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Short communication

PfKIN, an SNF1 type protein kinase of *Plasmodium falciparum* predominantly expressed in gametocytes<sup>1</sup>

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Phosphorylation of specific proteins by protein kinases is known as a major strategy by which a cell regulates protein and enzymatic activities in response to developmental, environmental, and metabolic signals [1]. Protein kinases constitute a large family of more than 100 regulatory proteins divided into two groups depending on which target residue is phosphorylated in the substrate: tyrosine or serine/threonine [2]. The serine/threonine protein kinase group has been further subdivided into nine subclasses [3] depending on their mode of regulation and substrate specificity. Among these subfamilies, the SNF1-related class is an expanding group of protein kinases [4]. The *Saccharomyces cerevisiae* SNF1 protein was first identified as a protein kinase playing a central role in the regulatory response of yeast to glucose availability [5]. Yeast SNF1 function was required

for the expression of a number of genes repressed by glucose [6]. Recently SNF1 homologues have been isolated from plants [7] and mammals [8]. The mammalian SNF1 homologue is the AMP-activated protein kinase which is known to play a central role in the regulation of lipid metabolism [9] and in cell response to stress [10]. Functional homologies between RKIN [7] and SNF1 as well as between AMPK [11] and SNF1 have been underscored by yeast *snf1* mutant complementation and biochemical assays, respectively.

*Plasmodium falciparum* is a protozoan parasite responsible for the most severe form of human malaria. Although phosphorylation and dephosphorylation mechanisms are reported as playing an important role during merozoite invasion [12] as well as during intraerythrocytic development [13], relatively little is known about reversible phosphorylation during the parasite life cycle but it is reasonable to assume that these processes should regulate many aspects of the parasite life both in the vertebrate and invertebrate hosts, and during the transition phases from one host to the other. The identification and characterization of

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protein kinase genes constitute an approach to gain an understanding on the processes regulated by phosphorylation.

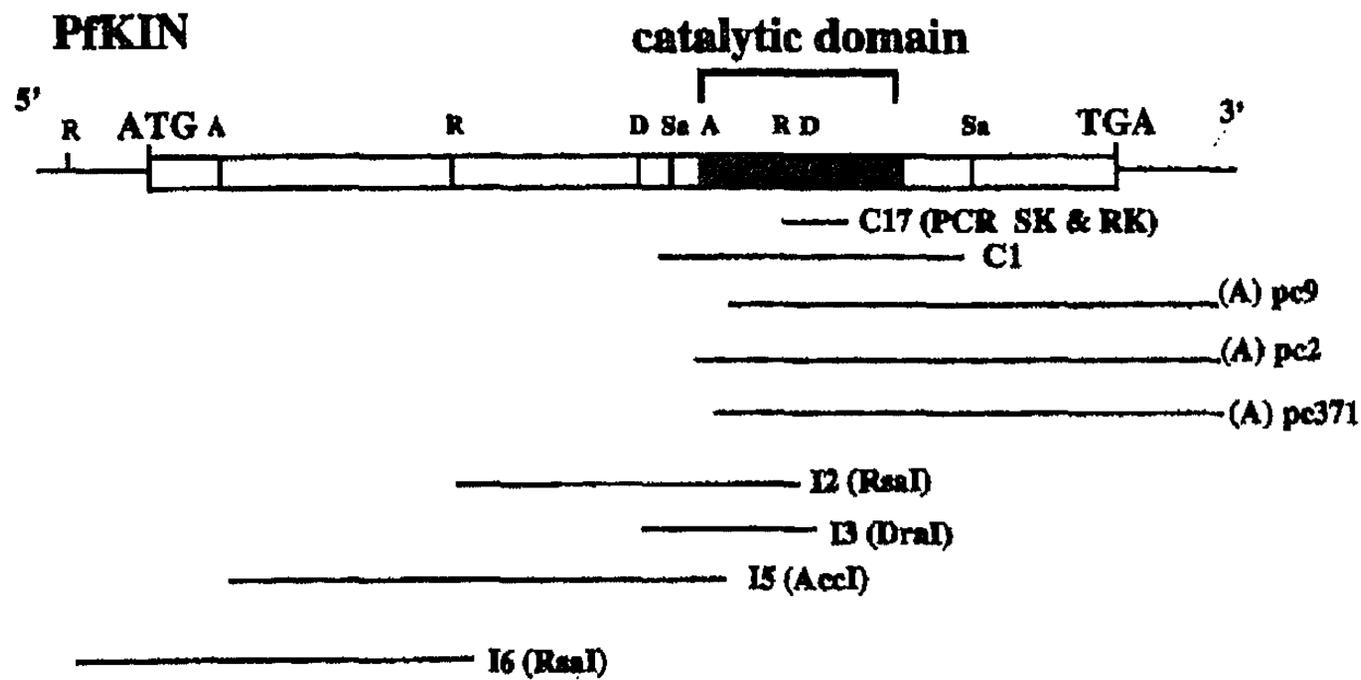
Using a pool of degenerate oligonucleotides deduced from amino acid consensus sequences of alignments of protein kinases, we amplified part of a novel malarial protein kinase gene related to SNF1. The oligonucleotide sense SK (5'-cggATC CAC MGN GAY BT-3') was derived from the conserved motif in subdomain IVB (IHRDL/V). The primer reverse RK (5'-ggaattCC RWA KCW CCA SAC RTC-3') was synthesized as a complementary oligonucleotide to the conserved motif in subdomain IX (DVWS/TF/YG). The primers SK and RK respectively contain a *Bam*HI and an *Eco*RI restriction site at their 5' extremity (underlined) for subsequent cloning. They were used in a 100- $\mu$ l PCR mixture containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM MgCl<sub>2</sub>, 0.05 mM dNTP and genomic DNA (500 ng) as template, with respect to the degeneracy ratio (RK/SK = 1.3). Amplification conditions were as follows: after 6 min of initial denaturation at 94°C, the samples were subjected to 40 cycles (94°C for 1 min, 37°C for 2 min, 63°C for 3 min). A single 198-bp PCR product of interest was obtained, cloned into the *Bam*HI/*Eco*RI-digested pUC19 plasmid and sequenced using the dideoxynucleotide chain termination method (Sequenase version 2.0 kit, USB) to verify it encoded a protein kinase. Then it was radio-labeled and used as a probe to screen a gametocyte stage cDNA library constructed in the vector pcDNAII [14]. Three positive clones (pc2, pc9 and pc371) were isolated with inserts ranging in size from 1100 to 1300 bp. Two of them encompassed the entire catalytic domain, but compared to other protein kinase genes the three inserts appeared to lack the amino terminus (the 5' end of the inserts contained no methionine). These truncated cDNA may have arisen from the annealing of the oligod(T) used for cDNA synthesis at sequences rich in adenines, blocking the progression of the reverse transcriptase (Fig. 1A). The screening of genomic libraries was unsuccessful, probably due to the extreme A + T content of the malaria genome [15] which makes it difficult to clone certain genes and to maintain them stably in standard prokaryotic

vectors and hosts [16]. Therefore, inverse PCR appeared to be a powerful tool for walking along the *P. falciparum* genome. Four different inverse PCR (I-PCR) reactions were carried out to reach the 5' end of the gene. Two independent clones of each I-PCR product were sequenced on both strands to minimize errors that might be introduced by the thermostable polymerase. In addition, overlapping inserts allowed us to confirm the sequence.

The PfKIN coding region has an AT ratio of 79%, whereas the 5'-untranslated region has 95% AT with poly(AT) repeats allowing formation of stem-loop structures. The AATAAA canonical sequence present 57 nucleotides 5' anterior to the poly(A) stretch could be a polyadenylation signal. PfKIN is encoded by a single copy gene carried on chromosome 13 that hybridizes to a 2300-bp mung bean fragment (data not shown). The size of the mung bean fragment correlates with the 2298-bp open reading frame obtained from the cloned PfKIN gene. The predicted 90 845-Da protein is highly basic with a calculated isoelectric point of 9.75. Lysine residues account for about 15% of the molecule.

The PfKIN-deduced protein sequence contains all the characteristic sequence motifs of the eukaryotic protein kinases, some of these being involved in ATP-binding, substrate recognition or catalysis. More particularly, the 15 residues conserved throughout the protein kinase family are present in PfKIN as well as the motifs DLKPEN and GSPFYTSPE in subdomains VIB and VIII, respectively, which are strong indicators of serine/threonine specificity (Fig. 1B). When compared to the other protein kinases, the PfKIN catalytic domain is identical to 40% with the SNF1 sequence from *S. cerevisiae* (and similar to 61%, if conservative substitutions were included). Outside the catalytic domain which corresponding to the N-terminus half of the enzyme, no significant amino acid sequence similarities to any other proteins were found. The 391-residue N-terminus region of PfKIN is much larger than for the other SNF1-related protein kinases (from 11 residues for RKIN to 52 residues for SNF1) suggesting that PfKIN may be modulated by signals different from those of other eukaryotes.

A



B

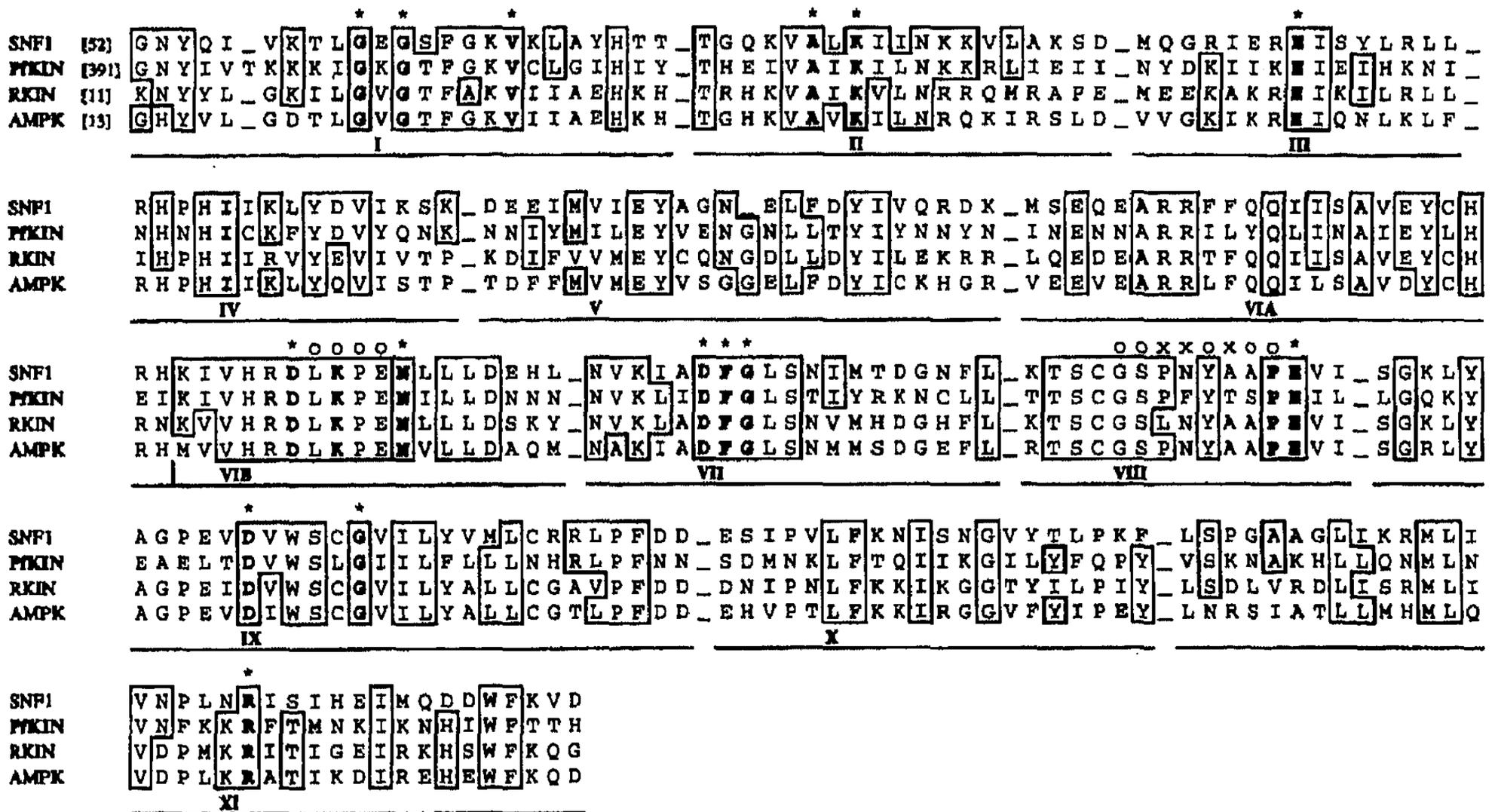


Fig. 1. (A) The PfKIN sequence represents a composite of sequences determined by cDNA (pc2, pc9 and pc371), genomic (C1) and inverse PCR product clones (I2, I3, I5 and I6) obtained from circularization of *P. falciparum* genomic DNA (1.5  $\mu$ g) with the indicated endonuclease in brackets. The thermolabile endonucleases used were inactivated for 10 min at 68°C before being diluted in 1 ml of ligation buffer. DNA molecules were circularized with 0.02 Weiss U/ $\mu$ l of T4 DNA ligase (Biolabs) at 15°C for 16 h, precipitated, and finally resuspended in 10  $\mu$ l of distilled water. Samples (5  $\mu$ l) were heated 30 min at 94°C just before amplification with the specific primers synthesized in opposite orientations to those normally used for PCR. Several restriction sites are shown: A, *AccI*; D, *DraI*; R, *RsaI*; Sa, *Sau3A*. (B) Multiple Alignment of amino acid sequences of catalytic domains related to SNF1. PfKIN was compared using the McVector software to the yeast SNF1 (*S. cerevisiae*, Ac. M13971 [5]), the plant homologue RKIN (*Secale cereale*, Ac. M74113 [7]) and the mammalian counterpart AMPK (rat, Ac. Z29486 [11]); identical amino acids are boxed. The 12 conserved subdomains [2] are indicated by Roman numerals. The positions of the 15 residues conserved throughout the protein kinase family are denoted by an asterisk. The motifs indicated by (O) are strong indicators of serine/threonine specificity. The number of additional amino flanking residues lying outside the catalytic domains are shown at the beginning of each sequence.

To investigate the expression of the PfKIN gene Northern blot analysis was performed and a weak signal was obtained only with RNA extracted from gametocytes (data not shown). Due to the low level of the signal, the expression of PfKIN was further analyzed by RT-PCR on RNA extracted from different developmental stages (Fig. 2). The specificity of expression was confirmed by performing in parallel RT-PCR with two stage-specific  $\beta$ -actin primers [17] which controlled both the quality of the RNA preparation and the level of genomic DNA contamination. A clear PfKIN signal was obtained only with gametocyte RNA. No signal was obtained on asexual stage RNA with PfKIN or the gametocyte specific  $\beta$ -actin primers and, conversely, the asexual stage-specific  $\beta$ -actin primers failed to amplify from the gametocyte RNA. These results concord with the fact that PfKIN clones were detected only in a gametocyte stage cDNA library, no clones were found by PCR on intraerythrocytic stage cDNA libraries.

Taken together the results strongly suggest that the PfKIN gene represents a new plasmodial member of the SNF1 protein serine/threonine kinase subfamily that is preferentially expressed in

gametocytes, the stage involved in the transmission of the malaria parasite from the human bloodstream to the mosquito midgut. The SNF1 protein kinase family is an expanding group of regulatory enzymes with a common function of protecting the cell from either environmental stress (as AMPK for the mammalian) or metabolic stress (as SNF1 for the yeast) by activating transcription of specific genes in response to the deleterious environment. On transition of mature gametocytes from the homeothermic vertebrate host to the poikilothermic insect vector, the malaria parasites have to quickly adjust to a biochemically different environment and a number of regulatory enzymes would predictably be activated or deactivated [18]. By analogy with other SNF1 type protein kinases, it is reasonable to think that PfKIN is one of these regulatory enzymes acting downstream from a signal pathway via an environmental stress constituted by the change of host. Hence, the activation of PfKIN protein induced by the change of host (say its phosphorylation) would lead to the stimulation of gene transcription allowing adaptation of the parasite to a different environment.

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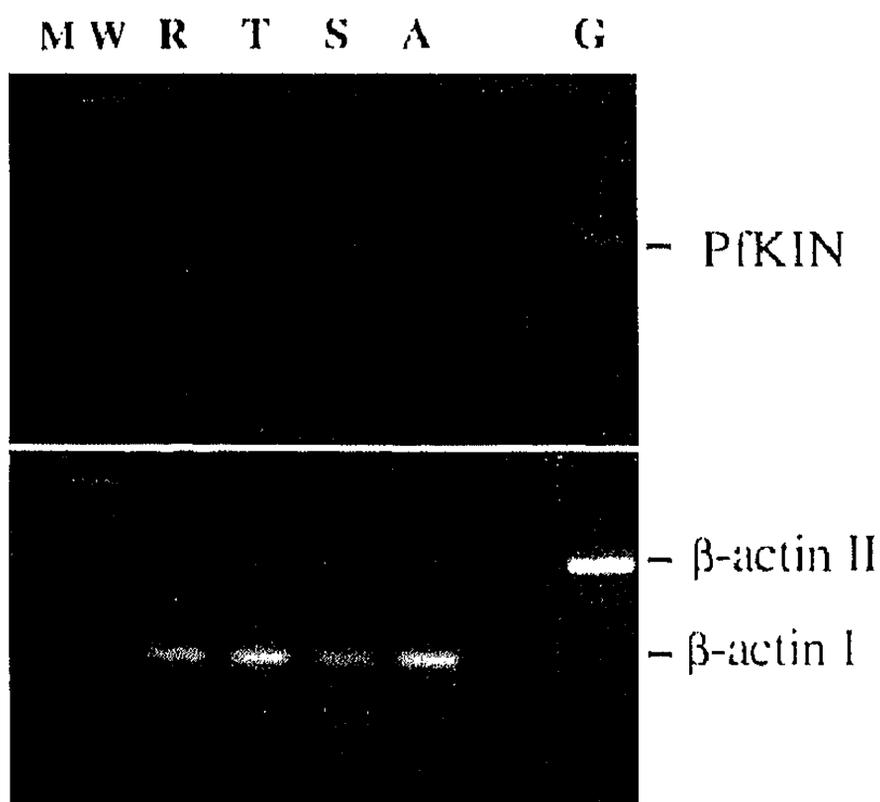


Fig. 2. PfKIN expression. RNA isolated from intraerythrocytic stages (ring, R; trophozoite, T; or schizont, S), mixed intraerythrocytic stages (A), or gametocyte stage (G) were amplified with either PfKIN primers,  $\beta$ -actin I (asexual RNA extraction control), or  $\beta$ -actin II (sexual RNA extraction control).

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