Analysis of CYC1, TPK2 and novel like molecule CYC2 of *Toxoplasma gondii*

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ABSTRACT

***Toxoplasma gondii* is causative agent of Toxoplasmosis which one of important pathogens of human and animals. In this study, we successfully cloned a novel gene containing one cyclin box motif, which has homology with cyclin Y (CCNY) in human, we described this new gene as CYC2. The results of this study describe the identification of the *T. gondii* CDK family, CYC1 and TPK2, and new gene CYC2. We produced antibody against CYC1 and CYC2, and we observed that their located in different compartments. By using anti-CYC1 antibody, the detected protein was located at the nucleus and apical complex of the parasites. By using anti-CYC2 antibody, protein was located at the dense granule. By using anti-TPK2 antibody, the signal still could not be detected in the parasites. Because these particular differences in the immunostaining patterns, their targets might be different. We will examine the function of them, which might be involved in the cell cycle of *T. gondii*.**

Key words: *Toxoplasma gondii*, CYC1, TPK2, CYC2, IFAT

INTRODUCTION

The phylum Apicomplexa, *T. gondii* is one of the most successful protozoan parasites in the world that infect a wide-range of warm-blooded vertebrates including human. This obligate intracellular parasite has a complex life cycle with three developmental stages: tachyzotes, bradyzoites and sporozoites. The parasite replicates by endodyogeny, an unusual form of binary fission in which daughter cells assembled within mother cells (Khan *et al*., 2002; Sibley *et al*., 2007). In human, *T. gondii* has responsibility in congenital neurological defects cases and an important opportunistic pathogen in immunocompromised individuals such as AIDS patients or after transplacental transmission to the fetus (Ke Hu, 2008; Luder, 2007).

There is a report about interaction between CYC1 and TPK2 in regulatory of cyclin dependent kinases (CDKs) of *T. gondii* by yeast two hybrid screening (Kvaal *et al*., 2002), which CYC1 contains two cyclin box motif and involved in the cell cycle, but the function is clearly unknown. Khan *et al*., 2002, reported about the cloning and molecular characterization of CDC2-related protein kinase TPK2 in *T. gondii*. They showed that TPK2 protein has kinase activity and can associates with different mammalian cyclins experimentally. A dominant-negative mutant of TPK2 arrests *T. gondii* replication suggested that TPK2 is an essential regulator of the *T. gondii* cell cycle.

There is no report about the function of gene that contain one cyclin box in eukaryotic cells, therefore in this study, we want to identify the function of new gene in *T. gondii*, which just contains one cyclin box motif. From data base, we found gene which sequence contains open reading frame of 766 bp, revealed 255 amino acids, predicted molecular size 29 kD. This gene has similarity with human cyclin family, we designated this as new gene namely CYC2. In case of human gene with one cyclin box motif (cyclin Y), there is report that the mutation of protein in gene could be causative agent of diseases such as Inflammatory Bowel Disease (IBD) (Weersma *et al*., 2009).

From the database, there are two cyclin like genes (CYC1 and CYC2). CYC1 was identified as a molecule, which binds to TPK2, but so far, there is no report about CYC2 function. Therefore, in this experiment, we want to identify the function of CYC2 in *T. gondii*.

The aims of this study were to analyze the cDNAs encoding the CDK family with evaluate their expression in mammalian cells and determine the localization of CYC1, TPK2 and CYC2 in
T. gondii.

MATERIALS AND METHODS

Gene Cloning

T. gondii RH strain was used in this experiment. The parasite cDNA were amplified by RT-PCR using the parasite cDNA as templates. Then the PCR product was subcloned in to pGEX vector.

Production of Recombinant Protein

The plasmids were transformed into *Escherichia coli* strain BL21. After addition of IPTG (final concentration 1mM) to induce recombinant protein, cells were collected and recombinant proteins were extracted and purified by GSH beads. Purified proteins were evaluated by SDS-PAGE and quantified by Coomassie assay kit.

Production of Polyclonal Antibody

Six weeks old female Balb/c and ICR mice were used in this study. For the primary immunization, the mice were injected subcutaneously with 10μg of antigen CYC1, TPK2 and CYC2 emulsified in equal volume Freund's complete adjuvant. Seven booster doses were given at interval 2 weeks with 10μg of the same antigen emulsified in equal volume of Freund's incomplete adjuvant.

Western Blot Analysis

Western blot was performed to analyze the recombinant protein of CYC1, TPK2 and CYC2 expressed 293T cells and *T. gondii* RH strain lysates. All of the recombinant proteins were suspended in same volume of SDS–PAGE sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue), heated at 96^0C for 5 min and applied on SDS polyacrylamide gel, and transferred to nylon membrane (Immobilon-P, Millipore), blocking with 1% skimmed milk in PBS-Tween, and the membranes were incubated for 1 hr at room temperature with anti-CYC1, anti-TPK2 and anti-CYC2 polyclonal antibodies, all diluted in 1% skimmed milk in PBS-Tween

1:100. After washed with PBS-Tween 3 times, the membranes were incubated for 1 hr with secondary antibody sheep anti-mouse IgG conjugated horseradish peroxidase (Amersham Biosciences) diluted in 1% skimmed milk in PBS-Tween (1:1000), washed 3 times with PBS-Tween. Then, the membranes were developed using DAB with mixture of 0.25mg/ml diaminobenzidine tetrahydrochloride (Sigma), 100mM Tris-HCl (pH7.5) and 0.05% H_2O_2. In this method, we used molecular mass standards (SeeBlue plus2 Pre-stained standard, Invitrogen).

Indirect Immunofluorescence Assay Test (IFAT)

Parasites were inoculated into host Vero cell and grown onto cover slips for 48 hrs. After incubated 2 days 37^0C, removed media and washed 3 times with PBS. Add fixing and permeablizing solution (3% Formaldehyde, 10x PBS and 0,2% Triton X-100), and incubated at room temperature for 15 min. Rinse 3 times with PBS and blocked 2 times with fresh 3% BSA, 10 min each. After rinse 3 times with PBS, incubated for 1 hr with anti-CYC2 diluted in 3% BSA (1:500) and anti-GRA1 diluted in 3% BSA (1:2000). Then, after rinse 3 times with PBS, the samples were incubated with mixture of two secondary antibody Alexa fluor 488-conjugated goat anti-mouse IgG antibody (green) and Alexa fluor 594 conjugated goat anti-rabbit IgG (red) diluted in 3% BSA (1:1000), incubated for 1 hr. For anti-CYC1, the staining procedure was the same as described above, except for anti-CYC1, staining did not use double staining with anti-GRA1, but after incubated with secondary antibody Alexa fluor 488-conjugated anti-mouse IgG antibody (green) 1:1000, the samples were stained for 5 min with Propidium Iodide (PI, red). Rinse 3 times with PBS and distilled water, mounting cover slip with mowiol on object glass, observed under immunofluorescent microscope and Leica TCS NT Confocal Laser Scanning Microscope (Leica, Germany).

RESULTS

Western Blot Analysis

We used Western blot analyses to confirm the production of the antibodies against CYC1, TPK2, and CYC2 . The result showed no specific bands could be detected in 293T cells transfected with empty vector (Fig. 1A, B and C lane 1), a specific band was detected approximately at 75 kDa and 78 kDa, 33 kDa, and 29 kDa in 293T cells transfected with CYC1, TPK2, and CYC2 (Fig. 1A, B and C lane 2), and also in *T. gondii* RH strain lysates, a weak band could be detected approximately at 78 kDa, 33 kDa, and 29 kDa (Fig. 1A, B and C lane 3). These results are consistent with the predicted size 66 kDa for CYC1, 33 kDa for TPK2, and 29 kDa for CYC2.

Indirect Immunofluorescence Assay Test (IFAT)

In order to determine localization of antibodies protein against CYC1, TPK2, and CYC2 of *T. gondii*, IFAT were carried out. By using anti-CYC1 antibody, protein was detected not only mainly in the nucleus but some parasites have some localization at apical complex of *T.gondii* (Fig 2). By using anti-CYC2 antibody, protein specifically expressed in the dense granule of the parasite (Fig 3). By using anti-TPK2 antibody, the signal still could not be detected in the parasites.

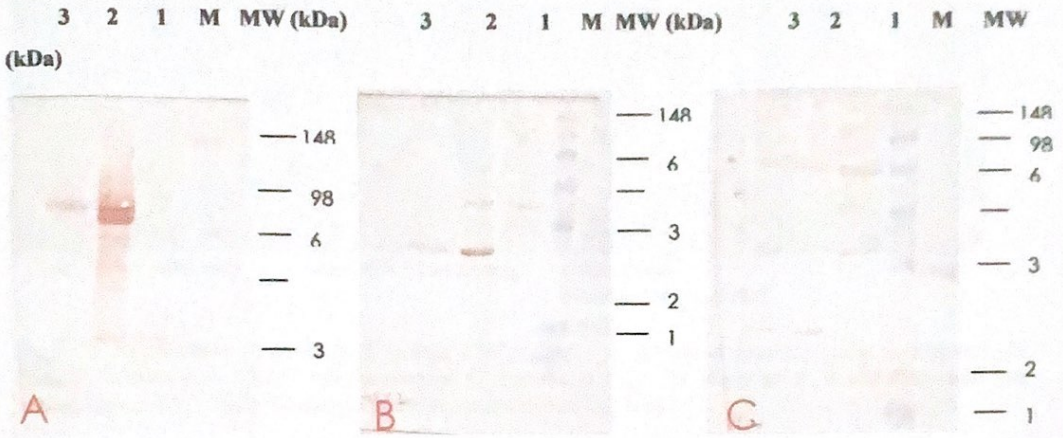

Fig.1. Western blot analysis showed that CYC1, TPK2 and CYC2 immunized sera could specifically detected in mammalian cells transfected with CYC1, TPK2 and CYC2. Lane M is molecular marker; lane 1 (A, B, C); 293T cells transfected with empty vector; lane 2 (A, B, C); 293T cell transfected CYC1, TPK2 and CYC2; lane 3(A, B, C); *T. gondii* RH strain lysates.

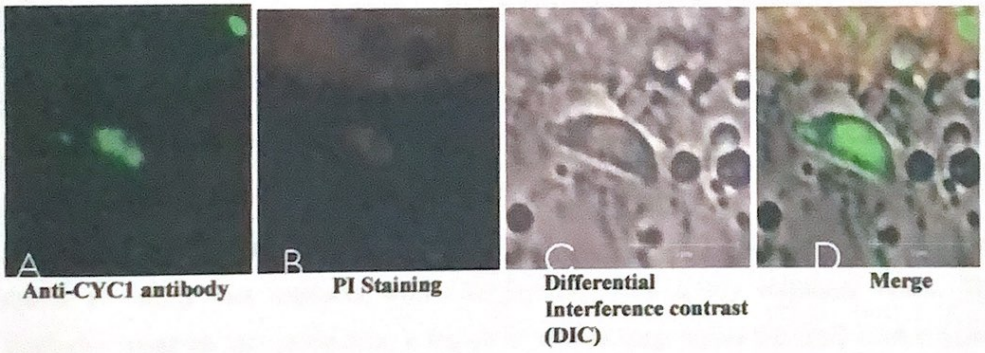

Fig.2. A. Localization of antibody *T. gondii* CYC1 stained in nucleus (green fluorescent) and apical complex (green fluorecent) B. Parasites stained with PI (red fluorescent). C. Parasite in DIC. D. Merge of A, B and C showed that location of antibody TgCYC1 stained in the nucleus and apical complex of *T.gondii*.

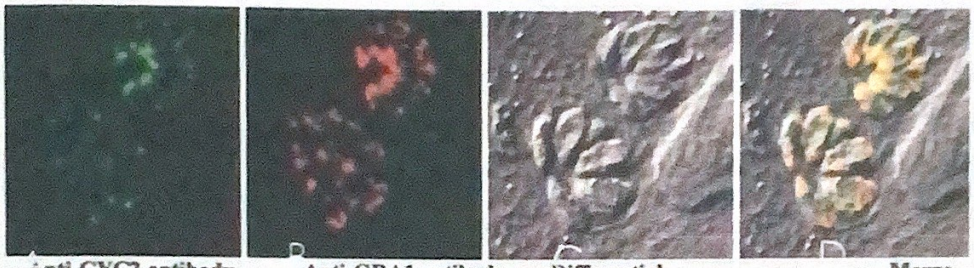

Fig.3. A. Localization of antibody *T. gondii* CYC2 stained in the dense granule (green fluorescent). B. Parasites stained with GRA1 (red fluorescent) C. Parasite in DIC. D. Merge of A, B and C showed that location of antibody TgCYC2 stained in the dense granule of *T.gondii.*

DISCUSSION

Cyclins are eukaryotic proteins that play an active role in controlling cell division cycles and regulate cyclin dependent kinases (CDKs) (Li *et al.,* 2009). Cyclin-dependent kinases (Cdk) are serine and threonine kinases, which control progression through the eukaryotic cell cycle (Bloom and Cross, 2007), with activating (cyclins) and inhibitory (Cdk inhibitors: CKIs) proteins, sub-cellular localization and phosphorylation.

In this study, we successfully analyzed CYC1, TPK2 and new molecule CYC2 of *T. gondii*, expressed in mammalian cells. As reported before, TgCYC1 has similarity to the cyclin H family and interacts with CDC2-related cdk family member, TPK2. This similarity based on the cyclin box, a region of cyclins responsible for cdc2-related kinase binding (Kvaal *et al.*, 2007). The cyclin box region of TgCYC1 is most similar to the H group of cyclins with the highest identity to the cyclin H homolog of Plasmodium (PfCYC1) (Le *et al.*, 2000).

The new molecule, CYC2 has similarity with cyclin Y and CCNY in human. Frank *et al.*, 2000, in their study reported that CCNY as susceptibility factors for Crohn's disease and ulcerative colitis. So far, there is no report about the function of CYC2 in eukaryotic cells.

From western blot result we reported that polyclonal antibodies protein could detect CYC1, TPK2 and CYC2 produced in mammalian cells specifically (Fig.1) TgCYC1 could be detected in two bands, one band at 75 kD and one band at 78 kD in mammalian cells (Fig 1A line 2), but only at 78 kD the *T.gondii* lysate was detected (Fig 1A line 3). This size is higher than predicted size at 66 kD, may be because of the post translation or modification of protein CYC1 in mammalian cells. TPK2 could be detected at 33 kD in mammalian cells (Fig.1B line 2) and the same size in the *T.gondii* lysate (Fig.1B line 3). This result agreement with Khan *et al.*, 2002, they reported two bands corresponded to TPK2-HA and endogenous cdk/CDC2 (approximately 33 kDa) appeared by western blot. TgCYC2 could be detected at 29 kD (Fig.1C line 2) and *T.gondii* lysate was detected in the same size (Fig.1C line 3). IFAT result showed the expression of protein detected by polyclonal antibody against CYC1 and CYC2. It showed that anti-CYC1 antibody, protein was located at not only the nucleus but also at the apical complex in some the parasites (Fig.2). This experiment suggested that CYC1 might be not only regulated at the nucleus division but also control apical complex division of *T. gondii*, but we still want to confirm about the localization of the anti-CYC1. TgCYC2 protein was founded in the dense granule of the parasite (Fig.3). The signal of anti-TPK2 antibody, protein still could not be detected in parasite. We still try to get the specific pattern of this protein. The immunostaining patterns of them are different that means their targets might be different, we will try to examine the function of them, which might be involved in the cell cycle of *T. gondii*. The totally function of CYC1, TPK2 and CYC2 still unknown. For further study, we want to determine more about the function of CYC1, TPK2 and CYC2 in *T. gondii*.

REFERENCES

Bloom, J., Cross, F.R., 2007. Multiple levels of cyclin specificity in cell cycle control. Nat. Rev. 8, 149–160.

Franke A., Balschun T., Karlsen TH., Hedderich J., May S., Lu T., Schuldt D., Nikolaus S., Rosenstiel P., Krawczak M., Schreiber S. 2008. Replication of signals from recent studies of Crohn's disease identifies previously unknown disease loci for ulcerative colitis. Jun;40(6):713-5

Hu K. 2008 "Organizational changes of the daughter basal complex during the parasite replication of *Toxoplasma gondii*.". *PLoS Pathogens* 4(1): e10.

Khan, F., Tang, J., Qin, C.L., Kim K. 2002. Cyclin-dependent kinase TPK2 is a critical cell cycle regulator in *Toxoplasma gondii*. Mol. Microbiol 45 : 321-332.

Kvaal, C.A., Radke, J.R., Guerini, M.N., White M.W. 2002. Isolation of a *Toxoplasma gondi*i cyclin by yeast two-hybrid interactive screen. Mol. Biochem. Parasitol. 120, 187–194.

Le Roch K., Sestier C., Dorin D., Waters N., Kappes B., Chakrabarti D., Meijer L., Doerig C. 2000. Activation of a Plasmodium falciparum cdc2-related kinase by heterologous p25 and cyclin H. Functional characterization of a P. falciparum cyclin homologue. J Biol Chem;275(12):8952_/8.

Li X., Wang X., Liu G., Li.Rui., Yu Long. 2009. Identification and characterization of cyclin X which activates transcriptional activities of c-Myc. Mol Biol Rep 36:97–103.

Lüder C.G.K. 2007. Survival Strategies of *Toxoplasma gondii*: Interference with Regulatory and Effector Functions of Macrophages. In *Protozoans in Macrophages*, Denkers EY, Gazzibelli RT (eds) Austin: Landes Bioscience.

Sibley LD., Charron AJ., Hakansson S., Mordue DG. 2007. Invasion and intracellular survival by Toxoplasma. In *Protozoans in Macrophages*, Denkers EY, Gazzibelli RT (eds) pp 46–24 Austin: Landes Bioscience.

Weersma RK., Stokkers PC., Cleynen I., Wolfkamp SC., Henckaerts L., Schreiber S., Dijkstra G., Franke A., Nolte IM., Rutgeerts P., Wijmenga C., Vermeire S. 2009. Confirmation of multiple Crohn's disease susceptibility loci in a large Dutch-Belgian cohort. Am J Gastroenterol. Mar;104(3):630-8.