasites at the trophozoite stage were washed twice in 10 volumes Phosphate Buffered Saline (PBS). The pellet was resuspended in PBS containing 0.05% (w/v) Saponin (Sigma). After an incubation of 2-3 min the suspension was centrifuged at 1500 g for 10 min. The pelleted trophozoites were washed 3 times with PBS.

2.4. Food Vacuole Isolation [Saliba et al., 1998]

Samples of parasitized red blood cells (pRBC), synchronized in the trophzoite stage with a parasitemia of 7 – 15 % were washed with cold PBS by centrifugation. The pelleted pRBCs were resuspended in PBS containing 0.05% (w/v) Saponin (Sigma). After 2-3 min incubation the sample was centrifuged at 1500 g for 10 min. The supernatant was discharged and the trophozoites were washed twice with ice cold PBS. To the trophozoites ice cold H_2O, pH 4.5 was added. This suspension was triturated four times through a 27-G 1.2cm needle and then centrifuged at 13000 rpm in a microfuge for 2 min. The pellet was resuspended in 300µl uptake buffer and triturated 2 times as above. 1.2 ml of Percoll solution was added and the samples were then centrifuged at 13000 rpm at 4^0C for 10 min. The pellet of the gradient contained the food vacuoles. They were twice washed with ice cold PBS in order to remove the Percoll.

Uptake buffer:
- 2mM $MgSO_4$
- 100mM KCl
- 10mM NaCl
- 25mM Hepes
- 25mM $NaHCO_3$, pH 7.4

Percoll solution: 42% Percoll (Sigma)
250mM Sucrose (Sigma)
2.5mM $MgCl_2$
pH 7.4

2.5. Spectrophotometric Method to Determine Vacuole Number

Serial diluted vacuole suspensions of a volume of 1 ml were made. The number of vacuoles in a volume of 0.1µl of these suspensions was counted in a Bright-Line haemocytometer. The OD_{662nm} of these dilutions was measured in a spectrophotometer (Shimadzu UV 160 I PC, dual bean). With these multiple values a standard curve plotting the counts in the haemocytometer versus the OD_{662nm} of the same dilutions was set up.

2.6. SDS-Polyacrylamide Gel Electrophoresis with *P. falciparum* Trophozoites and *P. falciparum* Food Vacuoles

Trophozoites were solubilized either in Urea sample buffer or in Glycerol sample buffer. After solubilization the sample was centrifuged at 13000 rpm for 10 min. The supernatant was transferred to a new tube, quick frozen in liquid nitrogen, thawed and loaded.

Food vacuoles were solubilized in Urea buffer. Immediately after adding the buffer the sample was centrifuged at 13000 for 10 min. The supernatant was transferred to a new tube and loaded.

As protein markers either a molecular weight standard, high range (BioRad, 161-030) or a prestained protein marker, broad range (New England Biolabs, 7707S) were used.

Electrophoresis was performed at 100, 150 or 200 V. Gels were used for Coomassie staining, Western blotting or autoradiography.

Separation gel:

Acrylamide concentration	10%	12.5%	15%
30% Acrylamide	10ml	12.5ml	15ml
1% Bisacrylamide	3.9ml	3.1ml	2.6ml
1.5M Tris-HCl pH 8.7	7.5ml	7.5ml	7.5ml
10% SDS	0.3ml	0.3ml	0.3ml
distilled H_2O	8.2ml	6.5ml	4.5ml
10% Am. persulfate	0.1ml	0.1ml	0.1ml
Temed	20µl	20µl	20µl

Stacking gel:

30% Acrylamide	1.67ml
1% Bisacrylamide	1.3ml
1M Tris-HCl pH 6.8	1.25ml
10% SDS	0.1ml
distilled H_2O	5.6ml
10% Am. persulfate	50µl
Temed	10µl

Urea sample buffer:

1% (w/v) SDS
8M Urea
1% (v/v) β-mercaptoethanol
0.1% (w/v) Bromphenol-blue
80mM Tris-HCl pH 6.8

4x Glycerol sample buffer:

68% (w/v) SDS
40% (v/v) Glycerol
0.7% (v/v) β-mercaptoethanol
0.1% (w/v) Bromphenol-blue
80mM Tris-HCl pH 6.8

1x Gel-running buffer:

Glycine	14.4g
Tris base	3.0g
SDS	1.0g
Distilled H_2O	to 1000ml
pH 8.3	

2.7. Coomassie Staining of SDS-Polyacrylamide Gels

Gels were stained in 10% (v/v) acetic acid, 25% (v/v) methanol and 0.5% (w/v) Coomassie Brilliant Blue R-250 for 1 hour. Destaining solution: 10%(v/v) acetic acid, 25% (v/v) methanol.

2. 8. Phosphorylation of Food Vacuole Proteins

Food vacuoles were obtained as described under 2.4. For the phosphorylation $10*10^6$ and $40*10^6$ food vacuoles were used. Experiments were carried out at 30^0C in 10mM Tris-HCl (pH 7.0)/0.25M Sucrose/5mM $MgCl_2$ (Phosphorylation buffer). Either no nucleotide, ATP, ADP or GTP at a final concentration of 50μM was added. The phosphorylation was started by addition of 100μCi [γ-^{32}P]-ATP of a specific activity of 3000Ci/mmol (Amersham). After 10 min the reaction was stopped by pelleting the food vacuoles in a microfuge. Food vacuoles were then washed 3 times in ice cold phosphorylation buffer. The food vacuole proteins were solubilized (as described under 2.6.) and loaded on a 10% SDS-polyacrylamide gel. After the run the gel was stained with Coomassie and dried in a BioRad gel-dryer. The dried gel was exposed to a Kodak BioMax MS Film in a cassette with intensifying screen at -70^0C for 72 hours.

2.9. Preparation of Competent *E. Coli* Cells

Five ml of L-Broth medium was inoculated with either 50 µl of bacterial strains XL-1 blue, JM 109 or JM 101 from glycerol stocks and grown overnight at 37°C with gentle agitation. One ml of the overnight cultures was used to inoculate 300 ml L-Broth medium in a 1 l flask. The bacteria were grown at 37°C till an $OD_{650\ nm}$ between 0.2 – 0.4 was reached. The cells were pelleted at 5000 rpm in a JA-10 Beckman rotor for 10 min at 4°C and resuspended in 40 ml of ice cold 60mM $CaCl_2$, 10mM Pipes (pH 7.2) by gentle pipetting. The suspension was left on ice for 20 min, transferred to Beckman JA-20 rotor tubes and centrifuged at 5000 rpm for 5 min. The pelleted cells were resuspended in 4 ml of 60mM $CaCl_2$, 10mM Pipes (pH 7.2), 15% glycerol and frozen in 200µl aliquots in liquid nitrogen and stored at -70°C.

L-Broth Medium:
- 5g Tryptone
- 2.5g Yeast Extract
- 5g NaCl
- In 500ml H_2O.
- Sterilized by autoclaving.

2.10. Transformation of *E.coli* with Plasmid DNA

Plasmids, (pGEX-3'-pfmdr1) and (pGEX-5'-pfmdr1) [Cowman et al., 1991 and Kracz et al., 1993], were a gift from Dr A. F. Cowman. pGEX-3'-pfmdr1 and pGEX-5'-pfmdr1, contained 3'-sequences (nucleotides 4252 to 4758; Genebank accession number M29154) and 5'-sequences (nucleotides 2024 to 2339) of the *P. falciparum* multidrug resistance (MDR) gene, which encodes for the P-glycoprotein

homologue 1 (Pgh1), cloned into the pGEX-3X vector (Pharmacia), respectively. Plasmid pGEX-3'-pfmdr1 encodes for a GST fusion protein containing amino acids 1252 to 1419 of Pgh1, while the fusion protein encoded by pGEX-3X-5'-pfmdr1 containes amino acids 510 to 614 of the Pgh1 N-terminal ATP-binding site.

Competent cells were thawed on ice. An aliquot of 100µl of thawed cells was transferred into a microfuge tube. Each plasmid was dissolved in 100µl TE-buffer, of which 10µl were added to 100µl aliquots of thawed competent cells. The DNA and the cells were mixed gently, incubated on ice for 60 min, and heat shocked at 42°C for 2 min. After the heat-shock 1ml of prewarmed L-Broth medium was added to each tube and incubated at 37°C for 1 hour. Ten, 20 and 50 µl of these cells were plated on L-Broth agar plates containing 0.5 mg/ml Ampicillin and incubated overnight at 37°C.

TE-buffer:	1mM EDTA, 10mM Tris-HCl pH 8.0
L-Broth Agar plates:	5g Tryptone, 2.5g yeast extract, 5g NaCl and 7.5g Bacto agar were resolved in 500ml distilled H_2O and autoclaved. Once the solution had cooled down, 5ml of Ampicillin solution (50mg/ml) was added and aliquots were poured in sterile Petri dishes. The L-Broth agar plates were sealed with Parafilm and stored at 4°C.

2.11. Preparation of Plasmid DNA

Four bacterial colonies were picked from each plate, with a sterile yellow Gilson tip. Five ml of L-Broth medium (containing Ampicillin to a final concentration of 0.1

mg/ml) was inoculated with the colonies. The cells were grown overnight at 37^{0}C. A volume of 0.5ml of the overnight cultures was mixed with 0.5ml sterile glycerol to produce glycerol stocks. The glycerol stocks were frozen in liquid nitrogen and stored at -70^{0}C. The rest of the overnight culture was centrifuged for 10min at 2000 rpm. The supernatant was discharged and the bacterial pellet was resuspended in 200µl of solution 1. The suspension was left at RT for 5min and was then transferred to a microfuge tube. Two µl of RNAse I (10mg/ml, Boehringer Mannheim) were added to each tube. The bacteria were lysed on ice with the addition of 400µl fresh solution 2. After an incubation of 5 min and occasional gentle mixing 300µl of solution 3 was added in order to precipitate genomic DNA and proteins. The tubes were kept on ice for 10 min and then centrifuged in a microfuge at 4^{0}C for 5 min. The supernatant with the plasmid DNA was pipetted into a new microfuge tube. This centrifugation was repeated in order to remove all genomic DNA and the supernatant was transferred to another microfuge tube. A volume of 600µl of isopropanol was added to each tube and they were incubated at –20^{0}C for 3 min. This was followed by a centrifugation for 5 min at RT. The plasmid DNA was washed twice with 70% EtOH and was then dissolved in 80µl TE-buffer.

Solution 1: 20mM Tris-HCl pH 8.0
10mM EDTA
50mM Glucose
H_2O to 200 ml

Solution 2: 0.2M NaOH
1% SDS
in H_2O

Solution 3: 3M Potassium acetate
pH 4.8

2.12. Enzymatic Digestion with Pst I, Hind III and Hinc II

Ten microliters of the plasmid DNA samples were pipetted into microfuge tubes. To these 2μl of buffer H (Boehringer Mannheim), 1μl of the corresponding restriction enzyme (either Pst I, Hind III or Hinc II) and 7 μl of distilled H_2O were added and mixed. The samples were digested at 37^0C; the digestion was stopped by adding 2μl of stop buffer after two hours. Ten microliters of the digested DNA samples were loaded together with 3μl of the uncut plasmid and 5 μl of a DNA marker. The electrophoresis was done with constant current of 40 mA for 50 min.

Stop-buffer: 10 mM EDTA (pH 7.5)
0.25 % Bromphenol Blue
0.25% Xylene Cyanole
30% Glycerol

DNA-marker: As marker λ phage DNA (48502 bp), completely digested by Hind III/EcoR I was used. (New England BioLab Catalogue 1926/87.)

bp:	21226
	5148
	4973
	4277
	3530
	2027
	1904
	1584
	1330
	983
	831
	564
	125

Restriction enzymes: Pst I, Hind III and Hinc II were from Boehringer Mannheim and were used at a concentration of 2 units/μg DNA.

Buffer H: SuRE/Cut Buffer H, Boehringer Mannheim.

2.13. Sequencing of the 2 Pfmdr1 Gene Fragments

Double-stranded plasmids were sequenced using the dideoxy chain-terminating method [Sanger et al., 1977] and the Cys™ Thermo Sequenase Dye Terminator Kit (Amersham Pharmacia Biotech) with Thermo Sequenase™ DNA polymerase (Amersham Pharmacia).

All reactions were performed according to the manufacturer's instructions and the plasmids were amplified on a GeneAmp PCR System 9700, (Perkin Elmer, Applied Biosystems).

A pGEX-3X forward Primer: (23 mer) 4 pmol/ul: 5'- GGG CTG GCA AGC CAC GTT TGG TG 3' and a reverse Primer: (23 mer) 4 pmol/ul: 5'- CGG GGA GCT GCA TGT GTC AGA GG- 3' were used as sequencing primers. The sequencing was done on an ALFexpress DNA Automated Sequencer (Amersham Pharmacia Biotech). A 5% polyacrylamide gel was used for the electrophoresis. The run was performed according to the manufacturer's operating procedure at 55 ^{0}C for 13 hours, using a standard gel cassette, 0.5 mm spacers and was controlled by ALFwin version 1.1 software (Amersham Pharmacia Biotech). The same software was used for the data processing.

2.14. Expression of the Pgh1-GST Fusion Proteins

One hundred ml of L-Broth medium containing 5 mg of Ampicillin was inoculated with 75 µl of the *E.coli* glycerol stocks containing either plasmid pGEX-3'-pfmdr1 or pGEX-5'-pfmdr1. This culture was incubated at 37^{0}C with gentle agitation (200 rpm) overnight. The overnight culture was added to 900 ml L-Broth medium, supplemented with Ampicillin and incubated as above. After an hour, the expression of the Pgh1-GST fusion proteins were induced by adding Isopropyl β-D-thiogalactopyranoside (IPTG, Promega) to a final concentration of 0.1mM. The incubation was continued for an additional 4-7 hours. The cells were then pelleted at 5000 rpm at 4^{0}C for 10 min. The supernatant was discharged and the pellet was quick frozen in liquid nitrogen. The centrifuge tubes with the pellets were stored at –70^{0}C.

2.15. Purification of the Native GST-Fusion Proteins

The cell pellets were thawed on ice, resuspended in 10 ml of PBS containing 1mM PMSF (Phenylmethylsulfonyl fluoride, Sigma) and Soybean Trypsin inhibitor (Sigma) at a final concentration of 20 μg/ml, by repeated pipetting and transferred to a 14-ml polypropylene tube. The cell suspensions were sonicated 4 times on ice for 20 sec (Power level 5, 50% duty cycle, output control 4) in an ultrasonic processor (Heat Systems Ultrasonics Inc., Model W-385). After sonication Triton X-100 was added to a final concentration of 1% and the lysate was incubated on ice with occasional mixing for 15 min. The lysate was poured into centrifugation tubes and was clarified at 10000 rpm for 10 min in a SS-34 rotor. The supernatant was pipetted to a new 14-ml tube and 1.5 ml of washed Glutathione-Agarose beads (Sigma) were added. This mixture was incubated at RT for 20 min on a rotating platform. The beads were collected by brief centrifugation and the supernatant was removed. The Glutathione-Agarose beads were then washed 5 times with ice cold PBS and resuspended in 1.5 ml elution buffer, 50mM Tris-HCl (pH 8.0) containing 5mM reduced Glutathione (Sigma). The beads were then incubated for 3 min on the rotating platform and pelleted. The supernatant was kept on ice. This elution was repeated and the supernatants were pooled. The purified fusion proteins were freeze dried and stored at -20^{0}C.

In order to quantify and analyze the eluted proteins, protein-determination assays and SDS-PAGE were performed.

2.16. Preparative Electrophoresis of the C-terminal-GST Fusion Protein

The pellet of 1 l culture of *E. coli* expressing the Pgh1-C-terminal-GST fusion protein was incubated with DNAse I (Boehringer Mannheim) at a concentration of 100µg/ml at 37°C for 1 hour. The DNAse I was removed by centrifugation and the pellet was dissolved in 500µl SDS-sample buffer. This sample was boiled and aliquots of 50µl were loaded on two large preparative 10% SDS-polyacrylamide gels.

2.17. Copper Chloride Staining of SDS-Polyacrylamide Gels

After the electrophoresis the gel was washed 3 times in distilled H_2O. The washed gel was then incubated in 0.3M $CuCl_2$ solution for 10 min. The protein bands were viewed against a dark background. The relevant band, for the C-terminal-GST fusion protein, a band at 44kD, was excised using a razor blade. These gel pieces were destained by incubation in 0.25M EDTA in 0.25M Tris-HCl (pH 9.0) with three changes of 10 min each.

2.18. Electro-Elution of the Denatured C-terminal-GST Fusion Protein from Gel Fragments

Elution of the C-terminal-GST fusion proteins was performed using the BioRad Electro-Eluter Model 422. Briefly, the destained gel pieces were equilibrated in elution buffer and cut into very small pieces with a razor blade. These gel fragments were then put in the elution-chambers of the assembled Electro-Eluter. Elution was done at 10mA/elution-chamber for 4 hours. The eluted and solubilized proteins were then carefully harvested with a plastic pipette and transferred into microfuge

tubes. In order to remove the SDS first the samples were dialyzed at RT against two L PBS overnight. Afterwards the samples were dialyzed against distilled H_20 at 4^0C with three changes of 12 hours each. From this dialyzed sample a protein determination was done. The proteins were freeze-dried and stored at -20^0C.

Protein elution buffer:	0.25mM Tris-HCl 192 mM Glycine 0.1% SDS pH 7.4
Dialysis tubing:	Cellulose Membrane (Sigma D-9777), exclusion size: 12000 Da

2.19. Preparation of Antibodies against a N-terminal Pgh1 Peptide

A peptide corresponding to the N-terminal amino acids 2-18 of Pgh1 was synthesized at the Eidgenössische Technische Hochschule (ETH) Zürich, Switzerland. To this peptide GKEQKEKKDGNLSIKEEV**C** a cysteine residue (bold, underlined) was introduced at the C-terminus. In order to induce antibodies against this short peptide it was necessary to couple the peptide to a carrier protein.

2.19.1. Activation of Ovalbumin with MBS (Maleimidobenzyl-N-hydroxysuccinimide Ester)

Twelve milligrams of pure ovalbumin (Sigma A-3686) were dissolved in 600µl PBS; 3.3mg of the crosslinker MBS were dissolved in 330µl dimethylformamide (DMF). A volume of 200µl of the MBS solution was added to the dissolved ovalbumin.

The activation was done at RT for 2 hours with gentle shaking. Afterwards the unbound maleimide was removed by dialysis against PBS at 4^{0}C overnight.

2.19.2. Coupling of the Pgh1 Peptide to the Activated Ovalbumin

An amount of 1.7 mg of peptide was dissolved in 20µl distilled water; and 600µl of the dialyzed ovalbumin-maleimide (3.13µg/µl) were added to the peptide. This solution was shaken gently at 4^{0}C for 20 hours. The crosslinking was analyzed by gel electrophoresis. Portions of 280µg/50µl of this antigen were stored at –70^{0}C and used for the immunizations.

2.20. Immunization of Rabbits

With each of the 3 antigens used in this study, namely, peptide 2-18 of Pgh1 [Pgh1 (2-18)], the N-terminal-ATP-binding site-GST fusion protein (ATP-GST) and the C-terminal-GST fusion protein (C-terminal-GST), a separate pair of rabbits was immunized. Before immunization 5 ml of blood was taken from each rabbit. The blood was incubated at 37^{0}C for an hour. After coagulation of the blood the serum was decanted. Red blood cells were removed by centrifugation. Preimmune serum was stored at –70^{0}C.

1st Injection:

An amount of 280 µg of Pgh1 (2-18) was dissolved in 290µl 0.85 % NaCl solution and 250µl of complete Freund's Adjuvant (CFA, Sigma) was added. This suspension was mixed vigorously and was then injected subcutanously at 4 different sites of New Zealand White rabbits. The freeze dried ATP-GST and C-terminal-GST fusion proteins were dissolved in H_2O to a concentration of 1.2µg/µl. A

volume of 200µl of these solutions was mixed with 1 ml of CFA, giving a final volume of 1.2 ml. This solution was divided into 4 aliquots, each of which was injected into the rabbit at different sites on the body.

1st and 2nd Boosts:

Subsequent boosts were done 4 and 8 weeks after the first injection. The same amount of antigen as in the first injection was given. As a solvent incomplete Freund's Adjuvant was used.

Bleeds:

Between 8 –12 days after the 1st boost each rabbit was bled from an ear vein. Five milliliters of blood was taken and incubated at 37°C. After coagulation of the blood the serum was decanted and stored at –70°C. The 2nd test bleeds were taken between day 7 and 12 after the 2nd boost and the terminal bleeds were performed between day 14 and 19 after the 3rd injection. All immune sera were stored at -70°C.

2.21. Dot blots

Anti Pgh1 (2-18) immune sera were first tested in dot blot experiments. Ovalbumin, activated ovalbumin and the Pgh1 (2-18) peptide were dissolved in PBS. 0.14µg (in 2µl) of ovalbumin, 0.17µg (in 2µl) of activated ovalbumin and 3µg (in 1.7µl) of the Pgh1 (2-18) peptide were pipetted onto Nitrocellulose membrane (Micro Separations, Inc.). The dried membrane was blocked in 5% milkpowder, 0.1% Tween-20 in PBS (MP/T/P) for two hours. The anti Pgh1 (2-18) immune serum was diluted 1:2000 with MP/T/P and incubated with the blocked membrane for two hours on a Belly Dancer® (Stovall Life Science Inc.). As negative control preimmune serum at a dilution of 1:2000 was used. After the incubation with the first antibodies

the membranes were washed three times for 10 min with MP/T/P. The membranes were afterwards incubated with a 2nd antibody (an anti-rabbit Ab coupled to HRP, Sigma A-0545) for 1 hour at a dilution of 1:4000. The membranes were then washed as follows:

1 times 15 min with MP/T/P

3 times 5 min with PBS/0.1% Tween-20

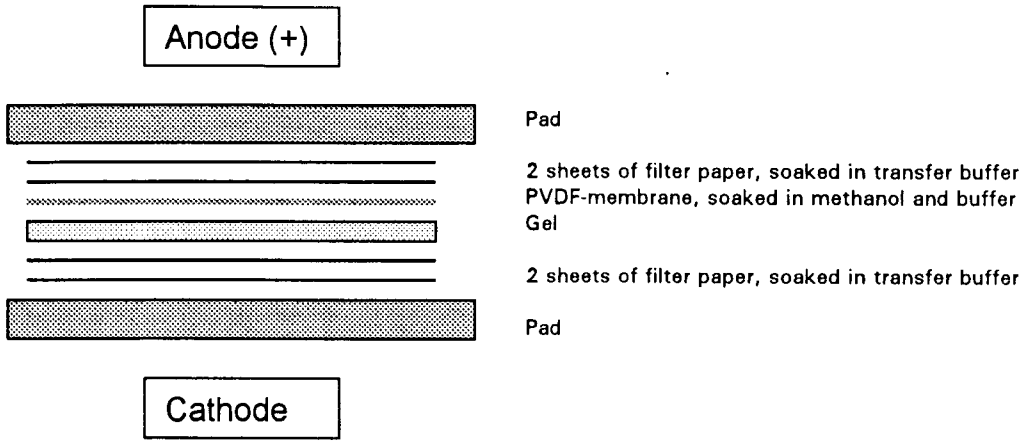
1 times 5 min with PBS.

Visualization of the 2nd antibody was done with the Pierce SuperSignal® CL-HRP chemiluminescent substrate. The membranes were exposed to a Dupont x-ray films.

2.22. Western blotting

PVDF-Plus membrane (Micro Separations Inc.) and filterpapers were sized and cut to fit the SDS-polyacrylamide gel. The filter papers were soaked in transfer buffer. The membrane was placed in 100% methanol and was then equilibrated with transfer buffer.

After gel electrophoresis the blotting cassette was assembled as below:



The transfer was done in the cold room (4^0C) overnight at 55 Volts.

Transfer buffer:

100 ml 10x Tris/Glycin solution (2M Glycine, 250mM Tris-HCl pH 8.2)
700 ml distilled H_2O
200 ml methanol
1 ml 10% (w/v) SDS

The efficiency of the transfer was checked by staining the PVDF-membrane with 0.2% (w/v) Ponceau S dye for 1 min. The membrane was rinsed briefly with distilled H_2O to remove excess stain. The membrane was photocopied and then destained with more distilled water. The membrane was blocked with MP/T/P as in the dot blot experiments. Anti Pgh1 (2-18) antisera were used at a dilution of 1:100 to 1:150. Anti ATP-GST and anti C-terminal-GST immunsera were used at dilutions of 1:750 – 1:1000. Incubations with the 1^{st} and 2^{nd} antibodies, wash cycles and visualization of the 2^{nd} antibody were performed as in dot blot experiments.

Western blot results were analysed using the Multi-Analyst PC Software from BioRad.

2.23. Image Analysis

A digitized image of the Western blots was acquired using the JASC Paint Shop Pro® software version 5.01. and a Hewlett Packard Scan Jet 5 P scanner. These data were saved as TIFF (Tag Image File Format) files and subsequently exported into the Bio-Rad Multi-Analyst® PC software.

The area of each of the Pgh1 bands was recorded as squares and ellipses. The intensity of the bands was measured as optical density (OD) at 400nm – 750nm. The mean of the products $area_{square} * OD_{square}$ and $area_{ellipse} * OD_{ellipse}$ were equated with the Pgh1 expression (formula 2.1.).

Formula 2.1. Pgh1 expression = $$\frac{area_{square} * OD_{square} + area_{ellipse} * OD_{ellipse}}{2}$$

Three separate Western blot experiments with the anti- ATP-GST and the anti-C-terminal-GST antisera were performed. The mean of each of these 3 data for the vacuolar Pgh1 expression was then calculated.

3. Results

3.1. Chloroquine Susceptibility of *Plasmodium Falciparum* Strains

In order to establish chloroquine susceptibility of the 4 clones and 4 strains, chloroquine dose-response experiments were performed. Parasites were exposed to different chloroquine concentrations for 2 days. The Malstat assay was used to determine the survival rate for each strain (materials and methods 2.2). The viability in percent was plotted versus the log chloroquine concentration. Curves and IC_{50} values were calculated using the GraphPad Prism software (figure 3. 1.). The chloroquine IC_{50} values were compared with the concentration of 30µg/l, the recommended plasma concentration which is required for treatment of susceptible *P. falciparum* [Wernsdorfer and McGregor, 1988 and Katzung, 1995] (figure 3.2.). D10, RSA3 and 3D7 were found to be chloroquine-sensitive with IC_{50} values of 27.36 (± 6.84) nM, 35.25 (± 14.62) nM and 27.25 (± 1.89) nM respectively. FAC8 [CQ-IC_{50}: 234.30 (± 69.01) nM], RSA11: [CQ-IC_{50}: 315.07 (± 53.12) nM], RSA15 [CQ- IC_{50}: 235.87 (± 42.96) nM], K1: [CQ-IC_{50}: 306.40 (± 44.83) nM] and W2 [CQ-IC_{50}: 398.93 (± 50.46) nM] were resistant. From these results two susceptible strains, namely D10 and 3D7 and three resistant strains FAC8, K1 and RSA11 were chosen for the following experiments. For the phosphorlylation experiments, RSA15 was used.

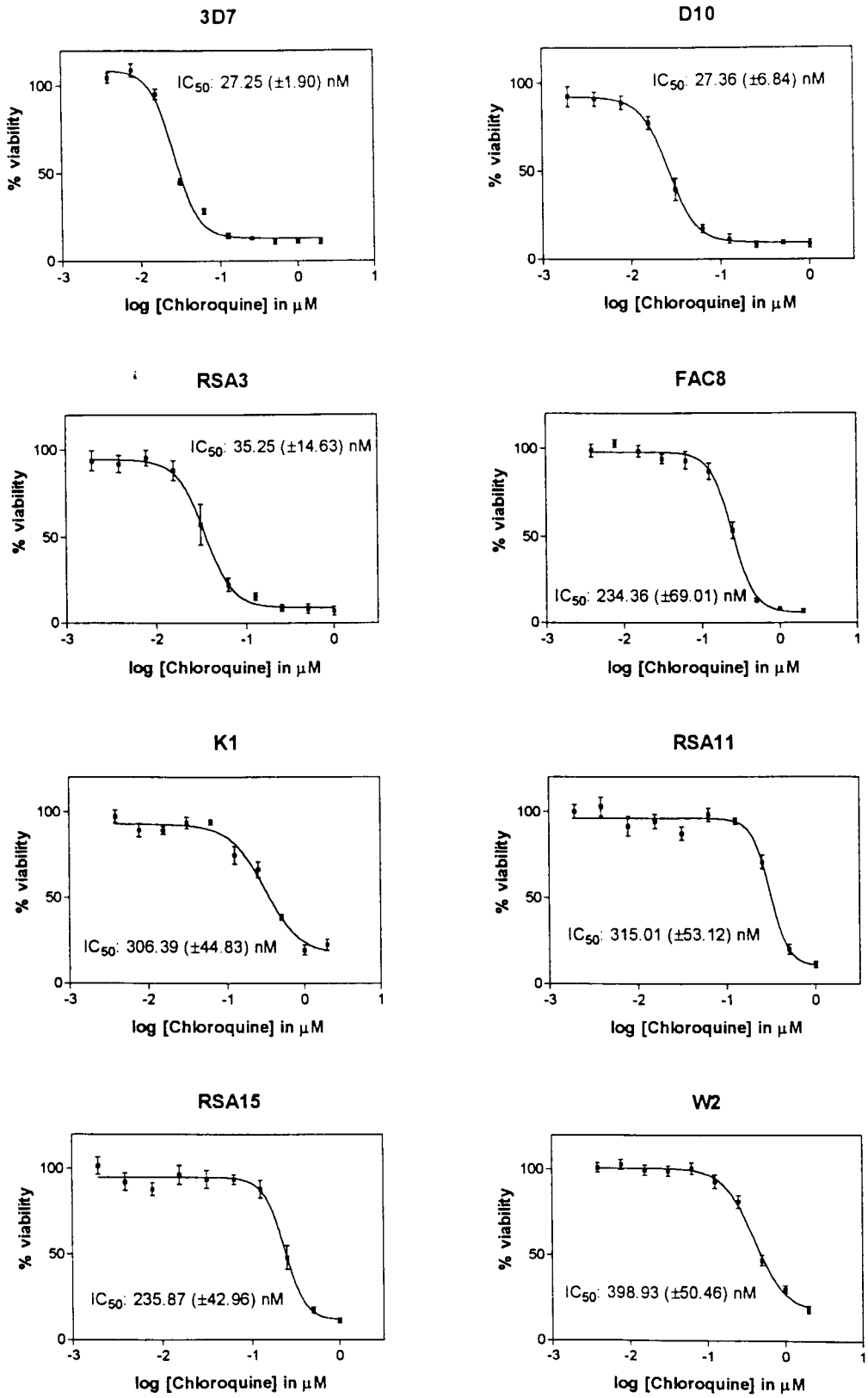


Figure 3.1. Chloroquine dose response curves for the *P. falciparum* clones D10, 3D7, FAC8, W2 and the RSA3, RSA11, RSA15 and K1 strains. The Chloroquine concentration range was 1µM – 1.95 nM for D10, RSA3, RSA11 and RSA15 and 2µM – 3.9 nM for 3D7, FAC8, W2 and K1 respectively. Each point represents the mean of at least 3 separate experiments. Each experiment was done in triplicate.

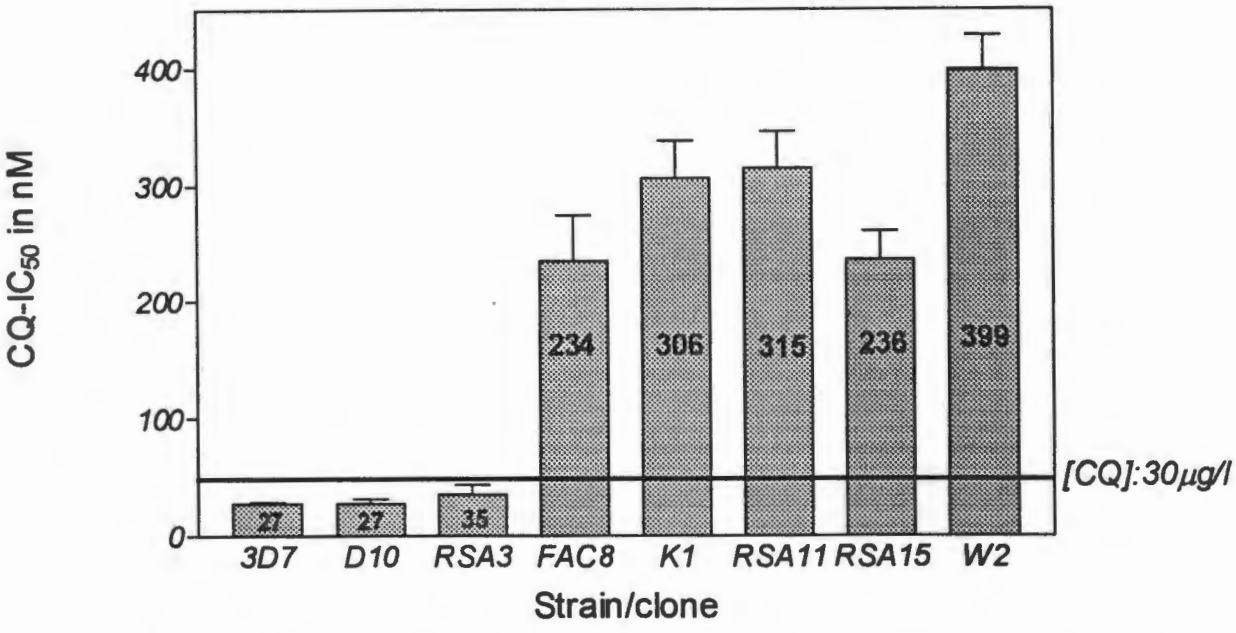


Figure 3.2. This bar chart shows the CQ IC_{50} values of the different strains/clones and compares them with the recommended therapeutic serum concentration of 30µg/l (bold line). The number in the bars indicates chloroquine IC_{50} values in nM.

3.2. Isolation of Food Vacuoles

A novel method of isolation of intact, pure and active food vacuoles of *P.falciparum* has been developed in our laboratory [Saliba et al., 1998]. Food vacuoles isolated from chloroquine-sensitive strains accumulate significantly more chloroquine than vacuoles isolated from chloroquine-resistant strains. Chloroquine uptake by these food vacuoles is Mg^{2+}, temperature and ATP dependent [Saliba et al., 1998].

For this project food vacuoles were prepared according to Saliba et al.. These food vacuoles were intact, active and free of contaminating membranes or organelles [Saliba et al., 1998].

3.3. Phosphorylation of Food Vacuoles

Studies involving protein kinase activators and inhibitors have shown that covalent modification of p-glycoprotein by phosphorylation may modulate its activity as a multidrug transporter [Aftab et al., 1994]. In order to examine if Pgh1 is phosphorylated on intact food vacuoles phosphorylation experiments were performed. Food vacuoles from the RSA15 strain were phosphorylated with [γ-^{32}P]-ATP, as described (materials and methods 2.8.) and analyzed on SDS-polyacrylamide gels. $10*10^6$ or $40*10^6$ food vacuoles were incubated with 100 µCi [γ-^{32}P]-ATP in the presence of 50 µM unlabeled ATP, ADP or GTP. Both ATP and ADP inhibited the labeling but GTP slightly stimulated the overall labeling (figure 3.3.). Under the conditions in our experiments there was no evidence that a protein with the size of Pgh1 was phosphorylated.

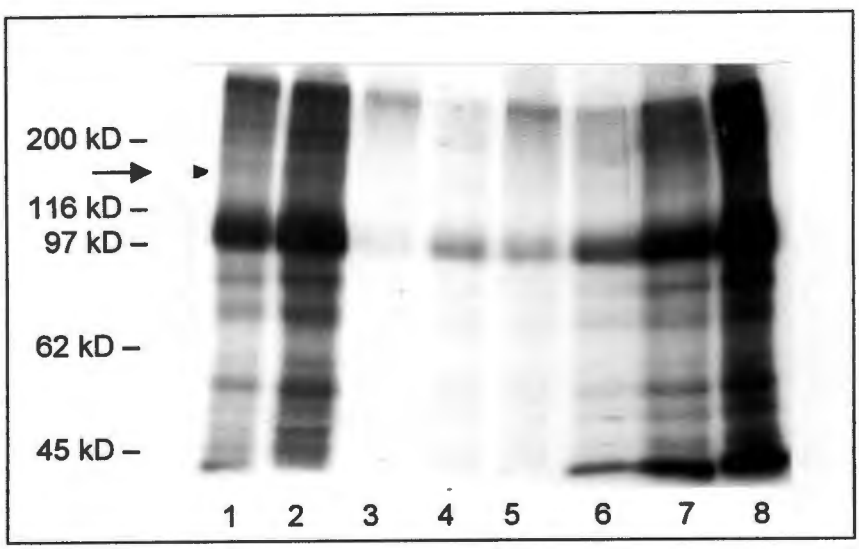


Figure 3.3. Autoradiograph. Phosphorylation of RSA15 food vacuole proteins. Effect of nucleotides on labeling with [γ-^{32}p]-ATP. Food vacuoles were subjected to phosphorylation in the absence of unlabeled nucleotide (lane 1 and 2), 50 µM ATP (lane 3 and 4), 50 µM ADP (lane 5 and 6) and 50 µM GTP (lane 7 and 8). Lanes 1,3,5 and 7 show the labeling of $10*10^6$ food vacuoles. In lane 2,4,6 and 8 proteins of $40*10^6$ food vacuoles were labeled. Molecular weight is indicated on the left. The arrow indicates the size of Pgh1.

3.4. Coupling of an N-terminal 18 Amino Acid Peptide of Pgh1 to Ovalbumin

In order to examine the level of Pgh1 expression on isolated vacuoles antibodies were raised against Pgh1. Antibodies were initially developed against a peptide of Pgh1. In order to induce antibodies against this N-terminal peptide (GKEQKEKKDGNLSIKEE, molecular mass of 2.059 kD) it was necessary to couple the peptide to a carrier protein. As a carrier protein, ovalbumin (43 kD) was chosen. Ovalbumin was activated with the conjugation reagent maleimidobenzyl-N-hydroxy succinimide ester (MBS).

The peptide was then coupled to this activated carrier protein, as described in materials and methods (2.20.a. and b.). Figure 3.4. shows a 10% SDS-PAGE of ovalbumin (lane 2 and 5), activated ovalbumin (lane 3 and 6) and the conjugates (lane 4 and 7). The shift in molecular weight of the conjugates comparing with the activated ovalbumin indicates that the peptide was coupled to the carrier protein. From this non-homogeneous shift it was estimated that between 0 and 9 peptide molecules were coupled to one activated ovalbumin molecule.

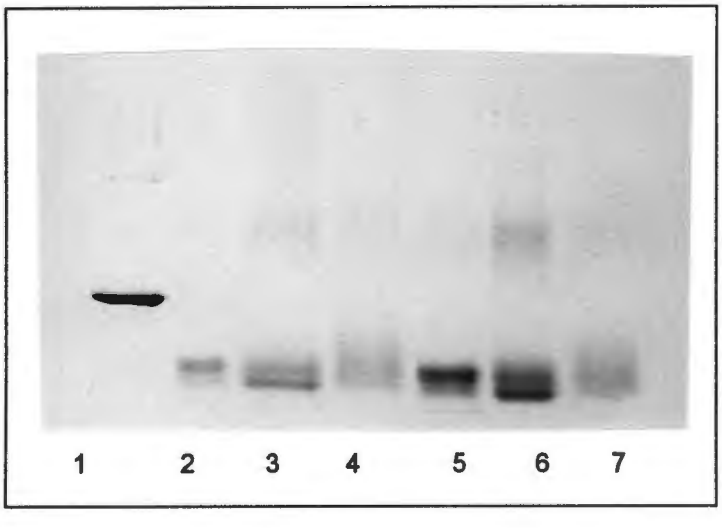


Figure 3.4. Coupling of a Pgh1 peptide (Aa 2-18) to ovalbumin.
Lane 2: 3μg of ovalbumin, lane 3: 3μg of MBS activated ovalbumin, lane 4: 3μg of conjugate, lane 5: 6μg of ovalbumin, lane 6: 6μg of MBS activated ovalbumin, lane 7: 6μg of conjugate. Lane 1: 3μg of BSA (Bovine Serum Albumin) was loaded as a control.

3.5. Recognition of Ovalbumin, Activated Ovalbumin and the N-terminal 18 Amino-Acid Peptide of Pgh1 by Anti-Pgh1 (2-18) Immunsera

Dot blot experiments were performed in order to test the immune response to the peptide of Pgh1. Aliquots of ovalbumin (0.14μg), activated ovalbumin (0.17μg) and the Pgh1 peptide (3μg) were dissolved in PBS and pipetted onto Nitrocellulose membranes. Membranes were incubated with anti-Pgh1 (2-18) immune sera and a negative pool from 2 rabbits. As 2nd antibody an anti-rabbit Ab coupled to HRP (Horseradish Peroxidase) was used.

After incubation of the membranes with the chemiluminescent substrates they were exposed to a x-ray film (materials and methods 2.22.). Figure 3.5. shows the antibody production against the carrier-protein and the peptide. Immune sera from both rabbits (1A and 1B) recognized the carrier-protein as well as the peptide. The overall antibody production of the rabbit 1A was higher than that of rabbit 1B. The negative pool did not recognize any of the antigens.

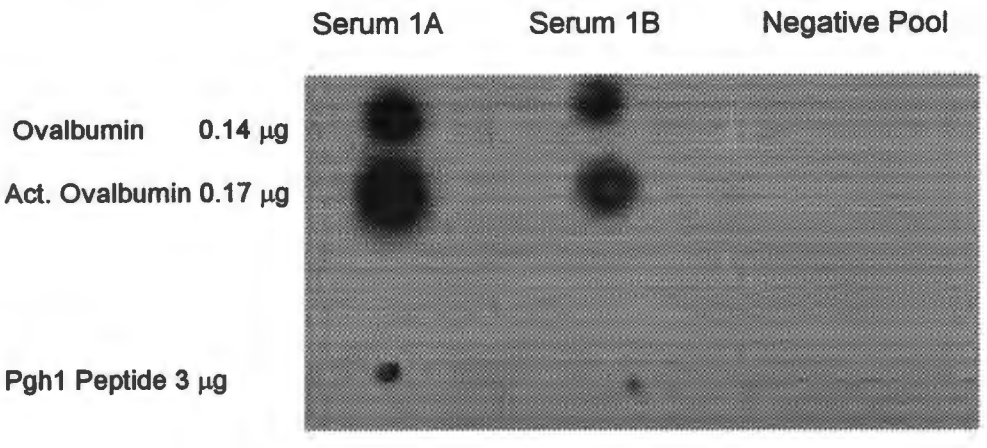


Figure 3.5. Recognition of carrier-protein and peptide by immune sera. Dot blot. Negative pool: Pooled pre-immune sera 1A and 1B.

3.6. Identification of Pgh1 by the Anti-Pgh1 (2-18) Immune Serum 1B

Western blot experiments showed that both anti-Pgh1 (2-18) immune sera recognized Pgh1. Although the overall production of antibodies of rabbit 1A was higher than that of rabbit 1B, serum of the latter was used in most Western blot experiments with the anti-Pgh1 (2-18) immune sera. Serum of rabbit 1A cross reacted with many proteins and was therefore used only in the first Western blot experiments (data not shown).

Figure 3.6. shows a representative immuno blot result with anti-Pgh1 immunserum 1B. This serum recognized Pgh1 in trophozoites as well in food vacuoles.

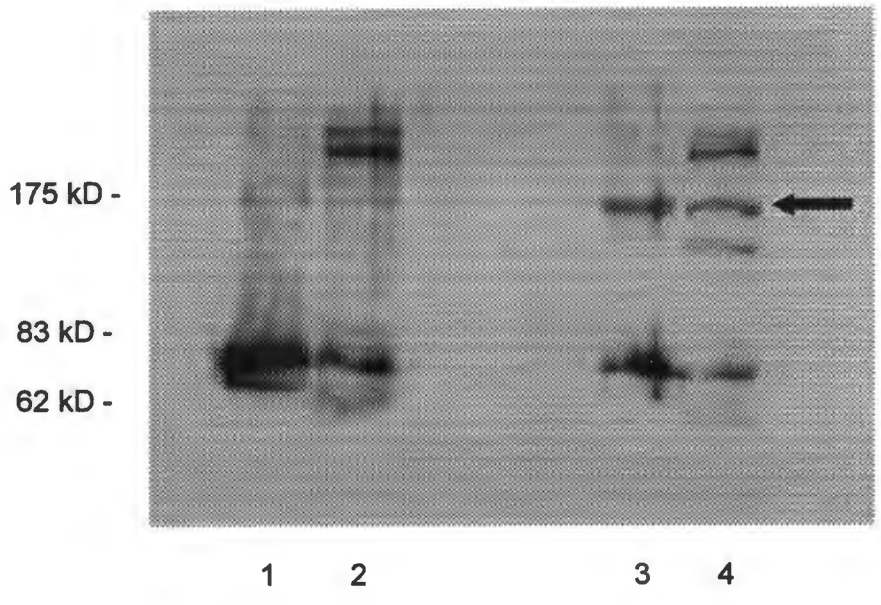


Figure 3.6. Identification of Pgh1 in D10 food vacuoles and FCR3 trophozoites by immune serum 1B. Proteins of $24*10^6$ food vacuoles of the *Plasmodium falciparum* clone D10 were separated by SDS-PAGE, transferred to PVDF membrane and incubated with preimmune serum 1B (lane1) and anti-Pgh1 (2-18) immune serum 1B (lane 3). On lane 2 and 4 proteins of trophozoites of the FCR3 strain were incubated with the preimmune serum 1B (lane2) and the immune serum 1B (lane 4). Pgh1 is indicated with an arrow, molecular weights are shown on the left.

Anti-Pgh1 1B immune serum was additionally affinity-purified against the Pgh1 (2-18) peptide. Immunoaffinity purification of the antibodies was done as in 'Antibodies a laboratory manuel ' by Ed Harlow and David Lane, 1st edition, 1988, Cold Spring Harbor Laboratory, pp 313, described [Harlow and Lane, 1988]. As affinity purification did not increase the specific recognition of Pgh1 in our experiments (data not shown) we determined to obtain antibodies against other epitopes of Pgh1. We received plasmids from Dr. A. F. Cowman from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, which encoded for GST fusion proteins containing either the N-terminal ATP- binding site of Pgh1 (amino acids 510 to 614) or the C-terminal portion of Pgh1 (amino acids 1252 - 1419) [Cowman et al., 1991 and Kracz et al., 1993]. This enabled us to produce recombinant fragments of Pgh1.

3.7. Digestion of the pGEX Vector with the Restriction Enzymes Pst I, Hind III and Hinc II

Competent cells of the *E.coli* strains XL-1 blue, JM 101 and JM 109 were transformed with plasmids, pGEX-3'- and pGEX-5'-pfmdr1, which encoded for the GST-Pgh1 fusion proteins as described in materials and methods 2. 10.. In order to clarify which clone of the *E.coli* strains used was successfully transformed with the vector; plasmid DNA from the transformed cells was extracted. The plasmids were subsequently digested by restriction enzymes.

The pGEX-3X (4952 bp) vector has 1 restriction site for Pst I (at 1905), 3 restriction sites for Hinc II (at 184,1605 and 4157) and none for Hind III; (figures 3.7.a. and b.). The 3'-DNA sequence of the pfmdr1 gene (nucleotides 4252-4758) which was introduced in the vector is not cut by Pst I or Hinc II. Hind III cuts the inserted 3'-pfmdr1 sequence once at 4428 [Foote et al., 1989].

None of the used restriction enzymes cuts the 5'-DNA sequence of the pfmdr1 gene (nucleotides 2024-2339) [Foote et al., 1989].

From this analysis it could be predicted that Pst I should cut the plasmids once; resulting in a single DNA band of 5267 bp for plasmid pGEX-5'-pfmdr1, and a band of 5459 bp for pGEX-3'-pfmdr1. Hind III would cut only pGEX-3'-pfmdr1. This should result in 1 single band of 5459 bp. Digestion of pGEX-5'-pfmdr1 with Hinc II, should result in 3 DNA fragments of 979, 1736 and 2552 bp (figures 3.7.a., 3.9. and 3.10.). Bands of 979, 1928 and 2552 bp should result when pGEX-3'-pfmdr1 was digested with Hinc II (figures 3.7.b. and 3.9.).

In figure 3.7.a. a restriction map of the pGEX-5'-pfmdr1 plasmid is demonstrated. Figure 3.7.b. shows a restriction map of pGEX-3'-pfmdr1.

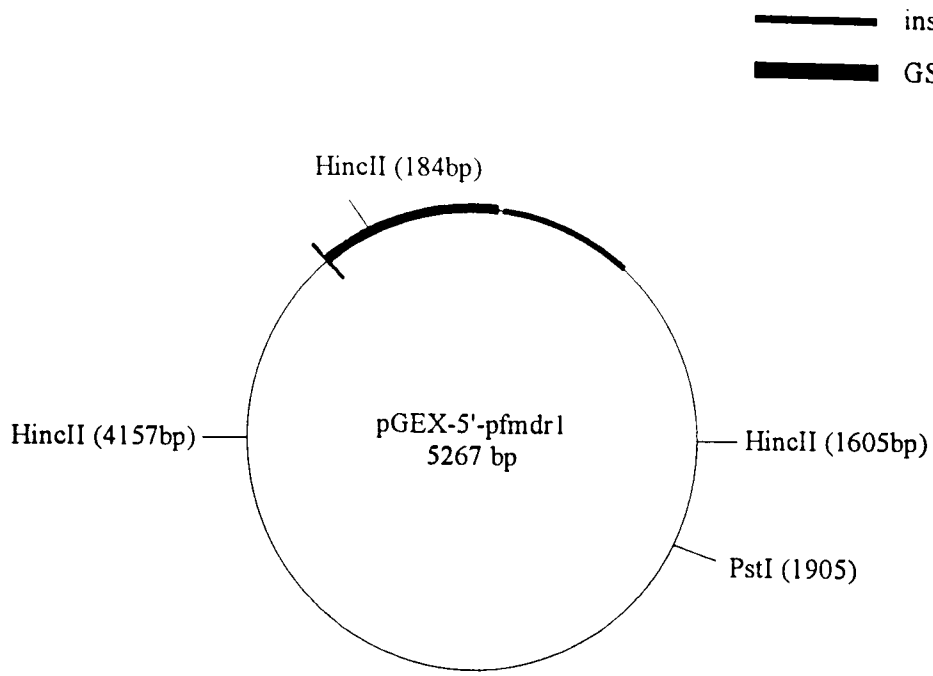


Figure 3.7.a. Restriction map of the pGEX-3X vector containing the 315 bp 5'-pfmdr1 insert.

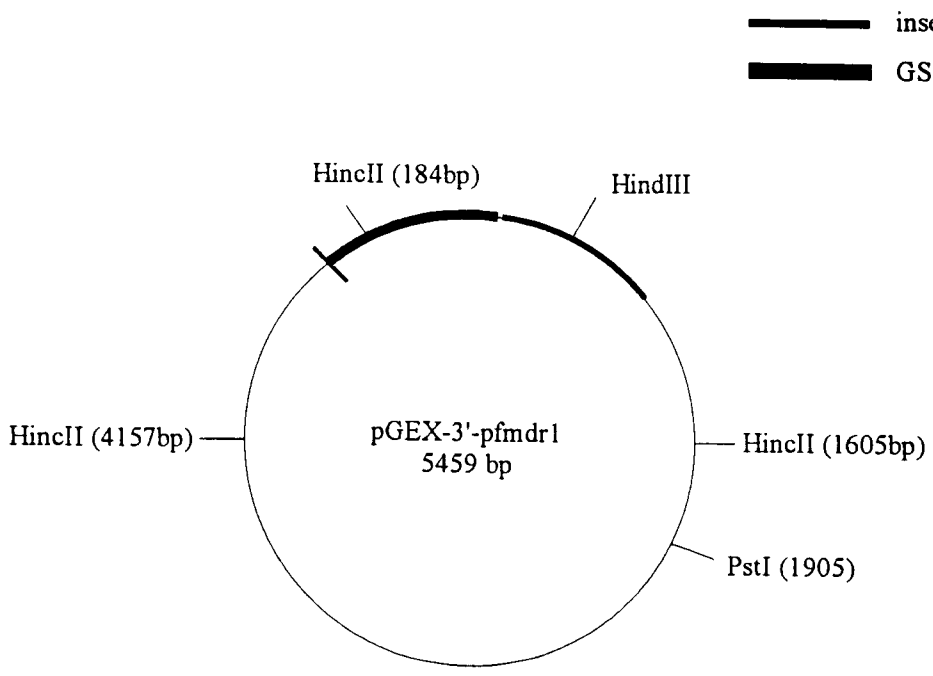


Figure 3.7.b. Restriction map of the pGEX-3X vector containing the 507 bp 3'-pfmdr1 insert.

In figure 3.8. the results of digestion with PstI and HindIII are shown.

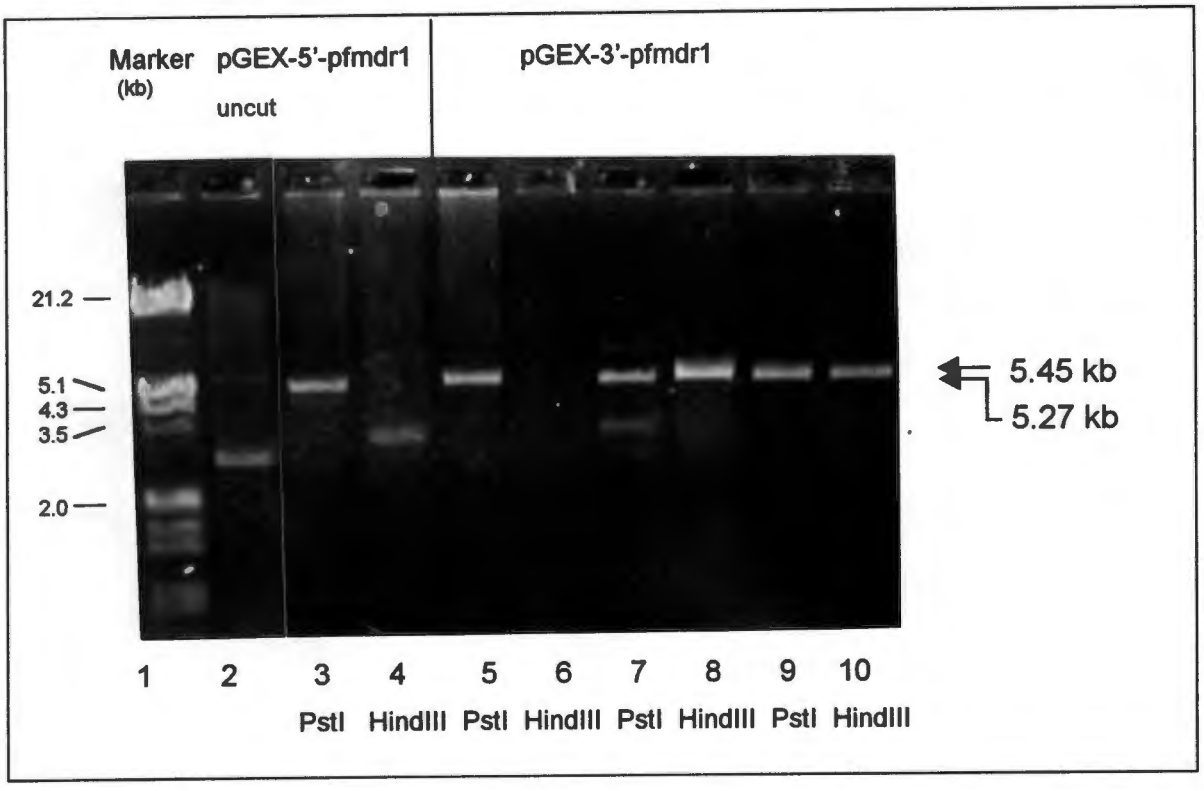


Figure 3.8. Digestion of pGEX-5'-pfmdr1 and pGEX-3'-pfmdr1 with PstI (lanes 3,5,7,9) and HindIII (lanes 4,6,8,10). 1 % Agarose gel.

Lane 1: Marker: λ Hind III/EcoR I

Lane 2: Uncut pGEX-5'-pfmdr1

Lanes 3 – 4: Digested pGEX-5'-pfmdr1; ATP clone B.

Lanes 5 - 10: Digested pGEX-3'-pfmdr1; Cterm clones.

Lanes 5 and 6 represent clone Cterm-A; lanes 7 and 8 clone Cterm-B; lanes 9 and 10 represent clone Cterm-C.

The arrows mark the cut Plasmid DNA.

Additional digestions were done with Hinc II. Electrophoresis on Agarose gels with the digested plasmids containing the clone ATP-B showed the predicted 3 bands, as well as the C-term clones A, B and C (figure 3.9.).

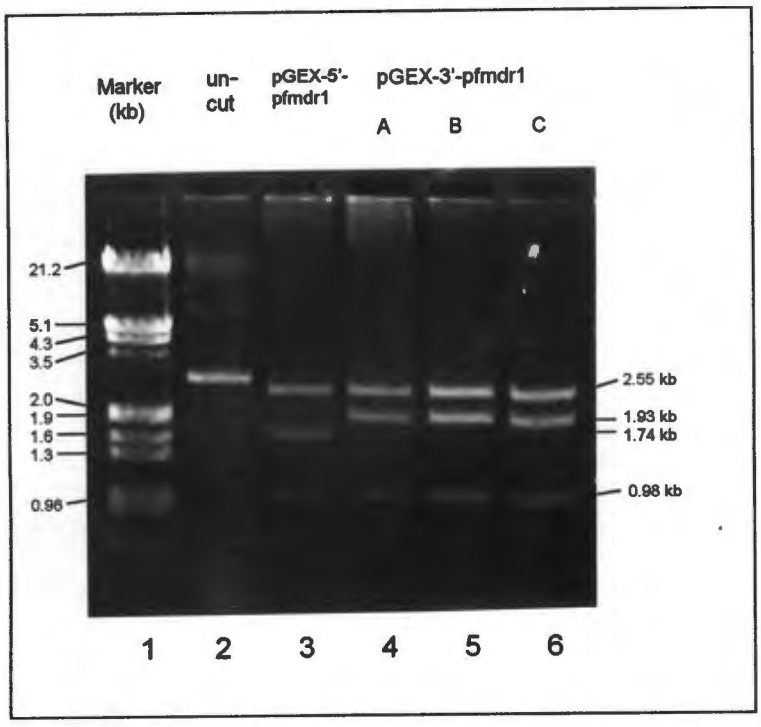


Figure 3.9. Digestion of pGEX-5'-pfmdr1 (lane 3) and pGEX-3'-pfmdr1 (lanes 4-6) with HincII. 1% Agarose Gel.

Lane 1: Marker: λ Hind III/EcoR I

Lane 2: Undigested pGEX-5'-pfmdr1

Lane 3: pGEX-5'-pfmdr1: ATP-B clone.

Lanes 4, 5 and 6: pGEX-3'-pfmdr1, C-term clones A, B and C, respectively.

In order to review the HincII digestion with the ATP-B clone an additional transformation with pGEX-5'-pfmdr1 was performed. Six clones were picked as in materials and methods (2.11.) described. Plasmid DNA of these clones , namely clone 1, 2, 3, 4, 5 and 6 were digested with HincII. All these clones showed the same digestion pattern as clone ATP-B (figure 3.10.).

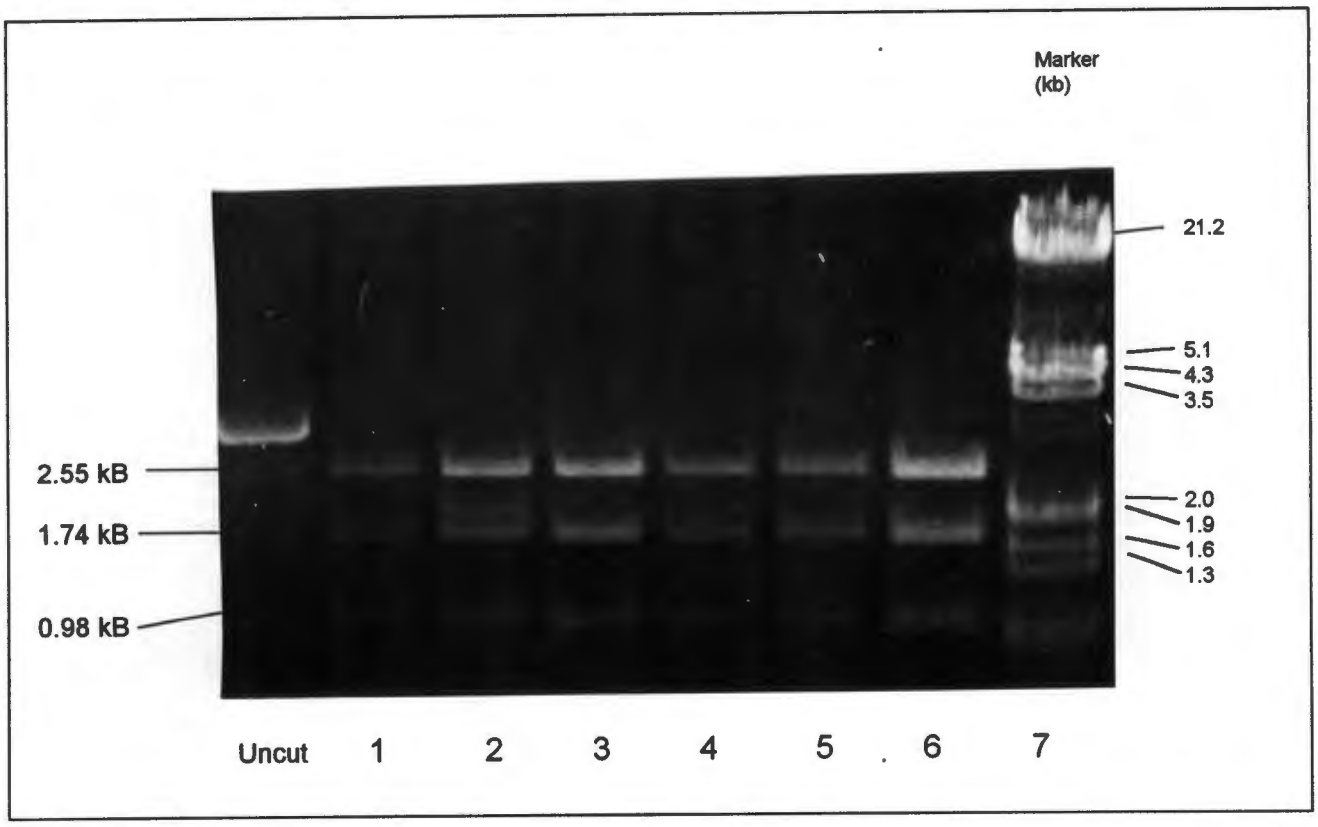


Figure 3.10. Digestion of 6 pGEX-5'-pfmdr1 clones with HincII.

Lanes 1 – 6 : Clones 1-6.

Lane 7: Marker: λ Hind III/EcoR I

Undigested plasmid DNA represents clone 6.

On the left the 3 DNA bands resulting from the digestion (3.7., page 40) are indicated.

In all 6 clones as well as in the ATP-B clone, the 2nd DNA band which contained the 5'-insert of the pfmdr1 gene had the same size (1736 bp). It could therefore be concluded that no random deletion of the inserted plasmid DNA happened during the cultureing of the transformed bacteria and that the ATP-B clone contained the correct insert. The ATP-B clone was used for the expression of the N-terminal ATP-binding site-GST fusion protein.

The identical results obtained from digestion with HincII of the Cterm clones A, B and C confirmed that they all contained the correct insert. The Cterm clone A was used for the expression of the C-terminal-GST fusion protein.

3.8. Sequencing of the Pfmdr1 Inserts of the pGEX-3X Vector

The sequence of the pfmdr1 inserts within plasmids pGEX-3'-pfmdr1 and pGEX-5'-pfmdr1 were confirmed by sequencing (materials and methods 2.13). In figure 3.11.a. the 5'-DNA sequence of the pfmdr1 gene encoding the N-terminal-ATP binding site is shown. The uppercase sequence corresponds to the 2024 – 2339 bp pfmdr1 gene fragment, [Gene bank accession number M29154 and Foote et al., 1989]. There was one base mismatch (shaded); it is indicated at position 249. This mismatch, a G instead of a A, does not alter the amino acid sequence. At the 5'-end, 4 nulceotides (lower case) from the cloning vector, encoding the amino acids PP are indicated. The 3'-end of this pfmdr1 sequence ends with the bases AG at position 320. The following nucleotides (lower case) are part of the multiple cloning site of the pGEX-3X vector [Gene bank accession number U13852]; (figure 3.11.a).

```
5' c c c  c CG AAT TCT ATG ACA TCA AAT GAA TTA TTA GAA ATG AAA AAA      45
    P    P   N   S   M   T   S   N   E   L   L   E   M   K   K

GAA TAT CAA ACT ATT AAA GAT TCT GAT GTT GTT GAT GTG TCC AAA            90
E   Y   Q   T   I   K   D   S   D   V   V   D   V   S   K

AAA GTA CTT ATA CAT GAT TTT GTA TCA TCA TTA CCA GAT AAA TAT           135
K   V   L   I   H   D   F   V   S   S   L   P   D   K   Y

GAT ACC TTA GTA GGT TCC AAT GCA TCC AAA TTA TCA GGT GGA CAA           180
D   T   L   V   G   S   N   A   S   K   L   S   G   G   Q

AAA CAA AGA ATA TCC ATT GCA AGA GCA ATT ATG AGA AAT CCT AAA           225
K   Q   R   I   S   I   A   R   A   I   M   R   N   P   K

ATT CTA ATT CTT GAT GAA GCT ACG TCT TCT TTA GAT AAT AAA TCT           270
I   L   I   L   D   E   A   T   S   S   L   D   N   K   S

GAG TAT TTA GTA CAA AAA ACA ATT AAT AAT TTG AAA GGA AAT GAA           315
E   Y   L   V   Q   K   T   I   N   N   L   K   G   N   E

AAT AGg gaa ttc atc gtg act gac tga 3'                               342
N   R   E   F   I   V   T   D   *
```

Figure 3.11a. Sequence of the 5' insert of the pfmdr1 gene.

Similar sequencing of the DNA insert encoding the C-terminal Pgh1-GST fusion protein was performed. The sequence corresponds to the 4252 – 4758 bp pfmdr1 gene fragment, [Gene bank accession number M29154 and Foote et al., 1989]. In figure 3.11.b. the obtained sequence is represented.

```
5'  TCA ATA GTT AGT CAA GAA CCC ATG TTA TTT AAT ATG TCC ATA TAT    45
     S   I   V   S   Q   E   P   M   L   F   N   M   S   I   Y

    GAA AAT ATC AAA TTT GGA AGA GAA GAT GCA ACA TTG GAA GAT GTT    90
     E   N   I   K   F   G   R   E   D   A   T   L   E   D   V

    AAA CGT GTT AGT AAG TTT GCT GCT ATA GAT GAA TTT ATC GAA TCA   135
     K   R   V   S   K   F   A   A   I   D   E   F   I   E   S

    TTA CCA AAT AAA TAT GAT ACA AAT GTT GGA CCA TAT GGT AAA AGC   180
     L   P   N   K   Y   D   T   N   V   G   P   Y   G   K   S

    TTA TCA GGT GGA CAA AAA CAG AGA ATA GCT ATA GCT AGA GCA TTA   225
     L   S   G   G   Q   K   Q   R   I   A   I   A   R   A   L

    TTA AGA GAA CCT AAA ATA TTA TTA TTA GAT GAA GCA ACA TCA TCA   270
     L   R   E   P   K   I   L   L   L   D   E   A   T   S   S

    CTT GAT TCC AAT TCT GAG AAA TTA ATT GAA AAA ACT ATT GTA GAT   315
     L   D   S   N   S   E   K   L   I   E   K   T   I   V   D

    ATT AAA GAT AAA GCT GAC AAA ACT ATT ATT ACT ATT GCC CAC AGA   360
     I   K   D   K   A   D   K   T   I   I   T   I   A   H   R

    ATT GCA TCT ATA AAA CGA TCA GAC AAA ATT GTG GTA TTT AAT AAC   405
     I   A   S   I   K   R   S   D   K   I   V   V   F   N   N

    CCT GAT CGA AAT GGA ACC TTT GTA CAG TCA CAT GGA ACA CAC GAT   450
     P   D   R   N   G   T   F   V   Q   S   H   G   T   H   D

    GAA TTA TTA TCA GCA CAA GAT GGA ATA TAT AAA AAA TAT GTA AAA   495
     E   L   L   S   A   Q   D   G   I   Y   K   K   Y   V   K

    TTA GCT AAA tga 3'                                           507
     L   A   K   *
```

Figure 3.11.b. Sequence of the 3'-insert of the pfmdr1 gene.

These results confirmed the results obtained in the digestion with the restriction enzymes. The ATP-B clone indeed contained the 5'-pfmdr1 DNA and the C-term clone A also contained the 3'-pfmdr1 DNA.

3. 9. Expression of the Pgh1-GST Fusion Proteins

Bacterial clones (*E. coli* strain JM 109) containing either pGEX-3'-pfmdr1 or pGEX-5'-pfmdr1 were cultured overnight as described in materials and methods (2.14.). With these precultures, 900 ml L-Broth medium was inoculated and incubated at 37 ^{0}C. After 1 hour 2 ml of the culture was removed. Afterwards the expression was induced by IPTG. The cultures were incubated for additional 4 – 7 hours. The uninduced and induced samples were analyzed by SDS-PAGE. Figure 3.12. shows a typical expression of the Pgh1 fragments in the pGEX-3X vector system. The expressed N-terminal-ATP-GST fusion protein had an approximate molecular size of 38 kD; the C-terminal-GST fusion protein had a molecular weight of 45 kD. The expression of the lactamase protein visible as a less intensive band of about 29 kD was also induced in both cases (figure 3.12.).

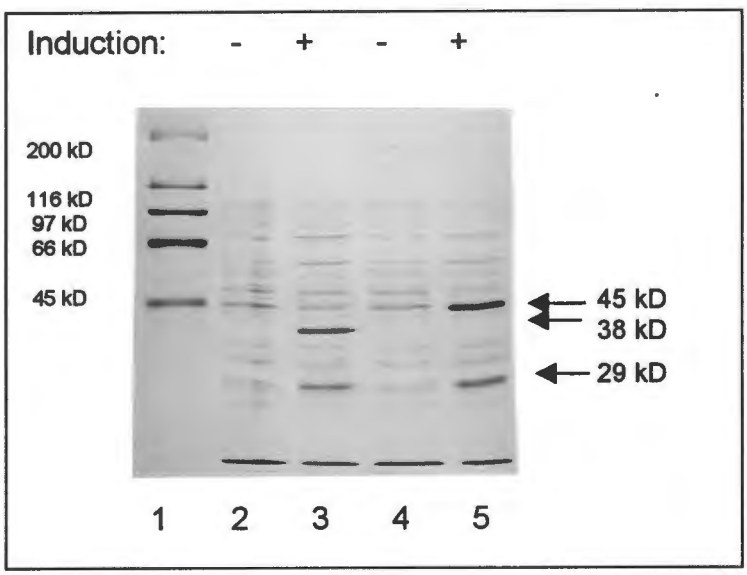


Figure 3.12. Expression of the Pgh1-GST fusion proteins. 12.5 %-PAGE.
Lane 1: Molecular weight marker. N-terminal ATP-binding site-GST fusion protein before induction (lane 2) and induced (lane 3). C-terminal-GST fusion protein uninduced (lane 4) and induced (lane 5). The arrows indicate the induced proteins (3.9.).

3.10. Purification of the Native GST Fusion Proteins

The GST-fusion proteins were purified by affinity chromatography. Bacterial cell extracts were incubated with glutathione immobilized on agarose beads. After the wash steps, the attached fusion proteins were released by adding excess glutathione (materials and methods 2.15.). The yield of the purification was determined by the Bio-Rad protein determination assay. Proteins were subsequently analyzed by SDS-PAGE. The yield of the N-terminal ATP-GST fusion protein was between 1.5-2 mg/l culture. SDS-PAGE analysis showed that approximately 60 % of the protein was degraded during the purification steps. Adding protease inhibitors to the extraction buffers and altering the sonication conditions had no influence on this degradation (figure 3.13.).

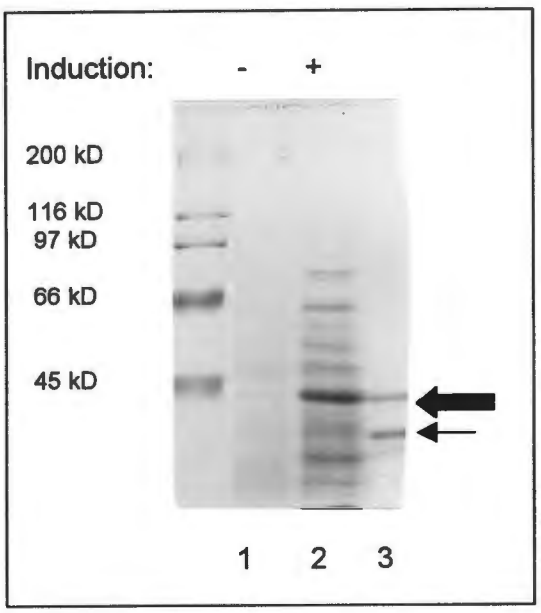


Figure 3.13. Purification of the ATP-GST fusion protein. 12.5% PAGE.
Lane 1: Bacterial extract of the transformed cells before induction.
Lane 2: Bacterial extract after induction of the N-terminal ATP-GST expression.
Lane 3: Purified fusion protein. The bold arrow marks the purified ATP-GST fusion protein. The small arrow marks the degraded fusion protein. Molecular weight markers are indicated on the left.

Due to the low solubility the yield of the C-terminal-GST fusion protein was low. From 1 l culture between 0.1 – 0.4 mg of protein was obtained. SDS-PAGE analysis of the purification of the C-terminal-GST fusion protein is represented in figure 3.14..

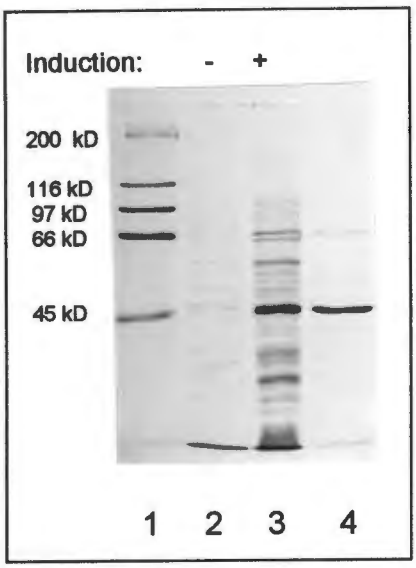


Figure 3.14. Purification of the C-terminal-GST fusion protein. 12.5% PAGE.
Lane 1: Molecular weight marker
Lane 2: Bacterial extract of the transformed cells before induction.
Lane 3: Bacterial extract after induction of the C-term-GST expression.
Lane 4: Purified C-terminal-GST fusion protein.

The purified fusion proteins represented in figures 3.13. and 3.14. were used for the subsequent immunizations of the rabbits 2A, 2B, 3A and 3B.

3.11. Purification of the C-terminal Pgh1-GST Fusion Protein by Electroelution

As the yield of the purification of the C-terminal-GST fusion was very low (3.10.), the Pgh1-C-terminal-GST fusion protein was also purified by electroelution. It was kept as a back up for further immunizations. This antigen was finally never used as the produced antiserum against the native C-terminal-GST fusion protein recognized Pgh1 well in Western blot experiments (3.13., 3.14., 3.15.).

Bacterial cell extracts from the C-term clone A containing the expressed C-terminal-GST fusion protein were separated by SDS-PAGE. After copper chloride staining, the bands with the fusion proteins were excised from the gel and decolorized as described in materials and methods (2.17.). The proteins were electroeluted and the samples subsequently dialyzed (materials and methods 2.18.).

From 1 l of bacterial culture 1.38 mg of the C-terminal-GST fusion protein was obtained. SDS-PAGE analysis showed that the eluted protein was pure (figure 3.15.).

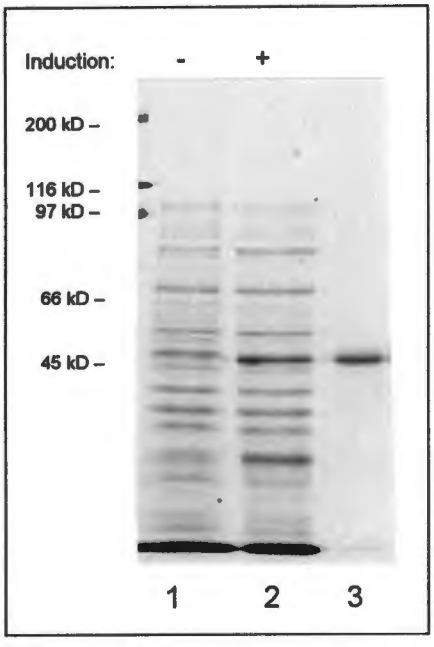


Figure 3.15. Purification of the C-terminal-GST fusion protein by electrocution.
Lane 1: Bacterial extract of transformed cells before induction.
Lane 2: Bacterial extract after induction of the Cterm-GST expression.
Lane 3: Purified C-terminal-GST fusion protein.
Molecular weights are indicated on the left.

3.12. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis of Trophozoite Proteins

In order to check the reactivity of the produced antibodies against the recombinant proteins, Western blot experiments were performed with the fusion proteins (data not shown) and with proteins from trophozoites. K1 trophozoites were extracted as described in materials and methods (2.6.) and separated on a 10% polyacrylamide gel and stained with Coomassie. Figure 3.16. shows a representative gel of separated proteins, which were used in Western blot experiments with trophozoites.

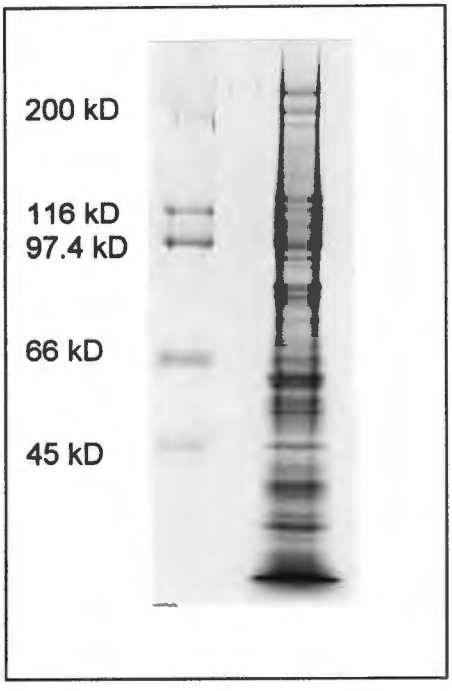


Figure 3.16. Coomassie stained 10% polyacrylamide gel of K1 trophozoites. Proteins were extracted with Urea sample buffer. Molecular weights are indicated on the left.

3.13. Detection of Pgh1 of Trophozoites from the Chloroquine Resistant *P. falciparum* Strain K1

The immune sera raised against the N-terminal ATP-binding site and the C-terminus of Pgh1 were tested against the fusion proteins (data not shown) and K1 trophozoite proteins. These experiments were mainly performed in order to verify which of the antisera would recognize the fusion protein and Pgh1 in Western blotting. Trophozoite proteins of K1 (figured in 3.16.) transferred on PVDF membranes were incubated with 4 pairs of preimmune- and immunsera.

In all experiments performed with the sera raised against the ATP-GST antiserum from rabbit 2B recognized Pgh1 better than the antiserum from rabbit 2A (figure 3.17.).

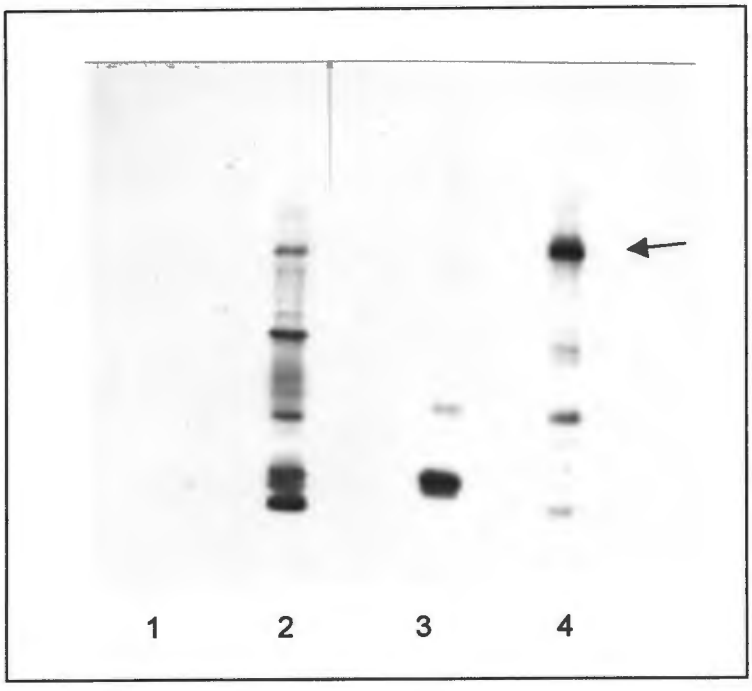


Figure 3.17. Detection of Pgh1 from K1 trophozoites by anti-ATP-binding site antisera. Western blot. Detection of antigen by chemiluminescence.
Lane 1: Incubation of trophozoite proteins with preimmune serum 2A.
Lane 2: Incubation of trophozoite proteins with immune serum 2A.
Lane 3: Incubation with preimmune serum 2B.
Lane 4: Incubation with antiserum 2B.
The arrow indicates the Pgh1-band.

Both antisera raised against the Pgh1-C-terminus-GST fusion protein (3A and 3B) recognized Pgh1 from K1 trophozoites (figure 3.18.).

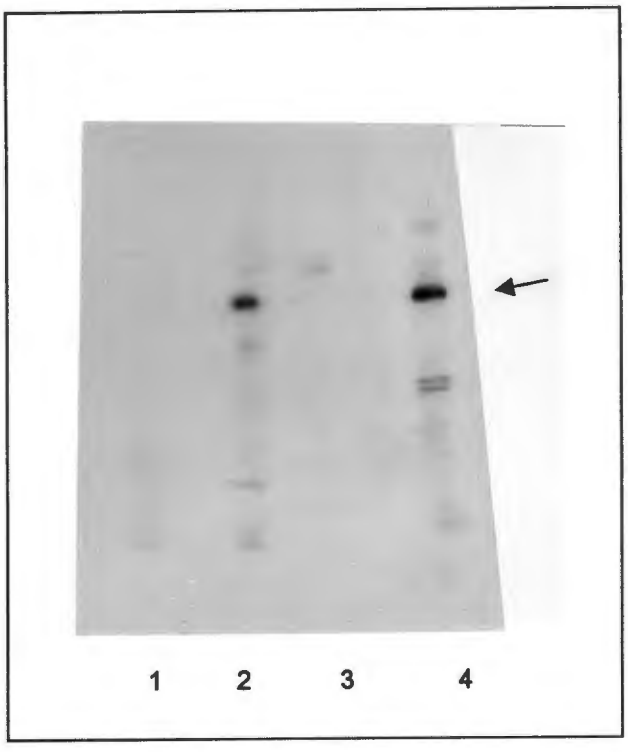


Figure 3.18. Detection of Pgh1 from K1 trophozoites by anti C-terminal antisera.
Western blot.
Lane 1: Incubated with preimmune serum 3A.
Lane 2: Incubation with antiserum 3A.
Lane 3: Incubation with preimmune serum 3B.
Lane: 4 incubation with antiserum 3B.

From these results the anti-ATP-binding site immune serum 2B and anti C-terminal immune serum 3A were selected and used in all following experiments.

3.14. Sensitivity of Anti-Pgh1 Antisera to Food Vacuole Proteins

Proteins from $3.2 * 10^6$, $6.4 * 10^6$ and $9.6 * 10^6$ food vacuoles of the K1 strain were extracted and separated by SDS-PAGE. This was followed by an electrophoretic transfer of the vacuolar proteins to a PVDF membrane and incubation with the anti-Pgh1 antisera (materials and methods 2.23.).

Antisera anti-ATP-binding site 2B and anti-C-terminal 3A both recognized Pgh1 in all extracts. The antiserum raised against the N-terminal ATP-binding site was overall more sensitive. On the other hand the antiserum against the C-terminus was more specific in recognition of Pgh1 (figure 3. 19.).

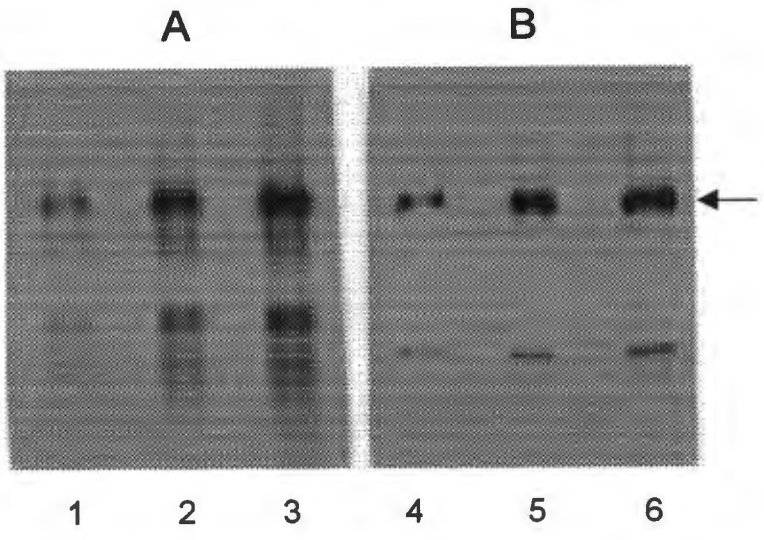


Figure 3.19. Sensitivity of the anti-Pgh1 antisera. Protein extracts from $3.2 * 10^6$ (lanes 1 and 4), $6.4 * 10^6$ (lanes 2 and 5) and $9.6 * 10^6$ (lanes 3 and 6) food vacuoles from the K1 strain were separated and transferred to a PVDF membrane. In panel A proteins were incubated with anti N-terminal-ATP-binding site antiserum. Panel B shows the incubation with the anti-C-terminal antiserum. The Pgh1 band is marked with an arrow.

3.15. Identification of Vacuolar Pgh1 of Chloroquine-Sensitive and Resistant *P.falciparum* Strains

Extracted proteins from 7.5 * 10^6 food vacuoles of the chloroquine-sensitive clones 3D7 and D10 and the resistant clone/strains FAC8 and the strains K1 and RSA11 were separated by SDS-PAGE. After the electrophoretic transfer the proteins were incubated either with the anti-N-terminal-ATP binding site antiserum 2B or with the anti C-terminal antiserum 3A. Antibody/antigen interactions were shown by chemiluminescence. In these immunoblot experiments Pgh1 was recognized in all strains/clones used. The intensity of the Pgh1 band was approximately the same for 3D7, D10, K1 and RSA11 probed with the 2B serum as well as with the 3A serum. Interactions of both of the raised antibodies to the extracted proteins from the chloroquine-resistant clone FAC8 gave a much stronger signal. This implies that at the vacuolar level FAC8 expresses the pfmdr1 product at a higher level compared to the other used *P.falciparum* clones and strains used here (figure 3.20.).

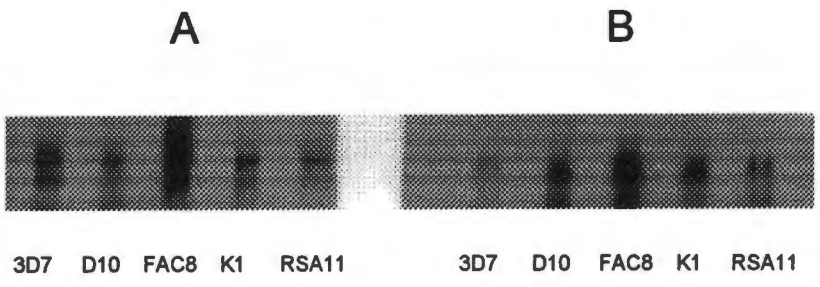


Figure 3.20. Expression of Pgh1 of food vacuoles. Western blot.
In Panel A the membrane was incubated with anti N-terminal ATP binding site antiserum. In panel B the membrane was incubated with the anti-C-terminal antiserum. In each lane proteins extracted from 7.5 * 10^6 food vacuoles were loaded.

The intensity of the expression of Pgh1 was subsequently quantified by using the Bio-Rad Multi-Analyst PC software.

3.16. Quantification of the Pgh1 Expression

In order to compare the level of the vacuolar Pgh1 expression of the 5 strains a precise method to quantify the Pgh1 bands obtained in Western blotting was sought. For this the Multi-Analyst® PC software from Bio-Rad was chosen (materials and methods 2.24.). With each of the 2 antibodies raised either against the N-terminal ATP-binding site of Pgh1 or against the C-terminus of Pgh1, 3 separate experiments were performed.

The level of Pgh1 expression of FAC8, was in all experiments much higher as for the other strains. It was taken as a reference and defined as a 100%. The Pgh1 expression of the other strains was compared to this 100%. In the figures below the vacuolar Pgh1 expression detected either with the anti-ATP (figure 3.21.a.) or the anti C-terminus antibody (figure 3.21.b.) is demonstrated for each strain in bar charts.

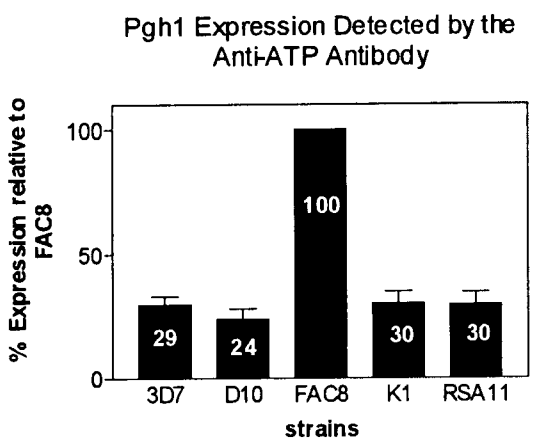


Fig.3.21.a. Expression of vacuolar Pgh1 detected by the antibody raised against the N-terminal ATP-binding site. The number in the bars indicates the expression relative to FAC8.

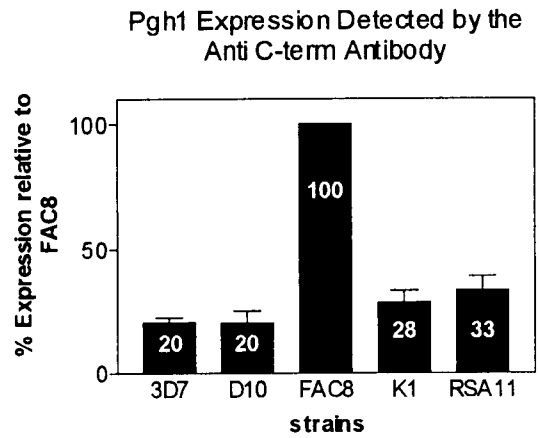


Fig.3.21.b. Expression of vacuolar Pgh1 detected by the antibody raised against the C-terminus. The number in the bars indicates the expression relative to FAC8.

With these experiments it was shown that the chloroquine-sensitive strains 3D7 and D10 and the resistant strains K1 and RSA11 expressed approximately the same amount of Pgh1 on the food vacuole. FAC8 expressed at least 3 times as much Pgh1 as the other strains and seems to be an exception.

4. Discussion

4.1. Production of Antibodies against Pgh1

4.1.1. Production of Antibodies against the N-terminal 18 Amino Acid Peptide of Pgh1

The N-terminal peptide of Pgh1 was successfully coupled to a carrier protein, ovalbumin, and this conjugate was used for the immunization of rabbits. In order to expose the N-terminus of the peptide to the immune system of the rabbits the peptide was coupled with its C-terminus to the carrier protein. Antibodies were raised and dot blot experiments showed that the peptide as well as the carrier protein is recognized by the immune sera.

These antibodies also recognized Pgh1 extracted from trophozoites and food vacuoles in Western blotting experiments. In order to obtain a clear detection of Pgh1 these antisera had to be used at a dilution of 1: 100. Under this condition a few other bands were also recognized by the immune- as well as by the preimmune sera. The antiserum was subsequently affinity purified against the peptide in order to increase its specificity. Unfortunately these purified antibodies lost nearly all the activity in Western blotting experiments and it was therefore decided to obtain other Pgh1 antigens.

4.1.2. Expression of the Pgh1-GST Fusion Proteins

After transformation of competent cells with plasmids encoding for two GST-Pgh1 fusion proteins, expression of the fusion proteins was successfully induced by IPTG. The predicted molecular weight of the N-terminal ATP-binding site fragment was 13.64 kD; the C-terminal fragment of Pgh1 had a predicted weight of 21.04 kD. Therefore the N-terminal ATP binding site-GST fusion protein (38 kD) was

approximately 7 kD smaller than the C-terminal-GST fusion protein (45 kD). This was in accordance with results obtained from digestion of the plasmid DNA with HincII where the second band of the ATP clone B (which contained the pfmdr1-GST DNA) was smaller than the size of the second band of the C-terminal clones (results 3.7.). Sequence analysis of the inserts within these plasmids also confirmed that the N-terminal fragment was smaller than the C-terminal fragment. Antibodies generated against both expressed gene products recognized Pgh1 in Western blotting.

4.1.3. Purification of the Pgh1-GST fusion Proteins

N-terminal ATP- and the C-terminal-GST fusion proteins were initially purified using glutathione (GSH) agarose beads. In all purifications at least 50% of the N-terminal ATP-GST fusion protein was degraded. Using milder sonication conditions, adding protease inhibitors as well as keeping the sample on ice did not influence this degradation. This mixture of degraded and non-degraded proteins was used for the immunizations of the rabbits.

The C-terminal-GST fusion protein did not show any degradation during purification. Despite the fact that the yield was low due to the low solubility of the protein, we produced sufficient quantity of the native protein for the immunizations. It was subsequently found out that with a shorter expression time this solubility could be increased. It was therefore speculated that this protein was expressed as inclusion bodies and the protein was subsequently purified by electroelution. This method produced a high yield of the C-terminal-GST fusion protein.

4.2. Western Blots with Antibodies Raised against the Pgh1-GST Fusion Proteins

Antiserum at a dilution of 1:1000 raised against both GST-Pgh1 fusion proteins recognized Pgh1 in Western blotting. In all experiments antiserum raised against the

N-terminal ATP-binding site-GST fusion protein cross-reacted with more proteins extracted either from trophozoites or food vacuoles than the antiserum raised against the C-terminal-GST fusion protein. This was not unexpected for the following reasons. The antigens to which the antibodies were raised up were of different quality and quantity. Whereas a shorter and partly degraded N-terminal ATP-binding site-GST fusion protein was produced in abundance and used as such for the injections, it was difficult to purify the C-terminal-GST fusion protein in our system. Only small amounts of highly pure non-degraded C-terminal antigen were used for the injections.

Nevertheless, the recognition of Pgh1 obtained with both polyclonal antibodies in Western blotting was sufficient for our purposes. Affinity purification was not considered necessary. A smaller band of approximately half the molecular size of Pgh1 was often recognized by the antisera. An example is shown in panel B of figure 3.19. (page 55). It was speculated that this might be a degradation product.

4.3. Recognition of Pgh1 on Trophozoites and Food Vacuoles of Different Strains

In our study Pgh1 was recognized in protein extracts of either trophozoites or food vacuoles from all examined strains. Proteins from trophozoites were mainly used in preliminary Western blotting experiments. This was done in order to conserve the food vacuole proteins which were more difficult to obtain. In Western blots with trophozoites it could not be ascertained if the recognized Pgh1 protein was of vacuolar origin or expressed at different sites in the parasite.

Both from literature [Cowman et al., 1991] and from the findings reported in this study it is clear that all *P. falciparum* strains thus far tested express Pgh1.

4.4. Expression of Pgh1 on Food Vacuoles

Pgh1 has been localized on the cell membrane and on the membrane of the digestive vacuole of the malaria parasite [Cowman et al., 1991]. From its likely orientation it is predicted that Pgh1 transports substrates either out of the cell, as do MDR proteins in cancer cells, or into the food vacuole.

With the exception of FAC8, all strains tested in this study expressed Pgh1 at a similar level in the vacuole. No correlation of chloroquine IC_{50} and the level of Pgh1 expression was observed.

The FAC8 clone was originally obtained from Dr. A.F. Cowman in 1994. It has been cultured in our laboratory since then. In our experiments, FAC8 consistently expressed at least three times more vacuolar Pgh1 than the other tested cell lines. In 1991 Cowman et al. reported that the FAC8 clone expresses Pgh1 at a higher level than other strains. Cowman and his colleagues showed that the extent of Pgh1 expression does not correlate with the chloroquine IC_{50}.

In this study two new strains, a chloroquine-resistant (RSA11) and a chloroquine-sensitive strain (D10), were added. They both were found to express Pgh1 at similar levels in highly purified, functional vacuoles.

4. 5. Phosphorylation of Food Vacuole Proteins

In order to determine whether Pgh1 is phosphorylated on intact food vacuoles, phosphorylation experiments were performed. Proteins from the chloroquine-resistant strain RSA15 were successfully labeled with [γ-^{32}P]-ATP. Approximately 10

different proteins were labeled by [γ-^{32}P]-ATP. As for all other strains tested, RSA15 expresses Pgh1 [L. Elandalloussi, personal communication, 1999]. Under the conditions of our experiments Pgh1 was not labeled by [γ-^{32}P]-ATP. Phosphorylation studies on isolated food vacuoles from the D10 strain indicated that Pgh1 is also not phosphorylated in this chloroquine-sensitive strain (data not shown).

Lim and Cowman [1993] successfully labeled Pgh1 by [^{32}P]orthophosphate as well as by [γ-^{32}P]-ATP. In their experiments they were labeling 10^6 food vacuoles isolated from FAC8 with 50 μCi [γ-^{32}P]-ATP at 30^0 C for 5 min. After the labeling, Pgh1 was immunoprecipitated.

In our experiments, $10*10^6$ or $40*10^6$ food vacuoles isolated from RSA15 were labeled with 100 μCi [γ-^{32}P]-ATP at 30^0 C for 10 min. Compared to the labeling of the other vacuolar proteins, our results indicate that Pgh1 is not phosphorylated. These observations might indicate that Pgh1 is either not regulated by phosphorylation or that under the conditions in our experiments Pgh1 is not active.

The overall labeling of the food vacuole with [γ-^{32}P]-ATP could be modulated by addition of cold ATP or ADP. Under these circumstances only a protein of about 100 kD was phosphorylated. It was not clear which protein this was.

In our experiments we found that GTP stimulated the radioactive labeling of the vacuolar proteins. This may be either because GTP had enhanced the activity of protein kinases or it might have reduced the activity of phosphatases.

4.6. Concluding Notes

In this study the expression of Pgh1 on food vacuoles isolated from the chloroquine-resistant strains FAC8, K1 and RSA11 and from the chloroquine-sensitive strains 3D7 and D10 was compared. With the exception of FAC8, the amount of Pgh1

expressed was equal for all the strains tested, detected either with the anti-N-terminal ATP-binding site or the anti C-terminal antibody. If Pgh1 plays a role in chloroquine resistance at the vacuolar level, this is clearly not due to differential expression of this protein in chloroquine-sensitive and resistant strains. The function of increased levels of Pgh1 in FAC8 vacuoles is unclear.

The phosphorylation experiments performed in this study provided no evidence that the phosphorylation of Pgh1 is different on food vacuoles isolated from chloroquine-sensitive and resistant strains.

The equal expression level could also lead to the conclusion that mutations on Pgh1 might alter the ability to transport chloroquine. Although Pgh1 acts as a transporter of a peptide, the pheromone a-factor, in yeast and mutations on the pfmdr1-gene were linked to reduced transport of this pheromone in the same system [Volkman et al., 1995], it is not clear whether Pgh1 is a direct transporter of chloroquine. Mutations on the pfmdr1-gene have not been linked to chloroquine resistance [Wellems et al., 1990; Haruki et al., 1994].

Substrate recognition by P-glycoproteins is a two-step process. The substrate first intercalates with the transmembrane segment of Pgp in the lipid bilayer. It must then be recognized as a substrate. Only once it is recognized as a substrate can it be transported. Seelig A. [1998] screened hundreds of chemically diverse compounds that had been tested as P-glycoprotein substrates [Seelig A. ,1998]. She detected potential elements responsible for substrate/P-glycoprotein interaction, as well as typical features for P-glycoprotein overexpression.

Two well-defined structural elements are required for recognition and interaction of a substrate with P-glycoproteins. One consists either of a.) two electron donor groups with a fixed spatial separation of 2,5 ± 0,3 Å, or b.) two electron donor groups with a fixed spatial separation of 4.6± 0.6 Å, or three electron donor groups with a fixed spatial separation of 4.6± 0.6 Å of the outer two groups. Applying these criteria,

chloroquine might, be only a very weak substrate for P-glycoproteins, if at all [A. Seelig, personal communication, 1999].

Foley et al. [1994] examined the binding of a photoactive analog of chloroquine to proteins of *P. falciparum.* This analog of chloroquine labeled two proteins of 33 and 42 kD size in their experiments, but not Pgh1. This is further evidence that chloroquine does not directly interact with Pgh1.

In our experiments FAC8 expressed 3 to 4 times more Pgh1 on food vacuoles compared to K1 and RSA11. Yet these strains have similar chloroquine IC_{50}'s. Considering that mutations on the pfmdr1-gene do not play a role in chloroquine resistance these different expression levels of three chloroquine-resistant strains also suggest that Pgh1 does not function as a direct chloroquine transporter in the food vacuole.

For future work it will be important to establish the amount of expression and exact distribution of Pgh1 within the malaria cell, and to compare these findings in chloroquine-resistant and sensitive strains. It is possible that there may be a differential expression of Pgh1 in various parts of the parasite. For example, it may specifically be the expression at the plasma membrane that correlates with the resistance phenotype.

5. References

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