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Subcellular localization of adenylate kinases in *Plasmodium falciparum*

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ABSTRACT

Adenylate kinases (AK) play a key role in nucleotide signaling processes and energy metabolism by catalyzing the reversible conversion of ATP and AMP to 2 ADP. In the malaria parasite *Plasmodium falciparum* this reaction is mediated by AK1, AK2, and a GTP:AMP phosphotransferase (GAK). Here, we describe two additional adenylate kinase-like proteins: PfAKLP1, which is homologous to human AK6, and PfAKLP2. Using GFP-fusion proteins and life cell imaging, we demonstrate a cytosolic localization for PfAK1, PfAKLP1, and PfAKLP2, whereas PfGAK is located in the mitochondrion. PfAK2 is located at the parasitophorous vacuole membrane, and this localization is driven by N-myristoylation.

Structured summary of protein interactions:
EXP-1 and **PfAK2** colocalize by fluorescence microscopy (View interaction)
PfAK2 and **SERP** colocalize by fluorescence microscopy (View interaction)

1. Introduction

Malaria still is a great socioeconomic and health problem responsible for 655 000 to around 1.24 million deaths and 216 million infections in 2010 [1,2]. Due to the absence of a vaccine and increasing resistance to current drugs, new chemotherapeutical approaches are urgently needed [3].

Adenylate kinase (AK, EC 2.7.4.3, ATP + AMP ⇔ 2 ADP) catalyzes the reversible high energy phosphoryl transfer from ATP to AMP forming ADP and is involved in energy, metabolic, and signaling pathways [4]. Two AKs (PfAK1, PfAK2) and a GTP:AMP phosphotransferase (PfGAK, EC 2.7.4.10, GTP + AMP ⇔ GDP + ADP) were characterized in the malaria parasite *Plasmodium falciparum* [5,6]. Recombinant PfAK1 displays the highest specificities for AMP and ATP as substrates, whereas PfGAK exhibits a preference for GTP and AMP [5]. Interestingly, PfAK2 can be N-myristoylated and forms a heterodimer with N-myristoyltransferase [6]. PfAK1 has been hypothesized to be located in mitochondria, since it contains an N-terminal amphipathic helix (residues 118–130) that could function as a mitochondrial import signal [5]. While most GAKs are located in the mitochondrial matrix, PfGAK has been predicted (PlasmoAP4.4, PlasmoDB) to be targeted to the apicoplast, a non-photosynthetic plastid found in *Apicomplexa*. For PfAK2, no target signals could be identified when using the prediction programs PlasmoAP and PlasMit [6].

As reported here, a new putative AK (PFA0530c, adenylate kinase-like protein 1, PfAKLP1) from *P. falciparum* is homologous to human AK6. Additionally, a second protein (PFI1550c, PfAKLP2) was predicted to belong to the AK family. We cloned and heterologously overexpressed these two AK-like proteins. Furthermore, we systematically studied the subcellular localization of all *Plasmodium* AK isoforms, and showed that N-myristoylation is required to target PfAK2 to the parasitophorous vacuole membrane (PVM).

2. Materials and methods

2.1. PCR amplification, sequencing, and subcloning of PfAKLP1 and PfAKLP2

In order to amplify the PfAKLP1-encoding sequence, a gametocyte as well as a blood stage cDNA library of *P. falciparum* 3D7 were successfully employed.

Abbreviations: aa, amino acid; AK, adenylate kinase; AKLP1, adenylate kinase-like protein 1; AKLP2, adenylate kinase-like protein 2; AP_5A, P1,P5-di(adenosine-5′) pentaphosphate; CRT, chloroquine resistance transporter; *E. coli*, *Escherichia coli*; GAK, GTP:AMP phosphotransferase; GP5A, P1,P5-di(guanosine-5′) pentaphosphate; hCINAP, human coilin interacting nuclear ATPase protein; IRBC, infected red blood cell; NCBI, National Center for Biotechnology Information; Ni-NTA, nickel-nitrilotriacetate; P-loop, phosphate binding loop with the canonic sequence GxxGxGxxT; PVM, parasitophorous vacuole membrane; SERP, serine-rich protein; TBST, Tris-buffered saline Tween-20

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Forward (5′-AACATATGAAAAGAAAAGTACCGAATATAAT-3′) and reverse (5′-AACTCGAGTATATATGAGAGAACCCAATTTTTTA-3′) primers (Eurofins MWG Operon) with *Nde*I and *Xho*I restriction sites (underlined) were used (PCR: 94 °C for 3 min; 94 °C for 30 s, 55 °C for 45 s, 72 °C for 90 s, 30 cycles; 72 °C for 10 min). The amplified sequence was cloned into a pGEM-T easy vector (Promega) for sequencing. The correct PfAKLP1 gene without stop codon (558 bp) was then subcloned into the expression vector pET24a (*aklp1*/*pET24a*) with a C-terminal hexahistidyl (His)-tag.

For amplification of the PfAKLP2-encoding sequence, two primers (5′-ATATGGATCCGAAACACTTCTACATAGCGAAATAT-3′ and 5′-ATATCTCGAGTT ACCTTATATAGGAAAGAACTTGG-3′, (Eurofins MWG Operon)) with restriction sites for *BamH*I and *Xho*I (underlined) were employed. This led to the amplification of an approximately 790 bp product by PCR (94 °C for 3 min; 94 °C for 30 s, 57 °C for 50 s, 72 °C for 120 s, 30 cycles; 72 °C for 10 min) from a gametocyte as well as a blood stage cDNA library of *P. falciparum* 3D7. The PCR product was cloned into a pGEM-T easy vector for sequencing. The fragment with the correct sequence was then subcloned into pET28a (Novagen) (*aklp2*/*pET28a*) with an N-terminal His-tag.

2.2. Heterologous overexpression and purification of PfAKLP1 and PfAKLP2

The construct *aklp1*/pET24a was transformed into *Escherichia coli* KRX cells (Promega). Cells were grown at 37 °C in Terrific Broth medium containing 50 μg/ml kanamycin. The overexpression was induced by 0.1% (w/v) rhamnose and the culture was further grown at 30 °C for 15 h. Cells were harvested and lysed by the addition of lysozyme and DNase and sonication in the presence of protease inhibitors. The recombinant protein was purified via a nickel-nitrilotriacetate (Ni–NTA) column (Qiagen).

Overexpression and purification of PfAKLP2 were carried out as described for PfAKLP1, but Protino^{R} Ni-TED resin (Macherey–Nagel) was used instead of Ni–NTA for the purification.

2.3. Gel filtration

PfAKLP1 and PfAKLP2 were further purified by gel-filtration chromatography on a HiLoad 16/60 Superdex 200 prep-grade column connected to an ÄKTA FPLC system (Amersham Pharmacia Biotech). The column was calibrated with a gel-filtration standard (Amersham Pharmacia Biotech) and equilibrated with buffer containing 50 mM sodium phosphate, 300 mM NaCl, pH 8.0. Proteins in eluate fractions were detected spectrophotometrically at 280 nm. The protein fractions containing PfAKLP1 and PfAKLP2 were concentrated with a 3 kDa viva spin column (Sartorius Stedim Biotech). Protein concentrations were determined using the Bradford protein assay [7]. Purity of the recombinant proteins was verified by SDS–PAGE.

2.4. Enzyme activity assays

The AK assay used is based on the determination of NDP with pyruvate kinase and lactate in a coupled assay system [8]. The enzyme assay mixture contained 200 μM NADH, 10 U/ml lactate dehydrogenase (Roche), 10 U/ml pyruvate kinase (Roche), 2.3 mM NMP, 0.8 mM NTP, and rate-limiting quantities of PfAKLP1 or PfAKLP2 in 1.5 mM $MgCl_2$, 90 mM KCl, 110 mM triethanolamine-HCl, pH 7.6, at 25 °C. The consumption of NADH (ε_{340nm} = 6.22 mM^{-1} cm^{-1}) was measured spectrophotometrically at 340 nm. In order to determine the substrate specificity, we tested AMP, CMP, UMP, IMP, and GMP as substrates while using ATP as the phosphate donor. The NTPs were also studied by testing ATP, GTP, ITP, and UTP as phosphate donors with AMP as described in [5,6].

2.5. Construction of the GFP fusion vector

In order to investigate the localization of all AKs in *P. falciparum*, we designed primers to insert *Bgl*II and *Avr*II restriction sites at the ends of the genes of PfAK1, PfAK2, PfGAK, and PfAKLP1. The genes were cloned into a pARL-1a+ shuttle vector, which contains a low-expression CRT promoter and allows episomal gene expression as described previously [11]. The transfection vectors containing the fusion constructs were verified by sequencing, transformed in *E. coli*, and purified from *E. coli* using a Qiagen Maxi Kit (Qiagen).

Because of an *Avr*II restriction site in PfAKLP2, we first introduced a silent mutation to remove the *Avr*II restriction site. Subsequently, we constructed a GFP fusion vector for the PfAKLP2 gene as described above.

2.6. Site directed mutagenesis

Glycine in position 2, the N-myristoylation site of PfAK2 [6], was mutated to an alanine. PCR for site-directed mutagenesis of PfAK2 was performed with a *Pfu* polymerase (Promega) using primers carrying the respective mutated codon: 5′-GAGATCT ATGGCATCATGTT ATAGTAGAAAAAAT-3′, 5′-ATTTTTTCTACTATAA CATGATGCCATAGATCTC-3′ (Eurofins MWG Operon); the mutated codon is underlined. PCR templates were removed by digestion with *Dpn*I, and the PCR product was subsequently transformed into competent *E. coli* XL1-Blue cells. The introduction of the correct mutation into the gene sequence was verified by sequencing, and a GFP-fusion construct of PfAK2^{G2A} was created as described above.

2.7. Parasite transfection and immunofluorescence

Blood stages of *P. falciparum* 3D7 were grown in continuous culture as described previously [9]. Electroporation was applied to transfect *Plasmodium falciparum* 3D7 with pARL-1a+ vectors containing the respective GFP-AK fusion constructs. Transfected parasites were selected under drug pressure with 2 nM WR99210 (kindly supplied by D. Jacobus, Princeton, New Jersey, USA) as described previously [10]. Live cell immunofluorescence images of the parasites were determined as described before [11].

2.8. Western blot analysis

The transgenic parasites were maintained as described [9]. Parasitized red blood cells with *P. falciparum* at the trophozoite stage (IRBC) were enriched using LD-columns (MACS, Miltenyi Biotec) [12]. IRBCs were harvested at 300 g for 3 min at room temperature and resuspended in 50 μl 10 mM Tris pH 7.4, containing protease inhibitors (Complete, Roche). IRBCs were lysed by three freezing-thawing cycles using liquid nitrogen, and the lysate was centrifuged at 50000×*g* for 30 min at 4 °C. The supernatant containing the erythrocyte cytosol and parasite cytosol was transferred to a new tube and centrifuged two more times in order to remove any remaining contamination with the membrane fraction. The membrane pellet derived from the first centrifugation of the lysate was washed once with 1 ml and twice with 100 μl of the Tris-buffer.

The Western blots were performed by using anti-GFP (1:1000, Roche), anti-Hsp70 (1:1000, T. Blisnick, Paris), and anti-Exp1 (1:500, Jude Przyborski) antibodies, respectively, followed by HRP-conjugated secondary anti-mouse antibodies (1:10000, Jackson ImmunoResearch).