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Plasmodium falciparum histidine triad protein and calmodulin modulates calcium homeostasis and intracellular proteolysis

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ABSTRACT

Calcium signaling has an essential role in fundamental processes of *Plasmodium* life cycle, including migration, cell invasion and parasite development. Two important players in calcium homeostasis, the Histidine Triad (HIT) protein that is implicated in calcium signaling in mammalian cells and calmodulin, which is a classic calcium sensor in eukaryotes are present in *Plasmodium falciparum,* however theirs function is unknown in the parasite. Here, we investigated the involvement of the *P. falciparum* Histidine Triad protein (PfHint-1) and calmodulin (PfCaM) in calcium signaling and intracellular proteolysis. For this, we targeted PfHint-1 with a hemagglutinin tail and overexpressed both proteins. We observed that PfHint-1 is expressed throughout the erythrocytic stages and partially colocalizes to the endoplasmic reticulum. Parasites overexpressing PfHint-1 displayed lower ER Ca^{2+} content and a higher $[Ca^{2+}]_{cyt}$ rise in the parasite cytosol upon Ca^{2+} addition to the extracellular medium after depletion of ER calcium store. PfCaM-overexpressing parasites exhibit a higher $[Ca^{2+}]_{cyt}$ rise after challenge with the calmodulin inhibitor, calmidazolium. The calcium-dependent proteolytic activity in PfCaM- and PfHint-1-overexpressing parasites was increased and correlated to alterations in calcium homeostasis. Taken together, our results indicate the participation of these proteins in *P. falciparum* fundamental cellular processes and highlights promising targets for the development of antimalarial drugs.

1. Introduction

Malaria is an important human parasitic infection that affected around 216 million people in 2016 leading to approximately 445 thousand deaths [1]. The disease is caused by the protozoan *Plasmodium* and the most serious symptoms are elicited by *Plasmodium falciparum*, that infects hepatocytes and erythrocytes in humans (reviewed in Ref. [2]).

Histidine triad proteins (HITs) are characterized by the presence of a HxHxHxx motif, where x designates a hydrophobic amino acid (reviewed in Ref. [3]). The first member of HITs was identified through Ca^{2+}-dependent hydrophobic interaction chromatography [4] and was reportedly able to inhibit the activity of protein kinase C (PKC) [5], which is currently a disputed function of this HIT. Although their function is largely unknown, some HITs were reported to bind and hydrolyze nucleotides [6,7], hence their name Hint (Histidine triad nucleotide binding, (reviewed in Ref. [3]).

Calcium is an important second messenger that has an essential role in fundamental processes of *Plasmodium* life cycle, such as invasion of red blood cells, egress, development and gliding motility (reviewed in Ref. [8]). It has been shown that malaria parasites are capable to maintain calcium concentration at

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nanomolar levels during their intraerythrocytic maturation [9]. This phenomenon is possible due to the micromolar extracellular calcium environment of the parasitophorous vacuole [10] and the parasite intracellular calcium stores, such as the endoplasmic reticulum (ER), mitochondria and acidic compartments, which participate in calcium homeostasis [11]. The available data about calcium signaling in *Plasmodium* suggests that some calcium effectors are unique, which converts them into novel therapeutic targets (reviewed in Ref. [8]).

Histidine triad protein 1 (Hint-1) has been described as a protein with similar properties to calmodulin and related Ca^{2+}-binding proteins. *P. falciparum* possesses one protein annotated as a PKC inhibitor (PfHint-1, PlasmoDB access number PF3D7_0817500), which has 46% identity to the human Hint-1 whose function is little known [4]. Calmodulin is a 17 kDa multifunctional calcium-binding protein, well conserved between *Plasmodium* species and eukaryotes (PfCaM, PlasmoDB access number PF3D7_1434200), with direct involvement with kinase signaling pathways [12]. This calcium sensor is expressed throughout intraerythrocytic stages of *P. falciparum* [13] and the employment of calmodulin inhibitors suggests that the protein might work as a calcium source and control calcium homeostasis in internal acidic stores [13]. Inhibition of PfCaM impairs parasites development, suggesting a critical role of the protein during the intraerythrocytic cycle of *Plasmodium* [14]. Herein, we show the Hint-1 of *P. falciparum* (PfHint-1) expression throughout the erythrocytic stages and their involvement in calcium homeostasis and proteolysis in the parasite. We also evaluate the function of PfCaM in calcium homeostasis and Ca^{2+}-dependent proteolysis in *P. falciparum.*

2. Materials and methods

2.1. P. falciparum culture and isolation

P. falciparum 3D7 strain was cultured in RPMI 1640 (Gibco, Carlsbad, USA) supplemented with 0.5% Albumax I (Life Technologies, Carlsbad, CA) according Trager and Jensen, 1976 [15]. The parasites were isolated from the infected erythrocytes (iRBC) by selective lysis with 0.01% saponin in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM NaH_2PO_4) followed by centrifugation at 2000 × g for 10 min. The isolated parasites were washed twice in PBS to remove the red cell membranes.

2.2. PfHint-1 knockin in P. falciparum

The 3′ genomic sequence of PfHint-1 (PlasmoDB access number PF3D7_0817500), without the stop codon, was amplified with the forward primer 5′- CGAGATCTTGCAGGAAAAGATGAAAATGGAGATTCAATTTTTG -3′ (BglII site underlined) and reverse primer 5′-AACTGCAGCACCAGGGGGCCATTTCATTTG -3′ (PstI site underlined). The amplified sequence was cloned into pcr2.1 vector, sequenced and subcloned into the knockin vector PTEX150-HA-Str, allowing homologous recombination of the PfHint-1 genomic locus with the plasmid and the resulting C-terminal fusion of PfHint-1 with hemagglutinin (HA) and strep tag (Str) [16]. Selection and cloning of PfHint-1 knockin parasites (PTEX-PfHint-1) were performed under 2.5 nM of WR99210. Insertion of the HA and Str tags in the PfHint-1 locus was verified in parasite clones by PCR employed the primers reverse 5′-GGATACGCATAATCGGGCAC-3′ and forward 5′-GGCGGATGAAGAAGAAAGAGC-3′ and western blot.

2.3. Episomal overexpression of PfHint-1 and PfCaM in P. falciparum

The PfHint-1 ORF (PlasmoDB access number PF3D7_0817500), without the stop codon, was amplified from cDNA of *P. falciparum* 3D7 strain using the forward primer 5′-TTTCCGCTCGAGATGGGTTTTAAAATAGTCTTTATATATTTAATTTTTGTTTGC-3' (XhoI site underlined) and reverse primer 5′-GCGAAAACGCGTACCAGGGGGCCATTTCATTTG-3' (MluI site underlined). The RNA extraction was performed using the Trizol (ThermoFisher Scientific, Waltham, MA, USA) and the cDNA was obtained using the RevertAid First Strand cDNA Synthesis kit (ThermoFisher Scientific, Waltham, MA, USA) following manufacturer's instructions. The amplified sequence was cloned into pcr2.1 vector, subcloned into the overexpression vector PEF-HA, yielding the PEF-PfHint-1 plasmid, with PfHint-1 under the control of the strong *Plasmodium* EF1α promoter [17]. Parasites were transfected and then selected by the maintenance of 2.5 nM WR99210 in RPMI 1640 supplemented with 0.5% Albumax I culture medium. The ORF of PfCaM (PlasmoDB access number PF3D7_1434200), codon-optimized for expression in *E. coli* by Genscript (www.genscript.com), was amplified with the forward primer 5′- CTCGAGATGGCGGATAAACTGACCG-3′ (XhoI site underlined) and the reverse primer 5′-ACGCGTTTTCGCAATCATCATTTTCACAAAT-3′ (MluI site underlined), cloned into pcr2.1 vector and subcloned into the PEF-HA vector, yielding the PEF-PfCaM plasmid, which was transfected in *P. falciparum.* To confirm the overexpression of PfHint-1 and PfCaM, Western blot assays and RT- PCR were performed.

2.4. Western blot of P. falciparum parasites

Parasites were isolated in PBS in the presence of a protease inhibitor cocktail (1 μM pepstatin, 10 μM E−64, 1 mM *ortho*-phenanthroline, 1 mM PMSF) at different stages during the third erythrocytic cycle. Parasites were lysed in 50 mM Tris, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA (pH 7.2) with the addition of the protease inhibitor cocktail described above. After lysis, the supernatant was submitted to SDS-PAGE, transferred to a PVDF membrane and blocked with 5% BSA in TBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.2). The membrane was cut in two and incubated with 1:2000 anti-HA, raised in rabbit (Sigma-Aldrich, St. Louis, MO) or anti-GAPDH raised in mouse (Sigma-Aldrich, St. Louis, MO) or 1:2000 anti-*Plasmodium falciparum* aldolase raised in mouse (Santa Cruz Bio-technology, CA, USA) in TBST +5% BSA, overnight, at 4 °C. After three washes in TBST, the membranes were incubated for 2 h at room temperature with anti-mouse IgG HRP conjugated (1:1000) or anti-rabbit IgG HRP conjugated (1:1000) (R&D Systems, MN, USA) in TBST +5% BSA. To detect the bands the membranes were incubated with SuperSignal WestPico substrate (ThermoFisher Scientific, Waltham, MA, USA).

2.5. Immunofluorescence of PfHint-1 knockin (PTEX-PfHint-1) parasites

Immunofluorescence was performed based on [18], using 0.24% NH_4Cl and 0.15% of glycine instead of $NaBH_4$ as autofluorescence quenching reagents. After fixation, quenching and block, the primaries antibodies anti-HA (rabbit) (1:50) and anti-PfBIP (mouse) (1:500) were incubated overnight at 4 °C. After three washings, the secondaries antibodies anti-IgG Rabbit Alexa Fluor 488, anti-IgG Rabbit Alexa Fluor 546 or anti-IgG mouse Alexa Fluor 488 (1:300) were incubated 2 h at room temperature. For mitochondria staining assays, the incubation with mitochondrial probe Mitotracker Red CMXROS (Life Technologies, Carlsbad, CA, USA) was performed before the fixation step, at 25 nM for 30 min, 37 °C in MOPS (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO4, 5.5 mM D-glucose, 50 mM MOPS, and 2 mM CaCl2, pH 7.2). DAPI (5 μg/mL) was incubated concomitantly with the secondary antibody. Images were

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