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ORIGINAL ARTICLE

In silico and *in vivo* studies on MAPK1 from *Plasmodium berghei*

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ABSTRACT
Objective of this study was to analyze Plasmodium berghei mitogen-activated protein kinase 1 (PbMAPK1) using in silico and in vivo approaches. In silico data indicated the conservation of functional domain and motifs required for typical MAP kinase in PbMAPK1, which was predicted to be nuclear-localized. However, PbMAPK1 did not contain any nuclear localization signal (NLS) and nuclear export signal (NES). From BLASTp analysis, PbMAPK1 was found to share the highest protein sequence similarity (93%) with MAPK1 from Plasmodium yoelli. Meanwhile, in vivo study revealed intraerythrocytic parasitemia development of P. berghei strain PZZ1/00 in male mice. The malaria parasites from the infected mice blood were successfully isolated and purified using 8% Percoll solution and spectrophotometric measurement showed that the parasite genomic DNA was of high purity with A_{260}/A_{280} ratio greater than 1.8. By using a specific primer pair namely PbMAPK1-F (5'-TATGTGGTCGCTTGGGTG-3') and PbMAPK1-R (5'-GAATCTTGGGATGCTTTA-3'), polymerase chain reaction (PCR) method successfully amplified a single band of ~250 bp corresponding to PbMAPK1 gene thereby validating some in silico data.
KEYWORDS: Malaria, *Plasmodium berghei,* signal transduction and mitogen-activated protein kinase

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INTRODUCTION

Plasmodium berghei is one of the malaria parasite infecting rodents. Others include *P. yoelli*, *chabaudi*, *P. knowlesi* and *P. vivax*. This unicellular protozoan which serves as a good model for the human parasites has a complex life cycle comprising of sexual and asexual stages [1]. A report by [2] stated that *P. berghei* strain ANKA and strain NK65 were isolated from *Anopheles dureni millecampsi* which originate from different forest galleries, River Kasapa and River Kisanga respectively. Those forest galleries are located in Lubumbashi, Republic of Congo. Other isolated *P. berghei* strains are LUKA, K173, KSP11 and SP11.

A family of protein serine/threonine kinases which are ubiquitous proline-directed is called mitogen-activated protein kinases (MAPKs). They play important roles in signal transduction pathways by controlling intracellular events including acute responses to hormones and major developmental changes in living organisms [3]. The organization of this protein family includes a G-protein acting as an upstream activator molecule for three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK [4]. The MAPK signaling pathway in malaria parasite was first investigated by [5]. In that study, the PfMAPK gene was expressed in *Escherichia coli* and it was found that the recombinant PfMAPK exhibited autophosphorylation on both the tyrosine and threonine amino acid residues within the TXY motif, and substrate-level phosphorylation on myelin basic protein.

There are two copies of MAPKs have been identified in human malaria parasite, *P. falciparum* namely MAPK1 and MAPK2 [6]. They share a peptide sequence identity of 41% in their catalytic domain. The TXY motif is conserved in PfMAPK1 (PlasmoDB identifier : PF14_0294) and PfMAPK2 (PlasmoDB identifier : PF11_0147) as TDY and TSH respectively. To the best of our knowledge, MAPK1 has been studied extensively in human malaria parasite but not in rodent malaria parasite. An extensive literature search did not reveal any established report on MAPK1 from *P. berghei*, the rodent malaria parasite. Therefore this study was carried out to gain an insight into functional characteristics of PbMAPK1 using bioinformatics tools and determine its existence *in vivo* using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Materials

A personal computer equipped with an AMD Turion 64x2 dual-core processor, 32 GB of RAM and an NVIDIA graphics card was used to perform *in silico* analyses with respect to the public databases and web based programs as presented in Table 1.

Table 1 Databases and web-based programmes used in the analysis of PbMAPK1

Analysis	Programme name	URL access
Sequence retrieval	PlasmoDB	http://www.plasmodb.org
Subcellular localization	SubLoc	http://www.bioinfo.tsinghua.edu.cn/SubLoc/
Nuclear localization signal	PredictNLS	http://www.predictprotein.org/
Nuclear export signal	NetNES	http://www.cbs.dtu.dk/services/NetNES/
Post translational modification	ScanProsite	htpp://www.expasy.org
Sequence similarity search	BLASTp (NCBI)	http://blast.ncbi.nlm.nih.gov/
Primer design	Primer 3Plus	http://www.bioinformatics.nl/primer3plus/

The parasite stock and 8-week male mice strain HSD were provided by School of Biosciences and Biotechnology, Faculty of Science and Technology and Animal House, Universiti Kebangsaan Malaysia respectively.

METHODS

In silico study was carried out using publicly available bioinformatics tools. Biological data of PbMAPK1 were retrieved from publicly available PlasmoDB database. On the other hand, the protein sequence of PbMAPK1 was retrieved from the PlasmoDB database in FASTA format and used in BLASTp analysis against the same database in order to determine the sequence similarity with its orthologues. The retrieved parasite protein sequence was also subjected to a series of computational analyses using various programmes including PROSITE [7] in order to predict post translational modification sites, SubLoc [8] for purpose of predicting protein subcellular localization, PredictNLS [9] for the prediction of nuclear localization and NetNES [10] to identify Leucine-rich nuclear export signals. Nucleotide sequence of PbMAPK1 was used to design the specific forward and reverse primers using Primer 3 Plus [11] programme for PCR reaction [12].

An *in vivo* study was carried out in order to determine existence of *PbMAPK1* gene in *P. berghei* strain PZZ1/00. The parasite was inoculated into a total of three 8-week male mice strain HSD intraperitoneally. At 70% parasitemia, which was monitored microscopically using Giemsa-stained blood films, the mice were sacrificed under chloroform anesthesia for blood sampling. The parasite was isolated from mice blood using Percoll 8% solution which was then subjected to genomic DNA extraction.

For isolation of genomic DNA, parasite cells were suspended in lysis buffer (Tris, EDTA, 2% SDS and Proteinase K) and subjected to phenol-chloroform methodology. Subsequently, cold ethanol solvent was used to precipitate parasite genomic DNA which in turn was determined its quantity and purity using spectrophotometry method at wavelength of A_{260} and A_{280}.

Determination of *PbMAPK1* gene *in vivo* was carried out using PCR method. Both parasite genomic DNA sample and a specific primer pair namely PbMAP1-F (5'-TATGTGGTCGCTTGGGTG-3') and PbMAP1-R (5'-GAATCTTGGGATGCTTTA-3'), were used in PCR reaction in order to amplify the *PbMAP1* gene. The reaction was performed using commercial PCR kit from Promega, USA. The reaction conditions were as follows: initial denaturation at 94^0C for 3 minutes, 33 cycles of denaturation at 94^0C for 1 minute, annealing at 53^0C for 1 minute and extension at 72^0C for 1 minutes, followed by a final extension at 72^0C for 10 minutes.

RESULTS

General characteristics of PbMAPK1

PlasmoDB database provides a wide range of biological data for PbMAPK1. Those useful biological data were retrieved, compiled and presented in Table 2. The functional domain and motifs which are required for a typical MAPK such as protein kinase domain, MAPK signature, serine/threonine kinase motif and ATP binding site were fully conserved in PbMAPK1. It is a strong likelihood that, similar to other

eukaryotic MAPKs, PbMAPK1 executes its catalytic activity when is activated by upstream activator molecules.

Table 2: Biological data of PbMAPK1 retrieved from PlasmoDB database.

PlasmoDB identifier	PB000806.03.0	
UniProt identifier	Q4YP23	
Protein length	373 aa	
Nucleotide length	1119 bp	
Protein length	599 aa	
Protein expression	Asexual stage	
Molecular weight	70.5 kDa	
Isoelectric point	9.68	
Biological process	Protein amino acid phosphrylation	
Molecular function	ATP binding and MAP kinase activity	
Domain/Motif	**Accession**	**Database**
Kinase-like_dom.	IPR011009	InterPro
MAP_kinase_CS	IPR003527	InterPro
Prot_kinase_cat_dom.	IPR003527	InterPro
Ser/Thr_prot_kinase_dom.	IPR003527	InterPro
PROTEIN_KINASE_ST	PS00108	PROSITE
MAPK	PS01351	PROSITE
PROTEIN_KINASE_ATP	PS00107.	PROSITE
PROTEIN_KINASE_DOM	PS50011	PROSITE
S_TKc	SM00220	SMART
Pkinase	PF00069	Pfam

Nuclear localization of PbMAPK1

The PlasmoDB database did not provide information on subcellular localization of PbMAPK1, thereby its protein sequence was analyzed using SubLoc, PredictNLS and NetNES programmes. Each functional protein is destined to a particular subcellular localization with respect to its specific function. *In silico* analysis showed that PbMAPK1 is nuclear-localized but did not contain any nuclear localization signal (NLS) and nuclear export signal (NES). However, this is in agreement with common characteristic of a typical MAP kinase protein whereby the nuclear localization is crucial for MAPK proteins to perform their specific functions.

Post translational modification of PbMAPK1

Most eukaryotic proteins undergo post translational modification after protein synthesis in order to extent the range of their functions which requires attachment of other functional groups including phosphate, myristate and carbohydrates. From protein sequence analysis, it was found that PbMAPK1 contains functional sites for phosphorylation, myristoylation and glycosylation processes (Table 3). It is likely that PbMAPK1 is regulated by these chemical modifications with regard to its activation.

Table 3: Post translational modification sites detected in PbMAPK1 protein sequence.

Phosphorylation sites	Glycosylation sites	Myristoylation sites
TqK	NVSK	GAfqNS
SnK	NETD	GLarSI
TfR	NSTD	GCimAE
TgK	NYTE	
TkR	NSTM	
StK	NNSR	
TiK	NETI	
SkK	NSSQ	
TiK		
SeK		
TfrE		
SfaD		
SrnE		
TikE		
RaknDqdiY		
KKfS		
KRiT		

Table 4 Protein sequence similarity between PbMAPK1 and its orthologues.

Orthologues	PlasmoDB identifier	Identity	E-value	Score
PyMAPK1	PY02176	93%	2.90E-183	1769
PcMAPK1	PCAS_101310	90%	7.10E-180	1737
PkMAPK1	PKH_131880	73%	1.10E-144	1405
PvMAPK1	PVX_084965	74%	2.60E-143	1392
PfMAPK1	PF14_0294	71%	1.70E-139	1356

Protein sequence similarity between PbMAPK1 and its orthologues

Protein sequence similarity is useful to useful to predict correspondence between two or more proteins. To evaluate the conservation of MAPK1 among *Plasmodium* orthologues, BLASTp analysis was performed which utilized protein sequences from *P. yoelli, P. chabaudi, P. knowlesi, P. vivax* as well as *P. falciparum*. As shown by Table 4, PbMAPK1 shared high protein sequence similarity with its orthologues of not less than 70%. It shared the highest protein sequence identity (93%) with MAPK1 from *Plasmodium yoelli*. When it was compared with PfMAPK1 of human malaria parasite, there was 71% identity between them which may be greater if PbMAPK1 is completely sequenced. This has made PbMAPK1 a good alternative for investigating MAPK pathway in malaria parasite.

Parasitemia development in *P. berghei*-infected mice

To determine PbMAPK1 experimentally, an animal model was used in order to maintain malaria parasite. *P. berghei* strain PZZ1/00 was inoculated into male mice. *In vivo* study showed that, through microscopic analysis on Giemsa-stained blood films, *P. berghei* infection developed rapidly within 15 days (Figure 1). The infected male mice were found to exhibit several common malaria symptoms such as shivering and cyclical occurrence of fever. At day 15 post-infection, 70% parasitemia was achieved, at which the male mice were sacrificed for blood sampling. The stable parasitemia development in mice was beneficial for parasite isolation from mice blood.

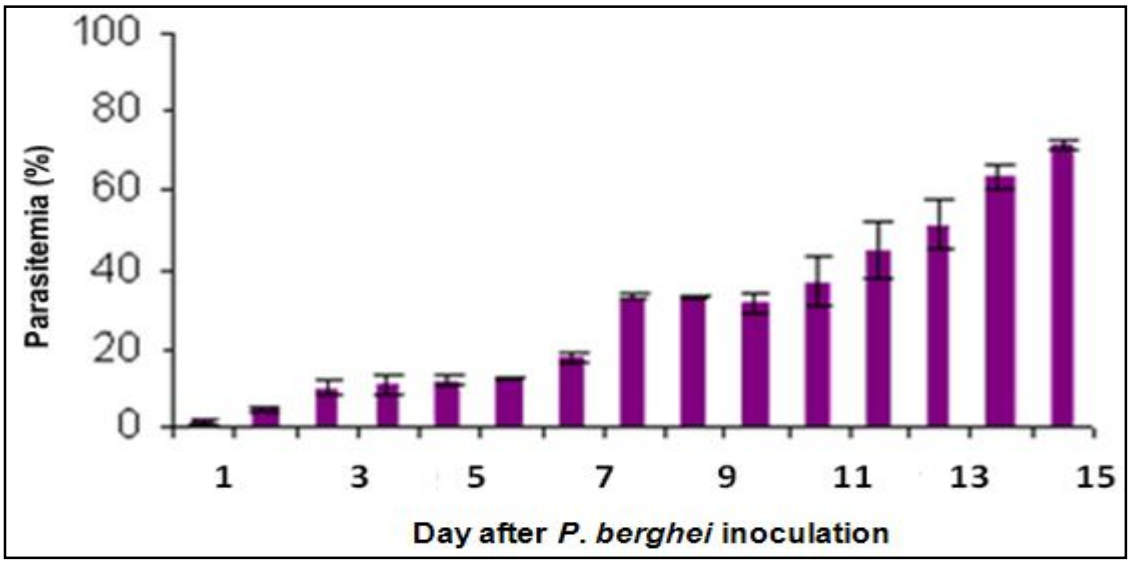

Figure 1 Development of parasitemia of *P. berghei* in male mice. Monitoring of the parasitemia was based on microscopic observation on Giemsa-stained blood films which were prepared from three experimental mice. Data is represented by mean ± S.E.M (n=3).

Genomic DNA and PCR amplification

The biological data of PbMAPK1 deposited in PlasmoDB database are corresponding to *P. berghei* strain ANKA. However, in the present study we used *P. berghei* strain PZZ1/00 to determine the existence of PbMAPK1 *in vivo*. It was previously expected that the strain factor does not affect the existence of the gene of interest. From primer analysis, the expected size of PCR product was only 250 bp despite the actual size of *PbMAPK1* gene was 1119 bp. The difficulty in obtaining greater expected size of PCR product was due to the presence of hairpin structure, dimer, false priming and also cross dimer in both sense and anti-sense DNA chains. Characteristics of primer pair for amplification of *PbMAPK1* gene were

summarized in Table 5. The successful extraction of parasite genomic DNA from *P. berghei*-infected mice erythrocyte at 70% parasitemia resulted in a sharp genomic DNA band (Figure 2a) and high spectrophotometric ratio of A_{260}/A_{280}, ±1.875. A single DNA band of *PbMAPK1* gene and positive control were successfully amplified using PCR method (Figure 2b). Both amplification products resulted from a single PCR amplification using same reaction profile but different primer pairs. There was no PCR product for negative control which used control DNA sample and primer pair of PbMAPK1-F/R.

Table 5 Analysis of primer pair using Primer 3 programme.

Primer	DNA sequence	Size (bp)	Tm (^{0}C)	GC (%)
PbMAP1-F	5'-TATGTGGTCGCTTGGGTG-3'	18	59.9	55.6
PbMAP1-R	5'-GAATCTTGGGATGCTTTA-3'	18	53.1	38.9

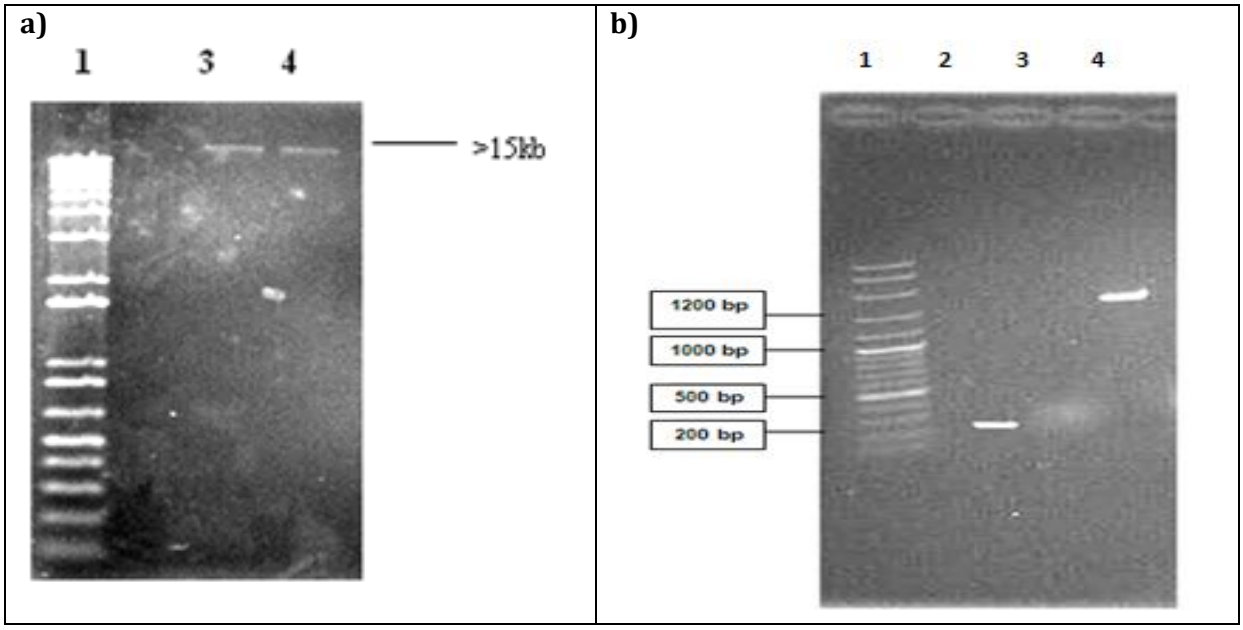

Figure 2 Agarose gel electrophoresis 1% (w/v) for parasite genomic DNA (a) and PCR products (b). For (a), well 1 represents 100 bp DNA marker and both well 3 and 4 represent parasite genomic DNA. For (b), well 1 represents 100 bp DNA marker, well 2 represents *PbMAPK1* gene, well 3 represents negative control and well 4 represents positive control.

DISCUSSIONS

MAPK signaling in rodent malaria parasite was previously investigated by [13]. In that study, by using mutant deletion model, PbMAPK2 was found to be crucial for male sexual differentiation and parasite transmission to the mosquito. In conjunction with that, we believe that there is a need to iniate the study on PbMAPK1 as well. In the present work, while functional characteristics of PbMAPK1 were studied using *in silico* approach, its presence in rodent malaria parasite was determined experimentally using PCR method.

The protein features of MAPK, such as the kinase domain, MAPK signature site, Serine/Threonine active sites and ATP binding sites are fully conserved in *P. berghei*. A protein domain corresponds to the functional part of a protein structure. It is characterized by independent protein folding and hydrophobic core [14] Domains, particularly those with enzymatic activities, may function independently or associate with larger multidomain protein. Other domains exist as binding sites in order to confer regulatory and specificity properties to multidomain proteins [14]. The conservation of the kinase domain, MAPK signature site, Serine/Threonine active sites and ATP binding site in Plasmodial MAPKs indicates that all Plasmodial MAPKs are similar to other eukaryotic MAPKs.

The nuclear localization of MAPK in *Plasmodium* parasite has been reported by previous research [5] whereby PfMAPK1 in COS-7 cells was predominantly localized at the nucleus. In this heterologous system, the basic stretches found in the PfMAPK1 are sufficient to target the protein in the nucleus where it accumulated in the nucleoli. This is in agreement with the mammalian MAPK where it localizes primarily

to the cytosol but after stimulation, MAPK rapidly and markedly accumulates in the nucleus. This nuclear localization is temporary, and MAPK redistributes to the cytosol when signaling is terminated [15-17]. For Hog1p MAP kinase, the recommencement of cytosolic localization postsignaling in cells is not perturbed by protein synthesis inhibitors and this indicates that the resynthesis of protein is not required for the cyctosolic localization [18]. Therefore, it is strongly believed that the cytosolic localization of *Plasmodium* MAPKs occur via nuclear export mechanism.

According to [19], post translational modification plays an important role in nuclear localization of cytosolic proteins. A study by [20] proposed that PfSUMO which is similar to ubiquitin may play an important role in nuclear localization of parasite intracellular components. This indicates that there is a relation between post translational modification and nuclear localization of cytosolic proteins in malaria parasite. Although the previous study [5] did not reveal any relation between post translational modification and nuclear localization of PfMAPK1, it is probable that PbMAPK1 can be regulated by post translational modification for the purpose of nuclear localization. Further experimental evidences are required to elucidate this issue.

It has been established that, matured erythrocytes circulating in blood system do not contain cellular nucleus [21]. In our study, the parasite was purely isolated from erythrocytes using Percoll 8% solution prior to DNA extraction. This is to avoid any possible contamination of parasite genomic DNA. Thus, the possibility of PCR method amplifying unspecific product from host genomic DNA was very low. Currently, the use of PCR for validating the preliminary *in silico* data is a common strategy [22]. Apart from minimizing failure of experimental works, the execution of *in silico* analysis prior to experimental works can also reduce the operational cost with respect to trial and error processes. This strategy is in agreement with the advantage of reverse vaccinology approach in which the identification of potential target genes using *in silico* approach is validated by normal experiment to test immune response [23].

CONCLUSIONS

We have reported biological features and existence of PbMAPK1 *in vivo* using a combination of *in silico*, animal model and PCR methods. Apart from providing a starting point for studying PbMAPK1 *in vivo*, this study also verifies that rodent malaria parasite can serve a good model for assisting the future work on MAPK signaling in malaria parasite.

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