Molecular & Biochemical Parasitology 162 (2008) 87–95

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Characterization of *Plasmodium falciparum* protein kinase 2

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ARTICLE INFO

Article history:
Received 24 March 2008
Received in revised form 28 July 2008
Accepted 28 July 2008
Available online 13 August 2008

Keywords:
Calcium calmodulin-dependent protein kinase
Merozoite
Phosphorylation
P. falciparum protein kinase 2
Plasmodium falciparum

ABSTRACT

A sustained elevation of free Ca^{2+} is observed on the rupture and release of merozoites of *Plasmodium falciparum* from the erythrocytes. The immunoelectron micrographs demonstrate that calmodulin is localized in merozoites. To elucidate the Ca^{2+} signal of *P. falciparum* invasion, we attempted to characterize *P. falciparum* protein kinase 2 (PfPK2), which is homologous to human calcium calmodulin-dependent protein kinase (CaMK). PfPK2 was purified as a fusion protein that was labeled with [γ-^{32}P]ATP; this labeling was then eliminated by phosphatase. This phosphorylation was eliminated when the putative catalytic lysine residue of PfPK2 was replaced with alanine. PfPK2 phosphorylated histone II_{AS} as a representative substrate in a Ca^{2+}- and calmodulin-dependent manner. Calmodulin antagonists inhibited the phosphorylation of PfPK2 *in vitro* and markedly decreased the parasitemia of ring forms in an invasion assay, whereas CaMKII-specific inhibitors had no effect. PfPK2 was localized in the merozoites in the culture of *P. falciparum*. Thus, purified PfPK2 possesses protein kinase activity in a Ca^{2+}- and calmodulin-dependent manner and the catalytic lysine of this protein was determined. These data suggest that PfPK2 is the *Plasmodium* protein kinase expressed in the merozoites during the invasion stage.

1. Introduction

Malaria is one of the most important infectious disease problems in humans, particularly in developing countries. *Plasmodium falciparum*, the most virulent human malaria parasite, is responsible for hundreds of millions of illnesses and more than one million deaths each year [1]. The life cycle of *P. falciparum* consists of sexual and asexual stages. The asexual blood stage is the most severe stage clinically [2]. Thus, it is necessary to investigate the mechanisms of invasion of erythrocytes by merozoites, as well as drugs that target this process.

One of the potential drug targets for malaria is the protein kinase of the parasite. Eukaryotic protein kinases form a large family of enzymes that have important roles in most cellular processes [3]. At least 65 protein kinases have been identified in the *P. falciparum* genome [4,5]. Except for a few of these protein kinases [6–10], the function, mechanism of regulation, and cellular targets of the kinases are unknown. Among the signals for eukaryotic protein kinases, a calcium signal is thought to be one of the most important ones.

There is a sustained elevation of free Ca^{2+} in merozoites of *P. falciparum* upon the rupture of erythrocytes and release of merozoites [11]. This increase is lost shortly after reinvasion of new erythrocytes (i.e., the early ring stage), which implies that the elevated free Ca^{2+} in merozoites plays an important role in the process of invasion. The invasion step has been investigated as clinically and pharmaceutically one of the most important targets in the blood stage of *P. falciparum*. Therefore, we attempted to unravel the functions of the protein kinases that are correlated to calcium and are supposed to be expressed during the invasion stage of *P. falciparum*.

Among the protein kinases of *P. falciparum*, five calcium-dependent protein kinases (CDPKs) are expected to be related to calcium signals. So far, the characterization of CDPKs has suggested that they play critical roles in gametogenesis or that they phosphorylate proteins of the host erythrocyte membrane *in vitro* [6,12]. Except for CDPKs, the *P. falciparum* genome encodes a protein homologous to human calcium/calmodulin-dependent protein kinase (CaMK), named *P. falciparum* protein kinase 2 (PfPK2). There has only been one report regarding PfPK2, which indicates that it is mainly localized in the parasite membrane [13]. The intraerythrocytic expression profiles and photolithographic oligo array of PlasmoDB indicate that PfPK2 (PFL1885c) mRNA is mainly expressed during the merozoite stage. During this stage, a moderately positive relationship between mRNA and protein abundance has been observed [14]. Calmodulin was shown to be localized at the apical end and underlying the plasma membrane of both free and intraerythrocytic merozoites by the electronmicroscopy

Abbreviations: PfPK2, *Plasmodium falciparum* protein kinase 2; CaMK, calcium calmodulin-dependent protein kinase; GST, glutathione *S*-transferase.

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doi:10.1016/j.molbiopara.2008.07.007

[15]. Thus far, no other gene homologous to human CaMK has been found in the *P. falciparum* genome except PfPK2. In this report, we attempted to characterize PfPK2. We showed that PfPK2 is the *Plasmodium* protein kinase localized in merozoites, and it may function with calmodulin in a calcium/calmodulin-dependent manner during invasion by *P. falciparum* merozoites.

2. Materials and methods

2.1. Plasmids

The entire PfPK2 open reading frame (ORF) was amplified by PCR using genomic DNA of *P. falciparum* clone 3D7 (provided by Malaria Research and Reference Reagent Resource Center, Manassas, VA, USA) as a template and the primers 5′-GCGCCTCGAGGGAGAAAAGATATCAGCAAT-3′ and 5′-GCGGATCCCTAATTCTGTGGGGGAGATC-3′. The amplified fragments were digested with XhoI/BamHI and cloned into the XhoI and BamHI sites of pBluescript II KS+ (Stratagene, La Jolla, CA, USA); the resultant plasmid was designated pBS-PfPK2. The pEU-GST-PfPK2 (Fig. 1B) was generated by inserting an XhoI-BamHI fragment of pBS-PfPK2 into pEU (CellFree Sciences, Yokohama, Japan) to express a glutathione *S*-transferase (GST) fusion protein. To generate pEU-GST-PfPK2KA (Fig. 1B), the lysine at position 140 of PfPK2 was replaced with alanine using a QuikChange site-directed mutagenesis kit (Stratagene) with the oligonucleotide 5′-CGTGTGGTTGTAGCAGAAGTTGATAAATC-3′ and its complementary oligonucleotide, according to the manufacturer's instructions.

2.2. Wheat germ cell-free protein synthesis system

At the transcription step, 2 μg of pEU-GST-GFP, pEU-GST-PfPK2, or pEU-GST-PfPK2KA was mixed with 18 μl of transcription mixture (transcription buffer with 2.5 mM NTP mix, 1 U/μl RNase inhibitor, 1 U/μl SP6 RNA polymerase; CellFree Sciences) and incubated at 37 °C for 6 h. Each mRNA that was generated was mixed with 10.8 μl of WEPRO1240G (CellFree Sciences) and 40 ng/μl creatine kinase (Roche, Germany), transferred into the bottom of the SUB-AMIX

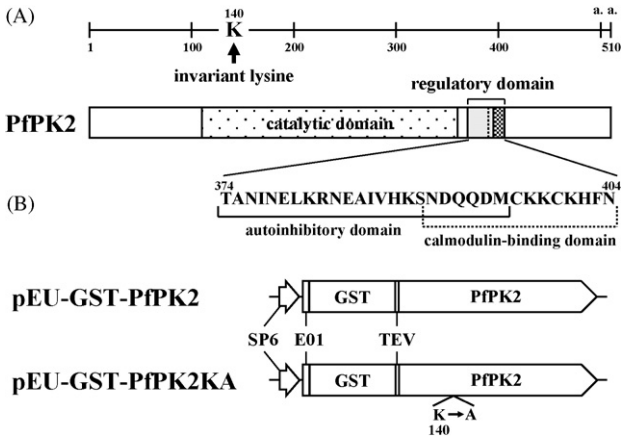

Fig. 1. Schematic diagram of the predicted amino acid sequence of PfPK2 and the expression plasmids. (A) The shaded areas represent catalytic and regulatory domains [autoinhibitory domain (AID) and calmodulin-binding domain (CBD)], which correspond to those of human CaMKIγ [28]. The putative autoinhibitory and calmodulin-binding domains are shown. The invariant catalytic lysine is shown by an arrow. The a.a. stands for amino acids. (B) pEU-GST-PfPK2 and pEU-GST-PfPK2KA used for the expression of GST-PfPK2 and GST-PfPK2KA, respectively, in the wheat germ cell-free protein synthesis system. The positions of the SP6 promoter (SP6), translational enhancer (E01), GST, and tobacco etch virus protease recognition site (TEV) are shown [44].

(CellFree Sciences) to form a bilayer, and incubated at 16 °C for 20 h. To analyze whether the generated protein was bound to Ca^{2+} or calmodulin at the translation step, the Ca^{2+} chelator and the calmodulin antagonist, i.e., 5 mM EGTA (Sigma–Aldrich, St Louis, MO, USA) and 0.66 mM W-7 (BIOMAL, Plymouth Meeting, PA, USA), or 0.05 mM calmidazolium (Sigma–Aldrich), were added to SUB-AMIX.

2.3. Purification of GST fusion proteins

The wheat germ extracts were mixed with 10 μl of 50% slurry of glutathione–sepharose beads (GE Healthcare, UK) for 2 h. The beads were then washed three times with buffer C [50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, and 1 tablet of protease inhibitor cocktail, EDTA-free, per 50 ml (Roche)]. Purified protein captured on the beads was separated by 10% SDS-PAGE and either silver stained (Fig. 2A) or immunoblotted (Fig. 2B) with rabbit antiserum containing anti-GST antibody, as described previously [16].

2.4. In vitro kinase assay

Purified GST-GFP, GST-PfPK2, or GST-PfPK2KA captured on glutathione–sepharose beads was rinsed twice with washing buffer (50 mM Tris–HCl, pH 9.0, and 2 mM DTT). Kinase assay reactions were performed with the purified GST fusion proteins at 37 °C for 30 min in a total volume of 50 μl of kinase buffer (50 mM Tris–HCl, pH 8.0, 200 mM NaCl, 50 mM $MgCl_2$, 0.1% Nonidet P-40, and 1 mM DTT) containing 5 μCi [γ-^{32}P]ATP. After incubation, the samples were washed with TNE buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) three times, and the phosphorylated proteins were separated by 10% SDS-PAGE. The proteins were stained with CBB, dried, and exposed to X-ray film [17,18].

To calculate the activity of substrate phosphorylation of PfPK2, histone II_{AS} (Sigma–Aldrich) was used as a representative substrate at the various concentrations of $CaCl_2$ and bovine calmodulin (99.3% similarity to that of *P. falciparum*). To evaluate the effects of CaMKII-specific inhibitor on the autophosphorylation and histone phosphorylation of PfPK2, GST-PfPK2 and histone II_{AS} were incubated with 28 μM KN-92 (Sigma–Aldrich), 28 μM KN-93 (Sigma–Aldrich), 33 μM myristoylated AIP (Alexis, Switzerland), 29 μM AIP (Alexis), 0.1, 1.0, 10 μM staurosporine (Sigma–Aldrich), or an equal volume of DMSO in the presence of 10 μM Ca^{2+}. The activity of PfPK2 in autophosphorylation and histone phosphorylation was measured by scintillation counting of pieces of dried gel corresponding to bands of GST-PfPK2 and histone three times.

2.5. Phosphatase treatment

After the *in vitro* kinase assays, GST fusion proteins captured on glutathione–sepharose beads were subjected to phosphatase treatment, as described elsewhere [17,18].

2.6. Parasite culture

P. falciparum 3D7 parasites were cultured in human erythrocytes at 3% hematocrit of human AB+ erythrocytes in RPMI 1640 medium supplemented with 0.5% Albumax II (Invitrogen, Carlsbad, CA, USA) [19,20].

2.7. Invasion assay

The late schizonts of *P. falciparum* 3D7 parasites were collected with percoll/sorbitol treatment and subjected to an invasion assay, as described elsewhere [21], in the presence of CaMKII inhibitor,