Molecular cloning, characterization and localization of PfPK4, an eIF-2 α kinase-related enzyme from the malarial parasite *Plasmodium falciparum*

Jörg J. MÖHRLE^{*1}, Yi ZHAO^{*2}, Barbara WERNLI^{*3}, Richard M. FRANKLIN and Barbara KAPPES^{*†4}

^{*}Department of Structural Biology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland, and [†]Swiss Tropical Institute, Socinstrasse 57, CH-4002 Basel, Switzerland

PfPK4, a protein kinase gene from the human malarial parasite *Plasmodium falciparum*, has been cloned utilizing oligonucleotide probing. The gene encodes a protein of a predicted length of 1123 amino acids, and within this amino acid sequence all the conserved regions characteristic of protein kinases can be identified. The catalytic kinase domain possesses highest identities (34–37%) with eukaryotic initiation factor-2 α (eIF-2 α) kinases, especially haem-regulated inhibitory (HRI) protein kinases. There are two kinase inserts in PfPK4, located at positions common to eIF-2 α kinases. The first insert separates kinase subdomains IV and VI by 559 amino acids, and the second subdomains VII and VIII by 41 amino acids. Both inserts are larger than their homologues in eIF-2 α kinases. The sequence of PfPK4 has one putative haemin-binding site. The recombinant protein, expressed in *Escherichia coli*, phosphorylates a synthetic peptide representing a substrate of eIF-2 α kinases. Autophosphorylation

and substrate phosphorylation are inhibited by haemin. Thus PfPK4 appears to be the first protozoan protein kinase related to eIF-2 α kinases and might be the first non-mammalian HRI kinase. Western blots indicated that the protein is expressed as major forms of 80 and 90 kDa. Whereas the 80 kDa form is present throughout the intraerythrocytic development and in merozoites, the two 90 kDa forms are only found in mature parasites. One of the latter is also present in the membrane fraction of erythrocytes harbouring segmenters. Confocal microscopy detected the protein distributed throughout the trophozoite, whereas it was found in discrete foci (punctate distribution) in segmenters. PfPK4 co-localizes with *P. falciparum* 83 kDa antigen/apical membrane antigen-1 at the apical complex in segmenters and merozoites, but does not co-localize with rhoptry-associated protein-1.

INTRODUCTION

Protein synthesis in eukaryotic cells is a highly regulated process. Protein phosphorylation is the major method of translational control [1]. One control mechanism involves the phosphorylation of the α subunit of eukaryotic translation-initiation factor-2 (eIF-2 α). eIF-2 mediates the binding of the charged initiator tRNA (Met-tRNA_i) to the 40 S ribosomal subunit in eukaryotic cells [2]. This reaction depends on GTP, which is hydrolysed to GDP before association of the 60 S ribosomal subunit with the 40 S ribosomal subunit complex. eIF-2 is then released from the ribosome as an inactive complex bound to GDP. eIF-2B, a guanine nucleotide exchange factor, catalyses the exchange of GDP for GTP. When eIF-2 α is phosphorylated at a specific serine residue (Ser⁵¹), the binding affinity of eIF-2(α P)–GDP for eIF-2B is much higher than that of eIF-2–GDP, and the GDP to GTP exchange is impaired. eIF-2B is available in much smaller amounts than eIF-2 and thus its concentration is limiting. eIF-2B activity is inhibited as soon as the amount of phosphorylated eIF-2 α exceeds the amount of eIF-2B, which is sequestered and removed from the initiation process.

Three forms of eIF-2 α kinase have so far been characterized [3]: the mammalian double-stranded RNA-dependent kinase

(PKR), the mammalian haem-regulated inhibitory (HRI) kinase (also called haem-controlled repressor) and the yeast general control non-derepressable (GCN2) kinase. PKR from man [4,5], mouse [6,7] and rat [8], HRI from rabbit [9] and rat [10], and the yeast GCN2 [11,12] kinase have been cloned and sequenced. The primary structures of the catalytic domains of these kinases are similar and reveal characteristics that distinguish them from other protein kinases. The most striking feature is the presence of a 35- (PKR) to 135- (HRI and GCN2) amino acid kinase insert sequence between kinase subdomains IV and VI. Another insert, situated between subdomains VII and VIII, is 13–30 amino acids long.

PKR is induced by interferon and activated by low concentrations of double-stranded RNA produced during replication of certain viruses [13,14]. PKR acts as part of the anti-viral defence mechanism that inhibits protein synthesis in virally infected cells. The yeast GCN2 kinase is activated by amino acid starvation. Phosphorylation of eIF-2 α is required for increased translation of GCN4, which is a transcriptional activator of more than 40 genes encoding enzymes in amino acid-biosynthetic pathways [15]. HRI is an eIF-2 α kinase that had originally been isolated and studied in reticulocytes. In these cells, HRI is controlled by haemin, the precursor of the prosthetic group of haemoglobin.

Abbreviations used: eIF-2, eukaryotic initiation factor-2; GCN2, general control non-derepressible-2; HRI kinase, haem-regulated inhibitory kinase; HRM, haem-regulatory motif; IPTG, isopropyl 1-thio- β -D-galactopyranoside; MSP-1, merozoite surface protein-1; NTA, nitriloacetic acid; PF83/AMA-1, *Plasmodium falciparum* 83 kDa antigen/apical membrane antigen-1; PfPK4, *Plasmodium falciparum* protein kinase 4; PKR, double-stranded RNA-dependent kinase; RACE, rapid amplification of cDNA ends; RAP-1, rhoptry-associated protein-1.

¹ Present address: Glaxo Wellcome SA, Bahnhofstrasse 5, CH-3322 Schönbühl, Switzerland.

² Present address: Department of Biological Chemistry, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109, U.S.A.

³ Present address: Bioreba AG, Christoph-Merian Ring 7, CH-4153 Reinach, Switzerland.

⁴ To whom correspondence should be addressed at the Swiss Tropical Institute.

The nucleotide sequence depicted in Figure 2 has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X94118.

Decreased haemin concentrations activate HRI leading to inhibition of protein synthesis [13,16,17]. This mechanism closely couples globin synthesis with the availability of haemin for haemoglobin biosynthesis. Mellor et al. [10] reported cloning of HRI from rat brain and found HRI mRNA in non-erythroid cells, which suggests that it has roles other than the control of haemoglobin synthesis. It is notable, however, that the level of HRI mRNA is much lower in non-erythroid tissues. Interaction of HRI with several heat-shock proteins (hsp90, hsp70 and p59) has been reported [18].

Surolia and Padmanaban [19] described the presence of haem-dependent eIF-2 kinase activity in the malarial parasite *Plasmodium falciparum*, acting in a similar way to rabbit reticulocyte HRI. An increase in eIF-2 α phosphorylation is observed after chloroquine treatment of rabbit reticulocyte lysate, parasitic lysate and parasites, coinciding with a decrease in protein synthesis. eIF-2 α phosphorylation is partially reversed after the addition of haemin to chloroquine-treated rabbit reticulocyte lysate and parasitic lysate.

In a general survey of *P. falciparum* protein kinase genes using degenerated oligonucleotide probes directed against conserved subdomains of Ser/Thr protein kinases [20], we isolated a cDNA clone the deduced amino acid sequence of which displayed the highest level of identity with eIF-2 α kinases. In the present paper we report the complete sequence of this putative *P. falciparum* eIF-2 α kinase gene, its stage-specific expression and localization in the parasite. The kinase was expressed in *Escherichia coli* and exhibited autophosphorylation and substrate phosphorylation activity which could be inhibited by haemin. This is the first identification of a protozoan eIF-2 α -related protein kinase and the first HRI isolated from a non-mammalian source.

MATERIALS AND METHODS

cDNA and genomic DNA libraries and screening

The strain *P. falciparum* K1 λ NM1149 cDNA library [21] and λ NM1149 *Eco*RI genomic DNA library [22] were kindly provided by J. Scaife (University of Edinburgh, Edinburgh, Scotland, U.K.). The ZAP cDNA library in λ ZAP vector (Stratagene) was constructed from malarial mRNA as described [23].

Approx. 2×10^5 recombinant phages from the λ NM1149 cDNA library were screened with end-labelled probe A, a 27-mer redundant oligonucleotide pool, 5'-TTC(T/A)GG(T/A)GC-(T/A)AT(A/G)TA(A/G)TC(T/A)GG(T/A)GT(T/A)CC-3', which corresponded to a protein kinase consensus sequence of subdomain VIII [20]. The sequences of the oligonucleotides were chosen following the codon usage in *P. falciparum* [24,25]. Hybridization and screening were carried out as described [23].

Probe B (5'-ATTATCATGAGCTATCATTTGTTGGTC-ATTTCC-3'), designed from the sequence of the clone identified by probe A, was used to screen the ZAP cDNA library by hybridization in $6 \times$ SSC at 50 °C and washing in $2 \times$ SSC/0.1% SDS at 50 °C (where $1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate).

Probe C (5'-TCACTCAAAAATGGATTGTGATTTTCT-CAGG-3'), designed from the sequence of the above clone, was used to screen the *Eco*RI genomic library under the same conditions as above.

Sequencing

The sequencing reactions were carried out with the USB kit following their protocols. Sequences were determined from both DNA strands.

Analysis of DNA and protein sequences

Analysis, comparison and sequence alignments of DNA and protein sequences were performed using the GCG sequence analysis software package, version 7.2 and version 8.0-open VMS [26,27].

Isolation of the 5'-end

Total RNA was isolated from *P. falciparum* as described [28]. Polyadenylated RNA was prepared using oligo(dT)-cellulose columns from Pharmacia following the manufacturer's protocol. First- and second-strand cDNA were then synthesized as in the protocols given in the Marathon RACE kit (Clontech [29]). A 1:200 dilution of this cDNA was used as template for the first PCR. The reaction mixture was 200 μ M dNTPs, 10 pmol of each primer and $10 \times$ *Taq* extender buffer (Stratagene). The primers were gene-specific primer 1 (GSP1; complementary to nucleotides 388–411; 5'-CACAACGTCAGTCTACTTCCACTACTATT-ATAACC-3') and anchor primer 1 (AP1), supplied with the Marathon RACE kit. A hot start was performed using 5 units of *Taq* polymerase (Boehringer) and 5 units of *Taq* extender (Stratagene). The PCR protocol was 30 cycles of 30 s at 92 °C/30 s at 60 °C/2 min at 72 °C and a termination cycle (30 s at 92 °C/30 s at 60 °C/10 min at 72 °C). The reaction product was diluted 1:100 and 1 μ l of it was used as a template for a second nested PCR. The reaction mixture was the same as above using a second gene-specific primer (GSP2; complementary to nucleotides 339–371; 5'-GCTTTACATTCATTTTATTAATGATCCACC-3') and anchor primer 2 (AP2) provided with the Marathon RACE kit. The protocol was the same as for the first round of PCR.

Using GSP3 (5'-TTCTTCATAAACTTCTTATTAATAAT-TGG-3'; complementary to nucleotides 302–329) and AP2 as nested primers, the diluted reaction products from the first round of PCR were subjected to a second PCR procedure using the same reaction mixture and cycling conditions as described above. Reaction products were cloned in vector pBluescript SK⁺ cut with *Eco*RV.

Cloning in the expression vector

To clone PfPK4 into the expression vector, PCR was carried out using the genomic clone PfPK4C in the pEMBL19 phagemid as a template with primer 4 (5'-CAACAATTATGGAACG-ATATAAAAG-3'; nucleotides 3028–3052), containing an *Nco*I site, and primer 5 (5'-AATGAGAATAATGGTGCAGAC-AAAAAGCTTAGGTAC-3'; complementary to nucleotides 3414–3437). Primer 5 introduced a *Hind*III site in-frame in front of the stop codon of the genomic clone. The reaction mixture was the same as described above. After a hot start, PCR was performed as above. The protocol was 10 cycles of 30 s at 92 °C/30 s at 57 °C/2 min at 72 °C, 20 cycles of 30 s at 92 °C/30 s at 54 °C/2 min at 72 °C and a termination cycle (30 s at 92 °C/30 s at 54 °C/10 min at 72 °C). The reaction product was cut with *Hind*III and *Nco*I and ligated into pBluescript SK⁺. To verify the sequence after PCR, this clone was completely sequenced. The genomic clone PfPK4C was cut with *Nco*I and *Hind*III, *Nco*I cutting the genomic clone at base 2854 and *Hind*III cutting inside the multiple cloning site of pEMBL19. The *Nco*I–*Hind*III-digested PCR product was ligated into this plasmid and the altered genomic clone PfPK4E (expression) was excised by digestion with the restriction enzymes *Eco*RI and *Hind*III and ligated into plasmid pET22(b) (Stratagene) creating pET-PfPK4E.

Figure 1 Schematic representation of the Pfk4 gene

Restriction sites shown are B = *Bgl*II and E = *Eco*RI. The coding sequences are represented by open boxes, and the 5'- and 3'-non-coding sequences are shown as black boxes. The ATG start codon and the TAG stop codon are indicated. Pfk4A, cDNA clone from the λ NM1149 cDNA library isolated with probe A; Pfk4B, cDNA clone from the λ ZAP cDNA library isolated with probe B; Pfk4C, genomic clone from the λ NM1149 genomic library; Pfk4, the composite of Pfk4C and the 5' sequence obtained by Marathon RACE.

Expression in *E. coli*, purification and subsequent kinase assay

The plasmid pET-Pfk4E was expressed in *E. coli* BL-21 (Stratagene) with induction by isopropyl 1-thio- β -D-galactopyranoside (IPTG). The expressed recombinant protein contained six histidine residues at its C-terminus. Expression and purification were carried out using the protocol described by Ross-MacDonald et al. [30].

Kinase assays were performed using the beads from a 50 ml culture; the beads were resuspended in 20 μ l of kinase assay buffer (20 mM Tris/HCl, pH 7.5, 5 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA) supplemented with 1 mM 1,4-dithio-D,L-threitol. Substrate phosphorylation was assayed by adding the synthetic oligopeptide P(45–56) (I-L-L-S-E-L-S-R-R-I-R) [31] to the kinase mixture to a final concentration of 2 mM. The reactions started by the addition of [γ -³²P]ATP (5 μ Ci/reaction; Amersham; specific radioactivity > 3000 Ci/mmol) were performed for 30 min at 30 °C and stopped by addition of 4 μ l of 1.5 M H₃PO₄. To test for haem-dependence of P(45–56) phosphorylation, haemin (Sigma) was added to the kinase mixture to a final concentration of 10 μ M; stock solutions of haemin were always prepared just before use. The reaction mixtures were spotted on to Whatman P81 phosphocellulose paper circles, which were dried for 45 min at ambient temperature, washed five times with 150 mM H₃PO₄, and dried under an infrared lamp. The circles were transferred to plastic scintillation vials and analysed for radioactivity in a liquid-scintillation spectrometer (Packard Instrument Company). Each assay was carried out in triplicate. The standard deviations associated with control protein kinase assays were about 10% of mean values.

Antisera to different fragments of Pfk4

To clone the sequence coding for the kinase subdomains VI–XI (amino acids 868–1049) into the expression vector, a PCR was performed with the 36-mer oligonucleotide probes pepB1 (5'-TGAGTTGGATCCAAACAATTAATTAAGGTCTTAA-3'; complementary to nucleotides 2670–2693) and pepB2 (5'-CACATAGGATCCCAAATGAGGATCTAGCAATACTT-3'; complementary to nucleotides 3192–3215) using the cDNA clone Pfk4A in the pEMBL19 phagemid as a template. To

clone the fragment C-terminal to subdomain XI (amino acids 1050–1123) the 36-mer oligonucleotide probes pepC1 (5'-CATCTCGGATCCACTGATTTTGCATTTAGCTTTAAC-3'; complementary to nucleotides 3216–3239) and pepC2 (5'-GTACCTGGATCCTTTGTCTGCACCATTATTCTCATT-3'; complementary to nucleotides 3414–3437) were used. All four oligonucleotides had 12 additional bases at the 5'-ends of the coding sequences in order to introduce *Bam*HI sites.

The PCR mixture was the same as described above; a hot start was carried out with 5 units of *Taq* polymerase (Boehringer) and 5 units of *Taq* extender (Stratagene). The protocol was 10 cycles of 30 s at 92 °C/30 s at 57 °C/2 min at 72 °C, 20 cycles of 30 s at 92 °C/30 s at 54 °C/2 min at 72 °C, and a termination cycle (30 s at 92 °C/30 s at 54 °C/10 min at 72 °C).

The resulting PCR fragments were cut with *Bam*HI and cloned into the expression plasmid pDS56/RBSII (6 $\times$ His) of Stueber et al. [32]; the plasmid containing the kinase subdomains was called p6His/pepB, the one containing the C-terminus p6His/pepC. The two constructed plasmids were expressed in *E. coli* strain SG13009 [33] with induction by IPTG, as described [34]. The expressed polypeptides contained six histidine residues at their N-terminus to facilitate purification. The histidine fusion proteins were partially purified by denaturing affinity chromatography on a Ni/nitriloacetic acid (NTA) column (NTA-resin (Qiagen) [32]), followed by preparative SDS/PAGE (Bio-Rad). To raise antisera against pepB and pepC, 500 μ g of the purified fusion proteins were mixed with complete Freund's adjuvant and injected into rabbits, which were boosted at monthly intervals with 500 μ g of fusion protein in Freund's incomplete adjuvant.

Parasite synchronization and protein preparation

P. falciparum isolate K1 (Thailand), adapted to grow in horse serum, was kindly provided by Dr. H. Matile (Hoffman–LaRoche, Basel, Switzerland). The techniques of parasite synchronization, isolation, lysis and protein preparation, as well as separation of the various cellular fractions, have all been described previously [35]. Parasites of four successive ages were harvested: (1) rings, (2) trophozoites, (3) schizonts and (4) segmenters.

	-68	AAGAATATTTCCTATTATATAGAAAGGCGACATCTCAGGATGTACTTAATAAAAACTTTTGTT	-1
1	1	ATGAAAAAACCGATACGTAGTAGTTATAAAGTTGGTAGTAGCAATAAATATCATCAAGAAAATATACAGATAATGAAAGGATAAGAAA	90
		M K K R I R S S Y K V G S S N K Y H K K N Y T D T N E K D K K	30
91	31	AAATATCTAGTTTATAAGAAAACAATAATAAAGAAANMTGCCGATATTAAGAAATTTCTGAACCTTTTAAACAAATTTTATAAGAAAG	180
		K Y R S Y S Y E K E K H I N E K M F L N D L N F L T N F N K	60
181	61	TTTTAGAAAGAAATCTTTGGTGATCATTTAATGAAATGAATGATAAAGCAGAAGATAATTATGATGGTTATATAAGTAGTGGAAGT	270
		F M K K N N S L V D H L M K M N D K A E D N Y D G Y N S S G S	90
271	91	AGATAATAATAATAAATGATGATGGTGTGAATTTGTGGTACTAAAGAGATACAGAAATAAAAAATATAGTGATATGATGATAATTAT	360
		R Y N N D I T A T D G V E L C T K R Y T N N K N N S D Y D N Y	120
361	121	AATAATAATAATAATGAAATAAAGATATTCCAATAAGAACATAATAAGATAATATTATTATTAATAATAATAACAACATAT	450
		N N N N N M K N K R Y S N K K H N N D N I I I N N N N N K Y	150
451	151	ACAGATGAAGGAAATATCGAAATAAAGATATAAAAGAAGATATACAAATGATTTATTAATATACAATTAATAATAATAATA	540
		T D E R K Y R N K S I K E D V D Y T N D Y N I Q L N N K	180
541	181	ATAAATAACAATCAAACAAAAATAAAATCGATACTATTAGAAATATTAGTCATGAGAAATAGGTAAATAAAAGTCGATGCTAGG	630
		I N N N Q T K T K N K I D T I R N I S H E K L G N N K S S S A R	210
631	211	AAITTTCTTTAATCAACACATCTCATATCCATACGATGCTCCATTTAGTGTATTTTTCGAGAAATCGCAGCTAATAGAGATATATAGTAA	720
		N L S L I Q T S H I P Y D A P L A D F L E N G R F L R T F E	240
721	241	AATATTCTCTTATAGGACAGGGAGGATTTGGTTCAGTTTATAAGATATCACATGATTAGAACCCTGATCTCCAACATATGCTGTGAA	810
		N I S L I G Q G G F G S V Y K V S H R L E P G S G T P Y A V K	270
811	271	TTTATTTATTTAAAGTAAAGTATAGATAATGTAAGTTCAAGGAGATATTTTCGAGAAATCGCAGCTAATAGAGATATATAGTAA	900
		F I Y L K V S S L D N V S S R Y R F R E I A N R D I Y S K	300
901	301	CATGTTGTTCGATATTTACATGGTGGTGAAGAACCTCAATTTTACCTATGATCTGATGCCAAAAGAAATCCAAACCTTAGTAA	990
		H V V R Y R Y T W W C E E P Q F L P M H L M P K E I Q N L V K	330
991	331	AAGAATAAGATACCTTTAAAAAAGACCTTACCAAAAAATAAGATATAGTAATAATGTATAAGTATAGTAAATAACAATAGT	1080
		K N K D T F K K R L T T K N K K Y S N N C I S D S N N N S	360
1081	361	AGTTGTTATATAGCTAGTGTATTAATTCAGTATTAAAGTAATTTATAGAAATAGAAATTTAGGATAAAAAAGAAAGAACAAAGCTCT	1170
		S C Y S A S S S I N S S I N S Y N R N M K L W I K K K E Q S P	390
1171	391	GATATGAAGATATAAAGAGGTTCTTAGAAGAAATAATGCTCCTAAATTTAGTTTATTTATAGTGATAACGATGTTTAACTATGAAGAT	1260
		D M K R Y K E V L R K N N A P N L V F Y S D N D G L T S K N	420
1261	421	AAGGAAATCTGAGAAAAACACAATCCATTTTGATGATAAAAAATTTTCGTCTTATATATAAAAAAGAAAGGATCATGATTAT	1350
		K E N P E K N H N P F L S D K N F S D S I Y K K K K S H D Y	450
1351	451	AAATCTAGCACCAACTTGA AAAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAGAAAG	1440
		N S S S H K L K K R K N K K K K K K K K K K K K K K K K	480
1441	481	GCTCAAGGAATATAGAGAAAGTGAAGACGTAAGGAAGAGATCACTTTCAATATAAAAAAGAAAGGAACAGTTTCTAAATTTATA	1530
		A Q G G I Y E E S E N D E G R D H F Q Y K K G K E Q F S K F I	510
1531	511	GGAAACATAATCTTATGGGTTTACACAAAGTTTCCAGAATATGATCTTTTGATATAAGTTTATTTAGTGGAAGAGATAGAGATCTT	1620
		G K L I L W V L H K V S K N M I L I M V I L S E E D R D L	540
1621	541	ATAGTTTTCGCCGATATGAAGAATCAAAATGGAATGAACCAAAATGATACGTATGATAATATGAACAATGAAATGTTTATTAAG	1710
		I V F A D N E S E N G N D Q Q M I R H D N M G N N E N V I I K	570
1711	571	CATAGAAATGAAGATGATAAAATGGATTTGGATGGTGACAAAAATGGATTTGGATGGTGATGGATGGTGATGGTGATGGTGATGGT	1800
		H R N E D <u>D K N G L D G D K N G L D G D K N G L D G D K N G</u>	600
1801	600	<u>TTGGATGGTGACAAAAAGCAATTTGGATGATAATAAAAGCAATTTGGATGATTATTAATGAACAAAAATTAATAGTTTAAACAGAAAC</u>	1890
		<u>L D G D K N E L D D N K K N E L D D L L M K Q K I N S L T R N</u>	630
1891	631	GATATGTTTAATATTGAGATGAGAACCAGCTCCTCATGCTACTAATAATATTAATAAATAAAGGTTGATTTAAATGGAGAATTAAC	1980
		D I V I N E I E N E N P A H T I N I K N K K L D N G E L T	660
1981	661	TATTATGATTACGTTGGAAAAATGAAGTCATACCAAAATCTCGAACCGAAACGAATGTTGAAGATATTAATACGAATGTTATGTTAAT	2070
		Y Y D A Y V G K N E V I P N S R T E T N V E S I N T N G M F N	690
2071	691	AATAAATTTCTGTATGAAGACGAGAGGGGAATATAAGAAAAAGAAACATGACCTGGGAGACAGCAAGAGGATGGTTTATAT	2160
		N K F S V M T K D G E G G E Y K K K E N M T W G D T K R D G L T	720
2161	721	GAAAAAGGAAACAGCAAAAGATGGTTTAGGTGTTATAAATGATATAACAATAAATAATATTTGAAATGATGATGACGATGATGATGAT	2250
		E N G K H E K D G L G V N K C I T N K Y I E N <u>D D D D D D D</u>	750
2251	751	GATGATGATAATAATAATAATAATATAGATGAAGGAAAAAGACCTAAGAAAGAAACAAAAATGCTATTACTAAGAGTATGA	2340
		<u>D D D N N N N N N N I</u> D E R K L L K K K Q K N A I T K G N E	780
2341	781	GATTGTGTAGCAAAATGGTACAAATAATAAGAGAGAAAGAAAGAGGATGATGATAATAAAAAATATGAAAAATAAAATCATAT	2430
		D L L A T N G T N N K E K R K K D D I N K N M E K I K S Y	810
2431	811	AAAAAGAAACACCACTTCTGAATTTTCTATAGTTTATTTATGACATGGAATTTGTATAAGGATATACCTTACGTAAATGGCTAGAT	2520
		K K K T P V P E F S I V L L Q M E L C K G Y T L R K W L D	840
2521	841	AGATCTACGAGAAAGTGACAAACCTTTACATTTTACTTATAGTATGATAAAAAATGAATCATCACTAGAAATTTGACTTATTTAAACAATTA	2610
		R S T R S D K P L H F T Y S D K K M N H P L E F D L F K Q L	870
2611	871	ATTAAAGGCTCTAAGAAATTTCACTGCTATGTTTATTTATGACATGGAATTTTGTATAAGGATATACCTTACGTAAATGGCTAGAT	2700
		I X G L K D H I A T C T F I H R D L K P E N I F V D N D N T Y	900
2701	901	TTAAAGATTOGAGATTTAGGATTAGTACGATTATTGTAAGAGAGAAAGAGAGAAAGGATTTTAAACAATATGATGTTTATAAAGATAAT	2790
		L K I G D L G L V R F I E E K K R E K D F N N I D D C Y K D N	930
2791	931	ATATATACAGATTAATTAACAGATTAAGTATGACATATGAACCAACCACTCAGGGAACCTAATAACTTTGAAGAACTTACAGCAGAGAA	2880
		I Y T D I N Q N R I T S Q I S I K G Q Q I I <u>E T P G Y T A P E</u>	960
2881	961	GOTGAGACTTTATGATGAAAAAGCAGATATATATAGTCAGCTTTTAATTTTATAGAATGTTATGTCACCGTTTACACAAATATG	2970
		G G A L C D E K A I I Y S A A L I L L L E L L C P R F T I M	990
2971	991	GAACGATATAAAGATTAATGATTTATATACGATACCTGATGATGATAAATAAAGTATGTAAGAAATCACTTAAACCCATGGTATTTTAAG	3060
		E Y R K R L N D F R N Y Y T V P D Y V K I H L N P W Y I L M	1020
3061	1021	TTACAAATGTCAAAAACCAACTACAGAGATAGACCATCGGCTGCTGATGTTATAGTAAAAATTAAGTATGCTAGATCTCATTTGACT	3150
		L Q M S K P N P A D R P S A A D V Y S K I K V L L D P H L T	1050
3151	1051	GATTTTCTTTAGCTTTTAACGATATTCAATATGACATATGAACCAACCACTCAGGGAACCTAATAACTTTGAAGAACTTACAGCAGAGAA	3240
		D F A F S F N D I H N E H M N K P P Q T T N N F E R I T D N	1080
3241	1081	AAGGACAAATTCGTAATAACAAGGTGTTGGATATGAAGATAAAGTAGAAAAATGAAGAAATACCTATTGAAAAAGGATTAATAGTAAT	3330
		K D K F V I Q S V V D M K N V E N E E I P I E K G L N S	1110
3331	1111	GTAGAGATATAAAAAATGAGAAATAAGTGGTCAGACAAATAGTGCAGCTGTGTAATTATATATATAGAGAGAAAGAAATTC	3407
		V E N I K N E N N G A D K *	1121

Merozoite preparation

Merozoites were prepared by a modified protocol of Freeman and Holder [36]. The parasites were synchronized twice by sorbitol treatment as described [37]. At 3 h after the second synchronization, the cultures were washed with digestion buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 10 mM glucose, 1 mM CaCl₂), resuspended to a haematocrit of 10% in digestion buffer, and treated with 1 mg/ml trypsin (type XI; diphenyl-carbamoyl chloride-treated; Sigma) for 60 min at 37 °C. The cells were pelleted by centrifugation at 820 g, and washed twice in complete RPMI 1640 medium. They were then resuspended in complete medium to a haematocrit of 5% and incubated for a further 24 h. Released merozoites were pelleted from the supernatant by centrifugation at 3300 g. The merozoite pellets were washed and resuspended in RPMI 1640 for preparation of microscope slides and in lysis buffer (50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1% Triton X-100) for Western blotting.

Affinity purification of antisera

Proteins pepB and pepC were expressed in *E. coli* and partially purified by denaturing affinity chromatography to Ni/NTA beads and SDS/PAGE, as described above. A 6 mg portion of each of the recombinant proteins was dialysed against 5 × 2 litres of coupling buffer 1 (CPI; 0.1 M NaHCO₃, 0.5 M NaCl, 0.1% SDS) and coupled to CNBr-activated Sepharose beads (CNBr-beads; Pharmacia) following the manufacturer's instructions. Peptides pepB and pepC coupled to Sepharose were used for affinity purification of the antisera as described by Harlow and Lane [38].

Immunoblotting

Specific fractions of *P. falciparum* and erythrocyte membranes were separated by SDS/PAGE (8% gel), and the resolved polypeptides blotted on to a Hybond-C membrane (Amersham) as described by Graeser et al. [39]. Affinity-purified anti-pepB and goat anti-rabbit-peroxidase conjugate (NORDIC) were used at a dilution of 1:5000. The blot was developed using the ECL system (Amersham) according to the protocol supplied by the manufacturer.

Confocal laser scanning microscopy

Parasitized erythrocytes were washed twice in RPMI 1640 at a haematocrit of 20%. Then 10 μ l of the cell suspension was fixed and permeabilized for 30 min with 20 μ l of 4% paraformaldehyde containing 0.1% Triton-X100 on microscope slides, which were precoated with poly-(L-lysine). The fixed cells were washed five times with PBS for 3 min each, followed by blocking of unspecific ligands with blocking solution [PBS containing 1% BSA (Sigma A 7030)] for 15 min. The parasites were double-labelled with affinity-purified α pepC antiserum at a dilution of 1:50 in blocking solution and mouse monoclonal α rhoptry-associated protein-1 (RAP-1) antibody 2.13 at a dilution of 1:1000 or mouse monoclonal α merozoite surface protein-1 (MSP-1) antibody 111.2, at a dilution of 1:100 for 60 min. Five 3 min washes

with blocking solution followed. The secondary antibodies donkey anti-rabbit-Cy5TM conjugate (Jackson ImmunoResearch Laboratories) at a 1:400 dilution and goat anti-mouse-FITC conjugate (Sigma Immuno Chemicals) at a 1:100 dilution were applied for 60 min. The slides were washed again five times with PBS, and mounted in 90% glycerol in 100 mM Tris/HCl, pH 8.0, containing 0.2% *o*-phenylenediamine.

Double-labelling with α pepC antiserum and α *P. falciparum* 83 kDa antigen/apical membrane antigen-1 (PF83/AMA-1) monoclonal rat antibody was carried out in four steps. The fixed and permeabilized parasitized erythrocytes were washed as described above, labelled with α pepC antiserum (dilution 1:50) for 60 min, and washed five times with blocking solution. The donkey anti-rabbit-Cy5TM conjugate was applied for 60 min, and the slides were washed again five times with blocking solution. Then the cells were labelled with the α PF83/AMA-1 monoclonal rat antibody 28G2 (concentration 25 μ g/ml) for 60 min, followed by five more washes. Rabbit anti-rat-FITC conjugate (Vector Laboratories), dilution 1:50, was applied for 60 min. The slides were washed with PBS and mounted as described. Microscope slides were examined on a confocal laser scanning microscope (NORAN, ODYSSEY XL). The rat monoclonal α PF83/AMA-1 antibody was a gift from A. W. Thomas (Biomedical Primate Research Centre, Rijswijk, The Netherlands), the mouse monoclonal α RAP-1 antibody 2.13 was a gift from R. Ridley (Hoffman-LaRoche), and the mouse monoclonal α MSP-1 antibody 111.2 was a gift from A. A. Holder (National Institute of Medical Research, Mill Hill, London, U.K.).

RESULTS

Isolation of the malarial PfPK4 gene

Screening of a λ NM1149 cDNA library with probe A under reduced stringency conditions resulted in the isolation of one positive clone, PfPK4A (Figure 1). This clone had the original *Eco*RI cloning sites of λ NM1149, and therefore this enzyme was used to excise the phage insert. Digestion resulted in a 1.8 kb fragment which was subcloned. The sequence hybridizing to probe A was located at position 1269–1295. We identified an open reading frame of 1762 bp terminating with a TAG stop codon at position 1763, but lacking the 5' ATG initiation codon. To isolate the complete PfPK4 gene, we screened a λ ZAP cDNA library with probe B, derived from the sequence of PfPK4A. A total of 24 positive clones were detected and six of them were analysed further; all contained the same 528 bp insert, PfPK4B. This 528 insert had an exact overlap to PfPK4A of 174 bp and an additional 354 bp 5' to the previously isolated clone. The sequence homologue to probe B is located at position 409–441 of PfPK4B.

Since screening of cDNA libraries did not yield the complete PfPK4 gene, a λ NM1149 *Eco*RI genomic library was screened with probe C (derived from PfPK4B), resulting in one positive clone, PfPK4C, which contained a 3218 bp insert. PfPK4C is identical with PfPK4A and PfPK4B in the overlapping sequences. The sequence of the probe used to identify this clone is located at nucleotides 1081–1112 of PfPK4C. Unfortunately, this clone did not contain the 5'-end of the gene and further screening of

Figure 2 Nucleotide sequence and predicted amino acid sequence of PfPK4

The nucleotide sequence represents a composite of sequences determined from genomic and cDNA clones and sequences obtained by PCR. The derived amino acid sequence is indicated in one-letter code beginning at the first methionine residue preceding a long open reading frame and terminating at the asterisk. The sequence targeted by probe A is boxed and printed in bold. Boxed sequences represent (1) heptamer amino acid repeats and (2) an aspartate/asparagine-rich structure in the kinase insert 1.

Figure 3 Alignment of the predicted amino acid sequence of PfPK4 with protein kinases with highest identities

The enzymes, organisms, accession numbers and fractions of the sequences aligned are as follows. PKR human, interferon-induced double-stranded RNA-dependent protein kinase from human, P19525, residues 266–531; PKR rat, double-stranded RNA-dependent eIF-2 kinase from rat, L29281, residues 235–493; PKR mouse, serine/threonine-protein kinase TIK from mouse, Q03963, residues 241–495; GCN2 yeast, protein kinase GCN2 from *Saccharomyces cerevisiae*, P15442, residues 529–905; HRI rabbit, haem-regulated eIF-2 γ kinase from rabbit, P33279, residues 169–571; HRI rat, haemin-sensitive initiation factor-2 α kinase from rat, L27707, residues 166–574; PIPK4, *P. falciparum* protein kinase 4, residues 238–1036. Residues identical with PIPK4 are boxed. The underlined regions I–XI represent the conserved kinase subdomains. Gaps (indicated by points) were introduced to optimize the alignment. The number of additional N-terminal and C-terminal flanking residues lying outside the alignments are shown at the beginning and end of each sequence.

Table 1 Kinase activity of recombinant PfPK4E and HRI

Substrate phosphorylation activity of PfPK4 and rabbit HRI were both set to 100%. Autophosphorylation and inhibition are shown as percentage of substrate phosphorylation. Control kinase assays were carried out with preparations from bacteria carrying the expression vector without the PfPK4 gene. Incorporation was about 2% of PfPK4 substrate phosphorylation activity under all the above conditions.

	Kinase activity (%)	
	PfPK4	HRI
Kinase	21	5
Kinase denatured	7	5
Kinase + haemin	1	2
Kinase + P(45–56)	100	100
Kinase + P(45–56) + haemin	38	31

Figure 4 Western blot with lysates from various fractions of the stages of the erythrocytic cycle of *P. falciparum*

The blot was probed with α pepB. I, Parasitic cytosol; II, parasitic membrane and organelle fraction; III, membranes of infected erythrocytes; C, membrane of uninfected erythrocytes as a control; M, merozoites; R, ring stage parasite; T, trophozoites; S, schizonts; Seg, segmenters. A 20 μ g amount of protein was loaded on each lane. The molecular-mass marker (10 kDa ladder) is indicated on the right.

cDNA, genomic and mung-bean endonuclease-digested genomic libraries (a gift from B. Müller-Hill, Institut für Genetik der Universität zu Köln, Köln, Germany [40]) was not successful. Therefore, to obtain the complete sequence of the PfPK4 gene, we applied the 5' Marathon RACE PCR method [29]. Using GSP1 and AP1 for the first round of PCR amplification and GSP2/AP2 for the second round, a PCR fragment of 236 bp was isolated. This fragment did not contain the 5'-end. From that sequence GSP3 was derived and used for PCR on the DNA amplified once with GSP1. This procedure resulted in a 258 bp fragment which did contain the 5'-end of PfPK4. Both 5'-RACE fragments overlapped by 103 nucleotides, and the extension from the end of PfPK4C was 313 bp in length. Both 5'-RACE reactions were performed in triplicate and individual clones of each were isolated and sequenced to avoid any sequence errors generated by PCR. The complete clone does not contain any intron sequences.

Properties of the nucleotide sequence

A total of 3475 nucleotides were sequenced. The open reading frame starts at nucleotide 1 with an ATG and ends with a stop codon TAG at nucleotide 3370 (Figure 2). There is a sequence of 68 nucleotides 5' to the start codon. The AT ratio of the coding region is 74.2%. In the sense strand the A content (45.0%) is higher than the T content (29.2%), and the content of G (16.9%) is higher than that of C (9%).

The nucleotide sequence upstream of the start codon deviates from the initiation consensus sequence AAAA/ATG, which is described for malarial genes [41]. The start codon is preceded by two adjacent stop codons at position -17 and -14.

Properties of the deduced PfPK4 amino acid sequence

The PfPK4 gene has an open reading frame of 1123 amino acids (Figures 2 and 3). The deduced PfPK4 protein sequence starts with M-K-K. The putative PfPK4 protein contains all the catalytic subdomains of eukaryotic protein kinases. Kinase subdomains VI and VIII are indicators of the substrate specificity of the protein kinase; the sequence of PfPK4 in both regions (D-L-K-P-E-N and G-T-P-G-Y-T-A-P-E) is consistent with the consensus sequence of serine/threonine kinases (D-L-K-P-N and G-T/S-X-X-Y/F-A-P-E) [20].

An unusual characteristic of the PfPK4 protein is the separation of the catalytic kinase subdomains by two large insertions, which are called kinase inserts in analogy with the insertions found in the catalytic domains of other protein kinases. The two kinase inserts of PfPK4 separate the catalytic subdomains IV and VI by 559 amino acids and the catalytic subdomains VII and VIII by 41 amino acids. A comparison of the insert sequences with sequences available in the protein databanks did not identify sequence similarities to any other proteins. The amino acid composition of the two kinase inserts differs considerably from that of the catalytic domain. Kinase inserts 1 and 2 have a significantly increased content of asparagine and lysine compared with the catalytic region. Kinase insert 1 contains a 4-fold repeat of the seven-residue motif D-K-N-G-L-D-G, followed by the sequence K-N-E-L-D-D-N-K-N-E-L-D-D, which is a variation of the previous repeat structure (amino acids 577–618) (Figure 2). Two further prominent features of the kinase insert are (a) the sequence of ten consecutive aspartates linked to six asparagines (amino acids 744–759) and (b) the clustered distribution of lysine between amino acids 443 and 479 (Figure 2).

From a search of the protein databases with the total kinase subdomains of PfPK4, minus the kinase inserts, the highest identity is with eIF-2 α kinases. The kinase subdomains are 36.6% identical with *Saccharomyces cerevisiae* GCN2 kinase, 36.4% identical with human PKR, 35.6% identical with rabbit HRI kinase, and 34% identical with murine PKR (Figure 3). These eIF-2 kinases also possess kinase inserts between subdomains IV–VI and VII–VIII. Kinase insert 1 is between 35 (mouse PKR) and 120 (HRI) amino acids in length, and kinase insert 2 between 13 (PKR) and 30 (HRI) amino acids in length. They are, however, smaller than the corresponding inserts of PfPK4 (see above). The sequences of PfPK4 outside the catalytic subdomains do not have significant homologies to proteins in the databases.

Expression in *E. coli* and kinase assays

The PfPK4C genomic clone was altered by PCR to remove the stop codon from the 3'-end and to create a *HindIII* restriction site just after the last codon of the open reading frame. This altered sequence was cloned into the expression vector pET22(b).

Figure 5 For legend see opposite page

The recombinant fusion protein contains six histidine residues at its C-terminus and is designated PfPK4E.

The purified product was active as a protein kinase and phosphorylates P(45–56), an undecamer peptide of the sequence I-L-L-S-E-L-S-R-R-I-R, containing the phosphorylation site of the eIF-2 α kinases (Ser⁵¹). We tested whether P(45–56) phosphorylation is haemin-dependent by adding haemin to the kinase assay mixture. A concentration of 10 μ M haemin in the kinase reaction mixture resulted in 61% inhibition of the ability of PfPK4 to phosphorylate P(45–56). Autophosphorylation of PfPK4E is also inhibited by the addition of haemin (Table 1). For comparison, HRI was assayed under the same conditions (Table 1).

Stage-specific expression and cellular distribution of PfPK4

To raise antisera against two fragments of PfPK4 we expressed the catalytic subdomains VI–XI (amino acids 876–1033; pepB) and the sequence C-terminal to subdomain XI (amino acids 1034–1123; pepC) of PfPK4 in *E. coli*. The recombinant polypeptides were partially purified by nickel affinity chromatography, further purified using the Bio-Rad SDS-prep system and then used to immunize rabbits (results not shown). The rabbit antisera were affinity-purified using the polypeptides as ligands.

The stage-specific expression and cellular distribution of PfPK4 during the parasitic life cycle was investigated. Parasites of synchronized stages were isolated at specific times after infection: ring stage (age 5–11 h), trophozoites (age 17–23 h), schizonts (age 27–33 h) and segmenters (age 37–43 h). Three cellular fractions from the parasite (cytosol, membrane and organelle fraction) and infected erythrocyte membrane fraction were prepared from each of these stages. These fractions and the lysate of isolated merozoites were separated by SDS/PAGE, blotted on to a Hybond-C membrane (Amersham) and probed with affinity-purified anti-pep B serum.

The anti-pepB serum recognized major bands of 80 and 90 kDa in Western blots of *P. falciparum* lysate. The 80 kDa form is present throughout intraerythrocytic development and in merozoites, the invasive form of the parasite. Two 90 kDa forms of slightly different electrophoretic mobility are only found in the membrane and organelle fraction of mature forms of the parasite. The 90 kDa form with the slowest electrophoretic mobility is also present in the membrane fraction of erythrocytes harbouring segmenters, which represent the latest stage of intraerythrocytic development of the malarial parasite before the rupture of the erythrocyte host (Figure 4).

Confocal microscopy was used to provide more information on the cellular distribution of PfPK4. These studies were carried out using the affinity-purified pepB and pepC antibodies. Both antibodies yielded the same results. However, the pepC antibody could be used at much higher dilution and was therefore used for all confocal microscopy studies presented here. Co-localization studies were performed using a mouse monoclonal antibody against RAP-1 (2.13), a mouse monoclonal antibody against MSP-1 (111.2) and a rat monoclonal antibody against PF83/AMA-1 (28G2) (Figure 5).

Within trophozoites the fluorescence of anti-PfPK4 serum is distributed throughout the whole parasite (results not shown).

During the schizont stage the PfPK4 protein is concentrated in foci (Figure 5B). This concentration is more pronounced in segmenters, resulting in the punctuate distribution of the protein in non-ruptured segmenters and in isolated merozoites (Figures 5A, 5C, 5D, 5E, 5F and 5G). This punctuate distribution is characteristic of proteins associated with rhoptries and the apical complex, such as RAP-1 and PF83/AMA-1. We therefore used α RAP-1 and α PF83/AMA-1 monoclonal antibodies for the co-localization studies. After double-labelling of segmenters and merozoites with α RAP-1 and α pepC, the foci of PfPK4 and RAP-1 were in close proximity, but did not co-localize (Figures 5A and 5F). When the same parasitic stages were double-labelled with α PF83/AMA-1 and α pepC, both PF83/AMA-1 and PfPK4 were co-localized at the apex (Figures 5C, 5D and 5E). The aggregation of PfPK4 and PF83/AMA-1 to discrete apical foci is only observed in segmenters; there is a more diffuse distribution of both proteins in the schizont stage of the parasite (Figure 5B). In merozoites the monoclonal antibody 28G2 detects PF83/AMA-1 at the apical end and to a lesser extent circumferentially on the surface of the merozoite [42]; PfPK4 is only detected at the apical end (Figure 5E). Our findings are in accordance with results of Narum and Thomas [42] and Crewther et al. [43] showing that PF83/AMA-1 and RAP-1 are in close vicinity at the apical complex, but do not co-localize.

DISCUSSION

In this study we isolated a malarial protein kinase gene, PfPK4, by homology probing, using an oligonucleotide probe corresponding to a short protein kinase sequence motif. The catalytic domain of PfPK4 possesses highest identities with eIF-2 α kinases.

The deduced amino acid sequence of PfPK4 contains all the characteristic sequence motifs described for the catalytic protein kinase domain involved in ATP binding, phosphotransfer, target amino acid specificity and catalysis [20,44]. Unusual, however, are the two large kinase inserts between kinase subdomains IV–VI and VII–VIII. Both kinase inserts correspond in their position to the kinase inserts of eIF-2 α kinases PKR, HRI and GCN2. Kinase insert 1 of PfPK4 is exceptionally large, 559 amino acids in length, which is about twice the size of the kinase domain without the kinase inserts. It is also considerably longer than the homologous inserts of PKR, HRI and GCN2, which are 35–120 amino acids in length. All eIF-2 α kinases share significant homology in the last few amino acids of kinase insert 1. This suggests a functional role for this insert. Deletion of six amino acids (L-F-I-Q-M-E, 361–336) of this region from PKR, corresponding to residues 822–827 of PfPK4 (L-L-L-Q-M-E), leads to a loss of autophosphorylation and eIF-2 α kinase activity [17]. Kinase insert 2 is 41 amino acids long and thus 10 amino acids longer than kinase insert 2 of HRI. Kinase insert 2 of eIF-2 α kinases and their kinase subdomains IX and X are likely to be involved in eIF-2 binding [3,9].

For binding of the regulators to eIF-2 α kinases, three different binding principles have been identified. PKR has two double-stranded RNA-binding motifs rich in basic amino acids in its first 160 amino acids [7,45,46]. GCN2 contains a 530-residue motif with homology to histidyl-tRNA synthetase C-terminal to the kinase domain [12], suggesting that GCN2 senses amino acid starvation by binding uncharged tRNA. HRI contains two

Figure 5 Confocal microscopy

Localization and co-localization of PfPK4, RAP-1, PF83/AMA-1 and MSP1. (A) A segmenter, stained for PfPK4 (red) and RAP-1 (green), with a composite of both labels on the right; (B) schizont; (C) segmenter; (D) and (E) free merozoites, stained for PfPK4 (red) and PF83/AMA-1 (green), with composites on the right; (F) a free merozoite, a composite of PfPK4 (red) and RAP-1 (green); (G) free merozoites stained for PfPK4 (red) and MSP-1 (green), with a composite of both labels on the right. The scale bars represent 2 μ m.

conserved haem-regulatory motifs (HRMs) [47] similar to those identified in other haem-binding proteins such as: erythroid δ -aminolaevulinic synthetase precursor [48]; HAP1, a yeast transcriptional factor for cytochrome genes [49]; haemopexin, a haem-binding serum glycoprotein [50]; catalase [51]. The HRM consists of R/K/N-C-P where Cys and Pro are invariant. In rabbit HRI there are two HRMs, Cys⁴⁰⁶-Pro⁴⁰⁷ and Cys⁵⁴⁸-Pro⁵⁴⁹ (rat HRI: Cys⁴⁰⁹-Cys⁴¹⁰, Cys⁵⁵¹-Pro⁵⁵²). The first HRM is located in kinase insert 1, and the second between kinase subdomains X and XI. We could not identify any of the double-stranded RNA-binding domains or a tRNA synthetase homology region on PfPK4. Like the haem-regulated catalase from *E. coli*, which contains a single HRM, there is a single C-P motif (Cys⁹⁸³-P⁹⁸⁴) between kinase subdomains IX and X, which could be considered an HRM.

We assayed recombinant PfPK4 for its autophosphorylation and substrate phosphorylation activity. Recombinant PfPK4 autophosphorylated and phosphorylated the substrate peptide of eIF-2 α kinases P(45–56) [31]. Like HRI, autophosphorylation and substrate-phosphorylation activity of PfPK4 are inhibited by haemin *in vitro*. A concentration of 10 μ M haemin inhibited autophosphorylation of recombinant PfPK4 to background level; P(45–56) phosphorylation was inhibited by 60%. Studies using α pepB antiserum for immunoprecipitation of parasite lysate also showed haemin-dependent P(45–56) phosphorylation activity of the immunoprecipitate *in vitro* (results not shown).

We used confocal microscopy to study localization of PfPK4, detecting it with antisera α pepB and α pepC. PfPK4 concentrated in foci inside the parasite in the schizont stage. During maturation of segmenters these foci are more pronounced, resulting in a punctuate distribution within the newly formed parasite. In merozoites these foci are close to the apical end. Since this punctuate distribution is observed with proteins associated with the rhoptry complex, we chose PF83/AMA-1 and RAP-1 for co-localization studies using monoclonal antibodies against both proteins. As observed by immunoelectron microscopy, RAP-1 is localized within the body of the rhoptry [43,52]; PF83/AMA-1 is localized more anteriorly within the rhoptry neck [43]. PfPK4 does not co-localize with RAP-1. After double-labelling of unruptured segmenters, PF83/AMA-1 is identified in the same foci as PfPK4. In free merozoites, PF83/AMA-1 is present at apical foci, but the processed 66 kDa form of PF83/AMA-1 is distributed on the surface of the whole merozoite [42]. In contrast with this distribution, PfPK4 is located only at the apex and not on the merozoite surface. Localization of PfPK4 at the apex of merozoites is identical with that of PF83/AMA-1. Since both antisera used for labelling PfPK4 were directed against C-terminal regions of the protein, these would detect an N-terminal processed form of PfPK4, and therefore we can exclude the possibility of an N-terminal processed form of PfPK4 being distributed on the merozoite surface in similar manner to PF83/AMA-1.

PfPK4 is expressed as major forms of 80 and 90 kDa during the intraerythrocytic development of the parasite. These forms probably arise through processing of the N-terminal region of the protein. Whereas the 80 kDa form of PfPK4 is solely restricted to the parasite, the upper one of the two 90 kDa forms was exported to the host compartment, namely the erythrocyte membrane, during segmentation.

The PfPK4 protein detected on Western blots of parasite lysate has an apparent size of 90 kDa at maximum, whereas the protein size calculated from the deduced amino acid sequence is 120 kDa. This discrepancy cannot be explained by well-known post-translational modifications. PCR on reverse transcripts gave no indication of RNA processing. The lower apparent molecular

mass may, however, be explained by unusual behaviour of the PfPK4 protein in SDS/polyacrylamide gels, as found for another malarial protein kinase, PfPK1. In this case, a 30 kDa reduction in apparent size was probably caused by the amino acid composition of the protein [34].

Like many other kinases, PfPK4 appears to be multifunctional. The association of PfPK4 with the host erythrocyte membrane during the final stage of parasite development within the erythrocyte may suggest involvement of PfPK4 in erythrocyte rupture. The localization of PfPK4 in the rhoptries suggests a possible role for this protein during invasion of the erythrocyte, formation of the parasitophorous vacuole or in early development of the ring stage. Erythrocytes contain small concentrations of free haemin, which has dissociated from haemoglobin into the cytosol and into the erythrocytic membrane; the level of free haemin is increased in aged erythrocytes and pathological cells of sickle cell anaemia and β -thalassaemia [53]. In the presence of haemin, PfPK4 is inhibited and does not regulate other processes by phosphorylation. Haem binding may therefore be a method whereby the parasite senses its environment during the invasion process. Whether PfPK4 is a true eIF-2 α kinase and thus regulates initiation of translation as suggested by its sequence homology, P(45–56) phosphorylation and haemin inhibition remains to be established.

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