

Molecular Cloning of *Plasmodium vivax* Calcium-Dependent Protein Kinase 4

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Abstract: A family of calcium-dependent protein kinases (CDPKs) is a unique enzyme which plays crucial roles in intracellular calcium signaling in plants, algae, and protozoa. CDPKs of malaria parasites are known to be key regulators for stage-specific cellular responses to calcium, a widespread secondary messenger that controls the progression of the parasite. In our study, we identified a gene encoding *Plasmodium vivax* CDPK4 (PvCDPK4) and characterized its molecular property and cellular localization. PvCDPK4 was a typical CDPK which had well-conserved N-terminal kinase domain and C-terminal calmodulin-like structure with 4-EF hand motifs for calcium-binding. The recombinant protein of EF hand domain of PvCDPK4 was expressed in *Escherichia coli* and a 34 kDa product was obtained. Immunofluorescence assay by confocal laser microscopy revealed that the protein was expressed at the mature schizont of *P. vivax*. The expression of PvCDPK4-EF in schizont suggests that it may participate in the proliferation or egress process in the life cycle of this parasite.

Key words: *Plasmodium vivax*, calcium-dependent protein kinase, schizont, EF-hand motif

Malaria, caused by the protozoan parasites, *Plasmodium* spp., is one of the most important infectious diseases that lead to high morbidity and mortality in most of the tropical and subtropical areas of the world [1]. Many efforts have been done to control and prevent malaria, but the global problems on malaria appear to be worsening [2,3]. The most important reason for this devastating situation is the emergence and spreading of drug resistance to currently affordable antimalarial drugs. Therefore, it is urgently needed to identify and characterize new potential targets for development of new antimalarial drugs.

A family of calcium-dependent protein kinases (CDPKs) is unique enzymes that are found in plants, green algae, ciliates, and apicomplexan parasites [4]. In the *Plasmodium* spp., mem-

bers of the CDPK family are likely to be the key regulators that mediate stage-specific cellular responses to calcium, a widespread secondary messenger that controls the progression of the parasite through its complex life cycles [5]. Moreover, CDPKs of the rodent malaria parasites, such as *Plasmodium berghei* and *Plasmodium yoelii* are known to be essential for parasite transmission to the mosquito [6]. Due to the absence of CDPKs in mammals, including human, they are considered as promising target molecules for antimalarial drug development [7,8]. In this study, we identified a gene encoding *Plasmodium vivax* CDPK4 (PvCDPK4) and characterized its molecular property and cellular localization of PvCDPK4 calmodulin region (EF-hand).

Blood samples infected with *P. vivax* were collected from indigenous malaria patients in South Korea. The parasite DNA was extracted from the whole blood using Nucleospin® blood kit (MACHEREY-NAGEL, Düren, Germany). By data-mining the recently updated *P. vivax* genome sequence database (PlasmoDB, <http://plasmodb.org>), we identified a gene encoding PvCDPK4 (Gene ID: PVX_000555). The full-length gene of PvCDPK4 was

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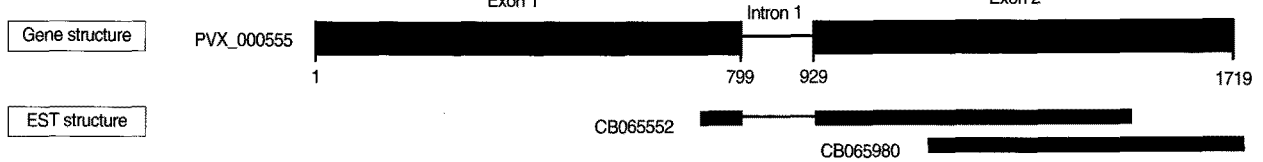
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amplified by PCR using the primers, 5'-ATGGGCCAAGAAA-TCTCCACGCCGAACGAC-3' and 5'-TTAATAGTTGCACAGC-TTGACGAGCATGTC-3' with *Taq* DNA polymerase (Takara, Otsu, Japan) and *P. vivax* genomic DNA under following condi-

tions: 95°C for 5 min, 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min 30 sec, and 72°C for 10 min for final extension. The resulting PCR product was purified from the gel using the Nucleospin extraction kit (MACHEREY-NAGEL), li-

A-a



A-b



B

PvCDPK4	MGQEI STP-----NDAR NERCKGYSYK NLRGSKAKGE	32
PfCDPK4	MGQEVSSV-----NWFK NERKKT-NKK S LKGGNERBE	31
PbCDPK4	MGQEMSTQ-----SDMQ NENKQK-MGR NLRGSKQKQNG	31
PfCDPK1	MCCSQSNN-----VQDF KTRRSKQ TNC NNYGSKQNN-	31
TgCDPK1	MGQESTL-----GGAA GEPRSKGAA QTSQGGP---	29
PfCDPK2	MGNELSVN-----KLR RKKKKKSLN-----TYGNT NEN-TSQSN DYKYDIN-	43
PfCDPK3	MNDLITKSK KQSCNITKY KCKKSDENK RRRKSSHYK NKSVVLGRSI MFKPKKELG ALKYKSKKE	70
PvCDPK4	QKQGLQSK KTAQNSFNS KLR-----PCMFQNS VVFNQY-KG IKL LKQSSFG EVILSRKRT	93
PfCDPK4	NKSSVGIK KIVENSFNS KLR-----PCMFQNS VVFNQY-KG IKL LKQSSFG EVILSRKRT	92
PbCDPK4	LKRSTSIK EIVKNSFNS KLR-----PCMFQNS VVFNQY-KG IKL LKQSSFG EVILSRKRT	92
PfCDPK1	-----KNSD LAIN-----PCMVKKE CKIGESY-FK VRL LKQSSFG EVILSRKRT	78
TgCDPK1	-----DH L HA?-----PCMFVQST AIFSDRY-KG QRL LKQSSFG EVILSRKRT	73
PfCDPK2	-----TSCI S--RGT T L E-----RKNLILCS SKLEDKY-II DER LKQSSFG CVYKQDEVT	131
PfCDPK3	IKTKKSKMI KNDKQNT L KSKSKSNFK SRKGFILST GNLEDFYNS KER LKQSSFG CVYKQDEVT	140
PvCDPK4	GHEYLIRVIS KKVVRKRT-----KQSLREVE L LKMLDHN I MKLYEFDN NYLYLSDVY	151
PfCDPK4	GHEYLIRVIS KKVVRKRT-----KESLREVE L LKMLDHN I MKLYEFDN NYLYLSDVY	150
PbCDPK4	GHEYLIRVIS KKVVRKRT-----KQSLREVE L LKMLDHN I MKLYEFDN NYLYLSDVY	150
PfCDPK1	RGEKALIRVIS KQVFRKRY I TNKIECDK IBEETVNE IS LKSLDHPNI IKLDFVFDK GYFVAVGFY	148
TgCDPK1	RQEDAVIRVIS KQVFRKRT-----KESLREVE L LKMLDHPNI MKLYEFDK GYFVAVGFY	151
PfCDPK2	NQYALIRVIS KDR LKRNDR-----FQGE IE LKMLDHPNI VCLYEFEDN NYLYLSDVY	149
PfCDPK3	KESEAVIRVIS KKLKRNDR-----FQGE ID LKMLDHPNI VCLYEFEDN NYLYLSDVY	155
PvCDPK4	SGGELFDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	221
PfCDPK4	TGGE L FDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	220
PbCDPK4	SGGELFDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	220
PfCDPK1	EGGELFDEII NRHRFYEDA ANIRKVLG I CYLIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	217
TgCDPK1	TGGE L FDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	200
PfCDPK2	SGE L FDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	218
PfCDPK3	TGGE L FDEII SRKRFYEDA ARIIRKVLG I TYMIRKVV HRDLKPEML LKTKNKEIMI IKI IDPGLST	264
PvCDPK4	HFEY-SRQKDKI GTAYTIA PDVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	290
PfCDPK4	HFEY-SRQKDKI GTAYTIA PDVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	289
PbCDPK4	HFEY-SRQKDKI GTAYTIA PDVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	289
PfCDPK1	FFSK-DKGLA DRDGTAYTIA PDVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	286
TgCDPK1	HFEA-SRQKDKI GTAYTIA PDVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	293
PfCDPK2	NLQTEGTFY-TRAGTPYVA PQVLDCKQK KCDIWSGVI MYTLSCGPP FYCDTINKV KRVKKECF	287
PfCDPK3	YFPHNRYEK TRAGTPYVA PQVLRGTDE KCDIWSGVI LYILLSCGPP FNGSNEYDIL KRVEYGRYF	334
PvCDPK4	DLPOFKI SD KAKLIRKML MYTSAVRISA RDALEHEWIR LMASKDNVSI DIPSLELSIT NIRQFQSTOK	360
PfCDPK4	DLPOFKI SD KAKLIRKML MYTSAVRISA RDALEHEWIR LMASKDNVSI DIPSLELSIT NIRQFQSTOK	359
PbCDPK4	DLPOFKI SD KAKLIRKML MYTSAVRISA RDALEHEWIR LMASKDNVSI DIPSLELSIT NIRQFQSTOK	359
PfCDPK1	DFMDKRSSE EAKLIRKML TVDYKSLTA KEALRSKVI KYARNINK-S DQK LCLAL NRRKTESOK	355
TgCDPK1	ELPQKRVSE SAKLIRKML TVYPSRISA RDALEHEWIT TYT-KEQISV DVPSLDAIL NIRQFQSTOK	338
PfCDPK2	YENDWCSSS DAKLIRKML TVYPSRISA RDALEHEWIT QMTKSH-EHV ELS-STLKL NLRKFKRNE	354
PfCDPK3	KGKEWNISE EAKLIRKML TVDADRICA SEALQRFVK KKYAFNMD KQD--TFVLE NFKYGLLXK	402
PvCDPK4	LAQAALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	430
PfCDPK4	LAQAALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	429
PbCDPK4	LAQAALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	429
PfCDPK1	LAQAALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	422
TgCDPK1	LAQAALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	408
PfCDPK2	LKRIALYMG SKLTTIDETK ELTKIKFQK RQEDQGLDR E L IGVKELL KLRGDTSDL DNAALEVEVD	412
PfCDPK3	FQKLAFTIA QQSNDVYK-LKSTFLAD EDGKATYK QLRKCG-----LEKQ G-LKLPNFD	450
PvCDPK4	QILS LKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	493
PfCDPK4	QILS LKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	492
PbCDPK4	QILS LKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	492
PfCDPK1	RI LKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	485
TgCDPK1	QVLDVDFKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	471
PfCDPK2	QVLRIDVDFKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	482
PfCDPK3	LLLDVDFKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	529
PvCDPK4	VSSDCWKTVL KEVDQNDGGE IDFKKIDML VKLQNY---	529
PfCDPK4	VSSDCWKTVL KEVDQNDGGE IDFKKIDML VKLQNY---	528
PbCDPK4	VSSDCWKTVL KEVDQNDGGE IDFKKIDML VKLQNY---	528
PfCDPK1	I SEQMNEVL GEVDQNDGGE IDFKKIDML VKLQNY---	524
TgCDPK1	VDSSTWVSE SEVDQNDGGE IDFKKIDML VKLQNY---	507
PfCDPK2	KAIT---SLL QVLDVDFKQSSFG EVILSRKRT L TVAIRKLL STERLEKAF LFRKQSSFG EVILSRKRT L LKQSSFG EVILSRKRT	513
PfCDPK3	RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI RQVNRVKKMI	563

Fig. 1. (A) Gene and domain structure of PvCDPK4. (a) The PvCDPK4 locus of *P. vivax* (CM000444: 475,102-476,845, PlasmoDB 7.0). The PvCDPK4 gene structure (nucleotides 1-1,590) comprises 2 exons. The black boxes in the PvCDPK4 gene represent exons of CDS. There are 2 expressed sequence tags (ESTs) in the region of the PvCDPK4 locus. (b) The domain structure of the PvCDPK4. The PvCDPK4 contains a diverse variety of protein domains and common patterns can be identified as PfCDPK4. The calcium-dependent protein kinase 4, encoded by PvCDPK4, is a 529 amino acid (aa) protein with 4-EF hand repeats known as calmodulin-like domain (CLD), which possesses N-terminal myristoylation motif and kinase domain (KD). (B) Sequence alignment of the deduced amino acid sequence of PvCDPK4 with other related proteins. The GenBank/EMBL database accession numbers are as follows: PfCDPK1, X67-288; PfCDPK2, X99763; PfCDPK3, AF10-60641; PfCDPK4, XM_001349042; PbCDPK4, AY555067; TgCDPK1, AF333958. Sequences were aligned with BioEdit ver 7.0 software (Tom Hall Isis Therapeutics, Isis Pharmaceuticals, California, USA). The numbers of amino acid residues are to the right of the sequences. Protein kinase ATP binding domain (grey box), serine/threonine kinase active site (open box), and 4 calcium binding EF-hand motifs (dark grey boxes).

gated into pCR2.1-TOPO Cloning Vector (Invitrogen, Carlsbad, California, USA) and then transformed into *Escherichia coli* TOP10 (Invitrogen). The plasmid DNA was extracted by Nucleospin plasmid kit (MACHEREY-NAGEL) and the nucleotide sequence of the insert was analyzed by automatic sequencing. The genomic sequence, transcript sequences and amino acid sequences were extracted from the PlasmoDB 7.0 released [9]. The Est2 genome [10] was used to align the transcript sequences with the genomic DNA sequence. We also searched for potential domains and motives on the coding sequences using the SWISS-MODEL workspace [11]. Then, the EF hand motif of PvCDPK4 was amplified by PCR using primers, PvCDPK-EF-F (5'-GGATCCATGGGGTCCAAACTCACAACC-3') and PvCDPK-EF-R (5'-AAGC-TTTCGCCGTCGTTGTTCTGGT-3'), under following conditions: 95°C for 5 min, 30 cycles of 94°C for 1 min, 67°C for 1 min and 72°C for 1 min 30 sec, and 72°C for 10 min for final extension. The amplified PCR product was ligated into pET28a expression vector (Merck, Darmstadt, Germany) and transformed into *E. coli* BL21 (DE3). Selected clones were grown and induced with 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG). The recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen) and analyzed by SDS-PAGE. The specific antiserum against recombinant PvCDPK4-EF (anti-PvCDPK4-EF) was produced by immunizing BALB/c mice with the purified protein (50 μ g) 3 times with 2 weeks intervals. The specificity of anti-PvCDPK4-EF was confirmed by immunoblot analysis and used in this study.

To determine the expression and confirm localization of PvCDPK4-EF in erythrocytic stage of *P. vivax*, indirect fluorescence assay (IFA) by confocal laser scanning microscopy was performed as previously described with some modification [12]. The *P. vivax*-infected blood smears were fixed with a 50% acetone-50% methanol for 10 min at -20°C and stored at -20°C until use. The slides blocked with 5% skim milk for 30 min at 37°C and washed with PBS. The slides were incubated for 30 min at 37°C with 1:100 diluted anti-PvCDPK4-EF in PBS and washed 3 times with PBS. The slides were then incubated for 30 min at 37°C with Alexa-Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, Oregon, USA). After washing 3 times with PBS, the slides were incubated with 25 μ g of propidium iodide (PI) (Molecular Probes) for 10 min at 37°C. They were mounted with 50% glycerol in PBS and observed under a confocal laser scanning microscopy (TCS NT, Leica, Germany).

The gene encoding PvCDPK4 was consisted with 1,754 bp

and contained an intron region (801-964 bp) in the middle of the sequence. We also analyze the gene structure of PvCDPK sequence in GenBank (PVX_000555) (Fig. 1A). It was slightly different with our sequence as shown in Fig 1A. Sequence analysis revealed that the PvCDPK4 gene contains a typical kinase domain (KD) and a calmodulin-like structure with 4-EF hand motifs that required for calcium-binding at C-terminus (Fig. 1A). Multiple sequence analysis of the deduced amino acid sequence of PvCDPK4 with other presently known plasmodial CDPKs revealed that PvCDPK4 showed the highest sequence identity to PbCDPK4 (93.0%) and PfCDPK4 (91.9%), but less than 50% to PfCDPK1 (44.5%), PfCDPK2 (34.5%) and PfCDPK3 (30.4%) (Fig. 1B).

The recombinant PvCDPK4-EF was expressed as soluble form in *E. coli* with the expected molecular weight of 34 kDa (Fig. 2). In the IFA result to determine the expression pattern of PvCDPK4 in the erythrocytic stage of *P. vivax*, interestingly, strong positive signals were identified in the schizont stage of the parasite, but not in other erythrocytic stages, including rings and trophozoites (Fig. 3, Panels II and III). Meanwhile, the negative control sera (Panel IV) did not show any reactions. The feature of PvCDPK4 specifically expressed in the schizont stage was similar to that of PfCDPK1 [13]. PfCDPK1 is exported from schizonts to the parasitophorous vacuole membrane (PVM) of *Plasmodium falciparum*, which would require the transfer of the protein across the parasite plasma membrane followed by insertion into the

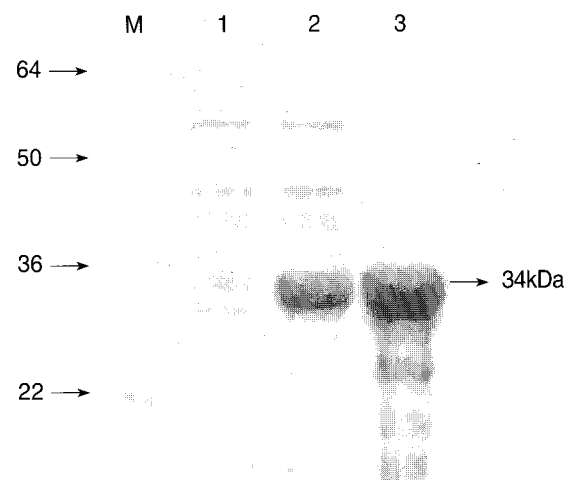


Fig. 2. Expression of PvCDPK4-EF. Expression and purification of the recombinant PvCDPK4-EF. The expression of recombinant protein was induced by adding IPTG to the final concentration of 0.5 mM and analyzed on SDS-PAGE followed by Coomassie blue staining. Lane 1, non-induced *E. coli*; Lane 2, IPTG-induced *E. coli*; Lane 3, Ni-NTA affinity purified recombinant protein. The recombinant PvCDPK4-EF protein was observed as approximately 34 kDa.

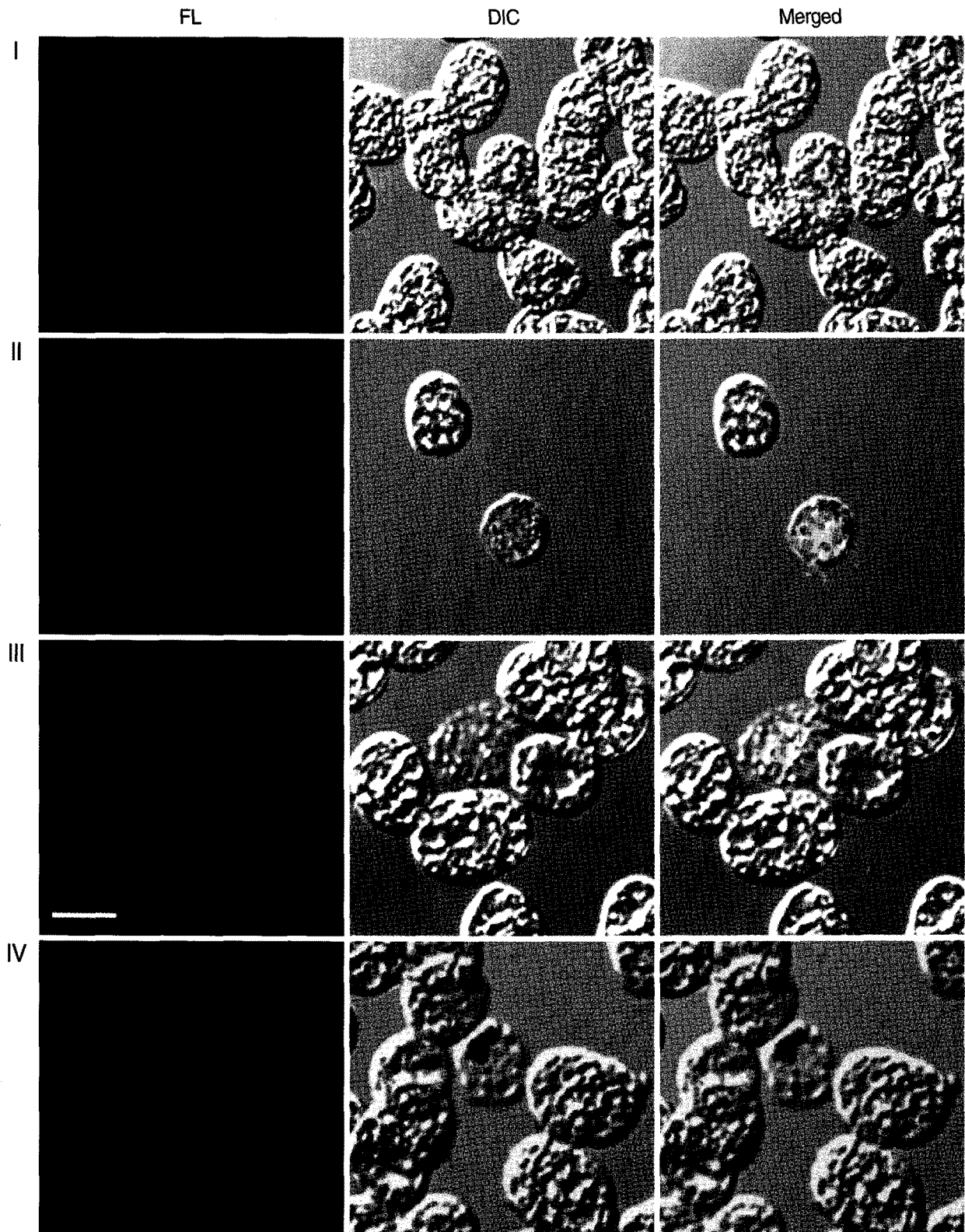


Fig. 3. Indirect fluorescent assay (IFA). *P. vivax*-infected blood smears were probed with anti-rPvCDPK4-EF followed by nuclear staining with propidium iodide (PI) and observed with confocal scanning laser microscopy. FL, fluorescence microscope; DIC, differential interference contrast microscope; Merge, merged image of FP and DIC. Panel I (positive control), probed with anti-MSP mouse monoclonal antibody and PI; Panel II and III, probed with anti-PvCDPK4-EF and PI; panel IV (negative control), probed with normal mouse serum and PI. Scale bars indicate as 5 μ m.

PVM [13]. PfCDPK1 and PfCDPK4 belong to the same class in phylogenetic analyses of calcium-dependent kinases in apicomplexans [5]. This might support the similar pattern of localization of PfCDPK1 [13] and PvCDPK4. It was also because we have analyzed EF-hand region of PvCDPK4 only. To report the evidences of the PvCDPK4-EF-hand expression, we searched for transcripts in the PlasmoDB database [9] and determined the structures of exon and intron of the PvCDPK4 gene using the alignment Est2 genome program [10]. In the case of PvCDPK4, we identified 2 transcripts, including EF-hand region that are likely to be partial fragments (Fig. 1A-a). One transcript (CB065552) begins at the position +715 relative to the start codon for PvCDPK4 and has a single intron. The other (CB065980) begins at the position +808 and has no intron. Both of them possess the unique calmodulin-like domain (CLD) containing 4 EF hand domains in the coding region in spite of the partially expressed sequences (Fig. 1A-a). Furthermore, another proof for the PvCDPK4 gene expression was found using the transcriptome profiling of *P. vivax* described by Bozdech, et al. [14]. This also proves that the PvCDPK4 gene was highly expressed throughout the intraerythrocytic cycle of 3 distinct *P. vivax* isolates. All these findings provide strong evidence that the calmodulin-like domain (CLD) of this gene is predominantly expressed in *P. vivax*.

It has been known that calcium controls many critical events in the complex life cycles of apicomplexan parasites, including protein secretion, motility, and development, and, therefore, plasmodial CDPKs has recognized emerging as important targets for malaria parasite development [5,6]. Calcium released from intracellular stores also governs egress of tachyzoites of *Toxoplasma gondii* [15]. *T. gondii* calmodulin-like domain protein kinase isoform 3 (TgCDPKif3) is activated by calcium in a dose-dependent manner and it mediates egress of the parasite through stimulation by calcium [16]. A specific expression in the mature schizont stage of *P. vivax* suggests that PvCDPK4 may participate in the development process and/or egress of the parasite. On the other hand, PfCDPK4 was detected on gametocytes [17] and high through-put proteome analyses in the rodent species, *P. berghei* and *P. yoelii*, suggested that CDPK4 is abundantly expressed in both the gametocyte and ookinete stages, and that it is also detectable in asexual parasite stages [6]. This fact suspected that PvCDPK4 may also serve in regulating the processes of sexual stage development, such as gametogenesis. However, we characterized only the EF-hand region of CDPK calmodulin-like domain in this study, thus it is difficult to say whether PvCDPK express in sexual stages or not. More complicated study would

be necessary in the future to determine the physiological significance of PvCDPK4.

In conclusion, this is the first report on characterization of *P. vivax* CDPK. PvCDPK4-EF was specifically expressed in the schizont stage, which suggests that it is possibly related to the proliferation or invasion of the parasite in the parasite's life cycle. PvCDPK would represent antimalarial drug targets, leading to the development of more stage-specific drugs.

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