Analysis of a novel cyclin like molecule CYC2 of *Toxoplasma gondii*

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ABSTRACT

In previously 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 that study described the identification of the *T. gondii* CDK family, CYC1 and TPK2, and new gene CYC2 by produced antibody against CYC1, TPK2, and CYC2, and then observed of their location in *T. gondii*. Subsequently, we concluded that the CYC2 was not involved in the cell cycle of *T. gondii* because of CYC2 had different cyclin box motif and protein localization. Recently, by yeast two-hybrid system, we found one protein, which could interact with CYC2 we named C2BP. A polyclonal antibody *Tg*CYC2 was produced with molecular weight 29 kDa and antibody *Tg*C2BP could be detected at 90 kDa, 70 kDa, 60 kDa, 50 kDa, which both were localized in the dense granule and secreted into parasitophorus vacuole (PV) after infection. The interaction of CYC2 with C2BP in the yeast two-hybrid system was confirmed by gluthathione S-transferase (GST) pull-down assay.

Key words: *Toxoplasma gondii*, CYC2, C2BP, IFAT, GST - Pull down Assay

INTRODUCTION

Toxoplasma gondii, the causative agent of toxoplasmosis is one of the unicellular parasites belongs to the phylum Apicomplexa which includes numerous obligate parasites of both human and veterinary importance together with Plasmodium, Cryptosporidium, and Neospora. Infections by this protozoan parasite are widespread in humans and warm-blooded animals. It caused clinical opportunistic pathogens in immunocompromised individuals such as HIV positive patients or organ transplant receivers and sometimes even in immunocompetent individuals (Botte *et al.*, 2008, Kim *et al.*, 2008, Lemgruber *et al.*, 2008).

T. gondii has three infectious stages i.e. tachyzoites, bradyzoites (contained in tissue cysts) and sporozoites (contained in sporulated oocysts) that are infectious for intermediate host (all warm-blooded animals including most livestock and humans), and definitive hosts (members of the

family Felidae, like domestic cats) (Tenter, 2009). The main transmission routes for toxoplasmosis to human are established by ingestion with raw or undercooked meat containing tachyzoites or bradyzotes in tissue cycts and also transplacental infection during pregnancy (Cook *et al.*, 2000). Recently, Tenter *et al.*, 2000 reported estimated 4 to 77 % of all people worldwide are infected with Toxoplasma.

Toxoplasma divided by binary fission called endodyogeny which a single chromosome replication during concurrent mitosis and parasite budding (White *et al.*, 2005). *T. gondii* is capable of invading any nucleated cell and replicates within a parasitophorous vacuole (PV) separate from the host endocytic pathway which has three organelles that secrete proteins sequentially during invasion and development of the PV (Black *et al.*, 2000, Coppens *et al.*, 2006). These organelles are the micronemes (secrete proteins for initial recognition and adhesion), rhoptries (secrete proteins during initial formation of the PV), and dense granules (secrete after PV formation). (Mercier C *et al.*, 2005).

In human, there is a gene with one cyclin box motif (cyclin Y). Weersma *et al.*, 2009 reported that the mutation of protein in that gene could be causative agent of diseases such as Inflammatory Bowel Disease (IBD). In the previous study, from database, we found a 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 (cyclin Y), we designated this as new gene namely CYC2.

In laboratory previous work, the yeast two-hybrid technique using CYC2 proteins as baits was performed to identify the partner protein that could interact with CYC2 in *T. gondii*. We found one protein namely C2BP, as a novel interacted with CYC2. Recently, we analyzed CYC2 and C2BP protein expression and localization in *T. gondii*. Their interactions were confirmed biochemically by GST Pull down Assay.

MATERIAL AND METHODS

Parasites and host cells

The RH strains of *T. gondii* and host Vero cells were used for this study. RH strain was maintained in our laboratory through serial passage in Vero cells grown in modified Eagles medium (Sigma-Aldrich, UK) supplemented with 5% fetal calf serum (FCS).

Animals

Six weeks old female BALB/c mice used in this experiment were purchased from CLEA, Japan. All of the animal experiments described here were conducted in accordance with the standard relating to the care and management of experimental animals of Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan.

Gene cloning

T. gondii CYC2 and C2BP cDNAs containing entire coding regions and N-terminal truncation were amplified by PCR from the parasite cDNA using the following specific primers, for CYC2 5'TTGTCGACGATGGGCTTTGCGGCCGCGGA 3',for the C2BP (1) 5'TTGAATTCATGTG GT TTTTGGCTCGCCTGGAC 3', C2BP (2) 5'TTTTGCGGCCGCGTGCCCGGGTTTGA ATAAAGAGG 3', C2BP (3) 5'TTGAATTCATGGCATTTCAAGGAGATGAC3', C2BP (4) 5' TTGAATTCATGGCATCTACAGCATCG 3', C2BP (5) 5' TTGAATTCATGGCTGCCGTTG TAGTC3'. The amplified cDNAs were subcloned into pGEX6P-2 vector (Pharmacia) with double digestion with enzymes and ligation using T4 DNA ligase (Invitrogen, USA). The plasmids CYC2 and C2BP were purified from transformed *Escherichia coli* (*E. coli*) DH5α cells by polyethylene glycol precipitation method. The purified plasmids were dissolved in sterile TE buffer and kept at -20°C until use. The integrity of the plasmids was determined by agarose gel electrophoresis after digestion with appropriate restriction enzymes. The DNA concentration was determined by measuring the optical density at 260 nm (OD_{260}) using an Ultrospec 2100 spectrophotometer (Amersham Biosciences, England).

Production of recombinant proteins

T. gondii CYC2 and C2BP cDNA containing entire coding sequences were subcloned in the pGEX6P-2 vector (Pharmacia) as described above. The plasmids were transformed into *E. 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 Gluthatione Sepharose Beads (Amersham Biosciences, Uppsala, Sweden). After being, dialysed with PBS, the recombinant proteins were evaluated by SDS-PAGE and quantified by Coomassie assay kit.

Production of Polyclonal Antibody

For the primary immunization, the mice were injected subcutaneously with 10 µg of antigen CYC2 and C2BP emulsified in equal volume Freund's complete adjuvant. Seven booster doses were given at interval 2 µg weeks 10 of the same antigen emulsified in equal volume of Freund's incomplete adjuvant.

Expression of CYC2 and C2BP in mammalian cells

The 293T cells were transfected with CYC2, C2BP and empty vector using the calcium precipitation method. The 293T cells were maintained with minimum essential medium (MEM) supplemented with 10% fetal calf serum. Forty µg of plasmid DNA were dissolved in 460 µl distilled H_2O, and 500µl of 0.5 M $CaCl_2$ was added and the resulting 1 ml mixture was incubated at room temperature for 1 hour. After 1 hr incubation at room temperature, 1 ml of HNP buffer (50 mM HEPES, pH7.1, 280 mM NaCl, 1.6 mM NaH_2PO_4) was added. Then, the mixture was added to the cells. After incubation 4 hrs, cells were washed with culture medium and finally cells were incubated in 10 ml culture medium for 48 hours in a 5% CO_2. Two days after transfection, the cells were lysed with RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, 0.1% Triton-X100, 1% Nonidet P-40). The lysates of transfected cells were dissolved in SDS-PAGE sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue), heated at 96°C for 5 minutes and separated on 12% polyacrylamide gel, and transferred onto membrane. Separated proteins were incubated with anti-CYC2 and anti-C2BP polyclonal antibodies dilution ratio of 1:100 in PBS containing 1% skimmed milk (PBS-M). Then, incubated 1 hr using sheep anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences) diluted in 1:1000 in PBS-M. Molecular mass standards (SeeBlue plus2 Pre-stained standard, Invitrogen) were used. Detection

was done with mixture of 0.25mg/ml diaminobenzidine tetrahydrochloride (Sigma), 100mM Tris, pH7.5 and 0.05% H_2O_2.

Transfection of C2BP in 293T mammalian cells

The plasmid C2BP was tranfected in 293T cells mammalian cells as described above. Cells were harvested 48 hrs after transfection and lysed with buffer (1 mM EDTA, 50 mM Tris–HCl pH 7.5, 10% Glycerol). After freeze – thawing 15 min each, sample were centrifuged 5 min and supernatants were obtained. Detection was carried out by chemiluminescence by using V5 antibody (1:1000) and HRP antimouse IgG (1:1000) as secondary antibody, respectively. After incubation 1 hr, specific reaction was detected by ECL, 10 sec and 1 min exposure. Then, the supernatant of cell lysate was used for GST pull-down assay.

GST Pull down Assay between CYC2 and C2BP

Two micrograms of GST or GST-CYC2 were mixed with 500 µg of C2BP lysate, in 500 µl binding buffer (50 mM Tris HCl pH 7.5; 1 mM EDTA ; 10% Glyserol). Each sample was mixed with 20 µl glutathione-Sepharose 4B beads and rotated for 1 hr at 4^0 C, and beads were recovered by centrifugation. The pellets were washed with 500 µl washing buffer same as binding buffer 5 times, dissolved in same volume of sample buffer and used for GST Pull down assay.

Indirect Immunofluorescence Assay Test (IFAT)

Parasites were inoculated into host Vero cell and grown onto cover slips for 24 hrs. After incubation, cells were washed 3 times with PBS. Fixing and permeablizing solution (3% Formaldehyde, PBS and 0.2% Triton X-100) or cold methanol was added and incubated at room temperature for 15 min. After rinsing 3 times with PBS, samples were blocked 2 times with fresh 3% BSA for 10 min each. After rinsing 3 times with PBS, incubated for 1 hr with anti-CYC2 and anti-C2BP diluted in 3% BSA (1: 200) and anti-GRA1 diluted in 3% BSA (1:10000). 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. Rinse 3

times with PBS and distilled water, cover slip was mounted with mowiol on object glass, observed under immunofluorescent microscope and Leica TCS NT Confocal Laser Scanning Microscope (Leica, Germany).

RESULTS

CYC2 molecule contains cyclin box motif at 110- 180 from full length 255 amino acid. The structure of CYC2 was shown in Fig 1A. By yeast two hybrid system we found one protein could interact with CYC2, we named C2BP which contain 12 repeated sequence (KE K/N A/V SVA F/S QGDDARVLTS G/D E/K G/E) located at 90- 330 amino acid from full length 672 amino acid, as shown in Fig.1B. Furthermore, we truncated of N-terminal within C2BP with 4 different truncation, C2BP 3-5, C2BP 3-4, C2BP 4, and C2BP 5, in order to find which binding site domain that might be important interact with CYC2 in *T. gondii,* as shown in Fig. 1C.

We used Western blot analyses to confirm the production of the antibodies against CYC2 and C2BP. The result showed no specific bands could be detected in 293T cells transfected with empty vector (Fig. 2A and 2B lane 1), a specific band was detected approximately at 29 kDa and 90 kDa, 70 kDa, 60 kDa, 50 kDa for C2BP in 293T cells transfected with CYC2 and C2BP (Fig. 2A lane 2 and Fig. 2B lane 2-6) and also in *T. gondii* RH strain lysates, band could be detected approximately at 29 kDa for CYC2 and 90 kDa for C2BP (Fig. 2A lane 3 and 2B lane 7).

CYC2 interacted with C2BP in the yeast two-hybrid system, of which the specific binding was proved by the GST pull-down assay, as shown in Fig. 3. Here, we used C2BP 3-5 as fractions that bind with CYC2. GST - CYC2 binding with C2BP was shown in Fig. 3A lane 3. C2BP lysate transfected in 293T cells as input protein was shown in Fig. 3A lane 1. GST alone in Fig. 3A. lane 2. The amount of GST alone and GST-C2BP was compared by Coomassie Blue staining. (Fig. 3B).

In order to determine localization of CYC2 and C2BP of *T. gondii*, IFAT were carried out. By using anti-CYC2 and anti-C2BP antibody, both protein specifically expressed in the dense granule (Fig. 4A and Fig. 5A) and secreted into parasitophorus vacuole (PV) (Fig. 4B and Fig. 5B).

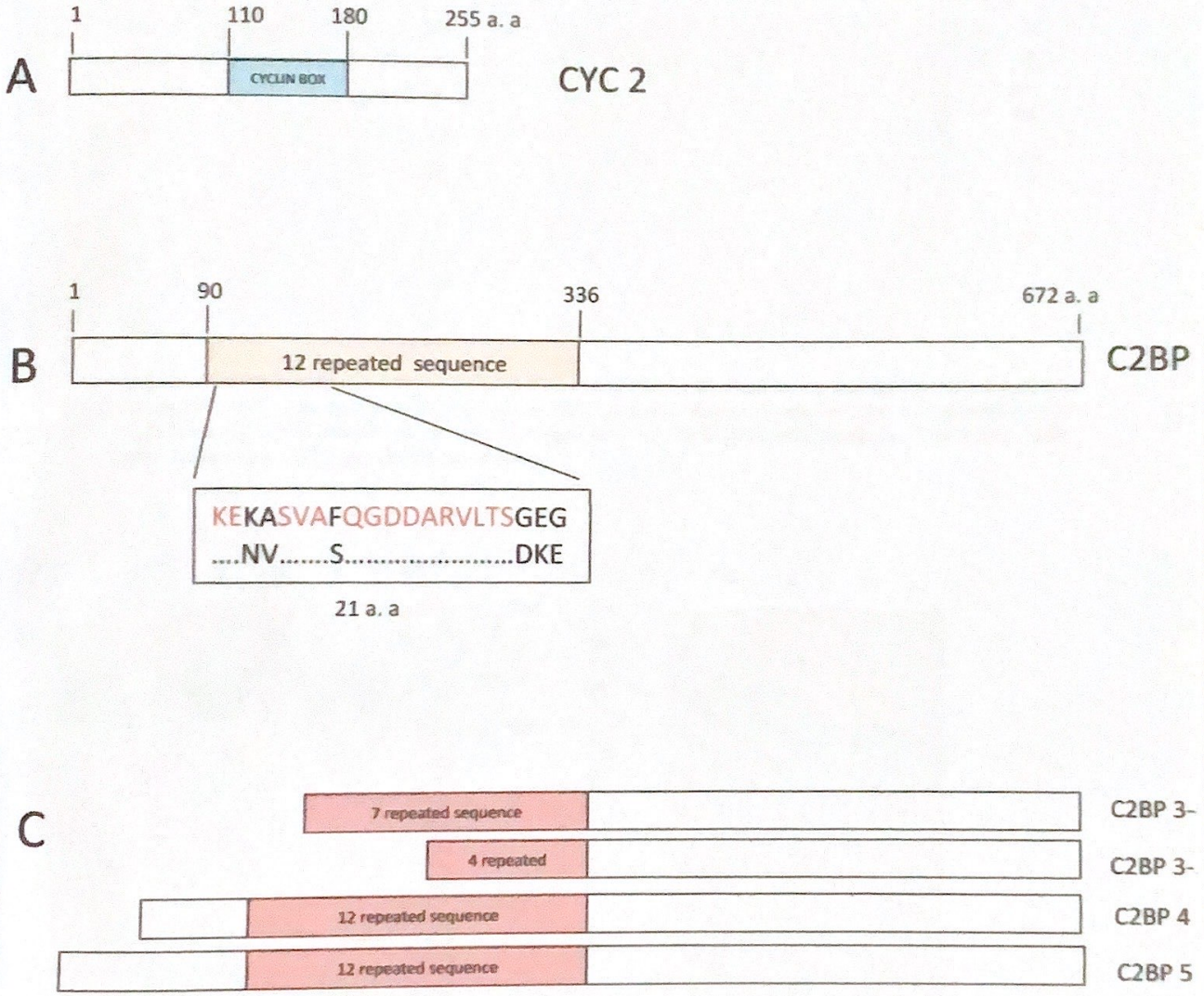

Fig. 1. (A) Structure of CYC2 with the cyclin box around 110 – 180 amino acid (B) Structure of C2BP with 12 repeated sequence. (C) Truncation of N-terminal within C2BP.

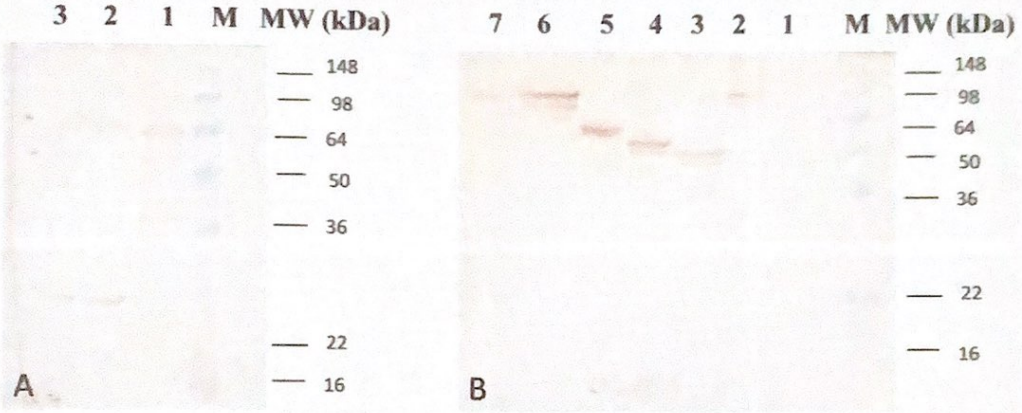

Fig. 2. Western blot analysis showed that CYC2 and C2BP immunized sera could specifically detected in mammalian cells transfected with CYC2 and C2BP. Lane M is molecular marker; lane 1 (A and B) is 293T cells transfected with empty vector; lane 2 (A) and lane 2-6 (B) is 293T cell transfected CYC2 and C2BP; lane 3A and lane 7B; *T. gondii* RH strain lysates.

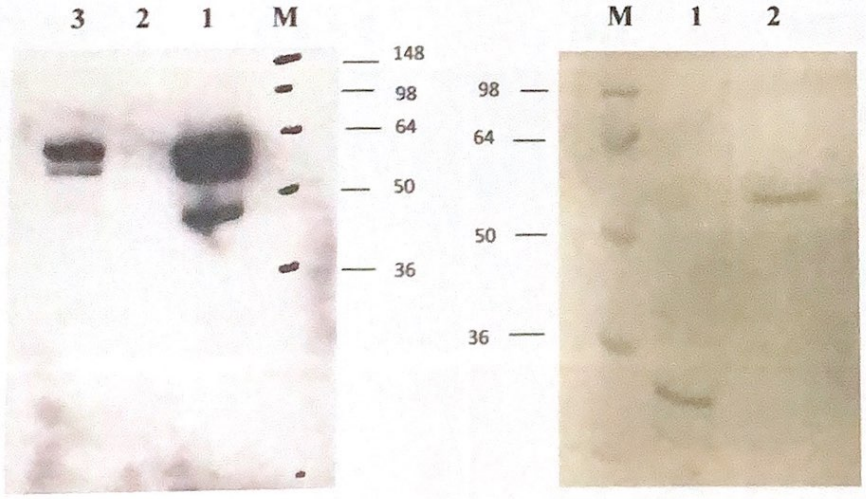

Fig. 3. (A). GST pull-down assay between CYC2 and C2BP were performed to proof the interaction of them. Lane M is molecular marker; lane 1 is input with C2BP lysate transfected in 293T cells; lane 2 is GST; lane 3 is GST-CYC2 binding with C2BP (B) Coomassie Blue staining. Lane M is molecular marker lane 1 is GST; lane 2 is GST-CYC2 binding with C2BP.

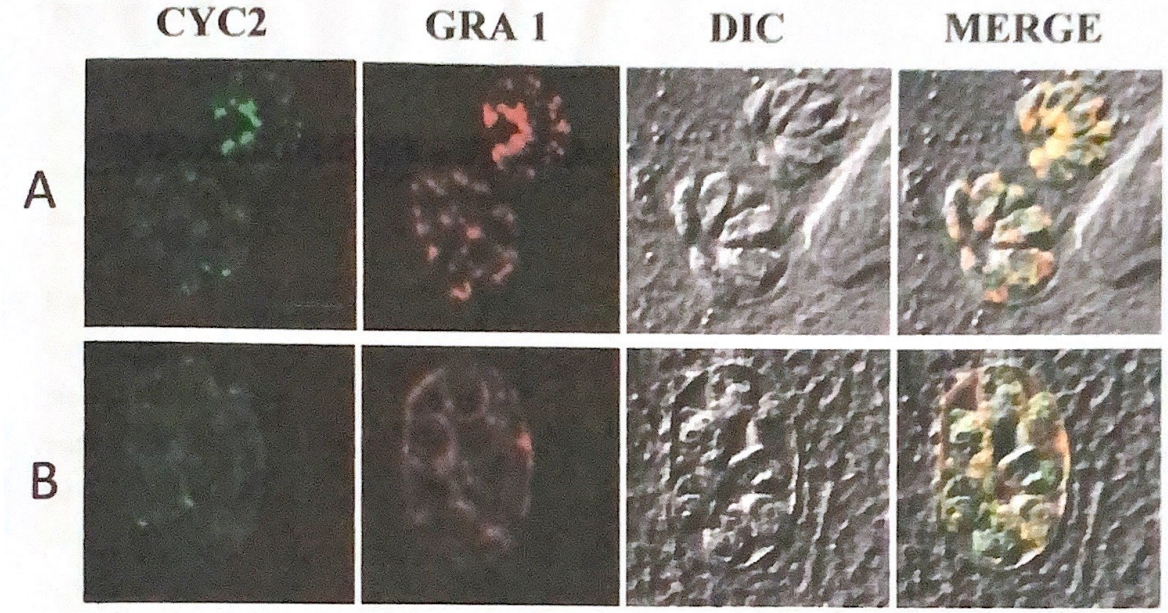

Fig. 4. Localization of CYC2, fixing with formalin, antibody expressed in the dense granule (A) and (B) with cold methanol, antibody protein secreted at parasitophorous vacuole (PV)

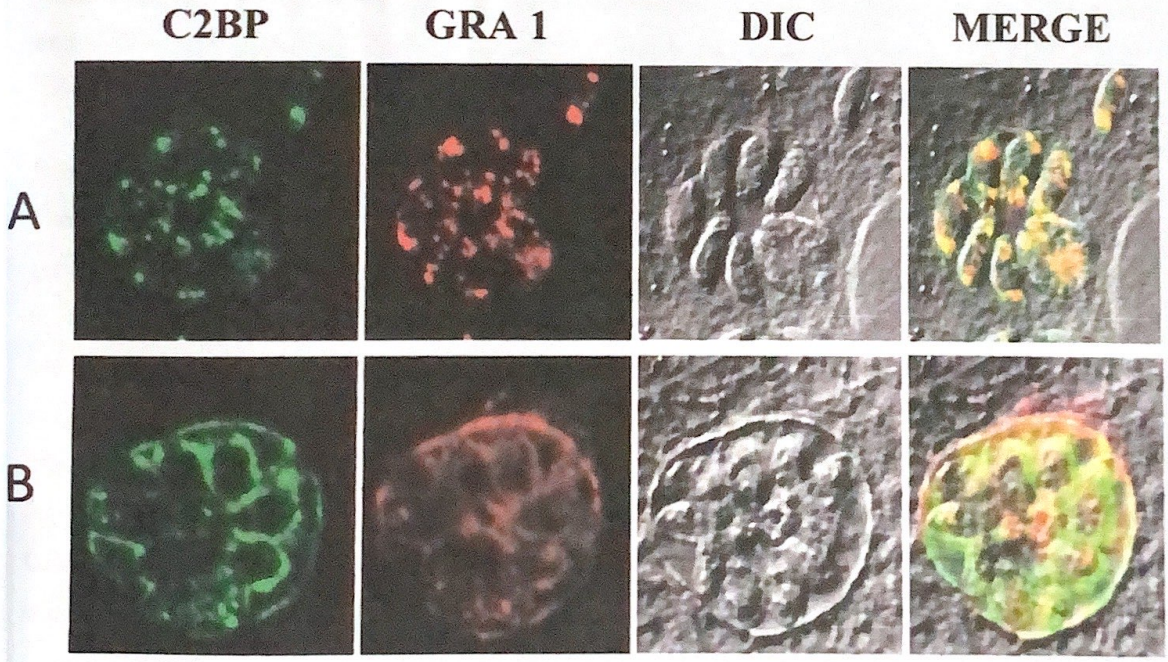

Fig. 5. Localization of C2BP, fixing with formalin, antibody expressed in the dense granule (A) and (B) fixing with cold methanol, antibody protein secreted at parasitophorous vacuole (PV).

DISCUSSION

The new molecule, CYC2 has similarity with cyclin Y (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 CCNY in eukaryotic cells. Here we determined the expression of CYC2 and C2BP and their localization in *T. gondii*.

C2BP was a prey protein to CYC2 in the yeast two-hybrid technique. Specific interaction between C2BP and CYC2 was confirmed by GST pull-down assay. We used C2BP 3-5 as a protein binding with GST-CYC2 because of the length C2BP 3-5 shortest than others C2BP truncated and contained 4 repeated sequence, as described above. Therefore, we want to examine the repeated sequence of the C2BP is important or not in order to find the binding site domain of C2BP. We found the interaction between CYC2 and C2BP by GST pull-down assay (Fig. 3A), C2BP was precipitated and detected by V5 antibody under the presence of GST-CYC2 fusion protein (Fig. 3A lane 3), while not bound to the beads in the GST only fraction without CYC2. (Fig. 3A lane 2). The size of C2BP expressed was similar with input (Fig. 3A lane 1). Using the same samples, Comassie Blue Staining was performed to compare the amount of the GST alone and GST-C2BP (Fig. 3B). The amount of GST alone was almost the same with GST-C2BP. These results suggested that *Tg*CYC2 is interacted with *Tg*C2BP.

During the entry of *T. gondii*, a parasitophorous vacuole (PV) is formed within the host cells and compartmentalized from the host cell cytoplasm by a PV membrane (PVM) within which the parasites multiply and grow (Ahn *et al.*, 2006). Dense granules release proteins from both the apical and posterior ends of the parasite after fusion with the plasma membrane and the proteins are among the most abundantly transcribed in *T. gondii* (Craver *et al.*, 2000). Some proteins of the latter group of structures including GRAs 3, 5, 7, and 8 are incorporated into the PVM, while others, such as GRAs 1, 2, 4, 6, and 9, are inserted into the membranes of a tubule network formed within the PV, the intravacuolar network (IVN) (Mercier *et al.*, 2005). Here, we used GRA 1 as a marker to confirm the localization of CYC2 and C2BP in the dense granule position and secreted on the PV of *T. gondii*.

In order to know the expression of CYC2 and C2BP, we carried out the western blot method. The result showed that polyclonal antibodies could detect CYC2 and C2BP produced in

mammalian cells specifically (Fig. 2). *Tg*CYC2 could be detected at 29 kD (Fig. 2A line 2) and *T. gondii* lysate was detected in the same size (Fig. 2A lane 3). *Tg*C2BP could be detected at 90 kDa, 70 kDa, 60 kDa, 50 kDa (Fig. 2B lane 2-6), and *T. gondii* lysate was detected in the same size as full length at 90 (Fig. 2B lane 7). From those results, molecular weight is higher from predicted size at 73, 67, 53 and 43 kDa, may be because of the post translational modification of protein C2BP in mammalian cells. IFAT result showed the expression of protein detected by polyclonal antibody against CYC2 and C2BP. It showed that both antibody, protein was located specifically expressed in the dense granule (Fig. 4A and Fig. 5A) and secreted into parasitophorous vacuole (PV) in *T. gondii* (Fig. 4B and Fig. 5B). Green fluorescence of CYC2 and C2BP overlapped with red fluorescence from GRA1-red fluorescent as revealed by merge image. Those results showed that CYC2 and C2BP had the same pattern, and may be they have similar function. The totally function of CYC2 and C2PB in *T. gondii* still not clearly understood. We need further study to determine exactly about the function of them in *T. gondii* or in others eukaryotic cells.

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