J. Protozool. Res. 18, 79-95 (2008)
Copyright©2008, National Research Center for Protozoan Diseases

The Influence of the regulation of *Toxoplasma gondii* TgMIC2 transgene on host cell infection.

Buates, S.[1, 2], Xuan, X.[2], Igarashi, M.[2], Sugimoto, C.[2] and Inoue, N.[2]*

[1]Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand, [2]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

*Corresponding Author: Dr. Noboru Inoue, D.V.M., Ph.D., National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

E-mail: ircpmi@obihiro.ac.jp Tel.: +81-155-495647 Fax: +81-155-495643

ABSTRACT

Toxoplasma gondii microneme protein 2 (TgMIC2), an apically stored adhesin, was shown to be a key participant involved in the initial attachment and invasion to a host cell. In this study, we had established the clonal line of *T. gondii* tachyzoites which over-expressed TgMIC2 using the tetracycline repressor (TetR)-based inducible gene expression system. The TgMIC2 over-expression significantly reduced tachyzoite propagation *in vitro* ($p < 0.002$) and significantly reduced the parasite virulence in mice ($p = 0.002$). The over-expression of exogenous TgMIC2 caused the increase of mRNA expression level of endogenous TgMIC2. Wild type parasites mainly expressed $TgMIC2^{115}$ protein, the microneme store and the surface form of TgMIC2. Over-expression of TgMIC2 induced production of $TgMIC2^{110}$, the cell surface form of TgMIC2 produced by N-terminal proteolytic processing of $TgMIC2^{115}$. Some of tachyzoites became spherical shape having disordered apical complex after TgMIC2 over-expression. TgMIC2 over-expression did not effect mRNA expression level of TgMIC2-associated protein (TgM2AP), but appeared to cause the aberrance of TgM2AP protein. In conclusion, our results reveal the novel findings on the importance of the regulation of TgMIC2 transgene expression on post-translational modification of TgMIC2 and TgM2AP, and parasite's morphology and propagation, with all together impacting the outcome of infection.

Key words: *Toxoplasma gondii*; TgMIC2; over-expression

INTRODUCTION

Toxoplasma gondii, an obligate intracellular parasite of the phylum Apicomplexa, is the etiological agent of toxoplasmosis in warm-blooded vertebrates including humans. Unlike most intracellular pathogens that require host cell functions for entry (e.g. endocytosis or phagocytosis), *T. gondii* tachyzoites actively invade host cells by its actin/myosin-powered motility (Dobrowolski and Sibley, 1996; Dobrowolski *et al*., 1997; Opitz and Soldati, 2002; Brossier and Sibley, 2005). The invasion process depends on strategically secretion of proteins from three distinct secretory organelles located in the apical complex, namely the micronemes, rhoptries, and dense granules (Carruthers and Sibley, 1997). The parasite initial attachment to the host cell via its apical end (anterior end) triggers the micronemes to secrete their contents (Carruthers and Sibley, 1999). One adhesive microneme protein, *T. gondii* microneme protein 2 (TgMIC2), is a member of the thrombospondin-related anonymous protein (TRAP) family that is unique for the phylum Apicomplexa (Brossier and Sibley, 2005). TgMIC2, the 115-kDa protein ($TgMIC2^{115}$), consists of (1) two well-defined extracellular adhesive domains [an N-terminal single integrin-like A/I domain and an M-domain which consists of five thrombospondin-1 (TSP-1)-like repeats and one degenerate repeat], (2) a transmembrane

domain (TMD), and (3) a short C-terminal cytoplasmic domain which is connected to the actin-myosin complex beneath the parasite plasma membrane via aldolase (Wan *et al*., 1997; Jewett and Sibley, 2003). In the parasite rough endoplasmic reticulum (ER), TgMIC2 forms a complex with another microneme protein namely TgMIC2-associated protein (TgM2AP) within 15 min after synthesis. TgM2AP helps TgMIC2 to transport throughout the secretory network (Rabenau *et al*., 2001). TgMIC2/TgM2AP, a hetero-hexameric complex of 450 kDa, contains three TgMIC2–TgM2AP entities that are associated via interactions between the TgMIC2 molecules (Jewett and Sibley, 2004). Following host cell contact, TgMIC2/TgM2AP complex is secreted from the micronemes and deposited on the parasite surface (Carruthers and Sibley, 1997; Carruthers *et al*., 1999; Carruthers and Sibley, 1999). This complex binds to host-cell receptors via the TgMIC2 adhesive domains. After binding, the complex is rapidly translocated to the parasite posterior end through an actin-myosin-dependent process (Carruthers *et al*., 1999). Ultimately, the complex is cleaved within the TgMIC2 transmembrane domain by the parasite derived-protease namely microneme protein protease 1 (MPP1) and released into the medium (Carruthers *et al*., 2000; Brossier *et al*., 2003; Zhou *et al*., 2004; Carruthers, 2006). The translocation and cleavage of TgMIC2/TgM2AP complex at the posterior end of the parasite surface result in gliding motility or entry into a host cell.

Extensive studies of the TgMIC2/TgM2AP complex reveal that TgMIC2 is a key component of the invasion machinery (Carruthers and Sibley, 1997; Brossier *et al*., 2003; Jewett and Sibley, 2003; Huynh *et al*., 2003, 2004, 2006). In TgMIC2 knock out (KO) parasites, there was a significant decrease in numbers of invading parasites (Huynh *et al*., 2004). TgM2APKO parasites demonstrated an 80% reduction in rapid cell invasion due to the reduction of TgMIC2 expression and impairment of TgMIC2 secretion with a proportion retained in the ER/Golgi (Huynh *et al*., 2003). The reduction of TgMIC2 expression led to mistrafficking of TgM2AP, markedly defective host attachment and invasion, the loss of helical gliding motility, and the inability to cause lethal infection in a murine model (Huynh and Carruthers, 2006).

To study function of essential genes without any pleiotropic effects except for the target gene, one of the widely used approaches is the tetracycline repressor (TetR)-based inducible gene expression system. The TetR system of *Escherichia coli* interferes with transcription, and has been optimized to tightly regulate gene expression in *Trypanosoma brucei* (Wirtz *et al*., 1999), *T. cruzi* (Taylor and Kelly, 2006), *Giardia lamblia* (Sun and Tai, 2000), *Leishmania donovani* (Yan *et al*., 2001), *Entamoeba histolytica* (Hamann *et al*., 1997; Ramakrishnan *et al*., 1997), *T. gondii* (Meissner *et al*., 2001), and *Trichomonas vaginalis* (Ortiz and Johnson, 2003). This system is driven by Tet-operator (TetO) and regulated by TetR. In the absence of tetracycline (Tet), TetR binds to TetO and blocks transcription from a promoter. In the presence of Tet, Tet binds to TetR, and causes conformational change of TetR resulting in its inability to bind TetO. Therefore, the transcription of the gene of interest can be artificially controlled. This system proves to be appropriate for conditional expression of the genes of interest including endogenous genes, mutated forms of endogenous genes, and exogenous genes. However, this system is not suitable to generate conditional KO in *T. gondii*. The necessity to keep the parasites in the presence of a drug during a prolonged period to maintain the expression of an essential gene led to generation of revertants that lost Tet-dependent regulation of gene expression (Meissner and Soldati, 2005). Recently, another TetR system which utilizes alternative TetR named YFP-TetR (TetR fusion with yellow fluorescent protein) has been developed (van Poppel *et al*., 2006). This YFP-TetR demonstrated to improve regulatory properties.

Since TgMIC2 is a central component of the invasion machinery, in this study, we were interested in over-expressing TgMIC2 using the TetR system to examine its effect on host cell infection and to study the

regulation of gene expression. Here, we show that the regulation of over-expression of the TgMIC2 inducible copy has the novel effects on the parasite's morphology and propagation, enhances the microneme protein protease 2 (MPP2)-mediated N-terminal proteolytic processing of TgMIC2, and causes the aberrance of TgM2AP protein expression. All of these effects impact the outcome of infection.

MATERIALS AND METHODS

Parasite strains, transfection and selections

T. gondii tachyzoites, RH strain wild type (RH-WT) and RH hypoxanthine-xanthine-guanine-phosphoribosyltransferase (*hxgprt*)$^{-}$, were grown in Vero cells, the African green monkey kidney cell line, and maintained in Eagle's minimum essential medium (MEM) (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% v/v heat inactivated fetal bovine serum (FBS) (Biosource International Inc., Camarillo, CA, U.S.A.) and 25 μg/ml gentamicin. Parasites were purified from infected monolayers by passing the scraped cells three times through a 27G needle and were subsequently filtered through a 5- μm pore size filter. To generate stable transformants, 5 x 10^7 freshly released RH *hxgprt*$^{-}$ parasites were transfected as described previously (Roos *et al*., 1994) and selected with 25 μg/ml mycophenolic acid (MPA) supplemented with 50 μg/ml xanthine (XT), a substrate for *hxgprt* (Donald *et al*., 1996). Positive selection against chloramphenicol acetyltransferase (CAT) was carried out using 20 μM chloramphenicol (Kim *et al*., 1993). The stable parasite cell lines emerged within 2-week period and were cloned by limiting dilution in 96-well plates (Roos *et al*., 1994).

Plasmids

The pTUB8TetR^{S}-HXGPRT plasmid is the TetR^{S} (synthetic TetR) containing plasmid. The p5RT70Tet4LacZ-CAT is the reporter plasmid containing the tetracycline (Tet)-inducible promoter and LacZ gene. The p5RT70Tet4-GFP is the regulatory plasmid containing the Tet-inducible promoter. All plasmids are kindly provided by Professor Soldati D. (Meissner *et al*., 2001).

Construction of Tet-inducibleTgMIC2 plasmid

The full-length TgMIC2 coding sequence was amplified from cDNA of RH-WT using Advantage™ HF 2 PCR kit (ClonTech Laboratories, Inc., Palo Alto, Calif) and 25 cycles of amplification with primers sense (5'-ATA TGC ATA AAA TGA GAC TCC AAC GCG AGG CCG-3') and antisense (5'-GCT TCG AAC TAC TCC ATC CAC ATA TCA CTA TCG-3'). The GFP gene in p5RT70Tet4-GFP plasmid was replaced with the PCR product encoding TgMIC2 to generate p5RT70Tet4-MIC2 plasmid. The inserted TgMIC2 sequence was confirmed using a BigDye Terminator V3.1 Cycle Sequencing kit (Applied Biosystem, Foster City, CA, USA), and an ABI PRISM 3100 automated DNA sequencer (Applied Biosystem). Thereafter, the TgMIC2 gene containing TetO cassettes from p5RT70Tet4-MIC2 plasmid was inserted downstream into the compatible sites in pBS(SK^{+})TUB-CAT-SAG1 to generate p5RT70Tet4-MIC2-CAT. The CAT gene was a selectable marker. Insertion of the p5RT70Tet4-MIC2-CAT into *T. gondii* genome was confirmed using the following two primer sets: primer set 1 (P1 or p5RT7OTet4: 5'-ATC TGC AGG GGC CCC CCC TCG ACG GTA TGG-3', and P2 or TgMIC2: 5'-GCT TCG AAC TAC TCC ATC CAC ATA TCA CTA TCG-3'), and primer set 2 (P3 or TUB 5'UTR: 5'-GCC TCG AGG ATA TGC ATG TCC GCG TTC GTG-3', and P4 or SAG1 3'UTR: 5'-GCA AGC TTC CCT CGG GGG GGC AAG AAT TGT-3').

TgMIC2 and TgM2AP antisera

The recombinant TgMIC2 and TgM2AP proteins containing entire coding sequences were produced as previously described (Dautu *et al*., 2007). The antisera against these two proteins were obtained

by immunization via intraperitoneal (ip) route of eight-week-old female BALB/c mice (CLEA, Tokyo, Japan) with 50 μg of TgMIC2 or TgM2AP protein emulsified in complete Freund's adjuvant. The mice were boosted by an ip injection of the same amount of the antigen emulsified in incomplete Freund's adjuvant on days 15 and 30 after the first immunization. Levels of specific anti-TgMIC2 and anti-TgM2AP antibodies in mice sera were determined as previously described (Ismael *et al*., 2003).

SDS-PAGE and Western blotting

Freshly released tachyzoites (1 x 10^7) were resuspended into 25 μl of RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) and lysed by sonication for 3-5 min in an ultrasonic tank. Pellet and supernatant were separated by centrifugation at 13,000 rpm at 4°C for 15 min. The supernatant was boiled in an equal volume of 2X SDS–PAGE sample buffer (4% SDS, 125 mM Tris HCl pH 6.8, 10% 2-β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) and run on a 10% or 12% SDS–polyacrylamide gels under reducing conditions (Laemmli, 1970). Western blotting was performed as described elsewhere (Hettmann *et al*., 2000). The PVDF membranes (Immobilon Millipore, U.S.A.) were blocked in PBS containing 10% FBS, 0.05% Tween-20 and then reacted with mouse polyclonal anti-TgMIC2 (dilution 1:100) or with mouse polyclonal anti-TgM2AP antiserum (dilution 1:100). The membranes were washed 3 times, and incubated with horseradish peroxidase–conjugated goat anti-mouse IgG (ICN Biomedicals, Inc., U.S.A.) (dilution 1:2,000). Results were detected by Western Lightning™ kit (PerkinElmer Life And Analytical Sciences, Inc., MA, USA) according to the manufacturer's protocols or a substrate solution containing 0.5 mg/ml diaminobenzidine and 0.005% H_2O_2.

Indirect Fluorescence Antibody Technique (IFAT)

Vero cell monolayers grown on 12-well glass slides were infected with freshly released tachyzoites (1 x 10^3 cells/well). The infected cells were cultured with or without 1 μg/ml anhydrotetracycline (ATc), a non-cytotoxic derivative of tetracycline. Four days post-infection, cells were fixed with absolute methanol for 30 min at room temperature followed by incubation for 1 h at 37°C with the primary antibody, mouse polyclonal anti-TgMIC2 antiserum (1:100 dilution). The slides were washed three times with PBS and then incubated for 1 h at 37°C with the secondary antibody, Alexa-Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, USA; 1:200 dilution). After washing three times with PBS, the slides were mounted with 50% glycerol in PBS and observed by confocal laser scanning microscopy (TCS NT, Leica, Germany).

Reverse transcriptase polymerase chain reaction (RT-PCR)

Ten μg of each total RNA sample extracted from parasites grown with or without 1 μg/ml ATc was treated with 200 units of RNase-free DNase I (Takara Bio Inc.) for 15 min at 37°C and the DNase I was inactivated by heating at 75°C for 5 min. The reverse transcription (RT) reaction was carried out using 2 μg of the RNA samples as recommended by the manufacturer. The polymerase chain reaction (PCR) was performed in a total volume of 50 μl containing 1 μl of template DNA (10 ng), 5 μl of dNTP mix (2 mM each dNTP), 5 μl of 10X PCR buffer, 10 μl of the primer mix (10 pmol/μl each primer), 0.4 μl of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, U.S.A.), and 28.6 μl of double distilled water (DDW). The amplification was performed in a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems), preceded by an initial denaturation at 95°C for 10 min, and programmed for 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. The primer pair used to amplify transcripts from the endogenous TgMIC2 gene was targeted to the 5'UTR (5'-CTC GCG ACA TTT GCT GCA GTT GTT TTC ACA-3') and the 3'UTR (5'-CAG CGC AAT CTG CTC AAA GCG

TCA GTG CTC-3') of the endogenous TgMIC2 mRNA. The transcripts from exogenous TgMIC2 gene were selectively amplified by using a sense primer to the TgMIC2 gene (5'-CCT TCG GAG GGA ACG ACT CCT GGT G-3') and an antisense primer corresponding to the 3'UTR of SAG1 (5'-CTC AAG AAG GAC CAT GTG GTC TCT C-3'), which is located downstream of TgMIC2 gene in the p5RT70Tet4-MIC2-CAT expression vector. The transcripts of TgM2AP gene were specifically amplified by using a sense primer (5'-ATG AAA CTC GCT GCC GTG TCC AGT G-3') and an antisense primer (5'-TTA CGC CTC ATC GTC ACT CGG CAG A-3'). Alpha-TUB transcript was used as an internal control of the RT-PCR analysis (sense primer: 5'-ATG AGA GAG GTT ATC AGC ATC CAC GTC GGC-3', antisense primer: 5'-TTA GTA CTC GTC ACC ATA GCC CTC CTC TTC-3').

Effect of TgMIC2 over-expression on *in vitro* propagation of the parasite

Vero cell monolayers were infected with 2 x 10^6 freshly released RH-WT or TgMIC2 transgenic parasites, and were kept at 37°C in 5% CO_2 with moist air with or without 1 μg/ml ATc (Meissner *et al*., 2001). Four days post-infection, the number of tachyzoites in the culture was determined by counting the number of freshly released tachyzoites and the tachyzoites in the culture supernatant using light microscopy.

Transmission electron microscopy

Tachyzoites in culture supernatant and freshly released tachyzoites were fixed with cold 2% glutaraldehyde in sodium cacodylate buffer (pH 7.4) overnight at 4°C, post-fixed with 1% OsO_4 in the same buffer for 2 h at 4°C after washing thoroughly with the same buffer, dehydrated in an ethanol series from 50% to 100%, and embedded in EPON812 resin (TAAB Laboratories, Berks, U.K.). Thin sections (approximately 80 nm thick) were cut on a Leica UCT ultramicrotome using a diamond knife, and doubly stained with uranyl acetate and lead citrate before examination with JEOL JEM2000EX electron microscope.

Virulence assay in mice

This experiment was designed as previously described (Meissner *et al*., 2002). Briefly, 250 freshly released tachyzoites (suspended in 0.5 ml of PBS) were injected into 2 groups of 6- to 8-week-old female BALB/c mice (CLEA, Tokyo, Japan) via intraperitoneal (i.p.) route. Before and after injection, one group of mice was received sterile drinking water supplemented with 0.1% sucrose. The other group of mice was received sterile drinking water supplemented with 0.1% sucrose and 0.2 mg ATc/ml. The effect of TgMIC2 over-expression was monitored by comparing the survival rate between two groups of mice. This experiment was conducted in accordance with the Standards relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (Hokkaido, Japan).

Statistical analyses

SPSS software was used for statistical analysis. The Two-tailed Student's *t* test was used for analysis of the number of tachyzoites in the culture (*in vitro* study). The Mann-Whitney *U* test was used for significance determination of a virulence assay. A p value of < 0.05 was considered significant for both tests.

RESULTS

Establishment of double transgenic parasites expressing $TetR^S$ and Tet-inducible TgMIC2

The application of the TetR-based inducible gene expression system for *T. gondii* requires cells expressing $TetR^S$. To establish the transgenic parasite expressing $TetR^S$, freshly released tachyzoites of RH *hxgprt*$^-$ strain *T. gondii* were transfected with pTUB8$TetR^S$-HXGPRT, and selected with MPA plus XT. The expression levels of $TetR^S$ from several independent clones were examined by SDS-PAGE followed by Western blotting (data not shown). The parasite clone expressing the highest level of $TetR^S$ was selected for

testing the activity of TetR^S using p5RT70Tet4LacZ-CAT, a reporter plasmid containing LacZ gene controlled by the Tet-inducible promoter. The Tet-dependent LacZ expression in the parasite clones was evaluated by X-Gal staining (data not shown). No difference in the number and the size of the plaque were observed between LacZ transgenic parasites with and without ATc (data not shown). In our other study using this system to regulate the expression of GFP, the morphology of GFP transgenic parasites with and without ATc was normal and no difference in the number of the parasite between these two groups was observed. This implies that TetR^S is not toxic to the parasite and has no invasion inhibitory to the parasite in the presence of ATc. It has to be noted that the highest level of TetR^S used in our study is lower than that of in the previous study (Meissner *et al*., 2001).

To over-express TgMIC2, the parasite clone exhibited the tightest Tet-regulatable LacZ expression was selected, and transfected with p5RT70Tet4-MIC2-CAT, a plasmid containing TgMIC2 gene controlled by the Tet-inducible promoter. The integrations of the TgMIC2 transgene from three independent clones were confirmed by genomic PCR. Insertion of TgMIC2 from p5RT70Tet4-MIC2-CAT was confirmed using the primers flanking the TetO containing promoter (P1) and TgMIC2 (P2) (Figs. 1A and 1B, lanes 3, 5, 7). A cassette of the selectable marker (TUB-CAT-SAG) from p5RT70Tet4-MIC2-CAT was amplified using the primers flanking TUB 5'-UTR (P3) and SAG1 3'-UTR (P4) (Figs. 1A and 1B, lanes 2, 4, and 6). It is likely that the P3 and P4 primers could also amplify genomic TUB 5'-UTR and SAG1 3'-UTR sequences of the parasite. It is also possible that the amplification could occur between TUB 5'-UTR and SAG1 3'-UTR sequences in the genome and in the integrated plasmid. Therefore, the bands with various sizes were detected and the band patterns among the three clonal lines differed from one another. These differences indicated that p5RT70Tet4-MIC2-CAT integrated into different sites of the parasite chromosome. Together, these results demonstrate that p5RT70Tet4-MIC2-CAT was properly integrated into the parasite genome.

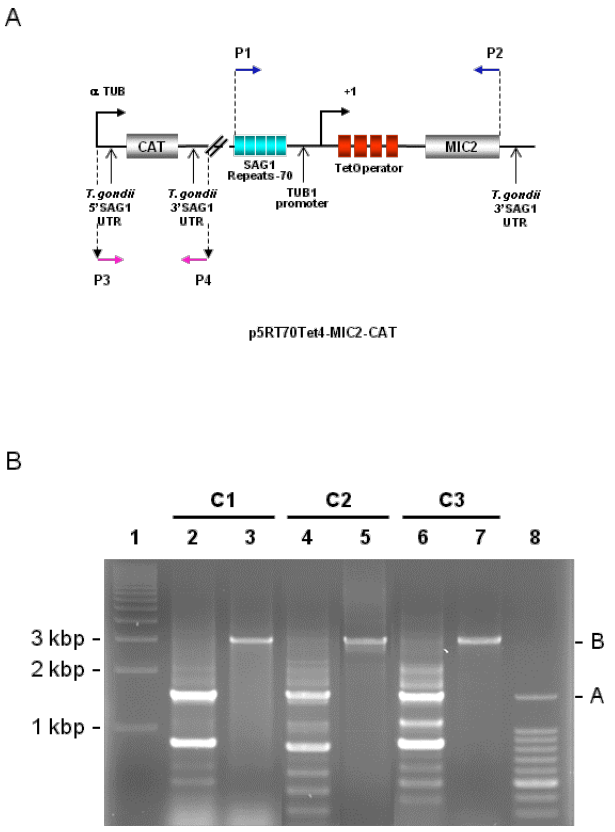

Fig. 1. Establishment of stable parasite cell lines expressing Tet-inducible TgMIC2 (A) Schematic representation of the expression plasmid used to express the TgMIC2 exogenous copy under the control of TetR –responsible promoter. The primers P1 and P2 as well as P3 and P4 were used to confirm the plasmid insertion in the parasite genome. (B) Detection of the integration of the TgMIC2 exogenous copy from three independent clones by genomic PCR. Lanes 3, 5, and 7 were the amplified products using the primers flanking the TetO containing promoter (P1) and TgMIC2 gene (P2). Lanes 2, 4, and 6 were the amplified products using the primers flanking TUB 5'UTR (P3) and SAG1 3'UTR (P4). Several bands were also detected in lane 2, 4, and 6. The P3 and P4 primers could possibly amplify genomic TUB 5'UTR and SAG1 3'UTR sequences of the parasite and the amplification was possibly to be occurred between the sequence in the parasite genome and the integrated plasmid. Lane 1 is a 1-kbp ladder DNA size marker and lane 8 is a 100-bp ladder DNA size marker.

Up-regulation of TgMIC2 protein expression after tetracycline induction

After establishment of the transgenic parasite with the inducible copy of TgMIC2, we at first examined the effect of over-expression of exogenous TgMIC2 gene at protein level. Normally, TgMIC2 mRNA is translated as the full-length TgMIC2 protein named $TgMIC2^{115}$ (Wan *et al*., 1997; Carruthers *et al*., 2000). However, it was reported that $TgMIC2^{115}$ is further processed by two parasite proteases, namely microneme protein protease 1 and 2 (MPP1 and MPP2), during invasion processes (Carruthers *et al*., 2000; Zhou *et al*., 2004). In primary processing (surface trimming), a short N-terminal extension of $TgMIC2^{115}$ is trimmed off by MPP2 to get the product $TgMIC2^{110}$. In the secondary processing (surface shedding), MPP1 cleaves at C-terminus of $TgMIC2^{115}$ and $TgMIC2^{110}$ to get the product $TgMIC2^{100}$ and $TgMIC2^{95}$, respectively. Together, four different forms of the TgMIC2 are produced after the proteolytic processing, namely $TgMIC2^{115}$ (the full-length TgMIC2), $TgMIC2^{110}$ (the N-terminal processing product of $TgMIC2^{115}$), $TgMIC2^{100}$ (the C-terminal processing product of $TgMIC2^{115}$), and $TgMIC2^{95}$ (the C-terminal processing product of $TgMIC2^{110}$). $TgMIC2^{115}$ exists as both microneme store and cell surface proteins and $TgMIC2^{110}$ exists as a cell surface protein, whereas $TgMIC2^{100}$ and $TgMIC2^{95}$ are released during an invasion process of the parasite.

In this study, the levels and forms of cellular TgMIC2 on the surface of RH-WT and TgMIC2 transgenic parasites with or without 1μg/ml anhydrotetracycline (ATc), a non-cytotoxic derivative of tetracycline, were determined by SDS-PAGE followed by Western blotting. As expected, RH-WT expressed only $TgMIC2^{115}$ regardless of the absence or presence of ATc (Fig. 2A, lanes 1 and 2). However, TgMIC2 migrating slightly faster than $TgMIC2^{115}$ was detected from the TgMIC2 transgenic parasite (Fig. 2A, lanes 3 and 4). This migration is reminiscent of the N-terminal processing of $TgMIC2^{115}$ that removes approximately 45 amino acids from the N-terminal (Carruthers *et al*., 2000). The sharp appearance and slightly faster mobility of the $TgMIC2^{110}$ band clearly distinguished it from $TgMIC2^{115}$ and the released forms, $TgMIC2^{100}$ and $TgMIC2^{95}$ (Fig. 2B, lane 3). It appeared that the transgenic parasite with ATc expressed higher amount of $TgMIC2^{115}$ and $TgMIC2^{110}$ than that of the transgenic parasite without ATc. The apparent greater intensity of both $TgMIC2^{115}$ and $TgMIC2^{110}$ in the transgenic parasite without ATc, compared with RH-WT without ATc, suggests a leaky phenotype of an inducible copy of TgMIC2. Thereafter, the levels of the released $TgMIC2^{100}$ and $TgMIC2^{95}$ in the culture supernatant were examined (Fig. 2C). Although the two released forms of TgMIC2 were not clearly separated, the levels of $TgMIC2^{95\text{-}100}$ of the transgenic parasite were clearly higher than those of RH-WT. The levels of the surface and the released forms of TgMIC2 were also examined in the other two independent clones and similar results were obtained (data not shown). The levels of cellular $TgMIC2^{115}$ and $TgMIC2^{110}$ protein expression were also confirmed by confocal laser scanning microscopy (CLSM). The visible difference of the fluorescence intensity was clearly distinct between the transgenic parasite with ATc and without ATC (Fig. 3). Together, both cellular ($TgMIC2^{115}$ and $TgMIC2^{110}$) and released ($TgMIC2^{100}$ and $TgMIC2^{95}$) forms of TgMIC2 were over-expressed in the *T. gondii* by tetracycline inducible expression system, although TgMIC2 expression was not tightly regulated.

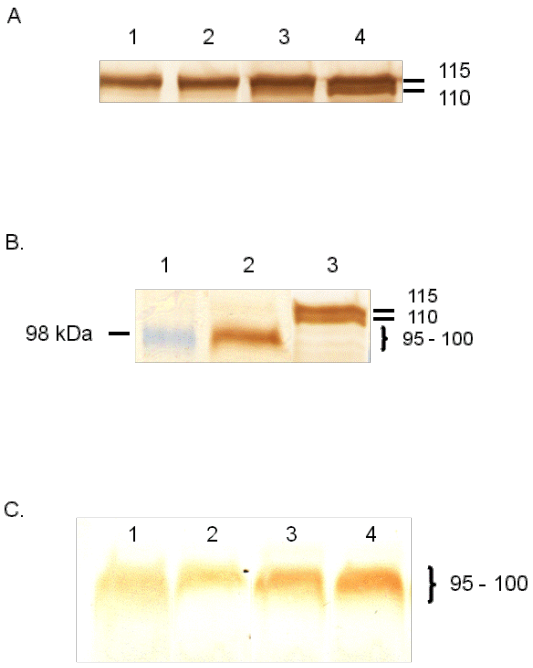

Fig. 2. Over-expression of TgMIC2 enhanced the N-terminal proteolytic processing of TgMIC2. (A) Tachyzoite lysate ($1x10^7$ cells) were examined by Western blotting using the mouse polyclonal anti-TgMIC2 antibody. The tachyzoites were cultured without (lanes 1 and 3) or with (lanes 2 and 4) ATc. $TgMIC2^{115}$ (115) was the major TgMIC2 species detected from RH-WT (lanes 1 and 2), while both $TgMIC2^{115}$ and $TgMIC2^{110}$ (110) were detected from TgMIC2 transgenic parasites. (B) Sizes of the cellular TgMIC2, $TgMIC2^{115}$ and $TgMIC2^{110}$, from tachyzoite lysate were compared with those of the released forms, $TgMIC2^{100}$ and $TgMIC2^{95}$, from the culture supernatant. (C) The released forms of TgMIC2, $TgMIC2^{100}$ and $TgMIC2^{95}$, were detected from the culture supernatant by Western blotting using the mouse polyclonal anti-TgMIC2 antibody. Separation of the two TgMIC2 species did not clear and designated as 95-100. Accumulation of the 95-100 in TgMIC2 transgenic parasites with ATc (lane 4) was observed compared to those of in the RH-WT (lanes 1 and 2) and TgMIC2 transgenic parasites without ATc (lane 3). Representative data from 3 independent experiments are shown.

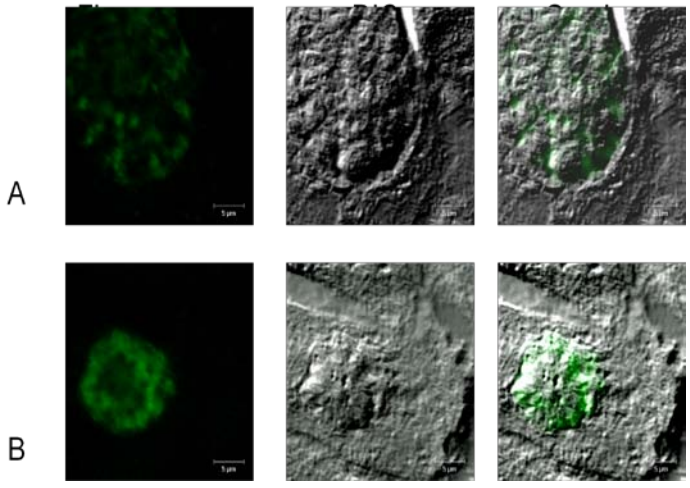

Fig. 3. Detection of the cellular TgMIC2 by indirect fluorescent antibody technique. The TgMIC2 transgenic parasites were grown without (row A) or with (row B) 1 μg/ml ATc. Four days after infection, the infected cells were fixed, and stained with the mouse polyclonal anti-TgMIC2 antibody. The fluorescence, differential interference contrast (DIC), and overlay images are obtained using confocal laser scanning microscopy. Since all images were photographed with the same detector sensitivity, fluorescence intensity of each images are comparable, and represent expression level of TgMIC2.

Modulation of endogenous TgMIC2 level by TgMIC2 over-expression

The levels of TgMIC2 mRNA in RH-WT and the TgMIC2 transgenic parasite with or without 1 μg/ml ATc were examined by RT-PCR. The α-TUB transcript was used as an internal control for equal amount of template DNA in each reaction. Surprisingly, the integration and over-expression of an inducible copy of TgMIC2 caused an up-regulation of endogenous TgMIC2 expression compared with that of RH-WT (Fig. 4, lane 2 and 3). The similar pattern of up-regulation in both endogenous and exogenous TgMIC2 mRNA expression was also observed by Northern blotting (Data not shown). A leaky phenotype of exogenous TgMIC2 mRNA expression was also observed (Fig. 4 lane 2). Therefore, the increased TgMIC2 protein levels in the transgenic parasite without ATc is probably due to both leaky expression and the up-regulated levels of endogenous TgMIC2 mRNA.

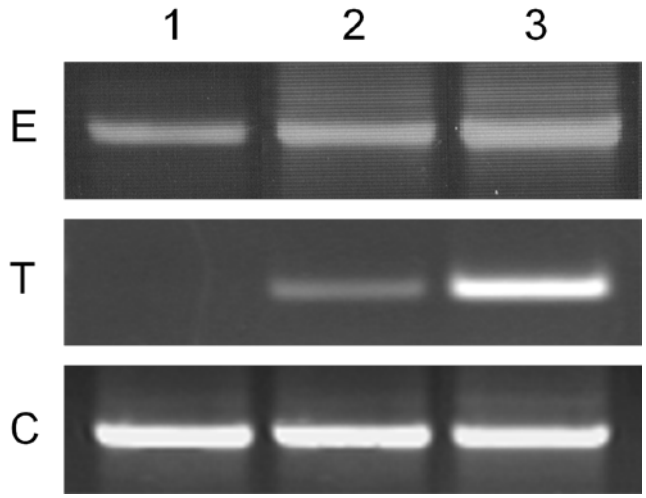

Fig. 4. Tetracycline-inducible expression of TgMIC2 transgene (panel T), and the compensatory modulation of the endogenous TgMIC2 expression (panel E). Total RNA isolated from RH-WT (lane 1) and TgMIC2 transgenic parasites (lanes 2 and 3) grown without (lane 2) or with (lanes 3) 1 μg/ml ATc were reverse transcribed, and examined by RT-PCR. Alpha-tubulin (α-Tub) was used as an internal control (panel C).

Effect of TgMIC2 over-expression on *in vitro* propagation of the parasite

Having demonstrated that the expression of TgMIC2 inducible copy in the transgenic parasite could be modulated by ATc treatment. We next determined whether induction of TgMIC2 over-expression had an effect on the parasite propagation inside the host cells. Vero cells were infected with RH-WT or the TgMIC2 transgenic parasite and were then cultured with or without 1 μg/ml ATc. The number of tachyzoites in the culture was determined at 4 days post-infection. At the concentration used, ATc had no effect on the viability of Vero cells and parasites as determined in the previous study (Meissner *et al*., 2001). There was no effect of ATc on the number of RH-WT parasites (Fig. 5). In contrast, the number of TgMIC2 transgenic parasites with ATc was significantly reduced compared with that of the transgenic parasite without ATc ($p < 0.002$). Similar results were obtained (data not shown) from the other two independent clones. These data demonstrate that induction of TgMIC2 over-expression significantly reduce tachyzoite's propagation inside the host cell.

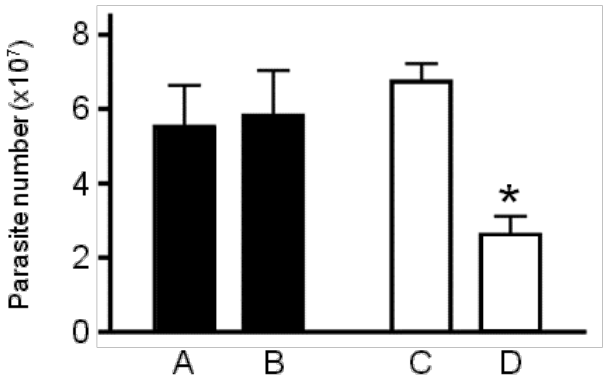

Fig. 5. Effect of TgMIC2 over-expression on *in vitro* propagation of the parasite. Vero cell monolayers were infected with 2 x 10^6 freshly released RH-WT tachyzoites (A and B) or with TgMIC2 transgenic tachyzoites (C and D), and were cultured without (A and C) or with (B and D) ATc for 4 days. Upon induction of TgMIC2 transgene, there was the significant reduction of the tachyzoite number (∗$P < 0.002$, two-tailed Student's *t*-test). Data are mean ± SE of 4 independent experiments.

Induction of the spherical cell phenotype by TgMIC2 over-expression

As we had observed the accumulation as well as less invasion of tachyzoites in the culture supernatant after TgMIC2 over-expression, we next examined the ultrastructure of the tachyzoites of RH-WT and TgMIC2 transgenic parasites with or without ATc using transmission electron microscopy. Compared with RH-WT (Figs. 6A and 6C), some of tachyzoites had an altered morphology (spherical shape) and fewer numbers of dense granules and micronemes at the apical end after the induction of TgMIC2 over-expression (Figs. 6B and 6D). A large number of dead parasites were also observed in the transgenic parasite with ATc (Fig. 6B). In transgenic parasites without ATc, abnormal cells could also be seen but the number was much fewer than those of transgenic parasites with ATc. This appears to be resulted from the leaky phenotype of TgMIC2 transgene expression

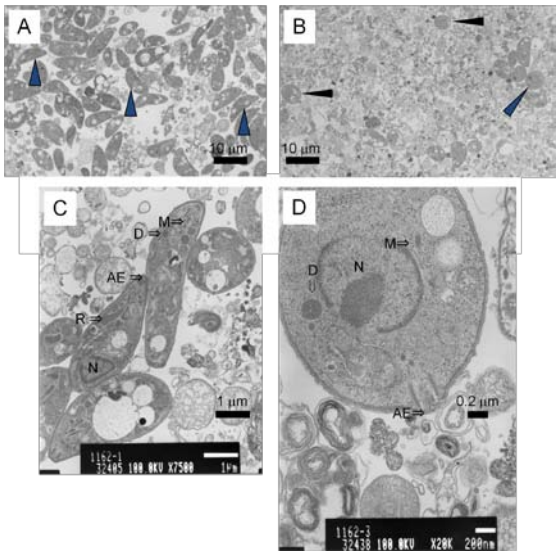

Fig. 6. Electron micrograph of RH-WT (A and C) and TgMIC2 over-expressed parasites (B and D). The RH-WT and TgMIC2 transgenic parasites were cultured with 1 µg/ml ATc for 4 days. Arrowheads in the panel A indicate typical crescent shape tachyzoites. Arrowheads in the panel B indicate spherical shape tachyzoite found after the TgMIC2 over-expression. Magnifications; Panels A and B: x800, Panel C: x7,500, Panel D: x20,000. AE: Apical end, D: Dense granule, M: Microneme, N: Nucleus, R: Rhoptory.

Effect of TgMIC2 over-expression on the expression of TgMIC2-associated protein (TgM2AP)

It has previously been established that TgMIC2 is physically associated with an accessory protein known as TgMIC2-associated protein (TgM2AP) within 15 min after synthesis, and remains associated with TgM2AP in the micronemes as well as on the parasite surface during invasion (Rabenau *et al.*, 2001; Jewett and Sibley, 2004). Since the TgMIC2/TgM2AP complex is a key participant in host cell attachment and invasion, we examined whether TgMIC2 over-expression altered the expression of TgM2AP using SDS-PAGE followed by Western blotting. The endogenous TgM2AP in RH-WT and transgenic parasites was detected by polyclonal anti-TgM2AP antiserum. TgM2AP is initially synthesized as a 43-kDa propeptide (pTgM2AP), which is normally processed at the N-terminus during transit through the golgi, resulting in a 40-kDa mature polypeptide (TgM2AP) (Rabenau *et al.*, 2001). In RH-WT, TgM2AP was processed and expressed normally (Fig. 7). While, weak signals of both pTgM2AP and TgM2AP were detected in transgenic parasites without ATc, a new cross-reactive molecule migrating at 64 kDa was observed. In transgenic parasites with ATc, only the 64-kDa species was detected as a more prominent signal. To assess the mRNA expression level of the endogenous TgM2AP, an RT-PCR analysis was carried out. The α-TUB transcript was used as an internal reference for comparison. The level of transcripts corresponding to endogenous TgM2AP remained constant among the sample tested (Fig. 8). These results indicate that over-expression of TgMIC2 appears to influence TgM2AP expression at the post-transcriptional or post-translational level.

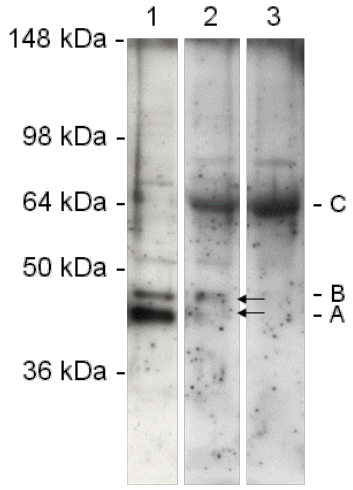

Fig. 7. Analysis of TgM2AP expression by Western blotting using mouse polyclonal anti-TgM2AP antiserum. Cell lysates were prepared from tachyzoites cultured without (lanes 1 and 2) or with (lane 3) 1 μg/ml ATc. Cell lysates of RH-WT (lane 1) and TgMIC2 transgenic parasite (lanes 2 and 3) were subjected for Western blotting. A, B and C indicate the position of mature TgM2AP, TgM2AP propeptide, and newly appeared 64 kDa cross-reactive protein, respectively.

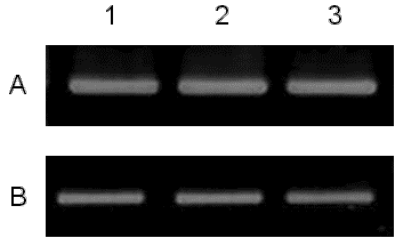

Fig. 8. Detection of TgM2AP by RT-PCR. Total RNA isolated from RH-WT (lane 1) and TgMIC2 transgenic parasites grown without (lane 2) or with (lane 3) 1 μg/ml ATc were reverse transcribed, and amplified using specific sets of primers. The alpha-tubulin (α-TUB) transcript was included as an internal control (panel C).

Decreased virulence *in vivo* by TgMIC2 over-expression

Having revealed in an *in vitro* experiment that the ability to propagate inside the host cell of the TgMIC2 transgenic parasite with ATc was significantly impaired, we next determined the effect of TgMIC2 over-expression on the virulence of the parasite *in vivo* by determining the acute infection in a mouse model. A typical virulent *T. gondii* strain has a lethal dose (LD_{100}) ranging between 1-10 parasites, and death can be monitored around 7-10 days after infection, regardless of the mouse strain used, while an typical avirulent strain has a LD_{100} of 10^3-10^5 parasites with death occurring within 10-20 days (Sibley and Boothroyd, 1992). Thus, a reduction in virulence can be observed via either longer survival period or spontaneous recovery. RH type I strain, a typical virulent strain, was the genetic background of the parasite in this study. This strain usually kills mice with the LD of a single infectious parasite (Pfefferkorn and Pfefferkorn, 1976). In this study, two groups of mice were infected with 250 TgMIC2 transgenic parasites. One group of mice was received sterile drinking water and the other received sterile drinking water with ATc. At day 8 after infection, all of the infected mice received only sterile drinking water had died, while all of the mice received sterile drinking water with ATc died at day 10 (Fig. 9). A delay to death of the mice received sterile drinking water with ATc was significant (p = 0.002). The same experiment was also done in RH-WT and all mice died at day 8 regardless of supplementation of ATc in sterile drinking water (Data not shown). These results indicate that over-expression of TgMIC2 transgene reduce virulence of the parasite in the mouse model.

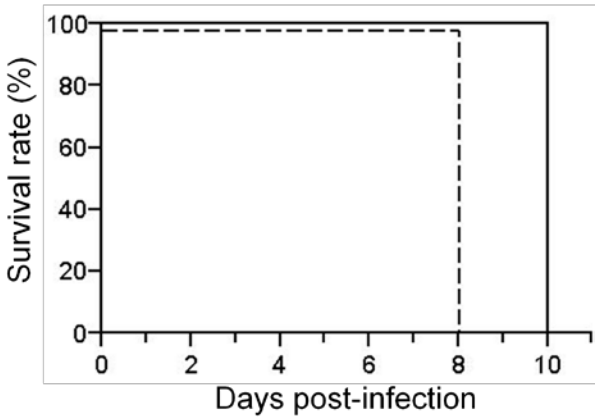

Fig. 9. Over-expression of TgMIC2 attenuates virulence of *T. gondii*. BALB/c mice were infected with 250 tachyzoites of TgMIC2 transgenic parasites. One group of mice (solid line) was given drinking water with ATc (0.2 mg/ml) for 3 days before infection until the end of the experiment. Another group of mice (dashed line) was given drinking water without ATc for the same period described above. TgMIC2 over expression significantly reduced virulence of the parasite in mice (P = 0.002, Mann-Whitney U test). Representative data from 3 independent experiments are shown.

DISCUSSION

T. gondii has devised a unique strategy to actively invade into a host cell. Active penetration is highly polarized and is initiated exclusively via the parasite apical end by secreting adhesins from micronemes (Carruthers and Sibley, 1997; Carruthers, 2002). Among the microneme adhesive proteins, TgMIC2 is potentially vital in successfully rapid host cell invasion. The presence of adhesive domains on TgMIC2 strongly indicates that this adhesin participates in host cell receptor recognition and binding (Wan *et al*., 1997; Carruthers *et al*., 1999; Huynh *et al*., 2003; Huynh *et al*., 2004). To further dissecting out the function and the regulation of gene expression of TgMIC2, the Tet-inducible gene expression system was utilized in

this current study. Our first attempt was to study both the effect of TgMIC2 over-expression and the importance of A/I domain on host cell attachment and invasion. Therefore, two Tet-inducible plasmids had been constructed. One plasmid contained the full-length TgMIC2 gene, and the other contained the TgMIC2 lacking of the A/I-domain (Δ A/I). Although parasite clones expressing the inducible copy of TgMIC2 were successfully established, we failed to isolate the parasite expressing Δ A/I (data not shown). Consistently, previous study also showed that recombinant parasites stably expressing the TgMIC2 with mutated transmembrane region could not be established (Opitz and Soldati, 2002). The lack of the A/I-domain or the proteolytic processing of the C-terminus region of TgMIC2 may possibly compromise parasite invasion, and following intracellular propagation. These findings support the notion that TgMIC2 is an essential gene.

Previous study reported that the major cell surface form of TgMIC2 in RH-WT was $TgMIC2^{115}$. The $TgMIC2^{110}$ (the N-terminal processing product of $MIC2^{115}$) was a major form found in the parasite which was arrested at the stage of apical attachment. It was proposed that the MPP2 protease might be restricted to the apical surface of the parasite. Arrest the parasite at the apical end would effectively trap TgMIC2 with MPP2, thereby increasing the likelihood of the surface trimming by the N-terminal processing (Carruthers *et al*., 2000). In this study, $TgMIC2^{115}$ was detected in RH-WT as a major cell surface form. A significant increase of $TgMIC2^{110}$ was observed in the transgenic parasite with ATc. This result indicates that TgMIC2 over expression enhances the surface trimming by the MPP2-mediated N-terminal proteolytic processing of $TgMIC2^{115}$. Two released forms of TgMIC2 ($TgMIC2^{100}$ and $TgMIC2^{95}$), the products of the surface shedding by MPP1, were also detected in the culture supernatant of RH-WT and the transgenic parasite. Although the separation of $TgMIC2^{100}$ and $TgMIC2^{95}$ was not clear in the Western blotting, the amount of the two forms of TgMIC2 was proportional to the amount of the $TgMIC2^{115}$ and $TgMIC2^{110}$ in the transgenic parasite. Since $TgMIC2^{115}$ and $TgMIC2^{110}$ are further proteolytically processed and released from the parasite during host cell invasion, accumulation of the two released forms of TgMIC2 in the culture supernatant is supposed to occur as a result of host cell invasion (Carruthers *et al*., 1999). Other studies using TgM2APKO (Huynh *et al*., 2003) and TgM2APΔpro (no propeptide) parasites (Harper *et al*., 2006), the abnormal of $TgMIC2^{115}$ proteolytic processing was also observed but in these two studies, it resulted from abnormal MPP1-mediated C-terminal proteolytic processing. Interestingly in our current study, TgMIC2 over-expression enhanced the expression level of endogenous TgMIC2 mRNA. This phenomenon might be due to the result of a compensation mechanism against TgMIC2 over-expression and following abnormal release of the surface TgMIC2.

The effect of TgMIC2 over-expression on host cell infection was determined *in vitro*. Compared with the propagation of RH-WT with or without ATc, over-expression of TgMIC2 significantly reduced *in vitro* propagation of the parasite. As *T. gondii* is an obligate intracellular parasite, this result indicates that the parasite ability in host cell invasion and/or intracellular propagation is hampered by TgMIC2 over expression. The same results were obtained from other two independent clones and each clone had integrated into different sites of the parasite genome. This implies that the effect on the parasite propagation is unlikely due to the effect from the other gene which can be read through when TgMIC2 is over-expressed. There is a contradiction between the fewer parasite number and higher amount of the released forms of TgMIC2 after TgMIC2 over-expression. For this contradiction, it is plausible that the proteolytic processes of $TgMIC2^{115}$ and $TgMIC2^{110}$ are abnormally activated in the TgMIC2 transgenic parasite with ATc regardless of the host cell invasion.

As we had observed the reduced invasion and increasing accumulation of tachyzoites in the culture

supernatant after TgMIC2 over-expression, the morphology of TgMIC2 transgenic parasites was compared with that of RH-WT by transmission electron microscopy. A large number of dead transgenic parasites with ATc were observed as compared with those of RH-WT. In addition, the morphology of some of transgenic parasites with ATc was not a typical crescent form but rather spherical shape. Apical complex of the spherical tachyzoites was totally disordered, because there were less dense granules, rhoptries and micronemes. The abnormal feature of the parasite appeared to reduce its ability to invade a host cell. Therefore, although the cell membrane and nucleus appear to be normal, we conclude that this spherical shape of the parasite is a course of cell death. Plausibly, the spherical shape of the parasite results in the slow invasion which might effectively trap TgMIC2 with MPP2 leading to the enhancement of MPP2-mediated processing.

Extensive studies demonstrated that TgMIC2 activity is most likely tightly linked to its association with TgM2AP. Upon induction of TgMIC2 over- expression, there was an apparent change of TgM2AP property at the protein level while the expression level of TgM2AP transcripts remained constant. This indicates that the effect of TgMIC2 over-expression on TgM2AP expression is most likely occurred at post-translational level. It is conceivable that a 64-kDa species detected in the transgenic parasite with or without ATc is an altered TgM2AP protein since it can react with the anti-TgM2AP antibody. In addition, while both pTgM2AP and TgM2AP are not detected in the transgenic parasite with ATc, the band intensity of the 64-kDa protein is increased in this transgenic parasite. Previously, the non-specific reaction at 62 kDa was observed in the Western blotting of TgM2AP (Rabenau *et al*., 2001). However, we did not observe such a non-specific band in our control sample. If the aberrant mobility of TgM2AP was modify by sizeable post-translational addition, such as N-linked or O-linked oligosaccharides, six N-glycosylation and many O-glycosylation sites would approximately require to increase the size to 64 kDa, respectively. However, only two N-glycosylation and thirteen O-glycosylation sites are predicted in TgM2AP. Although the basis of this alteration remains unknown and needs further investigation, it is likely that the TgM2AP alteration is one factor that diminishes the parasite propagation.

The *in vitro* result prompted us to investigate the effect of TgMIC2 over- expression on the virulence of the parasite in mice. Consistent with the *in vitro* result, whereas mice infected with the transgenic parasite without ATc demonstrated normal time-till-death kinetic as previously reported (Seng *et al*., 1999), mice infected with the transgenic parasite with ATc had a significant delay to death of ≈ 2 days. Since mice were infected with the number of the parasite which was normally used as previously described (Seng *et al*., 1999) and the transgenic parasite without ATc exhibit the normal time-till-death kinetic, this result implies that $TetR^S$ is not toxic to the parasite. A delay to death of mice likely reflects a slower course of infection due to the diminished ability of the transgenic parasite with ATc to propagate inside the host cell.

Contrary to our current study, the study by Huynh and Carruthers demonstrated that over expression of TgMIC2 resulted in significant increase of parasite invasion (Huynh and Carruthers, 2006). This study utilized a Tet-inducible transactivator system with a transcriptional transactivator (tTA) protein to regulate expression of genes containing TetO cassettes. Genes are constitutively transcribed till the addition of Tet dissociates the tTA from the operator cassettes, blocking further transcription. In this study, TgMIC2 protein was over-expressed and over-secreted in the parasite containing both endogenous and inducible copy of TgMIC2 gene (*mic*2e/*mic*2i). TgMIC2 over-expression resulted in significant increase of *mic*2e/*mic*2i parasite invasion. In contrast to our results which TgMIC2 over-expression caused the enhancement of TgMIC2 proteolytic processing and the aberrant TgM2AP expression, the TgMIC2 proteolytic processing and TgM2AP expression were likely to be normal in Huynh and Carruthers' study. It can be implied from the results of both studies that

the regulatory network between TgMIC2 and TgM2AP is closely linked and the altering expression of one protein effect the expression of the other interacting protein. The discrepancy between our current study and Huynh and Carruthers' study is possibly due to the use of the different plasmid which can result in the integration into different sites in the parasite genome; the number of the exogenous TgMIC2 copy in the transgenic parasite; and the level of TgMIC2 over-expression. These differences possibly influence distinct aspects on TgMIC2 itself and TgM2AP expression and also parasite's propagation. Moreover, we cannot rule out the possibility that the TgMIC2 over-expression may influence in the expression of the other gene with in turn has an effect on the TgMIC2 and TgM2AP expression.

Together, our current study brought up new implications on the importance of the regulation of TgMIC2 transgene expression on post-translational modification of TgMIC2 and TgM2AP as well as parasite propagation and morphology, with all together influencing the outcome of infection.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Soldati D., Imperial College of Science, Technology, and Medicine, London, United Kingdom, for providing pTUB8TetRS-HXGPRT, p5RT70Tet4LacZ-CAT and p5RT70Tet4-GFP plasmids. This work was made possible by a Grant-in-Aid for Scientific Research to N.I. and C.S., and in part, by a Grant-in-Aid for 21st Century COE Program from the Japan Society for the Promotion of Science.

REFERENCES

Brossier, F., Jewett, T.J., Lovett, J.L., Sibley, L.D., 2003. C-terminal processing of the *Toxoplasma* protein MIC2 is essential for invasion into host cells. J. Biol. Chem. 278: 6229-6234.

Brossier, F., Sibley, L.D., 2005. *Toxoplasma gondii*: microneme protein MIC2. Int. J. Biochem. Cell Biol. 37: 2266-2272.

Carruthers, V.B., 2006. Proteolysis and *Toxoplasma invasion*. Int. J. Parasitol. 36: 595-600.

Carruthers, V.B., Sibley, L.D., 1997. Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. Eur. J. Cell Biol. 73: 114-123.

Carruthers, V.B., Giddings, O.K., Sibley, L.D., 1999. Secretion of micronemal proteins is associated with toxoplasma invasion of host cells. Cell Microbiol. 1: 225-235.

Carruthers, V.B., Sibley, L.D., 1999. Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. Mol. Microbiol. 31: 421-428.

Carruthers, V.B., Sherman, G.D., Sibley, L.D., 2000. The *Toxoplasma* adhesive protein MIC2 is proteolytically processed at multiple sites by two parasite-derived proteases. J. Biol. Chem. 275: 14346-14353.

Carruthers, V.B., 2002. Host cell invasion by the opportunistic pathogen *Toxoplasma gondii*. Acta. Trop. 81: 111-122.

Dautu, G., Munyaka, B., Carmen, G., Zhang, G., Omata, Y., Xuenan, X., Igarashi, M., 2007. *Toxoplasma gondii*: DNA vaccination with genes encoding antigens MIC2, M2AP, AMA1 and BAG1 and evaluation of their immunogenic potential. Exp. Parasitol. 116: 273-282.

Dobrowolski, J.M., Sibley, L.D., 1996. *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasite. Cell. 84: 933-939.

Dobrowolski, J.M., Carruthers, V.B., Sibley, L.D., 1997. Participation of myosin in gliding motility and host

cell invasion by *Toxoplasma gondii*. Mol. Microbiol. 26: 163-173.

Donald, R.G., Carter, D., Ullman, B., Roos, D.S., 1996. Insertional tagging, cloning, and expression of the *Toxoplasma gondii* hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. Use as a selectable marker for stable transformation. J. Biol. Chem. 271: 14010-14019.

Hamann, L., Buss, H., Tannich, E., 1997. Tetracycline-controlled gene expression in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 84: 83-91.

Harper, J.M., Huynh, M.H., Coppens, I., Parussini, F., Moreno, S., Carruthers, V.B., 2006. A cleavable propeptide influences *Toxoplasma* infection by facilitating the trafficking and secretion of the TgMIC2-M2AP invasion complex. Mol. Biol. Cell. 17: 4551-4563.

Hamann, L., Buss, H., Tannich, E., 1997. Tetracycline-controlled gene expression in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 84: 83-91.

Hettmann, C., Herm, A., Geiter, A., Frank, B., Schwarz, E., Soldati, T., Soldati, D., 2000. A dibasic motif in the tail of a class XIV apicomplexan myosin is an essential determinant of plasma membrane localization. Mol. Biol. Cell. 11: 1385-1400.

Huynh, M.H., Carruthers, V.B., 2006. *Toxoplasma* MIC2 is a major determinant of invasion and virulence. PLoS. Pathog. 2: e84.

Huynh, M.H., Rabenau, K.E., Harper, J.M., Beatty, W.L., Sibley, L.D., Carruthers, V.B., 2003. Rapid invasion of host cells by *Toxoplasma* requires secretion of the MIC2-M2AP adhesive protein complex. Embo. J. 22: 2082-2090.

Huynh, M.H., Opitz, C., Kwok, L.Y., Tomley, F.M., Carruthers, V.B., Soldati, D., 2004. Trans-genera reconstitution and complementation of an adhesion complex in *Toxoplasma gondii*. Cell. Microbiol. 6: 771-782.

Ismael, A.B., Sekkai, D., Collin, C. Bout, D., Mevelec, M.N., 2003. The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. Infection and Immunity. 71: 6222-6228.

Jewett, T.J., Sibley, L.D., 2003. Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. Mol. Cell. 11: 885-894.

Jewett, T.J., Sibley, L.D., 2004. The *Toxoplasma* proteins MIC2 and M2AP form a hexameric complex necessary for intracellular survival. J. Biol. Chem. 279: 9362-9369.

Kim, K., Soldati, D., Boothroyd, J.C., 1993. Gene replacement in *Toxoplasma gondii* with chloramphenicol acetyltransferase as selectable marker. Science. 262: 911-914.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Meissner, M., Brecht, S., Bujard, H., Soldati, D., 2001. Modulation of myosin A expression by a newly established tetracycline repressor-based inducible system in *Toxoplasma gondii*. Nucleic. Acids. Res. 29: E115.

Meissner, M., Schluter, D., Soldati, D., 2002. Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. Science. 298: 837-840.

Meissner, M., Soldati, D., 2005. The transcription machinery and the molecular toolbox to control gene expression in *Toxoplasma gondii* and other protozoan parasites. Microbes. Infect. 7: 1376-1384.

Opitz, C., Soldati, D., 2002. 'The glideosome': a dynamic complex powering gliding motion and host cell invasion by *Toxoplasma gondii*. Mol. Microbiol. 45: 597-604.

Ortiz, D., Johnson, P.J., 2003. Tetracycline-inducible gene expression in *Trichomonas vaginalis*. Mol.

Biochem. Parasitol. 128: 43-49.

Pfefferkorn, E.R., Pfefferkorn, L.C., 1976. *Toxoplasma gondii*: isolation and preliminary characterization of temperature-sensitive mutants. Exp. Parasitol. 39: 365-376.

Rabenau, K.E., Sohrabi, A., Tripathy, A., Reitter, C., Ajioka, J.W., Tomley, F.M., Carruthers, V.B., 2001. TgM2AP participates in *Toxoplasma gondii* invasion of host cells and is tightly associated with the adhesive protein TgMIC2. Mol. Microbiol. 41: 537-547.

Ramakrishnan, G., Vines, R.R., Mann, B.J., Petri, W.A., Jr., 1997. A tetracycline-inducible gene expression system in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 84: 93-100.

Roos, D.S., Donald, R.G., Morrissette, N.S., Moulton, A.L., 1994. Molecular tools for genetic dissection of the protozoan parasite *Toxoplasma gondii*. Methods. Cell. Biol. 45: 27-63.

Seng, S., Nagasawa, H., Maki, Y., Yokoyama, M., Inoue, N., Xuan, X, Igarashi, I., Saito, A., Fujisaki, K., Mikami, T., Suzuki, N., Toyoda, Y., 1999. Increased susceptibility to *Toxoplasma gondii* infection in SAG-1 transgenic mice. Int. J. Parasitol. 29: 1433-1436.

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *Toxoplasma gondii* comprise a single clonal lineage. Nature. 359: 82-85.

Sun, C.H., Tai, J.H., 2000. Development of a tetracycline controlled gene expression system in the parasitic protozoan *Giardia lamblia*. Mol. Biochem. Parasitol. 105: 51-60.

Taylor, M.C., Kelly, J.M., 2006. pTcINDEX: a stable tetracycline-regulated expression vector for *Trypanosoma cruzi*. BMC. Biotechnol. 6: 32

van Poppel, N.F., Welagen, J., Duisters, R.F., Vermeulen, A.N., Schaap, D., 2006. Tight control of transcription in T*oxoplasma gondii* using an alternative tet repressor. Int. J. Parasitol. 36: 443-452.

Wan, K.L., Carruthers, V.B., Sibley, L.D., Ajioka, J.W., 1997. Molecular characterisation of an expressed sequence tag locus of *Toxoplasma gondii* encoding the micronemal protein MIC2. Mol. Biochem. Parasitol. 84: 203-214.

Wirtz, E., Leal, S., Ochatt, C., Cross, G.A., 1999. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 99: 89-101.

Yan, S., Myler, P.J., Stuart, K., 2001. Tetracycline regulated gene expression in *Leishmania donovani*. Mol. Biochem. Parasitol. 112: 61-69.

Zhou, X.W., Blackman, M.J., Howell, S.A., Carruthers, V.B., 2004. Proteomic analysis of cleavage events reveals a dynamic two-step mechanism for proteolysis of a key parasite adhesive complex. Mol. Cell. Proteomics. 3: 565-576.