out to have the greatest effect in our in vitro experiments. DS10 is a low molecular weight DS, but it was *not* the smallest molecule we tested. In general, low molecular weight polymers show weaker effects than high molecular weight polymers, but they have the advantage of being more easily absorbed into the body or incorporated into cells. Our data indicate that DS10 is the optimum size for maximum inhibitory efficiency, at least under in vitro conditions.

We propose that the major effect of DS on infection inhibition may be the disruption of *T. gondii* attachment. Specifically, DS may disrupt the interaction between host cell surface receptor molecules such as GAGs and a surface moiety of the *T. gondii* tachyzoites. One possible candidate for this moiety is the protein Surface Antigen 3 (SAG3). A previous report has described SAG3 binding to heparan sulfate and reported that recombinant SAG3 (rSAG3) binding to heparin-immobilized affinity beads can be completely eluted by a DS solution as the eluent (Jacquet et al. 2001).

DS molecules may also act on the other targets of *T. gondii* tachyzoites and/or on host cell targets. Further studies are needed to define the target moiety (moieties) of DS. The sulfated polysaccharides analyzed in this study (heparin, CSA, CSC, and DSs) did not irreversibly inactivate the entire infection machinery of *T. gondii* because inhibition was not completely blocked by the administration of these polysaccharides during the long-term incubation that lacked a wash step. GAGs on the surface of the host cells may contribute to the interaction with the individual targets of the *T. gondii* tachyzoite, and these polysaccharides may play diverse roles in host–parasite interactions. We previously reported that the P104 protein derived from *T. gondii* interacts with chondroitin sulfates and is involved in the infection event of *T. gondii* (Gong et al. 2012). The effect of chondroitin sulfates (CSA or CSC) is likely limited to the step of *T. gondii* infection that involves P104.

To determine whether the in vitro inhibitory effects of DS on *T. gondii* infection extended to in vivo conditions, we used a mouse model. In the animal experiment, DS10 inhibited *T. gondii* invasion (or growth) when simultaneously injected with *T. gondii* (Table 1). We also tried the oral administration of DS10. Oral administration of DS10 also inhibited *T. gondii* infection and extended the life of the infected mice (by approximately 1 day, unpublished data). We plan further experiments to definitively determine the most effective manner of DS administration; in these future studies, we will investigate not only the route of administration but also further investigate the molecular size of DS need for optimal effects.

It is anticipated that DS has the potential to become a novel type of drug for toxoplasmosis. As shown in this report, low molecular weight DS molecules not only inhibit the invasion of *T. gondii* tachyzoites to the host cell but also decelerate the growth inside the host cell. Then, we thought that it may be possible to moderate the symptom of acute toxoplasmosis by administration of DS. On the other hand, DS may be effective as prophylactic to decrease the risk of initial contact between parasites and host, especially at the case of oral infection by intake of the cyst or oocyst of *T. gondii*. To validate the potential of DS as the new type of drug for therapeutic or prophylactic of toxoplasmosis, we will continue with the further investigation.

Acknowledgments This study was supported by grant-in-aids for Young Scientists, Exploratory Research, Scientific Research on Innovative Areas (3308) from the Ministry of Education, Culture, Science, Sports, and Technology (MEXT) of Japan; Bio-oriented Technology Research Advancement Institution (BRAIN); and Program to Disseminate Tenure Tracking System from Japan Science and Technology Agency (JST).

References

Black MW, Boothroyd JC (2000) Lytic cycle of *Toxoplasma gondii*. Microbiol Mol Biol Rev 64:607–623

Carruthers VB, Håkansson S, Giddings OK, Sibley LD (2000) *Toxoplasma gondii* uses sulfated proteoglycans for substrate and host cell attachment. Infect Immun 68:4005–4011

Dobrowolski JM, Sibley LD (1996) *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. Cell 84: 933–939

Eustice DC, Feldman PA, Colberg-Poley AM, Buckery RM, Neubauer RH (1991) A sensitive method for the detection of beta-galactosidase in transfected mammalian cells. Biotechniques 11(739–40):742–743

Gong H, Kobayashi K, Sugi T, Takemae H, Kurokawa H, Horimoto T, Akashi H, Kato K (2012) A novel PAN/apple domain-containing protein from *Toxoplasma gondii*: characterization and receptor identification. PLoS One 7:e30169

Huynh MH, Carruthers VB (2006) *Toxoplasma* MIC2 is a major determinant of invasion and virulence. PLoS Pathog 2:e84

Jacquet A, Coulon L, De Nève J, Daminet V, Haumont M, Garcia L, Bollen A, Jurado M, Biemans R (2001) The surface antigen SAG3 mediates the attachment of *Toxoplasma gondii* to cell-surface proteoglycans. Mol Biochem Parasitol 116:35–44

Jones-Brando L, D'Angelo J, Posner GH, Yolken R (2006) *In vitro* inhibition of *Toxoplasma gondii* by four new derivatives of artemisinin. Antimicrob Agents Chemother 50:4216–4218

Katlama C, De Wit S, O'Doherty E, Van Glabeke M, Clumeck NS (1996) Pyrimethamine–clindamycin vs. pyrimethamine–sulfadiazine as acute and long-term therapy for toxoplasmic encephalitis in patients with AIDS. Clin Infect Dis 22:268–275

Kitajima S, Takuma S, Morimoto MS (1999) Tissue distribution of dextran sulfate sodium (DSS) in the acute phase of murine DSS-induced colitis. J Vet Med Sci 61:67–70

Ortega-Barria E, Boothroyd JC (1999) A *Toxoplasma* lectin-like activity specific for sulfated polysaccharides is involved in host cell infection. J Biol Chem 274:1267–1276

Ortega-Barria E, Pereira ME (1991) A novel *T. cruzi* heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. Cell 67:411–421

Pancake SJ, Holt GD, Mellouk S, Hoffman SL (1992) Malaria sporozoites and circumsporozoite proteins bind specifically to sulfated glycoconjugates. J Cell Biol 117:1351–1357

Reischl U, Bretagne S, Krüger D, Ernault P, Costa JM (2003) Comparison of two DNA targets for the diagnosis of toxoplasmosis by real-time PCR using fluorescence resonance energy transfer hybridization probes. BMC Infect Dis 3:7

Sugi T, Kato K, Kobayashi K, Pandey K, Takemae H, Kurokawa H, Tohya Y, Akashi H (2009) Molecular analyses of *Toxoplasma gondii* calmodulin-like domain protein kinase isoform 3. Parasitol Int 58:416–423

Tenter AM, Heckeroth AR, Weiss LM (2000) *Toxoplasma gondii*: from animals to humans. Int J Parasitol 30(12–13):1217–1258

Zhang JP, Stephens RS (1992) Mechanism of *C. trachomatis* attachment to eukaryotic host cells. Cell 69:861–869

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.

© 2013 Elsevier Ireland Ltd. All rights reserved.

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

were incubated with horseradish peroxidase-conjugated anti-GST antibody (1:5000) (Sigma-Aldrich, St. Louis, MO) in TPBS containing 3% skim milk for 1 h, washed with TPBS again, and visualized with an enhanced chemiluminescence (ECL) kit (GE Healthcare) and LAS 4000 mini imager (GE Healthcare).

2.6. In vitro kinase assay

In vitro kinase assay with GST-Pfcrk-1, GST-Pfcrk-1KA, and GST-GFP was performed as described elsewhere [16]. Briefly, purified GST-Pfcrk-1, GST-Pfcrk-1KA, or GST-GFP captured on glutathione-sepharose beads was rinsed three times with kinase washing buffer (50 mM Tris–HCl, 1 mM DTT, pH 8.0). Kinase assays were performed with the purified GST fusion proteins in 50 μl of kinase buffer (50 mM Tris–HCl, 1 mM DTT, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 20 μM ATP, pH 8.0) containing 1 μg/μl histone II_{AS} (Sigma-Aldrich) as a substrate and 5 μCi [γ-^{32}P]ATP at 37 °C for 30 min. To investigate the interaction between protein kinase and cyclin, 0.5 μg of recombinant cyclin A with a polyhistidine-tag (His-tag), cyclin B1 with a His-tag, and cyclin H proteins (expressed by *Escherichia coli*; Abcam, Cambridge, UK) was added to the reaction mix. After incubation, samples were centrifuged, supernatants were discarded, and the proteins on beads were separated by 10% SDS-PAGE under reducing conditions. The proteins in the gel were stained with Bio-Safe Coomassie (Bio-Rad), dried with a Multi Gel Dryer (Cosmo Bio, Tokyo, Japan), and exposed to X-ray film.

To investigate whether olomoucine inhibits the activity of Pfcrk-1, the protein was incubated in the same kinase buffer with 1.2 mM olomoucine (DMSO solution); in the control, only 1% DMSO was added. After SDS-PAGE, staining, and gel drying, histone phosphorylation activity of Pfcrk-1 was measured by scintillation counting of pieces of dried gel corresponding to the histone bands. Under each condition, with or without olomoucine, the kinase reaction was performed in triplicate, separated by SDS-PAGE, and measured by scintillation counting. These counts were standardized with protein volume determined from the histone band of the dried gel. After standardization, average values and standard errors were calculated and plotted.

2.7. Phosphatase treatment

After the *in vitro* kinase assays, GST fusion proteins captured on glutathione-sepharose beads in kinase buffer were subjected to phosphatase treatment with lambda protein phosphatase (New England Biolabs, Ipswich, MA), according to the manufacturer's instructions and previous reports [16,17]. Briefly, the proteins were boiled for 10 min to inactivate kinases, washed with 100 μl of NEBuffer (New England Biolabs) supplemented with 1 mM $MnCl_2$, and incubated in 50 μl of the same buffer with 400 IU of lambda protein phosphatase (New England Biolabs) at 30 °C for 30 min.

3. Results

3.1. Effects of cyclin-dependent kinase inhibitor on erythrocytic schizogony

We tested the effects of the CDK inhibitor, olomoucine, on erythrocytic schizogony of *P. falciparum* HB3 strain in GIA. Throughout the whole cycle, this inhibitor reduced the parasitemia in a dose-dependent manner (IC_{50}: 96.1 μM) (Fig. 1). We examined Giemsa-stained blood smears after incubation and ensured that all parasites were at the trophozoite or ring stage. Our results indicated that this CDK inhibitor affected the parasite erythrocytic cycle and decreased the number of parasites.

Furthermore, we investigated the point in the cycle at which this growth inhibition effect occurred by monitoring the time course of parasite asexual development from the ring stage to the trophozoite and schizont stages. At each time point, blood smears were made, stained, and observed by microscopy, and the number of the parasites was counted (Fig. 2). In the control culture, the population of parasites

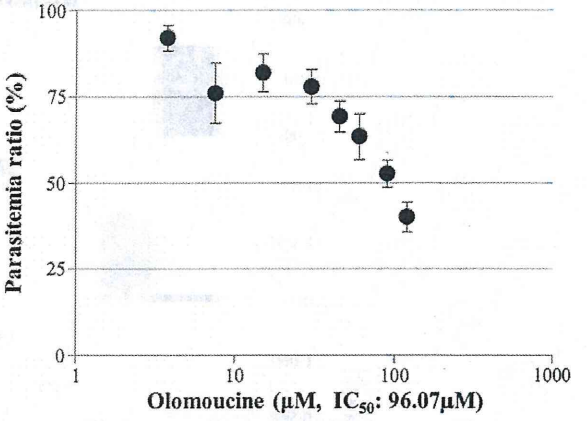

Fig. 1. Growth inhibition assay using the CDK inhibitor. A growth inhibition assay was performed to evaluate the effect of olomoucine, a CDK inhibitor, on the growth of *P. falciparum* in the asexual life cycle. For each drug concentration, the average percentage of parasitemia compared to the control culture (0.1% DMSO) was determined. Standard deviations are indicated.

changed from the first ring form (at 0 h post invasion) to an increased proportion of trophozoites with a black hemozoin pigment (at 24 h) followed by an increase in the proportion of multi-nuclear schizont parasites (at 38 h), and finally the appearance of the next ring form (at 44 and 50 h). On the other hand, in the presence of olomoucine, the transition from ring to trophozoite took place normally, but the transition from trophozoite to schizont was severely interrupted. When two-tailed t-test was performed to compare the trophozoite/schizont ratios at 38 h, there was a significant difference between these values ($p < 0.05$). However, when the ring/trophozoite ratios were compared at 24 h, these values didn't differ significantly ($p > 0.05$). These results suggested that this CDK inhibitor has the potential to inhibit the erythrocytic growth of *P. falciparum* during the trophozoite and schizont stages.

3.2. Expression and purification of Pfcrk-1 kinase domain

The Pfcrk-1 kinase domain and mutant protein were expressed as GST fusion proteins and purified using a wheat germ cell-free protein synthesis system (Fig. 3). Purified proteins were then electrophoretically separated on polyacrylamide gels and subjected to either silver staining (Fig. 4A) or immunoblotting with horseradish peroxidase-conjugated anti-GST antibodies (Fig. 4B). The purified proteins from the wheat germ extracts after the incubation of expression mixture with pEU-GST-GFP, pEU-GST-Pfcrk-1, or pEU-GST-Pfcrk-1KA contained a protein band of 54, 63, or 63 kDa, respectively, as detected by silver staining. These molecular weight values are similar to those calculated from the amino acid sequences. Protein bands around 50 kDa in GST-Pfcrk-1 and GST-Pfcrk-1KA lanes represent products that failed to complete expression or degradation products (the 50-kDa-band in GST-Pfcrk-1 lane was too weak to see in Fig. 4A). These proteins reacted with the anti-GST antibody, indicating successful protein expression. Bands of ~25 kDa in all lanes detected by silver staining represent the protein from the wheat germ expression system (see http://www.cfsciences.com/pdf/GST-tag_purification_ver1-1_En.pdf).

3.3. Protein kinase activity of recombinant Pfcrk-1 protein

Protein kinases have phosphorylation activity. To determine whether purified Pfcrk-1 protein has phosphorylation activity, an *in vitro* kinase assay was performed. When purified GST-GFP protein was incubated with histone protein and ATP in kinase buffer and separated by electrophoresis, autoradiography showed no distinct bands, indicating no phosphorylated protein (Fig. 5A, B, lane 1). On the other hand, when protein beads from the wheat germ extract incubated with pEU-GST-Pfcrk-1

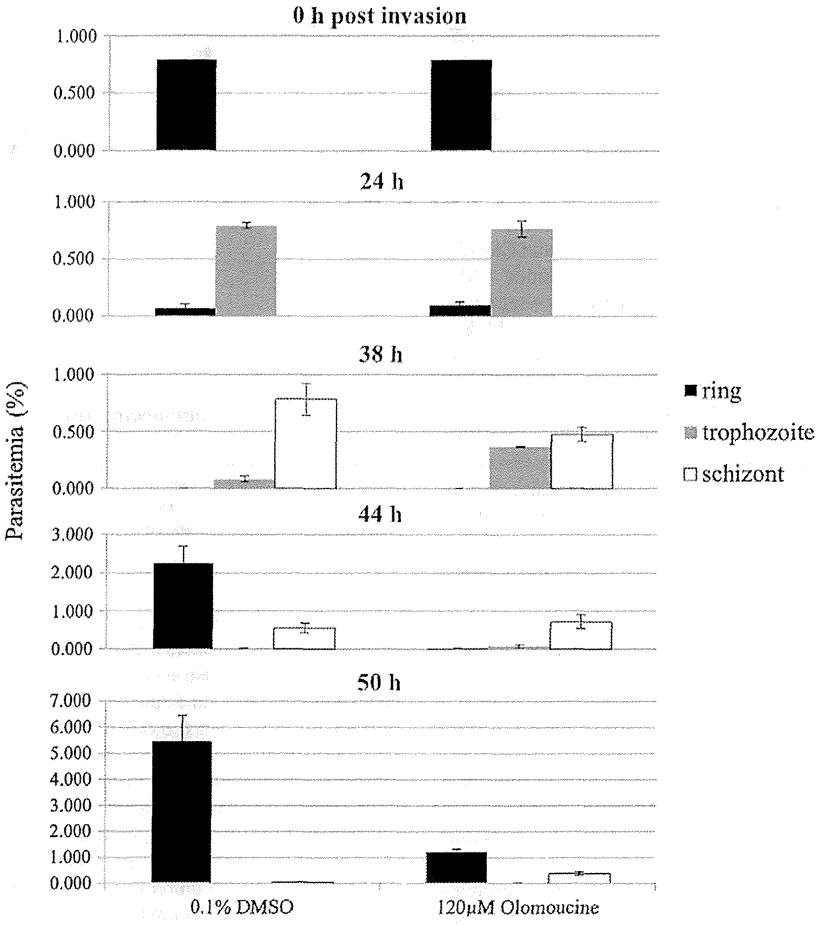

Fig. 2. Effects of the CDK inhibitor on the intraerythrocytic life cycle. At 0 h post-invasion, the CDK inhibitor olomoucine was added, and the time course of parasite development in the asexual life cycle was monitored by microscopy of blood smears stained with Giemsa. In each blood smear, 10,000 RBCs were counted and the proportions of each parasite stage (ring-form, trophozoite stage with hemozoin pigment, or schizont stage with divided multiple nuclei) were calculated. This assay was performed in triplicate, and means and standard deviations are indicated.

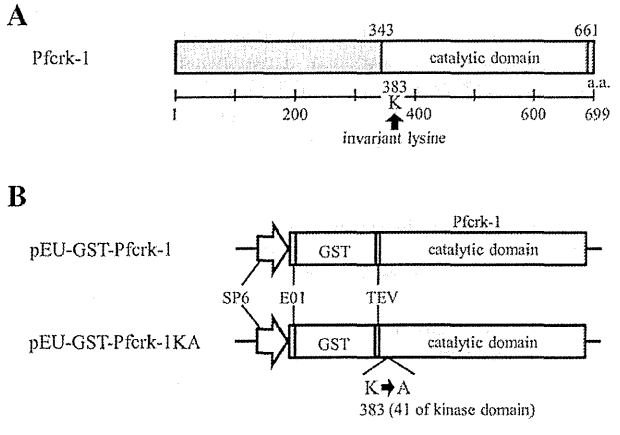

Fig. 3. Schematic diagram of Pfcrk-1 and the expression plasmids used. (A) Schematic diagram of Pfcrk-1, based on its amino acid sequence. The catalytic domains (from C343 to K661) are indicated, and the invariant lysine residue (K383) is indicated by an arrow. Amino acids (a.a.) are numbered. (B) Schematic diagrams of the pEU-GST-Pfcrk-1 and pEU-GST-Pfcrk-1KA plasmids used for the expression of GST-Pfcrk-1 and GST-Pfcrk-1KA, respectively, in the wheat germ cell-free protein expression system. The positions of the SP6 promoter (SP6), translational enhancer (E01), glutathione S-transferase tag (GST), and tobacco etch virus protease recognition site (TEV) are shown [30].

were subjected to this assay, protein bands of histone II_{AS} near 15 kDa were labeled by autoradiography (Fig. 5A, B, lane 2). These labels disappeared when proteins were treated with lambda protein phosphatase after the kinase reaction, indicating that histone proteins were phosphorylated by proteins from the wheat germ extract incubated with pEU-GST-Pfcrk-1 (Fig. 6A, B). Autophosphorylation, which is thought to be important for protein kinase activity, of Pfcrk-1 was not observed.

In the next step, we examined whether the invariant lysine of Pfcrk-1 was critical for this phosphorylation of histone. Protein kinases contain a number of invariant amino acid residues, some of which are indispensable for kinase activity [18]. Among them, an invariant lysine in subdomain II of the catalytic domain, corresponding to K383 of Pfcrk-1 (K41 of Pfcrk-1 kinase domain), is one of the most important residues involved in the phosphate group transfer reaction. Site-directed mutagenesis techniques have been used to substitute alternate amino acids, such as alanine, at this position in many protein kinases, and these substitutions result in loss of protein kinase activity. We used this technique to produce a kinase-negative mutant of Pfcrk-1 in which the invariant lysine was replaced by alanine. Histone proteins incubated with the proteins expressed from pEU-GST-Pfcrk-1KA were labeled by autoradiography to the same level as those with wild-type Pfcrk-1 (Fig. 5A, B, lane 3). Since there was little difference in the amounts of proteins between wild-type and mutant, the mutation of the invariant lysine residue of Pfcrk-1 did not alter the kinase activity. Many cyclin-dependent kinases

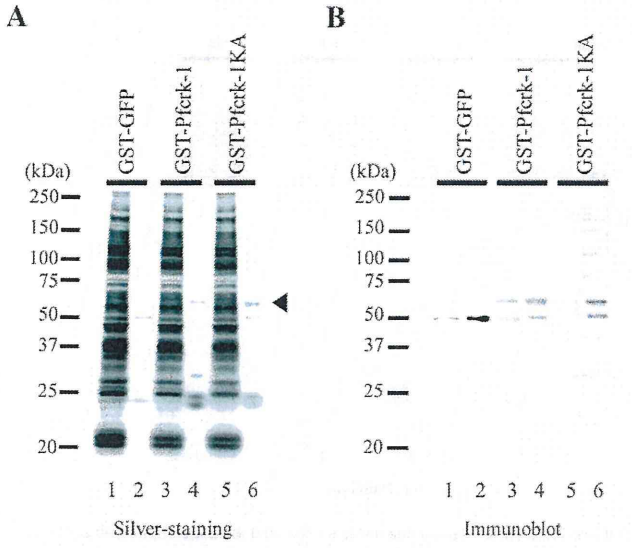

Fig. 4. Expression and purification of Pcrk-1 kinase domain and mutant. (A) Silver-stained GST-GFP, GST-Pfcrk-1, and GST-Pfcrk-1KA purified from the wheat germ cell-free protein expression system using the plasmid pEU-GST-GFP (lanes 1, 2), pEU-GST-Pfcrk-1 (lanes 3, 4), and pEU-GST-Pfcrk-1KA (lanes 5, 6), respectively. Lanes 1, 3, and 5 show total translation mixtures, from which GST fusion proteins were purified using glutathione-sepharose beads (lanes 2, 4, 6). These proteins were separated by SDS-PAGE under reducing conditions and subjected to silver staining. (B) An immunoblot of GST-GFP, GST-Pfcrk-1, and GST-Pfcrk-1KA purified from the wheat germ cell-free protein expression system using the plasmids pEU-GST-GFP (lanes 1, 2), pEU-GST-Pfcrk-1 (lanes 3, 4), and pEU-GST-Pfcrk-1KA (lanes 5, 6), respectively. Proteins loaded onto SDS-polyacrylamide gels were the same as in (A). After electrophoresis, these proteins were transferred onto a nitrocellulose membrane, followed by the detection of GST fusion proteins by anti-GST antibody conjugated with peroxidase. Molecular masses (kDa) are shown on the left.

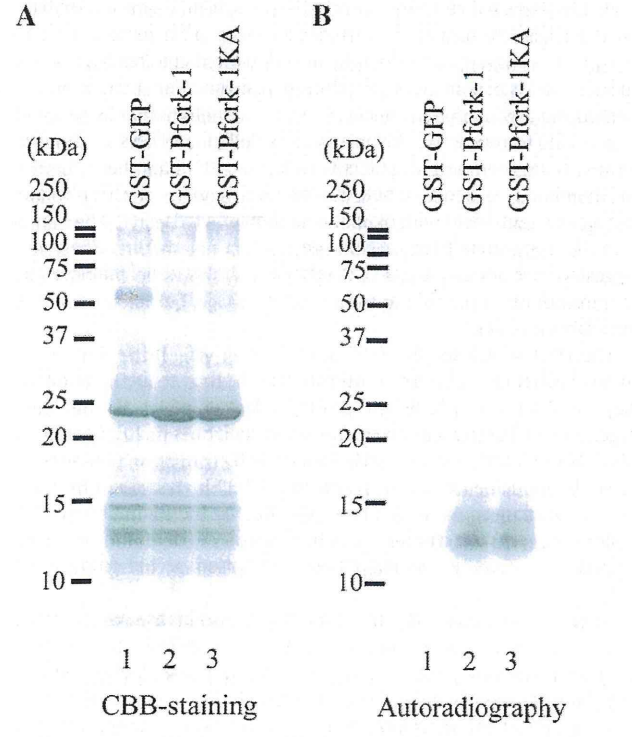

Fig. 5. *In vitro* kinase assay of the Pfcrk-1 kinase domain and mutant. (A) Purified GST-GFP (lane 1), GST-Pfcrk-1 (lane 2) or GST-Pfcrk-1KA (lane 3) was incubated in kinase buffer containing [γ-^{32}P]ATP, separated by SDS-PAGE under reducing conditions, and stained with CBB. (B) Autoradiography of the gel shown in (A). Molecular masses are shown on the left.

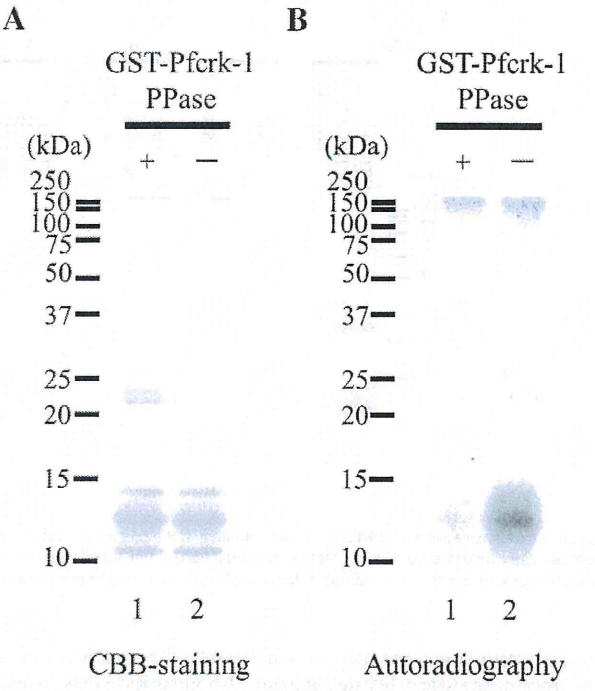

Fig. 6. *In vitro* kinase assay and phosphatase treatment of the Pfcrk-1 kinase domain. (A) After the incubation of purified Pfcrk-1 kinase domain with kinase buffer containing [γ-^{32}P]ATP, the labeled proteins were treated with (+) or without (—) lambda phosphatase, separated by SDS-PAGE under reducing conditions, and stained with CBB. (B) Autoradiography of the gel shown in (A). Molecular masses are shown on the left.

need interaction with cyclins, which are regulators of CDK. We next performed the same assay using recombinant cyclin proteins but kinase reaction with human cyclin protein A, B1, or H did not increase the phosphorylation level of histone by the proteins expressed from pEU-GST-Pfcrk-1 (Fig. 7A, B). Finally, we determined whether olomoucine, which inhibited the growth of *P. falciparum* at the intraerythrocytic stage of the life cycle, inhibits the kinase activity of Pfcrk-1. Purified GST-Pfcrk-1 kinase domain was incubated with histone proteins in kinase buffer in the presence of olomoucine, and these proteins were subjected to SDS-PAGE and Coomassie Brilliant Blue (CBB) staining. Phosphorylation of histone was quantified by scintillation counting; the phosphorylation level of histones incubated with olomoucine was shown to be lower than that of DMSO controls significantly (Fig. 8). These results suggested that this CDK inhibitor reduced the activity of expressed protein. Further investigations of both the activity of Pfcrk-1 and the association with CDK inhibitors are required.

4. Discussion

In this study, we investigated the effects of a CDK inhibitor, olomoucine, on the erythrocytic stage of the malarial parasite *P. falciparum* life cycle. This agent showed an inhibitory effect on parasite growth with an IC_{50} value of 96 μM. Previous studies investigated the growth inhibition by olomoucine on *P. falciparum* during the intraerythrocytic cycle, and estimated the IC_{50} concentrations [4–6]. In these studies, olomoucine reduced the growth of parasites by half at concentrations of ~10 μM. However, in the present study, 15 μM olomoucine did not markedly reduce parasitemia (at 15 μM, parasitemia decreased only to 82% of that in DMSO control cultures). In addition to HB3, other *P. falciparum* strains (3D7 and Dd2) were subjected to this assay and showed the same results as HB3.

This discrepancy between our observations and those of previous studies may have been due to differences in the assay methods used. In this study, we enumerated the parasites killed by the inhibitor. For

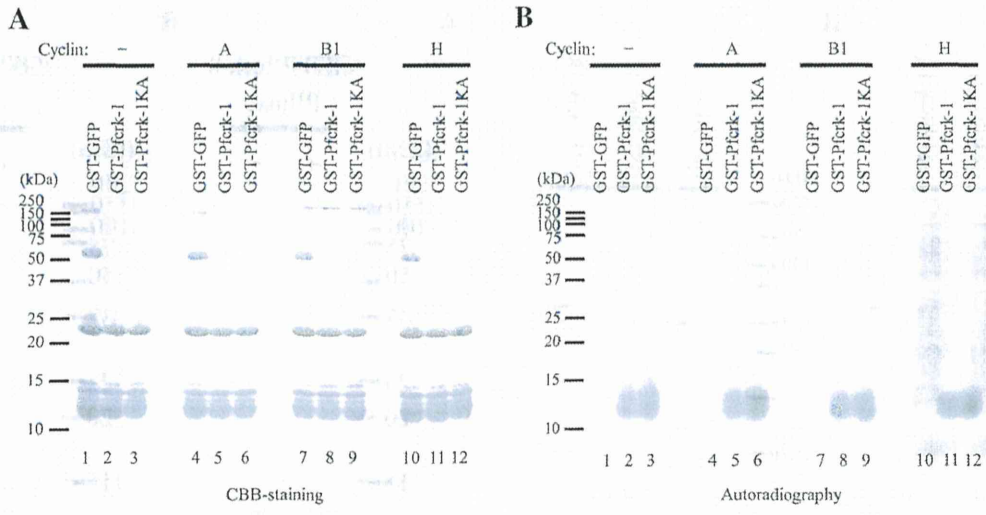

Fig. 7. *In vitro* kinase assay of the Pfcrk-1 kinase domain in the presence of cyclin proteins. (A) Purified GST-Pfcrk-1 kinase domain was incubated in kinase buffer containing human recombinant cyclin protein and [γ-^{32}P]ATP, separated by SDS-PAGE under reducing conditions, and stained with CBB. Names of cyclins used are described above (lanes 1–3: control, lanes 4–6: cyclin A, lanes 7–9: cyclin B1, lanes 10–12: cyclin H). (B) Autoradiography of the gel shown in (A). Molecular masses are shown on the left.

accurate estimation, we began the assay when the parasites were at the trophozoite stage, because during 48-h incubation every parasite would undergo erythrocyte invasion, an event in which the number of parasites increases. If the assay was started shortly after the parasites had invaded erythrocytes, some parasites—the growth of which was delayed for some reason—would not invade the next erythrocytes even if they were alive. In this case, it would not be possible to determine whether parasitemia was decreased by the growth inhibitory effect of the inhibitor or due to a lag of invasion. On the other hand, previous studies used the [^{3}H]-hypoxanthine incorporation assay, which evaluates the level of parasite biological activity, the way in which malarial parasites utilize exogenous hypoxanthine for nucleic acid synthesis and energy metabolism [19]. If some parasites did not die but only decreased their activity because of exposure to olomoucine, the IC_{50} value calculated from the number of parasites would be higher than that estimated from parasite activity.

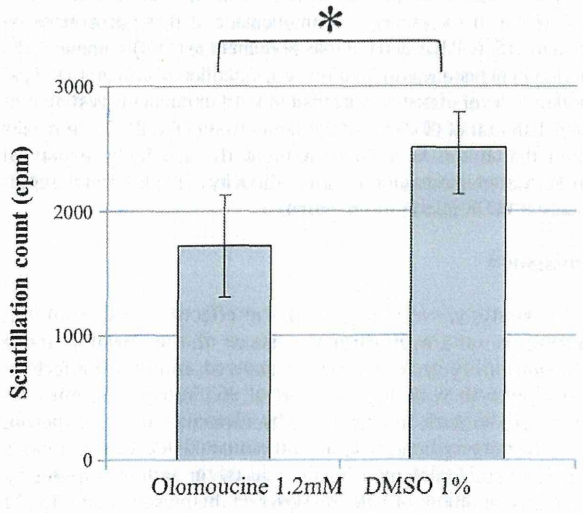

Fig. 8. Effects of CDK inhibitor on kinase activity of Pfcrk-1. Purified GST-Pfcrk-1 was incubated in kinase buffer containing [γ-^{32}P]ATP with or without the CDK inhibitor, olomoucine. After separation by SDS-PAGE under reducing conditions and staining with CBB, histone phosphorylation was assessed by scintillation counting of pieces of dried gel corresponding to histone bands. The assay was performed in triplicate and the counts were standardized to the protein volume of the histone band of the dried gel. After standardization, means and standard errors were calculated. One-tailed t-test was performed to compare these results ($p < 0.05$).

We next monitored the time course of asexual parasite development starting from the ring stage soon after merozoite invasion into RBC, through the trophozoite stage and schizont stage, and to the next ring stage, to determine at which stage of the cell cycle the inhibitor shows its effect on the parasite. The parasites can complete transition to trophozoites normally in the presence of this CDK inhibitor. However, at 38 h post-invasion, many trophozoites had not transitioned into schizonts and they had only a single nucleus in the treated culture, while the untreated cultures contained many schizont parasites with divided nuclei. Schizonts in untreated cultures at 38 h seemed to have many (>8) nuclei, but most of schizonts in treated cultures have only 2 nuclei. At 44 h post invasion, schizont parasites under the presence of olomoucine produced many nuclei. Then, we thought that some parasites who couldn't grow into schizonts were killed, and others were killed because their growth in schizonts was delayed. In another experiment, the inhibitor was added when all parasites were at the trophozoite stage; parasites treated with olomoucine showed a delay in the transition from the trophozoite to schizont stage (data not shown). These results suggested that olomoucine, a CDK inhibitor, shows no inhibitory effect on transition from the ring to trophozoite stage, but affects trophozoite and schizont stages.

The DNA synthesis phase (S phase), in which the genome copy number increases from 1*n* to 16*n* or 32*n*, begins at the trophozoite stage, and mitotic phase (M phase) takes place at the schizont stage, while DNA replication continues in schizonts [2,20]. Therefore, this CDK inhibitor may inhibit the cell cycle transition to S phase or M phase. Olomoucine has been shown to inhibit the transition from G1 phase to S phase in many cell lines, possibly by the inhibition of DNA replication [21,22]. Therefore, it will be necessary to determine whether olomoucine affects *Plasmodium* DNA replication at the trophozoite stage.

Next, we searched for the target protein kinase with which olomoucine interacts among the parasite proteins. The genome of *P. falciparum* is thought to contain six CDK homologs, and this kinase inhibitor was shown previously not to inhibit the activity of one of these homologs, Pfmrk [6]. Therefore, we investigated the activities of another CDK homolog, *P. falciparum* cdc2-related kinase 1 (Pfcrk-1). The open reading frame of this gene has a unique N-terminal region, the function of which has not been identified previously. Here, the catalytic domain of Pfcrk-1 was amplified using cDNA made from the total mRNA of

P. falciparum, expressed as a GST fusion protein by wheat germ protein expression system, and purified using GSH beads. This expression system is thought to be suitable for the synthesis of active malarial enzymes [23].

An *in vitro* kinase assay was performed with this purified protein to assess its protein kinase activity. The level of phosphorylation signal at histone proteins with GST-Pfcrk-1 kinase domain was higher than that with GST-GFP but the same as that with a kinase-negative mutant of Pfcrk-1. After the phosphatase treatment, the labeling of [γ-^{32}P]ATP was abolished. Therefore, the kinase activity of the synthesized Pfcrk-1 was as high as that of the KA mutant protein.

One reason why we observed the same level of activity between Pfcrk-1 and the mutant was that the mutation of Pfcrk-1 didn't result in a decrease in kinase activity because the activity of Pfcrk-1 was independent of the invariant lysine residue, but there is no previous report which supports our claim. Another reason was that the expressed Pfcrk-1 could not show enough phosphorylation activity because this protein was inactive in the absence of interaction with a regulator protein, such as a cyclin, which binds to CDK to form an active complex. According to the alignment data with *Homo sapiens* cdc2, the residues potentially important for binding with cyclins lie in the protein kinase catalytic domain [24]. Therefore, we repeated the kinase assay using several human cyclins, based on the observation that they activated recombinant *P. falciparum* kinases, PfPK5 and Pfmrk [4,25–27]. However, the activity of GST-Pfcrk-1 did not change in the presence of recombinant human cyclin A, B1, or H.

However, we were unable to demonstrate that this protein lacks activity, and so additional investigations are required; *e.g.*, it will be necessary to determine the activity of full-length Pfcrk-1 protein, or to perform *in vitro* kinase assay using other cyclins (human cyclins or parasite cyclin homologs) or substrate proteins other than histones. The activity of another mutant protein in which an invariant aspartic acid residue at subdomain VI, corresponding to Pfcrk-1 D480, was replaced with another amino acid remains to be examined [18,28].

At last, we performed the *in vitro* kinase assay to check whether olomoucine inhibits the activity of Pfcrk-1. This kinase inhibitor diminished the phosphorylation level, while it didn't show a significant effect at less than 1.2 mM. Olomoucine inhibits other plasmodial protein kinases, such as PfPK5 (IC_{50}: 15 μM) and PfPK6 (IC_{50}: 180 μM) [4,29], and there is a possibility that growth inhibition by this chemical is due to the inhibition of these kinases. Furthermore, PfPK5 is thought to be necessary to activate or maintain the parasite S phase [4]. In our report, we cannot say that olomoucine inhibits Pfcrk-1 activity enough. However, the interaction between Pfcrk-1 and olomoucine should be discussed after confirmation that this protein kinase has phosphorylation activity.

In this study, we investigated the function of *P. falciparum* cyclin-dependent kinases during the intraerythrocytic cycle by assessing the effects of a CDK specific inhibitor, olomoucine, on the parasites, and the activity of one of potential targets of the inhibitor, Pfcrk-1, the kinase catalytic domain of which was synthesized in a wheat germ expression system. Here, we investigated only one protein kinase inhibitor, and further studies using many other CDK inhibitors are required to estimate the functions of plasmodial cyclin-dependent kinases. The molecular characterization of parasite CDKs, not only Pfcrk-1 but also other CDK homologs, provides insight valuable to the search for targets of these inhibitors.

Conflict of Interest statement

The authors have declared that no competing interests exist.

Acknowledgments

This study was supported by Grants-in-Aid for Young Scientists, Exploratory Research, and Scientific Research on Innovative Areas (3308) from the Ministry of Education, Culture, Science, Sports, and Technology (MEXT), Research on Global Health Issues from the Ministry of Health, Labour and Welfare of Japan, Bio-oriented Technology Research Advancement Institution (BRAIN), Program to Disseminate Tenure Tracking System from Japan Science and Technology Agency (JST) and the Naito Foundation.

References

[1] Arnot DE, Gull K. The *Plasmodium* cell-cycle: facts and questions. Annals of Tropical Medicine and Parasitology 1998;92:361–5.
[2] Doerig C, Chakrabarti D, Kappes B, Matthews K. The cell cycle in protozoan parasites. Progress in Cell Cycle Research 2000;4:163–83.
[3] Enserink JM, Kolodner RD. An overview of Cdk1-controlled targets and processes. Cell Div 2010;5:11.
[4] Graeser R, Wernli B, Franklin RM, Kappes B. *Plasmodium falciparum* protein kinase 5 and the malarial nuclear division cycles. Molecular and Biochemical Parasitology 1996;82:37–49.
[5] Harmse L, van Zyl R, Gray N, Schultz P, Leclerc S, Meijer L, et al. Structure-activity relationships and inhibitory effects of various purine derivatives on the *in vitro* growth of *Plasmodium falciparum*. Biochemical Pharmacology 2001;62:341–8.
[6] Woodard CL, Li Z, Kathcart AK, Terrell J, Gerena L, Lopez-Sanchez M, et al. Oxindole-based compounds are selective inhibitors of *Plasmodium falciparum* cyclin dependent protein kinases. Journal of Medicinal Chemistry 2003;46:3877–82.
[7] Doerig C, Horrocks P, Coyle J, Carlton J, Sultan A, Arnot D, et al. Pfcrk-1, a developmentally-regulated cdc2-related protein-kinase of *Plasmodium falciparum*. Molecular and Biochemical Parasitology 1995;70:167–74.
[8] Rangarajan R, Bei A, Henry N, Madamet M, Parzy D, Nivez MP, et al. Pbcrk-1, the *Plasmodium berghei* orthologue of *P. falciparum* cdc-2 related kinase-1 (Pfcrk-1), is essential for completion of the intraerythrocytic asexual cycle. Experimental Parasitology 2006;112:202–7.
[9] Solyakov L, Halbert J, Alam MM, Semblat JP, Dorin-Semblat D, Reininger L, et al. Global kinomic and phospho-proteomic analyses of the human malaria parasite *Plasmodium falciparum*. Nature Communications 2011;2:565.
[10] Radfar A, Mendez D, Moneriz C, Linares M, Marin-Garcia P, Puyet A, et al. Synchronous culture of *Plasmodium falciparum* at high parasitemia levels. Nature Protocols 2009;4:1899–915.
[11] Boyle MJ, Richards JS, Gilson PR, Chai WG, Beeson JG. Interactions with heparin-like molecules during erythrocyte invasion by *Plasmodium falciparum* merozoites. Blood 2010;115:4559–68.
[12] Boyle MJ, Wilson DW, Richards JS, Riglar DT, Tetteh KKA, Conway DJ, et al. Isolation of viable *Plasmodium falciparum* merozoites to define erythrocyte invasion events and advance vaccine and drug development. Proceedings of the National Academy of Sciences of the United States of America 2010;107:14378–83.
[13] Persson KEM, Lee CT, Marsh K, Beeson JG. Development and optimization of high-throughput methods to measure *Plasmodium falciparum*-specific growth inhibitory antibodies. Journal of Clinical Microbiology 2006;44:1665–73.
[14] Kato K, Sudo A, Kobayashi K, Tohya Y, Akashi H. Characterization of *Plasmodium falciparum* protein kinase 2. Molecular and Biochemical Parasitology 2008;162:87–95.
[15] Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. Nature Protocols 2007;2:924–32.
[16] Kato K, Sudo A, Kobayashi K, Sugi T, Tohya Y, Akashi H. Characterization of *Plasmodium falciparum* calcium-dependent protein kinase 4. Parasitology International 2009;58:394–400.
[17] Kato K, Kawaguchi Y, Tanaka M, Igarashi M, Yokoyama A, Matsuda G, et al. Epstein–Barr virus-encoded protein kinase BGLF4 mediates hyperphosphorylation of cellular elongation factor 1 delta (EF-1 delta): EF-1 delta is universally modified by conserved protein kinases of herpesviruses in mammalian cells. Journal of General Virology 2001;82:1457–63.
[18] Hanks SK, Quinn AM, Hunter T. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 1988;241:42–52.
[19] Sherman IW. Biochemistry of *Plasmodium* (malarial parasites). Microbiological Reviews 1979;43:453–95.
[20] Mitra P, Deshmukh AS, Dhar SK. DNA replication during intra-erythrocytic stages of human malarial parasite *Plasmodium falciparum*. Current Science 2012;102:725–40.
[21] Abraham RT, Acquarone M, Andersen A, Asensi A, Belle R, Berger F, et al. Cellular effects of olomoucine, an inhibitor of cyclin-dependent kinases. Biology of the Cell 1995;83:105–20.
[22] Alessi F, Quarta S, Savio M, Riva F, Rossi L, Stivala LA, et al. The cyclin-dependent kinase inhibitors olomoucine and roscovitine arrest human fibroblasts in G1 phase by specific inhibition of CDK2 kinase activity. Experimental Cell Research 1998;245:8–18.
[23] Tsuboi T, Takeo S, Arumugam TU, Otsuki H, Torii M. The wheat germ cell-free protein synthesis system: a key tool for novel malaria vaccine candidate discovery. Acta Tropica 2010;114:171–6.
[24] Marcote MJ, Knighton DR, Basi G, Sowadski JM, Brambilla P, Draetta G, et al. A three-dimensional model of the Cdc2 protein kinase: localization of cyclin- and Suc1-binding regions and phosphorylation sites. Molecular and Cellular Biology 1993;13:5122–31.
[25] Waters NC, Woodard CL, Prigge ST. Cyclin H activation and drug susceptibility of the Pfmrk cyclin dependent protein kinase from *Plasmodium falciparum*. Molecular and Biochemical Parasitology 2000;107:45–55.

[26] Chen YQ, Jirage D, Caridha D, Kathcart AK, Cortes EA, Dennull RA, et al. Identification of an effector protein and gain-of-function mutants that activate Pfmrk, a malarial cyclin-dependent protein kinase. Molecular and Biochemical Parasitology 2006;149:48–57.
[27] Le Roch K, Sestier C, Dorin D, Waters N, Kappes B, Chakrabarti D, et al. Activation of a *Plasmodium falciparum* cdc2-related kinase by heterologous p25 and cyclin H — functional characterization of a *P. falciparum* cyclin homologue. Journal of Biological Chemistry 2000;275:8952–8.
[28] Yu JT, Zhao Y, Li ZX, Galas S, Goldberg ML. Greatwall kinase participates in the Cdc2 autoregulatory loop in *Xenopus* egg extracts. Molecular Cell 2006;22:83–91.
[29] Bracchi-Ricard V, Barik S, DelVecchio C, Doerig C, Chakrabarti R, Chakrabarti D. PfPK6, a novel cyclin-dependent kinase/mitogen-activated protein kinase-related protein kinase from *Plasmodium falciparum*. Biochemical Journal 2000;347:255–63.
[30] Sawasaki T, Ogasawara T, Morishita R, Endo Y. A cell-free protein synthesis system for high-throughput proteomics. Proceedings of the National Academy of Sciences of the United States of America 2002;99:14652–7.