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▣ ORIGINAL ARTICLE

Trichomonas vaginalis Metalloproteinase Induces mTOR Cleavage of SiHa Cells

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Abstract: *Trichomonas vaginalis* secretes a number of proteases which are suspected to be the cause of pathogenesis; however, little is understood how they manipulate host cells. The mammalian target of rapamycin (mTOR) regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. We detected various types of metalloproteinases including GP63 protein from *T. vaginalis* trophozoites, and *T. vaginalis* GP63 metalloproteinase was confirmed by sequencing and western blot. When SiHa cells were stimulated with live *T. vaginalis*, *T. vaginalis* excretory-secretory products (ESP) or *T. vaginalis* lysate, live *T. vaginalis* and *T. vaginalis* ESP induced the mTOR cleavage in both time- and parasite load-dependent manner, but *T. vaginalis* lysate did not. Pretreatment of *T. vaginalis* with a metalloproteinase inhibitor, 1,10-phenanthroline, completely disappeared the mTOR cleavage in SiHa cells. Collectively, *T. vaginalis* metallopeptidase induces host cell mTOR cleavage, which may be related to survival of the parasite.

Key words: *Trichomonas vaginalis*, 1,10-phenanthroline, mTOR cleavage, SiHa cell, metalloproteinase

INTRODUCTION

Trichomonas vaginalis is one of the major agents of non-viral sexually transmitted diseases in humans. Over 180 million people worldwide are annually infected by this parasite [1]. *T. vaginalis* commonly causes vaginitis and perhaps cervicitis in women as well as urethritis in both sexes [2]. Trichomoniasis also has been associated with premature membrane rupture, preterm delivery, low birth weight, preterm delivery, atypical pelvic inflammatory disease, infertility, predisposition to developing invasive cervical cancer, and increased susceptibility to HIV infection [1,3].

T. vaginalis is an extracellular protozoan parasite, thus it needs protease in order to hydrolyze organic components from the mucosa and extracellular matrix of its host cells into glucose, which is then transported into the parasitic cells [4]. *T. vaginalis* produces several kinds of proteases such as cysteine proteases, serine proteases, and metalloproteinases, which are important regulators of pathogenesis, invasion, and survival [4-7]. GP63 metalloproteinases are the major pathogenic agent of *Leishmania* spp., *Trypanosome cruzi*, and *Trypanosome brucei*, and play an important role in host-parasite interactions [8,9]. There are 48 members of the GP63 protease family in *T. vaginalis* [6]; however, the roles of *T. vaginalis* metallpoteinases are not well known until now.

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription [10]. mTOR is constitutively activated in the presence of growth factor and nutrients, and up-regulation of mTOR can be related to activation of AKT anabolism [11,12]. A recent study has shown that the *Leishmania* protease GP63 cleaves the mTOR, and then mTOR cleavage results in the inhibition of mTOR complex 1 (mTORC1) and concomitant activation of 4E-BP1 to promote *Leishmania* proliferation [13], because synthesis of type I IFN (IFN-α and IFN-β) is regulated by mTORC1

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and its downstream targets p70S6K1/2 [14]. *T. vaginalis* has abundant proteases, and phosphatidylinositol 3-kinase (PI3K)/protein kinase-B (AKT)/mTOR/S6K1 signaling is important in regulation of cell proliferation, differentiation, and cellular metabolism [15].

However, little is known about how *T. vaginalis* lives as a parasite and regulates host cell protein synthesis, which is considered to be the ultimate consequence of pathogenesis and effect of *T. vaginalis* infection on host protein synthesis. In the present study, we detected the presence of various types of metalloproteinases in *T. vaginalis* tachyzoites and elucidated the effect of *T. vaginalis* metalloproteinase on host cell mTOR in AKT/mTOR/S6K pathway. Thus, human cervical tumor cells (SiHa cells) were stimulated with live *T. vaginalis*, *T. vaginalis* excretory-secretory products (ESP) or *T. vaginalis* lysate, and then examined the post-translational modifications of mTOR, AKT, and p70S6K using western blot. In addition, we used the metallopeptidase inhibitor, 1,10-phenanthroline (1,10-PT), to confirm the roles of *T. vaginalis* metalloproteinases about the cleavage function of mTOR.

MATERIALS AND METHODS

T. vaginalis culture

The *T. vaginalis* T016 isolate was cultured in a glass, screw-capped tube containing Diamond's trypticase-yeast extract-maltose (TYM) medium (NAPCO, Winchester, Virginia, USA) supplemented with 10% heat-inactivated horse serum (Sigma-Aldrich, St. Louis, Missouri, USA) in 5% CO_2 at 37℃ for 24 hr. Cultured parasites were monitored for motility, and the viability of *T. vaginalis* was determined before each experiment using trypan blue staining (>99%).

Reverse transcription-PCR (RT-PCR) assay

Total RNA was extracted from *T. vaginalis* T016 isolate using Trizol Reagent (Invitrogen, Carlsbad, California, USA), and RNA concentrations were determined spectrophotometrically. RNA (1 μg) was reverse transcribed using the ReverTra Ace RT kit (Toyobo, Osaka, Japan). Following reverse transcription, cDNA was amplified by PCR using Ex Taq (Takara, Tokyo, Japan) under the following conditions: 5 min at 94℃, followed by 30 cycles of 30 sec at 98℃, 30 sec at 57℃, and 1 min at 72℃. The final elongation step was continued for 10 min at 72℃. The primer sequences were used as follows: GP63, MG aminopeptidase P-like metallopeptidase (MP50), M41 FtsH endopeptidase-like metallopeptidase (TVAG_243780.2), oligopeptidase A-like metallopeptidase (TVAG_477180), zinc carboxypeptidase-like metallopeptidase (TVAG_075740), and aspartyl aminopeptidase-like metallopeptidase (TVAG_392410) (Table 1). PCR products were electrophoresed through a 2% agarose gel and visualized with RedSafe (iNtRON Biotechnology, Seoul, Korea).

Construction of GP63 expression vector and bacterial expression

To construct pET32a-GP63 expression plasmids, the coding sequence of the *T. vaginalis* GP63 (GenBank accession no. GU356538.1) were amplified by PCR using Pfu-X DNA Poly-

Table 1. Primers used for detection of various kinds of metalloproteinases from *Trichomonas vaginalis*

Gene		Primer sequence	GenBank accession no.	PCR product size (bp)
GP63	(F)	ACGCTGTCCTTGCAATTCTT	GU356538.1	501
	(R)	TTGCGTTTTCTTTTGTGCAT		
MP50	(F)	TCTCGACTGCGGATTCTTCT	JF263458.1	304
	(R)	TCCGACGTGATGAGTCAAAC		
TVAG_243780.2	(F)	TGCAACAAATAGGGCAGACA	KF269491.1	598
	(R)	AGCCCTCTCAATGTCGTTTG		
TVAG_477180	(F)	TGTCCTCAAACCATGGAACA	XM_001583725.1	800
	(R)	ATTTGTCGGCAACAACATCA		
TVAG_075740	(F)	ATTCCCTGAGTGGGCTTTTT	XM_001583763.1	400
	(R)	TGGATGCGTACGAGCAACTA		
TVAG_392410	(F)	GTCGCATCAGAGCGTATTGA	XM_001327312.1	249
	(R)	CCATGCGTGAGCATTATCTG		
TVAG_077490	(F)	AGCCAACGAAACAGACTTCC	XM_001325199.1	201
	(R)	ATGATGTGATGGTGCTTGGA		
TVAG_371040	(F)	CGCCGAGACAAACAGAGAAT	XM_001300222.1	899
	(R)	GATCAGCACCATGAATCCAA		

merase (Solgent, Daejeon, Korea) from cDNA of *T. vaginalis* T016 with a pair of oligonucleotide primers: GP63 forward, 5´-CGCGGATCCATGATATTGTCTCTACTCCTCGTTG-3´ and reverse, 5´-CCCAAGCTTAATCGATAAATTTGCATCAGGTTCT-3´ (recognition sites for BamHI and HindIII, respectively are underlined). The PCR products were digested with the appropriate restriction enzyme and cloned into the BamHI/HindIII sites of the pET-32a (+) expression vector with N-terminal His-tag. The resulting plasmids were named pET32a-GP63. Recombinant plasmids were confirmed by restriction analysis and PCR sequencing (Solgent, Daejeon, South Korea). His-tagged GP63 protein was induced by 1 mM IPTG at 37˚C for 3 hr in BL21 (DE3) *Escherichia coli*. Finally, GP63 proteins in the supernatant were separated by 10% SDS-PAGE and visualized by Coomassie blue staining or detected by western blotting using the anti-His antibody.

Preparation of *T. vaginalis* lysate

To prepare *T. vaginalis* lysates, *T. vaginalis* were harvested in the logarithmic phase and washed 3 times in PBS (pH 6.2). *T. vaginalis* pellets were resuspended in PBS and lysed using a sonicator, and then centrifuged at 10,000 g for 30 min. The supernatant was collected and used as *T. vaginalis* lysate. The lysate was filtered through a 0.2-μm-pore-size filter and stored at -70˚C until required. The *T. vaginalis* lysate concentration was determined by the Bradford assay using bovine serum albumin (BSA) as the standard and stored at -70˚C until use.

Culture of SiHa cells

Human cervical cancer cell lines, SiHa cells, were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL, Grand Island, New York, USA) and antibiotic-antimycotic (Gibco BRL) in a 5% CO_2 at 37˚C.

Stimulation of SiHa cells with live *T. vaginalis*, *T. vaginalis* ESP, or *T. vaginalis* lysate

SiHa cell monolayers (1×10^6) were washed with PBS (pH 7.4) and live *T. vaginalis* were incubated with SiHa cell monolayers in the medium mixture (DMEM/TYM = 2:1) at a multiplicity of infection (MOI) of 2 for 16 hr. To investigate whether *T. vaginalis* ESP were critical for SiHa cell protein synthesis, we used the 6-well Transwell permeable supports system (Costar, Cambridge, Massachusetts, USA). These inserts, which have a porous (pore diameter 3 μm) membrane, serve as the upper chambers and ordinary tissue culture plate wells serve as the lower chambers. SiHa cells (1×10^6) was added to the lower chamber and medium containing *T. vaginalis* (2×10^6) was added to the upper chambers and the plates were then incubated for 16 hr. Otherwise, SiHa cells were treated with *T. vaginalis* lysate (100 μg/ml) for 16 hr.

To evaluate the effect of mTOR according to the incubation time and parasite load, SiHa cells were incubated with live *T. vaginalis* at MOI 2 for 0.5, 1, 2, 4, 8, 16 hr, or SiHa cells were infected with live *T. vaginalis* at MOI 0.5, 1, and 2 for 2 hr. At the indicated time points, cells were collected and washed in PBS.

Treatment of 1,10-PT-pretreated *T. vaginalis*

To determine whether *T. vaginalis* metalloproteinases are involved in the cleavage host mTOR, *T. vaginalis* (MOI 2) were treated for 30 min with 5 mM 1,10-phenanthroline (1,10-PT; Sigma-Aldrich) and the control group *T. vaginalis* were mock-treated in PBS. Then, *T. vaginalis* were washed with PBS and infected with SiHa cells for 16 hr.

Western blot analysis

Proteins were extracted using the PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea) supplemented with a complete cocktail of protease inhibitors (Roche, Basel, Switzerland) for 15 min on ice. After centrifugation at 14,000 g for 15 min at 4˚C, the supernatant was collected, and equal volumes of protein from each sample were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween-20 (TBST) and 5% skim milk. After being washed once in TBST, membranes were incubated overnight at 4˚C with the primary antibodies diluted in TBST supplemented with 5% BSA. The antibodies used included rabbit polyclonal anti-mTOR, rabbit polyclonal anti-AKT, rabbit polyclonal anti-p70S6K (all from Cell Signaling Technology Inc., Beverly, Massachusetts, USA), and mouse monoclonal anti-α tubulin (Santa Cruz Biotechnology, Santa Cruz, California, USA). Following 3 consecutive washes in TBST, membranes were incubated for 90 min with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) diluted 1:10,000 with incubation buffer, as described above. After extensive washing, bound secondary antibodies were visualized using an enhanced ECL

chemiluminescence detection kit (GE Healthcare, Little Chalfont, UK).

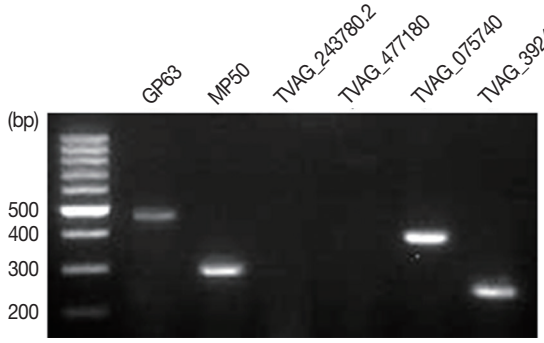

Fig. 1. *T. vaginalis* T016 isolate expressing several metalloproteinases. GP63 (501 bp), MP50 (304 bp), TVAG_243780.2 (598 bp), TVAG_477180 (800 bp), TVAG_075740 (400 bp), and TVAG_392410 (249 bp) metalloproteinase mRNA expression levels were detected by RT-PCR.

RESULTS

Identification of various *T. vaginalis* metalloproteinases

Previous studies demonstrated that metalloproteinase is the largest surface protease family and the second largest surface protein family in *T. vaginalis* [5], and some of these molecules may be involved in the damage to the host [16]. To better define the *T. vaginalis* T016 isolate metalloproteinases, GP63, MP50, TVAG_243780.2, TVAG_477180, TVAG_075740, and TVAG_392410 expression levels were detected by RT-PCR. Interestingly, GP63 (501 bp), MP50 (304 bp), TVAG_075740 (400 bp), and TVAG_392410 (249 bp) mRNA expression were detected. However, we could not detect any TVAG_243780.2 and TVAG_477180 mRNA (Fig. 1).

GP63 sequencing and analysis

We constructed the recombinant plasmid pET32a-GP63, and

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GU356538.1       1 atgatattgtctctactcctcgttgctcagctagatgtgcatacatgcaatcatgatcttgaaatgggtaacataacaagagaacttcaaggacccagttcatatcgcaa
pET32a-GP63      1 atgatattgtctctactcctcgttgctcagctagatgtgcatacatgcaatcatgatcttgaaatgggtaacataacaagagaacttcaaggacccagttcatatcgcaa

GU356538.1     111 tctcaaaagcactgaatggaaaagcatcagaataaaggtggattataactatattgacagaacaaaagatgatgcgcgaacatgtacatgggttggacaaatcgtaacat
pET32a-GP63    111 tctcaaaagcactgaatggaaaagcatcagaataaaggtggattataactatattgacagaacaaaagatgatgcgcgaacatgtacatgggttggacaaatcgtaacat

GU356538.1     221 tccaaggcgtaagatacacttgtaagagggagcatattttgacatattcaaaaattttggctattgaaggaacacttaataatgtcaaatcatatttagagagacttctt
pET32a-GP63    221 tccaaggcgtaagatacacttgtaagagggagcatattttgacatattcaaaaattttggctattgaaggaacacttaataatgtcaaatcatatttagagagacttatt

GU356538.1     331 acagtaattccattcaattataatattaattatgtggacttttgtagccgttatggatacgagacgaattttggcaaacaaagcgcatacggtgccgatatagtacttgc
pET32a-GP63    331 acagtaattccattcaattataatattaattatgtggacttttgtagccgttatggatacgagacgaattttggcaaacaaagcgcatacggtgccgatatagtacttgc

GU356538.1     441 agtactttcaagaccgagacaggataatgatgctactctcgcatcagctgctggattagcagaagagagaacatatcacagaccaatatttggtgtcgttttcttaaatc
pET32a-GP63    441 agtactttcaagaccgagacaggataatgatgctactctcgcatcagctgctggattagcagaagagagaacatatcacagaccaatatttggtgtcgttttcttaaatc

GU356538.1     551 caatggcagttcctgatacagcacaagatgaaaactcaaaggattcaagattcttttatacttgcgttcacgaaattttccatgctttgggtatttcgccatcatatttc
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pET32a-GP63    771 tgcagtgaagcactttggggttcaggaattccaaggagatgacagaaccttgccaatctggattagaaatcgaaaatgatggcggtcaaggaacagcaggaagtcatttag

GU356538.1     881 aagcaagatcttacatgtcagatttgatggttggcatgacaattcaaacaaaatctggaccatttgctagattgactgacgctgtccttgcaattcttttagacacaggt
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GU356538.1     991 aactacaaagttaactggagaatgggacaaccattagtatggggccacccagaatcaattgatggaaaaccaattccaaacttgcaattggtcctccacaaaatgtttt
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GU356538.1    1101 ccctaagtattatatgagagacttctcagttcaagaaacttggtatgcaagttttgatttcaaattctccggaccgttttctggacctgcacgtattagagcaagttata
pET32a-GP63   1101 ccctaagtattatatgagagacttctcagttcaagaaacttagtatgcaagttttgatttcaaattctccggaccgttttctggacctgcacgtattagagcaagttata

GU356538.1    1211 gcgaaaaacgattttatgatccgaaaggcattggagttggtacaacacaagtttatgatttcattccatttttatttcccaaattatgtttgtcctaagggacaggctatc
pET32a-GP63   1211 gcgaaaaacgattttatgatccgaaaggcattggagttggtacaacacaagtttatgatttcattccatttttatttcccaaattatgtttgtcctaagggacaggctatc

GU356538.1    1321 cttccatcaacaatgtcatattattaccacaaatgcggcgaatattcatgcgacggatataaatcgttcacaattcaagtcaacaaagataacattggaagacaatggga
pET32a-GP63   1321 cttccatcaacaatgtcatattattaccacaaatgcgggtacatattcatgcgacggatataaatcgttcacaattcaagtcaacaaagataacattggaagacaatggga

GU356538.1    1431 aagcgtcacatgcacaaaagaaaacgcaactaaggagttttctttgccattaggttactattccaggaaagtttcttgtgttgatcctgagagattctgcagatctgtga
pET32a-GP63   1431 aagcgtcacatacacaaaagaaaacgcaactaaggagttttctttgccattaggttactattccaggaaagtttcttgtgttgatcctgagagattctgcagatctgtga

GU356538.1    1541 aactgaacgagatgaagtttgttgttgacccattcgaccctaactcaaagcaactgagcagtgattctccaacaccaacagcaccagaatcaggaggatatgaaaatcca
pET32a-GP63   1541 aactgaacgagatgaagtttgttgttgacccattcgaccctaactcaaagcaactgagcagtgattctccaacaccaacagcaccagaatcaggaggatatgaaaatcca

GU356538.1    1651 actatatatggaagaaatgacgatcaaccagaatctggaggcgatggaaaagaaagttcaggtggaaattcgaagggaagagaagcaaaacagaatgcgaaaaagattat
pET32a-GP63   1651 actatatatggaagaaatgacgatcaaccagaatctggaggcgatggaaaagaaagttcaggtggaaattcgaagggaagagaagcaaaacagaatgcgaaaaagattat

GU356538.1    1761 aatcatctcaacttgctccgcagccgcagctttaattgtgattgcagtgattgttgctttcgctgtatatttcagctaagaaagcaaaagaaaacgcgcagaacgaagaac
pET32a-GP63   1761 aatcatctcaacttgctccgcagccgcagctttaattgtgattgcagtgattgttgctttcgctgtatatttcagctaagaaagcaaaagaaaacgcgcagaacgaagaac

GU356538.1    1871 ctgatgcaaatttatcgatttag
pET32a-GP63   1871 ctgatgcaaatttatcgatttag
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Fig. 2. The GP63 nucleotide sequences of recombinant plasmid pET32a-GP63 were compared with GenBank sequence (accession no. GU356538.1). Base homologies were indicated with blue, and base changes were shown in white. The total length of *T. vaginalis* T016 isolate GP63 region is 1,893 bp.

GP63 fragment was sequenced along both strands and compared to GenBank sequences (accession no. GU356538.1). As shown in Fig. 2, when the complete GP63 region nucleotide sequences were compared with the GU356538.1 sequences, they were 99.6% base pare identities. In the 1,893 bp of open reading frame, there were only 7 nucleotide differences.

Expression of recombinant GP63 protein

To identify the expression level of GP63 protein, IPTG-induced recombinant His-tagged GP63 protein bands were separated by 10% SDS-PAGE and visualized by Coomassie blue staining (Fig. 3A). The lysate of IPTG-induced pET32a-GP63 BL21(DE3) *E. coli* showed the specific high expression of 89 kDa His-tagged GP63 protein. However, uninduced pET32a-GP63 transfomed *E. coli* lysate did not expresse 89 kDa GP63 protein. Further, His-tagged GP63 protein band was also confirmed by western blotting using the anti-His antibody (Fig. 3B). These results clearly indicate that *T. vaginalis* metalloproteinase GP63 recombinant protein can highly expressed in vitro using a bacterial expression system.

Live *T. vaginalis* and *T. vaginalis* ESP induced cleavage of SiHa cell mTOR

The AKT/mTOR/p70S6K pathway regulates cell growth and proliferation via the regulation of protein synthesis [17]. To investigate the effect of *T. vaginalis* infection in the AKT/mTOR/p70S6K pathway, SiHa cells were incubated with live *T. vaginalis*, *T. vaginalis* ESP (transwell inside the live *T. vaginalis*), or *T. vaginalis* lysate for 16 hr, and then the posttranslational modification of mTOR, AKT, and p70S6K were observed by western blot. As shown in Fig. 4, live *T. vaginalis* and *T. vaginalis* ESP induced cleavage of host cell mTOR to generate approximately 85 kDa smaller protein band and the live *T. vaginalis* induced higher levels of mTOR cleavage than *T. vaginalis* ESP that even smaller band approximately 80 kDa appeared right beneath the 85 kDa band. Based on the reciprocal band intensity change between original larger mTOR band and cleaved smaller band, comparison of the mTOR-immunoreactive protein band sizes and the epitope (Ser2481 containing polypeptide) information for the antibody what we used, it is presumed that the cleavage of mTOR occurs at the N-terminal region of mTOR by *T. vaginalis*. Interestingly, AKT protein level was not affected by *T. vaginalis* but mTOR downstream target p70S6K had suffered degradation or cleavage by live *T. vaginalis* infection.

T. vaginalis induced the cleavage of SiHa cell mTOR time- and parasite load-dependent manner

In order to examine how fast *T. vaginalis* infection makes ef-

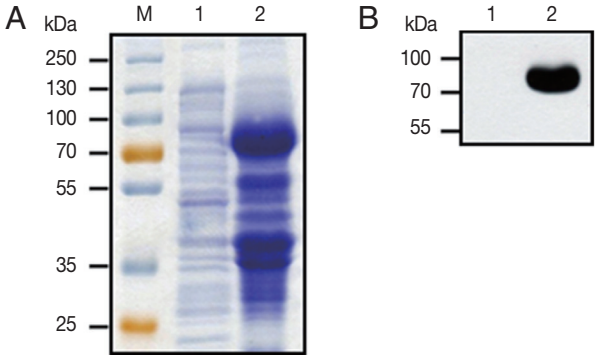

Fig. 3. Expression and identification of recombinant proteins. (A) Proteins were submitted to SDS-PAGE and revealed by Coomassie blue staining. M, protein molecular size marker; lane 1, pET32a-GP63 transfomed *E. coli*; lane 2, IPTG-induced pET32a-GP63 transfomed *E. coli*. (B) Western blot analysis of *E. coli* lysate with anti-His primary antibody. Lane 1, pET32a-GP63 transfomed *E. coli* lysate; lane 2, IPTG-induced pET32a-GP63 transfomed *E. coli* lysate.

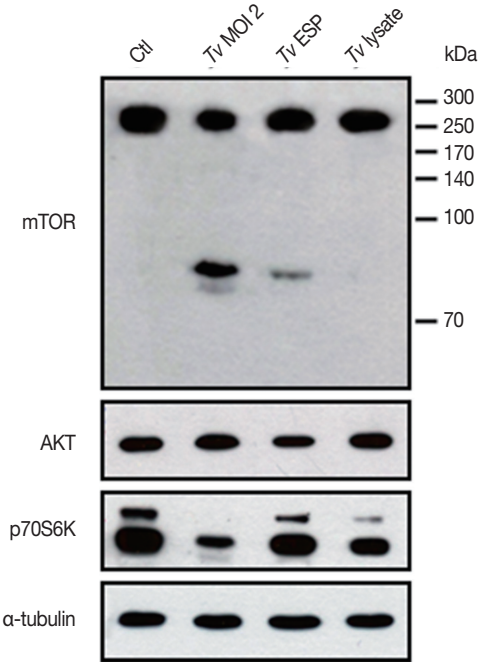

Fig. 4. Live *T. vaginalis* and *T. vaginalis* ESP induced cleavage of host cell mTOR. After SiHa cells were incubated with live *T. vaginalis* at a multiplicity of infection (MOI) 2, *T. vaginalis* ESP (transwell) or *T. vaginalis* lysate (100 μg/ml) for 16 hr, protein levels of mTOR, AKT, and p70S6K were shown by western blot. Anti-α-tubulin antibodies were employed to confirm equal loading of the cell extracts. A representative result of 3 independent replicates is shown.

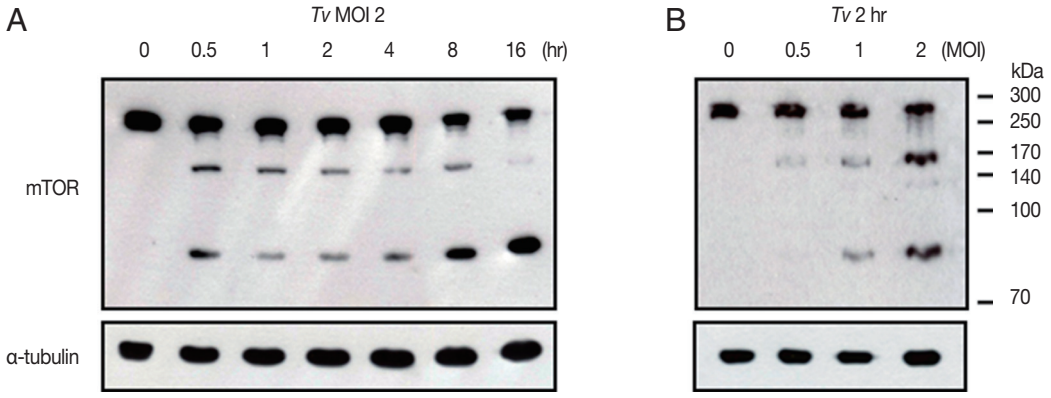

Fig. 5. *T. vaginalis*-induced cleavage of host cell mTOR is time- and parasite load-dependent manner. After SiHa cells were incubated with (A) *T. vaginalis* at MOI 2 for the indicated time points or (B) SiHa cells were infected with *T. vaginalis* at MOI 0.5, 1, and 2 for 2 hr, cells were collected, and levels of mTOR was measured. Anti-α-tubulin antibodies were employed to confirm equal loading of the cell extracts. A representative result of 3 independent replicates is shown.

fect on host cells, we performed time-course studies of mTOR cleavage after incubation of SiHa cells with live *T. vaginalis* for 0.5, 1, 2, 4, 8, and 16 hr. As shown in Fig. 5, *T. vaginalis* induced the cleavage of SiHa cell mTOR from 30 min after infection, and these cleavage phenomena were time-dependent, and prominently high levels of cleaved mTOR were detected after infection with *T. vaginalis* at 8 hr and 16 hr (Fig. 5A). Similarly, when the SiHa cells were infected with *T. vaginalis* at MOI 0.5, 1, and 2 for 2 hr, *T. vaginalis* induced mTOR cleavage in parasite load-dependent manner. A high level of cleaved mTOR was also detected in SiHa cells at *T. vaginalis* MOI 2 for 2 hr (Fig. 5B). Interestingly, Fig. 5A and 5B shows that, based on the infection time and infection strength, mTOR cleavage occurs in more than 2 phases, and in early infection, first N-terminal cleavage occurs to generate rather intermediate form of 170 kDa mTOR fragment. Then, as infection time elongates, this 170 kDa fragments get further cleaved to generate 85 kDa fragment of mTOR, and after 16 hr, most of 170 kDa intermediate mTOR fragment disappeared, and the band intensity of 85 kDa mTOR fragment become stronger.

Pretreatment of 1,10-PT inhibits *T. vaginalis*-induced cleavage of host cell mTOR

To determine whether the *T. vaginalis* metallopeptidase plays a vital role in cleavage of SiHa cell mTOR, live *T. vaginalis* (MOI 2) were treated for 30 min with 5 mM metallopeptidase inhibitor, 1,10-PT. After incubation finished, *T. vaginalis* was washed with PBS to remove the inhibitor from the surface of parasites, and then infected with SiHa cells for 16 hr, and cell morphology was observed by phase-contrast light microscopy. Some of live *T. vaginalis*-infected SiHa cells detached from the culture dish and were slightly darker than the control group (Fig. 6B, C). In addition, cells infected with 1,10-PT-pretreated *T. vaginalis* lost their lethal effect on cell survival, so that the infected host cells were morphologically similar with the control group (Fig. 6C). Our experimental results showed that the metallopeptidase inhibitor, 1,10-PT, was found to inhibit the mTOR cleavage significantly in SiHa cells (Fig. 6D). The 170 kDa and 85 kDa mTOR fragment bands completely disappeared by 1,10-PT pretreatment and regained the correct immune positive band size of intact mTOR protein. This is the first report about that the *T. vaginalis* metallopeptidase plays a vital role in cleavage of host cell mTOR.

DISCUSSION

It was known that *T. vaginalis* proteases are important regulators of pathogenesis, invasion, and survival [5,6], and *T. vaginalis* genome contains 13 families of metallopeptidases [5]. From this study, we tried to confirm whether *T. vaginalis* metalloproteinases are involved in host cell mTOR cleavage or not. In the present study, we detected several kinds of metalloproteinases in live *T. vaginalis* parasites. We also constructed pET32a-GP63 expression plasmids, and confirmed this His-tagged GP63 fusion protein was highly expressed. Furthermore, 1,10-PT-pretreated *T. vaginalis* did not induce SiHa cell mTOR cleavage. These findings indicate that *T. vaginalis* metalloproteinases are involved in mTOR cleavage, and when this activity is inhibited by 1,10-PT, *T. vaginalis* cannot induce SiHa cell mTOR cleavage.

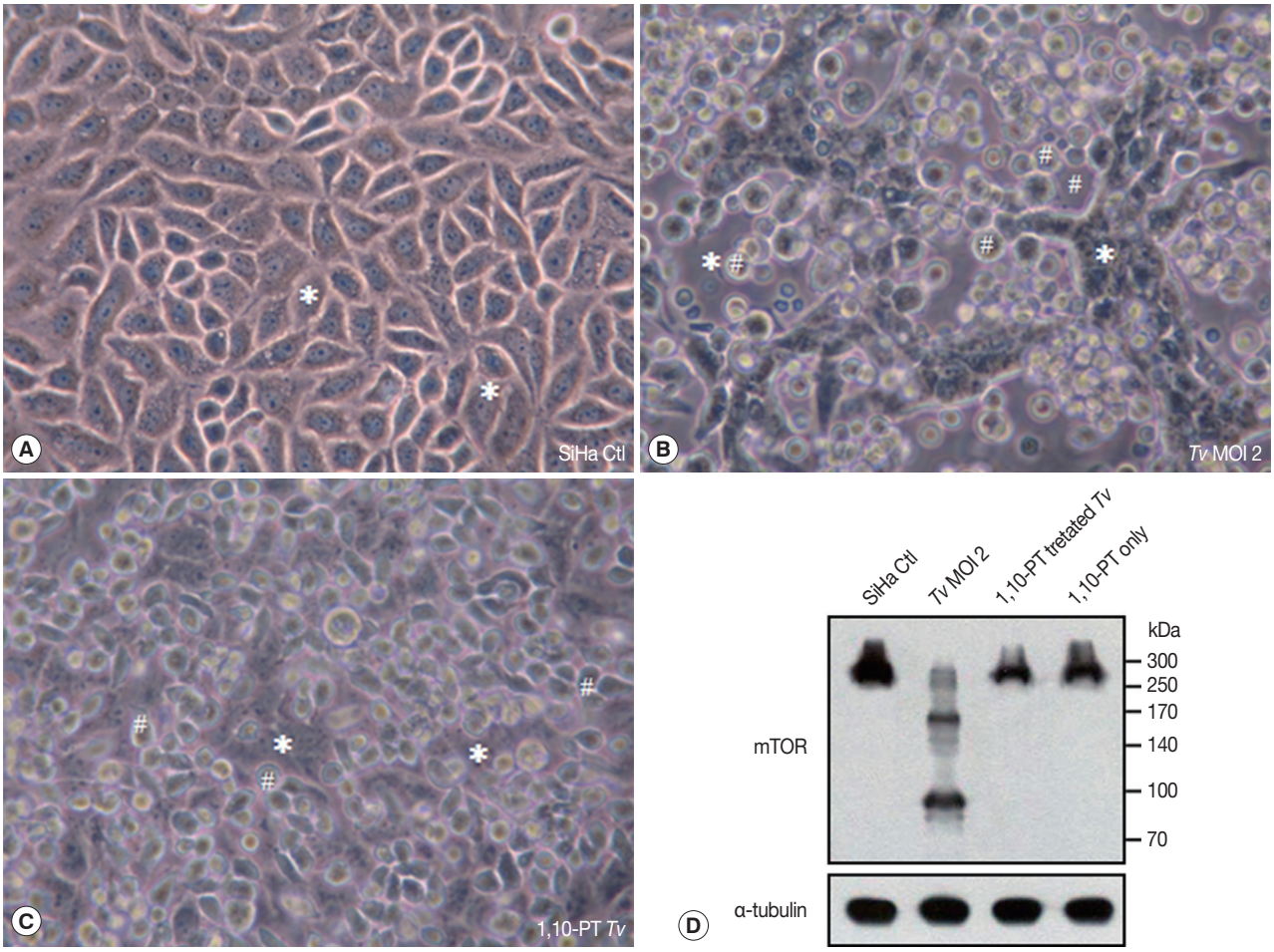

Fig. 6. Cleavage of host cell mTOR induced by *T. vaginalis* was inhibited with metallopeptidase inhibitor, 1,10-phenanthroline (1,10-PT). *T. vaginalis* (MOI 2) were treated for 30 min with 1,10-PT (5 mM), and the control group *T. vaginalis* were mock-treated in PBS, and then *T. vaginalis* were washed with PBS and infected with SiHa cells. After 16 hr later, (A-C) cells were visualized under phage-contrast microscope, and (D) western blot was performed in order to detect mTOR. Anti-α-tubulin antibodies were employed to confirm equal loading of the cell extracts. A representative result of 3 independent replicates is shown. *, SiHa cell; #, trophozoite of *T. vaginalis*.

The mTOR acts as a 'master switch' of cellular catabolism and anabolism, signaling cells to expand, grow, and proliferate [18]. Disruption of the AKT/mTOR pathway by *L. major* is dependent on the surface metalloprotease GP63 [18], and the *Leishmania* metalloprotease GP63 also cleaves the mTOR [13]. *Leishmania* metallopeptidase has been found to be involved not only in the cleavage and degradation of various kinases and transcription factors, but also to be the major molecule modulating host negative regulatory mechanisms [20]. In the present study, we confirmed that *T. vaginalis* contains zinc metallopeptidase with a zinc-binding motif of HEXXH (GP63), MG aminopeptidase P-like metallopeptidase (MP50), M41 FtsH endopeptidase-like metallopeptidase (TVAG_243780.2), oligopeptidase A-like metallopeptidase (TVAG_477180), Zinc carboxypeptidase-like metallopeptidase (TVAG_075740), and aspartyl aminopeptidase-like metallopeptidase (TVAG_392410).

T. vaginalis metalloproteinase is the largest surface protease family and the second largest surface protein family in *T. vaginalis* [5]; however, there was no report about the effects of mTOR in *T. vaginalis*-infected cells. We report here another case of host cell mTOR cleavage induced by live *T. vaginalis* and *T. vaginalis* ESP, except *Leishmania* infection. The host cell mTOR was cleaved as early as 30 min after *T. vaginalis* infection, and this mTOR cleavage occurred as time- and parasite burden-dependent manner. It may be important to understand the host-parasite relationship in *T. vaginalis*-infected SiHa cells. We need further studies to find out the mechanisms of mTOR cleavage by *T. vaginalis*.

PI3K/AKT/mTOR pathway plays an important role in cellular growth and survival [20]. The mTOR cleavage by *Leishmania* GP63 metalloproteinase inhibits host cell translation initiation, thus parasites survive and induce the pathogenesis of leishmaniasis [13]. From this study, we confirmed that *T. vaginalis* has various kinds of metalloproteinases by RT-PCR, sequencing analysis and western blot. *T. vaginalis* induced the SiHa cell mTOR cleavage as well as decreased the protein levels of p70S6K protein levels in comparison to uninfected control group. These data suggest that SiHa cell mTOR cleavage by *T. vaginalis* may induce the inhibition of host cell protein synthesis, parasite establishes host infection, and starts proliferation, which is a similar mechanism of *Leishmania* for its growth [13]. Base on the previous paper, *T. vaginalis* is strong inducer of host cell apoptosis [7]. It can be explained that host cell apoptosis by *T. vaginalis* could be related with disruption of PI3K/AKT/mTOR pathway according to our results and previous papers [22,23]. Namely, suppression of PI3K/AKT/mTOR/S6K pathway by β-caryophyllene oxide could facilitate downregulation of various cell survival genes and lead to apoptosis in tumor cells [22], and *M. citriodora* and its major constituent thymol inhibit the PI3K/AKT/mTOR pathway and induce apoptosis via both extrinsic and intrinsic pathways in human leukemia HL-60 cells [23].

Taken together, our results indicate that *T. vaginalis* metallopeptidase plays a vital role in cleavage of host cell mTOR by disruption of mTOR/p70S6K pathway, and *T. vaginalis* cleaves host cell mTOR probably to block various anti-parasitic events of its host which are under mTOR control and make profitable cellular environment for its survival. These findings raise an interesting possibility that agents that block mTOR cleavage could be therapeutically useful as an approach for treatment of trichomoniasis. Further investigation will shed light on the molecular mechanisms of mTOR cleavage by *T. vaginalis* GP63 and disruption of mTOR/p70S6K pathway.

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CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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