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Short sequence-paper

Sexual stage-specific expression of a third calcium-dependent protein kinase from *Plasmodium falciparum*[1]

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Abstract

A third calcium-dependent protein kinase (CDPK) gene has been isolated from the human malaria parasite *Plasmodium falciparum* by vectorette technology. The gene consists of five exons and four introns. The open reading frame resulting from removal of the four introns encodes a protein of 562 amino acid residues with a predicted molecular mass of 65.3 kDa. The encoded protein, termed PfCDPK3, consists of four distinct domains characteristic of a member of the CDPK family and displays the highest homology (46% identity and 69% similarity) to PfCDPK2, the second CDPK of *P. falciparum*. The N-terminal variable domain is rich in serine/threonine and lysine and contains multiple consensus phosphorylation sites for a range of protein kinases. The catalytic domain possesses all conserved motifs of the protein kinase family except for the highly conserved glutamic acid residue in subdomain VIII, which is replaced by a glutamine residue. The sequence of the junction domain comprising 31 amino acid residues is less conserved. The calmodulin-like regulatory domain contains four EF-hand calcium-binding motifs, each consisting of a loop of 12 amino acid residues which is flanked by two α-helices. Southern blotting of genomic DNA digests showed that the *Pfcdpk3* gene is present as a single copy per haploid genome. A 2900 nucleotide transcript of this gene is expressed specifically in the sexual erythrocytic stage, indicating that PfCDPK3 is involved in sexual stage-specific events. It is proposed that PfCDPK3 may serve as a link between calcium and gametogenesis of *P. falciparum*. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Malaria; Calcium-dependent protein kinase; Gametogenesis; *Plasmodium falciparum*

The resistance of *Plasmodium falciparum* to drugs and the resistance of mosquitos to insecticides have resulted in a resurgence of malaria in many parts of the world. Therefore, there is an urgent need for development of effective vaccines and new anti-malarial drugs. The identification of new targets for vaccine and drug development will be facilitated by a better understanding of the cellular and molecular processes at different stages of the parasite. The sexual erythrocytic stage is functionally very distinct from the asexual stage. It is responsible for trans-

Abbreviations: CDPK, calcium-dependent protein kinase; PfCDPK1, the first *P. falciparum* calcium-dependent protein kinase; PfCDPK2, the second *P. falciparum* calcium-dependent protein kinase; PfCDPK3, the third *P. falciparum* calcium-dependent protein kinase

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[1] Nucleotide sequence data reported in this paper are available in the GenBank, EMBL and DDJB databases under the accession number AF106064.

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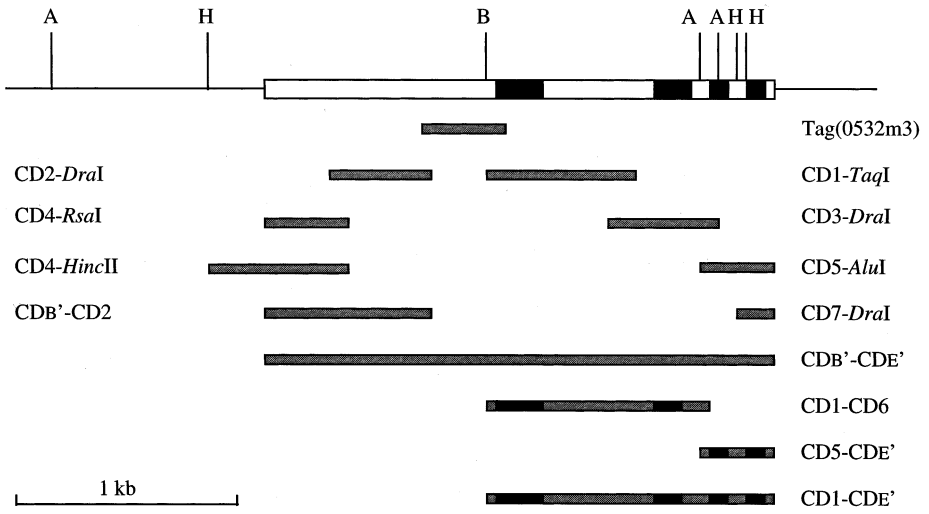

Fig. 1. A schematic representation of a partial restriction map of the *Pfcdpk3* gene and the overlapping PCR fragments used to determine the nucleotide sequence of *Pfcdpk3*. A, *Acc*I; B, *Bcl*I; H, *Hinc*II. The open boxes indicate the exon coding regions of the *Pfcdpk3* gene and the black boxes represent the introns. The fragments of CDB′-CD2 and CDB′-CDE′ are derived from genomic DNA. The fragments of CD1-CD6, CD5-CDE′ and CD1-CDE′ are RT-PCR products.

mission of the parasite to the mosquito, where exflagellation and fertilisation occur sequentially. Therefore, it is conceivable that more active biochemical and molecular events could be involved in the sexual stage than in the asexual stage. However, the number of sexual stage-specific proteins identified so far is very limited. Most of the sexual stage-specific genes isolated encode membrane proteins which are being assessed for their potential as transmission blocking vaccine candidates. Little is known about their biological function in the sexual stage. We are interested in the signal transduction pathways involved in the sexual differentiation of *P. falciparum*. The first step toward this goal is to identify the components, mainly protein kinases and phosphatases, of the signal transduction pathways. Recently, we have reported several sexual stage-specific genes encoding either protein serine/threonine phosphatases [1–3] or protein kinases (Li et al., unpublished). In this report, we describe the molecular cloning and characterisation of another novel gene encoding a third *P. falciparum* calcium-dependent protein kinase (CDPK) (PfCDPK3). PfCDPK3 is expressed specifically in the sexual stage, indicating that it may be important in regulating the processes of sexual stage development such as gametogenesis.

In the *P. falciparum* tag database, the nucleotide sequences of two DNA fragments (tag 0487m3 and tag 0532m3) were found encoding the same protein sequence with a high homology to the catalytic do-

Table 1
Characteristics of the nucleotide sequence of the *Pfcdpk3* gene

Segment	Size (bp)	A+T content (%)	Junction	Boundary sequence of exon/intron
5′ Untranslated	253	85.4 (216/253)		
Exon A	978	73.3 (717/978)	A/I	ATTGAGCATG:gtaataaaaa
Intron I	218	84.4 (184/218)	I/B	tattatatag:GTAAAGAAGG
Exon B	492	71.5 (352/492)	B/II	ATTATCCAAG:gtttttatat
Intron II	174	93.1 (162/174)	II/C	ttttctttag:AAATTAATAT
Exon C	75	56.0 (42/75)	C/III	ATTGGCCCAC:gtaataaaaa
Intron III	118	86.4 (102/118)	III/D	ttatttttag:ATACTTTATA
Exon D	99	63.6 (63/99)	D/IV	CGACGGAAAG:gtaatcataa
Intron IV	101	90.1 (91/101)	IV/E	ttttatttag:ATTGATTTTC
Exon E	42	78.6 (33/42)		

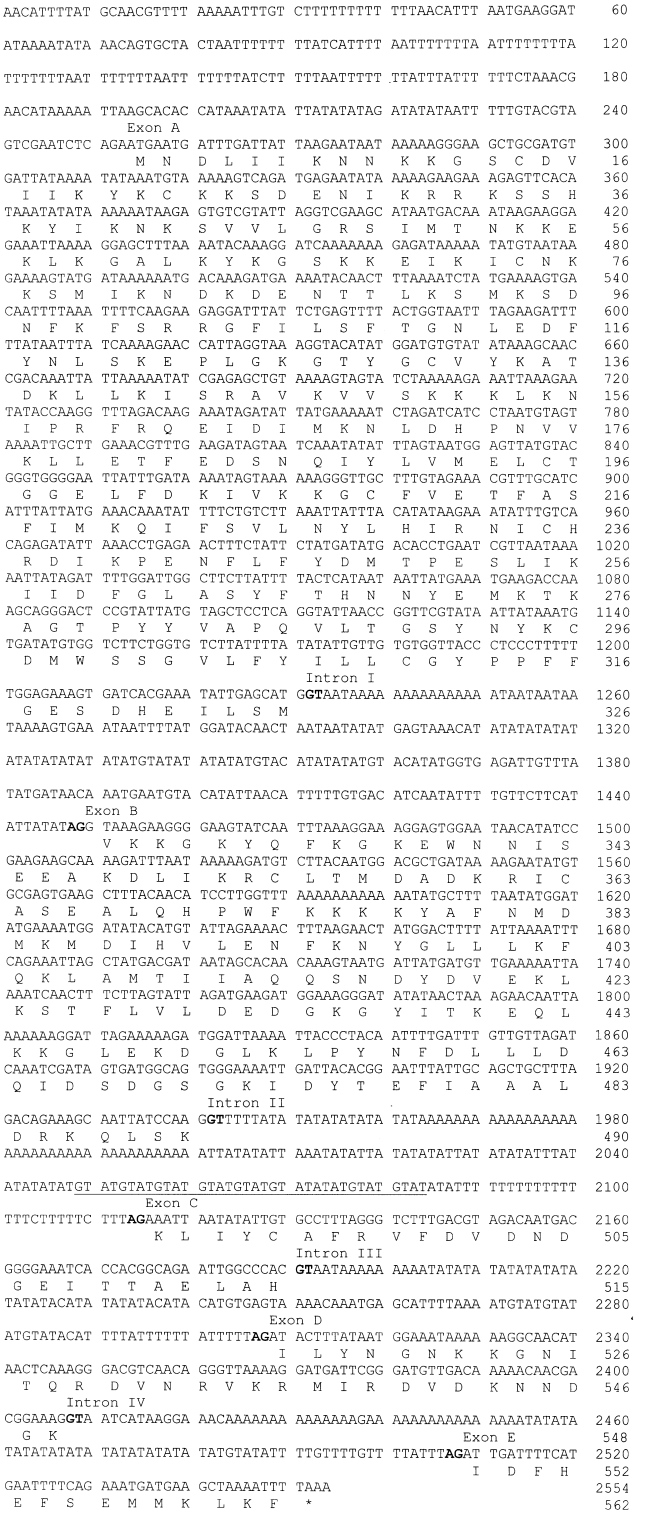

Fig. 2. Nucleotide and deduced amino acid sequences of the *Pfcdpk3* gene (single letter code). The nucleotides and the amino acid residues are numbered to the right of the sequence. The conserved dinucleotides of the exon–intron boundary sites are in bold. The repeated nucleotide sequence within intron II is underlined. The asterisk indicates the termination codon.

main of the CDPK family. Nucleotide sequence analysis revealed that the A+T content and codon usage of the tag sequences are typical of the coding region of *P. falciparum* genes. To isolate a full-length of the gene, two specific primers, CD1 (to obtain further sequence in the 3′ direction) and CD2 (to obtain further sequence in the 5′ direction), were constructed on the basis of the tag sequences and used in PCR to screen vectorette libraries [4]. Two fragments (CD1-*Taq*I and CD2-*Dra*I) (Fig. 1) were obtained and sequenced. The sequence data permitted construction of the CD3 and CD4 primers and subsequent screening of vectorette libraries. Two overlapping PCR products (CD4-*Rsa*I and CD4-*Hin*cII) were amplified with CD4, both containing a putative ATG start codon, whereas only one fragment (CD3-*Dra*I) was obtained with CD3. However, the sequence data of CD3-*Dra*I made it possible to construct the CD5 primer that gave rise to the CD5-*Alu*I fragment. Based on the sequence of CD5-*Alu*I, the CD7 primer was designed to produce the CD7-*Dra*I fragment. Analysis of the sequence revealed a putative TAA stop codon in CD7-*Dra*I. In order to confirm the sequence obtained from the overlapping fragments, a pair of primers, CDB′ and CDE′, were used to amplify the full-length gene from genomic DNA and the PCR product was sequenced in both strands (see Fig. 1).

The sequence derived from overlapping fragments consists of 2554 bp and contains five exons, four introns and a 5′ untranslated region (Fig. 2). The proposed coding region of the gene starts with an ATG codon at nucleotide 254 and terminates with a TAA codon at nucleotide 2551. The sequence and codon usage in the coding region are typical for a *P. falciparum* gene. The A+T contents of the 5′ flanking (253 bp) and four putative intron noncoding regions are characteristically higher than those of the exon coding regions (Table 1). The four proposed introns, ranging from 101 to 218 nucleotides in length, interrupt the coding region from

the middle to the C-terminus. Intron I is located in the middle of the gene, corresponding to subdomain X of the catalytic domain of the deduced kinase, whereas introns II, III and IV are located towards the 3′ end of the gene, corresponding to the calmodulin-like domain of the kinase. The highly conserved dinucleotides GT and AG, found at eukaryotic intron boundaries [5], define these intervening sequences. The sequences around the 5′ splice sites of these introns (GTA/TA/TT) show more conserved nucleotides, while the sequences around the 3′ splice sites (TA/TTAG) are fairly consistent with the consensus sequence found at the 3′ end of eukaryotic introns [5]. Long runs of poly(AT), poly(T) and poly(A) are present in the introns. A four nucleotide repetitive sequence, consisting of GTAT, occurs within intron II and repeats nine times. To verify the existence of these introns, reverse transcription (RT-) PCRs [2] were performed and the products sequenced (see Figs. 1 and 2). The data confirmed the precise exon–intron boundaries.

The open reading frame resulting from removal of the four introns encodes a protein of 562 amino acids (see Fig. 2) with a predicted molecular mass of approximately 65.3 kDa. Database searches revealed that the amino acid sequence of PfCDPK3 shares 50–69% similarity and 32–46% identity with kinases in the CDPK family. PfCDPK3 has the highest homology to PfCDPK2 (46% identity, 69% similarity), the second CDPK of *P. falciparum* [6], but only 40% identity and 64% similarity to PfCDPK1 (PfCPK), the first CDPK of the malaria parasite [7]. Fig. 3A shows a sequence alignment of all three *P. falciparum* CDPKs. PfCDPK3 is composed of four distinct domains characteristic of a member of the CDPK family: (1) a variable N-terminal segment, (2) a highly conserved protein kinase catalytic domain, (3) a junction domain, and (4) a calmodulin-like domain. The N-terminal domain, consisting of 116 amino acid residues, is not related to any previously described protein serine/threonine kinase and has several interesting features. Firstly, it is rich in serine/threonine (14% (16/116)) and lysine (24% (28/116)). Secondly, the N-terminal region contains multiple potential phosphorylation sites for a range of known protein kinases [8], suggesting that the PfCDPK3 activity may also be regulated by reversible phosphorylation of the N-terminal segment. Thirdly, in contrast to those of the known kinases in the CDPK family, the N-terminal segment is among the larger extensions of the CDPKs yet described. The kinase catalytic domain of PfCDPK3 is composed of 264 residues and contains all 11 conserved subdomains of the protein kinase family. PfCDPK3 has almost all of the characteristic features of a kinase [9,10]. These include (corresponding to the residue numbers for the bovine cAPK-α catalytic subunit): the glycine loop G50-X-G52-X-X-G55, forming part of the ATP-binding site; D166, N171 and D184, which are also thought to be a sequence motif implicated in ATP-binding; the triad composed of the side chain of K72, D184 and E91, that is close to the ATP γ-phosphate and plays a critical role in recognition of the phosphate; the catalytic loop R165-D166-X-X-X-X-N171, involved in catalysis and in guiding the peptide substrate into the proper orientation for catalysis; D220 and R280, involved in the stabilisation of kinases; and A206, which is diagnostic of the catalytic domain of protein kinases. In addition, PfCDPK3 also contains almost invariant amino acids corresponding to F185, G186, W222 and G225 whose functions are still unclear. The se-

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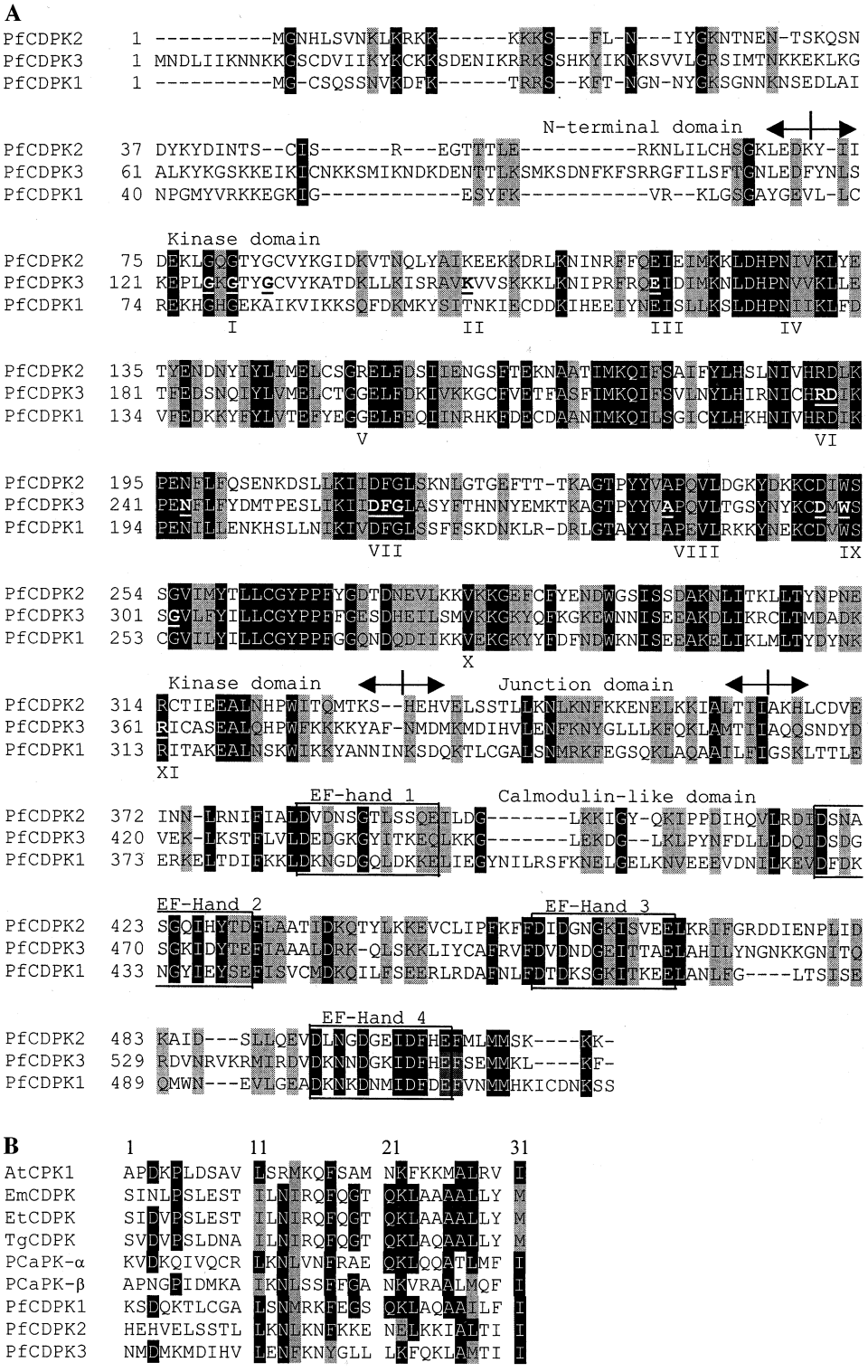
Fig. 3. (A) Alignment of the predicted amino acid sequence of PfCDPK3 with those of PfCDPK1 and PfCDPK2. The GenBank/EMBL database accession numbers are as follows: PfCDPK1, X67288; PfCDPK2, X99763; PfCDPK3, AF106064. Sequences were aligned with the CLUSTAL W (1.60) multiple sequence alignment programme. The amino acid residues are numbered to the left of the sequence. Identical residues are highlighted with solid black and conservative changes shaded with grey. The 11 canonical subdomains of protein kinases [11] are indicated by roman numerals. The residues conserved in the catalytic domain of the protein kinase family are underlined with bold in PfCDPK3. The boundaries of the variable, kinase, junction and calmodulin-like domains are shown. The four calcium-binding EF-hand motifs are boxed. (B) Alignment of amino acid sequences in junction regions of the protozoan CDPKs. EmCDPK, *E. maxima* [24]; EtCDPK, *E. tenella* [24]; TgCDPK, *T. gondii* (accession no. AF043629); PCaPK-α and PCaPK-β, *P. tetraurelia* [14]; and PfCDPK1, PfCDPK2 and PfCDPK3, *P. falciparum* [6,7]. AtCPK1 from *A. thaliana* [17] acts as a reference. Sequences were aligned with the CLUSTAL W (1.60) multiple sequence alignment programme. Identical residues in more than half of the CDPKs are highlighted with solid black and conservative changes shaded with grey.


A
PfCDPK2 1 ----------MGNHLSVNKLKRKK------KKKS---FL-N---IYGKNTNEN-TSKQSN
PfCDPK3 1 MNDLIIKNNKKGSCDVIIKYKCKKSDENIKRRKSSHKYIKNKSVVLGRSIMTNKKEKLKG
PfCDPK1 1 ----------MG-CSQSSNVKDFK------TRRS--KFT-NGN-NYGKSGNNKNSEDLAI
N-terminal domain
PfCDPK2 37 DYKYDINTS--CIS------R---EGTTTLE----------RKNLILCHSGKLEDKY-II
PfCDPK3 61 ALKYKGSKKEIKICNKKSMIKNDKDENTTLKSMKSDNFKFSRRGFILSFTGNLEDFYNLS
PfCDPK1 40 NPGMYVRKKEGKIG------------ESYFK----------VR--KLGSGAYGEVL-LC
Kinase domain
PfCDPK2 75 DEKLGQGTYGCVYKGIDKVTNQLYAIKEEKKDRLKNINRFFQEIEIMKKLDHPNIVKLYE
PfCDPK3 121 KEPLGKGTYGCVYKATDKLLKISRAVKVVSKKKLKNIPRFRQEIDIMKNLDHPNVVKLLE
PfCDPK1 74 REKHGHGEKAIKVIKKSQFDKMKYSITNKIECDDKIHEEIYNEISLLKSLDHPNIIKLFD
I II III IV
PfCDPK2 135 TYENDNYIYLIMELCSGRELFDSIIENGSFTEKNAATIMKQIFSAIFYLHSLNIVHRDLK
PfCDPK3 181 TFEDSNQIYIVMELCTGGELFDKIVKKGCFVETFASFIMKQIFSVLNYLHIRNICHRDIK
PfCDPK1 134 VFEDKKYFYIVTEFYEGGELFEQIINRHKFDECDAANIMKQILSGICYLHKHNIVHRDIK
V VI
PfCDPK2 195 PENFLFQSENKDSLLKIIDFGLSKNLGTGEFTT-TKAGTPYYVAPQVLDGKYDKKCDIWS
PfCDPK3 241 PENFLFYDMTPESLIKIIDFGLASYFTHNNYEMKTKAGTPYYVAPQVLTGSYNYKCDMWS
PfCDPK1 194 PENILLENKHSLLNIKIVDFGLSSFFSKDNKLR-DRLGTAYYIAPEVLRKKYNEKCDVWS
VII VIII IX
PfCDPK2 254 SGVIMYTLLCGYPPFYGDTDNEVLKKVKKGEFCFYENDWGSISSDAKNLITKLLTYNPNE
PfCDPK3 301 SGVLFYILLCGYPPFFGESDHEILSMVKKGKYQFKGKEWNNISEEAKDLIKRCLTMDADK
PfCDPK1 253 CGVILYILLCGYPPFGGQNDQDIIKKVEKGKYYFDFNDWKNISEEAKELIKLMLTYDYNK
X
Kinase domain
Junction domain
PfCDPK2 314 RCTIEEALNHPWITQMTKS--HEHVELSSTLLKNLKNFKKENELKKIALTIIAKHLCDVE
PfCDPK3 361 RICASEALQHPWFKKKKYAF-NMDMKMDIHVLENFKNYGLLLKFQKLAMTIIAQQSNDYD
PfCDPK1 313 RITAKEALNSKWIKKYANNINKSDQKTLCGALSNMRKFEGSQKLAQAAILFIGSKLTTLE
XI
EF-hand 1
Calmodulin-like domain
PfCDPK2 372 INN-LRNIFIALLDVDNSGTLSSQELLDG-------LKKIGY-QKIPPDIHQVLRDIDSNA
PfCDPK3 420 VEK-LKSTFLVLDEDGKGYITKECLKKG-------LEKDG--LKLPYNFDLLLDQIDSDG
PfCDPK1 373 ERKELTDIFKKLDKNGDGQLDKKELIEGYNILRSFKNELGELKNVEEEVDNILKEVDFDK
EF-Hand 2
EF-Hand 3
PfCDPK2 423 SGQIHYTDFLAATIDKQTYLKKEVCLIPFKFFDIDGNGKISVEELKRIFGRDDIENPLID
PfCDPK3 470 SGKIDYTEFIAAALDRK-QLSKKLIYCAFRVFDVDNDGEITTAELAHILYNGNKKGNITQ
PfCDPK1 433 NGYIEYSEFISVCMDKQILFSEERLRDAFNLFDTDKSGKITKEELANLFG----LTSISE
EF-Hand 4
PfCDPK2 483 KAID---SLLQEVDLNGDGEIDFHEFMLMMSK----KK-
PfCDPK3 529 RDVNRVKRMIRDVDKNNDGKIDFHEFSEMMKL----KF-
PfCDPK1 489 QMWN---EVLGEADKNKDNMIDFDEFVNMMHKICDNKSS
B
1 11 21 31
AtCPK1 APDKPLDSAV LSRMKQFSAM NKFKKMALRV I
EmCDPK SINLPSLEST ILNIRQFQGT QKLAAAALLY M
EtCDPK SIDVPSLEST ILNIRQFQGT QKLAAAALLY M
TgCDPK SVDVPSLDNA ILNIRQFQGT QKLAQAALLY M
PCaPK-α KVDKQIVQCR LKNLVNFRAE QKLQQATLMF I
PCaPK-β APNGPIDMKA IKNLSSFFGA NKVRAALMQF I
PfCDPK1 KSDQKTLCGA LSNMRKFEGS QKLAQAAILF I
PfCDPK2 HEHVELSSTL LKNLKNFKKE NELKKIALTI I
PfCDPK3 NMDMKMDIHV LENFKNYGLL LKFQKLAMTI I

quences (DIKPEN) in subdomain VI and (GTPYYVAPQ) in subdomain VIII indicate that PfCDPK3 is a serine/threonine kinase rather than a tyrosine kinase [11]. However, a highly conserved E residue in the APE motif of subdomain VIII is replaced by a Q residue in PfCDPK3. A similar change was also found in PfCDPK2 [6]. It is not clear how this change would influence the kinase activity. The junction domain of PfCDPK3 comprises 31 amino acid residues and separates the catalytic and calmodulin-like domains. Sequence comparison of all known protozoan CDPKs showed less conservation in this region for PfCDPK3 (Fig. 3B). The junction region is thought to contain an autoinhibitory motif which acts as a pseudosubstrate, interacting with the normal substrate-binding region of the enzyme to block entry of the substrate and, therefore, inhibiting kinase activity [12,13]. The binding of Ca^{2+} to the calmodulin-like domain is believed to release the pseudosubstrate from the active site of the catalytic domain by a mechanism that involves binding of the calmodulin-like domain to the autoinhibitory motif, therefore activating the kinase [14,15]. However, the basic-X-X-S/T consensus motif recognised by CDPKs [16] does not exist in the junction region of PfCDPK3. The sequences of the three *P. falciparum* kinases in the junction region are identical in only four of 31 residues, and all differ in at least 22 of 31 residues from the highly conserved junction sequences of plant CDPKs. This divergence of junction sequences within *P. falciparum* is similar to the situation in *Paramecium* [17] but is in sharp contrast to that in *Arabidopsis*, in which the junction region is strongly conserved; many *Arabidopsis* CDPKs are more than 90% identical in this region [18]. The LRVI sequence is thought to mediate an intramolecular interaction between the calmodulin-like domain and the junction region of the *Arabidopsis* CPK1 (AK1) [14]. However, only the I residue exists in PfCDPK3, although there are two residues (L and I) conserved in PfCDPK2 (see Fig. 3B). The calmodulin-like regulatory domain of PfCDPK3 consists of 151 residues and contains four putative EF-hand (helix-loop-helix) calcium-binding motifs, which are very similar to the EF-hands of calmodulin and other calcium-binding proteins. Each EF-hand of PfCDPK3 contains nearly all the requirements of such calcium-binding sites: six oxygen-containing ligands at positions 1, 3, 5, 7, 9 and 12, an invariant G residue at position 6 and a conserved aliphatic residue at position 8. The only exception is the highly conserved E residue at position 12 in the first EF-hand, which is replaced by a Q residue (see Fig. 3A). The refinement of the crystal structure of calmodulin has delineated the crucial role of this conserved E residue in calcium-binding [19]. In PfCDPK1, mutation of the conserved E residue to either K or Q in EF-hand 1 is deleterious and dramatically reduces the sensitivity of the Ca^{2+}-induced conformational change and the Ca^{2+}-dependent activation [20]. Therefore, it would be of great interest to examine whether the replacement in EF-hand 1 of PfCDPK3 would influence the Ca^{2+}-binding and the Ca^{2+}-dependent enzyme activity. Each of the four calcium-binding sites is flanked by residues predicted to form α-helices (data not shown), as expected in a calcium-binding EF-hand. The presence of four EF-hands suggests that calcium would directly bind to PfCDPK3 and regulate its activity.

To investigate the structural organisation of the

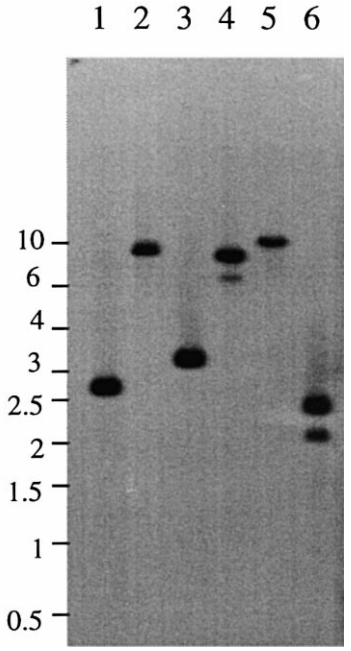


Fig. 4. Southern blot analysis of the *Pfcdpk3* gene. Four μg of genomic DNA from *P. falciparum* clone 3D7A was digested with restriction enzymes, electrophoresed on a 1.0% agarose gel, transferred onto a nylon membrane and probed with the CDB′-CD2 fragment of *Pfcdpk3*. Lanes 1–6 correspond to digests with *Acc*I, *Bam*HI, *Bcl*I, *Eco*RI, *Eco*RV and *Hin*cII. The sizes of 1 kb DNA markers are given in kb to the left.

Pfcdpk3 gene in the *P. falciparum* genome, 3D7A genomic DNA was digested with a number of restriction enzymes and analysed by Southern blotting. Hybridisation of the CDB′-CD2 (see Fig. 1) probe revealed a single band in digests with *Acc*I, *Bam*HI, *Bcl*I and *Eco*RV, respectively, consistent with the restriction map (Fig. 4), suggesting strongly that Pfcdpk3 is encoded by a single copy gene in the parasite genome. However, two bands (one predominant band and the other faint) were detected in digests with *Eco*RI and *Hin*cII, respectively, contradictory to the restriction map (lanes 4 and 6 in Fig. 4). The intensity of the faint bands decreased with higher stringency washing conditions (data not shown), indicating the presence of CDB′-CD2-related gene(s) in the *P. falciparum* genome. Indeed, two genes encoding PfCDPK1 and PfCDPK2, respectively, have recently been isolated from *P. falciparum* [6,7]. Whether the extra bands detected on the Southern blot represent these or other related gene(s) is unknown. Therefore, it is concluded that CDPKs exist as a multigene family in *P. falciparum*. A similar situation has been found in some plant species [21] and in the ciliated protozoan *Paramecium* [17]. So far, at least 20 CDPKs have been found in *Arabidopsis thaliana* [18,21], nine in maize [21,22], three in rice [23,24], three in soybean [25,26] and three in *Paramecium tetraurelia* [17]. CDPKs have also been isolated from other plants including mungbean, carrot, sweet potato and zucchini [21] and protozoans such as *Eimeria tenella* and *Eimeria maxima* [27] and *Toxoplasma gondii* (accession no. AF043629) as well as the unicellular algae *Chlamydomonas moewusii* [21].

In order to obtain some information on how *Pfcdpk3* mRNA levels are regulated during parasite development and differentiation, a Northern blot containing equal quantities of total RNA prepared from cultures enriched in stage III to stage V gametocytes and from mixed asexual erythrocytic stages was probed with the CDB′-CD2 fragment. A single transcript of approximately 2900 nucleotides in size was detected only in the lane containing the sexual stage RNA, migrating between the 28S and 18S ribosomal RNA bands (Fig. 5A). The result suggests that PfCDPK3 is involved in sexual stage-specific events. To exclude the possibility that *Pfcdpk3* could crossreact with the other two related genes (*Pfcdpk1* and *Pfcdpk2*) on the Northern blotting, the same blot was hybridised with the PCR fragment (equivalent to the coding region of the CDB′-CD2 fragment for PfCDPK3) of *Pfcdpk1* and *Pfcdpk2*, respectively (Fig. 5B,C). Interestingly, PfCDPK1 expressed not only in the asexual stage as described previously [7] but also in the sexual stage (see Fig. 5B), whereas PfCDPK2 expressed predominantly in the sexual stage (see Fig. 5C) rather than in the asexual stage [6]. In maize, it has been shown that a CDPK is specifically and developmentally expressed in pollen and required for germination and pollen tube growth [22]. In rice, expression of a CDPK gene has also been reported to be spatially and temporally regulated during seed development [23]. Interestingly, treatment of *Plasmodium berghei* and *P. falciparum* with Ca^{2+} antagonists such as TMB-8 (an inhibitor of intracellular Ca^{2+} release) and W-7 (a calmodulin inhibitor) strongly inhibited exflagellation, but

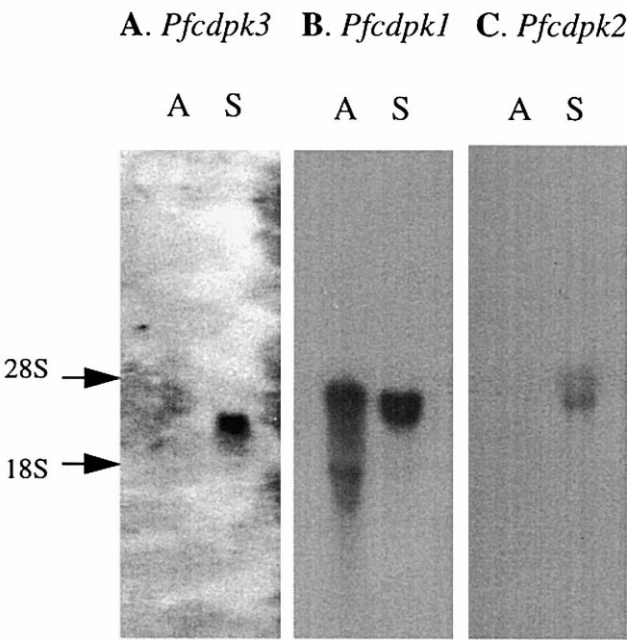


Fig. 5. Northern blot analysis of the *Pfcdpk3* gene. Ten μg of total RNA extracted from asexual erythrocytic stages (A) and sexual erythrocytic stage (S) of *P. falciparum* (3D7A) was fractionated in a denaturing formaldehyde gel, blotted onto a nylon membrane and hybridised to radiolabeled probes. The positions of *P. falciparum* rRNA subunits (18S and 28S) are indicated by arrows. A, B and C are autoradiographs of the membrane probed with the *Pfcdpk3* gene (CDB′-CD2), the *Pfcdpk1* gene and the *Pfcdpk2* gene, and exposed for 18 h, 24 h and 24 h, respectively.

EGTA (a Ca^{2+} chelator) and nicardipine (a Ca^{2+} channel inhibitor) had no effect, indicating that mobilisation of the parasite internal resources of Ca^{2+} is a prerequisite for exflagellation [28]. It has also been shown that DNA synthesis and axoneme formation in male gametocytes may be regulated by Ca^{2+}/calmodulin [29]. Taken together, we propose that PfCDPK3 (probably PfCDPK2 as well) may serve as a link between Ca^{2+} and gametogenesis of *P. falciparum*. Identification of the upstream regulators and downstream substrates of PfCDPK3 will afford new insight on the regulatory mechanisms of sexual stage-specific processes. In addition, CDPK does not seem to exist in vertebrates, PfCDPK3 may, therefore, represent a promising target for development of new anti-malarial drugs.

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References

[1] D.A. Baker, J.L. Li, A family of PP2 phosphatases in *Plasmodium falciparum* and parasitic protozoa: reply, Parasitol. Today 15 (1999) 124.
[2] J.L. Li, D.A. Baker, Protein phosphatase β, a putative type-2A protein phosphatase from the human malaria parasite *Plasmodium falciparum*, Eur. J. Biochem. 249 (1997) 98–106.
[3] J.L. Li, D.A. Baker, A putative protein serine/threonine phosphatase from *Plasmodium falciparum* contains a large N-terminal extension and five unique inserts in the catalytic domain, Mol. Biochem. Parasitol. 95 (1998) 287–295.
[4] J.L. Li, K.J.H. Robson, J.L. Chen, G.A.T. Targett, D.A. Baker, Pfmrk, a MO15-related protein kinase from *Plasmodium falciparum*: gene cloning, sequence, stage-specific expression and chromosome localization, Eur. J. Biochem. 241 (1996) 805–813.
[5] R.A. Padgett, P.J. Grabowski, M.M. Konarska, S. Seiler, P.A. Sharp, Splicing of messenger RNA precursors, Annu. Rev. Biochem. 55 (1986) 1119–1150.
[6] P.M. Farber, R. Graeser, R.M. Franklin, B. Kappes, Molecular cloning and characterization of a second calcium-dependent protein kinase of *Plasmodium falciparum*, Mol. Biochem. Parasitol. 87 (1997) 211–216.
[7] Y. Zhao, B. Kappes, R.M. Franklin, Gene structure and expression of an unusual protein kinase from *Plasmodium falciparum* homologous at its carboxyl terminus with the EF hand calcium-binding proteins, J. Biol. Chem. 268 (1993) 4347–4354.
[8] P.J. Kennelly, E.G. Krebs, Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases, J. Biol. Chem. 266 (1991) 15555–15558.
[9] D.R. Knighton, J. Zheng, L.F. TenEyck, F.A. Ashford, N.H. Xuong, S.S. Taylor, J.M. Sowadski, Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase, Science 253 (1991) 407–414.
[10] H.L. DeBondt, J. Rosenblatt, J. Jancarik, H.D. Jones, D. Morgan, S.H. Kim, Crystal structure of cyclin-dependent kinase 2, Nature 363 (1993) 595–602.
[11] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52.
[12] A.C. Harmon, B.C. Yoo, C. McCaffery, Pseudosubstrate inhibition of CDPK, a protein kinase with a calmodulin-like domain, Biochemistry 33 (1994) 7278–7287.
[13] J.F. Harper, J.F. Huang, S.J. Lloyd, Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain, Biochemistry 33 (1994) 7267–7277.
[14] J.F. Huang, L. Teyton, J.F. Harper, Activation of a Ca^{2+}-dependent protein kinase involves intramolecular binding of a calmodulin-like regulatory domain, Biochemistry 35 (1996) 13222–13230.
[15] B.C. Yoo, A.C. Harmon, Intramolecular binding contributes to the activation of CDPK, a protein kinase with a calmodulin-like domain, Biochemistry 35 (1996) 12029–12037.
[16] D.M. Roberts, A.C. Harmon, Calcium-modulated proteins: targets of intracellular calcium signals in higher plants, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 375–414.
[17] K. Kim, L.A. Messinger, D.L. Nelson, Ca^{2+}-dependent protein kinases of *Paramecium*. Cloning provides evidence of a multigene family, Eur. J. Biochem. 251 (1998) 605–612.
[18] E.M. Hrabak, L.J. Dickmann, J.S. Satterlee, M.R. Sussman, Characterization of eight new members of the calmodulin-like domain protein kinase gene family from *Arabidopsis thaliana*, Plant Mol. Biol. 31 (1996) 405–412.
[19] Y.S. Babu, C.E. Bugg, W.J. Cook, Structure of calmodulin refined at 2.2 A resolution, J. Mol. Biol. 204 (1988) 191–204.
[20] Y. Zhao, S. Pokutta, P. Maurer, M. Lindt, R.M. Franklin, B. Kappes, Calcium-binding properties of a calcium-dependent protein kinase from *Plasmodium falciparum* and the significance of individual calcium-binding sites for kinase activation, Biochemistry 33 (1994) 3714–3721.
[21] J.S. Satterlee, M.R. Sussman, Unusual membrane-associated protein kinases in higher plants, J. Membr. Biol. 164 (1998) 205–213.
[22] J.J. Estruch, S. Kadwell, E. Merlin, L. Crossland, Cloning

and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase, Proc. Natl. Acad. Sci. USA 91 (1994) 8837–8841.

[23] T. Kawasaki, N. Hayashida, T. Baba, K. Shinozaki, H. Shimada, The gene encoding a calcium-dependent protein kinase located near the *sbe1* gene encoding starch branching enzyme I is specifically expressed in developing rice seeds, Gene 129 (1993) 183–189.

[24] D. Breviario, L. Morello, S. Giani, Molecular cloning of two novel rice cDNA sequences encoding putative calcium-dependent protein kinases, Plant Mol. Biol. 27 (1995) 953–967.

[25] J.F. Harper, M.R. Sussman, G.E. Schaller, C. Putnam-Evans, H. Charbonneau, A.C. Harmon, A calcium-dependent protein kinase with a regulatory domain similar to calmodulin, Science 252 (1991) 951–954.

[26] J.Y. Lee, B.C. Yoo, A.C. Harmon, Kinetic and calcium-binding properties of three calcium-dependent protein kinase isoenzymes from soybean, Biochemistry 37 (1998) 6801–6809.

[27] P.P.J. Dunn, J.M. Bumstead, F.M. Tomley, Sequence, expression and localization of calmodulin-domain protein kinases in *Eimeria tenella* and *Eimeria maxima*, Parasitology 113 (1996) 439–448.

[28] F. Kawamoto, R. Alejo-Blanco, S.L. Fleck, R. Kawamoto, R.E. Sinden, Possible roles of Ca^{2+} and cGMP as mediators of the exflagellation of *Plasmodium berghei* and *Plasmodium falciparum*, Mol. Biochem. Parasitol. 42 (1990) 101–108.

[29] F. Kawamoto, H. Fujioka, R.I. Murakami, Syafruddin, M. Hagiwara, T. Ishikawa, H. Hidaka, The roles of Ca^{2+}/calmodulin- and cGMP-dependent pathways in gametogenesis of a rodent malaria parasite, *Plasmodium berghei*, Eur. J. Cell Biol. 60 (1993) 101–107.