

# Involvement of the GP63 protease in infection of *Trichomonas vaginalis*

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**Abstract** There are 48 members of the GP63 protease family in *Trichomonas vaginalis* according to our annotations; 37 of them are predicted to be transmembrane proteins. Because the GP63 protease family is the largest surface protease family and the second largest surface protein family, they are most likely to be involved in the interactions between *T. vaginalis* and the host cell surfaces, or otherwise participate in infection. We performed a preliminary study on the functions of GP63 in *T. vaginalis* (TvGP63). We demonstrated the cell surface localization of one highly expressed member of TvGP63 using indirect immunofluorescence assays in both isolate T016 and isolate 30236. The specific inhibitor of TvGP63

protease, 1,10-phenanthroline, was found to significantly inhibit the destruction of HeLa cells, whereas another chelator, EDTA, could not. Further tests showed that 1,10-phenanthroline did not inhibit the adherence of *T. vaginalis* cells to HeLa cells. The results presented in here suggest that GP63 protease plays a vital role in *T. vaginalis* infection process, but may not be related to the adherence of parasitic cells to their hosts.

## Introduction

*Trichomonas vaginalis* is the major causative agent of non-viral vaginitis, which is a sexually transmitted disease in humans, and there are more than 170 million cases reported worldwide every year. Women who are infected with *T. vaginalis* will not only get vaginitis but may also have a higher risk of premature delivery, low birth rate, atypical pelvic inflammatory disease, infertility, predisposition to developing invasive cervical cancer, and increased susceptibility to HIV infection (Minkoff et al. 1984; Cotch et al. 1997; Zhang and Begg 1994; Kharsany et al. 1993; Grodstein et al. 1993; Yap et al. 1995; Sorvillo and Kerndt 1998; Sorvillo et al. 2001). *T. vaginalis* can infect the urogenital tracts of both sexes. In men, it can cause nongonococcal urethritis and chronic prostatitis (Soper 2004). The mechanisms of the disease caused by *T. vaginalis* have become a hot topic of study in recent years.

In 1985, Krieger et al. (1985) reported that the cytopathogenic mechanism that *T. vaginalis* uses is contact-dependent, where adherence to a cell is the first step leading to cytotoxicity. Further experiments have indicated that adherence is also cell-specific and species-specific. *T. vaginalis* can adhere to human vaginal epithelial cells (hVECs) and produce cytotoxic effects, but neither

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adherence nor cytotoxicity has been observed when *T. vaginalis* is exposed to human vaginal fibroblasts or bovine vaginal epithelial cells. Similarly, the bovine parasite *Tritrichomonas foetus* had no cytotoxic effects on hVECs (Gilbert et al. 2000). Four proteins—AP65, AP51, AP23, and AP33—have been reported to be adhesins that bind *T. vaginalis* to its host cells. Antibodies against these adhesins can inhibit the binding of live parasites to vaginal epithelial cells and protect vaginal epithelial cells from contact-dependent cytotoxicity (Arroyo et al. 1992). As a result, subsequent research activities have mostly focused on AP65, which has been found to be a primary adhesin. Antisense silencing of the AP65 gene resulted in lower levels of adherence of parasitic cells to vaginal epithelial cells (Mundodi et al. 2004). In addition to the presence of the four adhesins, cysteine proteinase (CP) activity is also necessary for cytoadherence (Arroyo and Alderete 1989). TVCP30 and TVCP62, in particular, have been reported to be related to cytoadherence (Arroyo and Alderete 1995; Hernandez et al. 2004; Mendoza-Lopez et al. 2000).

There have been numerous studies about how *T. vaginalis* causes diseases, although little is clearly illustrated yet. *T. vaginalis* lives as a parasite and utilizes nutrients from its host for survival, which is considered to be the ultimate consequence of pathogenesis. It hydrolyzes organic components from the mucus and extracellular matrix of its host cells into glucose, which is then transported into the parasitic cells (Asami and Nakamura 1955; ter Kuile and Muller 1995) where nucleotides from the host are used for DNA synthesis (Gonzalez-Lazaro et al. 2005). We assume that cell surface proteins are probably critical for preliminary interactions between parasites and mucosa/extracellular matrices and thus may be related to subsequent cytotoxicity. In this study, we performed differential expression analyses on surface proteins from three major developmental stages of *T. vaginalis*—trophozoite, pseudocyst, and ameboid—and found that GP63 proteases appeared differentially expressed. As the largest surface protease family and the second largest surface protein family in *T. vaginalis* (Hirt et al. 2007), a TvGP63 has been previously noted as a possible factor (Carlton et al. 2007) and always participates in the pathogenesis of protozoan parasites.

GP63 proteases were first detected in *Leishmania*. According to crystal structure analyses of GP63 proteases in *Leishmania major*, they are zinc metallopeptidase with a zinc-binding motif of HEXXH (Joshi et al. 2002). The primary function of GP63 protease proteolytic activity is related to the cleavage of host cell macromolecules, either for self-protection or to provide nutrition for the parasite (Chaudhuri et al. 1989b). GP63 proteases are the major pathogenic agent of *Leishmania* spp. (Joshi et al. 2002) and play an important role in host–parasite interactions (Bouvier

et al. 1995). Evidence has suggested that GP63 proteases facilitate the attachment of the promastigote form of the parasite to host cell surface receptors, such as the fibronectin receptor (Chang and Chang 1986; Russell and Wright 1988; Soteriadou et al. 1992). In addition, GP63 proteases interfere with the host complement system (Russell 1987) and facilitate the survival of the amastigote form inside macrophages (Chaudhuri et al. 1989b; McGwire and Chang 1994; Seay et al. 1996). GP63 proteases have also been reported to play a role in the pathogenicity of two other parasites, *Trypanosoma cruzi* and *Trypanosoma brucei*. *T. brucei* GP63 proteases are predominantly expressed in the bloodstream from of the parasite and may protect bloodstream trypanosomes against complement-mediated lysis by imparting protein-processing capabilities to *T. brucei* surface. Neutralization assays have indicated that anti-GP63 serum significantly inhibits infection by *T. cruzi* (El-Sayed and Donelson 1997; Cuevas et al. 2003; Kulkarni et al. 2009).

We performed protease domain comparisons among all related species, and the results indicated that TvGP63 is quite different from the GP63 of other species and has the shortest protease domain. It also lost the glycosylphosphatidylinositol (GPI) structure at its C-terminus, which attaches to cell surface proteins in organisms such as trypanosomatids (Englund 1993). However, the results of recent genome sequencing have demonstrated that *T. vaginalis* is a very special species where a great number of GP63 genes were found, which bear very little similarity to their orthologs (Carlton et al. 2007), although this does not necessarily mean that the functions of these genes have changed. We assume that the function of GP63 proteases in the cytotoxicity of *T. vaginalis* has been preserved and then performed the following assays. We used the specific inhibitor of GP63 protease, 1,10-phenanthroline (Chaudhuri et al. 1989a; Seay et al. 1996), in our series of experiments, hoping to collect useful information about the function of TvGP63. We discovered that GP63 protease participated in the destruction of HeLa cells, but found that the inhibition of GP63 protease did not affect the adherence of parasite cells to HeLa cells.

## Materials and methods

### Parasite culture

We obtained the *T. vaginalis* T016 isolate from Prof. J. F. Alderete and the *T. vaginalis* 30236 isolate from Chang Gung University. *T. vaginalis* T016 was cultured in Diamond's trypticase–yeast extract maltose medium (TYM) with 10% heat-inactivated horse serum for 36 h at 37°C. *T. vaginalis* 30236 was cultured in TYM with 10%

heat-inactivated horse serum for 24 h at 37°C. Cultures in the logarithmic-growth phase were used for all assays.

#### Annotation and classification of GP63 protease in *T. vaginalis*

We annotated 48 proteins to be GP63 proteases using Hmmpfam (Krogh et al. 1994) based on the 69 proteins that have been previously identified as GP63 or leishmanolysin by TIGR. Hmmpfam compares one or more sequences to the pfam database of profile hidden Markov model of GP63 in order to identify known domains within the sequences. The gathering threshold of the local search model was used as the cutoff score.

We built a phylogenetic tree from the 48 proteins using the neighbor-joining (NJ) method in Clustal W (<http://www.ch.embnet.org/software/ClustalW.html>). The robustness of the phylogenetic inferences was tested via bootstrapping, using 100 replications of the data based on 50% majority rule consensus. Sequence identity values were calculated using the software MEGA 3.1.

#### Differential expression analysis

We used three expressed sequence tag (EST) libraries that represented the three main developmental stages of *T. vaginalis*—trophozoite, pseudocyst, and ameboid (<http://txpress.cgu.edu.tw/>)—for our differential expression analysis. Paired comparisons of gene expression levels were carried out between the three libraries using IDEG6 (Romualdi et al. 2003) and the standard  $p < 0.05$ . Because surface proteins are the most probable proteins to be involved in host–parasite interactions, we chose all potential cell surface proteins as candidates for analysis. Transmembrane proteins were annotated using TMHMM and Phobius (Kall et al. 2007). We sought to verify the differential expression results of TvGP63-b, but failed due to the fact that the EST level of TvGP63 was too low to be verifiable through RT-PCR.

#### Gene expression and antibody generation

The sequence of *tvgp63* gene was obtained from isolate T016 based on PCR amplification (forward primer: ATGA TATTGTCTCTACTCCT; reverse primer: CTAAATCGA TAAATTTGCAT; designed according to the gene TVAG\_367130 based on TIGR ID in isolate G3). The sequence was submitted to GenBank (GenBank ID: GU356538). As characterized by the result of Protscale antigen prediction (<http://www.expasy.org/cgi-bin/protscale.pl>), we used the hydrophilic segment of the coding sequence between positions 757 and 1749 to generate polyclonal antibodies. This segment was amplified using Pyrobest DNA

Polymerase to avoid mutations and inserted between restriction sites *NdeI* and *HindIII* of pET-28b. The purified recombinant TvGP63 were injected into rabbits to generate polyclonal antibodies. The antibodies were purified from the anti-serum using ammonium sulfate.

#### Western blot assays

Approximately  $4 \times 10^7$  mid-log phase *T. vaginalis* 30236 cells were collected, washed in PBS (pH 6.2), and lysed in 400  $\mu$ l lysis buffer (8 M urea, 4% CHAPS) along with the protease inhibitor cocktail (Sigma). The supernatant was centrifuged at 13,000 rpm for 10 min at 4°C to remove insoluble material. After electrophoresis, proteins were transferred to a nitrocellulose membrane, blocked with 5% skim milk in PBS-0.1% Tween 20 (TBST) for 18 h at 4°C, and incubated with the anti-synthetic peptide antibody (anti-TvGP63, at 1:2,000 dilution) for 18 h at 4°C. Membranes were washed five times with PBST, incubated with peroxidase-conjugated secondary antibody (at 1:3,000 dilution; Amersham) for 2 h at 25°C, and developed using the chemiluminescence system (Sigma). Normal rabbit serum was used as negative control (at 1:3,000 dilution).

#### Indirect immunofluorescence assays

For the indirect immunofluorescence assays, approximately  $1 \times 10^7$  cells were fixed with 6 ml 3.5% of paraformaldehyde for 7 min at 37°C. We collected the parasites at low centrifugation speed and suspended the cells in 1 ml 3.5% paraformaldehyde. We added an aliquot of 200  $\mu$ l onto a gradient microscope slide and air-dried the slides at room temperature for 15 min. The slides were washed in PBS (pH 7.4) and incubated with anti-TvGP63 antibodies (at 1:200 dilution) for 30–45 min at 37°C and washed in PBS again. The parasites were incubated with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins (at 1:100 dilution) for 30 min at 37°C and washed. Finally, the parasites were incubated with DAPI (1:2,500 dilution) for 10 min at 37°C and washed. Slides were examined using laser confocal microscopy (Olympus BX51) and photographed.

#### Antibody inhibition experiment

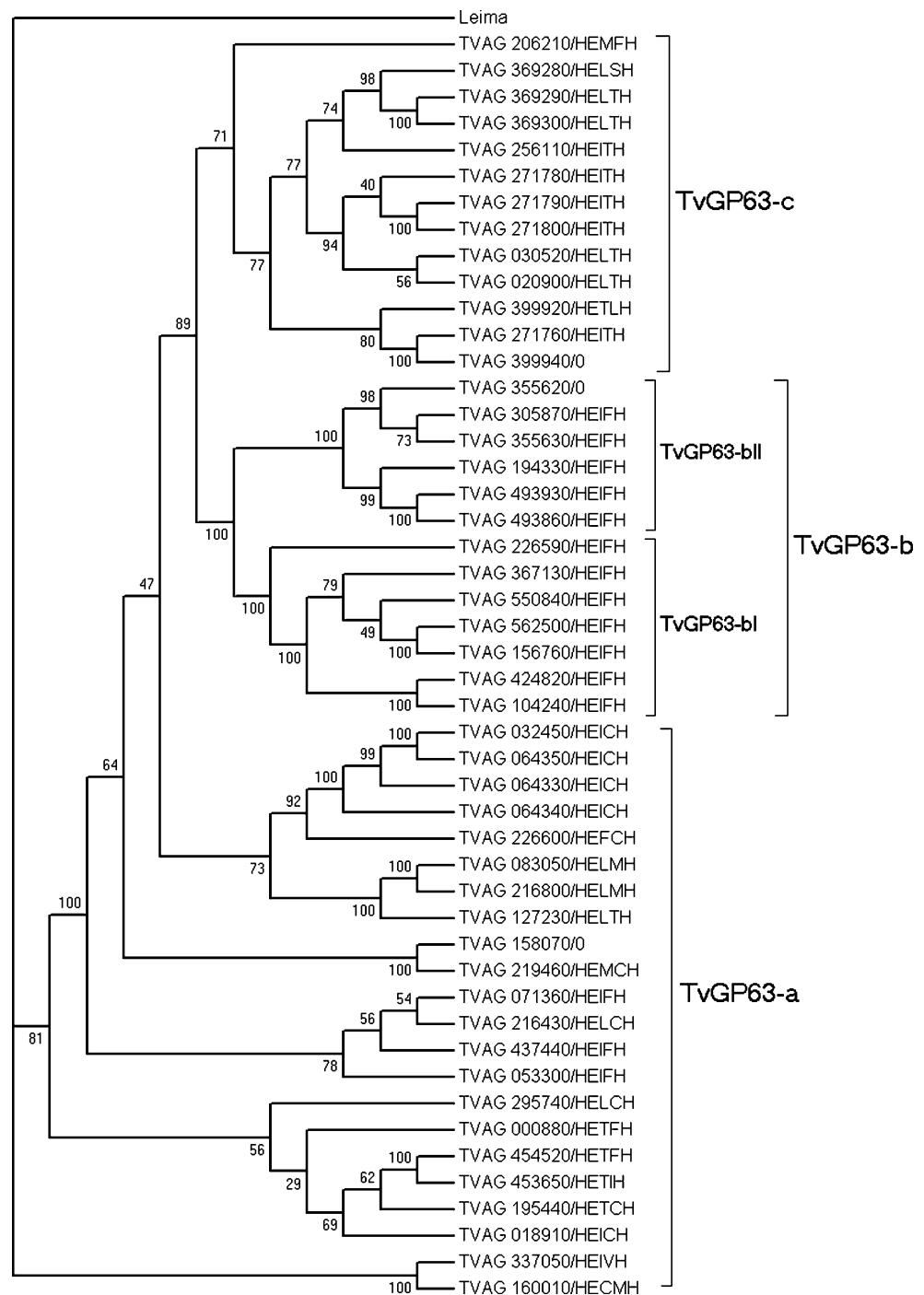
HeLa cells were cultured in 24-well plate with Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, and 100  $\mu$ g/ml of streptomycin in 5% CO<sub>2</sub> atmosphere at 37°C. Logarithmic growth phase *T. vaginalis* was collected and washed three times in PBS (pH 6.2). The parasites were divided into three groups. One group was

incubated with anti-TvGP63 antibodies at concentrations of 250, 500, 1,000, and 2,000 ng/ml. The second group received the same treatment using unrelated antibodies. The third group was incubated without any antibodies. HeLa cell monolayers were washed with PBS (pH 7.4). Parasite cells were incubated with HeLa cell monolayers in the medium mixture (DMEM/TYM=2:1) in 5% CO<sub>2</sub> atmosphere at 37°C at a ratio of 5:1. The cells were examined 4 h after incubation.

#### Effect of 1,10-phenanthroline

The mid-log phase parasites were collected and washed three times in PBS (pH 6.2) and divided into three groups. One was treated with 1,10-phenanthroline ranging from 1 to 11 mM; a second was treated with EDTA and the same concentration of 1,10-phenanthroline; the control group was incubated in PBS (pH 6.2) without 1,10-phenanthroline or EDTA. All the incubations were performed at 37°C for

**Fig. 1** Phylogenetic tree of TvGP63. The tree was built using NJ method in clustal W. A sequence of *Leishmania major* was used as an outgroup. Two groups can be evidently detected, which we named as TvGP63-b and TvGP63-c. The remaining sequences were grouped into TvGP63-a to facilitate description. TvGP63-b can be further divided into two subgroups according to the phylogenetic tree: TvGP63-bI and TvGP63-bII. Names for each sequence is formed by the accession number as “TVAG\_XXXXXX” and the zinc-binding motif “HEXXH”, with “/” to separate the two parts. If there is no zinc-binding motif, “0” is used



30 min. HeLa cell monolayers were washed with PBS (pH 7.4) and the parasite cells incubated with HeLa cell monolayers in the mixture medium (DMEM/TYM=2:1) in 5% CO<sub>2</sub> atmosphere at 37°C. In the adherence experiment, after 30 min of incubation with HeLa cell monolayers, the unbounded parasites were collected from each well. PBS (100 μl, pH 6.2) was added to each tube to resuspend the parasites and the cells were counted.

## Results and discussion

### Classification of *T. vaginalis* GP63

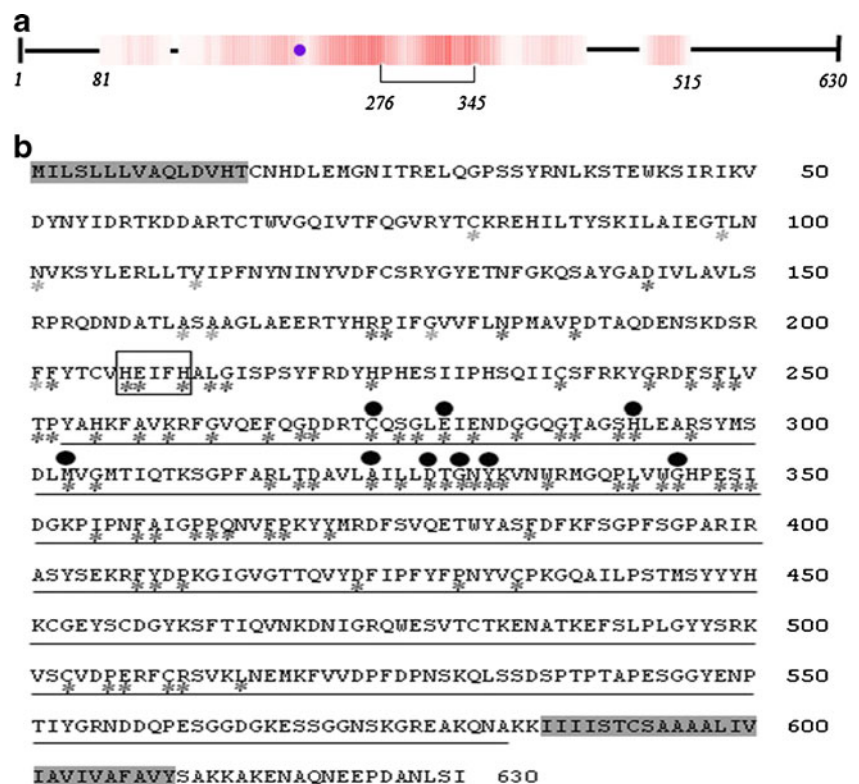
From the three EST libraries (trophozoite, pseudocyst, and ameboid stages), we identified TvGP63, one of the differentially expressed membrane proteins (data not shown). Only a segment of TvGP63, about 70 amino acids long, has the conserved protease motif when compared to GP63 proteases of non-pathogenic protists, pathogenic protists, animals, and plants (Electronic Supplementary Material (ESM) Online Resource 1). Unlike trypanosomatids, *T. vaginalis* TvGP63 has no GPI anchorage but a transmembrane domain at its C-terminus. In fact, *T. vaginalis* is special in that many of its genes have very little similarity with their orthologs (Carlton et al. 2007). We used the program HmmPfam to annotate all versions of

TvGP63 and found 48 family members. Two newly duplicated groups were detected among TvGP63 proteases: TvGP63-b and TvGP63-c. The remaining sequences were grouped into TvGP63-a because its sequences were more primitive (Fig. 1).

According to the NJ tree of the 48 amino acid sequences, *tvgp63* may have experienced five to eight duplication events and form three main groups. The three TvGP63 groups are conserved between amino acid positions 81 and 515 (the amino acid positions mentioned in our paper correspond to the sequence of TVAG\_367130), and the conserved sites are mostly centered in the positions immediately behind the zinc atom-binding motif HEXXH (Fig. 2a). There are 90 highly conserved amino acid sites which appear in more than 75% of all TvGP63 sequences (Fig. 2b).

We investigated gene expression, degrees of conservation, and the potential cellular locations of protein in each group (Table 1). There were 11 members of TvGP63-a (out of 22) expressed according to the EST data. However, only six members (out of 13) of TvGP63-b and two members of TvGP63-c (out of 13) were expressed. Among these three groups, the members of TvGP63-b were the most conserved. They had the highest average amino acid identity value, 56.3%, and the lowest average  $\omega$  value ( $\omega=K_a/K_s$ ), 0.1230. Also, all members of TvGP63-b had a uniform pattern for their zinc-binding motif HEIFH (Fig. 1), while the motifs of

**Fig. 2** Sequence features of TvGP63 protease family. **a** General scheme of sequence conservation (heat scale) along the whole amino acid sequence over all TvGP63. The blue spot indicates the zinc-binding motif. The segment from 276AA to 345AA is conserved among the GP63 proteases of other species. **b** Detailed sequence information among all TvGP63 proteases. Signal peptide (1AA–15AA) and the transmembrane peptide (586AA–610AA) are shaded. The open box highlighted the active protease site, which contains a zinc-binding site. Asterisks indicate positions that are conserved among the three GP63 groups of *T. vaginalis*. Solid circles above the amino acid sites indicate the sites conserved among all species in our analysis. The underlined segment is what was used to prepare the polyclonal antibody





**Table 1** Basic information of TvGP63

Subfamily	Member	Identity (%)	AA <sup>a</sup>	GC content (%)	Omega <sup>b</sup>	TM domain	No. of expressed genes
TvGP63-a	22	28.9	556	36.11	0.2037	18	11
TvGP63-b	13	56.3	610	36.33	0.1230	10	6
TvGP63-c	13	45.9	473	33.41	0.1261	9	2

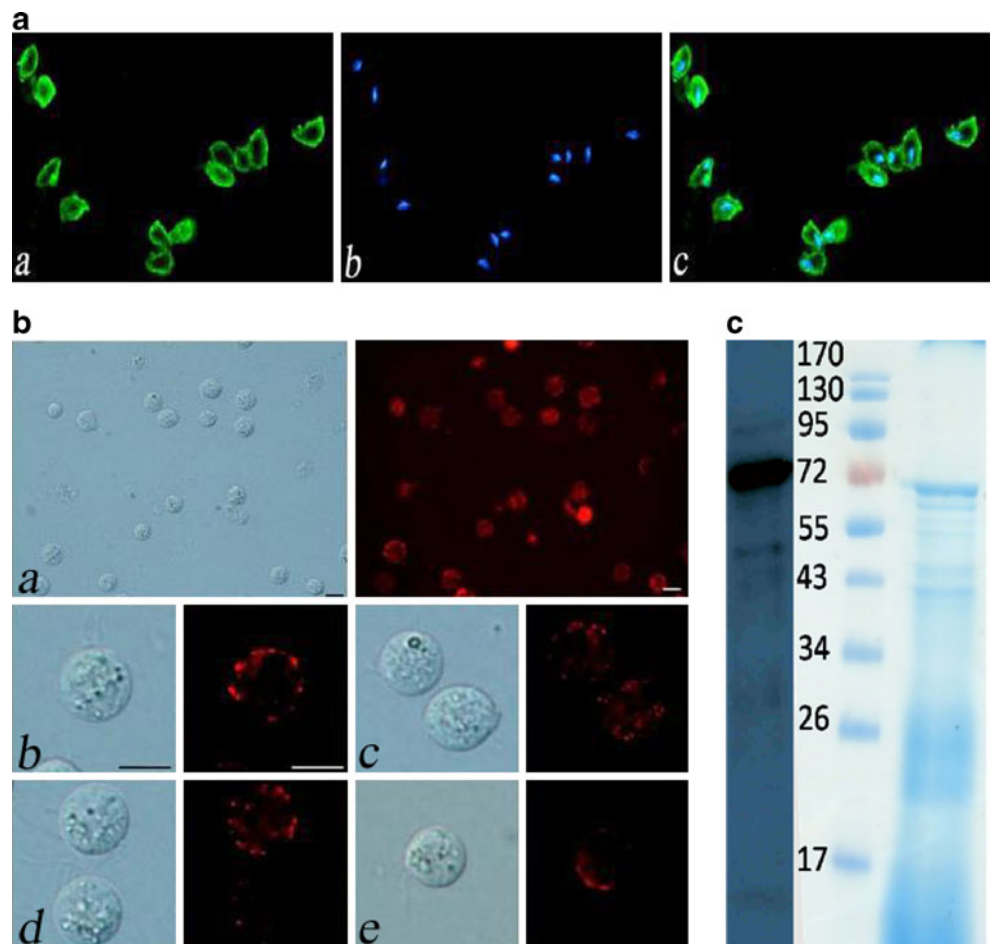
<sup>a</sup> Average protein length for each subfamily

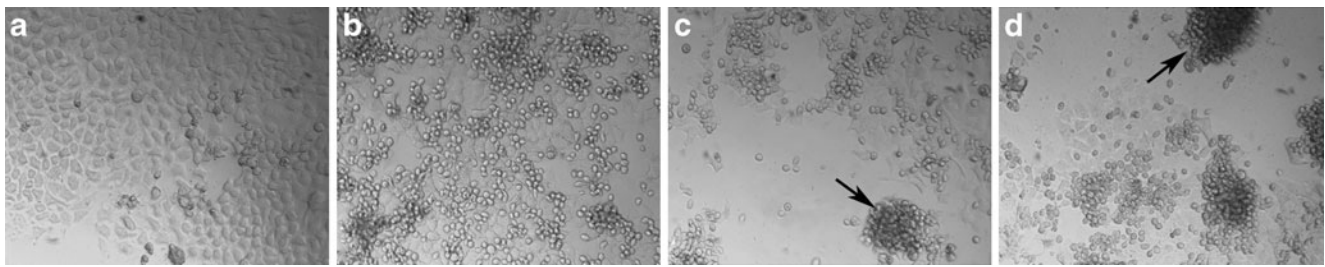
<sup>b</sup> Omega value or  $K_a/K_s$  was calculated using the YN method in paml

the other two groups varied greatly. Three amino acids in the motif, two H and one E, form catalytic sites. Although the remaining two sites, I and F, do not contribute significantly to protease function, their degree of variation is influenced by the adjacent active sites. In conclusion, TvGP63-b is the group that has evolved most recently and is also the group that has the highest degree of conservation. TvGP63-b also has the largest number of expressed ESTs, 46 in all, while TvGP63-a and TvGP63-c have only 26 and 5, respectively (ESM Online Resource 2). Coincidentally, the differentially expressed member of TvGP63 and TVAG\_367130 also belong to TvGP63-b.

In order to study the function of TvGP63, we chose to use TVAG\_367130 in the following experimental assays. The CDS sequence of TVAG\_367130, taken from T016 isolate, was cloned and expressed for preparing antibodies. The cloned TVAG\_367130 from T016 isolate resembles its homolog from G3 isolate (nucleotide acid identity value, 96.9%) more than the TvGP63-b sequences from isolate G3, and the observation suggests that TvGP63 homologs from different *T. vaginalis* isolates may also be quite conserved. We paid particular attention to TvGP63-b and further divided TvGP63-b into two subgroups according to the phylogenetic tree, TvGP63-bI and TvGP63-bII (Fig. 1). TVAG\_367130 and six other members constituted

**Fig. 3** Demonstration of the presence and localization of TvGP63. **a** Indirect immunofluorescence assays on the trophozoite form of isolate T016. The parasites were probed with antibody against TvGP63: FITC (*a*), DAPI (*b*), and FITC + DAPI (*c*). **b** Indirect immunofluorescence assays of the trophozoite form of isolate 30236. The phase contrast images are shown in *a–e* and the indirect immunofluorescence on the *right*. The same antibody was used for T016. Scale bar, 100  $\mu$ m. **c** Result of Western blotting. Polyacrylamide gel (15%) SDS-PAGE was used for electrophoresis. Total protein (10  $\mu$ g) was loaded per lane. The *lanes* show (from the *left*): the blot, Tv protein extract, the size markers, and the extract stained with Coomassie brilliant blue





**Fig. 4** Results from inhibition experiments. *a* Intact monolayer without *T. vaginalis*. *b* Group treated with the inhibitor 1,10-phenanthroline (5 mM). *c* The mock group. *d* Group treated with 5 mM EDTA. Arrowheads in *b* and *c* show the clumps formed by the parasites, and

this is considered as the indicator of cytotoxicity. The images were captured 4 h after adding parasites to the HeLa cell monolayers; more than 50% of the HeLa monolayers were destructed (*c*, *d*)

TvGP63-bI, and the average amino acid identity value among the seven members was 75.6%. TVAG\_367130 expressed the largest number of ESTs in the trophozoite form. Its potential full length is 630AA, and TMHMM and Phobius predicted it to be a transmembrane protein, with its C terminal embedded in the membrane (Fig. 2b). With a combination of differential expression, high mRNA levels, and high degrees of conservation, TVAG\_367130 is qualified to be our antigen for antibody production to study cellular location and function, representing GP63 proteases of *T. vaginalis*.

#### Expression and localization of TvGP63

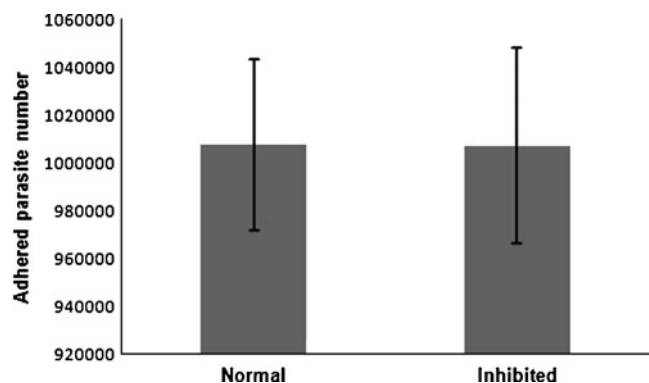
We used the specific rabbit polyclonal IgGs against TVAG\_367130 to test GP63 protease expression. Before doing this, the sensitivity and specificity of the anti-TVAG\_367130 serum was tested, and we observed a single major band in the blot (Fig. 3c and ESM Online Resource 3). The region cloned and expressed to prepare antibodies only has an average amino acid similarity value of 45.1% with the remaining 47 members (49.0% with members of TvGP63-bII and 79.3% with the remaining six members of TvGP63-bI). Therefore, the members of TvGP63-bI would be the most likely sequences to interfere in Western blotting. Although we saw other minor bands in the blot, they are most likely unprocessed and degraded forms of TVAG\_367130.

Proteins which are localized on the cell surface always have greater opportunities to participate in interactions between parasites and their hosts, and this is why we chose membrane proteins as the subjects of our differential expression analysis. We first used TMHMM and Phobius to predict transmembrane proteins and then chose those proteins that were differently expressed. TvGP63 was such a protein. To verify the cellular localization of TvGP63, we carried out indirect immunofluorescence assays on fixed parasites using the specific rabbit antibody against TVAG\_367130. TvGP63 was detected on the plasma membranes of both the T016 (Fig. 3a) and 30236 isolates (Fig. 3b).

Patches of TvGP63 were clearly detected on the cell surface of isolate 30236, and a clear outer ring was detected in isolate T016. We used the trophozoite form of the two isolates, T016 and 30236, and both assays were able to detect the cell surface localization of TvGP63, and also discovered TvGP63 in the cytosol. We considered this result to be normal since all proteins are processed in the cytosol before moving to their final positions. In conclusion, the results presented here indicate that TvGP63 was expressed in both T016 and 30236 isolates and also localized on their cell surfaces.

#### Effect of 1,10-phenanthroline

In order to study the function of GP63 protease in *T. vaginalis*, we utilized antibodies against the highly expressed GP63 gene to inhibit trichomonal cytotoxicity. It was found that antibodies against TvGP63 and the control group that was given unrelated antibodies against a human protein can at most delay trichomonal cytotoxicity for about 2 h even at the highest concentration of 2,000 ng/ml in our assays. On the



**Fig. 5** Results from adherence assays. Two groups of parasitic cells with different treatments were used. The inhibited group was incubated with 5 mM 1,10-phenanthroline and the control group was mock-treated in PBS. The *y*-axis displays the number of *T. vaginalis* cells that adhered to HeLa monolayer. Eleven pairs of data were used. The detailed number for each group is supplied in ESM Online Resource 4

other hand, antibodies against TvGP63 failed to make any significant difference compared to the control group in time delay. This result was really discouraging, but may still have been acceptable considering the following reasons. First, there are 48 members of the GP63 protease family in *T. vaginalis*, so antibodies against only one TvGP63 may have very limited influence and may need to be present in very large quantities to have a significant inhibitory effect on *T. vaginalis*' cytotoxicity. Second, there were two kinds of TvGP63 according to our predictions—some may have been located on cell membranes (37 members are potential membrane proteins) and others may have been secreted out of the parasite cells (11 members are potential secreted proteins). Experimental evidences have suggested that soluble extract antigen of *T. vaginalis* isolates from the symptomatic women have cytotoxic activity (Malla et al. 2008). We do not have enough knowledge concerning which kinds of GP63 are effectively involved in the parasite cytotoxicity, and since there are so many members of the GP63 family, studies based only on one specific TvGP63 may not really be able to generate any significant results. We changed our tactics and chose to try to study all members of TvGP63. A specific inhibitor of the GP63 family, which can inhibit all TvGP63, 1,10-phenanthroline (Chaudhuri et al. 1989a; Seay et al. 1996), was chosen as the most favorable reagent for studying the functions of the GP63 protease family in *T. vaginalis*.

In the following experiments, we found that the use of the GP63 inhibitor 1,10-phenanthroline could prevent the destruction of HeLa cells by *T. vaginalis* to a significant degree (Fig. 4). We used different concentrations of 1,10-phenanthroline (ranging from 1 to 11 mM) and the same concentrations of a negative control (EDTA) to perform inhibition assays. We observed that the cytotoxicity of *T. vaginalis* could be inhibited by 1,10-phenanthroline at a concentration of 1 mM, while EDTA was not able to inhibit cytotoxicity, even at a concentration of 11 mM (data not shown). We continued observing the cells for more than 12 h and found that the inhibitory effect of 1,10-phenanthroline was sustained without killing the parasites. Our results suggested that GP63 may participate in the killing of HeLa cells.

Does GP63 facilitate the adherence of parasite cells to HeLa cells? To address this question, we incubated the parasites with 5 mM 1,10-phenanthroline. We did not observe differences between the test and control (ESM Online Resource 4; the parasite-to-HeLa cell ratio is 5:1). The *p* value of a *t* test on adhered parasite number of the two groups was 0.978, which is >0.05 (Fig. 5). As a result of our experiments, we drew a preliminary conclusion that TvGP63 may not influence the adherence of the parasites to HeLa cells. We observed that *T. vaginalis* cells remained

alive after both incubations, and because our adherence evaluation experiment was carried out in quite a short time, the results were unlikely to have been influenced by the growth of *T. vaginalis*.

Based on previous studies, it was thought that the parasites need protease in order to hydrolyze the mucosa and extracellular matrix proteins of host cells. However, the mechanisms of how the protease actually works have remained unclear. Surface protease is thought to be the type of protease most likely to be involved in parasite–host interactions. Through our preliminary study of the function of TvGP63, we came to the conclusion that TvGP63 is likely necessary for the cytotoxicity of *T. vaginalis*, but may not influence cytoadherence. Whether TvGP63 has any relationship with mucus or extracellular matrix hydrolysis needs to be examined further. Many other questions need further investigation as well, such as how secreted GP63 protease and surface GP63 protease differ in function and how they may interact with other surface proteins in *T. vaginalis* or with a host cell's surface coating. Because of the great variety of TvGP63 in existence, and the limitations of our experiments, we were not able to generate very elaborate findings. Nevertheless, this preliminary study furthers our understanding of the pathogenesis of *T. vaginalis*, and we hope that this will inspire more in-depth studies.

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