Parasitology International 62 (2013) 423–430

Contents lists available at SciVerse ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Characterization of *Plasmodium falciparum* cdc2-related kinase and the effects of a CDK inhibitor on the parasites in erythrocytic schizogony

Tatsuya Iwanaga [a], Tatsuki Sugi [a], Kyousuke Kobayashi [a], Hitoshi Takemae [a], Haiyan Gong [a], Akiko Ishiwa [a], Fumi Murakoshi [a], Frances C. Recuenco [a], Taisuke Horimoto [a], Hiroomi Akashi [a], Kentaro Kato [a,b,*]

[a] Department of Veterinary Microbiology, Faculty of Agriculture, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
[b] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form 27 March 2013
Accepted 11 May 2013
Available online 18 May 2013

Keywords:
Cyclin
Olomoucine
Pfcrk-1
Plasmodium falciparum

ABSTRACT

The cell cycle of *Plasmodium* is unique among major eukaryotic cell cycle models. Cyclin-dependent kinases (CDKs) are thought to be the key molecular switches that regulate cell cycle progression in the parasite. However, little information is available about *Plasmodium* CDKs. The present study was performed to investigate the effects of a CDK inhibitor, olomoucine, on the erythrocytic growth of *Plasmodium falciparum*. This agent inhibited the growth of the parasite at the trophozoite/schizont stage. Furthermore, we characterized the *Plasmodium* CDK homolog, *P. falciparum* cdc2-related kinase-1 (Pfcrk-1), which is a potential target of olomoucine. We synthesized a functional kinase domain of Pfcrk-1 as a GST fusion protein using a wheat germ protein expression system, and examined its phosphorylation activity. The activity of this catalytic domain was higher than that of GST-GFP control, but the same as that of a kinase-negative mutant of Pfcrk-1. After the phosphatase treatment, the labeling of [γ-^{32}P]ATP was abolished. Recombinant human cyclin proteins were added to these kinase reactions, but there were no differences in activity. This report provides important information for the future investigation of *Plasmodium* CDKs.

1. Introduction

Plasmodium parasites cause malaria, a severe disease characterized by acute fever and anemia in vertebrate animals, including humans. This disease caused an estimated 650 thousand deaths in 2010, mainly in Africa (http://www.who.int/mediacentre/factsheets/fs094/en/), and it represents a serious barrier to the social and economic progress of many second and third world countries. For the treatment of malaria, antimalarial agents that inhibit growth of the parasites have been used. However, drug-resistant parasites have been reported to appear every year, making necessary development of novel drugs and new therapeutic approaches. Therefore, there is a great deal of research interest regarding the molecular biology of *Plasmodium* parasites.

The intraerythrocytic cycle of the *Plasmodium* parasite differs from the normal cell cycle of eukaryotes. In typical eukaryotes, chromosomes replicate once per cell cycle. On the other hand, the parasite performs multiple rounds of DNA replication at one cycle of erythrocytic schizogony. The multiple replications at the trophozoite stage are thought to produce highly polyploid chromosomes before the schizont stage when asynchronous nuclear divisions appear to intervene between rounds of DNA replication [1,2].

Cyclin-dependent kinases (CDKs) play an important role in cell cycle progression. Every eukaryote has CDKs and the function of CDKs is thought to be highly evolutionarily conserved. Therefore, CDKs are expected to play an important role even in the complicated cell cycle of *Plasmodium* parasites. The CDK coordinates with a regulatory protein, cyclin. Cyclin binds at the regulatory domain of CDK, and thus forms a cyclin–CDK complex. Then, this active form of CDK phosphorylates substrate proteins to regulate the activities of these proteins. The transition from one cycle stage to the next requires the switching of these activities of substrate proteins. Therefore, this kinase family is thought to orchestrate progression of the entire cell cycle. For example, in the cell cycle of *Saccharomyces cerevisiae*, in which CDK functions are well known, Cdk1 is inactive during G1 phase, because of the lack of cyclins and the presence of cyclin-dependent kinase inhibitor proteins (CKI). At the late stage of G1 phase, when B-type cyclins increase and CKIs are degraded, Cdk1 that binds to cyclin, is phosphorylated by cyclin-dependent kinase activating kinases (CAKs), and phosphorylates many substrate proteins. Some of these molecules play a role in transition from G1 phase to S phase, while others play a role in DNA replication [3].

A number of CDK homologs have been identified in the genome of *Plasmodium falciparum*, which shows the most severe pathogenicity in humans. However, little was known and characterized about their function. To determine the functions of CDKs in the asexual growth of *P. falciparum*, we investigated how an inhibitor of CDKs, olomoucine, affected the growth of the parasite. Olomoucine, a substituted purine

* Corresponding author at: National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan. Tel.: +81 155 49 5645; fax: +81 155 49 5646.
E-mail address: kkato@obihiro.ac.jp (K. Kato).

1383-5769/$ – see front matter © 2013 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.parint.2013.05.003

derivative, is a highly specific CDK inhibitor, which inhibits mammal CDK1 and CDK2. In previous studies, this chemical was shown to inhibit *P. falciparum* growth throughout the asexual life cycle [4–6]. We monitored the time course of the asexual development of parasites from the ring stage to the trophozoite and schizont stages, and observed at which stage(s) of the cell cycle the inhibitor exerts an effect on the parasites.

Moreover, for searching potential targets of olomoucine, we characterized one of the *Plasmodium* CDK homolog, Pfcrk-1. Pfcrk-1, *P. falciparum* cdc2-related kinase ("cdc2" means "cell division control protein 2"), has the greatest similarity with the p58-GTA protein kinase (CDK11). A previous study indicated that this kinase is expressed at higher levels in gametocytes than in asexual blood stages, but some studies using reverse genetics showed that intraerythrocytic growth of *P. falciparum* and *Plasmodium berghei*, a murine malaria parasite, requires Pbcrk-1, the homolog in *P. berghei* [7–9]. The function of this kinase has never been revealed. In this study, using a wheat germ protein expression system, we synthesized a catalytic kinase domain of Pfcrk-1. The activity of Pfcrk-1 as a kinase was examined by *in vitro* kinase assay using mammalian histone protein.

2. Materials and methods

2.1. Parasite culture

The *P. falciparum* clone HB3, provided by the Malaria Research and Reference Reagent Resource Center (MR4), was maintained in culture flasks with human AB + erythrocytes (1% hematocrit) as described elsewhere [10]. Cultures were synchronized by the addition of 5% D-sorbitol (Wako Pure Chemical Industries, Osaka, Japan). For tighter synchronization, we added 30 IU/ml heparin (Mochida Pharmaceutical, Shinjuku, Japan) to inhibit the invasion of parasites, and washed with medium 4 h before sorbitol treatment to allow schizonts to rupture and merozoites to invade new erythrocytes [11,12]. The parasitemia and parasite stages were checked daily by microscopy of Giemsa-stained blood smears.

2.2. Growth inhibition assay

A growth inhibition assay (GIA) to assess the IC_{50} of the protein kinase inhibitor was performed. We used flow cytometry for the quantification of the parasites because we can estimate the parasitemia more objectively than microscopy. Parasites at synchronized trophozoite stage were exposed to the agents for 48 h, a period in which all parasites can complete one erythrocytic cycle from the trophozoite stage to the next trophozoite stage. Parasites were cultured with various drug concentrations in 96-well plates at 0.5–1.0% parasitemia and 1% hematocrit. The protein kinase inhibitor olomoucine (Calbiochem, San Diego, CA) is soluble in dimethyl sulfoxide (DMSO), and all assays were carried out using a control culture treated with 0.1% DMSO (*i.e.*, the same concentration as in the assayed cultures). After 48-h incubation, parasitemia of the cultures was determined by flow cytometry as described elsewhere [13]. Briefly, 100 μl of 10 μg/ml ethidium bromide (Nippon Gene, Chiyoda, Japan) in phosphate-buffered saline (PBS) was mixed with 25 μl of parasite culture and incubated for 1 h in the dark at room temperature. After centrifugation, the supernatant was discarded, cells were resuspended in 700 μl of PBS, and samples were analyzed using a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA). Parasitemia was evaluated using the WinMDI 2.9 software (The Scripps Research Institute; http://facs.scripps.edu/software.html) by gating for intact erythrocytes by side scattering and forward scattering parameters and subsequent determination of the proportion of ethidium bromide-positive cells, indicating infected RBCs. Each parasitemia value was also validated by microscopy of blood smears.

To investigate the effects of the kinase inhibitor on the parasite life cycle, we examined the time course of the development of parasite cultures by microscopy of blood smears. Once a culture plate was taken out of the incubator, the growth of parasites was delayed. We prepared as many culture plates as necessary to take samples from the same culture, and at each sampling time we removed one plate from the incubator and sampled once. Each sample was used to make a thin blood smear, which was stained with Giemsa, and the numbers of parasites at the ring, trophozoite (parasites at this stage have a hemozoin pigment), and schizont (parasites at this stage have multiple nuclei) stages, were counted under a microscope. The proportion of each stage of parasites in 10,000 RBCs was calculated. All examinations were performed in triplicate.

2.3. Plasmids

Total RNA was isolated from *P. falciparum* clone HB3. First-strand cDNA was amplified from this RNA. The catalytic domain (kinase domain) of Pfcrk-1 (PFD0865c; PlasmoDB) open reading frame (ORF) was amplified by PCR using the total cDNA as a template, an iProof High-Fidelity PCR Kit (Bio-Rad, Berkeley, CA), and the primers 5′-TACTCGAGGATGGGAAAAGGGCATGATGT-3′ (primer A, *Xho*I restriction site is underlined) and 5′-TAGGTACCTCATGAATGGAATT GGATATTATTTTGG-3′ (primer B, *Kpn*I site is underlined). The amplified fragments were digested with restriction enzymes. The plasmid, pEU-GST-PfPK2 [14], was digested with the same enzymes and PfPK2 site was replaced with Pfcrk-1 fragment. The product was designated pEU-GST-Pfcrk-1. To generate KA mutant of pEU-GST-Pfcrk-1, pEU-GST-Pfcrk-1KA, the lysine at position 383 of Pfcrk-1 (corresponding to position 41 of the Pfcrk-1 kinase domain) was replaced with alanine by the overlap extension PCR method [15]. First PCR was performed with primer A and the oligonucleotide 5′-CTAGAAAAATTTTTTAGTTTC GCCAAGGCAACGATCTTTTTCG-3′ (primer C, targeted mutation site is underlined), or with the oligonucleotide 5′-CGAAAAAGATCGTTGCCTTG GCGAAACTAAAAAATTTTTCTAG-3′ (primer D, targeted mutation site is underlined) and primer B using pEU-GST-Pfcrk-1 as a template. The amplified fragments were mixed, and a second PCR was performed with primers A and B using the mixed fragments as a template. The products of second PCR were digested with restriction enzymes and cloned into the *Xho*I and *Kpn*I sites of pEU-GST-Pfcrk-1 vector to replace Pfcrk-1 site with the mutated fragment.

2.4. Wheat germ cell-free protein expression system

Protein expression of the Pfcrk-1 kinase domain and green fluorescent protein (GFP) with a glutathione S-transferase (GST)-tag was performed with a WEPRO1240 Expression Kit in accordance with the manufacturer's protocol (CellFree Sciences, Yokohama, Japan). Aliquots of 2 μg of each plasmid (pEU-GST-Pfcrk-1, pEU-GST-Pfcrk-1KA, and pEU-GST-GFP) were mixed with 20 μl of transcription mixture containing transcription buffer, 2.5 mM NTP mix, 1 U/μl RNase inhibitor, and 1 U/μl SP6 RNA polymerase, and incubated for 6 h at 37 °C. Each generated mRNA was mixed with 20 μl of 40 ng/μl creatine kinase supplied with the WEPRO 1240 kit, transferred into 400 μl of SUB-AMIX translation buffer to form a bilayer, and incubated at 16 °C for 18 h.

2.5. Purification of GST fusion proteins and western blot

Aliquots of 100 μl of a 10% slurry of glutathione-sepharose beads (GE Healthcare, Buckinghamshire, UK) in PBS were mixed with the translation mixture and incubated with gentle agitation at 4 °C for 2 h. The beads were then washed five times with 1 ml of PBS. Purified protein captured on the beads was subsequently separated by 10% SDS-PAGE under reducing conditions and either subjected to silver staining (the amount of protein loaded was adjusted for each expression level) or transferred onto nitrocellulose membranes (Bio-Rad). The membranes were blocked with 3% skim milk in PBS containing 0.1% Tween 20 (Wako) (TPBS) for 30 min at room temperature. After rinsing twice with one wash for 5 min in TPBS, the membranes