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Plasmodium falciparum expresses a multidrug resistance-associated protein

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Abstract

Plasmodium falciparum proteins that efflux toxic metabolic products such as oxidised glutathione (GSSG) are possible targets for anti-malarial drug development. Proteins capable of transporting GSSG and glutathione conjugates include the multidrug resistance-associated transporters (MRPs). A gene, PFA0590w, encoding a MRP homologue, has been identified in *P. falciparum*. Here we show the presence of full-length mRNA (5.5 kb) of this *Pf*MRP in trophozoites by RT-PCR and Northern blotting. A polyclonal anti-*Pf*MRP antibody generated against two unique, hydrophilic peptides in the predicted sequence produced a strong immunoreactive protein band of 210–215 kDa on Western blots of schizonts of chloroquine-sensitive and chloroquine-resistant strains, confirming expression of *Pf*MRP protein. Using confocal microscopy the protein was seen to be localised at the edge of the schizonts with no obvious staining of the food vacuole. We suggest that *Pf*MRP may act as the GSSG transporter in the parasite plasma membrane.
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Keywords: *Plasmodium falciparum*; Drug resistance; ATP-binding cassette transporter; Multidrug resistance-associated protein; Glutathione

The identification of potential drug targets in *Plasmodium falciparum* with the aim of developing an effective therapy for the treatment of malaria is one of the key objectives of the Malaria Genome Sequencing Project (see http://www.plasmodb.org). *P. falciparum* transport proteins that regulate the movement of toxic metabolic products such as oxidised glutathione (GSSG) [1] out of the parasite are one possible target for anti-malarial drug development. In all organisms studied, ATP-binding cassette (ABC) transporters translocate a wide range of molecules across membranes against a concentration gradient [2–6]. Among these, multidrug resistance-associated (MRP or ABCC) transporters primarily function as organic anion transporters [4] with substrates that include GSSG and glutathione conjugates.

At least nine genes containing both an ATP binding cassette and at least one predicted transmembrane spanning domain have been identified in *P. falciparum* [7,8]. At present *Pf*MDR1, the protein encoded by the PFE1150w (aka *Pfmdr1*) gene (NC_004326), is the only one known to be involved in drug resistance [5,9]. *Pf*MDR1 is a homologue of the human P-glycoprotein. The genome of *P. falciparum* contains another gene, PFA0590w (NC_004325), which has been annotated as a 1822 amino acid full-length ABC transporter (CAB63558). We have previously noted that his putative protein appears to be an MRP homologue [10] here called *Pf*MRP. It is predicted to contain two nucleotide

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binding domains (NBDs), designated NBD_1 (675–904 amino acids) and NBD_2 (1581–1797 amino acids). Based on transmembrane prediction analysis by HMMTOP software [11], it also contains two membrane spanning domains, MSD_1 (125–482 amino acids) and MSD_2 (1171–1454 amino acids), each with six putative α-helical transmembrane segments. The domains are ordered as $\{MSD_1–NDB_1–MSD_2–NDB_2\}$ and the $–NH_2$ and –COOH terminals are on the same side of the membrane as the NBDs, presumably in the cytoplasm. The predicted protein lacks the additional NH_2-terminal MSD (MSD_0) which is present in human MRP1-3 transporters but is absent in MRP4-5. The function of the MSD_0 in MRP1 has been examined and it appears that it is not a determinant domain for MRP1 function and cellular trafficking [12]. We here report exploration of the expression of this gene and its protein product.

Materials and methods

Multiple alignments of archived sequences including PFA0590w were generated using Clustal-W (http://www2.ebi.ac.uk/clustalw) [13] and visualised using Genedoc (http://www.psc.edu/biomed/genedoc/).

mRNA for the central region of PFA0590w, 1545–2267 bp, was detected using the primers 5′-GTA AAT ATT AAA CGT TTA AAT-3′ and 5′-CCA ACT ATT TTG AGG AAC ATA-3′. (Primers were synthesised by MWG-Biotech, Milton Keynes). Total mRNA was extracted from the *P. falciparum* A4 strain derived from the endothelial binding line of the chloroquine resistant strain ITO4 [14] and cultured as previously described [15]. It was reverse transcribed using the SUPER RT enzyme with random hexamer primers and amplified with SUPER *Taq* DNA polymerase (HT Biotechnology, Cambridge). Full-length mRNA was detected using the primers 5′-TAT GAC GAC ATA TAA AGA AAA TGT TG-3′ and 5′-CCA TCT AGA TCA GTG ATG GTG ATG ATG GTC GTC CAT TTC TAA CAA ATG TGA-3′. Total mRNA was extracted, DNAse-treated (DNA-*free* kit, Ambion), and reverse transcribed using the Thermoscript RT kit (Gibco Life Technologies). PCR amplification was carried out with PLATINUM *Pfx* DNA polymerase (Invitrogen).

A ^{32}P-labelled *pfmrp* DNA probe was synthesised by random primed DNA labelling starting from the gel purified central RT-PCR fragment of *pfmrp* gene using α-^{32}P-dCTP (3000 Ci mmol^{-1}, 10 μCi μl^{-1}) (Amersham), and 2 U of large fragment of *E. coli* DNA polymerase I (Klenow fragment) (Gibco Life Technologies). Unincorporated nucleotides were removed by the QIAquick nucleotide removal kit (Qiagen). Total RNA (20 μg) was fractionated on a 1.5% formaldehyde denaturing agarose gel and then hybridised with the ^{32}P-labelled *pfmrp* probe.

A polyclonal anti-*Pf*MRP antibody was raised in rabbits against two unique, hydrophilic peptides of the putative *Pf*MRP protein (^{215}CSNNNHLQNPDAFY^{228} and ^{1420}YASGIIKLYKEKNYV^{1434}) (Covalab, Lyon, France). The first is located on the external face of the predicted structure between transmembrane segments 2 and 3 and the second is located on the cytoplasmic face between transmembrane segments 11 and 12. Parasites were isolated from infected erythrocytes by 0.05% saponin permeabilisation, collected by centrifugation at 1500*g* for 10 min, and washed four times with phosphate-buffered saline (PBS). The amount of protein was determined using the Bio-Rad protein assay. Parasites were solubilised in reducing sample application buffer (2% SDS, 0.1 M Tris–HCl, pH 7.5, 10% glycerol, 0.1 M DTT, and 0.1% bromophenol blue) and boiled. Ten micrograms of total protein was loaded per well. The secondary antibody was goat anti-rabbit IgG peroxidase conjugate (Sigma) visualised using Pierce Supersignal West Pico chemiluminescent substrate.

Human erythrocytes infected with schizonts were fixed in 4% paraformaldehyde at room temperature for 45 min, gently precipitated by centrifugation, and applied onto 0.01% poly-L-lysine coated coverslips. The cells were then blocked for 45 min with 5% bovine serum albumin in PBS-Tween containing 0.1% Triton followed by overnight incubation with anti-*Pf*MRP antibody at 4 °C. The slides were washed with PBS-Tween twice for 2 min at room temperature and FITC-conjugated anti-rabbit IgG antibody was applied for 30 min at room temperature, followed by washing the coverslips twice with PBS-Tween. The coverslips were allowed to air-dry and mounted in Vectashield (VectorLabs) containing DAPI (4′,6′-diamidino-2-phenylindole).

Results and discussion

We have previously compared the predicted protein from PFA0590w with members of the ABC protein superfamily [10]. It shows strong resemblance with several MRP homologues: *Cp*ABC2 in the parasite *Cryptosporidium parvum* (AAL59847, 14% identical residues; 32% conservative substitutions), MRP2 in human (NP_000383, 13% identical residues; 30% conservative substitutions), the yeast bile transporter Ybt1p in *Saccharomyces cerevisiae* (NP_013052, 13% identical residues; 28% conservative substitutions), and *At*MRP2 in *Arabidopsis thaliana* (NP_181013, 11% identical residues; 30% conservative substitutions). Alignments of the predicted amino acid sequence of NBD_1 and NBD_2 from PFA0590w with corresponding NBDs from selected ABC transporters are shown in Fig. 1. For comparison, this protein shares only 7% overall sequence identity with *Pf*MDR1. By analogy with *Pf*MDR1 we suggest this protein be called *Pf*MRP.

RNA corresponding to PFA0590w has previously been detected using oligonucleotide probes on arrays with greatest expression in the trophozoite or early schizont stage [16,17]. In the present study, the presence of full-length mRNA (≈5.5 kb) corresponding to *Pf*MRP was confirmed by RT-PCR analysis using primers corresponding to the 5′ and 3′ ends of the putative gene (Fig. 2A). Northern blot analysis also revealed the presence of a *PfMRP* transcript (≈6 kb) in the trophozoites but not in the ring-stage of *P. falciparum* clone A4 (Fig. 2A-c), confirming the results of RT-PCR analysis. Thus, *PfMRP* is present at the RNA level in *P. falciparum.* PFA0590w contains no introns, consistent with prediction and the array data [17].

Protein fragments corresponding to *Pf*MRP were not detected in either of the published investigations of the *P. falciparum* proteome [18,19]. We have performed immunoblotting analysis to investigate expression of *Pf*MRP throughout the erythrocytic asexual life cycle stages of several *P. falciparum* chloroquine-sensitive and chloroquine-resistant strains using a polyclonal anti-*Pf*MRP antibody. There is a strong immunoreactive

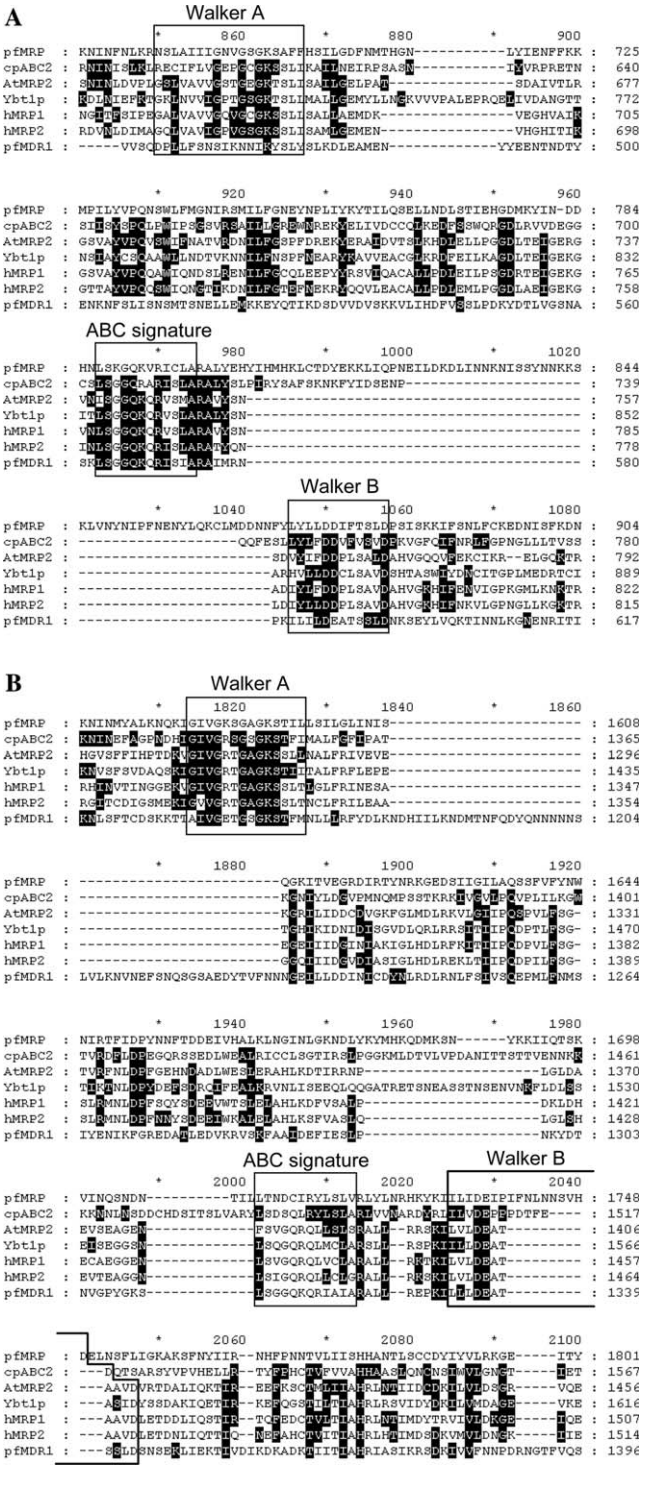

Fig. 1. Alignment of the predicted amino acid sequence of NBD_1 and NBD_2 of *Pf*MRP with corresponding NBDs of selected ABC transporters: *Cp*ABC2 from *C. parvum*, *At*MRP2 from *Arabidopsis thaliana*, the yeast bile transporter Ybt1p from *S. cerevisiae*, and MRP1 and MRP2 from human and *P. falciparum* MDR1. Each NBD contains three conserved motifs: Walker A, the ABC signature, and Walker B. Note the additional ≈60 amino acids which are present between the ABC signature and the Walker B in NBD_1 of *Pf*MRP1 but are absent in the other ABC transporters. Interestingly, *Pf*MDR1 also has an additional ≈40 amino acids which are present between the Walker A and the ABC signature but are absent in the other ABC transporters.

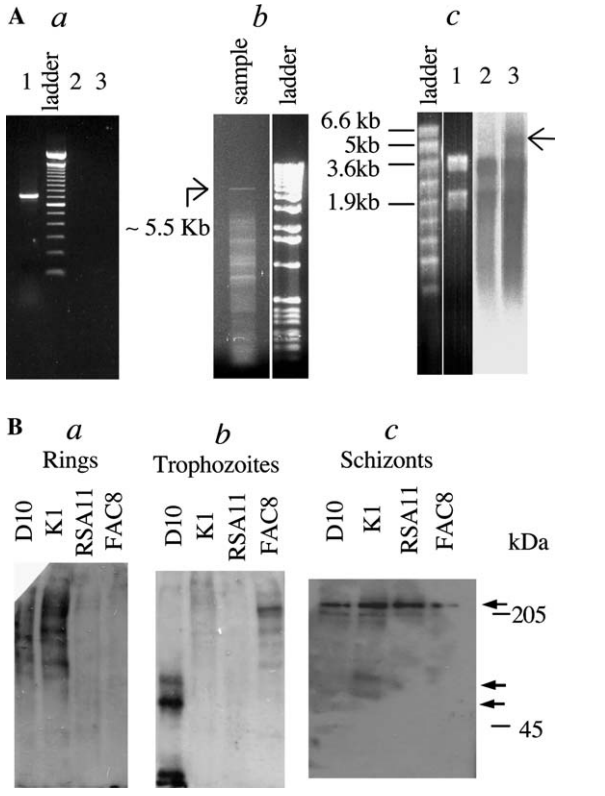

Fig. 2. Expression of (A) PFA0590w mRNA and (B) *Pf*MRP protein. (A-a) The central region from 1545–2267 bp. Lanes shown are: (1) the sample showing a single band at the expected position; (ladder) a 100 bp sizing ladder; (2) sample not reverse transcribed; and (3) water control. (A-b) The full-length mRNA. In the sample the band at the expected size, 5.5 kb, is indicated by the arrow. The 1000 bp ladder is also shown. (A-c) Northern blot detection. (1) Ethidium bromide staining of the total RNA. (2) Northern blot of RNA extracted from ring stage parasites; and (3) Northern blot of RNA from trophozoites as in lane 1. The ladder shows 0.28–6.58 kb RNA markers (Promega). The arrow indicates the band thought to correspond to *pfmrp*. The two prominent bands present in all of lanes 1–3 correspond to ribosomal RNA which is expected to be more than 97% of the total. (B) Immunoblotting analysis of samples taken at three stages of the erythrocytic asexual life cycle of chloroquine-sensitive (D10) and chloroquine-resistant (K1, RSA11, and FAC8) *P. falciparum* strains using a polyclonal anti-*Pf*MRP antibody. One of two experiments with the same results.

band corresponding to a protein of 210–215 kDa in the schizonts of both chloroquine-sensitive and chloroquine-resistant strains (Fig. 2B-c). This is similar in size to the predicted molecular weight of *Pf*MRP (≈215 kDa) and confirms the expression of *Pf*MRP in parasites. It also suggests that *Pf*MRP is not glycosylated. The 210–215 kDa immunoreactive band was also detected at the trophozoite stage in one of the chloroquine-resistant *P. falciparum* strains, FAC8 (Fig. 2B-b). It was not detected at the ring stage in any strain (Fig. 2B-a) nor was it detected in samples of erythrocyte protein obtained from uninfected erythrocytes (data not shown). Interestingly, two or three, strong, low-molecular weight bands (<100 kDa), presumably degraded

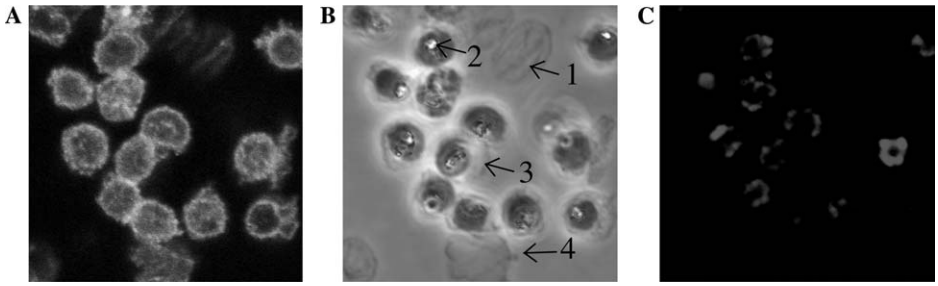

Fig. 3. Localisation of *Pf*MRP in *P. falciparum* schizonts from strain A4. (A) Confocal image of schizont-infected erythrocytes stained using the anti-*Pf*MRP antibody and FITC-labelled secondary antibody. (B) Transmitted light image of the same field. (C) Confocal image of the DAPI-stained schizont nuclei in the same field. Images were captured using a Leica TCS 4D confocal microscope. Arrows in (B) indicate: 1, uninfected human erythrocyte; 2, parasite food vacuole; 3, unstained plasma membrane of an infected human erythrocyte; and 4, stained, disrupted membrane of a presumably formerly infected erythrocyte.

protein products, were consistently detected in the trophozoites from the chloroquine-sensitive strain, D10 (Fig. 2B). These may represent degradation products of fully formed *Pf*MRP. If so this would imply greater expression when compared to trophozoites from the chloroquine-resistant strains (Fig. 2B). This raises the possibility that *Pf*MRP may degrade more readily in trophozoites of chloroquine-sensitive parasites when compared to the trophozoites of chloroquine-resistant parasites. No immunoblotting bands were detected when pre-immune serum was used instead of *Pf*MRP antiserum (data not shown).

The localization of *Pf*MRP was investigated in paraformaldehyde-fixed parasites using the anti-*Pf*MRP antibody. Transmitted light images of the schizont-infected erythrocytes show the location of the food vacuole as a white area with dark spots representing the haemozoin crystals (Fig. 3B). Uninfected erythrocytes are easily distinguished in these images by the absence of any concentrated dark material. Schizont-infected erythrocytes could also be identified by staining them with DAPI (4′,6′-diamidino-2-phenylindole), which fluoresces blue in contact with DNA distinguishing the nucleated parasites from the anuclear erythrocytes (Fig. 3C). Schizonts show a very distinctive pattern of fluorescence staining with anti-*Pf*MRP with the majority of reactivity surrounding what seems to be the plasma membrane of the parasite (Fig. 3A). No obvious staining is associated with the food vacuole of the parasite. The evidence for staining of the red blood cell plasma membrane of infected cells in this study is ambiguous. Some clearly stained parasites were seen to be inside erythrocytes with little if any staining of their plasma membranes (e.g., 3 in Fig. 3B). By contrast membranes from erythrocytes apparently disrupted so having lost their parasites (e.g., 4 in Fig. 3B) were sometimes seen to be stained. No immunofluorescence staining was seen when the paraformaldehyde-fixed schizont-infected erythrocytes were exposed to pre-immune serum (data not shown).

In conclusion, we have provided evidence which indicates that *P. falciparum* contains an MRP homologue called here *Pf*MRP. This protein appears to be located on the plasma membrane of the parasite. *Pf*MRP is the first MRP transport protein to be identified in *Plasmodium falciparum*. The structural similarity of *Pf*MRP to human MRP2 and the parasitic *Cp*ABC2 has important functional implications as it suggests that *Pf*MRP may be the GSSG transporter in the parasite plasma membrane.

References

[1] H. Atamna, H. Ginsburg, The malaria parasite supplies glutathione to its host cell—investigation of glutathione transport and metabolism in human erythrocytes infected with *Plasmodium falciparum*, Eur. J. Biochem. 250 (1997) 670–679.
[2] H.W. van Veen, W.N. Konings, The ABC family of multidrug transporters in microorganisms, Biochim. Biophys. Acta 1365 (1998) 31–36.
[3] W. Saurin, M. Hofnung, E. Dassa, Getting in or out: early segregation between importers and exporters in the evolution of ATP-binding cassette (ABC) transporters, J. Mol. Evol. 48 (1999) 22–41.
[4] P. Borst, R. Oude Elferink, Mammalian ABC transporters in health and disease, Annu. Rev. Biochem. 71 (2002) 537–592.
[5] S.A. Peel, The ABC transporter genes of *Plasmodium falciparum* and drug resistance, Drug Resist. Updat. 4 (2001) 66–74.
[6] M. Ouellette, D. Legare, B. Papadopoulou, Multidrug resistance and ABC transporters in parasitic protozoa, J. Mol. Microbiol. Biotechnol. 3 (2001) 201–206.
[7] M. Dean, S. Shulenin, T. Annilo, Z.Q. Chen, in: HGM2003 Workshop, 2003, p. Presentation 49..
[8] A.C. Szeto, J. Perez-Rosado, I. Ferrer-Rodriguez, J. Vega, C. Torruella-Thillet, A.E. Serrano, Identification and expression analysis of ABC genes in *Plasmodium yoelii* and *P. berghei*, Parasitol. Res. 92 (2004) 1–11.
[9] M.B. Reed, K.J. Saliba, S.R. Caruana, K. Kirk, A.F. Cowman, Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*, Nature 403 (2000) 906–909.
[10] A. Klokouzas, S. Shahi, S.B. Hladky, M.A. Barrand, H.W. van Veen, ABC transporters and drug resistance in parasitic protozoa, Int. J. Antimicrob. Agents 22 (2003) 301–317.
[11] G.E. Tusnady, I. Simon, Principles governing amino acid composition of integral membrane proteins: application to topology prediction, J. Mol. Biol. 283 (1998) 489–506.
[12] E. Bakos, R. Evers, G. Szakacs, G.E. Tusnady, E. Welker, K. Szabo, M. de Haas, L. van Deemter, P. Borst, A. Varadi, B.

Sarkadi, Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain, J. Biol. Chem. 273 (1998) 32167–32175.

[13] J.D. Thompson, D.G. Higgins, T.J. Gibson, Clustal-W—improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, Nucleic Acids Res. 22 (1994) 4673–4680.

[14] A.R. Berendt, D.L. Simmons, J. Tansey, C.I. Newbold, K. Marsh, Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*, Nature 341 (1989) 57–59.

[15] T. Tiffert, H.M. Staines, J.C. Ellory, V.L. Lew, Functional state of the plasma membrane Ca^{2+} pump in *Plasmodium falciparum*-infected human red blood cells, J. Physiol. (Lond.) 525 (2000) 125–134.

[16] Z. Bozdech, M. Llinas, B.L. Pulliam, E.D. Wong, J. Zhu, J.L. DeRisi, The transcriptome of the intraerythrocytic developmental cycle of *Plasmodium falciparum*, PLoS Biol. 1 (2003) E5.

[17] K.G. Le Roch, Y. Zhou, P.L. Blair, M. Grainger, J.K. Moch, J.D. Haynes, P. De La Vega, A.A. Holder, S. Batalov, D.J. Carucci, E.A. Winzeler, Discovery of gene function by expression profiling of the malaria parasite life cycle, Science 301 (2003) 1503–1508.

[18] L. Florens, M.P. Washburn, J.D. Raine, R.M. Anthony, M. Grainger, J.D. Haynes, J.K. Moch, N. Muster, J.B. Sacci, D.L. Tabb, A.A. Witney, D. Wolters, Y.M. Wu, M.J. Gardner, A.A. Holder, R.E. Sinden, J.R. Yates, D.J. Carucci, A proteomic view of the *Plasmodium falciparum* life cycle, Nature 419 (2002) 520–526.

[19] E. Lasonder, Y. Ishihama, J.S. Andersen, A.M.W. Vermunt, A. Pain, R.W. Sauerwein, W.M.C. Eling, N. Hall, A.P. Waters, H.G. Stunnenberg, M. Mann, Analysis of the *Plasmodium falciparum* proteome by high-accuracy mass spectrometry, Nature 419 (2002) 537–542.